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# Accumulation of theanderose in association with development of freezing tolerance in the moss *Physcomitrella patens*

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**Abstract**

Mosses are known to have the ability to develop high degrees of resistance to desiccation and freezing stress at cellular levels. However, underlying cellular mechanisms leading to the development of stress resistance in mosses are not understood. We previously showed that freezing tolerance in protonema cells of the moss *Physcomitrella patens* was rapidly increased by exogenous application of the stress hormone abscisic acid (ABA) [Minami, A., Nagao, M., Arakawa, K., Fujikawa, S., Takezawa, D., 2003a. Abscisic acid-induced freezing tolerance in the moss *Physcomitrella patens* is accompanied by increased expression of stress-related genes. J. Plant Physiol. 160, 475–483]. Herein it is shown that protonema cells with acquired freezing tolerance specifically accumulate low-molecular-weight soluble sugars. Analysis of the most abundant trisaccharide revealed that the cells accumulated theanderose ($G^6$-α-glucosyl sucrose) in close association with enhancement of freezing tolerance by ABA treatment. The accumulation of theanderose was inhibited by cycloheximide, an inhibitor of nuclear-encoded protein synthesis, coinciding with a remarkable decrease in freezing tolerance. Furthermore, theanderose accumulation was promoted by cold acclimation and treatment with hyperosmotic solutes, both of which had been shown to enhance cellular freezing tolerance. These results reveal a novel role for theanderose, whose biological function has been obscure, in high freezing tolerance in moss cells.


*Keywords:* *Physcomitrella patens*; Bryopsida; Freezing tolerance; Abscisic acid; Sucrose; Theanderose

## 1. Introduction

Many plants growing in temperate and frigid zones are able to acquire resistance to freezing stress in response to decreases in air temperatures over mid-fall and early winter. This phenomenon, called cold acclimation, is associated with dynamic physiological changes within the plant cells (Levitt, 1980; Sakai and Larcher, 1987). These changes include expression of genes and activation of enzymes, which are crucial for cells to undergo biochemical and morphological changes leading to acquirement of resistance not only to low temperature but also to mechanical and osmotic stress imposed by growth of extracellular ice crystals (Thomashow, 1998).

Cold-acclimated plants typically accumulate substantial amounts of compatible solutes, such as soluble sugars (Ristic and Ashworth, 1993; Wanner and Junttila, 1999), amino acids (Lalk and Dörfflung, 1985; Wanner and Junttila, 1999) and glycine betaine (Kishitani et al., 1994), which are thought to play a role in protection of cells from freezing injury. Among these compatible solutes, soluble sugars

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are most commonly detected in various species of land plants that have undergone seasonal cold acclimation, including angiosperms, gymnosperms, and lower land plants such as bryophytes (Sakai, 1961; Rütten and Santarius, 1992).

Mono-, di-, and oligo-saccharides are most commonly found to be accumulated in the cold-acclimated plants. It is suggested that these low-molecular-weight sugars serve mainly as (1) osmolytes against hydraulic pressure across cellular membranes generated by extracellular ice crystals (Kacperska, 1993) and (2) protectants for cellular membranes and enzymes from irreversible damage caused by freezing (Strauss and Hauser, 1986; Anchordoguy et al., 1987; Crowe et al., 1987). Exogenous application of high concentrations of mono- and di-saccharides has been shown to increase freezing tolerance of plant cells. For instance, preincubation with glucose (**1**) increased survival after cryopreservation of cabbage leaf cells (Jitsuyama et al., 1997), and preincubation with sucrose increased survival after cryopreservation of Jerusalem artichoke cells (Harris et al., 2004). It has also been reported that exogenous application of sucrose (**3**) improves freezing tolerance of protoplasts in *Arabidopsis thaliana* (Uemura and Steponkus, 2003). The Arabidopsis *sfr4* mutant showed levels of freezing tolerance similar to those of wild-type (WT) plants when grown at ambient temperatures but, unlike WT plants, failed to increase freezing tolerance when cold-acclimated. The decreased freezing tolerance in the *frs4* mutant has been attributed to its reduced accumulation of glucose (**1**) and sucrose (**3**) (Uemura et al., 2003). These facts suggest critical roles for these sugars as compatible solutes in freezing tolerance.

In addition to mono- and di-saccharides, it has been demonstrated that the levels of a few oligosaccharides increase during cold acclimation, typically in cereals and woody plants, both of which develop high degrees of freezing tolerance in winter. Galactosyl-sucrose oligosaccharides such as raffinose and stachyose are commonly found to accumulate during seasonal cold acclimation in woody plants, along with development of freezing tolerance to levels so high that some plants can even tolerate to freezing in liquid nitrogen without cryoprotectants (Sauter et al., 1996). These oligosaccharides might be critical for protection of plant cells against severe dehydration caused by freezing, since these sugars are also known to be accumulated in developing seeds of higher plants that develop high degrees of desiccation tolerance during maturation (Koster and Leopold, 1988). Recent studies have indicated that transgenic plants over-accumulating raffinose have higher degrees of freezing tolerance than do wild-type plants (Taji et al., 2002; Pennycooke et al., 2003). It has also been reported that the *Arabidopsis* ecotype C24, which accumulates more raffinose (**4**) than does the Columbia ecotype, shows greater freezing tolerance than that of the Columbia ecotype (Klotke et al., 2004). Cereals, on the other hand, accumulate low-molecular-weight fructooligosaccharides (fructans), such as 1-kestose and nystose, during cold acclimation. Transgenic plants with larger amounts of fructans than those of wild-type plants exhibited improved freezing tolerance (Hisano et al., 2004).

The plant cold acclimation process often accompanies transient or sustained increase in endogenous levels of abscisic acid (ABA), a 'stress hormone' involved in various water-related stresses such as desiccation. Since exogenous application of ABA to plant cells provokes freezing tolerance without cold acclimation, ABA has been postulated to play a key role in the development of freezing tolerance in plants (Chen and Gusta, 1983). A few studies have shown that ABA treatment resulted in accumulation of sugars as well as freezing tolerance of plant cells (Orr et al., 1986; Barvo et al., 1998). Thus, increased ABA levels during cold acclimation might affect accumulation of soluble sugars that are necessary for protection of cells from freezing, although regulatory mechanisms of sugar accumulation by ABA have not been clarified.

We previously reported that protonema cells of the moss *Physcomitrella patens* grown under normal culture conditions have relatively low levels of freezing tolerance, with an $LT_{50}$ value, the lethal temperature causing 50% mortality, of around −2 °C. Treatment with 10 μM of ABA resulted in development of freezing tolerance in the cells with changes in $LT_{50}$ from −2 to −10 °C after one day and to over −15 °C after two days (Minami et al., 2003a; Nagao et al., 2005). These results indicated that ABA provoked dramatic changes in the intracellular environment leading to freezing tolerance in the protonema cells, similar to cold-acclimated higher plant cells. Our recent examination has revealed that the content of free soluble sugars drastically increased in response to ABA, along with rapid degradation of starch in chloroplasts and increases in cellular osmotic concentrations (Nagao et al., 2005). Analysis of sugar components resulted in identification of specific oligosaccharides that accumulated during ABA treatment in *P. patens* protonema cells. Here, we report results of structural analysis of major oligosaccharide that accumulates in close association with ABA-induced freezing tolerance.

## 2. Results

### 2.1. Changes in profiles of soluble sugars induced by ABA treatment

The effect of ABA on the profiles of soluble sugar composition in *P. patens* protonema cells in association with development of freezing tolerance was analyzed by thin-layer chromatography (TLC) (Fig. 1). The major component of sugars in non-treated cells was thought to be a disaccharide because it was mobilized to a position similar to that of sucrose. This disaccharide was indeed identified as sucrose (**3**) by subsequent high-performance liquid chromatography (HPLC) analysis following digestion with invertase giving rise to glucose (**1**) and fructose (**2**) (data not shown). Treatment with different concentrations of

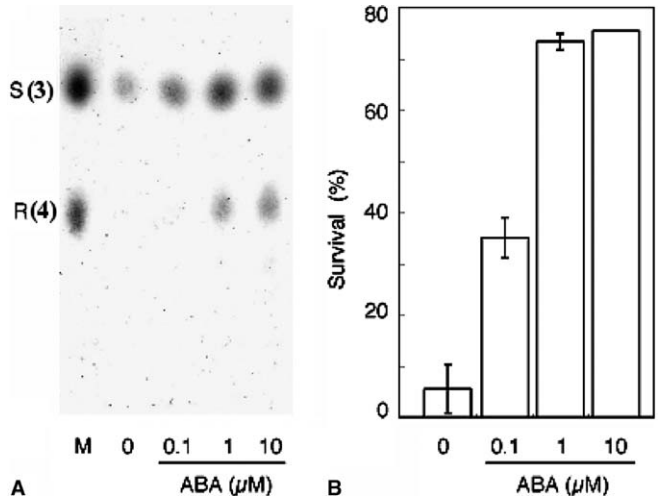


Fig. 1. Accumulation of soluble sugars in association with increase in freezing tolerance by treatment with ABA in moss protonema cells. (A) Total soluble sugars extracted by EtoH–$H_2O$ (4:1) were analyzed by thin-layer chromatography using $CH_3CN$–$H_2O$ (3:1) as solvent. Sugars from control cells (0) and cells treated for one day with 0.1, 1, and 10 μM ABA were analyzed. Sucrose (S) (**3**) and raffinose (R) (**4**) were used as markers (M). (B) ABA-induced freezing tolerance of the protonema cells. Protonema cells that had been treated for one day with 0.1, 1 or 10 μM ABA or not treated were frozen to −5 °C and thawed at 4 °C overnight. Survival of the cells was determined by measurement of electrolyte leakage from damaged cells.

ABA resulted in increase in freezing tolerance and changes in sugar accumulation profiles. ABA increased the amount of sucrose (**3**) and also induced accumulation of a putative oligosaccharide mobilized to a position similar to that of the trisaccharide raffinose (**4**).

We analyzed ABA-induced changes in sugar compositions by HPLC. The results shown in Fig. 2A are sugar composition profiles of non-treated protonema cells and cells that had been treated for one day with 10 μM ABA. The analysis resulted in identifications of two refraction peaks corresponding to putative oligosaccharides of which levels were dramatically increased by ABA. Of these, a putative trisaccharide (**6**) (peak 2) was so prominent that its levels, which were very low in non-treated cells, exhibited dramatic increase by 10 μM ABA treatment for one day (*peak 2* shown in Fig. 2A). Its HPLC retention time was distinct from that of known stress-induced trisaccharides, i.e., raffinose (**4**) and 1-kestose. ABA treatment also increased levels of sucrose (**3**), which already existed in the non-treated control cells.

We previously reported that the freezing tolerance of *P. patens* protonema cells was increased to its maximal level after 2 days of 10 μM ABA treatment (Nagao et al., 2005). Time course analysis of sugar accumulation indicated that the putative trisaccharide (**6**) reached maximum level after 2 days, while sucrose (**3**) levels were saturated within one day (Fig. 2B). The increase in the putative trisaccharide (**6**) accumulation induced by ABA was 13-fold after one day and 30-fold after 2 days. In contrast, the

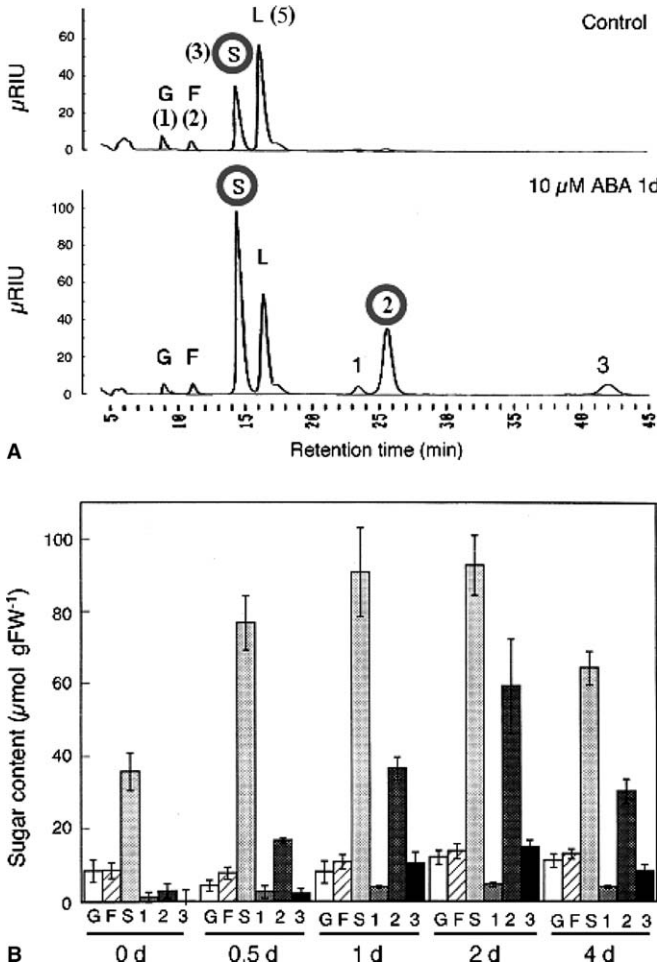


Fig. 2. HPLC analyses of sugar components in *P. patens* protonema cells. The cells were treated with 10 μM ABA and sugars extracted with 80% ethanol were analyzed by HPLC. (A) Chromatograms of sugars extracted from control cells (upper panel) and cells treated with ABA for one day (lower panel). Area of peaks indicates magnitude of refractive index. G, glucose (**1**); F, fructose (**2**); S, sucrose (**3**); L, lactose (**5**) (internal standard). Putative oligosaccharides are indicated by 1–3. The letters representing sugars that accumulated in the ABA-treated cells are circled. (B) Time-dependent accumulation of soluble sugars during ABA treatment determined by HPLC. Sugars from protonema cells treated with ABA for the indicated time periods were quantified by analysis of HPLC peaks ($n = 3$). G, F, S, 1, 2 and 3 correspond to those shown in panel A.

increase in sucrose (**3**) accumulation induced by 10 μM ABA treatment was as much as 2.5-fold in comparison with non-treated control cells.

### 2.2. Chemical analyses of the trisaccharide induced by ABA treatment

In order to understand molecular features of the trisaccharide induced by ABA, we purified this trisaccharide (**6**) and determined its chemical structure. Mass spectrometry of trisaccharide (**6**) revealed that it has a molecular weight of 504. Hydrolysis to monosaccharides by trifluoroacetic acid (TFA) resulted in detection of glucose (**1**) and fructose (**2**) at an approximate ratio of 2:1 as revealed by TLC.

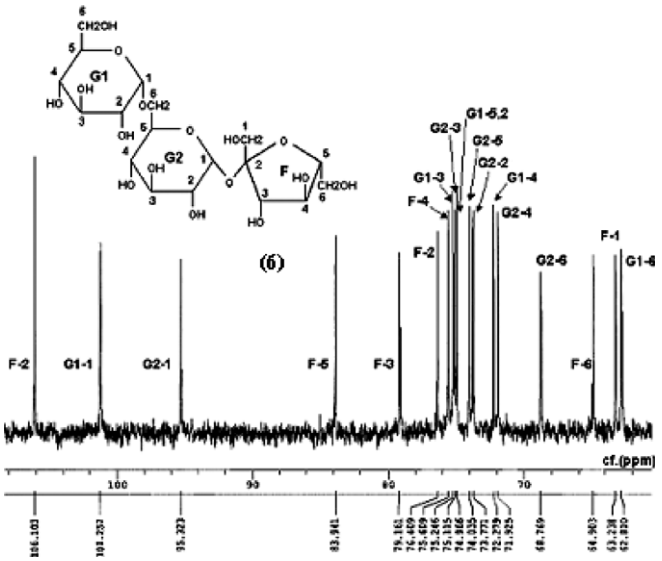


Fig. 3. $^{13}$C NMR spectrum of the ABA-induced trisaccharide (**6**), showing 18 carbon signals. Of these, four signals, G1-1, G2-1, G2-6 and F-2, shifted toward the lower magnetic field, indicating that they are involved in inter-linkage between monomers. The chemical shifts of all carbon signals were identical to those of authentic theanderose (**6**).

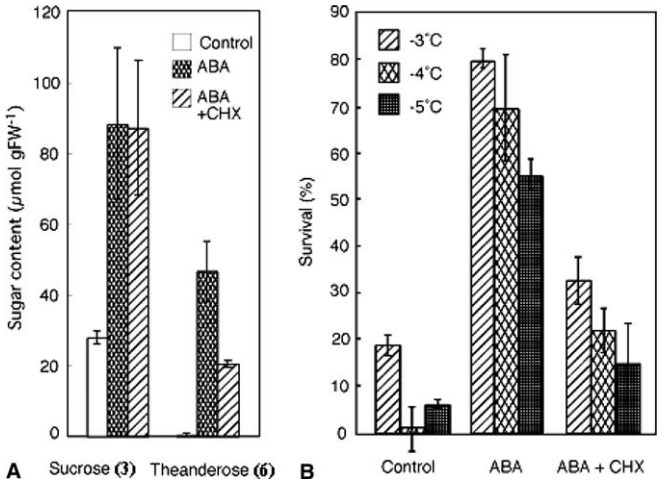


Fig. 4. Effect of protein synthesis inhibitors on ABA-induced sugar accumulation. Cultured protonema cells were pre-incubated for 1 h with 5 μM cycloheximide (CHX) and then transferred onto a medium containing 1 μM ABA and the same concentration of CHX. After one-day treatment at 25 °C, the cells were used for sugar component analysis by HPLC (A) and freezing test (B) as described in the legends of Figs. 1 and 2. The results were compared with those of protonema cells untreated (control) or treated with 1 μM ABA only (ABA).

Digestion by 0.5 M acetic acid at 80 °C for 1 h followed by gas chromatography analysis indicated that it has an isomaltose moiety (data not shown). Further analysis by $^{13}$C NMR spectroscopy resulted in its identification as theanderose (**6**) ($G^6$-α-glucosyl sucrose) (Fig. 3). The structure was also confirmed by $^1$H NMR spectroscopy and methylation analyses (data not shown).

### 2.3. Production of theanderose through de novo synthesis of proteins

We previously found that the ABA-induced increase in freezing tolerance of *P. patens* protonema cells is effectively inhibited by cycloheximide (CHX) but not by chloramphenicol or lincomycin, indicating that the cells require de novo synthesis of nuclear-encoded proteins for development of freezing tolerance (Minami et al., 2003b). Thus, the effect of cycloheximide (CHX) on ABA-induced accumulation of sugar composition was examined. The results indicated that accumulation of theanderose (**6**), but not that of sucrose (**3**), was inhibited by 5 μM CHX, which effectively reduced ABA-induced freezing tolerance (Fig. 4A and B). These results indicated that ABA-induced production of theanderose (**6**) requires de novo synthesis of nuclear-encoded proteins.

### 2.4. Effects of cold, hyperosmosis and salt stress on theanderose production

We analyzed the effects of environmental stress conditions (i.e., cold and osmotic stress) on accumulation of soluble sugars. Freezing tolerance of *P. patens* protonema cells has been shown to be increased by cold treatment for several days without ABA, although the tolerance levels achieved by cold treatment for 7 days were much lower than those achieved by ABA treatment for one day (Minami et al., 2005). Examination of the effects of cold treatments on accumulation of sugars revealed that cold-increased freezing tolerance accompanied accumulation of sucrose (**3**) and theanderose (**6**) (Fig. 5). Acclimation

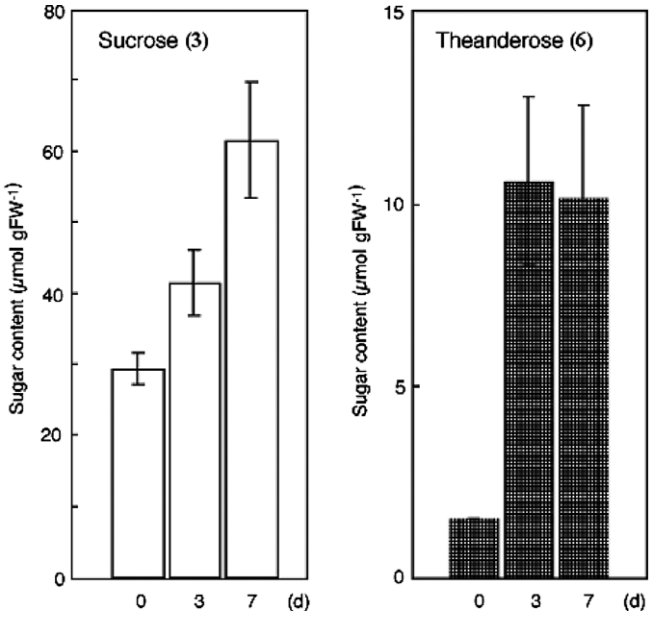


Fig. 5. Effect of incubation at low temperature on accumulation of soluble sugars in *P. patens* protonema cells. Protonema cells cultured at 25 °C were transferred onto a fresh medium and incubated at 0 °C for the indicated time periods. Soluble sugars were extracted and analyzed by HPLC as described in the legend of Fig. 2.

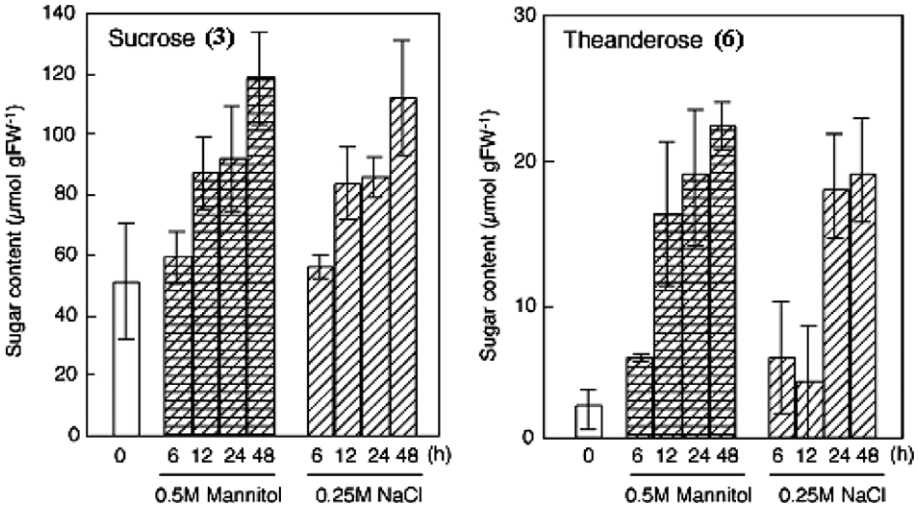


Fig. 6. Effect of hyperosmotic solutes on accumulation of soluble sugars in *P. patens* protonema cells. Protonema cells cultured on the BCD medium were transferred onto a medium containing either 0.5 M mannitol or 0.25 M NaCl and cultured for the indicated time periods under the same light and temperature conditions. Soluble sugars in the cells were analyzed by HPLC as described in the legend of Fig. 2.

at 0 °C for one week resulted in accumulation of sucrose and theanderose to 61 and 10 μmol $gFW^{-1}$, respectively. We also examined accumulation of the sugars during treatments with hyperosmotic concentrations of mannitol and NaCl, which have been shown to increase freezing tolerance of protonema cells (Minami et al., 2003a). Fig. 6 shows that treatment with both of the solutes resulted in increased accumulation of both sucrose (**3**) and theanderose (**6**). The levels of sucrose (**3**) after 2-day treatment with these solutes were in the range of 112–119 μmol $gFW^{-1}$, while the levels of theanderose were 19–22 μmol $gFW^{-1}$.

## 3. Discussion

Mosses do not possess established epidermal tissues and they cannot retain water under water-stress conditions. However, mosses are in general known to have cells with high stress tolerance. For instance, moss species growing in a natural environment with severe winter possess high degrees of tolerance to freezing and dehydration, allowing several species to dominate in habitats where higher plants can hardly grow, such as some areas of Continental Antarctica (Robinson et al., 2000; Burch, 2003). However, the mechanisms by which these mosses acquire high degrees of freezing tolerance are not understood. Mosses growing in various European habitats have also been shown to possess substantial degrees of freezing tolerance, and it has been shown that these moss species exhibit higher degrees of freezing tolerance in winter than in summer (Rütten and Santarius, 1992). Increase in freezing tolerance in these mosses appeared to be associated with accumulation of soluble sugars, especially that of sucrose (**3**), indicating that it might play a protective role in winter survival. However, it has been shown that variations in the levels of sucrose (**3**) in different parts of tissues are not necessarily correlated with extents of freezing tolerance in the moss *Plagiomnium* species, indicating that the sucrose (**3**) level might not be a critical determinant of freezing tolerance (Rütten and Santarius, 1993). The role of other sugar species such as oligosaccharides in freezing tolerance in moss species has not been determined.

In this study, we identified the trisaccharide theanderose as a compatible solute that accumulated in close association with development of freezing tolerance in *P. patens* protonema cells. The amount of sucrose (**3**) was also increased, but it reached almost maximal level between 12 and 24 h, to which time freezing tolerance had not reached the maximal level. Theanderose (**6**), on the other hand, gradually accumulated in ABA-treated protonema cells and reached maximum level, accounting for 31% of total soluble sugars on a weight basis, after 2 days, at which time the cells had acquired maximum levels of freezing tolerance with an $LT_{50}$ value below −15 °C (Fig. 2) (Nagao et al., 2005). Considering that the amount of sucrose (**3**) is 48% of the total soluble sugars, theanderose (**6**) and sucrose (**3**) are major compatible solutes of which amounts are increased by ABA in *P. patens*. We previously showed that one-day treatment of *P. patens* protonema cells with 10 μM ABA increases osmotic concentration of the cells from 0.3 osmol to around 0.5 osmol and that the extent of cellular dehydration caused by extracellular ice crystals was less severe in ABA-treated cells than in non-treated cells (Nagao et al., 2005). Accumulation of sucrose (**3**) and theanderose (**6**) in ABA-treated cells indicates that both sugars might have served as osmoprotectants against hydraulic pressure imposed by growth of extracellular ice crystals under freezing conditions.

Sucrose (**3**), glucose (**1**), fructose (**2**), and oligosaccharides such as raffinose (**4**) and stachyose are commonly

found soluble sugars that accumulate along with development of freezing tolerance in higher plants. It has been suggested that these sugars not only serve as osmoprotectants but also play a role in protection of cellular membranes by interacting with the lipid bilayer (Anchordoguy et al., 1987; Crowe et al., 1987, 1988). The roles of oligosaccharides in cellular freezing tolerance are not clearly understood, though their accumulation is characteristic to plants that develop high degrees of freezing tolerance such as cold-acclimated woody plants (Sauter et al., 1996).

Oligosaccharides have been proposed to play physiological roles in desiccation tolerance in seeds of higher plants. In imbibed seeds of soybean, pea and corn, loss of desiccation tolerance was associated with decreased levels of sucrose and oligosaccharides such as raffinose (**4**) and stachyose (Koster and Leopold, 1988). It is proposed that the role of these oligosaccharides is to prevent crystallization of sucrose (**3**), thus facilitating glass formation, or vitrification, within the cell, leading to protection of membrane phospholipids (Williams and Leopold, 1989; Koster, 1991). It has been shown by Smythe (1967) that sucrose-derivative oligosaccharides possessing an $O^6$-glucosyl link such as raffinose (**4**), stachyose, gentianose, and neokestose, when doped in sucrose solution, alter legitimate growth of sucrose crystals. Theanderose (**6**), which also has a *link* in the glucose-6 position, has been reported to contribute to formation of a *c*-axis-elongated sucrose crystal (Morel du Boil, 1992). Thus, theanderose, which accumulated in association with enhancement of freezing tolerance in *P. patens* protonema cells, might play a role in protection of cellular membranes under the condition of freezing-induced dehydration by promoting glass formation within the cells.

Experiments with CHX, revealed that ABA-induced accumulation of theanderose (**6**) requires protein synthesis of nuclear-encoded genes (Fig. 4). CHX also had a negative effect on ABA-induced freezing tolerance. Interestingly, CHX did not inhibit ABA-induced accumulation of sucrose (**3**), a major sugar component in ABA-treated cells. This indicated that increased accumulation of sucrose (**3**) does not require protein synthesis and that the presence of sucrose (**3**) alone is insufficient for development of maximal freezing tolerance (Minami et al., 2003b). It is likely that theanderose (**6**) production is catalyzed by newly synthesized protein translated from mRNA induced by ABA treatment. Biosynthetic pathways for theanderose (**6**) production in plants are not understood. In vitro, theanderose (**6**) can be generated from sucrose (**3**) by transglucosylation reaction catalyzed by α-glucosidase (Nakao et al., 1994; Sugimoto et al., 2003). Considering that production of theanderose (**6**) requires de novo synthesis of proteins, it is likely that the genes for theanderose (**6**) production are induced by ABA. Our analysis of mRNA revealed that expression of two genes encoding α-glucosidase was dramatically increased by ABA treatment (data not shown). Catalytic activity of the gene products has not been determined.

Theanderose (**6**) is known to be present in small amounts in cane sugar and honey molasses. However, it is not known whether it had been synthesized in plants or had been made artificially during the sugar or honey processing. Moreover, none of its biological functions in plants have been described. Thus, the results shown in this paper provide evidence of its novel function in protection of cells from stress caused by freezing. Isolation of genes encoding the enzyme catalyzing transglucosylation reaction that gives rise to theanderose might lead to establishment of methods for generating transgenic plants with increased freezing tolerance.

## 4. Experimental

### 4.1. Plant material and treatments

Protonemata of *P. patens* were grown on cellophane-overlaid 0.8% agar plates of modified BCD medium (Ashton et al., 1979) in a controlled-environment growth chamber at 25 °C under continuous illumination (light intensity, 50–80 $\mu E/m^2/s$). Treatment of protonemata with ABA, low temperature, NaCl, and mannitol was carried out as described previously (Minami et al., 2003a). For treatments with protein synthesis inhibitors, protonemata were preincubated in a liquid BCD medium containing either 10 μM cycloheximide or 100 μM chloramphenicol for 1 h at 25 °C, transferred onto an agar plate of BCD medium containing the same concentrations of inhibitors and ABA, and further incubated at 25 °C for one day.

### 4.2. Measurement of freezing tolerance

Freezing tolerance was estimated by equilibrium freezing of protonema cells, followed by measurement of electrolyte leakage as described by Minami et al. (2003a) with slight modifications. Colonies of protonema tissues were placed in the bottom of a test tube containing 0.5 mL of distilled $H_2O$ and the tubes were placed in a cooling bath (type F26, Julabo, Germany). The tubes were kept at −1 °C for 10 min, seeded with ice, and then further kept at −1 °C for 1 h. The tubes were then cooled at a rate of 0.04 °C $min^{-1}$. At desired temperatures, the tubes were removed from the bath and thawed overnight at 4 °C in the dark. Distilled $H_2O$ (2 mL) was added to each tube, and the tubes were gently shaken for 1 h at room temperature in the dark. Electrolyte leakage from the tissues was then measured using a conductivity meter. Electrolyte leakage from unfrozen tissues and tissues freeze-thawed twice by liquid nitrogen were also measured. All of the tissues were then boiled for 10 min and shaken again for 2 h to measure amounts of total cellular electrolytes. Percent survivals of the freeze-thawed cells were calculated by assuming that electrolyte leakage from unfrozen cells was 0% and that from cells frozen twice at liquid nitrogen was 100%.

### 4.3. Analysis of sugars by high-performance liquid chromatography

Protonema tissues were homogenized at 0 °C in 5 mL of EtoH–$H_2O$ (4:1) with 20 mM lactose (0.5 mL) as an internal standard. The homogenate was centrifuged at 14,000*g* for 5 min at 4 °C, and the supernatant was recovered. After evaporation of ethanol, the samples were dissolved in distilled $H_2O$ (10 mL) and then freeze-dried for storage. The freeze-dried samples were dissolved in distilled $H_2O$ (0.5 mL) and centrifuged at 14,000*g* for 5 min at 4 °C to remove any debris before column injection. Sugars were analyzed by HPLC using the $NH_2$P-50 4E column (Shodex, Japan) at 30 °C with $CH_3CN$–$H_2O$ (3:1) (Kanto Chemical, Japan) as a mobile phase. Peaks of sugars were detected by an RID-6A refractive index detector (Shimadzu Co., Japan). Fractions of sugar samples were collected, and the collected fractions were subjected to further analysis.

### 4.4. Thin-layer chromatography

Sugar samples extracted by EtoH–$H_2O$ (4:1, v/v) were dried, dissolved in water, and subjected to ascendant chromatography on a thin-layer silica gel plate. The samples were developed by $CH_3CN$–$H_2O$ (3:1). The mobilized sugar spots were visualized by spraying 50% $H_2SO_4$ in EtoH supplemented with 0.1% (v/w) 1-naphtrol followed by baking at 110 °C. For mono-sugar analysis, the sugar samples were hydrolyzed by trifluoroacetic acid (TFA) at 110 °C and analyzed by TLC using $CH_3CN$–$H_2O$ (85:15).

### 4.5. NMR analyses

HPLC-purified sugar samples were dried, dissolved in deuterium oxide (1 mL, D, 99.9%, Cambridge Isotope Laboratories, MA), and used for NMR analyses. The NMR spectra were recorded using an FT NMR spectrometer (model JMN-AL300, JOEL, Tokyo, Japan) operating at 300.4 MHz for $^1H$ and 75.45 MHz for $^{13}C$. The $^1H$ and $^{13}C$ chemical shifts were calibrated against the $^1H$ and $^{13}C$ signals of 3-(trimethylsilyl)-1-propane-sulfonic acid sodium salt (Aldrich, St. Louis, MO) dissolved in the sugar samples, respectively. The NMR scans of this analysis were carried out at 160 for $^1H$ NMR and 16,000 for $^{13}C$ NMR, respectively. The $^1H$ and $^{13}C$ signal assignments of theanderose were done in comparison with previous data obtained by using authentic theanderose (Wako Pure Chemicals, Japan).

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2006.01.031.

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