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Abscisic acid-induced rearrangement of intracellular structures associated with freezing and desiccation stress tolerance in the liverwort *Marchantia polymorpha*

Khaleda Akter[a], Masahiro Kato[a], Yuki Sato[a], Yasuko Kaneko[a,b], Daisuke Takezawa[a,b,*]

[a] Graduate School of Science and Engineering, Saitama University, Shimo-Okubo 255, Sakura-ku, Saitama 338-8570, Japan
[b] Institute for Environmental Science and Technology, Saitama University, Shimo-Okubo 255, Sakura-ku, Saitama 338-8570, Japan

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ABSTRACT

The plant growth regulator abscisic acid (ABA) is known to be involved in triggering responses to various environmental stresses such as freezing and desiccation in angiosperms, but little is known about its role in basal land plants, especially in liverworts, representing the earliest land plant lineage. We show here that survival rate after freezing and desiccation of *Marchantia polymorpha* gemmalings was increased by pretreatment with ABA in the presence of increasing concentrations of sucrose. ABA treatment increased accumulation of soluble sugars in gemmalings, and sugar accumulation was further increased by addition of sucrose to the culture medium. ABA treatment of gemmalings also induced accumulation of transcripts for proteins with similarity to late embryogenesis abundant (LEA) proteins, which accumulate in association with acquisition of desiccation tolerance in maturing seeds. Observation by light and electron microscopy indicated that the ABA treatment caused fragmentation of vacuoles with increased cytosolic volume, which was more prominent in the presence of a high concentration of external sucrose. ABA treatment also increased the density of chloroplast distribution and remarkably enlarged their volume. These results demonstrate that ABA induces drastic physiological changes in liverwort cells for stress tolerance, accompanied by accumulation of protectants against dehydration and rearrangement and morphological alterations of cellular organelles.

Introduction

Drought and freezing cause cellular dehydration, which can severely damage plant growth. Plants sense changes in environmental water status by which they develop a mechanism for tolerating stress caused by dehydration. It has been suggested that abscisic acid (ABA), a sesquiterpenoid compound, plays a crucial role in dehydration tolerance in plants. ABA is present ubiquitously in seed plants and plays an important role in stress tolerance of vegetative tissues as well as seeds (Hartung and Davies, 1988). Under conditions of stress, biosynthesis and release from the site of synthesis of ABA are increased, resulting in closure of stomata and increase in dehydration tolerance of vegetative cells (Hartung, 1983; Schroeder et al., 2001). During seed maturation, the increased endogenous ABA accelerates dormancy and desiccation tolerance of embryos (Zeevaart and Creelman, 1988). In both vegetative and reproductive tissues, ABA plays a role in triggering membrane depolarization (Thiel et al., 1992), changes in cellular metabolism and expression of a number of genes encoding proteins that protect cellular membranes and proteins from stress-induced damage (Chandler and Robertson, 1994).

It has been shown that ABA is present not only in seed plants but also in non-seed plants such as ferns and bryophytes and that it is likely to play a role in triggering desiccation tolerance of vegetative cells (Takezawa et al., 2011). In mosses, representing the major bryophyte group, the effect of ABA on stress tolerance has been documented in detail. In the moss *Funaria hygrometrica*, protonemal tolerance to rapid desiccation was shown to be induced by slow desiccation, accompanying increases in endogenous ABA levels (Werner et al., 1991). In *Atrichum undulatum*, ABA has been shown to enhance the tolerance of photosystem II to desiccation and increase NPQ upon rehydration (Beckett et al., 2000). The role of ABA has been more extensively examined in the model plant *Physcomitrella patens*. Freezing and desiccation tolerance of its protonemata was induced by exogenous ABA, accompanied by

Abbreviations: EST, expressed sequence tag; gFW, gram fresh weight; LEA, late embryogenesis abundant; RH, relative humidity.

* Corresponding author at: Graduate School of Science and Engineering, Shimo-Ohkubo 255, Sakura-ku, Saitama 338-8570, Japan. Tel.: +81 48 858 3409.
E-mail address: takezawa@mail.saitama-u.ac.jp (D. Takezawa).

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accumulation of proteins similar to late embryogenesis abundant (LEA) proteins (LEA-like proteins) and their transcripts (Minami et al., 2003; Khandelwal et al., 2010). Since LEA proteins that accumulate in maturing seeds have been suggested to play a role in acquisition of desiccation tolerance (Tunnacliffe and Wise, 2007), ABA-induced accumulation of LEA-like proteins in *P. patens* is thought to be crucial for its freezing and desiccation tolerance. In addition to these proteins, low-molecular-weight soluble sugars such as sucrose accumulated by ABA have been suggested to function as protectants during freezing and hyperosmotic stress (Nagao et al., 2005, 2006).

A role of ABA in stress tolerance of liverworts, another group of bryophytes, has also been suggested. Desiccation tolerance was shown to be induced by exogenous ABA in some liverwort species including *Exormotheca holstii* and *Riccia fluitans* (Hellwege et al., 1994; Hartung et al., 1994). In contrast to mosses, information on the characteristics of ABA responses in liverworts has been limited due to the lack of a model system that enables study of the physiological changes associated with stress tolerance under controlled environmental conditions. Furthermore, molecular mechanisms for ABA sensing and response in liverworts have not been characterized. In a previous study, we used cultured cells of *Marchantia polymorpha* as a model system and demonstrated the presence of a conserved ABA signaling pathway in the liverworts (Tougane et al., 2010). However, the role of ABA in developmental changes and stress tolerance of intact liverwort plants has not been elucidated. Previous studies have indicated that thalli of *M. polymorpha*, though it contains endogenous ABA at levels comparable to those of seed plants, show little response to exogenous ABA and do not induce desiccation tolerance unless they are encapsulated with a high concentration of sucrose (Li et al., 1994; Hellwege et al., 1994; Pence, 1998; Pence et al., 2005).

In the present study, we show that gemmae, which are vegetative clones produced on the thalli of *M. polymorpha*, have high sensitivity to ABA, and they are an ideal system to analyze ABA-induced changes in stress tolerance. Gemmalings of *M. polymorpha* develop freezing and desiccation tolerance by treatment with exogenous ABA in association with accumulation of low-molecular-weight soluble sugars and *LEA*-like transcripts. Furthermore, structural analysis revealed that the liverwort cells undergo drastic changes in cellular morphology during the ABA treatments, which have not been described previously.

Materials and methods

Plant materials and pretreatment conditions

Aseptic culture of *Marchantia polymorpha* L. accession Takaragaike-1 was maintained and propagated through gemmae growth using M51C agar medium (pH 5.5) containing 1% (w/v) sucrose under continuous light at 22 °C as described previously (Tougane et al., 2010). Gemmae produced in the gemma cup of thalli were used for abscisic acid (ABA) and sugar pretreatment as follows: gemmae were placed in test tubes containing the M51C liquid medium with different concentrations of sucrose and ABA and cultured for 3 d with gentle shaking under continuous light at 22 °C. Obvious plasmolysis or cell collapse caused by treatment with up to 0.3 M sucrose was not observed. Pretreatment of mature thalli was carried out for 3 d in the same way as done for the gemmae.

Freezing and desiccation treatment

For freezing treatment, pretreated gemmalings in the test tube were rinsed once in distilled water and placed in a liquid bath in which the temperature was controlled. When the temperature had reached −1 °C, ice was seeded using a toothpick cooled in liquid nitrogen. The samples were frozen at a rate of −2.4 °C/h to −3 °C and −5 °C. After thawing at 4 °C for 5 h in the dark, the gemmalings were transferred onto M51C agar medium and grown in continuous light at 22 °C for 7 d to determine survival rates. For desiccation treatment, the pretreated gemmalings were placed on sterile cellophane sheets in petri dishes and dried in a laminar flow for 1 h to remove excess water and then exposed to descending relative humidities (RHs) in desiccators with different salt solutions at 22 °C: saturated KCl solution (86% RH), saturated NaCl solution (75% RH) and silica gel for 4 and 15 h in the dark at 22 °C. The desiccated gemmalings were rehydrated using sterile water and transferred onto M51C agar plates. They were cultured in continuous light at 22 °C and survival rates were determined after 7 d. The gemmalings that stayed at least partially green and could make rhizoids were considered as surviving gemmalings.

Estimation of ABA-induced sugar accumulation

Both gemmalings and mature thalli were used for sugar extraction. The pretreated tissues were collected after washing with distilled water 3 times to remove the external sugar. After water had been removed by using tissue paper, the gemmalings were weighed and kept at −80 °C until use. Preparation of the soluble sugar fraction was carried out as described previously (Nagao et al., 2006). The total sugar was quantified by the anthrone-sulfuric acid assay (Yemm and Willis, 1954). The sugar sample was mixed with 0.2% (w/v) anthrone in 75% (v/v) sulfuric acid and boiled for 10 min, and absorbance at 620 nm was measured with glucose as a standard. Analysis of sugars by thin-layer chromatography (TLC) was carried out using silica gel plates as described previously (Nagao et al., 2006).

Protein gel electrophoresis

The pretreated gemmalings were collected after washing with double distilled water and weighed after removal of water using tissue paper. Protein extraction, preparation of boiling-soluble fractions and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were carried out as described previously (Minami et al., 2005). Boiling-soluble proteins corresponding to 20 μg of total soluble proteins were electrophoresed on 10% (w/v) SDS-polyacrylamide gels followed by silver staining.

RNA gel blot analysis

RNA extraction and RNA gel blot analysis were carried out as essentially described by Minami et al. (2003). Four micrograms of total RNAs isolated from gemmalings were electrophoresed in formaldehyde-containing gel and transferred onto a nylon membrane. The blotted RNA was hybridized with the radiolabeled cDNA probes. The membranes were subjected to high stringency washing in 0.2 × SSC and 0.2% SDS at 60 °C for 30 min and exposed to X-ray film to detect signals.

Light and electron microscopy

The pretreated gemmalings were fixed in 2% (v/v) 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% (w/v) 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 light microscopy, sections were stained with toluidine blue. For electron microscopy, ultra-thin sections were