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Role of Abscisic Acid in Drought-Induced Freezing Tolerance, Cold Acclimation, and Accumulation of LTI78 and RAB18 Proteins in *Arabidopsis thaliana*[1]

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To study the role of abscisic acid (ABA) in development of freezing tolerance of *Arabidopsis thaliana*, we exposed wild-type plants, the ABA-insensitive mutant *abi1*, and the ABA-deficient mutant *aba-1* to low temperature (LT), exogenous ABA, and drought. Exposure of *A. thaliana* to drought stress resulted in a similar increase in freezing tolerance as achieved by ABA treatment or the initial stages of acclimation, suggesting overlapping responses to these environmental cues. ABA appears to be involved in both LT- and drought-induced freezing tolerance, since both ABA mutants were impaired in their responses to these stimuli. To correlate enhanced freezing tolerance with the presence of stress-specific proteins, we characterized the accumulation of RAB18 and LTI78 in two ecotypes, Landsberg erecta and Coimbra, and in the ABA mutants during stress response. LT- and drought-induced accumulation of RAB18 coincided with the increase in freezing tolerance and was blocked in the cold-acclimation-deficient ABA mutants. In contrast, LTI78 accumulated in all genotypes in response to LT and drought and was always present when the plants were freezing tolerant. This suggests that development of freezing tolerance in *A. thaliana* requires ABA-controlled processes in addition to ABA-independent factors.

Cold hardiness of temperate plants has been shown to be a genetically determined (Nilsson-Ehle, 1912) but complex phenomenon, composed of separable inherited traits (Palta and Simon, 1993). Some plant species are able to tolerate freezing stress by an adaptive process, cold acclimation, which is triggered by low, nonfreezing temperatures (Levitt, 1980). In addition to LTs, D has been reported to lead to enhanced freezing tolerance in winter wheat, rye, and spinach (Tyler et al., 1981; Cloutier and Siminovitch, 1982; Guy et al., 1992). The phytohormone ABA has been suggested to mediate the responses of plants to these environmental stimuli (Chen et al., 1983). The involvement of ABA in stress adaptation is supported by several lines of evidence: (a) endogenous ABA levels have been shown to increase during both LT (Daie and Campbell, 1981; Chen et al., 1983; Lalk and Dörffling, 1985; Guy and Haskell, 1988, Lång et al., 1994) and D (Wright, 1977; Guerrero and Mullet, 1986; Bray, 1988; Lång et al., 1994). (b) Plants develop freezing tolerance when treated with ABA under nonacclimating conditions (Chen and Gusta, 1983; Lång et al., 1989). (c) An ABA-deficient mutant (*aba-1*) (Koornneef et al., 1982) was shown to be impaired in cold acclimation and this defect could be complemented by addition of exogenous ABA (Heino et al., 1990). (c) Several of the proteins induced by LT or D are induced by exogenously added ABA (Cattivelli and Bartels, 1992; Palva, 1994).

A variety of physiological alterations, such as changes in carbohydrate, lipid, and protein composition, have been correlated with cold acclimation (Levitt, 1980; Sakai and Larcher, 1987), although a clear role for any of these changes in development of freezing tolerance is yet to be demonstrated. However, protein synthesis appears to be required for development of freezing tolerance (Chen et al., 1983), and accumulation of several distinct polypeptides has been observed during cold acclimation (Guy, 1990; Thomashow, 1990). Consequently, accumulation of these novel proteins has been suggested to play a role in the observed increase in freezing tolerance (Cloutier, 1984; Meza-Basso et al., 1986; Guy et al., 1987; Mohapatra et al., 1987; Kurkela et al., 1988; Gilmour et al., 1988; Lång et al., 1989; Perras and Sarhan, 1989).

As originally proposed by Weiser (1970) these acclimation-related alterations in protein profiles seem to occur at the level of gene expression. This has led to isolation and characterization of several of the corresponding genes from different plant species (Guy, 1990; Thomashow, 1990; Cattivelli and Bartels, 1992; Palva, 1994). Structural analysis of these genes has revealed that several of the corresponding LTI proteins (Gilmour et al., 1992; Guo et al., 1992; Houde et al., 1992; Lång and Palva, 1992; Neven et al., 1993; Ouellet et al., 1993; Wolfraim et al., 1993; Welin et al., 1994) share conserved motifs with the RAB/LEA/DHN-protein family (Close et al., 1989; Dure et al., 1989; Skriver and Mundy, 1990), a family of proteins synthesized during

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Abbreviations: CO-1, Coimbra; D, drought; LE, Landsberg erecta; LT, low temperature; LT_{50}, temperature at which 50% of plants died; LTI, low temperature induced; MBP, maltose-binding protein; RAB, responsive to ABA; ψ_w, total water potential.

seed maturation and in response to water stress in vegetative tissues. In addition to sequence similarity, these proteins also share other properties; they are hydrophilic and remain soluble during boiling in aqueous solution in contrast to the bulk of thermolabile noninduced proteins (Close et al., 1989; Jacobsen and Shaw, 1989; Lin et al., 1990). The RAB/LEA/DHN polypeptides are thought to play a role in tolerance to water stress. Relatedness of many of the LTI proteins to this family of proteins may suggest a protective function during freeze-induced cellular dehydration. Proteins from cold-hardened tissue have been tested in vitro in functional assays, and the results suggest a protective effect on thylakoid membranes or enzyme activities at subzero temperatures (Hincha et al., 1990; Lin and Thomashow, 1992). However, little is known about the majority of the induced proteins, and their in vivo function in freezing tolerance remains to be elucidated.

In this study we have characterized the role of ABA in development of freezing tolerance in *Arabidopsis thaliana*, triggered by different environmental stimuli (LT and D) and exogenous ABA. To accomplish this we assessed the freezing tolerance of two ecotypes of *A. thaliana*, LE and CO-1, an ABA-insensitive mutant (*abi1*) (Koornneef et al., 1984), and an ABA-deficient mutant (*aba-1*) (Koornneef et al., 1982). We further investigated whether the observed increase in freezing tolerance could be correlated with the accumulation of two stress-induced proteins, RAB18 and LTI78.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Plant material used in the experiments consisted of two ecotypes of *Arabidopsis thaliana* (L.) Heynh., LE, originating from Poland, and CO-1, originating from Coimbra, Portugal, as well as two ABA mutants in the LE background, an ABA-insensitive mutant *abi1* (Koornneef et al., 1984), and an ABA-deficient mutant *aba-1* (Koornneef et al., 1982). Seeds of the *abi1* mutant were germinated on filters moistened with MS-2 medium (Murashige and Skoog, 1962; Flow Laboratories) containing ABA (10 μM) to confirm that all germinated seedlings indeed harbored the *abi1* mutation. All *abi1* plant material presented in this paper originated from such selected seedlings.

Soil-grown plants were raised as described elsewhere (Lång et al., 1994). For axenic growth, *A. thaliana* plants (referred to as in vitro plants) were grown for 2 weeks on MS-2 medium in 24-well tissue culture plates as described previously (Lång et al., 1989). LT treatment (4/2°C day/night) and deacclimation (transfer to 22°C) of the soil-grown plants and plants grown in vitro were done as described previously (Lång et al., 1989, 1994). To facilitate application of ABA and ensure uniform water-stress conditions, only plant material grown in vitro was used for the exogenous ABA and D treatments. Application of exogenous ABA was carried out as described earlier (Lång et al., 1989) with a final concentration of 60 μM in the medium. The plants were exposed to water stress by removing the lids of the tissue culture plates in a growth cabinet, thus lowering the RH from >80 to 70%. ψ_w of the plants was measured as described previously (Lång et al., 1994).

Determination of Freezing Tolerance

Freezing tolerance of soil-grown *A. thaliana* was estimated by scoring the extent of the irreversible damage to rosette leaves caused by exposure to a subzero temperature cycle with a minimum of −10°C. Temperature was first lowered (2°C/h) from the cold-acclimation temperature of 4°C to −3°C. To initiate extracellular ice nucleation the plants were misted with 0°C tap water and kept at −3°C for 1 h. The temperature was subsequently lowered (2°C/h) to −10°C, held constant for 3 h, and then elevated (2°C/h) back to 4°C. The state of the plants was evaluated visually after 2 d of recovery at room temperature, which was determined to be the best time for scoring, because the irreversible damage was clear and no significant regrowth had occurred.

Freezing tolerance of plants grown in vitro was determined by controlled freezing of excised plants (Lång et al., 1989), and the extent of freezing injuries was determined both visually and by ion leakage (Sukumaran and Weiser, 1972).

Production of Antibodies

Partial cDNA for *rab18* (Lång and Palva, 1992) lacking 90 bp of the coding region from the 5′ end was ligated into the expression vector pMAL-p (New England Biolabs, Inc., Beverly, MA). The truncated RAB18 polypeptide was produced as part of a hybrid protein with MBP in *Escherichia coli* and transported to the periplasm of the bacteria. Partial cDNA (620 bp) of the *lti78* gene (Nordin et al., 1991, 1993), corresponding to the 3′ end of the coding region and encoding a hydrophilic domain of the LTI78 polypeptide, was ligated into the expression vector pMAL-c. The fragment expressed in pMAL-c produced a hybrid protein located in the cytosol of *E. coli*. Purification of the MBP-RAB18 and MBP-LTI78 hybrid proteins was achieved by affinity chromatography of periplasmic and cytosolic fractions, respectively, on an amylose resin bed as described by the manufacturer (New England Biolabs). Pooled fractions containing the hybrid protein were concentrated with dry dialysis in PEG 6000 (Fluka Chemie AG, Buchs, Switzerland). Separation of degradation products from intact hybrid protein was accomplished by preparative SDS-PAGE on 12% polyacrylamide gel. The band representing intact hybrid protein was excised from the gel, cut into small pieces, and washed in PBS, pH 7.5, for 10 min prior to homogenization and powderization in liquid N_2. The fine powder was then lyophilized and, after solvation, injected intramuscularly into rabbits to produce antisera against the hybrid proteins.

Immunoblots

Plant tissue samples (leaves and stems but not roots) from plants grown in vitro were immediately frozen in liquid N_2 after harvesting. Proteins were extracted from 150-mg (fresh weight) samples by homogenization on ice in

150 μL of cold protein extraction buffer (50 mM Tris·Cl, pH 7.2, 250 mM Suc, 5 mM EDTA, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1 mM PMSF, 30 μM pepstatin, 50 μM leupeptin). The homogenate was clarified twice by centrifugation in a microfuge at 13,000 rpm for 10 min at 4°C, and the protein concentration of the supernatant was determined by the dye-binding assay of Bradford (1976). Protein samples (50 μg) were separated by SDS-PAGE on 8 to 18% polyacrylamide gradient gels (Laemmli, 1970). The separated polypeptides were electroblotted from gels onto 0.2-μm nitrocellulose membranes (Schleicher & Schuell) for 4 h at 0.4 mA or overnight at 0.2 mA. Prior to blocking, the membranes were stained with 0.5% Poinceau Red in 5% acetic acid to monitor successful transfer and to verify that all lanes had equal amounts of protein. The membranes were then cut in two at the 40-kD region, and the upper part was used for detection of LTI78 protein and the lower part was used for RAB18 detection with the respective antisera. Anti-rabbit IgG-alkaline phosphatase conjugate (Promega, Madison, WI) served as a secondary antibody and 4-nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma) were used as substrates for color development.

RESULTS

D Stress Results in Increased Freezing Tolerance of *A. thaliana*

Since both freezing and D tolerance appear to involve a common component, tolerance to cellular dehydration, it is conceivable that even the stimuli that induce these stress responses overlap. To test this hypothesis plants grown in vitro were exposed to progressive D stress (70% RH) for the times indicated in Figure 1 and their freezing tolerance was examined (Fig. 1A). ψ_w of wild type (LE), *abi1*, and *aba-1* were measured during the course of treatment. After 1 d of treatment the ψ_w had decreased from about −0.5 to −0.91, −1.04, and −1.24 MPa and on the 2nd d of treatment to −1.50, −2.7, and −3.5 MPa, respectively (Lång et al., 1994), indicating drastic changes in water status of the ABA mutants. Comparison of D-induced freezing tolerance to that obtained by ABA and the early stages of LT treatment (Fig. 1, B and C) of the ecotypes LE and CO-1 showed that all three treatments seemed to confer comparable levels of freezing tolerance (down to −7°C) to *A. thaliana* grown in vitro.

ABA Is Required for the Development of Full Freezing Tolerance

ABA has been implicated as a signal molecule in plant responses to different environmental stimuli (Chen et al., 1983). To assess the significance of ABA in development of freezing tolerance triggered by LT and D, we determined the freezing tolerance of the ABA-insensitive (*abi1*) and the ABA-deficient (*aba-1*) mutants. LTI freezing tolerance was assessed visually for plants grown in vitro (Fig. 1B) and by ion-leakage measurement (data not shown) and for the soil-grown plants by visual assessment of irreversible damage to rosette leaves. Our results indicate both temporal and quantitative differences in LTI cold acclimation between the genotypes of *A. thaliana*. The ABA-insensitive mutant *abi1* was impaired in development of freezing tolerance compared to the wild type (LE). The *abi1* mutant showed a delayed cold-acclimation phenotype (Fig. 1B) with gradually increasing freezing tolerance but lacked the early rapid increase in tolerance, characteristic of the wild type (Fig. 1B). Furthermore, *abi1* plants never quite reached the same level of tolerance as did wild-type plants during the acclimation period studied.

As previously reported (Heino et al., 1990; Gilmour and Thomashow, 1991) the ABA-deficient mutant *aba-1* was

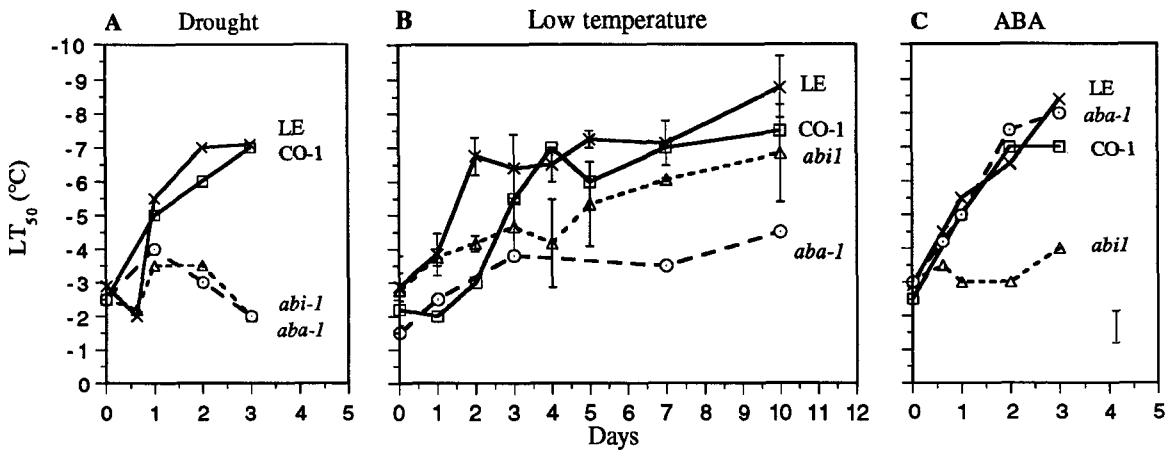

Figure 1. Induction of freezing tolerance of in vitro grown *A. thaliana* ecotypes LE and CO-1 and the ABA mutants *abi1* and *aba-1* by D (A), LT (B), and ABA (C) treatments. The killing temperatures (LT_{50}) obtained derive from visual estimations of excised plants exposed to freezing temperatures in a controlled temperature bath. X, LE (12 plants/point in A and C, 48 plants/point in B [average of four different frost tests]); △, *abi1* (12 plants/point in A and C, 36 plants/point in B [average of three different frost tests]); ⊙, *aba-1* (12 plants/point in A, B, and C); □, CO-1 (12 plants/point in A, B, and C). For the sake f clarity the SE is shown for only ecotype LE and the *abi1* mutant in B.

severely affected in development of LTI freezing tolerance (Fig. 1); the *aba-1* plants were practically unable to survive −4°C. Similar results confirming the observed differences in tolerance between the genotypes were obtained from cold-acclimation studies in soil-grown plants (data not shown).

Exposure of plants grown in vitro to water stress (70% RH) resulted in enhanced freezing tolerance of both ecotypes (LE and CO-1) but not of the ABA mutants *abi1* and *aba-1* (Fig. 1A). This suggests that ABA may be involved in D-induced freezing tolerance. In accordance with a previous report by Koornneef et al. (1989), both ABA mutants were more sensitive to water stress than either of the wild types (see ψ_w values above); the wild types showed first signs of water stress on the 3rd d of treatment when ABA mutants appeared to be already wilted.

Since freezing tolerance can be induced directly by exogenous ABA (Fig. 1C; Lång et al., 1989), we wanted to test whether the *abi1* mutation would define a component in this ABA-response pathway. To study this we compared freezing tolerance of the *abi1* mutant to that of the wild type following exogenous ABA application (Fig. 1C). The results showed that *abi1* plants were impaired in development of freezing tolerance, in contrast to the wild-type and the *aba-1* mutant plants, which showed a clear increase in tolerance in response to exogenous ABA (Fig. 1C).

A Southern Ecotype of *A. thaliana* Exhibits Delayed Cold-Acclimation Response

To identify components of the cold-acclimation pathway in *A. thaliana* we screened for ecotypes that would show a differential response to LT acclimation. Based on preliminary screenings of several ecotypes of *A. thaliana* from different geographical regions, the ecotype CO-1 from Portugal was identified as a possible candidate showing impaired cold acclimation. Determination of freezing tolerance after LT treatment of in vitro plants revealed a somewhat slower cold acclimation in the ecotype CO-1 compared to the rapid acclimation response of the LE ecotype (Fig. 1B). A similar delay in acclimation of CO-1 was also observed in soil-grown plants (data not shown). Differences in development of freezing tolerance were most marked during the LT response. Tolerance of both LE and CO-1 ecotypes appeared to develop similarly in response to both mild D stress and ABA application (Fig. 1, A and C).

Accumulation of RAB18 Correlates Temporally with Freezing Tolerance

Determination of freezing tolerance induced by three treatments (D, LT, and ABA) revealed differential development of tolerance in the four genotypes tested (LE, CO-1, *abi1*, *aba-1*). This spectrum of different tolerance levels provided us with a useful system for attempts to correlate accumulation of LT-responsive proteins to freezing tolerance of *A. thaliana*. For this purpose we raised antisera against two LTI proteins, RAB18 and LTI78, for which the genes have been previously characterized and their expression analyzed at the mRNA level (Nordin et al., 1991, 1993; Lång and Palva, 1992). The regulation of the *rab18* gene has been shown to be ABA mediated in vegetative tissue, with transcripts abundantly accumulating in wild-type plants during ABA treatment and D and to a lesser extent at LT but being almost undetectable in ABA mutants (Lång and Palva, 1992; Lång et al., 1994). Exposure of plants to LT resulted in weak but detectable amounts of the RAB18 polypeptide in both LE and CO-1 ecotypes (Fig. 2), followed by a slight decrease after 1 d of deacclimation (data not shown). The effect of deacclimation on RAB18 levels was studied in a separate experiment, which confirmed the slow decrease in the amount of this protein after transfer to normal growth temperatures (Table I). We could not detect any clear-cut difference in the accumulation of RAB18 between LE and CO-1 during the first days of cold acclimation because of the weakness of the signal (Fig. 2). Both ABA mutants were defective in RAB18 induction. In the *abi1* mutant, accumulation of this protein was delayed, showing a weak increase in RAB18 amounts above the background levels after 5 and 7 d of treatment, whereas in the ABA-deficient mutant *aba-1* no RAB18 was detected at all during LT treatment (Fig. 2).

Application of exogenous ABA resulted in a massive accumulation of the RAB18 protein in both ecotypes LE and CO-1, as well as in the *aba-1* mutant (Fig. 2). The *aba-1* mutant seemed to accumulate RAB18 even faster and to a higher level than did LE, which is in accordance with the increased levels of *rab18* transcripts observed in this mutant during ABA treatment (Lång et al., 1994). As expected, the *abi1* mutant did not respond significantly to exogenous ABA, although some accumulation of RAB18 was seen on the 3rd d of treatment (Fig. 2), indicating that the mutation may be somewhat leaky to prolonged exposure to high levels of ABA.

Under conditions of progressive D stress (70% RH, Fig. 2) RAB18 accumulated to high levels in both ecotypes LE and CO-1. No trace of the protein could be seen in the *aba-1*

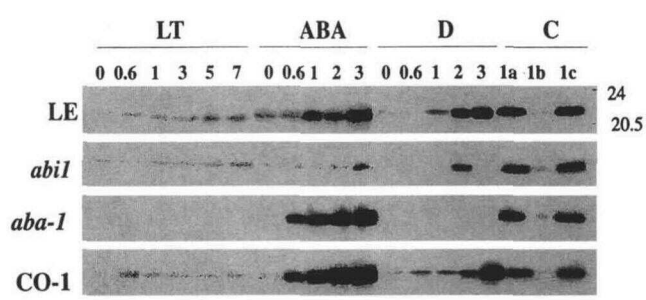

Figure 2. Immunological detection of RAB18 accumulation in in vitro grown *A. thaliana* ecotypes LE and CO-1 and the ABA mutants *abi1* and *aba-1* during LT, D, and ABA treatments. Plants were exposed to LT, D (70% RH), and exogenous ABA (60 μM final concentration of ABA in media) for the times indicated (days). The control lanes (C) consist of the samples from 1-d ABA-treated LE (1a), *abi1* (1b), and *aba-1* (1c). Equal amounts of protein (50 μg) from plant extracts were loaded on 8 to 18% polyacrylamide gradient gels, separated by SDS-PAGE, and transferred to nitrocellulose membranes. The 22-kD RAB18 protein was detected with RAB18 antisera as described in "Materials and Methods."

Table I. *The effect of deacclimation on levels of RAB18 and LTI78 proteins in A. thaliana, ecotype LE grown in vitro*

LT_{50} of in vitro plants was determined after 5 d of LT treatment and subsequent deacclimation for 1 and 3 d. The strength of the signal of the respective proteins was evaluated by scanning bands from an immunoblot (data not shown). In the case of LTI78 the uppermost band (140 kD) was chosen for this purpose. The area under the densitogram peaks was cut out and weighed to get a value of the intensity of the band. The band intensity of the respective band from 5-d LT-treated LE ecotype was given the arbitrary value of 1.00.

Protein	Treatment			
	LT (0)[a]	LT (5)[b]	DA (1)[c]	DA (3)[d]
LTI78	0.00	1.00	0.47	0.27
RAB18	0.12	1.00	n.d.[e]	0.91
LT_{50}	−2.5°C	−6.5°C	−2.5°C	−2.5°C

[a] 22°C day/night. [b] Five days at 4°C/2°C day/night. [c] DA, Deacclimation; 1 d at 22°C after 5-d LT treatment at 4°C/2°C day/night. [d] Three days at 22°C after 5-d LT treatment at 4°C/2°C day/night. [e] n.d., Not determined.

mutant, whereas *abi1* showed a weak and transient accumulation of RAB18 on the 2nd d of treatment (Fig. 2). In conclusion, accumulation of RAB18 protein appears to be mediated by ABA, which is in accordance with what is known about expression of the gene at the transcriptional level (Lång and Palva, 1992).

To correlate accumulation of RAB18 protein in *A. thaliana* with increase in freezing tolerance, data from selected times of freezing tolerance assays and western analysis (Figs. 1 and 2) are summarized in Table II and from a separate experiment in Table I. LTI accumulation of RAB18 seemed to precede the development of freezing tolerance. The level of this protein decreased during deacclimation, although not as abruptly as the decrease in freezing tolerance (Table I). Comparison of levels of RAB18 and freezing tolerance reveal that plants can become freezing tolerant without large amounts of RAB18, as was evident from the LT treatment (Table II). The strong increase in RAB18 during D stress probably reflects the high levels of ABA reported (Bray, 1988; Lång et al., 1994). However, comparison of the temporal pattern of RAB18 accumulation and development of freezing tolerance shows that this accumulation, whether strong or weak, seemed to coincide with the onset of freezing tolerance development.

LTI78 Is Present in Freezing-Tolerant Plants

Immunodetection with LTI78 antisera showed induction of a distinct set of bands (Fig. 3), ranging from 80 to 140 kD in plants exposed to LT, D and ABA. A similar pattern was seen in soil-grown plants at LT (data not shown). These bands represent polypeptides with molecular masses between the size of the LTI78 polypeptide predicted from the genomic sequence of the *lti78* gene (78 kD) (Nordin et al., 1993) and the size of the in vitro translation product of the gene as estimated from SDS-PAGE, 140 or 160 kD, respectively (Nordin et al., 1991; Horvath et al., 1993).

A strong increase in the anti-LTI78 reactive bands was observed in response to LT in all four genotypes (Fig. 3). A clear decrease in LTI78 levels was evident after 1 and 3 d of deacclimation in the LE ecotype (Table I). LTI78 also accumulated in response to ABA and D stress but considerably less than at LT. The D-induced accumulation of LTI78 was similar in LE and CO-1, somewhat weaker in *abi1*, and much weaker in the *aba-1* mutant, suggesting that part of the D induction of this protein could be ABA mediated (Nordin et al., 1991). The decrease of LTI78 in *abi1* on the 3rd d of D treatment (Fig. 3) is presumably due to loss of soluble proteins observed in the wilting plants.

LTI78 was clearly accumulating in response to LT in an ABA-independent fashion but was also somewhat responsive to ABA and D stress. Comparison of the amounts of LTI78 with freezing tolerance of the wild-type plants during LTI acclimation and deacclimation (Table II, Fig. 3) reveals that LTI78 levels do correlate with the tolerance. However, as shown by the mutant studies, LT treatment resulted in accumulation of LTI78 in all genotypes, regardless of their capacity to develop freezing tolerance (Table II, Fig. 1). The protein was present in as high amounts in the

Table II. *Freezing tolerance of A. thaliana plants grown in vitro compared with RAB18 and LTI78 protein levels at normal growth temperatures and during D, LT, and ABA treatments.*

Relative amount of protein is indicated by estimation from Figures 2 and 3 on a scale from − (no protein detected) to +++ (maximal accumulation of protein). Freezing tolerance (FT) is presented as LT_{50} values from Figure 1.

Mutant or Ecotype	Treatment											
	D[a]			LT[b]			ABA[c]			NGT[d]		
	RAB18	LTI78	FT	RAB18	LTI78	FT	RAB18	LTI78	FT	RAB18	LTI78	FT
LE	++	+	−7.0	+	+++	−7.4	++	+	−6.5	−	−	−2.9
abi1	±	+	−3.5	±	+++	−4.1	±	+	−3.0	−	−	−3.0
aba-1	−	−	−3.0	−	+++	−3.8	+++	+	−7.5	−	−	−3.0
CO-1	+	+	−6.0	+	+++	−6.3	+++	+	−7.0	−	−	−2.5

[a] Two days at 22°C with 70% RH. [b] Three days at 4°C/2°C day/night. [c] Two days at 22°C with 60 μM ABA. [d] NGT, Normal growth temperatures; grown at 22°C day/night.

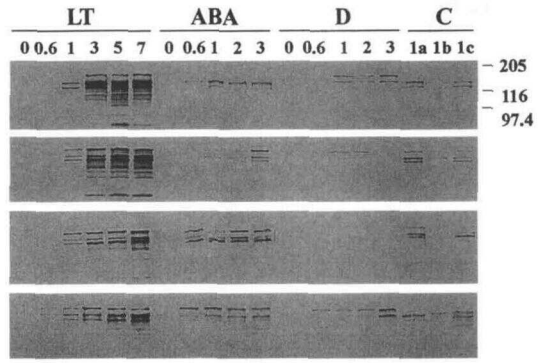

Figure 3. Immunological detection of LTI78 accumulation in in vitro grown *A. thaliana* ecotypes LE and CO-1 and the ABA mutants *abi1* and *aba-1* during LT, D, and ABA treatments. Plants were exposed to LT, mild water stress (70% RH), and exogenous ABA (60 μM final concentration in media) for the times indicated (days). The control lanes (C) consist of the samples from 1-d ABA-treated LE (1a), *abi1* (1b), and *aba-1* (1c). Equal amounts of protein (50 μg) from plant extracts were loaded on 8 to 18% polyacrylamide gradient gels, separated by SDS-PAGE, and transferred to nitrocellulose membranes. The 140-kD LTI78 protein was detected with LTI78 antisera as described in "Materials and Methods."

cold-acclimation-impaired ABA mutants as in the wild types at LT. Nevertheless, these data may suggest that the LTI78 protein represents a component of an ABA-independent part of the acclimation process. We conclude that LTI78 alone is not sufficient to confer freezing tolerance but, on the other hand, is always present when plants have become freezing tolerant.

DISCUSSION

Mild D stress has been reported to increase freezing tolerance in some plant species, such as spinach (Guy et al., 1992) and rye (Cloutier and Siminovitch, 1982). To test whether this is a general phenomenon in herbaceous plants, we determined freezing tolerance of *A. thaliana* in response to D treatment. The results show that *A. thaliana* does indeed develop freezing tolerance when exposed to D stress. The extent of freezing tolerance achieved by exposure to D was of similar magnitude as observed in LT- and ABA-treated plants (Fig. 1). D-induced freezing tolerance is probably not a result of decreased ice formation due to lower water content in the stressed tissue, since fully rehydrated plants have been shown to retain their tolerance (Guy et al., 1992); rather, these data suggest that both LT and water stress induce overlapping adaptive responses. Freezing causes formation of ice crystals in extracellular spaces of plant tissue, resulting in cellular dehydration (Levitt, 1980; Sakai and Larcher, 1987). Our results bring forth the similarity in the adaptive mechanisms toward freezing and water stress in plants and can be viewed as supporting evidence for the notion that freeze-induced dehydration is a major stress to the plant at subzero temperatures (Levitt, 1980). The overlapping nature of these two stress adaptations is further supported by the apparently common role of ABA in mediating these responses. Failure of the ABA mutants to fully increase their freezing tolerance in response to D stress suggests that ABA is indeed involved in D-induced freezing tolerance. However, both *aba-1* and *abi1* mutants are known to be more sensitive to dehydration than wild type, because the mutations also affect stomatal closure (Koornneef et al., 1989). Thus, the possibility that the wilting sensitivity of the ABA mutants may hamper their ability to respond like wild-type plants cannot be excluded.

Involvement of ABA in the LTI freezing tolerance was evident from the mutant data (Fig. 1B), because both *abi1* and *aba-1* mutants were clearly impaired in cold acclimation. The results (Fig. 1, A and B) suggest that ABA-controlled processes are required for development of both LT- and D-induced freezing tolerance. The mutant studies indicate that the ABA-insensitive mutant *abi1* is defective in this response. Similarity in freezing tolerance of LT-treated soil-grown plants (data not shown) and plants grown in vitro confirms that the same response mechanisms to LT are operational in both growth systems.

Our results showing that the *aba-1* mutant is impaired in acclimation are in accordance with previous reports (Heino et al., 1990; Gilmour and Thomashow, 1991). However, our results demonstrating that the *abi1* mutant is deficient in cold acclimation differ from those of Gilmour and Thomashow (1991), who found no difference in the development of freezing tolerance of the *abi1* and LE wild type. The reason for this discrepancy is unclear. One possible explanation could be that we found the mutant to be somewhat unstable; unless the seeds were germinated in the presence of ABA, we did occasionally observe ABA-sensitive seedlings. We recently provided further evidence for the role of ABA in both D-induced and LTI freezing tolerance in *A. thaliana* (Lång et al., 1994). Endogenous levels of ABA were shown to increase in response to both stimuli. The LTI increase of ABA was only transient in wild-type LE, whereas in the *abi1* mutant ABA remained at the elevated level throughout the LT treatment (Lång et al., 1994). As suggested by the data presented in Figures 1 and 2 the *abi1* mutant seems to be somewhat responsive to ABA. Thus, the increased ABA levels in this mutant following LT exposure, together with the proposed leakiness of the mutant, may explain the increase in RAB18 levels seen after 5 and 7 d of LT treatment. The *aba-1* mutant shows enhanced sensitivity to ABA, with higher levels and faster accumulation of both *rab18* mRNA (Lång et al., 1994) and protein (Fig. 2) in the ABA treatment, compared to LE wild type.

Since LTI proteins are thought to carry out the biochemical functions associated with enhanced freezing tolerance, the presence of these proteins should correlate with the tolerance. To facilitate such analysis we characterized a southern ecotype of *A. thaliana*, CO-1, which exhibited a delayed acclimation phenotype. Furthermore, the freezing tolerance achieved was somewhat less than that of the LE ecotype. This CO-1 ecotype should be useful in studies of molecular mechanisms of freezing tolerance, since it does not suffer from possible complications of pleiotropic mutations. Comparison of LE and CO-1 ecotypes suggested that the observed differences in freezing tolerance seem

to correlate with differences in RAB18 accumulation, although this analysis was hampered by the weakness of RAB18 signal in immunoblots of LT-treated plants. We did not detect any clear-cut differences in levels of LTI78 between the ecotypes LE and CO-1, although they differ in the onset of freezing tolerance. The temporal pattern of RAB18 accumulation (LE and CO-1 versus the ABA mutants) correlates with the induction of freezing tolerance in *A. thaliana*. The comparisons presented in Tables I and II also reveal that *A. thaliana* appears to become freezing tolerant at LT, although only modest accumulation of RAB18 is evident, whereas D stress results in high levels of accumulation of the protein. On the other hand, low levels of RAB18 might be sufficient for effective cold acclimation. This difference in RAB18 levels most likely reflects the differences in endogenous levels of ABA under these conditions (Lång et al., 1994) and may suggest that the primary function of RAB18 is in tolerance to cellular dehydration caused by water stress rather than direct involvement in freezing tolerance.

LTI78 was shown to accumulate at LT in an ABA-independent fashion, since it was also accumulating strongly in both ABA mutants. This protein was present in much lower amounts in plants exposed to D or exogenous ABA. Thus, LTI78 levels do not reflect endogenous ABA levels or the water status of the cell, since the largest increase in LTI78 was observed at LT, where ABA levels are only transiently increased and ψ_w is much less affected than during D stress (Lång et al., 1994). The rapid and strong induction of LTI78 protein synthesis (Fig. 3) as well as *lti78* gene expression (Nordin et al., 1993) suggests an important function in adaptation to LT. Lowering of temperature alone affects the biochemistry and physiology of the cell, including activities of important enzymes (Cattivelli and Bartels, 1992) and membrane fluidity (Sakai and Larcher, 1987). It can be speculated that vital enzymes or proteins may need protection to function properly at low but nonfreezing temperatures prior to any cellular dehydration. LTI78 could be involved in this aspect of the cold-acclimation process. The presence of LTI78 alone appears not to be sufficient to confer freezing tolerance to plants, but, on the other hand, LTI78 is always present when plants have become freezing tolerant.

The discrepancy between the size deduced from the DNA sequence (78 kD) and the observed molecular mass of 140/160 kD, together with the complex pattern of anti-LTI78 reactive bands in the western filters, suggests post-translational modification of the LTI78 polypeptide. Alternatively, at least some of the bands may derive from proteolysis.

The results presented in this paper demonstrate that ABA-insensitive and -deficient mutants are defective in both LTI and D-induced cold acclimation, indicating that ABA-controlled processes are required for acquisition of full freezing tolerance in plants. We further suggest that freezing tolerance in *A. thaliana* is dependent on ABA controlled factors, like RAB proteins, in addition to ABA-independent processes such as synthesis of LTI78 and related proteins.

In future studies we will examine the function of RAB18 and LTI78 by overexpressing or blocking expression of these genes in plants with sense/antisense constructions along with localization of the proteins in the cell.

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