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Cold acclimation in the moss *Physcomitrella patens* involves abscisic acid-dependent signaling

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ABSTRACT

Overwintering plants develop tolerance to freezing stress through a cold acclimation process by which the cells provoke internal protective mechanisms against freezing. The stress hormone abscisic acid (ABA) is known to increase freezing tolerance of plant cells, but its role in cold acclimation has not been determined. In this study, we used ABA-insensitive lines of the moss *Physcomitrella patens* to determine whether cold acclimation in bryophytes involves an ABA-dependent process. Two ABA-insensitive lines, both impaired in ABA signaling without showing ABA-induced stress tolerance, were subjected to cold acclimation, and changes in freezing tolerance and accumulation of soluble sugars and proteins were compared to the wild type. The wild-type cells acquired freezing tolerance in response to cold acclimation treatment, but very little increase in freezing tolerance was observed in the ABA-insensitive lines. Analysis of low-molecular-weight soluble sugars indicated that the ABA-insensitive lines accumulated sucrose, a major compatible solute in bryophytes, to levels comparable with those of the wild type during cold acclimation. However, accumulation of the trisaccharide theanderose and of specific LEA-like boiling-soluble proteins was very limited in the ABA-insensitive lines. Furthermore, analysis of cold-induced expression of genes encoding LEA-like proteins revealed that the ABA-insensitive lines accumulate only small amounts of these transcripts during cold acclimation. Our results indicate that cold acclimation of bryophytes requires an ABA-dependent signaling process. The results also suggest that cold-induced sugar accumulation, depending on the sugar species, can either be dependent or independent of the ABA-signaling pathway.

Introduction

Freezing stress causes severe damage to the structural organization and physiological mechanism of plant cells. Many of the terrestrial plants growing in the temperate zone develop tolerance to freezing in winter through a process known as cold acclimation by which plants acquire freezing tolerance in response to exposure to non-freezing low temperatures for a certain period. Earlier studies indicated that cold acclimation is associated with a combination of biochemical and physiological changes within plant cells, including the accumulation of compatible solutes such as soluble sugars and other metabolites (Levitt, 1980; Sakai and Larcher, 1987). More recent molecular studies have indicated that cold acclimation is accompanied by the expression of a number of genes, including those encoding late embryogenesis abundant (LEA) proteins, that is mediated by the function of cold-regulated transcription factors (Pearce, 1999; Thomashow, 1999; Xin and Browse, 2000; Seki et al., 2001; Van Buskirk and Thomashow, 2006). Cold acclimation is a phenomenon observed not only in angiosperms but also in other classes of land plants. Bryophytes, representing the basal land plant lineage, also have the capacity to acclimate to cold temperatures. It has been shown that gametophytes of bryophytes growing in natural habitats exhibit greater levels of freezing tolerance in winter than in summer, though the level of tolerance differs among species (Rütten and Santarius, 1992a). Artificial cold acclimation can also induce freezing tolerance (Hudson and Brustkern, 1965). These facts suggest that cold acclimation is a phenomenon

Abbreviations: DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride.

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common to both vascular and non-vascular land plants, providing evolutionary insights into the adaptation process of plants for surviving subzero temperatures they are exposed to in the terrestrial environment. However, whether or not the mechanism of cold acclimation is identical in higher and lower land plants is yet to be determined.

In angiosperms, the phytohormone abscisic acid (ABA), which triggers cellular responses to water stress, has been suggested to play a role in the cold acclimation process. Exogenous ABA treatment at ambient temperatures has been shown to increase freezing tolerance in cultured plant cells (Chen et al., 1983). ABA enhances the expression of a number of stress-responsive genes that are also induced during cold acclimation (Zeevart and Creelman, 1988). In addition, transient increase in endogenous ABA levels during cold acclimation has been observed in various plant species (Daie and Campbell, 1981; Lalk and Dörffling, 1985; Smolenska-Sym et al., 1995; Jouve et al., 2000). These results indicate that increased levels of ABA during cold acclimation might have triggered the induction of genes necessary for freezing stress tolerance.

In contrast to a considerable understanding of the molecular signaling involved in cold acclimation in angiosperms, little is known about mechanisms of cold responses in bryophytes, including the role of ABA. We have previously reported that the freezing tolerance of protonemata of the moss *Physcomitrella patens* was enhanced by artificial cold acclimation (Minami et al., 2005). The increase in freezing tolerance was accompanied by the accumulation of transcripts encoding LEA-like proteins and soluble sugars, sucrose and theanderose (Minami et al., 2005; Nagao et al., 2006). Since the accumulation of these proteins and sugars was also induced by exogenous ABA, it was thought that ABA mediates the cold acclimation process in *P. patens*. However, results of direct measurement of ABA indicated that endogenous ABA levels did not increase in the protonemata during cold acclimation (Minami et al., 2005). In addition, the amounts of accumulated ABA in tissues and the medium during cold acclimation were below the threshold level and not sufficient to increase freezing tolerance or up-regulate ABA-induced genes (Minami et al., 2005). From these results, it was speculated that cold acclimation in *P. patens* is not accompanied by endogenous ABA accumulation but shares a part of the ABA-signaling process to increase expression of genes rendering freezing tolerance.

The aim of the present study was to clarify the role of the ABA signaling in cold acclimation that leads to the development of freezing tolerance in bryophytes. We have characterized the cold responses of two ABA-insensitive lines of *P. patens*, both deficient in the early signaling process of ABA responses. The results indicate that cold acclimation in mosses is heavily dependent on ABA signaling.

Materials and methods

Plant materials and growth conditions

Protonema tissue of *Physcomitrella patens* (Hedw.) Bruch & Schimp was grown on cellophane-overlaid 0.8% agar plates of modified BCD medium (Ashton et al., 1979) supplemented with 0.5% glucose. The protonemata were cultured in a controlled-environment growth chamber at 25 °C under continuous illumination (35 μmol photons m^{-2} s^{-1}). For ABA treatment, the protonemata were transferred onto BCD medium containing ABA and cultured under the same conditions. Cold treatment was carried out by transferring colonies of protonemata onto fresh agar plates of BCD medium and incubating them at 0 °C (on ice) under continuous light for different times as described previously (Minami et al., 2005). Special care was taken to prevent water loss from the culture, which might cause desiccation or osmotic stress during cold acclimation.

Freezing stress treatment

Freezing tolerance was estimated by equilibrium freezing of protonema tissue as previously described (Nagao et al., 2006). After thawing, electrolyte leakage from the tissues was determined by an electro-conductivity meter to estimate the survival rate. Alternatively, the protonemata were stained for 1 h on 0.5% Evans Blue after thawing, and the number of stained and unstained cells was counted for estimation of survival.

Soluble sugar extraction and analysis

Protonemata were weighed, frozen and crushed using a mortar and a pestle for soluble sugar extraction. Samples were suspended in 80% (v/v) ethanol and insoluble materials were removed by centrifugation at 14,000 × *g* for 10 min at 4 °C. The supernatant was dried and suspended in H_2O. After removal of water-insoluble material by centrifugation, the supernatants were quantified by the anthrone-sulfuric acid assay using glucose as a standard (Yemm and Willis, 1954). Analysis of sugars by high performance liquid chromatography (HPLC) was carried out by using the NH2P-50 4E column (Shodex, Japan) at 30 °C with 70% (v/v) acetonitrile as a mobile phase. Peaks of sugars were detected by an RID-6A refractive index detector (Shimadzu Co., Japan) (Nagao et al., 2006).

Protein gel electrophoresis and immunoblot analysis

Proteins were extracted from the protonema cells by the procedure described previously (Minami et al., 2005). SDS-polyacrylamide gel electrophoresis was used for protein analysis. Protonema cells were homogenized and extracted with a solution containing 50 mM Tris–Cl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF) on ice. After centrifugation at 14,000 × *g* for 10 min at 4 °C, protein concentrations in the supernatants were determined. The proteins adjusted to equal concentrations were boiled for 3 min, and insoluble materials were removed by centrifugation at 14,000 × *g* for 10 min at 4 °C. These boiling soluble proteins corresponding to 20 μg of total soluble proteins were used for gel electrophoresis. The crude proteins and boiling-soluble proteins were electrophoresed in 12% (w/v) SDS-polyacrylamide gels. For immunoblot analysis, the electrophoresed proteins were transferred onto a polyvinylidene difluoride membrane and detected with the anti-17B9 antibody as described previously (Tougane et al., 2010).

Electron microscopy

Protonemata cultured on BCD medium were fixed in 2% glutaraldehyde in 0.05 M potassium–phosphate (K–P) buffer (pH 7.0) for 2 h at room temperature and then overnight at 4 °C. After rinsing with the K–P buffer, the specimens were incubated in 2% osmium tetroxide in the K–P buffer for 2 h at room temperature. The specimens were then dehydrated in an acetone series and embedded in Spurr's resin. For electron microscopy, ultra-thin sections were cut and stained with 2% (w/v) uranyl acetate for 10 min and lead citrate for 5 min. The specimens were observed at 100 kV using a Hitachi H-700H electron microscope.

RNA gel blot analysis

RNA extraction and RNA gel blot analysis were carried out as described by Minami et al. (2003). Four micrograms of total RNAs isolated from protonema cells were electrophoresed in a formaldehyde-containing gel and transferred to a nylon membrane. The blotted RNAs were hybridized with the radiolabeled