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Original Research

Extracellular ice management in the frost hardy horsetail *Equisetum hyemale* L.

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ABSTRACT

Formation of extracellular ice at specific positions in the plant interior is a common and probably essential component of plant cold hardiness. Studies on extracellular freezing in spore-bearing plants are, however, scarce. In this study, extracellular ice formation in the cold hardy horsetail *Equisetum hyemale* L. is analyzed. Horsetails show an extensive system of intercellular air spaces which are probably crucial for internal ice storage during winter. Previous studies emphasized the spacious pith cavity as the main place for ice crystal growth. Shoots were studied during summer and in the frozen state in winter, after natural acclimatization, by using digital (incident light) microscopy, Scanning Electron Microscopy and Cryo Scanning Electron Microscopy. It was shown that the vallecular canals also contain a large share of ice bodies under freezing conditions. The vallecular canals, which are directly seated within the cortex and whose interior is directly connected to the cortex via gaps in the canal wall, were often and rapidly filled with ice. The pith cavity also contained ice, depending on the position along the shoot and the internode. The carinal canals contained almost no ice crystals. Furthermore, some ice crystals were detected in the intercellular spaces of the chlorenchyma and the substomatal chamber. The stomatal antechamber, however, was always ice-free, probably due to the presence of water-repellent wax crystals. The results of this study support available evidence for the crucial role of pre-existing extensive lacunae for extracellular ice formation in *E. hyemale*. Furthermore, the findings indicate that anatomical details of canal structure and position are important for the pattern of extracellular ice accumulation.

1. Introduction

Cold hardiness is a crucial asset for plants to survive subzero conditions, and various mechanisms are involved. The formation of extracellular ice has long been recognized as a common process during plant freezing (Gusta and Wisniewski, 2013; Levitt, 1980; Molisch, 1897; Pearce, 2001; Prillieux, 1869). Buildup of extracellular ice bodies is placed into the category of freezing avoidance which sums up all physical mechanisms which control the formation of ice in the plant interior (Gusta and Wisniewski, 2013). Whereas freezing tolerance means processes of physiological adaptation, such as changes of membrane composition, the role of freezing avoidance mechanisms is to prevent harmful ice crystal formation within living cells (Gusta and Wisniewski, 2013). Other aspects of freezing avoidance are, for example, the presence of ice nucleators/anti-nucleators and ice barriers, or deep supercooling.

Extracellular ice formation was observed in various plant tissues and organs. A common event is freezing of the xylem content (Utsumi et al., 1998) which occurs quite rapidly (Hacker and Neuner, 2007; Neuner et al., 2010). Extracellular ice formation was also observed in leaves, including conifer needles (Ball et al., 2004; Hacker and Neuner, 2007; Roden et al., 2009), petioles of herbaceous plants (McCully et al., 2004) and buds (Ashworth et al., 1989; Ishikawa et al., 1997). Ice shows a lower water potential than liquid water: below 0 °C, the water potential of ice will decrease about −1.2 MPa with each degree Kelvin decrease in temperature (Rajashekar and Burke, 1982). An ice body will therefore attract water from the surroundings, thereby dehydrating the living cells, depressing their freezing temperature further and making their content strongly viscous (Franks, 1985). Extracellular ice bodies forming in cold hardy plants can be quite massive, and the water stored in them is usually completely absorbed by the living cells after thawing (McCully et al., 2004).

The essential aspect of freezing avoidance is the ability of the cold hardy plant to store ice at specific sites. For example, whereas ice forms randomly in the leaves of freezing sensitive *Eucalyp-*

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tus species, occurrence of ice is limited to specific positions in the leaves of frost hardy species (Ball et al., 2004). Basic components for limiting the spreading of ice are tissue structure, cell walls, and membranes. Cell walls are suitable to stop a moving ice front because water inside a finely porous material (pores in the nm range) freezes at a much lower temperature compared to unconfined water (Ashworth and Abeles, 1984). Thus, finely porous material can prevent entrance of a moving ice front. Also hydrophobic layers, including artificial ones, can limit spreading of ice (Fuller et al., 2003; Kuprian et al., 2016).

Freezing avoidance and freezing tolerance are coupled mechanisms and both are essential for the ability of cold hardiness. Improving our knowledge of freezing processes in plants is thus essential for understanding cold hardiness, which is a highly relevant aspect of plant evolution (Zanne et al., 2014), ecology (Larcher et al., 2010; Taschler and Neuner, 2004) and agriculture (Lindow et al., 1978; Wisniewski et al., 2014). In the last years, however, the numbers of studies considering freezing avoidance in plants have declined (Wisniewski et al., 2014). Furthermore, gymnosperms and angiosperms are preferentially studied with respect to internal ice formation, whereas spore bearing plants were seldom considered. An outstanding example for a cold hardy spore-bearing plant is the horsetail species *Equisetum hyemale* L., subgenus *Hippochaete* (Husby, 2013). Horsetails are assigned to the monilophytes, a spore-bearing vascular plant group which includes – besides horsetails – the whisk ferns, and eusporangiate and leptosporangiate ferns (Smith et al., 2006).

Equisetum hyemale L., common name "winter scouring rush", shows a wide circumpolar distribution, being native throughout temperate Asia, North America, South America and Europe (Hauke, 1963). Its shoots reach heights from about 0.5 m to 1 m. Usually, *E. hyemale* grows in moist habitats, such as wetlands, riparian environments, swamps or moist forests (Husby, 2013). Horsetails show a unique and characteristic morphology and anatomy (Hauke, 1963, 1979). The hollow stem is organized into nodes and internodes and shows ridges and furrows. As characteristic for plants of moist habitats which are tolerant of flooding, horsetails show an extensive internal system of intercellular air spaces (Bierhorst, 1971). Although the various species of *Equisetum* differ with respect to site soil moisture demand, the structure of their internal canals is similar, consisting of carinal and vallecular canals and a more or less spacious central pith. The carinal canals represent the space originally occupied by protoxylem, and were suggested to support water transport by supplying a low-conductivity pathway (Leroux et al., 2011; Xia et al., 1993).

Horsetails thus represent a special plant construction by showing an extensive and strictly organized intercellular system, consisting of a spacious pith cavity and various longitudinal canals. All canals are more or less circular in cross-section and run continuously along each internodal segment. The shoots of *E. hyemale* persist for about two years (Hauke, 1963), and ice accumulation within their canal system under subzero conditions was reported (Niklas, 1989; Schaffner, 1908). There is evidence that the pith cavity represents the essential site for extracellular ice formation (Niklas, 1989). Because in cold hardy plants extracellular ice formation often takes place at preferred intercellular spaces which are subsequently expanded by the growing ice body and finally occupy the space left by the shrinking dehydrating tissue (McCully et al., 2004), the already extensive canal system of *E. hyemale* appears to be a predestined location for ice storage. Analyzing extracellular ice formation in the canal system of frost hardy *E. hyemale* may contribute to understand the importance and role of intercellular space architecture for freezing avoidance. In this contribution we studied extracellular freezing in *E. hyemale* and the extracellular distribution of ice within its shoots in detail.

2. Material and methods

2.1. Plant material and sample collection

Plants of *Equisetum hyemale* var. *robustum* from the inner courtyard of the State Museum of Natural History Stuttgart (SMNS; 48.793308° latitude, 9.190340° longitude), cultivated as potted plants, and field-grown plants from the Botanical Garden of the Technical University Dresden, Germany (51.040074° latitude, 13.771024° longitude), were used throughout the work. The plants received natural precipitation and potted individuals were additionally watered ad libitum to keep the substrate moist. The plants cultivated in Stuttgart were used for digital microscopy (DM) studies and Scanning Electron Microscopy (SEM), while the plants obtained from the Botanical Garden in Dresden were analyzed with Cryo-Scanning Electron Microscopy (Cryo-SEM).

2.2. Acclimation and freezing

Observations of ice formation were conducted during the winter season, after all plants were acclimated at their growing site under natural conditions. For freezing, acclimated plants were either 1) put into a freezer, or 2) plants were harvested after natural freezing in the field, under subzero conditions. For artificially freezing, whole plants including pot were put into a custom-built freezer (Fryka, Esslingen, Germany), which was set to 0 °C. To protect the root zone, the pot was kept within a polystyrene box. Then, the temperature was decreased to −10 °C, with a cooling rate of 2 °C h^{-1}. With this cooling rate, which is in the range of that experienced by plants in nature (Steffen et al., 1989), plants should have enough time to perform extracellular ice formation. Samples were harvested from the frozen material after keeping it under −10 °C for 16 h.

2.3. Microscopic analysis

2.3.1. Digital microscopy

During the summer, samples were collected from the six potted plants at the SMNS, which were supplied from the same breeder (Pflanzmich GmbH, Hamburg, Germany), and visualized and analyzed with the digital microscope (Keyence VHX-500F, with VH-Z250R and VH-Z20R, Keyence Corp.). During winter, acclimated and unfrozen as well as frozen plants were studied. Artificially frozen samples were harvested with a pre-cooled razor blade. During the transport as well as during the analysis samples were kept frozen on pre-cooled copper plates (−10 °C) in a small cooling box (True North® mini-cooler, Heathrow Scientific, LLC, Vernon Hills, Illinois, U.S.). For both summer and winter, for frozen as well as unfrozen plants, 10–15 shoot samples taken from different shoot heights were studied.

2.3.2. Scanning electron microscopy

For SEM analysis during summer, samples from current year and older shoots were dehydrated for 10 min in absolute methanol before putting them into absolute ethanol for 30 min. This procedure was repeated, according to Talbot and White (2013), before critical point drying (CPD) was applied using a Leica EM CPD300 (Leica Microsystems GmbH, Wetzlar, Germany). Dried samples were subsequently mounted by an adhesive conducting tape on a stub, and sputtered with gold, and examined in the SEM Zeiss EVO LS 15 (Carl Zeiss Microscopy GmbH, Jena, Germany).

2.3.3. Cryo-scanning electron microscopy

Cryo-fixation with subsequent Cryo-SEM allows for preserving and observing the distribution of ice, liquid water and gas spaces