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A freezing-sensitive mutant of *Arabidopsis*, *frs1*, is a new *aba3* allele

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Abstract. To investigate the molecular mechanisms controlling the process of cold acclimation and to identify genes involved in plant freezing tolerance, mutations that impaired the cold acclimation capability of *Arabidopsis thaliana* (L.) Heynh. were screened for. A new mutation, *frs1* (*freezing sensitive 1*), that reduced both the constitutive freezing tolerance as well as the freezing tolerance of *Arabidopsis* after cold acclimation was characterized. This mutation also produced a wilty phenotype and excessive water loss. Plants with the *frs1* mutation recovered their wild-type phenotype, their capability to tolerate freezing temperatures and their capability to retain water after an exogenous abscisic acid (ABA) treatment. Measurements of ABA revealed that *frs1* mutants were ABA deficient, and complementation tests indicated that *frs1* mutation was a new allele of the *ABA3* locus showing that a mutation in this locus leads to an impairment of freezing tolerance. These results constitute the first report showing that a mutation in *ABA3* leads to an impairment of freezing tolerance, and not only strengthen the conclusion that ABA is required for full development of freezing tolerance in cold-acclimated plants, but also demonstrate that ABA mediates the constitutive freezing tolerance of *Arabidopsis*. Gene expression in *frs1* mutants was altered in response to dehydration, suggesting that freezing tolerance in *Arabidopsis* depends on ABA-regulated proteins that allow plants to survive the challenges imposed by subzero temperatures, mainly freeze-induced cellular dehydration.

Key words: Abscisic acid – *Arabidopsis* (mutant *frs1*) – Cold acclimation – Dehydration – Freezing tolerance – *frs1* mutant

Introduction

Freezing temperatures are one of the most important environmental constraints limiting the geographical distribution and productivity of agricultural crops (Levitt 1980). In fact, modest increases (1–2 °C) in the freezing tolerance of crop species would have a dramatic impact on agricultural production (Steponkus et al. 1998). Plants from temperate regions survive freezing temperatures ranging from −5 °C to −30 °C depending on the species. In general, the maximum freezing tolerance of these plants is induced in response to low non-freezing temperatures (0–10 °C), an adaptive process known as cold acclimation (Levitt 1980; Guy 1990).

The process of cold acclimation is very complex and involves numerous biochemical and physiological changes, including increased levels of sugars, soluble proteins, proline, and organic acids, the appearance of new isoforms of proteins, and alterations in lipid membrane composition (Hughes and Dunn 1996). Since most of these changes are presumed to be regulated at the gene expression level, during recent years much effort has been made to identify genes, in both monocotyledonous and dicotyledonous species, whose expression is induced in response to low temperatures (for reviews, see Hughes and Dunn 1996; Thomashow 1999). However, only a few of these genes have been shown to have a role in cold acclimation and freezing tolerance (Artus et al. 1996; Jaglo-Ottosen et al. 1998; Liu et al. 1998). In fact, looking for low-temperature-inducible genes is likely to identify genes not directly involved in cold acclimation but related to other adaptive processes occurring simultaneously (Jarillo et al. 1993; Leyva et al. 1995). Furthermore, components of cold acclimation and freezing tolerance that are constitutively active

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Abbreviations: ABA = abscisic acid; DMSO = dimethylsulfoxide; LT_{50} = low temperature at which plant survival percentage is 50%

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or involve a decrease in gene expression are difficult to uncover. A method that can circumvent many of these limitations is the classical mutant analysis. By using this genetical approach, it may be possible to identify most genes involved in cold acclimation and freezing tolerance. Moreover, the phenotypes shown by the mutants can suggest specific functions for the cognate genes.

Recently, various *Arabidopsis* mutants have been identified which show an altered response to cold acclimation and freezing tolerance. Warren et al. (1996) isolated seven nonallelic sensitive-to-freezing (*sfr*) mutants, which only acquired partial freezing tolerance after cold acclimation. The *sfr* mutations were recessive or co-dominant (in one case), consistent with the expectation that they should be loss-of-function mutations. A preliminary characterization (McKown et al. 1996) revealed that four *sfr* mutations, *sfr3*, −4, −6 and −7 reduced or blocked anthocyanin accumulation during cold acclimation. Moreover, *sfr4* prevented the cold-induced increase in sucrose and glucose levels, and both *sfr4* and *sfr7* altered fatty acid composition after cold acclimation. On the other hand, Xin and Browse (1998) identified a series of *Arabidopsis* mutant lines that were constitutively freezing tolerant in the absence of cold acclimation. Allelism tests identified mutations in at least six different loci. The characterization of one of these mutants, named *eskimo1* (*esk1*), revealed that it was produced by a single recessive mutation. The *esk1* mutant accumulated high levels of proline, but did not exhibit constitutive increased expression of cold-regulated genes. Ishitani et al. (1998) isolated a mutant of *Arabidopsis*, designated as *hos1* (for high expression of osmotically responsive genes), that showed reduced freezing tolerance compared to wild-type plants when non-acclimated. The *hos1* mutation was recessive, and caused overexpression of several cold-responsive genes, early flowering, and constitutive vernalization. Finally, Lee et al. (1999) have reported the characterization of a genetic locus in *Arabidopsis*, *HOS2*, which was defined by three mutants (*hos2-1*, *hos2-2*, *hos2-3*). The *hos2* mutations were recessive and resulted in enhanced expression of different low-temperature-regulated genes. However, when compared with wild-type plants, the *hos2* mutants were defective in developing freezing tolerance during cold acclimation.

In order to identify new genes whose products are required in cold acclimation and freezing tolerance, we isolated a series of freezing-sensitive (*frs*) *Arabidopsis* mutants that were impaired in their capability to tolerate freezing temperatures after being cold-acclimated. Here, we report the characterization of one mutant line (*frs1*) that also showed a reduced constitutive freezing tolerance and a wilty phenotype. Abscisic acid (ABA) measurements demonstrated that *frs1* mutants were ABA deficient, and complementation tests revealed that the *frs1* mutation corresponded to a new allele of the *ABA3* locus. In addition, we investigated whether the decreased freezing tolerance of *frs1* could be correlated with low-temperature- and/or drought-regulated gene expression. Based on all these data, the involvement of ABA in cold acclimation and freezing tolerance is discussed.

Materials and methods

Plant material, growth conditions and treatments

Arabidopsis thaliana (L.) Heynh., ecotype Landsberg *erecta* (L*er*) and the ABA-deficient (*aba*) and -insensitive (*abi*) mutants *aba1-1*, *aba3-2* and *abi1-1* were kindly provided by Maarteen Koornneef (Wageningen Agricultural University, The Netherlands). The ecotype Columbia (Col) as well as M_2 seeds from ethyl methanesulfonate-treated (EMS) populations of L*er* were purchased from Lehle Seeds (Round Rock, Tex., USA).

Plants were grown at 20 °C under long-day photoperiods (16 h of cool-white fluorescent light, photon flux of 70 μmol m^{-2} s^{-1}) in pots containing a mixture of soil and vermiculite (3:1 w/w) and irrigated with mineral nutrient solution once a week (Haughn and Somerville 1986). All treatments were performed on 3-week-old plants. Low temperature treatments were given by transferring plants to a growth chamber set to 4 °C for different periods of time under the light and photoperiodic conditions described above. In order to perform the freezing experiments under conditions as close as possible to natural conditions, the plants were not induced to nucleate. Therefore, the plants were exposed to 4 °C for 30 min in darkness and subsequently the temperature was lowered by 2 °C per hour. The final desired freezing temperature was maintained for 6 h and then the temperature was increased again to 4 °C at the same rate. After thawing at 4 °C for 4 h in the dark, the plants were returned to their original growth conditions (see above). Freezing tolerance was scored 2 weeks later as the capacity of plants to survive, i.e. to resume growth. Under our experimental conditions, and with plants that were not nucleated, the variation in supercooling before ice formation was most likely low as reflected by the low variation in the results obtained. Water loss was induced by exposing rosettes, previously detached from their root system, to an air stream within a flow chamber. The rate of water loss was determined as the percentage of initial weight lost after different times of exposure. For ABA treatments, plants were sprayed with a 10 μM solution of ABA once a day during 7 d. In all cases, an ABA stock solution in Dimethyl Sulfoxide (DMSO) was prepared and control experiments were given by using water containing the same final concentrations of DMSO as the corresponding ABA solution.

Genetic analyses

The dominance of the *frs1* mutation was determined by reciprocally crossing *frs1* plants to wild-type plants and analysing the freezing tolerance of the F_1 progeny. To study the segregation of freezing tolerance, some F_1 hybrids were allowed to self-fertilize and the resulting F_2 plants analyzed for freezing sensitivity. Furthermore, some F_2 progenies derived from selfing individual F_1 hybrids were grown to maturity and the seeds used to generate F_3 families. The genotype of each F_2 plant at the *frs1* locus was confirmed by checking the freezing phenotype of plants from the F_3 families.

For genetic mapping of the *frs1* mutation, a mutant plant was crossed to a wild-type plant of the Col ecotype and an F_1 hybrid was allowed to self-fertilize. A total of 95 F_2 plants was used to map the *frs1* mutation. A single leaf from each F_2 plant was sampled and stored at −70 °C. After sampling, the plants were self-fertilized to produced F_3 seeds, and the genotype of each F_2 plant at the *frs1* locus was determined by analyzing the freezing tolerance of 30 F_3 plants derived from the corresponding F_2 plant. Genomic DNA from 24 F_2 plants that showed the mutant phenotype (i.e. freezing sensitivity) was extracted as described by Cenis (1992). The chromosome location of the *frs1* mutation was determined by co-segregation of freezing sensitivity with two types of polymerase chain reaction (PCR)-generated molecular markers: co-dominant cleaved amplified polymorphic sequence (CAPS) markers (Konieczny and Ausubel 1993) and simple sequence-length polymorphic

(SSLP) markers (Bell and Ecker 1994), using the Kosambi mapping function (Kosambi 1944).

For complementation tests, crosses were performed between the two mutant parents (*frs1* and *aba3-2*) in both directions, and F_1 hybrids tested for freezing sensitivity. Allelism was confirmed in the F_2 progeny.

Determination of ABA

Rosette leaves from 3-week-old plants were used to extract ABA as described by Gómez-Cadenas et al. (1996). Abscisic acid was purified by reverse-phase HPLC using a Hewlett–Packard 1090 chromatograph (Palo Alto, Calif., USA). Dried samples were dissolved in 1.5 mL of 20% methanol (v/v) and loaded into a Rheodyne injector (Cotati, Calif., USA) with a 2-ml sample loop. The eluents of the mobile phase were water (1% acetic acid) as solvent A and methanol as solvent B. A 40-min gradient from 20 to 100% of solvent B in solvent A was used at a flow-rate of 1.5 mL min^{-1} on a column (250 mm long, 4.6 mm i.d.) packed with Nucleosil C18 (10 μm). The UV detector was set to 254 nm. Fractions of 1.5 mL were collected and analyzed on a scintillation counter. Those showing the presence of [^{3}H]ABA (see Gómez-Cadenas et al. 1996) were selected, mixed, dried and redissolved in 1 mL of methanol. To obtain a calibration curve for quantification of ABA in samples, an aliquot of 100 ng (2 mL) of hexadeuterated ABA was added as an internal standard to an ABA-dilution series. Samples and ABA dilutions were derivatized to the methylester with trimethylsilyldiazomethane for 10 min at room temperature. A 2-μL sample was injected (splitless 1.5 min) at a temperature of 250 °C into a Hewlett–Packard 5890 gas chromatograph equipped with a capillary column HP-1 (12.5 m long, 0.2 mm i.d.; 0.33 μm cross-linked methylsilicone gum). The carrier gas was helium at a column head pressure of 60 kPa. The oven was set at 80 °C for 1 min, then increased at a rate of 12 °C min^{-1} to 250 °C and maintained at that temperature for 3 min. The detection was performed in a Hewlett–Packard Mass Spectrometer 5989A under the Negative Chemical Ionisation modality, with methane in the selected ion monitoring (SIM) mode of the ions: 278 atomic mass units (amn) for ABA-methyl ester and 284 amu for hexadeuterated ABA-methyl ester. The source and quadrupole were set at 150 and 100 °C, respectively. The chromatograms for ions 278 and 284 were extracted and integrated for quantification by reference to the calibration curve.

Analysis of RNA

Total RNA was isolated from 3-week-old rosette leaves following the method of Logeman et al. (1987). The RNA-blot hybridizations were performed according to Medina et al. (1999). The *RCI1A* gene-specific probe consisted of a 0.2-kb DNA fragment corresponding to the 3′ untranslated region (Jarillo et al. 1994). The *RCI2A* and *RCI2B* specific probes were 0.3- and 0.2-kb DNA fragments from the 3′ noncoding regions, respectively (Capel et al. 1997). The probe for *KIN1* (Kurkela and Franck 1990) was a 0.7-kb fragment obtained by PCR from genomic DNA of L*er* ecotype by using the primers: 5′-GGCACCACACTCCCTTTAGC-3′ and 5′-GAATATAAGTTTGGCTCGTC-3′. The *COR47* probe was a 0.4-kb *Apa*I-*Sac*I fragment obtained from the corresponding cDNA (Gilmour et al. 1992). The probe for *RAB18* (Lang and Palva 1992) was a 0.25-kb fragment obtained by PCR from genomic DNA of the L*er* ecotype by using the primers: 5′-TCCATATCC-GAAACCGGACT-3′ and 5′-ACGTACCGAGCTAGAGCTGG-3′. The probes for *LTI78* and *LTI65* (Nordin et al. 1993) were 1.0-kb and 0.5-kb fragments obtained by PCR from genomic DNA of the L*er* ecotype using the following primer pairs: 5′ -CGGGATT-TGACGGAGAACCA- 3′ and 5′-ACCATAATACATCAAA-GACG-3′, and 5′-GGAGTGAAGGAGACGCAACAAG-3′ and 5′-ACCCCAAATCTTCAGTTCCCAG-3′, respectively. In all cases, equal RNA loading was controlled with a probe consisting of a 0.3-kb *Eco*RI fragment from the *18S rDNA* (Tremousaygue et al. 1992). The hybridization signals were quantified as described by Capel et al. (1997). The RNA samples from each experiment were analyzed in at least two independent blots, and each experiment was repeated at least twice.

Results

Isolation of mutants

We established a freezing protocol that could clearly distinguish non-acclimated from cold-acclimated L*er* *Arabidopsis* plants. In this assay, non-acclimated 3-week-old plants had an LT_{50} (temperature at which the survival percentage is 50%) of −5 °C and were completely killed when exposed to −7 °C for 6 h (Fig. 1). After 7 d of acclimation at 4 °C, nearly 100% of plants survived freezing at −7 °C for 6 h, 50% of plants survived freezing at −8 °C, and were completely killed at −10 °C (Fig. 1). This pattern of cold acclimation suggested that it might be possible to identify mutant plants that remain as freezing sensitive (*frs*) after being cold-acclimated and, therefore, are impaired in the development of freezing tolerance.

M_3 families derived from 300 EMS-mutagenized L*er* M_2 seeds were used for the screening. Twenty 3-week-old plants from each family were acclimated at 4 °C for 7 d, and then analyzed for their ability to survive after being exposed for 6 h at −7 °C. In one family, all the plants analyzed were unable to survive the screening conditions (Fig. 2A) and this phenotype was maintained in the M_4 progenies. This mutant line was named *frs1* and backcrossed to wild-type plants four times. Phenotypic analyses of F_1 and F_2 progenies showed that the *frs1* mutant line was caused by a recessive mutation in a single nuclear gene (data not shown).

Freezing tolerance and pleiotropic phenotypes of frs1 mutant plants

When 3-week-old *frs1* plants were exposed to freezing temperatures for 6 h without being cold-acclimated,

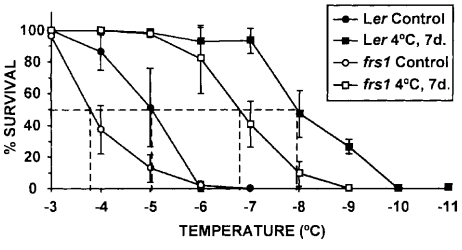

Fig. 1. Freezing tolerance of non-acclimated and cold-acclimated wild-type L*er* and *frs1* mutant plants of *Arabidopsis thaliana*. Plants were grown under long-day photoperiods, acclimated 7 d at 4 °C, or non-acclimated (control), and frozen as described in *Materials and methods*. Freezing tolerance was estimated as the percentage of plants surviving each specific temperature. Data are means ± SE from three separate experiments

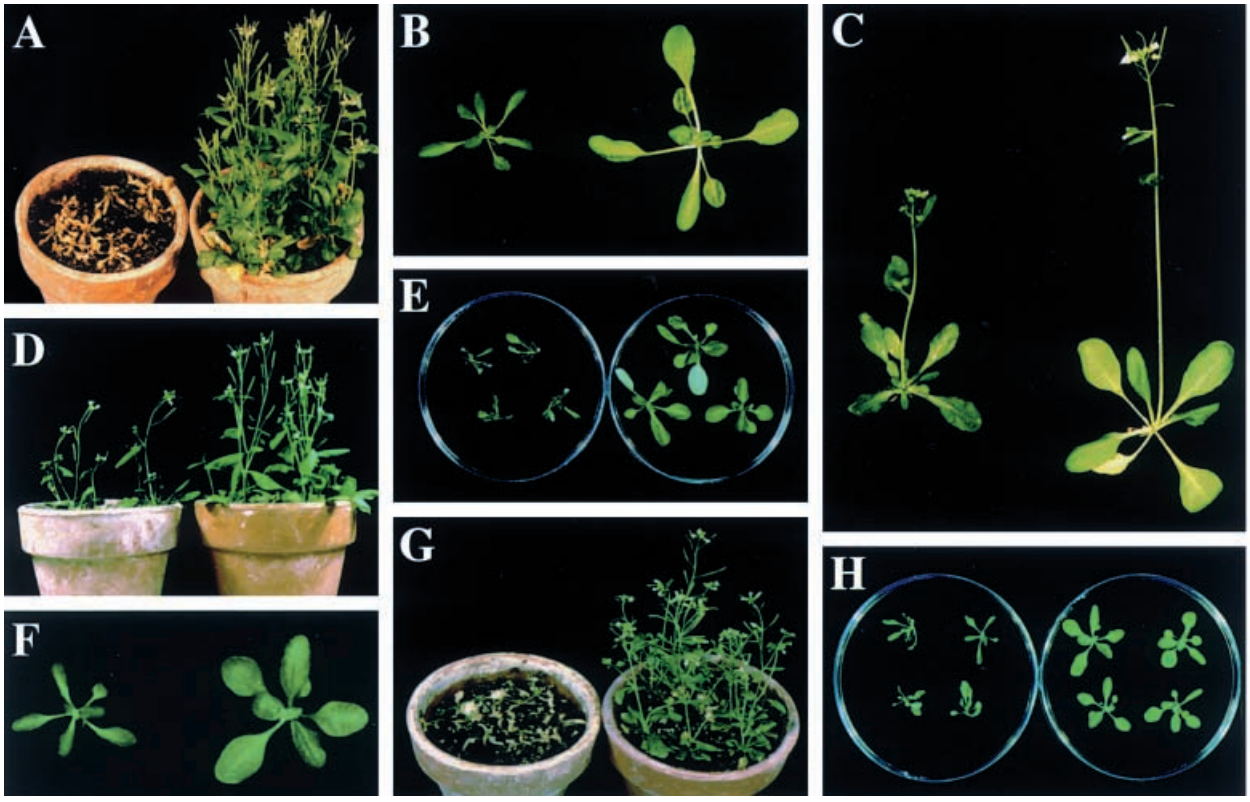

Fig. 2A–H. Phenotype of *frs1* mutant plants. **A** Three-week-old *frs1* (*left*) and L*er* (*right*) plants after being acclimated 7 d at 4 °C, exposed 6 h at −7 °C, and recovering for 2 weeks at 20 °C under long-day photoperiods. **B** Seventeen-day-old *frs1* (*left*) and L*er* (*right*) plants grown at 20 °C under long-day photoperiods. **C** Four-week-old *frs1* (*left*) and L*er* (*right*) plants grown as described in **B**. **D** Five-week-old *frs1* (*left*) and L*er* (*right*) plants grown as described in **B**. **E** Seventeen-day-old *frs1* (*left*) and L*er* (*right*) rosettes after being exposed to dehydration for 40 min. **F** Seventeen-day-old *frs1* rosettes after being sprayed once a day for 7 d with a 10-μM solution of ABA (*right*) or with water containing the same concentration of the ABA solvent DMSO (*left*). **G** Three-week-old *frs1* plants after being sprayed with ABA (*right*) or the ABA solvent DMSO (*left*) as described in **F**, and then acclimated, exposed to freezing and allowed to recover as described in **A**. **H** Seventeen-day-old *frs1* rosettes after being treated with ABA (*right*) or the ABA solvent DMSO (*left*) as described in **F**, and then dehydrated as described in **E**

100% survived at −3 °C, around 50% survived at −4 °C and none survived at −7 °C (Fig. 1). On the other hand, after 7 d of acclimation at 4 °C, 100% of *frs1* plants survived at −5 °C, around 50% at −7 °C and none at −9 °C (Fig. 1). Taken together, these results indicate that both the constitutive freezing tolerance and the freezing tolerance that is generated by cold acclimation are lower in *frs1* mutants than in L*er* wild-type plants.

Under standard growing conditions (see *Materials and methods*), *frs1* plants showed a darker green colour and were smaller than wild-type plants (Fig. 2B,C). Furthermore, *frs1* plants displayed a wilty phenotype (Fig. 2D). In fact, mutant plants detached from their root system and exposed to dehydration in a flow chamber lost water much faster than wild-type plants (Fig. 2E). In a time course experiment, while 3-week-old *frs1* plants lost 50% of their fresh weight after 35 min in the flow chamber, L*er* plants at a similar developmental stage could stand 90 min before such a loss took place (Fig. 3). All these phenotypes exhibited by the mutant plants were recessive and co-segregated with freezing sensitivity during backcross experiments (data not shown), indicating that they were pleiotropic effects of the *frs1* mutation.

The frs1 mutant line is deficient in ABA

The wilty phenotype displayed by *frs1* plants suggested that they could be affected in ABA content (Koornneef et al. 1998). In fact, *frs1* mutants rescued part of their wild-type morphological phenotype when treated with ABA (Fig. 2F). In addition, after treatment with ABA the *frs1* plants partially recovered their capacity to tolerate freezing temperatures and retain water (Fig. 2G,H). Table 1 shows the LT_{50} values of non-acclimated and cold-acclimated 3-week-old plants from L*er* and *frs1* that had been previously treated with 10 μM ABA or with the ABA solvent (DMSO). As a control for the treatment, plants from the ABA-deficient *Arabidopsis* mutant *aba1-1*, which has been described as being impaired in freezing tolerance (Heino et al. 1990; Gilmour and Thomashow 1991) were also analyzed (Table 1). Non-acclimated ABA-treated L*er* plants showed LT_{50} values very similar to those of the control (DMSO-treated) plants, while the LT_{50} values of non-acclimated ABA-treated *frs1* plants were reduced with respect to control plants by more than 1 °C, almost reaching the values of wild-type plants. In the case of cold-acclimated plants, L*er* did not show any reduction

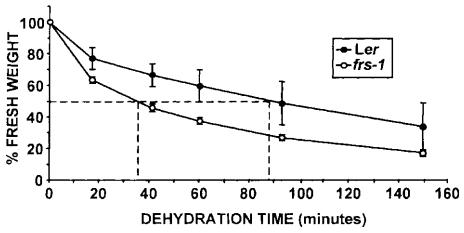

Fig. 3. Rate of water loss in wild-type L*er* and *frs1* mutant plants. Plants were detached from their root systems and exposed to dehydration in a flow chamber for different times. The rate of water loss was estimated as the percentage of fresh weight lost at each specific time. Data are means ± SE from three separate experiments

Table 1. Freezing-tolerance recovery of wild-type L*er*, and *frs1* and *aba1-1* mutants after ABA treatment. Plants were non-acclimated or acclimated for 7 d at 4 °C, and treated with 10 μM ABA or with the ABA solvent (DMSO) once a day for 1 week before freezing. Table shows LT_{50} values. The data are means ± SE for two separate experiments

Genotype	Non-acclimated		Cold acclimated	
	DMSO	ABA	DMSO	ABA
L*er*	−5.1 ± 0.6	−5.6 ± 0.4	−7.9 ± 0.1	−7.7 ± 0.0
frs-1	−4.1 ± 0.2	−5.3 ± 0.1	−6.9 ± 0.3	−7.6 ± 0.4
aba1-1	−4.2 ± 0.6	−5.3 ± 0.4	−5.2 ± 0.4	−7.8 ± 0.1

in LT_{50} after being treated with ABA with respect to control plants, suggesting that the LT_{50} value reached by exposure to 4 °C for 7 d is the minimum value L*er* plants can attain in our experimental conditions. However, when *frs1* mutant plants were treated with ABA, their LT_{50} values decreased with respect to control plants, reaching the values of wild-type L*er* plants. As expected, the LT_{50} values obtained for the *aba1-1* plants treated with ABA were, in all cases, similar to those of the wild-type plants (Heino et al. 1990). Table 2 shows the time, in minutes, that 3-week-old plants from L*er* and *frs1* required to lose 50% of their fresh weight when exposed to dehydration in a flow chamber after being treated with 10 μM ABA or DMSO. Three-week-old *aba1-1* plants, which have been reported to be affected in their rates of water loss (Koornneef et al. 1982), were also analyzed as a control for the treatment (Table 2). In the case of L*er* plants, the ABA treatment increased their capacity to retain water less than 2-fold. However, this increase was much higher (6-fold) in the case of the *frs1* mutant, which nearly recovered to wild-type values. The results obtained for the *aba1-1* plants after ABA treatment were, as expected (Koornneef et al. 1982), very similar to those of *frs1*.

These results indicate that the *frs1* mutant line could be deficient in ABA. Abscisic acid measurements in rosette leaves from 3-week-old L*er* and *frs1* plants confirmed this hypothesis (Table 3). The ABA content of *aba1-1* mutant plants was also measured as a control (Table 3). The levels of ABA in *frs1* mutant plants were much lower (ca. 83%) than in wild-type plants, and similar to those shown by the *aba1-1* mutant.

Table 2. Recovery of the rate of water loss in wild-type L*er*, and *frs1* and *aba-1* mutants after ABA treatment. Plants were sprayed once a day for 1 week with 10 μM ABA or with the ABA solvent (DMSO). Plants were detached from their root systems and placed in a flow chamber to provoke dehydration. Values indicate time required (in minutes) to lose 50% of fresh weight. The data are means ± SE for two separate experiments

Genotype	DMSO	ABA
L*er*	117.8 ± 3.1	198.7 ± 5.3
frs1	41.1 ± 11.1	183.8 ± 5.3
aba1-1	28.2 ± 1.2	180.5 ± 2.0

The mutation that produces the frs1 phenotypes is a new allele of the ABA3 locus

The F_2 progenies derived from crosses between *frs1* mutant plants and the Col ecotype were used to map the *frs1* mutation with SSLPs and CAPS molecular markers. The *frs1* mutation was mapped on the top of chromosome 1 between SSLP and CAPS markers nga63 (6.2 cM ± 2.5 SE) and g2395 (2.2 cM ± 1.5 SE), respectively. The *ABA3* locus had been mapped to this chromosomal region (Léon-Kloosterziel et al. 1996) which indicated that *frs1* could be allelic for *ABA3*. Allelism tests among *frs1* and *aba3-2* mutants (Léon-Kloosterziel et al. 1996) using freezing sensitivity as a criterion, confirmed that they represented mutations at the same locus (data not shown). Léon-Kloosterziel et al. (1996) have reported two different *ABA3* alleles, *aba3-1* in the ecotype Col and *aba3-2* in L*er*. Since the *frs1* mutant line was isolated from an independent M_2 population, it must represent a new allele of the *ABA3* locus that we designated *aba3-3*. When compared, both *aba3-2* and *aba3-3* mutant plants had the same ABA levels and showed a similar phenotype including a similar tolerance to both freezing and rate of water loss (data not shown).

Cold- and drought-regulated gene expression in the frs1 mutant line

To investigate whether the reduced capacity of *frs1* mutant plants to tolerate freezing was correlated with changes in the expression of cold- and/or drought-regulated genes, the accumulation of transcripts corresponding to several genes whose expression is differentially regulated by low temperatures and/or dehydration was assayed in *frs1* plants by RNA-blot experiments (*RCI1A*, Jarillo et al. 1994; *RCI2A*, *RCI2B*, Capel et al. 1997; *KIN1*, Kurkela and Franck 1990;

Table 3. Abscisic acid contents of wild-type L*er*, and *frs1* and *aba1-1* mutants. Abscisic acid was measured by GC-MS. The data are means ± SE for two separate experiments; range in parenthesis

Genotype	ABA [μg (g DW)$^{-1}$]
L*er*	0.11 ± 0.05 (0.07–0.14)
frs1	0.02 ± 0.01 (0.03–0.02)
aba1-1	0.04 ± 0.01 (0.03–0.05)

COR47, Gilmour et al. 1992; *RAB18*, Lang and Palva 1992; *LTI78*, *LTI65*, Nordin et al. 1993). Total RNA was extracted from L*er* and *frs1* plants that had been exposed to cold (24 h at 4 °C) or dehydration conditions (loss of 50% fresh weight), and hybridized with the indicated probes (see *Materials and methods*).

Figure 4A shows the expression patterns of the genes analyzed in control plants and in plants exposed to low temperature. As expected, the expression levels for all genes were higher in cold-treated than in untreated control L*er* plants. In *frs1* mutants, the expression levels of all genes were also higher in cold-treated than in control plants. However, while the levels of *RCI1A*, *KIN1* and *LTI78* transcripts in *frs1* plants exposed to low temperature were similar to those obtained in cold-treated L*er* plants, the levels of *RCI2A*, *RCI2B*, *COR47*, *RAB18* and *LTI65* transcripts were reduced. Furthermore, the levels of *KIN1*, *COR47*, *RAB18* and *LTI78* transcripts were lower in control *frs1* plants than in the corresponding L*er* plants. The expression patterns of the genes analyzed in control and dehydrated plants are shown in Fig. 4B. With the exception of *RCI1A*, whose expression was not induced by dehydration, as expected (Jarillo et al. 1994), the levels of mRNAs corresponding to the rest of the genes were higher in dehydrated than in control L*er* and *frs1* plants. Nevertheless, the expression levels of these genes were consistently lower in dehydrated *frs1* plants than in the corresponding L*er* plants, being almost undetectable in the case of *RCI2B* and *LTI65*. As in the experiment with low-temperature-treated plants (Fig. 4A), the levels of *KIN1*, *COR47*, *RAB18* and *LTI78* transcripts were also lower in control *frs1* plants than in control L*er* plants.

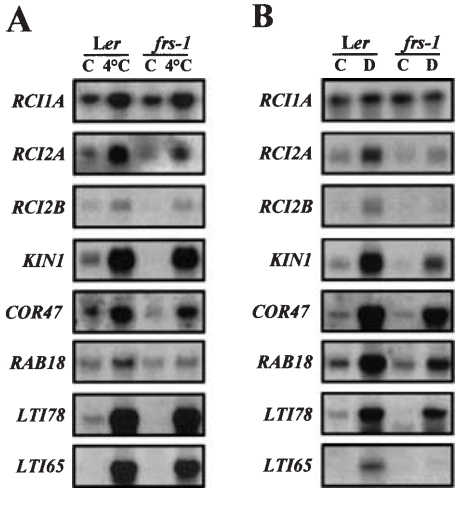

Fig. 4A,B. Accumulation of transcripts corresponding to different cold- and drought-regulated genes in L*er* and *frs1* mutant plants. **A** Gene expression patterns in 3-week-old rosette leaves from control plants (*C*) and plants exposed for 1 d at 4 °C (*4 °C*). **B** Gene expression patterns in 3-week-old rosette leaves from control plants (*C*) and plants exposed to dehydration until they had lost 50% of their fresh weight (*D*)

Discussion

We have isolated a mutant line of *Arabidopsis* showing reduced freezing tolerance after cold acclimation. The mutation, designated *frs1*, segregated in a simple Mendelian fashion and displayed impaired freezing tolerance also in non-acclimated plants. This indicated that the *frs1* mutation affects both the constitutive freezing tolerance of *Arabidopsis* and the freezing tolerance that is induced by cold acclimation. In addition, *frs1* mutant plants were slightly darker green and showed an elevated loss of water in dehydration conditions leading to a wilty phenotype. This phenotype was similar to that reported for ABA-deficient and -insensitive mutants (Koornneef et al. 1998). In fact, as in the case of *aba1-1* (Heino et al. 1990), *frs1* plants partially recovered the wild-type capacity to tolerate freezing temperatures as well as the wild-type rate of water loss after treatment with ABA. These results strongly suggested that *frs1* was ABA-deficient, which was confirmed by ABA measurements. Since the *frs1* mutation mapped on the same chromosomal region as the locus *ABA3* (i.e. top of chromosome 1; Léon-Kloosterziel et al. 1996), we performed complementation assays with *aba3-2* mutants that demonstrated that the *frs1* mutation was a new allele of the *ABA3* locus. We designated this new allele *aba3-3*. The locus *ABA3* seems to encode a function required for the conversion of ABA-aldehyde to ABA, which is the last step in the proposed pathway of ABA biosynthesis (Schwartz et al. 1997). Lesions in the conversion of ABA-aldehyde to ABA may result from a defect in the aldehyde-oxidase apoprotein or in the molybdenum cofactor (Moco) required by the aldehyde oxidase (Leydecker et al. 1995; Marin and Marion-Poll 1997). Presumably, the mutations so far isolated in *ABA3* affect the introduction of a desulfo moiety into the Moco (Schwartz et al. 1997).

Although the issue of whether ABA has a fundamental role in the cold-acclimation response beyond its general role in plant growth and development is still a matter of discussion for some authors (Thomashow 1999), different results suggest that ABA has an important role in mediating plant responses to different environmental stimuli, including low temperatures and water stress. In this way, endogenous ABA levels are increased by low temperature and dehydration exposure (Bray 1988; Guy and Haskell 1988; Lang et al. 1994), and exogenous treatment with ABA induces the development of freezing and drought tolerance (Chen and Gusta 1983; Orr et al. 1986; Lang et al. 1989; Mantylä et al. 1995). Furthermore, as mentioned above, the *aba-1* mutant was shown to be less tolerant to freezing temperatures and to have a higher rate of water loss than wild-type plants (Koornneef et al. 1982; Heino et al. 1990; Gilmour and Thomashow 1991), and the majority of cold-inducible genes are also responsive to ABA and/or water stress (for reviews, see Hughes and Dunn 1996; Thomashow 1999). Thus, low, including freezing, temperatures and water stress induce overlapping adaptive responses. In fact, the adaptation of a plant to survive low and freezing temperatures involves, at least, the adaptation to cellular

dehydration (Pardossi et al. 1994). Our results, showing that a mutation in the *ABA3* locus leads to an impairment of freezing tolerance and an elevated rate of water loss under dehydration conditions, are in agreement with a common role for ABA in mediating plant responses to freezing and water stress, and constitute additional evidence for the overlapping nature of these two stress adaptations. Abscisic acid mediates cold acclimation and is required for a full development of freezing tolerance in *Arabidopsis*. Interestingly, the *frs1/aba3-3* mutant also shows a reduced freezing tolerance in non-acclimated plants, which indicates that ABA mediates not only cold acclimation but also the constitutive freezing tolerance of *Arabidopsis*. In addition, our results suggest that different pathways leading to freezing tolerance co-exist in *Arabidopsis*, since the *frs1/aba3-3* mutant still displays a certain capacity to tolerate freezing temperatures before and after cold acclimation. The possibility, however, that the reduced ABA levels still present in the mutant plants could be responsible for this capability cannot completely be excluded.

To obtain some information on how ABA mediates cold acclimation and freezing tolerance, we analyzed the expression patterns of different genes the expression of which is regulated by low temperature, ABA and water stress in mutant plants exposed to both cold and dehydration. In the case of low-temperature exposure, only *RCI2A*, *RCI2B*, *COR47*, *RAB18* and *LTI65* showed lower transcript accumulation in *frs1/aba3-3* than in wild-type plants. This is in agreement with previous studies indicating that the gene expression in response to low temperature would not only be mediated by ABA (Hughes and Dunn 1996; Thomashow 1999). However, when *frs1/aba3-3* plants were exposed to dehydration, all transcripts, except those from *RCI1A* which is not regulated by dehydration (Jarillo et al. 1994), showed much lower accumulation than in the corresponding L*er* plants, indicating that gene expression in the *frs1/aba3-3* mutant line is mainly affected under dehydration stress. These results are consistent with the fact that most of the gene expression regulated by water stress is responsive and mediated by ABA (Shinozaki and Yamaguchi-Shinozaki 1997), and suggest that the diminished freezing tolerance of *frs1/aba3* plants after cold acclimation might be due to an impaired capacity to tolerate the cellular dehydration produced at subzero temperatures (Levitt 1980). On the other hand, the *frs1/aba3-3* mutant line displays a reduced accumulation of several transcripts in non-stressful conditions when compared to the wild-type L*er*. This impairment in the constitutive gene expression of *frs1/aba3-3* could be related to its reduced constitutive tolerance to freezing, suggesting that other ABA-dependent factors not related to dehydration are also involved in this tolerance in *Arabidopsis*.

Multiple signalling pathways are involved in cold acclimation leading to physiological and morphological adaptations that allow plants to survive freezing. This complexity is consistent with the data obtained so far from analyses of low-temperature-regulated gene expression (Thomashow 1999) and previous mutants (McKown et al. 1996; Warren et al. 1996; Ishitani et al. 1997, 1998; Xin and Browse 1998; Lee et al. 1999). The results presented in this paper constitute the first report showing that a mutation in *ABA3* leads to an impairment in freezing tolerance in both cold-acclimated and non-acclimated plants. These data strengthen the conclusion that ABA-controlled processes are required for the acquisition of full freezing tolerance in acclimated plants, and demonstrate that ABA also mediates the constitutive freezing tolerance of *Arabidopsis*. We further suggest that freezing tolerance in *Arabidopsis* is dependent, in part, on ABA-regulated proteins that would allow the plant to survive the different challenges imposed by subzero temperatures, mainly the freeze-induced cellular dehydration. The molecular characterization of mutants altered in freezing tolerance will help to identify the signalling components that mediate the ABA response in relation to cold acclimation and freezing tolerance.

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