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Peroxidation due to cryoprotectant treatment is a vital factor for cell survival in *Arabidopsis* cryopreservation

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ABSTRACT

Cryopreservation can be a safe and cost-effective tool for the long-term storage of plant germplasm. In *Arabidopsis*, the ability to recover from cryogenic treatment was lost as growth progressed. Growth could be restored in 48-h seedlings, whereas 72-h seedlings died after cryogenic treatment. Why seedling age and survival are negatively correlated is an interesting issue. A comparative transcriptomics was performed to screen differentially expressed genes between 48- and 72-h seedlings after exposure to cryoprotectant. Among differentially expressed genes, oxidative stress response genes played important roles in cryoprotectant treatment, and peroxidation was a key factor related to cell survival. Seedlings underwent more peroxidation at 72-h than at 48-h. A comprehensive analysis indicated that peroxidation injured membrane systems leading to photophosphorylation and oxidative phosphorylation damage. Furthermore, the apoptosis-like events were found in cryogenic treatment of *Arabidopsis* seedlings. 48- and 72-h seedlings underwent different degrees of membrane lipid peroxidation during cryoprotectant treatment, and reducing the injury of oxidative stress was an important factor to successful cryopreservation. This study provided a novel insight of genetic regulatory mechanisms in cryopreservation, and established an excellent model to test and evaluate the effect of exogenous antioxidants and conventional cryoprotectants in plant cryopreservation.

1. Introduction

Cryopreservation, the storage of viable cells, tissues, organs, and organisms at ultralow temperatures, usually in LN, has successfully preserved various plant species [2–4]. It is considered as an important tool for long-term germplasm conservation that requires minimal space and maintenance. Controlled freezing, vitrification and dehydration-encapsulation are commonly used basic methods in plant cryopreservation [5], and vitrification has a wide range of application in cryopreservation because it is rapid and easy to perform. Vitrification is characterized by forming a glass that avoids ice-caused damage during cryogenic storage. The vitrification procedure mainly includes preculture, osmoprotection, dehydration with vitrification solution, rapid cooling, rapid warming, dilution of the vitrification solution, and plating rewarmed bio-materials for recovery [6]. Osmoprotection increases cell osmolarity and decreases osmotic damage arising from the vitrification solution, and dehydration prevents lethal injury from ice during rapid cooling [6]. Volk and Walters [7] elucidated three important functions of vitrification solutions: replacing cellular water, altering the freezing behavior of water remaining in cells, and impeding water loss on drying. Cryoprotectants are important for cell survival, however, they can also cause some complex stresses, such as osmotic injury and dehydration stress [8]. Prior research provides evidence that some stress-related genes and proteins are induced during cryopreservation. Some genes were specifically up regulated in response to cryoprotectant treatment in *Arabidopsis* shoot tips, mainly involved in dehydration responses [9]. Basu [10] detected

Abbreviations: AFLPa, mplified fragment length polymorphism; BLAST, basic local alignment search tool; EST, expressed sequence tag; FDA, fluorescein diacetate; LN, liquid nitrogen; MDA, malondialdehyde; MS, Murashige and Skoog [1]; NCBI, National Center for Biotechnology Information; PVS, plant vitrification solution; qRT-PCR, quantitative real time-PCR; ROS, reactive oxygen species; TBA, thiobarbituric acid; TCA, trichloroacetic acid; TDFs, transcript-derived fragments.

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that *los4* (low expression of osmotically responsive genes) was induced in this process. Xu [11] found that the abundance of some heat and boiling stable proteins changed significantly during *Arabidopsis* cryopreservation, and identified that these proteins were induced by PVS2. Additionally, some proteins with a positive effect on slowing the rate of ice crystal formation and growth were found in cryopreserved plants. COR15 effectively protects the cold-labile enzyme, lactate dehydrogenase, against freeze inactivation and has potent cryoprotective activity [12]. Antifreeze proteins (AFPs) inhibit ice crystal growth by binding ice nuclei, which decreases the freezing point of the cytoplasm, thereby allowing survival at subzero temperatures [13,14]. Guo [15] found that AFPs were induced during low-temperature preculture before cryopreservation in longan (*Dimocarpus longan*). Cryoprotectants possess both advantageous and disadvantageous aspects for plant cells during cryopreservation, and enhancing advantageous functions or decreasing disadvantageous effects has important significance for improving plant cryopreservation. Revealing the mechanisms of abiotic stress originating from cryoprotectants will help us improve plant cell survival after cryogenic treatment that may enhance success of cryopreservation.

Generally, some materials such as seed, pollen and shoot tip with characteristic of dense cytoplasm, high meristematic activity and low water content were usually cryopreserved by vitrification [16]. In addition, callus, suspension cell and seedling of some species regarded as important germplasm resources were difficult to be cryopreserved due to the features of higher water content, vigorous metabolic activities, complicated biological processes and sensitive stress response. Therefore, using suitable plant materials to uncover the dynamic process of metabolism, stress response and signaling transduction has important significance in cryobiological theory and plant cryopreservation practical operation. *Arabidopsis* is a good species to study plant cryopreservation at the molecular level. Previous studies mainly used *Arabidopsis* young seedlings which ages were not beyond 72-h to investigate the ATPase activities of plasma membrane [17], the protective effect of vitrification on freeze injury [18], some cryopreservation-related proteins [11], and the DNA methylation patterns in cryopreservation [19]. In this study, *Arabidopsis* seedlings cultured on the MS medium up to 48 h were found regrowing well, whereas seedlings cultured 72 h lost their growth ability after cryogenic treatment. The seedling age and the survival ratio had a significant negative correlation, and the latter decreased along with the extension of the growing period. To understand the mechanism of this correlation, comparative transcriptional profiling of seedlings was analyzed in this paper. At the mRNA level, the comparison of the gene expression patterns can partly verify the molecular mechanisms among plants of varying developmental status, stress response and signaling transduction pathways during cryoprotectant treatment, and provide a novel understanding of genetic regulatory mechanisms in cryopreservation process.

2. Materials and methods

2.1. Plant materials

The *Arabidopsis thaliana* (ecotype Col-0) seeds were kindly provided by Prof. Hong-Quan Yang (Shanghai Jiao Tong University, Shanghai, China). The seeds were sterilized with 70% ethanol solution for 15 s and 20% NaClO + 0.01% Tween 20 for 10 min, rinsed with sterile water 6 times, and then plated on MS medium with 3% sucrose and 0.9% agar and maintained at 4 °C for 48 h. Finally, the seeds were cultured in a growth cabinet with an 8/16 h light–dark cycle, 25 °C/20 °C day–night temperature, and 150 μmol m^{-2} light intensity. The seeds and seedlings at different growth periods (48, 51, 54, 57, 60, 63, 66, 69 and 72 h) were taken as samples for cryogenic treatment. The survival ratio of seedlings was calculated after 15 d of recovery.

$$\text{Survival ratio}\,(\%) = \frac{\text{Alive seedlings}}{\text{Total seedlings}} \times 100\%$$

For RNA and MDA extractions, 48- and 72-h seedlings without treatments were designated as CK (Check), and the seedlings treated with osmoprotection and dehydration (but no LN) were designated as T (Treated), thus 4 samples were defined as 48-h CK, 48-h T, 72-h CK, and 72-h T.

2.2. Cryogenic treatment procedure

The cryogenic treatment (short term preservation)/cryopreservation (long term preservation) procedure was as described by Wang and He [19] adapted from Sakai et al. [6]. The complete procedure included the following steps:

(1) Osmoprotection: 30–40 seedlings were immersed in 1 ml loading solution (MS liquid medium + 2 M glycerol + 0.4 M sucrose) at room temperature for 20 min.
(2) Dehydration: The loading solution was replaced by the vitrification solution PVS2 (30%, w/v glycerol, 15%, w/v ethylene glycol, and 15%, w/v dimethyl sulfoxide in MS liquid medium with 0.4 M sucrose) at 0 °C for 50 min.
(3) Rapid cooling and warming: Seedlings after dehydration were rapidly plunged into LN, held for 1 h (cryogenic treatment) or long term (cryopreservation), and rewarmed in 40 °C water bath for 1 min.
(4) Dilution: PVS2 was replaced by the unloading solution (MS liquid medium + 1.2 M sucrose) for 40 min, and the solution was replaced with fresh unloading solution every 10 min.
(5) Recovery: The seedlings were cultured on MS medium under the same conditions as for seedling growth.

2.3. Viability staining

For viability staining, 48- and 72-h seedlings were collected after each of 4 steps: culture in a growth cabinet, osmoprotection + dehydration treatment, dilution and 24-h recovery. Analysis of cell viability was performed using the FDA staining method [20]. Experimental samples were stained with 0.01% (w/v) FDA (BBI, Markham, Ontario, Canada) in acetone for 20 min in the dark. Cell viability staining was observed using Olympus fluorescence microscope (BX51TRF). Cell death status was examined using the Evan's blue staining method [21], the seedlings were submerged in 0.05% (w/v) Evan's blue (BBI) water solution for 50 min, then washed with distilled water for 3 times, and photographs were captured by Olympus digital system. Each sample for FDA and Evan's blue staining was repeated 5 times.

2.4. RNA extraction and cDNA synthesis

The cDNA-AFLP method was as described by Zhang et al. [22] adapted from Bachem et al. [23,24]. The samples used in cDNA-AFLP were mixed samples. A mixed sample contained about 200 seedlings. Total RNA was extracted from CK and dehydrated seedlings using RNAiso Plus (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer's instructions. The total RNA was purified using the DNaseI and RNase inhibitors (TaKaRa). Double-stranded cDNA was synthesized using the M-MLV RTase cDNA Synthesis Kit (TaKaRa) according to the manufacturer's instructions.