Ultrastructure of Mesophyll Cells in Two Moss Species under Cryogenic Stress and Recovery Growth Conditions (Postprint)

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Abstract

This study compared the ultrastructural characteristics of two moss species from different substrates beneath the No. 1 Glacier—the aquatic-terrestrial Anomobryum auratum and the rock-surface terrestrial Tortula desertorum—under normal temperature, ultra-low temperature stress, and post-stress recovery conditions, to explore the cold resistance mechanisms of mosses in high-cold ice margin areas at the ultrastructural level. Following ultrasonic cleaning, samples were divided into three groups: -80 °C ultra-low temperature stress, room temperature recovery culture, and normal temperature control. Ultrathin sections were prepared through treatment with reagents including glutaraldehyde, phosphate buffer, osmium tetroxide, ethanol, and acetone, and ultrastructural observations and analyses were conducted after staining. At room temperature, moss mesophyll cell structures were intact and clearly defined. Following -80 °C ultra-low temperature stress treatment, ultrastructural changes in mesophyll cells included: most cells did not exhibit plasmolysis, but showed blurred plasma membrane structures and cytoplasmic contraction; organelles were damaged or even disintegrated; and the numbers of starch grains, lipid droplets, and vacuoles increased substantially. During room temperature recovery, mitochondrial numbers increased, and the structural integrity of various organelles improved compared with the ultra-low temperature stress state. Based on ultrastructural analysis, it is hypothesized that these changes facilitate rapid restoration of physiological functions; the -80 °C ultra-low temperature stress did not completely eliminate the physiological functions of the mosses, and recovery remained possible. The leaf cell wall thickness of the rock-surface terrestrial Tortula desertorum (1 100~1 300 nm) was greater than that of the aquatic-terrestrial Anomobryum auratum (200~700 nm); the leaf cell walls of Tortula desertorum were thicker than those of Anomobryum auratum, leading to the inference that the organelle stress resistance capacity of Tortula desertorum is also stronger. This demonstrates that these two moss species from the No. 1 Glacier possess extremely

strong cold resistance, and their unique cold resistance mechanisms are related not only to the structural and functional integrity of starch grains and organelles at the ultrastructural level, but also to their habitats.

Full Text

Ultrastructure of Mesophyll Cells in Two Moss Species Under Ultra-Low Temperature Stress and Recovery

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Abstract

This study compares the ultrastructural characteristics of two moss species from different substrates at the terminus of Glacier No. 1: *Anomobryum auratum* (growing on aquatic soil) and *Tortula desertorum* (growing on rocky surfaces) under normal temperature, ultra-low temperature stress, and post-stress recovery conditions to explore the cold resistance mechanisms of mosses in alpine ice-marginal zones at the ultrastructural level. Samples were ultrasonically cleaned and divided into three groups: -80°C ultra-low temperature stress, room temperature recovery culture, and normal temperature control. Ultra-thin sections were prepared using glutaraldehyde, phosphate buffer, osmium tetroxide, ethanol, acetone, and other reagents, then stained for ultrastructural observation and analysis. At room temperature, moss mesophyll cells exhibited intact and clear structures. After -80°C ultra-low temperature stress, most mesophyll cells in both species showed no plasmolysis but displayed blurred plasma membrane boundaries and cytoplasmic contraction; organelles were damaged or even disintegrated; and the numbers of starch granules, lipid droplets, and vacuoles increased substantially. During room temperature recovery, mitochondrial numbers increased and organelle integrity improved compared with the stress state. Based on submicrostructural analysis, these changes presumably adapt cells for rapid physiological recovery. The -80°C ultra-low temperature stress did not completely eliminate physiological function, as recovery remained possible. The leaf cell wall thickness of rock-dwelling *T. desertorum* (1,100–1,300 nm) was greater than that of aquatic-soil *A. auratum* (200–700 nm). The thicker cell walls in *T. desertorum* suggest stronger stress resistance capacity in its organelles. These two moss species from Glacier No. 1 exhibit extremely strong cold resistance, and their unique cold-resistance mechanisms relate not only to the structural and functional integrity of starch granules and organelles at the ultrastructural level but also to their habitats.

Keywords: glacier, ultra-low temperature stress, growth restoration, moss, submicroscopic structure

Introduction

The high-altitude ice-marginal zone of Glacier No. 1 in the Tianshan Mountains, Xinjiang, features harsh climate conditions with extreme cold, strong winds, and poor water retention. The aridity index is 1.25, relative humidity ranges from 60% to 70%, vegetation communities are relatively simple, and the area belongs to the permanent ice-marginal zone margin (An Lizhe et al., 2000). *Anomobryum auratum* and *Tortula desertorum* are dominant groups in the typical alpine tundra vegetation belt of this region. Previous researchers have conducted anatomical studies on xerophytic mosses and bryophytes from the Glacier No. 1 area (Wang Hong et al., 2000, 2008, 2014, 2016, 2017, 2017a, b; Zeng Jian et al., 2015; Chen Qiuyan et al., 2016), identifying structural characteristics of mosses growing in extreme environments, particularly the morphological features that are similar yet distinct among related species within the same family or genus, which hold potential taxonomic and ecological significance.

Research on ultrastructural changes in plants under stress has traditionally focused on angiosperms (Yan Jiqiong, 1999; Thomson & Moeller, 1983). He Tao et al. (2005) studied the ultrastructure of chloroplast starch granules in five alpine plants from Daban Mountain in the northeastern Qinghai-Tibet Plateau, finding abundant starch granules in leaf mesophyll cell chloroplasts. This variation in starch granules represents an adaptation to stress and results from long-term 胁迫 from the unique ecological conditions of the Qinghai-Tibet Plateau. Wen Zhibin et al. (2016) noted that under mild drought stress, cellular organelles show slight damage, while under severe stress, chloroplast structures become disorganized and mitochondria degrade, with differential responses among different tissues and even among cells within the same tissue. Studies indicate that chloroplast structures exhibit varying degrees of damage under senescence, disease, and severe stress conditions (Wilttenbaeh et al., 1980; Crawford & Wildens, 1996; Cheeseman, 1988; Asada, 1999). Recent ultrastructural studies on mosses under stress have primarily examined salt stress. Liu Weiguo et al. (2016) investigated the ultrastructure of *Syntrichia caninervis*, a dominant species in the Gurbantunggut Desert, under different NaCl concentrations, observing no significant changes at low concentrations compared with unstressed controls. At moderate concentrations (200, 300 $mmol \cdot L^{1}$), leaf cells began plasmolyzing, large vacuoles ruptured, chloroplasts deformed, and lipid droplets appeared. At high concentrations (400, 500 $mmol \cdot L^{1}$), plasmolysis intensified, chloroplast lamellar structures disappeared and disintegrated, and mesophyll cells died, leading to the conclusion that damage to chloroplast membrane structures is closely related to mesophyll cell death under salt stress. Wannson (1976) analyzed lipid components in bryophytes, finding that electron microscopy revealed numerous osmium accumulations in the cytoplasm of gametophores, with massive numbers of cytoplasmic lipid droplets when triglyceride content was high and fewer when content was low. Thomson & Moeller (1983) studied effects of acid rain on bryophyte ultrastructure. Wei Meili and Zhang Yuanming (2009) examined the microscopic and submicroscopic structure of leaf cells in *Syntrichia caninervis*

from biological soil crusts. Michele & Cauley (1992) investigated submicroscopic structures in *Funaria hygrometrica* under freezing stress, noting that cell membrane integrity, matrix status, and organelle morphological changes significantly impact water retention and drought resistance. By observing and comparing the ultrastructural characteristics of leaf cells from two moss species inhabiting different habitats in the Glacier No. 1 area, this study explores the cold resistance mechanisms of mosses to provide reference for research on drought and cold resistance mechanisms in alpine plants.

Materials and Methods

Experimental Materials: *Anomobryum auratum* (aquatic soil-dwelling, elevation 3,553 m, voucher specimen No. 20160823017) and *Tortula desertorum* (rock surface-dwelling, elevation 3,563 m, voucher specimen No. 20160823018) were collected in August 2016 from the swampy area at the terminus of the eastern branch of Glacier No. 1 in the Tianshan Mountains, Xinjiang. Voucher specimens are stored at the Herbarium of the College of Life Science and Technology, Xinjiang University (XJU). Green portions were placed in clean penicillin bottles with a small amount of distilled water. After leaves expanded, samples were degassed until submerged and cleaned twice using an ultrasonic cleaner (Model: KQ-250DE).

More than 60 individuals of each species were cleaned with a KQ-250DE ultrasonic cleaner, then subjected to -80°C ultra-low temperature stress for 24 h. Half of the stressed materials were then placed at room temperature (approximately 20°C) for 24 h recovery culture.

Leaves from the middle-upper portions of plants under normal, stressed, and recovery conditions were selected and placed in EP tubes. Fixation was performed with glutaraldehyde solution (3.00% glutaraldehyde, 0.2 mol · L ^{1} Na HPO , 0.2 mol · L ^{1} NaH PO , pH 7.4), followed by rinsing with pH 7.4 phosphate buffer, osmium tetroxide fixation, phosphate buffer rinsing, ethanol dehydration, acetone series replacement, acetone and embedding agent (Epon:DDSA:MNA:DMP-30 = 13:9:7:0.4) series infiltration, and embedding with Epon 812 resin. Samples were polymerized in a warm oven, then sectioned into ultra-thin slices (110–120 nm thickness) using a LEICA EM UC7 microtome. After staining with uranyl acetate and lead citrate, samples were observed and photographed under a JEOL 1230 transmission electron microscope.

2.1 *Anomobryum auratum*

Under normal temperature conditions, *A. auratum* leaf cells exhibited clear plasma membranes. Leaf cell wall thickness measured 1,100–1,300 nm, with weak plasmodesmata between adjacent cell walls and wavy, uneven inner cell walls. The cytoplasm was contracted, with sparse lipid droplets present. Chloroplasts were centrally located, round, relatively large (approximately 2 per cell), and contained some osmiophilic granules or grana; starch granules were essen-

tially unobservable. The intergranal membrane system was relatively clear, with chloroplast stromal lamellae concentrated in the central region and low stacking degree. The nucleus was large (second only to chloroplasts in volume) with uniform nucleoplasm and a small, slightly dark-stained nucleolus. Mitochondria were present near chloroplasts and the nucleus. The intracellular vacuole area was extensive, featuring one irregularly shaped large central vacuole with numerous dispersed small vacuoles of varying sizes throughout the cytoplasm (Plate I: A, B).

After -80°C ultra-low temperature stress, the cell membrane appeared irregular with indistinct boundaries, though no plasmolysis occurred. Chloroplasts contracted irregularly, with stromal lamellae disintegrating and transforming into multiple starch granules that formed carbohydrate storage structures; plastoglobuli aggregated from grana remained within chloroplasts. No nuclei were observed in the examined images. Other organelles suffered varying degrees of damage or even disintegration. Mitochondrial membranes were destroyed, cristae morphology became blurred, and entire mitochondria ruptured and disintegrated. The central large vacuole diminished or disappeared, with vague vacuolar wall boundaries (Plate I: C, D).

After recovery culture, images revealed that organelles in the cytoplasm began recovering, including reappearing chloroplasts with clearly visible stromal lamellae and initially formed plastoglobuli or plastoglobuli-like granules. Larger plastoglobuli-like granules further expanded. Mitochondrial numbers increased substantially, with clearly observable cristae. Vacuolar volumes also gradually increased (Plate I: E, F).

2.2 *Tortula desertorum*

Cells exhibited small, irregular lumens with vacuoles. Leaf cell wall thickness measured 200–700 nm. The plasma membrane was clear, with spherical lipid droplets of various sizes, some vacuoles, endoplasmic reticulum, and proplastids distributed throughout the cytoplasm. Chloroplasts were large, nearly round, and centrally located, bounded by clear membranes containing numerous stromal lamellae, osmiophilic granules, vacuoles, and relatively large plastoglobuli. The intergranal membrane system was clear, with slightly swollen intergranal membrane grooves making appearance and layering more distinguishable. Stromal lamellae were unevenly distributed with low stacking density. Clear mitochondria with observable cristae were distributed in the cytoplasm. No central large vacuole was observed in the examined region, though numerous irregular small vacuoles and some cisternae were present (Plate I: G, H).

Under -80°C ultra-low temperature stress, no plasmolysis occurred. Extremely thick cell walls protected the cell membrane periphery, with wavy inner cell wall portions. The cytoplasm contracted substantially. Stromal lamellae in chloroplasts essentially disintegrated, becoming unrecognizable, though some lipid droplets appeared. Mitochondria were faintly visible. Due to damage

to various biomembrane systems, the cell interior filled with numerous vague vesicular vacuoles and organelle residues scattered throughout the cytoplasm. Vacuoles became diffuse (Plate I: I, J).

After recovery culture, plasmolysis remained absent, and the wavy curvature of inner cell walls decreased substantially. Lipid droplets of varying sizes remained visible in the cytoplasm. Chloroplasts began recovering, with very clear stromal lamellae observed, enlarged thylakoids, and entire chloroplasts undergoing reorganization, with plastoglobuli reappearing. Faint endoplasmic reticulum and mitochondria were visible. The tonoplast was indistinct, and other cellular components remained to be restored (Plate I: K, L).

Note: 1–2. *Anomobryum auratum* normal ×4,430, ×11,000; 3–4. *A. auratum* -80°C ultra-low temperature stress ×4,430, ×13,800; 5–6. *A. auratum* recovery ×9,420, ×13,800; 7–8. *Tortula desertorum* normal ×13,800, ×19,400; 9–10. *T. desertorum* -80°C ultra-low temperature stress ×27,700, ×13,800; 11–12. *T. desertorum* recovery ×19,400, ×16,600. PM: plasma membrane; CW: cell wall; PD: plasmodesmata; M: mitochondria; Ch: chloroplast; E: chloroplast envelope; G: granum; G: starch granule; L: lipid droplet; P: plastoglobulus; F: intergranal membrane system; ER: endoplasmic reticulum; V: vacuole; t: tonoplast; PP: proplastid; N: nucleus; NE: nuclear envelope.

Plate I Leaf cross-sectional submicroscopic structures of two moss species

3.1 Cell Wall Thickening and Plasmolysis

This study observed thickened cell walls in leaf cells of both moss species, with relatively thick plasma membranes. The leaf cell wall thickness of rock-dwelling *T. desertorum* (1,100–1,300 nm) exceeded that of aquatic-soil *A. auratum* (200–700 nm), with *T. desertorum* possessing slightly thicker walls. Under -80°C ultra-low temperature stress, most leaf cells in both species showed no plasmolysis, with only a few cells exhibiting slight plasmolysis, unclear plasma membranes, and varying degrees of cytoplasmic contraction. Yan Jiqiong (1999) proposed that plant injury activates hydrolytic enzymes that degrade cell wall components, triggering defense mechanisms to repair damage. Thickened cell walls reduce water evaporation, while wax and other appendages on primary walls also prevent water loss. We infer that the degree of cell wall thickening in these mosses relates not only to species but also to capacity to resist harsh cold environments, with greater thickening conferring stronger resistance to extreme conditions like severe cold and drought. This parallels Fang Qiang' en et al. (2015), who reported that alfalfa root crown bud cell walls thickened significantly during early winter. During cold acclimation, frequent plasma membrane exocytosis releases macromolecular substances from cytoplasmic vesicles (including small vacuoles) outside the plasma membrane to participate in cell wall construction, increasing wall rigidity. This adaptive change prevents mechanical damage from freezing-induced water loss. Under normal conditions, neither moss species showed plasmolysis, suggesting its absence is a fundamen-

tal condition for recovery. Plasmolysis under stress may indicate cell death and inability to recover. Therefore, after ultra-low temperature stress and recovery culture, these mosses can restore organelle function and normal metabolic activity, demonstrating their capacity to withstand -80°C ultra-low temperature stress and recover growth.

3.2 Substances in Chloroplasts

Under normal growth conditions, chloroplasts in both moss species contained numerous plastoglobuli. However, under low temperature stress, chloroplast structures suffered extensive damage, with plastoglobuli numbers decreasing dramatically or disappearing, then increasing again during recovery. We hypothesize that plastoglobuli are closely associated with chloroplast structural recovery and may determine chloroplast numbers. Chen Yan et al. (2003) demonstrated that thylakoids degrade under stress, with degradation products accumulating as plastoglobuli that appear in heavily damaged plant cells. In our results, the stacking degree of chloroplast stromal lamellae decreased in both species under low temperature stress. Chen Yan et al. showed that stress reduces photosynthetic phosphorylation, thereby decreasing lamellar stacking and exacerbating membrane structural damage.

Additionally, starch granules appeared in chloroplasts of both species during recovery after ultra-low temperature stress, consistent with He Tao et al. (2005). Chloroplasts produce starch through photosynthesis during daylight for mitochondrial use or cellular storage. The Glacier No. 1 region experiences low temperatures year-round, slowing plant physiological metabolism and favoring starch accumulation. Starch granules maintain thylakoids in a high-sugar environment to prevent degradation, while starch hydrolysis releases energy to help resist low temperatures when light is insufficient (He Ruoyun, 1995). Zhou Yonghui et al. (2016) also proposed that increased and enlarged starch granules represent responses to multiple stress conditions including low temperature, low pressure, and high radiation. We hypothesize this is likely a self-protective measure for mosses adapting to cold extreme environments, though our experimental results show little correlation with substrate conditions.

Under low temperature stress, both species exhibited thylakoid swelling and low chloroplast stacking, with most stromal lamellae disintegrating but lipid droplets appearing. Rock-dwelling *T. desertorum* also showed cisternae. Thylakoid swelling was also observed by Du Junhua et al. (2001) in studies of alpine plants. Although drought, salinity, and high radiation can also cause thylakoid swelling that may lead to chloroplast disintegration, such phenomena are not normal or long-term. In normal high-altitude plants, swollen thylakoids can temporarily store CO from respiration and O from photosynthesis, ensuring survival in low CO and O environments. Mosses from Glacier No. 1 are also higher plants from high altitudes, and their swollen thylakoids may represent an adaptation to low partial pressures of CO and O . Wu Xueming (1997) demonstrated that lipid droplet appearance relates to reactive oxygen species

scavenging, representing results of combined low temperature and high radiation effects and accumulation of thylakoid degradation products. Zhou Yonghui et al. (2016) reported that long-term low temperature causes thylakoid swelling, membrane rupture, and even disappearance in plateau plants. Under the year-round low temperature and high radiation extremes of Glacier No. 1, both moss species showed thylakoids and lipid droplets. Drought and salt stress also induce lipid droplets and thylakoid swelling (Bai Zhiying et al., 2009; Liu Weiguo et al., 2016), leading us to hypothesize this as one strategy for adapting to their growth environment.

3.3 Mitochondrial Changes

Mitochondria are the powerhouses of cells. During recovery after -80°C ultra-low temperature stress, numerous mitochondria appeared in leaf cells of both species, consistent with Chen Yan et al. (2003). Increased mitochondria after low temperature stress ensure energy supply to support various steps in the recovery process. Chloroplasts are more sensitive to ultra-low temperature stress than mitochondria, with structures more easily damaged under stress conditions, consistent with Wan Liqiang et al. (2009).

3.4 Central Vacuole Changes

The central vacuole is a water-soluble system and the organelle most susceptible to change in cold environments. This study found that the original central large vacuole disappeared under ultra-low temperature stress, replaced by small, diffuse vacuoles with significantly increased numbers. We hypothesize this relates to self-protective measures activated in cold environments. Vacuoles can engulf and hydrolyze cytoplasm and other organelles, increasing cell sap concentration and lowering freezing points to prevent freezing damage (Chen Xuwei et al., 2005; Yang Fengxian et al., 2001).

Although these two moss species from Glacier No. 1 in the Tianshan Mountains inhabit different substrates, they share some common structural characteristics under low temperature stress. Most notably, they show no or only slight plasmolysis after ultra-low temperature stress—a crucial condition for recovery of viability, allowing restoration of basic physiological functions. Some leaf cells suffered varying damage, including irregular chloroplast contraction, stromal lamellae disintegration, mitochondrial and other organelle breakdown, and transformation of the central vacuole into numerous small vacuoles. These changes generally align with ultrastructural alterations reported in other studies of plants under low temperature stress (Du Junhua et al., 2001; Zhang Jing and Zhu Weimin, 2012). Microscopic images demonstrate that both moss species can recover physiological function after ultra-low temperature stress, reflecting their exceptional capacity to adapt to extremely harsh environments. The cell wall thickness of rock-dwelling *T. desertorum* (1,100–1,300 nm) exceeds that of aquatic-soil *A. auratum* (200–700 nm). During ultra-low temperature stress, *T. desertorum* leaf cell inner walls appeared wavy. Comparative ultrastructural

observations suggest that *T. desertorum* possesses slightly stronger recovery capacity and organelle stress resistance than *A. auratum*, though whether this relates to their growth conditions requires more systematic future research.

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Note: Figure translations are in progress. See original paper for figures.