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A study on ice crystal formation behavior at intracellular freezing of plant cells using a high-speed camera

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ABSTRACT

Intracellular ice crystal formation (IIF) causes several problems to cryopreservation, and it is the key to developing improved cryopreservation techniques that can ensure the long-term preservation of living tissues. Therefore, the ability to capture clear intracellular freezing images is important for understanding both the occurrence and the IIF behavior. The authors developed a new cryomicroscopic system that was equipped with a high-speed camera for this study and successfully used this to capture clearer images of the IIF process in the epidermal tissues of strawberry geranium (*Saxifraga stolonifera* Curtis) leaves. This system was then used to examine patterns in the location and formation of intracellular ice crystals and to evaluate the degree of cell deformation because of ice crystals inside the cell and the growing rate and grain size of intracellular ice crystals at various cooling rates. The results showed that an increase in cooling rate influenced the formation pattern of intracellular ice crystals but had less of an effect on their location. Moreover, it reduced the degree of supercooling at the onset of intracellular freezing and the degree of cell deformation; the characteristic grain size of intracellular ice crystals was also reduced, but the growing rate of intracellular ice crystals was increased. Thus, the high-speed camera images could expose these changes in IIF behaviors with an increase in the cooling rate, and these are believed to have been caused by an increase in the degree of supercooling.

1. Introduction

Cryopreservation techniques are used in various fields, including food and medical engineering. The most appropriate cryopreservation temperature depends on the properties of the object being preserved, and the preservation period varies with temperature. Cryopreservation involves placing living materials such as food, biological organs, and tissues under a low temperature, thus reducing putrefaction by bacteria and plant respiration [25]. However, since living materials contain water, they are also frozen spontaneously through supercooling when placed at a temperature below the solidification point. Therefore, cryopreservation techniques can be classified into two categories depending on whether the living materials are frozen, with frozen preservation generally being considered to allow an extended preservation period.

The suitability of frozen preservation depends on the state of the living materials during cryopreservation. Mazur [19,20] previously described a general relation between the cooling rate and survival rate of freezing cells, which applies to both animal and plant cells. At a slow cooling rate, the inside of a cell becomes dehydrated because of the difference in chemical potential between the extracellular ice crystals and the intracellular solution [16,19]. Although absolute dehydration is lethal to a cell, partial dehydration increases the survival rate of freezing cells. As the cooling rate increases, intracellular freezing occurs as a result of supercooling following extracellular freezing [19,23], which is also lethal to cells [1,16,23,31,34]. However, a further increase in the cooling rate can lead to vitrification preservation, which significantly increases the survival rate of freezing cells [19,27]. At present, however,

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vitrification preservation can only be applied to micro-objects, making it difficult to prevent the occurrence of intracellular freezing when living materials are frozen.

Frozen preservation is used for food storage, where it is unnecessary to keep the living material alive. Rapid freezing can preserve fish and meat for a long time period with a less decline of the quality [12,18], and so is used, for example, to store tuna. However, it is difficult to apply cryopreservation techniques to plant foods, such as vegetables and fruits, as intracellular ice formation leads to a decline in their texture because of mollification of the tissue [5,13]. Plant cells are surrounded by cell walls, and it is believed that the mollification of the tissue is caused by injury to the cell wall when ice crystals form both outside and within a cell [9].

Based on this information, the authors consider that the ability to visualize intracellular ice crystal formation (IIF) behavior to understand the point at which intracellular freezing begins and how IIF progresses, is the key to developing improved cryopreservation techniques.

Studies have captured images of intracellular freezing in animal and plant cells and tissues since the 1930s [6]. Most of these studies have investigated the effect of intracellular freezing on the survival rate or level of injury to the cells [2,17,19,21,29], or relate to vitrification preservation [30,33], with fewer studies examining IIF behavior. However, Modilibowska and Rogers [22] filmed intracellular freezing in plant tissues, and reported the behavior outside and inside the cells upon intracellular freezing; Asahina [3] provided a detailed report on the freezing process in plant cells, and recently, Stott and Karlsson [32] investigated IIF using a high-speed camera. All of these previous studies used images that captured the phenomenon known as “flashing,” whereby the inside of the cell darkens upon freezing. However, the authors consider that it is necessary to capture the progress of IIF and to investigate its behavior in even more detail. It is known that intracellular freezing begins with the invasion of an ice nucleus from outside the cell [24,31]. However, it is thought that ice nucleation may occur not only as a result of extracellular factors, but also intracellular factors, with heterogeneous or homogeneous nucleation inside the cell.

To gain a better understanding of the IIF behavior, the authors developed a new cryomicroscopic system, which comprised an inverted microscope, a cooling stage, and a high-speed camera with a maximum frame rate of 2000 frames per second (fps) and a resolution of 512 × 512 pixels. Plant tissues consisting of a single cell layer were selected for specimens to examine in this system because they were easy to obtain in a living state. The above-mentioned system successfully captured images of IIF in these plant tissues.

This study was performed to investigate the IIF behavior in further detail using high-speed camera images of intracellular freezing acquired at different cooling rates. First, the progression of intracellular freezing was observed and patterns in the location and formation of intracellular ice crystals were determined to evaluate the effects of cooling rate on the occurrence of intracellular freezing and IIF. The ice crystals formed inside the cells due to intracellular freezing deform the cells. Thus, the relation between the degrees of supercooling and cell deformation due to intracellular freezing was shown to be the effect of cooling rate on IIF behavior. In addition, using the high-speed camera images, the growing rate and grain size of intracellular ice crystals were also evaluated. The results show that an increase in the cooling rate caused an increase in the degree of supercooling and a decrease in the degree of cell deformation and indicate that the degree of cell deformation decreases due to a decrease in the grain size of intracellular ice crystal with the increase in the degree of supercooling.

2. Materials and methods

2.1. Cryomicroscopic system

The cryomicroscopic system used in this study consisted of an inverted microscope (IX71, Olympus) equipped with a cooling stage (HCD301, INSTEC) and a high-speed camera (Phantom V4.2, Vision Research) (Fig. 1).

The plant specimens were irradiated with light from a 100-W halogen lamp (12V100WHAL-L, Olympus) passed through a condenser (IX2-LWUCD, NA: 0.55, Olympus). Intracellular freezing in the plant specimens was then captured by the high-speed camera through an objective lens (SLCPlanFl 40x Olympus). The high-speed camera could capture images at a maximum frame rate of 2000 fps and a resolution of 512 × 512 pixels using Phantom Camera Control software package (Version9.0.640.0-C PhCon:640).

Cooling was performed by pouring combination air using liquid nitrogen and a heater (STC200, Instec) onto the cooling stage. The cooling rate was controlled by a computer and varied from −1 °C/min to −100 °C/min. Nitrogen gas was also flowed onto the cooling stage to prevent the observation window from clouding.

2.2. Plant materials

Epidermal tissues on the abaxial surface of the leaves of strawberry geranium (*Saxifraga stolonifera* Curtis) plants obtained from the campus of Kanagawa Institute of Technology, Atsugi, Japan, were used as specimens. The leaves were removed from their leafstalk and placed in a dish filled with water to prevent them from drying.

Initial trials to investigate the usefulness of our cryomicrosopic system for observing intracellular freezing behavior were carried out using specimens obtained in early summer 2007 (Figs. 2 and 3).

Specimens to investigate intracellular freezing behaviors in the tissues were obtained between late December 2014 and mid-January 2015, and in early December 2015. The strawberry geranium plants grew at the campus of Kanagawa Institute of Technology, which is located in a humid subtropical region. The meterological station at neighboring Ebina city recorded an average air temperature of 7 °C and an average day length of 172 h.

2.3. Experimental method

Each specimen was cut into a 10 mm^2 section and placed onto a cover glass with distilled water to prevent it from drying. A second cover glass with silicon grease applied around the edges was then placed over the specimen. The cover glasses were placed on the cooling stage and cooled from room temperature (approximately 22 °C) to the temperature at which intracellular freezing occurred at cooling rates that ranged from −1 °C/min to −100 °C/min.

The shutter of the high-speed camera was pressed when half of the cells (approximately seven cells) within the observation area had experienced intracellular freezing. Thus, intracellular freezing in each specimen was captured at 1000 fps for 2 s before and after pressing the shutter. The experiments were repeated at least 10 times at each cooling rate.

All images were analyzed using Image J (v1.46) [26,28].

2.4. Definition of the degree of supercooling

The temperature at which intracellular freezing occurred (*Ti*) was defined using the temperature when the shutter of the high-speed camera was pressed. *Ti* was then converted into the degree of supercooling (*Ts*), by subtracting the temperature at the release of supercooling from the solidification point of water (0 °C). Thus, the *Ts* was defined as follows: