Title

Confocal cryomicroscopic analysis and cryodynamics of endoplasmic reticulum in herbaceous plant cells

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ABSTRACT

Plants that survive subzero temperatures in winter enhance their freezing tolerance upon exposure to non-freezing temperature, a phenomenon termed cold acclimation. Although the mechanism of enhanced freezing tolerance has been widely examined at the molecular level, the understanding of cell physiological aspects remains poor. In our previous study, confocal cryomicroscopic observation of leaf epidermal cells of *Allium fistulosum*, the cold-hardy Welsh onion, showed that extracellular freezing seems to induce cessation of streaming and development of vesiculation of the endoplasmic reticulum (ER). Further analysis of this unique cryobehavior of ER may lead to a better understanding of how plant cells survive freezing. We confirmed that ER vesiculation occurred in root cells of *Arabidopsis thaliana*, which suggested that freeze-induced vesiculation of ER might be conserved in herbaceous plants. Because the ER membrane dynamics in Welsh onion cells were more clearly observed than those in *Arabidopsis* cells, the Welsh onion system was used for more detailed analyses. Average velocities of ER membranes were quantitatively analyzed and resultant Arrhenius plots showed that extracellular freezing rapidly decreased ER streaming, which had ceased completely at -5°C in both non-acclimated and cold-acclimated cells. After thawing, only in cold-acclimated cells, ER networks quickly recovered at 2°C . Furthermore, during supercooling at -5°C , only ER of cold-acclimated cells maintained streaming and networks. These results indicated that ER networks in cold-acclimated cells are more stable at low temperature, and extracellular freezing results in the breakdown of cold-stable ER networks in cold-acclimated cells. One possibility is that the breakdown of ER networks is involved in an enhanced freezing tolerance mechanism.

Keywords: freezing tolerance, confocal cryomicroscopy, endoplasmic reticulum, cryodynamics, herbaceous plants

1. Introduction

Freezing tolerance is an important feature in plants living in areas that experience subzero temperatures in winter, and the cold acclimation process enhances freezing tolerance (Levitt, 1980). Many physiological changes during cold acclimation that are supported by

gene expression are necessary for plant cells to tolerate freezing and to overwinter (Steponkus et al., 1993, Thomashow, 1999, Seki et al., 2002, Yamazaki et al. 2008a, Minami et al., 2009, Takahashi et al., 2012). Many studies of cold acclimation in a wide range of plants have helped to understand freezing tolerance and overwintering mechanisms. Many reports in this research area have been published in *Environmental and Experimental Botany* (for example, Hekneby et al., 2006, Repo et al., 2008, Walker et al., 2008, Charrier and Améglio, 2011, Pagter et al., 2011). Although considerably less frequent than genetic and biochemical reports, cryomicroscopy studies are important to elucidate how plant cells survive low temperature and freezing because they provide vital visualization of the mechanistic response.

Several cryoelectron microscopy studies have shown that abnormal structures, distinct from the lamellar phase, appear in the plasma membrane during freezing (Steponkus et al., 1993, Fujikawa et al., 1999). On the basis of these results, when freeze-induced dehydration causes the plasma membrane and the internal endomembranes to come into close apposition, both membranes are hypothesized to undergo a transition into abnormal structures. Given that the survival frequency during freezing stress is negatively correlated with the frequency of appearance of abnormal structures in the plasma membrane, the plasma membrane is thought to be the primary site of injury induced by extracellular freezing (Steponkus et al., 1993, Fujikawa et al., 1999). An additional cryoelectron microscopy study reveals that when freezing-tolerant mulberry cortical parenchyma cells are frozen, for example at -5°C , multiplex lamellae (MPL) are formed by the fusion of endoplasmic reticulum (ER) vesicles (Fujikawa and Takabe, 1996). The MPL completely cover the area beneath the plasma membrane and are composed of a parallel array of sheet-like ER cisternae. This cryodynamic process is completed within 10 min of freezing at -5°C and is quickly reversed upon thawing. Although freeze-induced formation of MPL is hypothesized to play a role in avoiding the close apposition of membranes, including the plasma membrane (Fujikawa and Takabe, 1996), the actual role remains unknown.

Living cells cannot be observed with an electron microscope (for example, cells must be fixed chemically or by other means), which presents a limitation in electron microscopic approaches to the understanding of how living cells survive freezing. In modern biology, confocal microscopic techniques are essential to reveal the dynamics of membranes and proteins in living cells, which are stained with a fluorescent dye or tagged with a fluorescent protein. Thus, to determine how plant cells survive freezing, live-cell imaging with a confocal

microscopic system is an essential approach. In our previous studies, we sought to combine confocal microscopy with a cryostage, which allowed more detailed observation of membranes, for example ER and the plasma membrane, when using specific fluorescent dyes (Yamazaki et al., 2008b, Tanino et al., 2013). In addition, we used *Allium fistulosum*, the cold-hardy Welsh onion, as a model system to examine the mechanism of freezing tolerance in cold-hardy plants (Tanino et al., 2013). Cold-acclimated *A. fistulosum* can survive below -40°C and the sheath epidermis consisting of a single cell layer can be easily separated by hand as an intact sheet from the leaf.

In the present study, we focused the cryodynamics of ER. In eukaryotic cells, the ER is a continuous membrane system including cisterna and tube forms, and ER tubes interconnect to form a reticular network (Shibata et al., 2006, Du et al., 2004). Furthermore, in plant cells, the dynamic streaming of ER networks has been observed in subperipheral cytoplasm and transvacuolar strands (Ueda et al. 2013). Interestingly, we observed previously that freeze-induced cell deformation seemed to stop ER streaming and ER vesiculation subsequently occurred via the breakdown in the ER network (Tanino et al., 2013). By contrast, in woody plants small vesicles originating from smooth ER are altered into MPL in a process of extracellular freezing (Fujikawa and Takabe, 1996). In the present study, we focused on the cryodynamics of ER membranes. We investigated whether freeze-induced ER vesiculation also occurs in *Arabidopsis* root cells as a representative dicotyledon. Our cryomicroscopic observations led to important novel findings. i) The ER network in cold-acclimated cells is more stable and active at low temperature. ii) Extracellular freezing causes the breakdown of cold-stable ER networks in cold-acclimated cells. And iii) upon thawing, cold-acclimated cells recover ER streaming velocity more rapidly than non-acclimated cells. These physiological phenomena may be important for plant cells to survive freezing and to overwinter.

2. Materials and methods

2.1. Plant materials

As previously described (Tanino et al., 2013) with slight modifications, seeds of Welsh onion (*A. fistulosum*), collected from plants growing outdoors at Iwate University, Morioka,

Japan, were germinated at 22°C under continuous light at 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in vermiculite supplemented with modified Hoagland solution (Uemura et al., 1995). After growth for 4 months, plants were cold-acclimated by exposure to 2°C for 4 weeks under a 12 h photoperiod at 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$. For observation of ER membranes in the root of *Arabidopsis thaliana*, we used a transgenic line (GFP-HDEL) expressing the green fluorescent protein (GFP) and an ER-retention signal, HDEL (Hayashi et al., 2001, Matsushima et al., 2003). Seeds of transgenic plants were planted on half-strength Murashige and Skoog medium with 0.8% agar and grown at 23°C for 10 days under a 16 h photoperiod at 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Plants were cold-acclimated by exposure to 2°C for 7 days under a 12 h photoperiod at 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

2.2. Visualization of ER membranes

The ER membranes of Welsh onion cells were stained with 1 μM ER-Tracker™ Green solution (Molecular Probes, Eugene, OR, USA), dissolved in buffer containing 1 mM MES/KOH (pH 5.6) and 1 mM CaCl_2 , by immersing the epidermal layer of both non-acclimated and cold-acclimated samples in the solution for 20 min at room temperature, followed by washing three times in the buffer solution without ER-Tracker. To confirm that only living cells were observed during freezing, we used the FM4-64 lipophilic fluorescent dye (Molecular Probes), which strongly stains all endomembranes only when the plasma membrane is irreversibly broken. Samples that were stained with ER-Tracker were immersed in buffer containing 1 mM MES/KOH (pH 5.6), 1 mM CaCl_2 and 8 μM FM4-64. Samples were observed with a confocal laser scanning microscopy with the emission filters of Em01-R488-25 for ER-Tracker and GFP and Em01-R405/568-25 for FM4-64 (Semrock, Rochester, NY).

2.3. Confocal cryomicroscopy

All confocal microscopic observations were performed on a cryostage (THMS600, Linkam, Guildford, Surrey, UK) using a microscope (BX-61, Olympus, Tokyo, Japan) fitted with a confocal scanning unit (CSU 10, Yokogawa, Tokyo, Japan) as previously described (Tanino et al., 2013). Images were captured with the image analysis software SlideBook

(Intelligent Imaging Innovations, Denver, CO USA). The temperature was lowered at a rate of $1^{\circ}\text{C min}^{-1}$ from 20°C to 0°C and $0.08^{\circ}\text{C min}^{-1}$ from 0°C to -15°C with ice-nucleation of the sample induced with an LN2 cooled aluminum peg around -0.5°C . When samples were observed under the supercooling condition, cooling was performed at $0.08^{\circ}\text{C min}^{-1}$ from 0°C to -5°C . For observation of the thawing process, samples were heated to 2°C at $1^{\circ}\text{C min}^{-1}$.

2.4. Analysis of average velocity of ER membranes

To quantitatively analyze the velocity of ER membrane movement, the KbiFlow plug-in, which is contained in the KBI plug-in package (available for free download at <http://hasezawa.ib.k.u-tokyo.ac.jp/zp/Kbi/ImageJKbiPlugins>), for ImageJ software (<http://rsb.info.nih.gov/ij/>) was used (Ueda et al., 2010). The KbiFlow plug-in, based on optical-flow analysis, makes it possible to estimate not only the velocity and direction, but also the distribution, of ER streaming in a wide field. After capturing 100 time-lapse images with 386×280 pixels ($0.491 \mu\text{m pixel}^{-1}$) at an interval of 1200 ms, a sequence of time-lapse images was treated with the Gaussian smoothing filter (SD: three frames) along the temporal axis, and the first and last images were removed. The KbiFlow plug-in then generates a velocity map by calculating the average velocity of 16×16 pixels in 98 time-lapse images.

3. Results

3.1. Cryodynamics of ER membranes

In general, at non-freezing temperatures, ER membranes formed a network and streamed in the cytoplasm. During freezing, ER networks collapse and many freeze-induced ER vesicles (FIERVs) are observed in both non-acclimated and cold-acclimated cells (Tanino et al., 2013), (for example, of the development of FIERVs in cold-acclimated onion cells, Fig. 1A and B and Supplementary Video S1). To investigate occurrence of this phenomenon in other plant species without specific fluorescent dyes, we used transgenic GFP-HDEL *Arabidopsis* plants. In these transgenic plants, ER membranes were observed as ER bodies of spindle-form rather than ER networks (Hayashi et al., 2001). We now confirmed the presence of ER bodies in both non-acclimated and cold-acclimated root cells before freezing (Fig. 1C to E). During freezing

1 at -3°C , ER bodies were greatly reduced, and many vesicles appeared in both non-acclimated
2 and cold-acclimated cells of *Arabidopsis* roots, which seemed to be comparable to FIERVs of
3 onion epidermal cells (Fig. 1F to H). Thus, FIERV formation is not a phenomenon specific to
4 Welsh onion cells. Because the ER membrane dynamics in Welsh onion cells were more
5 clearly observed than those in *Arabidopsis* cells, the Welsh onion system was used for more
6 detailed analyses.

7 In our previous report, when temperature gradually decreased and freezing started at
8 -0.5°C , the velocity of ER streaming rapidly decreased just after the freeze-induced cell
9 deformation, which occurred at -1.8°C , and ER streaming ceased completely at -4°C (Tanino
10 et al., 2013). Many FIERVs occurred simultaneously with cessation of ER streaming, and
11 therefore the decrease in velocity of ER streaming was expected to result in formation of
12 FIERVs. On the other hand, it was unclear whether freezing induced the cessation of ER
13 streaming, because the velocity of ER streaming decreased as temperature dropped. Thus, the
14 average velocity of ER membranes at each specified temperature was quantitatively analyzed
15 with the KbiFlow plug-in package for ImageJ software (Ueda et al., 2010).

16 One-hundred time-lapse images captured at intervals of 1200 ms were necessary to
17 calculate the average velocity of ER membranes in the captured area, and therefore the
18 temperature was temporarily held at each specified temperature only when images were
19 captured. Temperature gradually decreased from 20°C and freezing started by -1°C . The
20 images at -1°C were captured after freeze-induced cell deformation was confirmed. As shown
21 in Fig. 2A, the velocities of ER membranes in non-acclimated cells were almost the same as
22 those of cells cold-acclimated for 4 weeks over a wide range of temperatures, and both ER
23 velocities gradually decreased as temperature decreased. The temperature-dependent decrease
24 in velocity did not appear to differ before and after freezing. However, the Arrhenius plot
25 showed a distinct break in that the velocity of ER membranes rapidly decreased after freezing
26 (Fig. 2B). Although the velocities of ER membranes were measured up to -15°C , the values
27 below -5°C were almost constant (data not shown). Time-lapse images showed that some
28 FIERVs slightly fluctuated at the same place even after freezing, and therefore the velocity
29 values were not zero even below -5°C , even though the streaming of ER membranes seemed to
30 completely stop in time-lapse images (Supplementary Video S1). The velocity analysis of ER
31 membranes confirmed that freezing rapidly decreased ER streaming, which completely
32 stopped by -5°C .

3.2. Dynamics of ER membranes after freeze-thawing

We observed morphological changes of FIERVs at 2°C after thawing from -15°C. In this experiment, we observed only living cells that were confirmed with FM dye staining, which can strongly stain all endomembranes only when the plasma membrane is irreversibly broken. This is because the survival frequency of non-acclimated onion cells at -15°C, which was evaluated by FM dye staining, is about 50% (Tanino et al., 2013). Interestingly, the membrane dynamics in cold-acclimated cells was remarkably different from that in non-acclimated cells. To clearly show the time-dependent morphological changes of ER membranes after thawing, three images at different time points were taken with three different colors of red, green and blue, respectively, and subsequently the three images were merged. In the merged image, the ER membranes that did not move were colored white. For example, images of non-acclimated cells captured at 58, 59 and 60 min after thawing were colored red, green and blue, respectively, but after merging the three images only white pixels were observed (Fig. 3A), which showed that ER membranes did not move in non-acclimated cells even at 60 min after thawing. In cold-acclimated cells, however, FIERVs started to move just after thawing at 1 min, because some ER membranes were colored in the merged images at 1, 2 and 3 min after thawing. In addition, colored network structures were observed in the merged images at 10, 11 and 12 min after thawing, thus ER streaming gradually recovered and simultaneously FIERVs reformed ER networks within 10 min (Fig. 3B). Within 60 min after thawing, the ER dynamics appeared to recover to almost the same level observed before freezing. To quantitatively analyze this phenomenon, the average velocity of ER membranes was calculated using the images captured at 2°C from 60 min after thawing (Fig. 4). Whereas the velocity in non-acclimated cells was at the same level at -5°C, that in cold-acclimated cells was at the same level at about 2°C before freezing (compare Figs. 2A and 4).

3.3. Dynamics of ER membranes during supercooling

To further study the temperature sensitivity of ER networks and separate the response to low temperature from the freezing process, ER membranes were observed during supercooling at -3°C and -5°C. In non-acclimated cells, even at -3°C, supercooling resulted in the collapse of ER networks and the formation of many small ER vesicles (Fig. 5). In cold-acclimated cells, however, ER networks were maintained and no small vesicles were

observed even at -5°C . The average velocities of ER membranes during supercooling were compared with those during freezing. In both non-acclimated and cold-acclimated cells, the velocities in the supercooling condition decreased more gradually than those in the freezing condition as temperature decreased (Fig. 6). Furthermore, during supercooling, the velocities in cold-acclimated cells decreased more gradually than those in non-acclimated cells, in that the velocities in non-acclimated and cold-acclimated cells at -5°C were 67% and 86% of the value at 0°C , respectively, although the p-value of T-test for this difference was ca. 0.07. The results of Fig. 5 and 6 indicated that the temperature sensitivity of ER networks differed between non-acclimated and cold-acclimated cells, and the ER network in cold-acclimated cells was more cold-stable.

4. Discussion

In the present study, we confirmed that freeze-induced vesiculation of ER membranes was not a phenomenon specific to Welsh onion (Fig. 1). It is probable that the phenomena observed cryomicroscopically in Welsh onion are similarly observed in a wide range of herbaceous plant cells, although, of course, more observations in other plants are needed. Given that Welsh onion cells show significant cold acclimation (Tanino et al., 2013) and the large cells in the single epidermal layer are clearly and easily observed even during freezing, Welsh onion represents an ideal experimental system to investigate how herbaceous plant cells survive and overwinter during freezing. Our cryomicroscopic observations led to several important novel findings. i) The ER network in cold-acclimated cells is more stable and active at low temperature. ii) Extracellular freezing results in the breakdown of cold-stable ER networks in cold-acclimated cells. And iii) upon thawing, cold-acclimated cells recover ER streaming velocity more rapidly than non-acclimated cells.

Although the cryodynamics of ER membranes in cold-acclimated cells were seemingly similar to those in non-acclimated cells during the cooling and freezing processes (Fig. 2), ER networks in non-acclimated cells but not in cold-acclimated cells could not be recovered at 2°C after freeze-thawing (Fig. 3). One possibility for this phenomenon is that freezing and/or thawing causes irreversible injury to non-acclimated cells, and consequently the cells cannot recover ER networks at 2°C . However, this was not the case, because we observed only cells in which the plasma membrane integrity was maintained even after freeze-thawing.

1 In addition, we confirmed that ER networks in non-acclimated cells were recovered at room
2 temperature after freeze-thawing (data not shown). On the other hand, even if the plasma
3 membrane is intact, it is possible that the ER networks in non-acclimated cells are more
4 cold-unstable and, consequently, are reversibly damaged. Thus, another possibility is that the
5 difference in ER network recovery between non-acclimated and cold-acclimated cells is
6 related to a difference in the cold-stability of ER networks. In fact, supercooling experiments
7 clearly revealed that ER networks in cold-acclimated cells were more cold-stable than those
8 in non-acclimated cells (Figs. 5 and 6).

9 On the other hand, the cold-stable ER networks in cold-acclimated cells were
10 undoubtedly broken by extracellular freezing, but not by low temperature, because the
11 breakdown of ER networks was not observed in the supercooling condition (Fig. 5). Given
12 that the survival frequency of cold-acclimated cells is about 100% at -15°C in the
13 cryomicroscopic system (Tanino et al., 2013), the cryodynamics of ER membranes does not
14 result from irreversible freezing injury. These results indicate two possibilities: i)
15 cold-acclimated cells sense the extracellular freezing and its sensing is finally linked to the
16 breakdown of cold-stable ER networks; or ii) the extracellular freezing causes the reversible
17 injury of ER networks which can be quickly recovered after thawing. In either case, the
18 molecules which control the morphology and streaming of ER membranes are involved in the
19 cryodynamics of ER membranes.

20 It is known that the morphology and streaming of ER membranes are maintained by
21 many proteins, including cytoskeleton proteins and molecular motors, in a wide range of
22 eukaryotes (Dabora and Sheetz, 1988, Waterman-Storer and Salmon, 1998, Prinz et al., 2000,
23 Estrada et al., 2003). In plant cells, actin filaments are crucial for organization of the ER
24 network (Staehelin, 1997, Boevink et al., 1998, Tamura et al., 2005, Nakano et al., 2009), and
25 some membrane proteins, such as reticulon, GNL1/ERMO1 and SEC24a/ERMO2, may
26 regulate ER morphology (Nziengui et al., 2007, Tolley et al., 2008, Nakano et al., 2009). In
27 addition, ER streaming is driven primarily by the ER-associated myosin XI-K, a member of a
28 plant-specific myosin, and dynamic interactions among ER, actin filaments, and myosins
29 determine the morphology and streaming patterns of the ER membranes (Ueda et al., 2010).
30 In cells of the relatively non-cold-hardy onion *Allium cepa*, chilling treatment causes the
31 networks and strands of ER to transform into the vesicle-like structures, and pharmacological
32 studies indicate that these changes might result from the influence of low temperature on the
33 interaction of myosins and actin filaments (Quader et al., 1989). Thus, a better understanding

of how the stability of actin filaments is regulated before and after freezing is crucial to understanding the dynamics of ER membranes during freezing and freeze-thawing.

Some actin-binding proteins cause depolymerization of actin filaments. The actin depolymerizing factor (ADF)/cofilin protein family contributes to filament severing, and in *adf4* mutant cells the frequency of actin filament severing decreases and the actin filament length and longevity increase (Henty et al., 2011). Conversely, some actin-binding proteins increase the stability of actin filaments. In *Theilungiella salsuginea*, the dehydrins TsDHN-1 and TsDHN-2 stabilize the cytoskeleton under stress conditions, and furthermore, their phosphorylation is involved in this stabilization (Rahman et al., 2011).

In cold-acclimated cells, the cryodynamics of ER membranes may be related to actin-binding proteins and their regulating factors. Interestingly, in wheat the induction of an active ADF during cold acclimation is correlated with increased freezing tolerance (Ouellet et al., 2001). In addition, partial disruption of actin filaments may promote freezing tolerance during preservation of carrot cell suspensions in liquid nitrogen (Morisset et al., 1993). From the viewpoint of overwintering, the quick recovery of ER membrane dynamics after freeze-thawing is also important for plant cells to survive during winter, because it may enable the rapid recovery of many metabolic activities and, in early or late winter, there are many days in which the temperature increases above 0°C during the day. Although it remains unknown why the freeze-induced ML structures of ER membranes, which are observed in trees with cryoelectron microscopy (Fujikawa and Takabe, 1996), were not observed in herbaceous plants under the present experimental condition, in future studies elucidation of ER cryodynamics may result in greater clarity of the mechanism of enhanced freezing tolerance and overwintering.

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Figure legends

Fig. 1. Confocal microscopic observation of ER membranes in living cells during freezing.

ER membranes were observed in Welsh onion epidermal cells cold-acclimated (CA) for 4 weeks (A, B) ($n = 6$), and *Arabidopsis* root cells non-acclimated (C, D, F, G) ($n = 2$) or cold-acclimated for 7 days (E, H) ($n = 5$) before freezing at 2°C (A, C–E) or during freezing at –3°C (B, F–H). Temperature was decreased at the rate of 0.08°C min^{–1}. ER membranes were labeled with the specific fluorescent dye ER-Tracker Green, or with GFP and the ER-retention signal HDEL. Bar indicates 10 µm.

Fig. 2. Quantitative analyses of ER dynamics during cooling and freezing. In non-acclimated (NA) and 4-week cold-acclimated (CA) Welsh onion epidermal cells, ER membranes were observed as temperature gradually decreased from 20°C. The cooling rate was 1°C min^{–1} from 20°C to 0°C and 0.08°C min^{–1} from 0°C to –15°C. To allow the capture of 100 time-lapse images, the temperature was temporarily maintained at each specified temperature. Freezing had started when temperature decreased to about –1°C, and the images at –1°C were captured after freeze-induced cell deformation was confirmed. The average velocities of ER membranes were quantitatively analyzed with the KbiFlow plug-in for ImageJ software (A), and an Arrhenius plot was constructed from these kinetic values (B). In panel A, each point represents the mean ± s.e. ($n = 4–5$).

Fig. 3. Dynamics of ER membranes after freeze-thawing. Morphological changes of ER membranes at 2°C after thawing from freezing at –15°C were observed. After reaching –15°C, samples were heated to 2°C at 1°C min^{–1} and held at 2°C. A) To show the time-dependent morphological changes of ER in non-acclimated (NA) cells at 60 min after thawing, three images captured at 58, 59 and 60 min were colored with red, green and blue, respectively, and then the three images were merged. The picture before freezing are shown as a control. B) In cells cold-acclimated (CA) for 4 weeks, morphological changes of ER at 1, 10, 30 and 60 min after thawing are shown by merging three differently colored images. The picture before freezing are shown as a control. Both observations of NA and CA cells were performed 6 times, respectively. Bars indicate 20 µm.

Fig. 4. Quantitative analyses of ER dynamics after freeze-thawing. Time-lapse images of ER

membranes in non-acclimated (NA) and 4-week cold-acclimated (CA) cells were captured at 2°C from 60 min after thawing. The average velocity of ER membranes was calculated with the KbiFlow plug-in for ImageJ software. Each bar represents the mean $\pm$ s.e. ($n = 5-7$).

Fig. 5. Dynamics of ER membranes during supercooling. To show the time-dependent morphological changes of ER in non-acclimated (NA) and 4-week cold-acclimated (CA) cells during supercooling at -3°C or -5°C , three images captured at 0, 1 and 2 min after reaching the specified temperature were colored red, green and blue, respectively, and then the three images were merged. The cooling rate was $0.08^{\circ}\text{C min}^{-1}$ from 0°C to -5°C . Bar indicates 20 μm .

Fig. 6. Quantitative analyses of ER dynamics during supercooling. Time-lapse images of ER membranes in non-acclimated (NA) and 4-week cold-acclimated (CA) cells were captured at -3°C or -5°C during supercooling. After the average velocity of ER membranes was calculated with the KbiFlow plug-in for ImageJ software, the values in the supercooling condition were compared with those in the freezing condition. Asterisks indicate a statistically significant difference between supercooling and freezing conditions based on a Student's *t* test (*, $P < 0.05$). The cooling rate was $0.08^{\circ}\text{C min}^{-1}$ from 0°C to -5°C . Each bar represents the mean $\pm$ s.e. ($n = 3-5$).

Supplementary movie 1. ER cryodynamics in cold-acclimated cells. ER in Welsh onion cells cold-acclimated for 4 weeks was observed during extracellular freezing. Observations were performed from 2 to -15°C and temperature was decreased at the rate of $0.08^{\circ}\text{C min}^{-1}$. Images were captured at 30 s intervals. Bar indicates 50 μm .

Figure(s)

Fig. 1

Fig. 2

A Nonacclimated cells

R: 58 m

G: 59 m

B: 60 m

R: 58 m, G: 59 m, B: 60 m

R: 0 m, G: 1 m, B: 2 m

at 2°C after freeze/thawing

at 2°C before freezing

B Cold-acclimated cells

R: 1 m, G: 2 m, B: 3 m

R: 10 m, G: 11 m, B: 12 m

R: 30 m, G: 31 m, B: 32 m

R: 60 m, G: 61 m, B: 62 m

R: 0 m, G: 1 m, B: 2 m

at 2°C after freeze/thawing

at 2°C before freezing

Fig. 3

Fig. 4

Fig. 5

Fig. 6