Protoplasma (2010) 239:57–67
DOI 10.1007/s00709-009-0083-2

ORIGINAL ARTICLE

Cytological and physiological changes in orthodox maize embryos during cryopreservation

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Received: 23 June 2009 / Accepted: 22 October 2009 / Published online: 11 November 2009

Abstract Cytological and physiological changes during cryopreservation were studied in maize embryos at 35 days after pollination (DAP). Both dehydration and freezing caused cytological damage, such as plasmolysis, swelled mitochondria, increased heterochromatin, and nuclear shrinkage. Dehydration alone slightly impaired plasma membrane integrity while a drastic increase in electrolyte leakage was observed after freezing of embryos with moisture content above 23%. Damage to cellular ultrastructure and plasmalemma integrity was negatively related to moisture content in unfrozen embryos and positively related in frozen embryos. The pattern of changes in activity of antioxidant enzymes differed from one another during dehydration and/or freezing–thawing treatment. Dehydration increased activity of ascorbate peroxidase (APX) and glutathione reductase (GR) but decreased activity of superoxide dismutase (SOD) and dehydroascorbate reductase (DHAR). Freezing further decreased GR and SOD activity and resulted in extremely low DHAR activity. Embryos at intermediate moisture contents had low catalase (CAT) activity before freezing but highest CAT activity after freeze–thaw. Both dehydration and freezing promoted membrane lipid peroxidation which resulted in an approximately threefold increase at most in the malondialdehyde content in postthaw embryos. Changes in viability of postthaw embryos can be closely related to damage in cellular ultrastructure and plasmalemma integrity but directly related neither to antioxidants nor lipid peroxidation levels.

Keywords Antioxidant enzymes · Cryoinjury · Cryopreservation · Desiccation tolerance · Electrolyte leakage · Orthodox seeds · Ultrastructural studies · *Zea mays* L.

Abbreviations

APX	Ascorbate peroxidase
CAT	Catalase
DAP	Days after pollination
DHAR	Dehydroascorbate reductase
GR	Glutathione reductase
MC	Moisture content
MDA	Malondialdehyde
ROS	Reactive oxygen species
SOD	Superoxide dismutase

Introduction

Cryopreservation is a safe efficacious means of securing ex situ plant germplasm (Stanwood and Bass 1981). Moreover, it has been regarded as the only technique available for long-term germplasm conservation of recalcitrant seeds (Hor et al. 2005). However, cryoexposure often causes lethal injury in most recalcitrant seeds (Walters et al. 2008). Understanding the mechanism of cryoinjury is critical to resolving this problem, but to date, this elucidation has rarely occurred. One of the factors causing this situation is that seeds of most recalcitrant

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species are available only over a short period around the fruiting season of the plant. It is very difficult to get enough seeds at uniform maturity for experiments at any one time.

There are at least two reasons for using orthodox seeds in cryopreservation studies: (1) the comparison between recalcitrant and orthodox seeds is an important method of understanding seed recalcitrance; in this case, orthodox seeds are used as a control for recalcitrant seeds; and (2) in some cases, such as imbibed seeds and developing seeds, orthodox seeds can be used as a substitute for recalcitrant seeds. Compared with developing seeds, imbibed orthodox seeds such as tomato (Grout 1979), lettuce (Kaurin and Stushnoff 1985) and maize (Boucaud and Cambecedes 1988; Isaacs and Mycock 1999) have been subjected to more intensive cryobiological study. Although mature orthodox seeds usually can be desiccated to low-moisture contents and cryopreserved, they are sensitive to desiccation and liquid nitrogen exposure at early developmental stages (Vertucci and Farrant 1995; Kermode and Finch-Savage 2002). It was found that maize embryos could not be cryopreserved during seed development until 29 days after pollination (DAP), while those at 35 DAP had only limited cryotolerance with cryobehavior similar to recalcitrant seeds (Wen and Song 2007a). Therefore, developing orthodox seeds can be a model for recalcitrant seeds in cryoinjury studies. In this paper, we investigated the physiological basis of cryoinjury using developing maize embryos: subcellular damage, plasmalemma integrity perturbation, and antioxidant system profile changes, which were comparatively studied.

Material and methods

Plant material

Maize (*Zea mays* L. cv. "Nongda 108") was cultivated in Menglun, Mengla, Xishuangbanna, Southwest China, where information on climate is available (Wen and Song 2007b). Ears at 35 DAP were harvested manually for this study. The methods used to avoid natural pollination and to conduct controlled artificial pollination, as well as isolating the embryos, have been described in detail by Wen and Song (2007a).

Moisture content determinations

Moisture content of the maize embryos, expressed on a percentage fresh weight basis, was determined gravimetrically after oven drying at 103±2°C for 17 h. Eight sets of five embryos were used for each of the determinations.

Desiccation, cryopreservation, and viability assessment of embryos

Embryos were excised from developing seeds at 35 DAP and desiccated by holding over activated silica gel. Harvesting at different time periods resulted in ten moisture levels between 56% and 3.7%.

A dehydration and rapid freeze–thaw protocol was employed for cryopreservation in this study. After dehydration, 60 embryos at each moisture level were cultured directly as the control nonfrozen samples; another 60 embryos were placed into 2-ml cryovials and then plunged into liquid nitrogen. After 3 days of cryostorage, the cryovials were removed from the liquid nitrogen and immersed into 4 l of 40°C sterilized water for rapid thawing prior to culturing.

The embryos were cultured on double filter paper saturated with sterilized deionized water in clean Petri dishes (50-mm diameter). All cultures were incubated at 25± 1°C in a temperature-controlled incubator with an alternating photoperiod of 14 h light/10 h dark with light intensity of 66.25 μmol m^{-2} s^{-1} provided during daylight by fluorescent tubes. The cultures were monitored and watered regularly for 4 weeks. Survival was scored as the percentage of embryos showing an apparently visible elongation of root or shoot after culture and emergence as the percentage of embryos showing normal growth of both root and shoot.

Ultrastructural studies

The changes occurring in maize embryonic axes during cryopreservation were investigated using transmission electron microscopy. Ten embryonic axes were excised from postthaw embryos and their nonfrozen counterparts at each moisture level. They were then fixed in 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.0) at 4°C for at least 24 h, postfixed in osmium tetroxide followed by dehydration in graded acetone solutions and embedding in epoxy resin. The radicle portion of the maize embryo was chosen for ultrastructural observation in this study since embryo culture studies had shown that it is the most cryosensitive component in the whole embryonic axes (Wen and Song 2007a; Wen et al. 2009). The embryonic axes were first cut into 1-μm semithin sections and then cut into 50–70 nm ultrathin sections with an LKB-V ultramicrotome. The ultrathin sections were double-stained with uranyl acetate and lead citrate and finally viewed and photographed with a JEM-1011 transmission electron microscope.

Determination of electrolyte leakage

Eighteen embryos in each treatment were used to determine electrolyte leakage. Three were immersed in 20-ml deion-

ized water (Millipore, Molsheim, France) in a test tube. The conductivity of the bathing solution was measured by a conductivity meter (DDS-307, Shanghai Precision and Scientific Instrument Co., Ltd., China) at 0, 2, 5, 8, and 24 h of incubation. The 0-h reading was used to determine initial conductivity ($C_{initial}$). The tubes were then heated in a boiling bath for 30 min to kill the embryos, and the total conductivity (C_{total}) was measured after the solution cooled.

The relative intactness of plasma membranes was expressed as the leakage percentage of electrolytes. This was determined by the following formula:

$$\text{Electrolyte leakage } (\%) = (C - C_{initial}) \div (C_{total} - C_{initial}) \times 100$$

where C = the conductivity at a fixed time; $C_{initial}$ = the initial conductivity; C_{total} = the total conductivity. The means and standard deviation (SD) were calculated for the six replicate samples at each moisture content.

Sample preparation before biochemical analyses

One hundred to 150 postthaw embryos and their nonfrozen counterparts at each moisture level were ground to a fine powder using a mortar and pestle under liquid nitrogen and stored at −80°C until the extraction of crude enzymes.

For the catalase (CAT; EC1.11.1.6) assay, soluble proteins were extracted by homogenizing 0.1 g (fresh weight) powdered sample in 3 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 1% polyvinylpyrrolidone, with the addition of 1 mM ascorbic acid in the case of ascorbate peroxidase (APX; EC1.11.1.11) and GR (EC1.6.4.2) assays. For the superoxide dismutase (SOD; EC1.15.1.1) assay, the potassium phosphate buffer (pH 7.0) contained 1 mM EDTA, 0.05% (*v*/*v*) Triton X-100, and 2% polyvinylpyrrolidone. In the dehydroascorbate reductase (DHAR; EC1.8.5.1) extraction, 0.1 g (fresh weight) powdered sample was homogenized in 3 ml of 50 mM Tris–HCl buffer (pH 7.2) containing 0.3 M mannitol, 1 mM EDTA, 0.1% (*w*/*v*) bovine serum albumin (BSA), and 0.05% cysteine. The homogenate was centrifuged twice (15 min each time) at 15,000×*g* at 4°C, and the supernatant was used for the spectrophotometric and electrophoretic enzyme assays described below. The SOD supernatant was also used for malondialdehyde (MDA) determination. Protein content was determined according to the method of Bradford (1976) with BSA as the standard.

Spectrophotometric determination of antioxidant enzyme activities and indices of oxidative damage

Standard enzymatic assays were performed at 25°C using a DU 800 Spectrophotometer (Beckman Coulter, USA) according to the following methods and using the volumes described: total SOD as Ginnopolitis and Ries (1977) measured in a 3-ml total volume; total CAT as Aebi (1984) in a 3-ml total volume; total GR as Schaedle and Bassham (1977) in a 1-ml total volume; total APX as Nakano and Asada (1981) in a 3-ml total volume; and total DHAR as Arrigoni et al. (1992) in a 1-ml total volume.

Lipid peroxidation was measured as the concentration of thiobarbituric-acid-reactive substances in embryo homogenates, equated with MDA as described by Heath and Packer (1968) but modified by Hodges et al. (1999). The products were quantified from the second derivative spectrum with standards prepared from 1,1,3,3-tetraethoxypropane. One and half milliliters of freshly prepared reagent (0.5% (*w*/*v*) 2-thiobarbituric acid in 15% (*w*/*v*) trichloroacetic acid) was added to 300 μl of sample. The assay mixture was heated at 95°C for 15 min, and then the tubes were cooled immediately in tap water. After cooling and then centrifuging for 10 min at 3,000×*g*, the absorbance of the supernatant was measured at 450-, 532-, and 600-nm wavelengths.

Electrophoretic determination of antioxidant enzyme activities

In-gel analyses of antioxidant enzyme activities were performed at 4°C on nondenaturing polyacrylamide gels using a Mini-Protein II electrophoresis cell (Bio-Rad, Hercules, CA, USA) according to the following procedures: CAT isozymes as Anderson et al. (1995; 7.5% T, 3% C, notation of Righetti 1983); APX isozymes as Lee and Lee (2000; 10% T, 3% C); isozymes of SOD as Beauchamp and Fridovich (1971; 12% T, 3% C); and GR isozymes as Anderson et al. (1995; 7.5% T, 3% C).

Results

Viability change in maize embryos during desiccation and freezing

As reported in a previous study (Wen and Song 2007a), maize embryos reach physiological maturity at 44 DAP. At 35 DAP, embryos had an initial moisture content of about 56% and had acquired considerable desiccation tolerance and some cryotolerance, i.e., 85% emergence retained after desiccation to 8% moisture content, and 80% survival was achieved in postthaw embryos within a narrow moisture range between 5% and 8%. However, neither tolerance was at its maximum. Dehydration for 12 h to about 6.0% MC made no difference to survival and caused slight damage to emergence, while further desic-

cation appeared to decrease both parameters (Fig. 1). The critical effect of moisture content on cryopreservation success made dehydration prior to freezing a necessary procedure. None of the immature 35 DAP embryos could survive cryoexposure at the initial moisture content. As moisture content decreased, emergence and survival in postthaw embryos increased progressively. Dehydration for 7 h to 11% moisture content significantly enhanced survival and emergence. Dehydration for 12 h prior to freezing resulted in peak emergence and survival in postthaw embryos. Further dehydration resulted in obvious desiccation damage and subsequent freezing of these overdehydrated embryos further decreased viability (Fig. 1). These results show that control of moisture content prior to freezing is the key to cryopreservation.

Ultrastructural changes in embryonic cells

The radicle portion of maize embryos was investigated in this study. Electron microscopy revealed that: the radicle cells were fully turgid in the initial state at this developmental stage; dense cytoplasm filled the cell volume; the plasmalemma was intact and contiguous with the cell wall; the mitochondria had normal appearance; and the nuclear envelope was intact, and nucleoli were clear (Fig. 2a–d). However, conspicuous changes were caused by freezing embryos at their initial moisture content, which was as high as 56% on a fresh basis. The frozen samples showed a mosaic of cells with various injuries. Symptoms included: plasmolysis, mitochondrial condensation, increased heterochromatin, nuclear shrinkage, and chromatin condensation as well as rupture in cell wall, cell membrane, and nuclear envelope (Fig. 2e–h). Dehydration prior to freezing can decrease damage that cells suffer during a subsequent freezing–thawing cycle. As an example of this, rupture of the cell membrane was found only on frozen samples hydrated beyond 40% MC (fresh basis) and rupture of the nuclear envelope only on frozen samples hydrated beyond 30% MC. The embryos frozen at moisture contents between 6% and 15% demonstrated mild injury; main symptoms exhibited were plasmolysis, heterochromatin increase and folding of cell wall, and nuclear distortion in some cells (Fig. 2n–o). However, dehydration itself can be a stress resulting in desiccation damage, with its severity increasing as desiccation progresses. A slight heterochromatin increase and mitochondrial swelling can be observed in some cells from dehydrated samples beyond 30% MC (pictures not shown), while cellular shrinkage was popular when dehydrated to 23% MC (Fig. 2i). Cells responded to desiccation in a quantitative manner; for an example, heterochromatin increase became more and more evident as dehydration proceeding in drying samples (Fig. 2i–k) and condensed in overdehydrated samples, i.e., MC<5% (Fig. 2l). Subsequent freezing imposed additional stress on these dehydrated embryos and resulted in more obvious symptoms; chromatin condensation in dehydrated samples (Fig. 2m–o) and coalescence of lipid bodies in overdehydrated samples (Fig. 2p) were observed only after freezing. Meanwhile, ruptures in cell walls, cell membranes, and nuclear envelop were found only in postthaw embryos (Fig. 2f). Although plasmolysis was frequently observed in dehydrated samples, large gaps between cell wall and cell membrane were found only in overdehydrated samples. However, such large gaps were relatively popular in frozen samples. Comparison of frozen samples (Fig. 2m–p) with their nonfrozen counterparts (Fig. 2e–h) at the same moisture level revealed that freezing induced or increased plasmolysis. At the same time, cell distortion, folding of cell wall, severe plasmolysis, and chromatin condensation were detected only in overdehydrated samples (Fig. 2l) before freezing while these symptoms were more common in postthaw embryos (Fig. 2e–h, m–p).

Damage to the integrity of the plasma membrane during dehydration and freezing–thawing

Results showed that electrolyte leakage from dehydrated and/or frozen embryos was a function of incubation time and that damage to the integrity of plasma

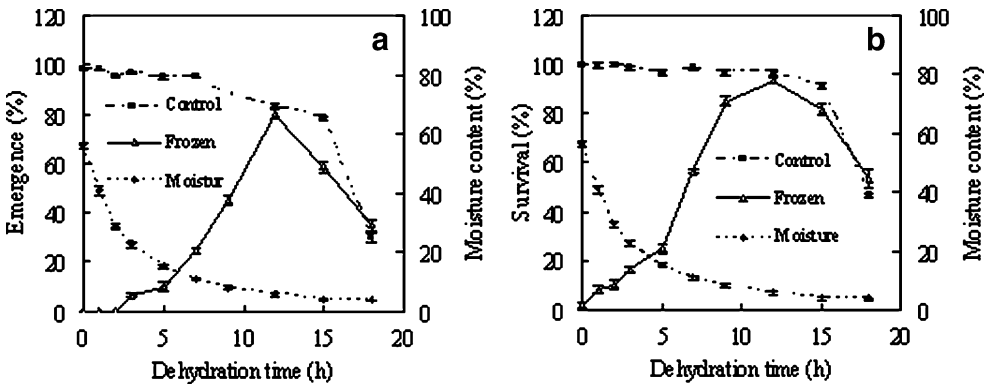


Fig. 1 Changes in emergence (**a**) and survival (**b**) of maize embryos after dehydration and/or freezing. The moisture content is expressed as means ± SD of eight replicates of five embryos and the emergence and survival as means ± SD of six replicates of ten embryos

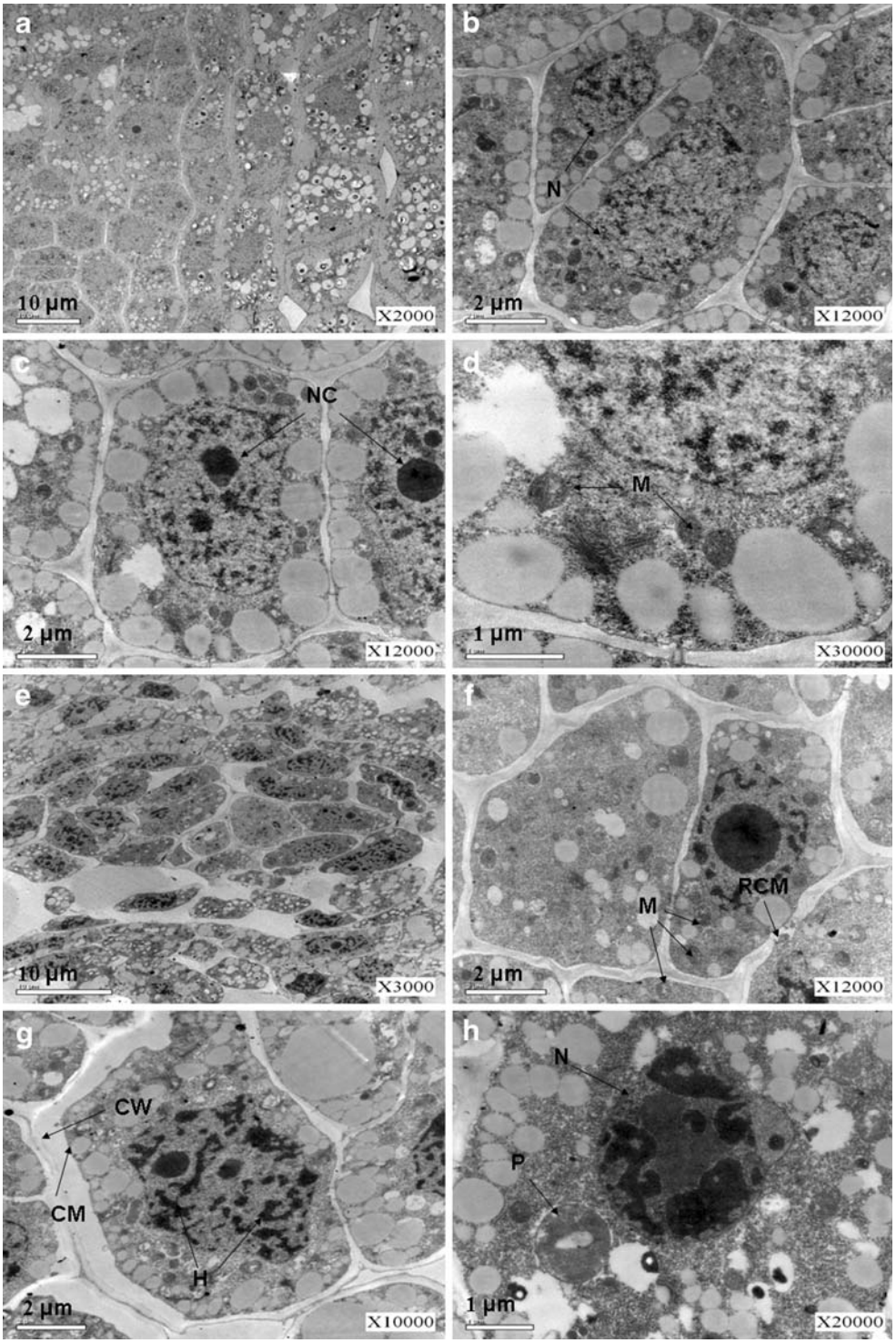
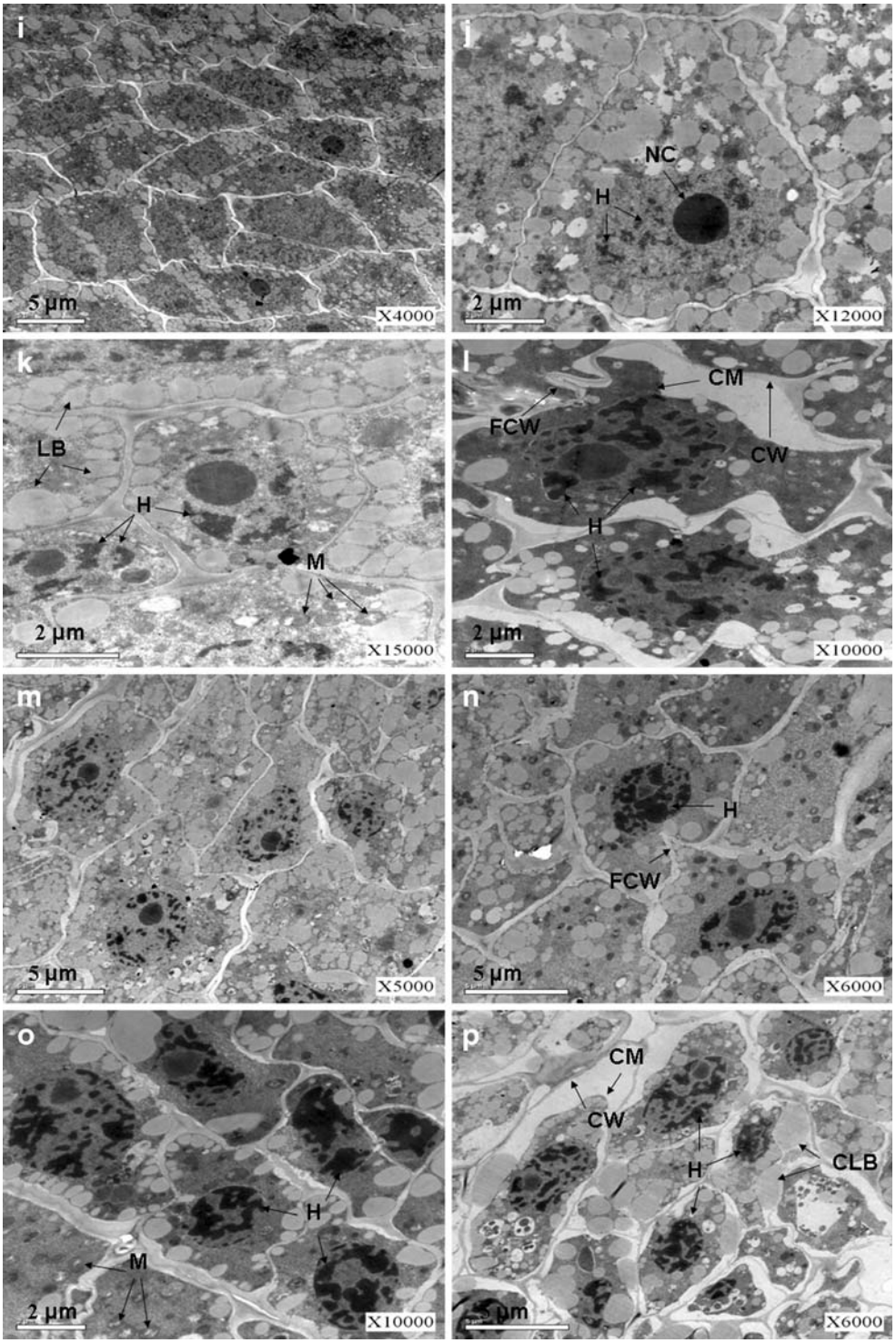


Fig. 2 **a**–**h** Electron micrographs of sections of the radicle of maize during cryopreservation. **a**–**d** Nondehydration and nonfrozen control (56% MC); **e**–**h** frozen embryos at initial moisture level of 56%. *N* nucleus, *NC* nucleolus, *CW* cell wall, *CM* cell membrane, *M* mitochondrion, *P* plastid, *H* heterochromatin, *RCM* ruptured cell membrane. **i**–**p** Further electron micrographs of sections of the radicle of maize during cryopreservation. **i**–**l** Dehydrated embryos, **m**–**p** dehydrated and frozen embryos, among them, **i** and **m** at 23% MC, **j** and **n** at 11% MC, **k** and **o** at 8% MC, **l** and **p** at 4% MC. *CW* cell wall, *CM* cell membrane, *NC* nucleolus, *M* mitochondrion, *LP* lipid body, *H* heterochromatin, *FCW* folding of cell wall, *CLB* coalesced lipid body

membranes resulted mainly from freezing (Fig. 3). As maize embryos dehydrated, electrolyte leakage of imbibed embryos increased. Dehydration alone, shown by the *dashed lines* in Fig. 3, however, only slightly damaged the plasma membrane, demonstrating that the plasma membrane of maize embryos at this stage was relatively desiccation tolerant. As an example, 24-h incubation following 18-h dehydration produced 18% electrolyte leakage, double that of the nondehydration control sample (Fig. 3d). In contrast, freezing of highly hydrated embryos caused severe electrolyte leakage (Fig. 3), which closely correlated to the viability loss in postthaw embryos (Fig. 1). Samples frozen at 30% moisture content produced 20% electrolyte leakage after 2-h incubation

Fig. 2 (continued)



and 60% leakage after 5-h incubation (Fig. 3). Dehydration for 5 h to 15% moisture content prior to freezing was a critical turning point, which greatly reduced electrolyte leakage of postthaw embryos. Freezing did not influence electrolyte leakage in samples below 10% moisture content. The difference in electrolyte leakage between frozen and unfrozen samples at 10–15% moisture content was evident only when incubation exceeded 8 h (Fig. 3).

Antioxidant enzyme activity analysis and detection of lipid peroxidation products

Both dehydration and freezing influenced antioxidant enzyme activity, with the response being different for each antioxidant enzyme. Dehydration of embryos to 10–30% moisture content greatly enhanced APX activity while overdehydration almost depressed activity to its initial

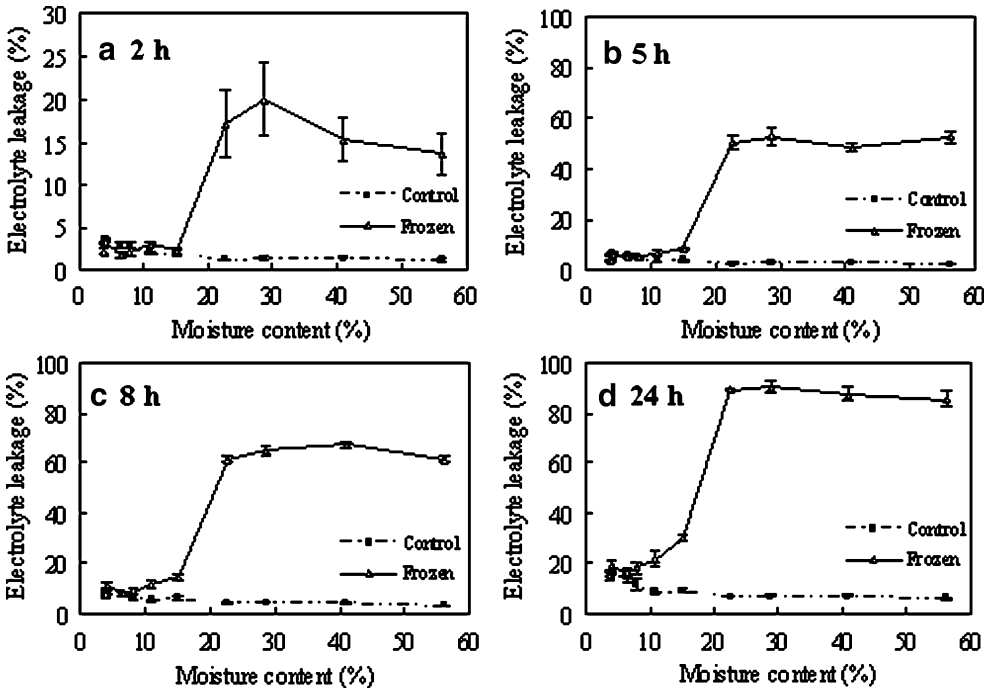


Fig. 3 Electrolyte leakage of maize embryos after imbibition for 2 h (**a**), 5 h (**b**), 8 h (**c**) and 24 h (**d**). Electrolyte leakage is expressed as relative conductivity. Values are expressed as means ± SD of six replicates. Note that *Y*-axis in **a** had a maximum scale of 30%, different from that in **b**–**d**

level. Freezing increased APX activity in samples at high-moisture content, but this declined in low-moisture content samples. Hence, postthaw samples had largely equal APX activity when dehydrated below 30% moisture content (Fig. 4a). These results were strongly supported by isozyme profiles (Fig. 5a). Moderate dehydration slightly depressed CAT activity, but these samples had the highest CAT activity after a freezing–thawing cycle (Figs. 4b and 5b). GR activity was induced by intensive desiccation, but regardless of moisture content, its activity was depressed to similar levels after freezing and thawing (Figs. 4c and 5c). DHAR activity was the lowest of all antioxidant enzyme investigated. Activity decreased when samples were desiccated to 6–30% moisture content and freezing deactivated DHAR activity in all samples to an extremely low level (Fig. 4d). SOD activity largely decreased as dehydration progressed, and freezing resulted in an additional decrease which was minimized as moisture content declined (Fig. 4e). The gel isozyme profiles for SOD in frozen samples were not as intense as in unfrozen samples (Fig. 5d).

As expected, both dehydration and freezing increased lipid peroxidation as indicated by MDA production. As the moisture content decreased during dehydration, the MDA content in embryos increased progressively, and there was a further increase after freezing. This latter additional increase was evident except for embryos below 4% MC. At most, a threefold increase in MDA content was detected in dehydrated and frozen samples compared with their controls (Fig. 4f).

Discussion

Before they are able to tolerate exposure to cryogenic temperatures, developing orthodox maize seeds/embryos are as cryosensitive as recalcitrant seeds (Wen and Song 2007a). This study used maize embryos at 35 DAP, when some survived cooling in liquid nitrogen, but only after dehydration, to investigate the lesions imposed during cryopreservation. Obvious biochemical and ultrastructural changes were detected in dehydrated and/or frozen embryos. There is little doubt that cryoinjury is a complex problem which needs comprehensive analysis.

Desiccation and freezing impose a double stress during cryopreservation. As our study used a dehydration and immersion into liquid nitrogen within cryovials, followed by rapid thawing, lesions from dehydration and freezing were evaluated in tandem and were found to harm maize embryos differentially. As moisture content declined, dehydrated embryos exhibited more and more obvious symptoms of damage. There was a slight heterochromatin increase and mitochondrial swelling even when only mild dehydration was employed. Cellular shrinkage and plasmolysis occurred after dehydration to 23% moisture content, and cell distortion and nuclear shrinkage were observed in samples which experienced intensive desiccation. The subsequent freezing–thawing treatment inflicted additional lesions; their severity strongly correlated with the moisture content of the samples. Freezing resulted in serious injuries such as severe plasmolysis, rupture of the nuclear envelope, mitochondrial and nuclear condensation

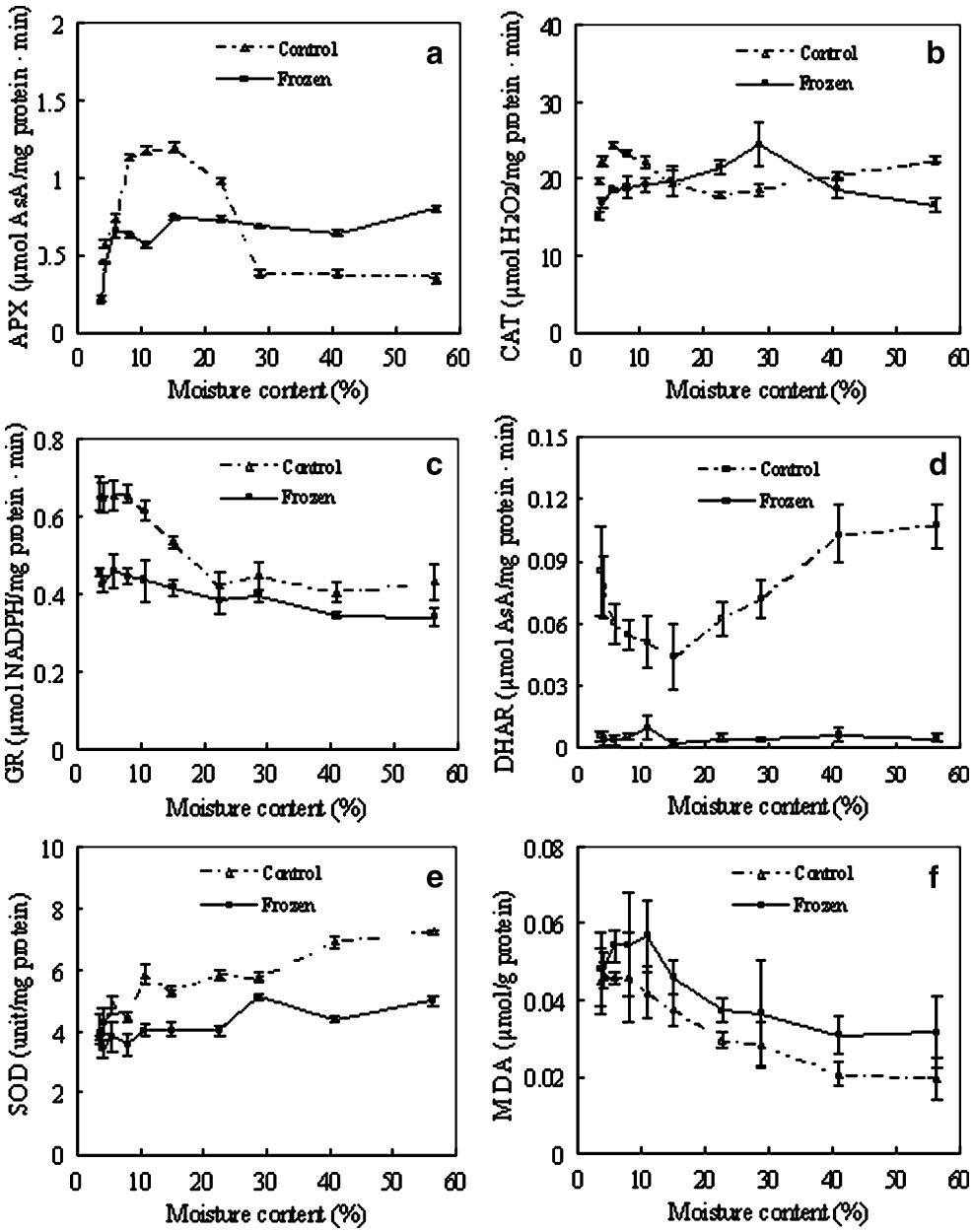


Fig. 4 Changes in APX (**a**), CAT (**b**), GR (**c**), DHAR (**d**), SOD (**e**) enzyme activity, and MDA content (**f**) in maize embryos after desiccation and/or freezing. Values are expressed as means ± SD of four replicates

in highly hydrated embryos, and cell and nuclear distortion in overdesiccated embryos. This indicates that cryopreservation is a conflict between freezing and desiccation injury. Minor symptoms of damage only occur in embryos at intermediate moisture contents of between 6% and 15%, emphasizing the necessity of moderate dehydration prior to freezing. The additional lesions from freezing were also demonstrated by an increased electrolyte leakage, decreased SOD activity, and increased MDA production. The damage from dehydration was negatively related to moisture content while that from freezing was positively related to moisture content. This confirmed the previous report (Wen and Song 2007a) for embryos at this particular developmental stage that desiccation clearly decreased survival and emergence only when the moisture content fell below 6%, while freezing caused drastic viability loss in embryos hydrated beyond 15% moisture content.

Histocytological study has been used to investigate damage occurring during desiccation and freezing. However, recent studies revealed that some ultrastructural changes were artifacts of aqueous fixation: the description of the phenomenon formerly referred to as sign of desiccation stress, including separation of cell wall and the plasmalemma, membrane irregularities, and distortions of cellular substructures, could be induced during aqueous fixation, instead of during desiccation (Wesley-Smith 2001). In spite of this, aqueous fixation has continued to be used in desiccation studies until very recently, such as

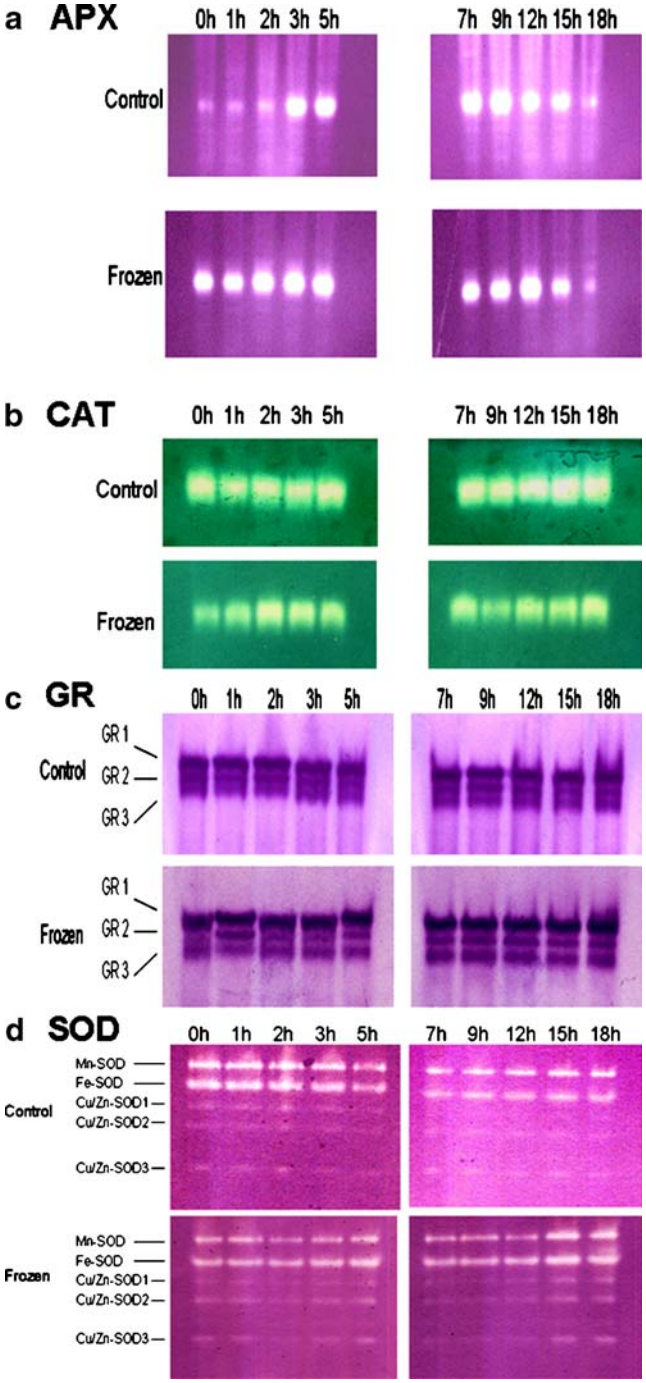


Fig. 5 Native gels stained for the activity of APX (**a**), CAT (**b**), GR (**c**), and SOD (**d**) in maize embryos after 0- to 18-h dehydration and/or freezing

Vicré et al. (2004), Moore et al. (2006), and Liu et al. (2007). In the present study, the symptoms observed in Fig. 2e–h, including plasmolysis, mitochondrial condensation, increased heterochromatin, nuclear shrinkage, and chromatin condensation as well as rupture in cell membrane and nuclear envelope, should not be this case because they were observed in samples frozen at initial moisture level (approximately 56% MC on the fresh basis). In dehydrated samples, cell shrinkage and wall folding were maintained; straightening did not take place during fixation (Fig. 2i–p). Severe plasmolysis happened in overdehydrated samples before freezing (Fig. 2l), but this is more common in postthaw samples at all moisture levels (Fig. 2m–o) and most conspicuous in overdehydrated and frozen samples (Fig. 2p). Although artifacts of aqueous fixation could not be excluded for this phenomenon, at least it can be an evidence to prove that cytoplasm greatly reduced in volume, which was also harmful (Iljin 1957; Walters et al. 2002).

The plasma membrane is a primary target of cellular injury during cryopreservation, and increase in membrane permeability corresponding to the viability decrease has frequently been detected following freezing–thawing cycles (Fujikawa 1995; Grout et al. 1980; Gusta et al. 1982; Lepock et al. 1984). In our study, dehydration down to 8% moisture content prior to freezing resulted in only a limited increase in electrolyte leakage, indicating that orthodox maize embryonic cells are characterized by a plasmalemma that is relatively tolerant to dehydration. A drastic increase in electrolyte leakage was detected in postthaw embryos hydrated beyond 15% MC (Fig. 3), and this coincided with the hydration level at which only a quarter of the embryos survived freezing (Fig. 1 and also Wen and Song 2007a). We suggest that this is the threshold of freezable moisture content for successful freezing of maize embryos at this developmental stage. The lethal electrolyte leakage and ultrastructural changes seen were most possibly caused by intracellular and/or extracellular ice crystal formation. Vertucci et al. (1991) found that the presence of a sharp peak in the warming thermogram at 0°C, indicating that ice formation had occurred at this temperature, invariably predicated a lethal response for frozen *Landolphia kirkii* embryonic axes while Mazur (1984) suggested that lethal intracellular ice crystal formation might be the major factor causing cryopreservation failure.

It has been shown that the freeze–thaw cycle promoted reactive oxygen species (ROS) production (Benson 1988; Benson and Bremner 2004) and consequently sped up the accumulation of cytotoxic metabolic products, such as MDA, in frozen–thawed material (Benson et al. 1992; Fleck et al. 1999). Oxidative stress caused by ROS production can be an important agent of cryoinjury. Our study showed perturbed antioxidant enzymes in maize embryos during cryopreservation, with each antioxidant enzyme having characteristic responses to dehydration and the freezing–thawing treatment—the activity of some enzymes increased while others decreased. This resembles the chilling stress-induced changes in the leaves of cucumber where the profile of cellular antioxidant systems was changed (Lee and Lee 2000). As demonstrated by this

study, the imbalance in the pro-oxidant to antioxidant ratio in cells will lead to elevated level of peroxidants and increased MDA content (Fig. 4f). Although it was difficult to establish a close correlation between viability and antioxidant enzyme activity in this study, the potential injury derived from changes in antioxidant levels can be drastic. It has been shown that the loss in seed viability can be closely related to a decrease in antioxidant enzyme activities and/or promotion of lipid peroxidation in naturally and artificially aged seeds; for example, this has been shown for *Fagus* seeds under conventional storage (Pukacka and Ratajczak 2005) and seeds of cotton (Goel et al. 2003) and sunflower (Bailly et al. 1996, 1998; Kibinza et al. 2006) during artificial aging and neem seeds under cryogenical storage (Varghese and Naithani 2008). This indicates that oxidative stress is characterized by secondary and cumulative nature. We suggest that perturbations in antioxidant enzymes are a potential and important, but not a lethal, factor for orthodox maize during short-term cryotreatment.

Acknowledgements We are grateful to the National Natural Science Foundation of China (grant no. 30571526), the President's Foundation of the Chinese Academy of Sciences, and Key Laboratory of Tropical Horticultural Plant Resources and Genetic Improvement (Hainan University), Ministry of Education, for funding the research that resulted in this publication.

Conflict of interest The authors declare that they have no conflict of interest.

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