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Low temperature acclimation and treatment with exogenous abscisic acid induce common polypeptides in *Arabidopsis thaliana* (L.) Heynh

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Summary. Exogenously applied abscisic acid (ABA) induced frost hardening of *Arabidopsis thaliana* (L.) Heynh. The freezing tolerance of *A. thaliana* plantlets treated with ABA (15 mg/l) at a non-acclimating temperature (20 °C) appeared to increase even more rapidly than following a low temperature (4 °C) acclimation. Analysis of in vivo-labelled soluble proteins by two-dimensional gel electrophoresis revealed several low temperature – or ABA – induced proteins, which where not produced in non-acclimated plants. A subset of these proteins was induced by both low temperature and ABA treatments, suggesting that they might be directly involved in the frost hardening process in *A. thaliana*.

Key words: Abscisic acid – Cold acclimation – *Arabidopsis thaliana* – Protein synthesis – Frost-hardiness

Introduction

The hormone abscisic acid (ABA) is involved in many important physiological processes in plants. ABA seems to have a role in such diverse processes as development and germination of seeds, root geotropism and induction of dormancy (Walton 1980; Addicott 1982). In particular, ABA appears to be involved in the responses of plants to adverse environmental conditions, such as water stress, chilling and freezing stress (Hiron and Wright 1973; Boussiba et al. 1975; Rikin et al. 1979; Bornman and Jansson 1980; Daie and Campbell 1981; Chen et al. 1983; Mundy and Chua 1988).

Many plant species are capable of cold-hardening: they can increase their tolerance to freezing stress (Levitt 1980). The hardening process can be initiated by exposing the plants to low but non-freezing temperatures (Graham and Patterson 1982). Two lines of evidence indicate that ABA is involved in the induction of cold-hardiness. Firstly, low temperature treatment of plants has been shown to result in accumulation of ABA (Kacperska-Palacz 1978; Daie and Campbell 1981). In a wild cold hardy potato species, *Solanum commersonii,* the endogenous ABA level was shown to increase rapidly during early stages of cold acclimation followed by a decline to the initial level. In contrast, no such increase of ABA level was observed in the frost-sensitive cultivated potato *S. tuberosum* (Chen et al. 1983). Secondly, exogenously applied ABA has been reported to induce frost-hardiness at non-acclimating temperatures in several plant species, e.g. stem-cultured *S. commersonii* (Chen et al. 1979, 1983), in suspension cultures of *Brassica napus* (Orr et al. 1986), winter wheat, winter rye and bromegrass (Chen and Gusta 1983; Reaney and Gusta 1987), in callus cultures of *Lotus corniculatus* (Keith and McKersie 1986), as well as in seedlings of alfalfa (Rikin et al. 1975). Consequently, it has been suggested that the low temperature-induced increase in the endogenous ABA content is the trigger for induction of frost-hardiness in plants (Chen et al. 1983).

Protein synthesis appears to be a prerequisite for cold-hardening (Chen et al. 1983). Furthermore, cold acclimation has been correlated with the induction of new proteins in several plant species (Graham and Patterson 1982; Cloutier 1983, 1984; Guy et al. 1985; Meza-Basso et al. 1986; Guy and Haskell 1987; Kurkela et al. 1988). At least part of this induction appears to be at the transcriptional level, indicating altered gene expression during cold acclimation. (Guy et al. 1985; Meza-Basso et al. 1986; Kurkela et al. 1988). However, the causal relationship between changes in protein patterns and increased freezing tolerance is far from clear.

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As exogenous ABA can be used to induced freezing tolerance, it has been of interest to analyze proteins induced by this treatment. This work, which has been carried out mainly by employing cell cultures, shows that new proteins are indeed induced by ABA treatment (Robertson and Gusta 1986; Robertson et al. 1987, 1988; Johnson-Flanagan and Singh 1987). Furthermore, comparison of low temperature and ABA-induced polypeptides shows that some of these appear to be common to both treatments (Tseng and Li 1987; Robertson et al. 1988).

Recently, we have shown that *Arabidopsis thaliana*, a small weed in the Cruciferae family, is able to acclimate to cold and we demonstrated the induction of new proteins during the acclimation process (Kurkela et al. 1988). In this communication, we have employed *Arabidopsis thaliana* to elucidate the role of exogenously applied ABA in frost-hardiness and the induction of soluble proteins of this plant. The results show that freezing tolerance can be induced by exogenously added ABA and that this induction is even more rapid than by low temperature treatment. We also show that both cold acclimation and ABA treatment induce a set of common polypeptides, suggesting that these proteins may be involved in development of freezing tolerance in *Arabidopsis thaliana*.

Materials and methods

Plant material and growth conditions

Seeds of *Arabidopsis thaliana* (L.) Heynh race Columbia (Rédei 1970) were surface-sterilized in 7.5% hypochlorite solution (containing 0.1% of Tween 20) and rinsed several times in sterile distilled water. The seeds were then planted in 24-well tissue culture plates, each well containing 1 ml of gelatine-solidified MS medium (Murashige and Skoog 1962; Flow laboratories), supplemented with 2% sucrose; the plates were then incubated in a growth chamber at 20 °C with a 16 h photoperiod. Abscisic acid (ABA) was first dissolved in 75% ethanol and then diluted with liquid MS-medium to obtain an aqueous stock solution. This ABA stock solution was added directly to each well to obtain the final concentration indicated and the growth was continued as above. Control plants were grown either as above (without ABA addition) or exposed to a low temperature treatment: 4 °C day/2 °C night, 14 h photoperiod, for times indicated.

Assay of freezing tolerance

To determine the degree of freezing tolerance, plants (without roots) were wrapped in Miracloth (Calbiochem) and placed in test tubes in a controlled freezing bath. Extracellular freezing of the plant material was initiated at −1.5 °C by touching the samples with a frosted wire. After a 1 h equilibration period the bath temperature was decreased by 2 °C/h. Samples were withdrawn at 1 °C intervals and thawed on ice overnight. Freezing damage was determined by measuring the electrolyte leakage, essentially as described by Sukumaran and Weiser (1972): Deionized water (20 ml) was added to each tube and the samples were extracted for 1 h at 25 °C. The conductivity of the resulting solution was measured. To obtain a value for 100% leakage, the samples were subsequently frozen by submerging them in liquid nitrogen followed by a re-extraction with the original solution for 1 h and by conductivity measurement. Plants showing 50% (LT_{50}) or more electrolyte leakage were considered killed. Visual determination of frost damage was performed by observing the loss of turgidity and the degree of water soaking in the thawed plantlets. The results from the visual tests were in agreement with the results obtained from electrolyte leakage measurements and were used for additional support to determine the LT_{50} values.

Radio-labelling and extraction of soluble proteins

In vivo labelling of plant proteins was performed (with plants growing on tissue culture plates) by adding [^{35}S]-methionine (1,000 Ci/mmol) directly to the growth medium (100 µCi/well) and incubating for a further 12 h. Plant samples (100–200 mg) were homogenized with a glass rod in microcentrifuge tubes on ice with 100 µl extraction buffer (50 m*M* Tris-HCl pH 7.2, 250 m*M* sucrose, 5 m*M* EDTA, 10 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 10 m*M* β-mercaptoethanol, 1 m*M* phenylmethylsulfonylfluoride, 30 µ*M* pepstatin, 50 µ*M* leupeptin, 15 µ*M* aprotinin). The insoluble material was removed by two centrifugations in a microcentrifuge (13,000 × g, 10 min). The supernatant was then treated with DNase and RNase (50 µg/ml) for 5 min on ice and the proteins were precipitated with 5 volumes of cold aceton containing 10 m*M* β-mercaptoethanol for 2 h at −20 °C. The protein precipitate was collected by centrifugation as above, dried and resuspended in 100 µl of O'Farrel lysis buffer [9.5 *M* urea, 5% β-mercaptoethanol, 2% Nonidet NP-40, 2% ampholytes; O'Farrell (1975)].

Electrophoretic analysis of proteins

The radio-labelled proteins were separated by two-dimensional electrophoresis essentially as described by O'Farrell (1975). First dimension equilibrium isoelectric focusing (IEF) was done in tubes with 1.5 mm ID containing a mixture of ampholytes (pH 5–7 1.6% and pH 3.5–10.0 0.4%) (LKB). Second dimension was run in SDS-polyacrylamide slab gels (Laemmli 1970) using a 10%–18% linear acrylamide gradient. For fluorography, coomassie blue stained gels were treated with enhancer (Enlightning, Du Pont), dried and exposed to X-ray films at −80 °C.

Results

Effect of ABA on frost tolerance

To test whether exogenous ABA could induce freezing tolerance in *Arabidopsis thaliana* and substitute for acclimation by low temperature treatment, ABA was added to the growth medium of axenically grown 10-day-old plantlets. Control plants were grown without ABA at 20 °C or at 4 °/2 °C (cold-acclimated control). As shown in Fig. 1, the increase in freezing tolerance in both ABA treated and low temperature-acclimated plants is already evident after 2 days, although the cold-acclimated plants seem to exhibit a somewhat slower rate of adaptation. After 4 days of hardening, the frost killing temperature (LT_{50}) had decreased to about −7 °C in both cases, after which the ABA treated plants showed no further increase in freezing tolerance. However, the cold-acclimated

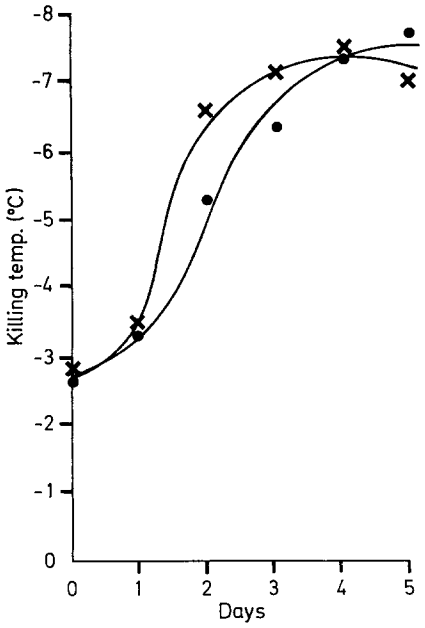

Fig. 1. Effect of low temperature acclimation and growth in the presence of exogenous ABA on freezing tolerance of *Arabidopsis thaliana*. At day 0, the plants were either transferred to cold acclimation conditions (•) or 15 mg/l ABA was added to the growth medium (×). Control plants were grown at 20 °C. At times indicated plant samples were withdrawn and their freezing tolerance was assayed by measuring the electrolyte leakage as described in Materials and methods

plants exhibited a further slow increase in tolerance to about −8 °C. The same increase in freezing tolerance was observed with plants subjected to simultaneous low temperature and ABA treatments (data not shown). The non-acclimated controls could not survive temperatures below −3 °C.

The degree of freezing tolerance induced by ABA treatment was dependent on ABA concentration. At concentrations below 2 mg/l, ABA had apparently no effect on frost-hardiness, whereas concentrations above 25 mg/l resulted in extensive wilting of the plantlets. Fifteen mg/l of ABA appeared to be optimal for hardening, and during the 5 days of ABA treatment the plantlets did not show any apparent differences to the control plants. However, after 6 days of ABA treatment, the first symptoms of wilting became evident. The cold-acclimated controls exhibited no visible changes except a slightly reduced rate of growth.

Effect of ABA on induction of proteins

We have previously shown that several new proteins are induced by low temperature treatments of *A. thaliana* (Kurkela et al. 1988). Seven cold-induced polypeptide species could be detected by one-dimensional SDS-polyacrylamide gel electrophoresis. To increase the resolution of this analysis, two-dimensional electrophoresis was employed with isoelectric focusing in the first dimension. The proteins were labelled in vivo by [^{35}S]-methionine, and the soluble protein fraction was isolated and subjected to electrophoretic analysis. The comparison between proteins from low temperature-acclimated and control plants indicated the presence of several changes in the protein patterns (Fig. 2A, B). We could reproducibly detect 11 low temperature-induced polypeptides (with apparent M_r values of 150, 2 × 80, 69, 58, 57, 45, 33, 32, 30 and 20 kD) (Fig. 2A, B; Table 1). These induced polypeptides appear to include six of those (150, 80, 69, 58, 45 and 30) detected earlier by one-dimensional gel electrophoresis. Several other proteins were produced in somewhat higher amounts in the low temperature-treated plants but were also present in substantial amounts in the control plants (data not shown).

Table 1. Induction of soluble proteins in *A. thaliana* seedlings following low temperature acclimation or ABA treatment

Apparent M_r of polypeptide	Control plants (20 °C)	Low temperature (4 °C/2 °C) treated plants	ABA (15 mg/l, 20 °C) treated plants
150	−[a]	+	+
2 × 80	−	+	+
69	−	+	−
58	−	+	+
57	−	+	−
45	−	+	+
36	−	−	+
33	−	+	−
32	−	+	+
30	(−)	+	+
27	−	−	+
21–22	−	−	+
20	−	+	+

[a] Only proteins which are induced, +, either by ABA treatment or cold acclimation or by both treatments are shown; (−) indicates that small amounts of protein are present in control plants

To elucidate the effect of ABA on induction of protein synthesis, ABA treated plants were in vivo-labelled and subjected to similar two-dimensional electrophoretic analysis. Growth of the plants in the presence of exogenous ABA resulted in induction of 15 new polypeptide species (Fig. 2C). A subset of eight of the ABA induced proteins (150, 2 × 80, 58, 45, 32, 30, 20 kD) was apparently also induced by low temperature treatment (Fig. 2B, C; Table 1). In addition to these common polypeptides, ABA treatment led to induction of several ABA-specific polypeptides (most notably those with apparent M_r of 36, 27, 21–22 kD) not present in cold-acclimated or control plants (Fig. 2C; Table 1). Similarly, three of the polypeptides appeared to be low temperature-specific, as they were not induced by ABA treatment (Fig. 2).

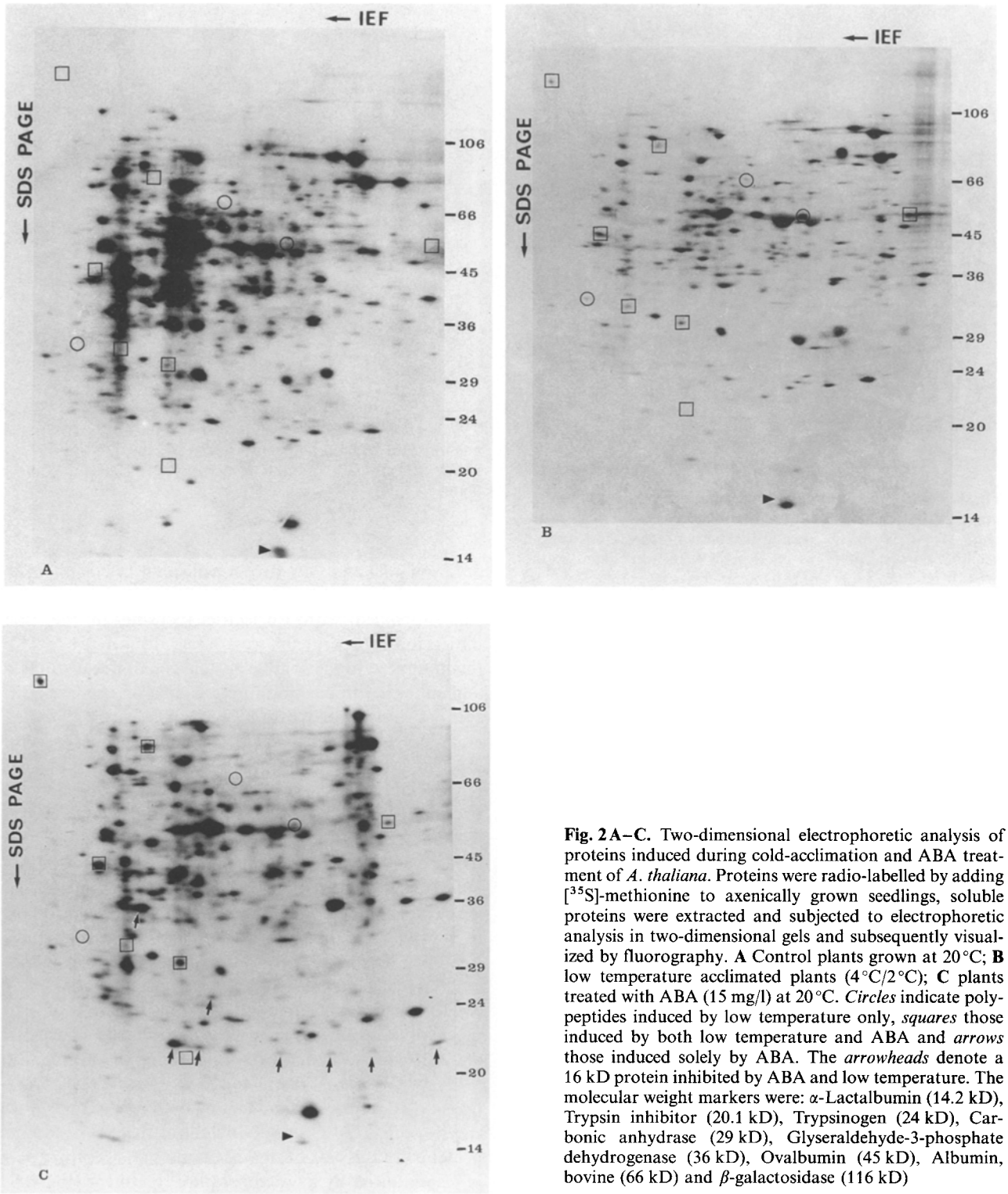

Fig. 2A–C. Two-dimensional electrophoretic analysis of proteins induced during cold-acclimation and ABA treatment of *A. thaliana*. Proteins were radio-labelled by adding [^{35}S]-methionine to axenically grown seedlings, soluble proteins were extracted and subjected to electrophoretic analysis in two-dimensional gels and subsequently visualized by fluorography. **A** Control plants grown at 20 °C; **B** low temperature acclimated plants (4 °C/2 °C); **C** plants treated with ABA (15 mg/l) at 20 °C. *Circles* indicate polypeptides induced by low temperature only, *squares* those induced by both low temperature and ABA and *arrows* those induced solely by ABA. The *arrowheads* denote a 16 kD protein inhibited by ABA and low temperature. The molecular weight markers were: α-Lactalbumin (14.2 kD), Trypsin inhibitor (20.1 kD), Trypsinogen (24 kD), Carbonic anhydrase (29 kD), Glyseraldehyde-3-phosphate dehydrogenase (36 kD), Ovalbumin (45 kD), Albumin, bovine (66 kD) and β-galactosidase (116 kD)

In addition to the induction of new polypeptides, both low temperature and ABA treatment resulted in reduced synthesis of several polypeptides present in non-acclimated controls. Most prominent was the repression of a 16 kD polypeptide caused by both low temperature and ABA treatments (Fig. 2A, B, C).

Discussion

Our results indicate that freezing tolerance in *Arabidopsis thaliana* can be induced by exogenously added ABA at a normal growth temperature (20 °C). Thus, ABA can substitute for the low temperature treatment in inducing

frost-hardiness in *A. thaliana*. The degree of freezing tolerance induced was about the same (LT_{50} −7 °C) with both treatments and comparable to that obtained in other herbaceous plants following low temperature acclimation (Chen et al. 1979; Guy and Haskell 1987).

In this study we have employed axenically grown whole plants. Due to the small size of *A. thaliana*, the plantlets could be grown in tissue culture plates in small amounts of growth media. This facilitates both the controlled application of ABA to the plants and in vivo labelling of plant proteins. Furthermore, the use of whole plants represents a more natural approach to study the effect of ABA on frost-hardiness. In studies employing cell cultures (Chen and Gusta 1983; Robertson et al. 1987, 1988; Johnson-Flanagan and Singh 1987), the putative effects caused by ABA-induced developmental, tissue-or organ-specific functions on freezing tolerance would have been omitted.

The degree of freezing tolerance obtained by ABA treatment was dependent on ABA concentration in the growth medium, 15 mg/l resulting in maximal tolerance. Similar values have been obtained previously with stem-cultured *S. commersonii* plants (Chen et al. 1983; Tseng and Li 1987). In contrast, more than ten times higher ABA concentration was required to obtain maximal frost-hardiness in suspension cultures of wheat, rye and bromegrass (Chen and Gusta 1983) as well as those of *Brassica napus* (Orr et al. 1986). This could reflect a difference in ABA uptake and/or metabolism between whole plants and cell suspension cultures, although we cannot rule out the possibility that the difference is due to different plant species used. Preliminary uptake studies in *A. thaliana* with [^{14}C] ABA indicate that the plantlets do indeed rapidly take up ABA applied to the growth medium (data not shown).

The frost-hardening process in *A. thaliana* appears to be rapid (Kurkela et al. 1988); a clear increase in freezing tolerance was obtained already after one day exposure to low temperature. Addition of exogenous ABA to the plants resulted in an even more rapid increase in freezing tolerance than that achieved with low temperature treatment. If indeed the accumulation of ABA in the plant tissues serves as an internal trigger for the cold-acclimation process (Chen et al. 1983), the uptake of exogenous ABA could result in more rapid accumulation of ABA, thereby explaining the observed differences. In contrast, in low temperature acclimation, the primary trigger is the shift in temperature that in turn would trigger the increase in the endogenous ABA concentration. Therefore, application of exogenous ABA would circumvent the first step in the acclimation process and result in enhanced rate of frost-hardening. The role of ABA as a trigger in the acclimation process is further substantiated by the results showing that the effects of low temperature and ABA on freezing tolerance are not additive. The same degree of tolerance was obtained with low temperature, ABA or low temperature + ABA treatments.

Low temperature treatment has been shown to correlate with induction of new polypeptides in *A. thaliana* (Kurkela et al. 1988) as well as in several other plant species that can be cold-hardened (Cloutier 1983, 1984; Guy et al. 1985; Meza-Basso et al. 1986; Guy and Haskell 1987). In *A. thaliana* we showed that synthesis of several new polypeptides was rapidly induced following exposure of the plants to low temperatures and persisted throughout the acclimation period (Kurkela et al. 1988). Furthermore, at least part of this induction was apparently at the transcriptional level. In this study we have extended the earlier work by employing two-dimensional gel electrophoresis. We could reproducibly detect 11 new cold-induced polypeptides that appeared to correlate with the onset of acclimation and that might be involved in the increased freezing tolerance. However, there is no a priori reason to believe that any particular induced polypeptide provides increased freezing tolerance to the plants.

For example, many of these polypeptides could be induced merely as a response to growth of plants at a lower temperature regime. As discussed, ABA can circumvent the low temperature requirement in frost-hardening. Therefore, proteins that provide increased freezing tolerance to the plants should be induced by both acclimation treatments. Comparison of the protein patterns obtained by two-dimensional gel electrophoresis following low temperature or ABA treatment suggested that a subset of the induced polypeptides was common to both treatments. Eight polypeptides were induced by both low temperature and exogenous ABA, and could serve as good candidates for proteins that are more directly involved in the frost-hardening process.

Common proteins induced by ABA and cold treatment in alfalfa (Robertson and Gusta 1986), bromegrass (Robertson et al. 1987, 1988) and in *Brassica napus* (Johnson-Flanagan and Singh 1987) have also been shown to correlate to increased frost-hardiness. Whether any of these proteins correspond to those induced in *A. thaliana* is not known.

Low temperature treatment also induced proteins which were not detected in ABA treated plants (Table 1). It therefore appears that these proteins are not involved in actual frost-hardening process but are rather part of an adaptation system for growth of the plants in a lower temperature regime. In addition, several ABA-specific proteins were detected (Table 1). As these proteins are not low temperature-induced, they probably represent proteins involved in other ABA-controlled processes.

Cold-acclimation also appears to result in reduction of synthesis of existing proteins (Guy et al. 1985; Meza-Basso et al. 1986). One of these proteins that exhibit

reduced synthesis during low temperature acclimation has been identified as the small subunit of D-ribulose-1,5-bisphosphate carboxylase in *Brassica napus* (Meza-Basso et al. 1986). Decreased synthesis of several polypeptides following either low temperature or ABA treatment was also evident in *A. thaliana*. The most prominent change was in the amount of a 16 kD polypeptide (Fig. 2). Whether these changes have a role in frost-hardening or merely reflect adaptation of the plant metabolism to slower growth is not clear. We cannot rule out the possibility that repression of some freezing sensitive protein could enhance the freezing tolerance of the plant.

In conclusion, ABA can substitute for low temperature acclimation and induce freezing tolerance in *Arabidopsis thaliana*. ABA appears to induce a subset of proteins also induced by low temperature treatment, suggesting that these proteins may have a direct role in the frost-hardening process. However, to confirm this, isolation of acclimation deficient mutants becomes necessary. This work is now in progress.

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