Protoplasma (2009) 236:29–37
DOI 10.1007/s00709-009-0044-9

ORIGINAL ARTICLE

Cytological and physiological changes related to cryotolerance in orthodox maize embryos during seed development

Bin Wen · Ruling Wang · Songquan Song

Received: 9 October 2008 / Accepted: 21 April 2009 / Published online: 12 May 2009

Abstract Cytological and physiological changes were studied in orthodox maize (*Zea mays* L.) embryos following the acquisition of cryotolerance to liquid nitrogen during seed development. It was found that the embryonic cells at radicle portion were hydrated at all stages investigated, but those at early stages contained fully functional organelles, which disappeared at last developmental stages, and reserve materials accumulated intensively during seed development. Total soluble sugar content in the embryos had a steady rise on fresh weight and moisture weight basis; meanwhile, soluble and heat-stable proteins increased progressively in their number and contents as embryos matured. These cytological and biochemical changes had good correspondence with acquisition of cryotolerance in maize embryos.

Keywords Seed development · Desiccation tolerance · Cryotolerance · *Zea mays* L.

Abbreviation

DAP	Days after pollination
W_{50FS}	The upper limit of moisture content allowing 50% postthaw embryos to survive

Introduction

The term "orthodox seeds" (Roberts 1973) was used to define the category of seeds which are tolerant to desiccation and freezing and bankable, but seeds do acquire cryotolerance during development. Though orthodox seeds at maturity usually can be desiccated to low moisture content and accomplish cryopreservation, they could be sensitive to desiccation and liquid-nitrogen exposure at early developmental stages. It was found that maize embryos were cryosensitive until 26 days after pollination (DAP) during seed development, while those at 29 DAP had only very limited cryotolerance, which gradually increased in the subsequent maturation and to its peak at 44 DAP (Wen and Song 2007a). Changes in cryotolerance of seeds or embryos during seed development were also reported in recalcitrant Chinese fan palm (Wen and Song 2007b) and sycamore (Daws and Pritchard 2008) though they exhibited different patterns.

What events and process happened during seed development? Which of them were involved in acquisition of seed cryotolerance? What is the physiological basis of cryotolerance development? As mechanism on cryotolerance development has been rarely elucidated so far, only some implies can be inferred from studies on desiccation tolerance development (for example, *Oryza sativa*; Still et al. 1994), cold acclimation of vegetative tissues (for example, *Betula pubescens*; Rinne et al. 1998), and in vitro induction of stress tolerance (for example, *Asparagus officinalis* L.; Jitsuyama et al. 2002). These studies reported notable changes in cytological and biochemical aspect, especially those concerning cellular dedifferentiation, "switch-off" of metabolism, vitrification, presence and efficient operation of antioxidant systems, and accumulation of protective molecules, including soluble sugars and late embryogenesis abundant proteins (reviewed by Vertucci and Farrant 1995; Pammenter and Berjak 1999; Berjak and Pammenter 2008 etc.). As maize is a model plant whose seeds were frequently used in the study of desiccation tolerance development (for examples, Bochicchio

B. Wen (✉) · R. Wang · S. Song
Xishuangbanna Tropical Botanical Garden,
The Chinese Academy of Sciences,
Mengla,
Yunnan 666303, China
e-mail: wenb@xtbg.org.cn

et al. 1988, 1991, 1994; Chen and Burris 1990; Thomann et al. 1992; Bernac et al. 1997; Perdomo and Burris 1998) and acquisition of cryotolerance has been described previously in maize embryos (Wen and Song 2007a), it is worthy to conduct subsequent study to investigate further the physiological basis of cryotolerance development using this material.

Materials and methods

Plant material

Ears of maize (*Zea mays* L. cv. "Nongda 108") were manually collected between 17 and 53 (DAP) from cultivated plants in Menglun, Mengla, Xishuangbanna, China in 2004. Avoidance of natural pollination, conduction of controlled artificial pollination, harvest of ears in different development stages, isolation of the embryos, and determination of moisture content were described in detail before (Wen and Song 2007a).

Ultrastructural studies

The changes occurring during seed development were followed using transmission electron microscopy. Ten fresh embryonic axes in each development stage were excised and fixed in 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH7.0) at 4°C for at least 24 h, then postfixed in osmium tetroxide followed by dehydration in a graded acetone and embedding in epoxy resin. The radicle portion of maize embryos was chosen for ultrastructural observation in this study. They were first cut into 1-μm semithin sections and then cut into 50–70 nm ultrathin sections with LKB-V ultramicrotome. The ultrathin sections were double stained with uranyl acetate and lead citrate, finally viewed and photographed with the JEM-1011 transmission electron microscope.

Collection of biochemical materials

Two hundred to 300 fresh embryos in each development stage were isolated directly into liquid nitrogen. They were ground to a fine powder in liquid nitrogen with a mortar and pestle and stored at −80°C in a freezer until the extraction of soluble sugars and soluble proteins.

Extraction and determination of soluble sugar

About 0.1 g (fresh weight) fine powder of sample was placed in a mortar and pestle, 5 ml deionized water, and a few drops of ether added. The mixture was homogenized and then washed using 80°C deionized water into a beaker to a total volume of 30 ml. The mixture was heated in a water bath at 80°C for 30 min. After cooling down, saturated neutral lead acetate solution was added drop by drop to remove proteins until no more white precipitate formed. After centrifugation at 5,000×*g* for 15 min, the supernatant was transferred into a tube containing 1.0 g sodium oxalate powder to remove the lead acetate. Following another centrifugation at 5,000×*g* for 15 min, the transparent solution was used to determinate soluble sugar contents.

The modified anthrone reagent (Fairbairn 1953) was made by dissolving 1.0 g anthrone into 1,000 ml 72% (*v*/*v*) cool sulfuric acid, and glucose was used to establish a standard curve. After oscillation, the mixture of 1.0 ml soluble sugar solution and 5.0 ml anthrone reagent was heated for 10 min in a boiling water bath, cooled down, and the OD value read at 620 nm in a DU-800 ultraviolet light/visible light spectrophotometer (Beckman Coulter, USA). Six determinations were used for each sample.

Extraction of soluble proteins and heat-stable proteins

About 0.2 g (fresh weight) fine powder of sample was placed in a cool mortar and pestle and 3.0 ml extraction buffer (50 mM of Tris-HCl (pH7.0), 10 mM of NaCl, 1 mM of DTT, and 2 mM of phenylmethanesulfonyl fluoride) was added. The mixture was vigorously homogenized on ice and then centrifuged twice at 15,000×*g* at 4°C, 15 min each time. The supernatant was collected and half of this extract was used as "soluble proteins". The other half was boiled at 95°C for 10 min in a water bath, cooled down in tap water for 10 min, and then centrifuged at 15,000×*g* at 4°C for 15 min to remove precipitated proteins. The supernatant was collected and used as "heat-stable protein" analysis (Thierry et al. 1999).

Determination of protein contents

Protein content of each sample was determined according to Bradford (1976) using the Coomassie Brilliant Blue method. A protein standard curve was established using dilution of bovine serum albumin (BSA; BioRad, Fraction V) prepared in solubilization solution. Four determinations were used for each sample.

SDS polyacrylamide gel electrophoresis of soluble and heat-stable proteins

Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Each lane was loaded with 10 μg of soluble proteins on 7.5–18% gradient acrylamide gel, or 6 μg of heat-stable proteins on 12% constant acrylamide gel. The gels were run at 200 V (Bio-Rad Mini

Proteian II apparatus) following the manufacturer's instructions and then stained overnight with colloidal Coomassie Blue G-250.

Results

A previous study (Wen and Song 2007a) has described basic changes of maize seeds and embryos during development in detail; therefore, only a brief summary is given here. A main change took place in embryo size between 17 and 23 DAP, which made embryo size multiplied by three times. Seeds and embryos steady increased their dry weights and reached the peak at 44 DAP. Subsequently, dry weights remained constant essentially while fresh weight began to decline. The moisture contents of seeds and embryos had been on decline all the time from 17 to 53 DAP.

In the previous study, ears of maize were harvested at 3 days interval between 17 and 53 DAP (Wen and Song 2007a). Developing maize embryos at each stage were rapidly dehydrated to nine moisture levels over activated silica gel, and half of embryos at each moisture level were subsequently plunged in liquid nitrogen and thawed in 40°C water after 3 days cryostorage, the other half used as their nonfrozen control. The resultant embryos were cultured on double filter paper saturated with sterilized deionized water in clean Petri dishes to assess their desiccation tolerance and cryotolerance. Survival was scored as percentage of embryos showing an apparent elongation of root or shoot after culture. Embryos at 17 DAP had gotten complete germinability but only very limited desiccation tolerance. They were cryosensitive until 26 DAP. Maize embryos increased desiccation tolerance and cryotolerance gradually during following development, reached the maximum of desiccation tolerance at 38 DAP and that of cryotolerance at 44 DAP. Since then, desiccation tolerance and cryotolerance remained at their maximum till full maturity.

Ultrastructural observation of cells in the developing embryos

In the previous study, we found that lots of postthaw embryos developed shoot but failed to develop root, especially at relatively high moisture content, while contrasting case rarely happened. These indicated that the radicle portion is more cryosensitive than plumule portion, so the radicle portion of maize embryos was intensively investigated in this study. Electron microscopy reveal that radicle cells were fully hydrated in all stages investigated, the dense cytoplasm filled the cell volume and pushed the plasma membrane to the cell wall, for developing maize embryos had rather high moisture contents at their initial status, ranging from 70% to 50% on a fresh basis. But notable changes can be found in appearance and intracellular structure corresponding to acquisition of cryotolerance. Cells at 26 DAP were large in size, contained abundant mitochondria, endoplasmic reticulum, and vacuole, indicating active metabolism. The regularly shaped nuclei possessed compact nucleoli. The mitochondria are well-differentiated and have plenty of crystal membranes; numerous ribosome can be found around endoplasmic reticulum; vacuoles are large and numerous, some filled with clusters of electron-dense material and some are transparent. Meanwhile, well-developed starch grains and lipid bodies are visible. Cells in the central region were compact and smaller with a large nuclear-to-cytoplasmic ratio, while those outside the center were larger and highly vacuolated and they were loosely organized with large intercellular space (Fig. 1a–d). Samples at 44 DAP were still hydrated, with round plasma membrane and expanded cytoplasm, but organelles concerning metabolism disappeared except sporadic mitochondria, indicating a very low level of metabolism; embryonic cells were arrested at an inactively metabolic status ready for maturation drying. The cells were full of reserve material in the cytoplasm—starch grains increased, lipid bodies were smaller but conspicuously increased, and protein bodies, which were presumably formed by deposit of storage proteins in vacuoles, presented greatly in these cells. The nuclear membranes were clearly definable. The plasma membrane was intact and enclosed a densely packed cytoplasm. Mitochondria were rare and endoplasmic reticulum was invisible. Cells in the central region were smaller and more compact than those outside the center, they had relatively uniform appearance with a globule shape, sporadic mitochondria are visible, and vacuole are filled with dense clusters of electron-dense material, while cells outside the center contain more lipid bodies, starch grains, and protein bodies and a compact layer of peripheral lipid bodies lined the inner surface of the plasma membrane. Generally, cells outside the center accumulated more reserve than those in the central region (Fig. 1e–h).

Changes in contents of soluble sugars during cryotolerance development

Maize embryos exhibited obvious changes in soluble sugar contents during seed development (Fig. 2). Expressed on the basis of dry weight of embryos, total soluble sugar content in the embryo had a fall period before 29 DAP and a rise period after 38 DAP roughly. The overriding pattern of dry weight accumulation that occurs during development may mask the relationship between soluble sugar accumulation and cryotolerance increase. A rather clear pattern presented itself when total soluble sugar content was expressed on the basis of the fresh weight, or especially

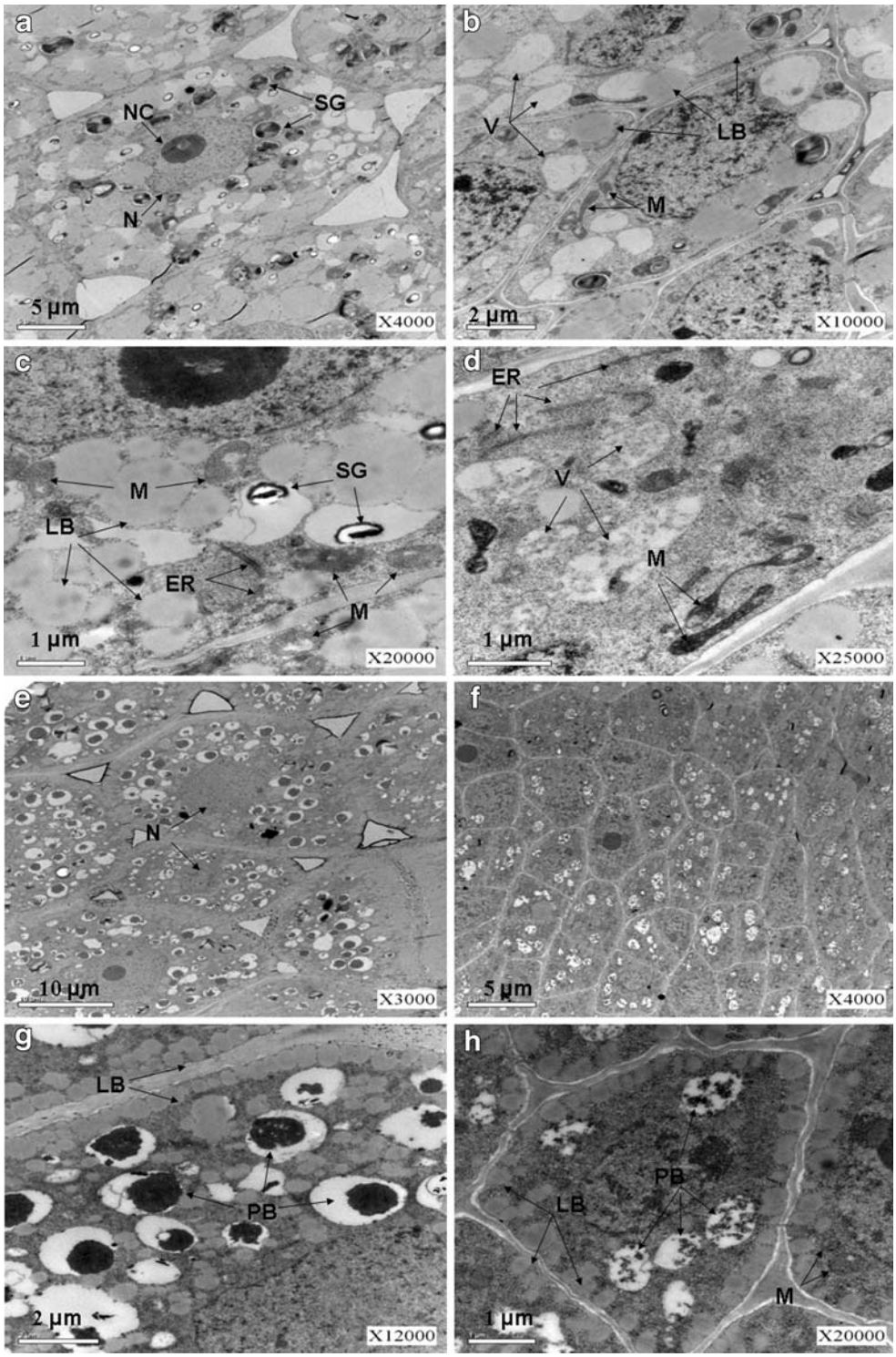

Fig. 1 Electron micrographs of sections of radicle of maize embryos during seed development. **a**–**d** At 26 DAP, **e**–**h** at 44 DAP. **a**, **c**, **e**, **g** Rim sections; **b**, **d**, **f**, **h** Central section. *N* nucleus, *NC* nucleoli, *M* mitochondria, *V* vacuole, *ER* endoplasmic reticulum, *SG* starch grain, *LB* lipid body, *PB* protein body. Note: Cells at 26 DAP were large in size, contained abundant mitochondria, endoplasmic reticulum, and vacuole, while those at 44 DAP were full of starch grains, lipid bodies, and protein bodies. At both stages, cells in the center are more compact and isodiametric that those outside the center

on the basis of moisture weight of the embryos. Its value increased steady in the whole development investigated. For example, soluble sugar content on a basis of moisture weight increased from 33 to 68 mg/g during period from 17 to 29 DAP, as embryos transited from cryosensitive to cryotolerance phase. It was 98 mg/g at 38 DAP when desiccation tolerance in embryos peaked and 122 mg/g at 44 DAP when cryotolerance peaked (Fig. 2). In this way, total soluble sugar content in embryos corresponded rather well with cryotolerance development in this period. After 44 DAP, soluble sugar content had a further increase, but this resulted more from moisture loss than from accumulation of soluble sugar, since maturation drying onset at this time.

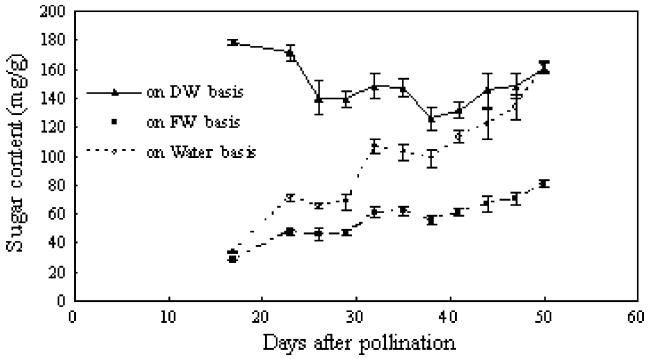

Fig. 2 Changes in content of soluble sugars in maize embryos during seed development. All values are expressed as means ± SD of six replicates

Analysis of soluble proteins and heat-stable proteins of embryos during cryotolerance development

The total soluble protein contents in the maize embryos progressively increased in the whole development no matter expressed on the basis of dry weight, fresh weight, or moisture weight of the embryos. This trend was the most outstanding when expressed on the basis of moisture weight (Fig. 3a). For example, the soluble protein content on a basis of moisture weight was 9 mg/g at 17 DAP, 24 mg/g at 29 DAP, 44 mg/g at 38 DAP when desiccation tolerance peaked, and 57 mg/g at 44 DAP when cryotolerance peaked in maize embryos. At the same time, the composition of the soluble proteins also changed. The embryos are mainly composed of high molecular weight proteins of 98 to 32 kDa before 26 DAP. In the subsequent development, the number and content of soluble low molecular weight proteins especially those below 30 kDa accumulated intensively. There were new protein bands appearing on SDS-PAGE corresponding to transition of maize embryos from cryosensitive to cryotolerant phase at 29 DAP and peaking of cryotolerance at 44 DAP (Fig. 4).

Similar results were obtained for heat-stable proteins in the embryos during seed development. The total content of heat-stable proteins had a steady rise on the basis of dry weight, fresh weight, and moisture weight of the embryos (Fig. 3b). For example, on a basis of moisture weight, the heat-stable protein content in the maize embryo was 0.78 mg/g at 17 DAP and 5.7 mg/g at 29 DAP, the value multiplied by more than sevenfold during this period. It reached 12 mg/g when desiccation tolerance peaks at 38 DAP and 17 mg/g when cryotolerance peaks at 44 DAP. Heat-stable proteins in maize embryo are mainly of molecular weights below 32 kDa. Bands for these proteins are very few within 29 DAP, but since 32 DAP on, a lot of new protein bands emerged and the heat-stable proteins became much more abundant. A new band at 24 kDa on SDS-PAGE, which became conspicuous only after 41 DAP had good correspondence with peaking of cryotolerance, can be specific to cryotolerance (Fig. 5).

In the previous study, W_{50FS}, the upper limit of moisture content allowing 50% postthaw embryos to survive, was used to describe the change in cryotolerance of maize embryos during development. In order to analyze the quantitative relationship between protective molecules and cryotolerance, we calculated protective molecule contents (on a moisture basis) at moisture content equal to W_{50FS} and found that soluble sugar content and soluble and heat-stable protein content at W_{50FS} were quiet constant since 38 DAP on, they were 7.02±0.77, 3.48±0.62, and 1.04±0.15 mg/g water, respectively, though soluble sugar content and soluble protein content were much higher at 32 and 35 DAP (Fig. 6).

Discussion

Development of orthodox seeds can be divided into three phases: histodifferentiation, cell expansion and reserve deposit, and maturation drying (Bewley and Black 1994). This paper followed maize embryo development from

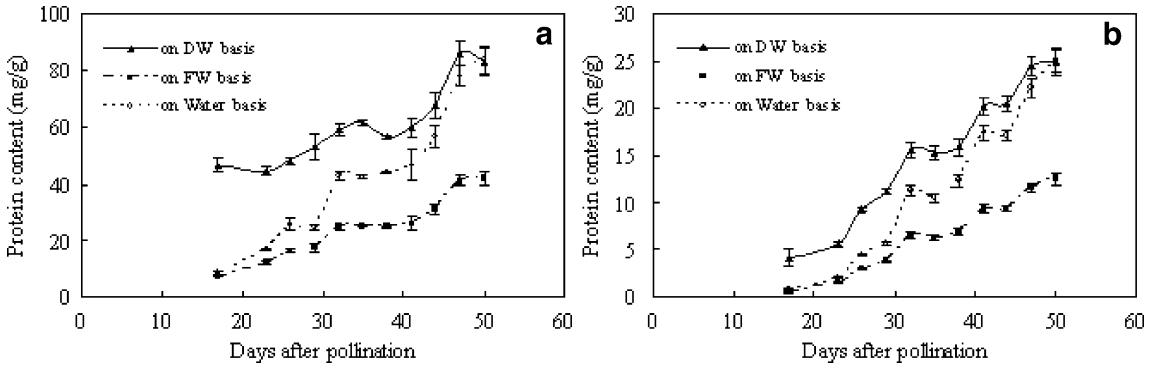

Fig. 3 Changes in content of soluble proteins (**a**) and heat-stable proteins (**b**) in maize embryos during seed development. All values are expressed as means ± SD of four replicates

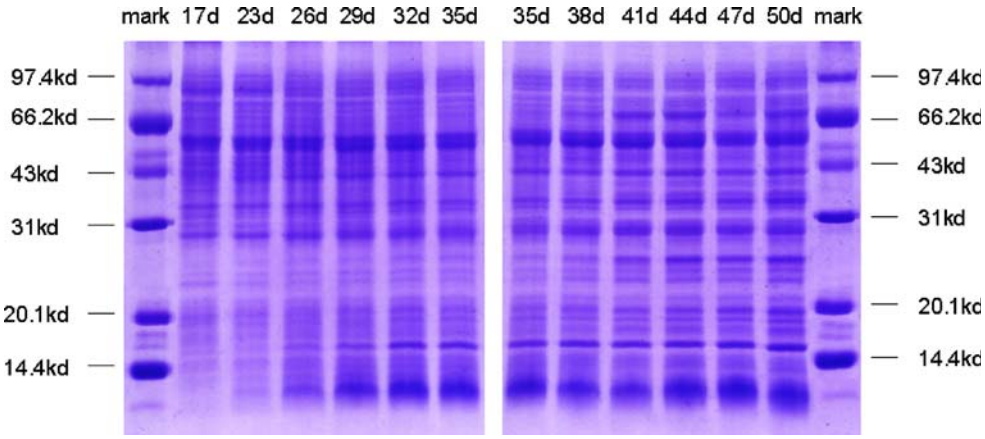

Fig. 4 SDS-PAGE of soluble proteins in maize embryos during seed development from 17 to 50 DAP. Aliquots of the protein extract were electrophoresed on 7.5–18% gradient acrylamide gel and a total of 10 μg of proteins was loaded in each *lane*. Molecular mass markers and their molecular masses are shown on both sides

histodifferentiation end to maturation drying onset to investigate events and process corresponding to cryotolerance. In concomitant with cryotolerance development, maize embryos underwent notable changes in cytological and biochemical aspects. Embryonic cells were hydrated at all stages investigated, but had a decrease in metabolism-related organelles and an increase in reserve-related organs during cryotolerance development. Total soluble-sugar content in the embryos demonstrated a progressive rise on fresh weight and moisture weight basis; meanwhile, soluble and heat-stable proteins in their number and contents increased steadily as embryos matured. These changes had good correspondence with acquisition of cryotolerance in maize embryos, and all of them presumably contributed to cryotolerance; however, it is hard to discern from the studies presented here which is critical. Therefore, we suppose that acquisition of cryotolerance is a multifactor result of all these events and process.

Ultrastructural observation revealed that intracellular ultrastructure is closely related to cryotolerance. On the one hand, embryonic cells from recalcitrant seeds are always hydrated and possess fully functional organelles, for example, *Aesculus hippocastanum* (Farrant and Walters 1998) and *Podocarpus henkelii* (Dodd et al. 1989); on the other hand, mitochondria, endoplasmic reticulum, and vacuole were found being reconstituted in orthodox seeds losing desiccation tolerance and cryotolerance during germination, such as imbibed maize embryos (Crèvecoeur et al. 1976; Isaaca and Mycock 1999) and oats embryos (Sargent et al. 1981). In addition, it was reported that pregrowth of sycamore cell suspensions under osmotic stress reduced cellular vacuole level and improve cryotolerance, but the same procedure could neither reduce cellular vacuole level nor improve cryotolerance in soybean suspended cells (Pritchard et al. 1986a, b). This correlation also exists in vegetative tissues; for an example, cytological changes in phloem parenchyma cells of living bark of black locust are closely related to the seasonal cycle of frost resistance (Pomeroy and Siminovitch 1971). During development, maize embryos underwent an evident dedifferentiation of intracellular organization, a great reduce of cellular membranes, and a 'switch-off' of cellular metabo-

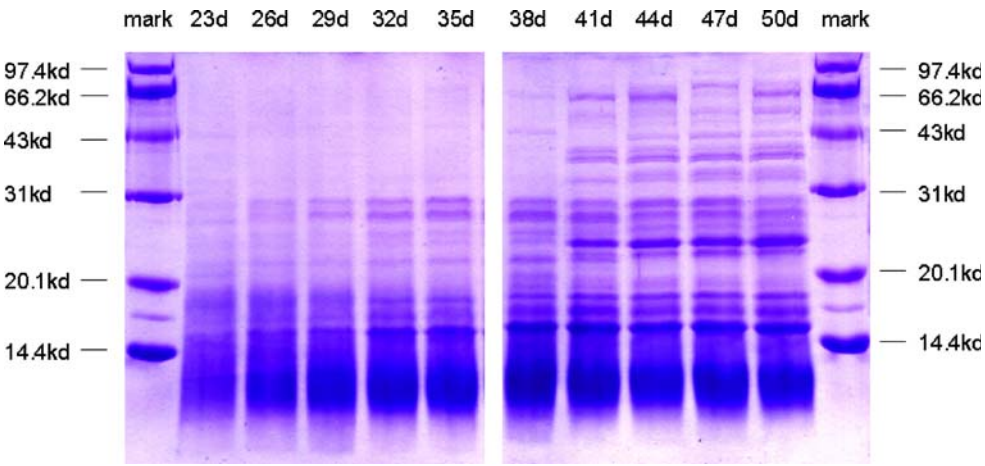

Fig. 5 SDS-PAGE of heat-stable proteins in maize embryos during seed development from 23 to 50 DAP. Aliquots of the protein extract were electrophoresed on 12% constant acrylamide gel and a total of 6 μg of proteins was loaded in each *lane*. Molecular mass markers and their molecular masses are shown on both sides

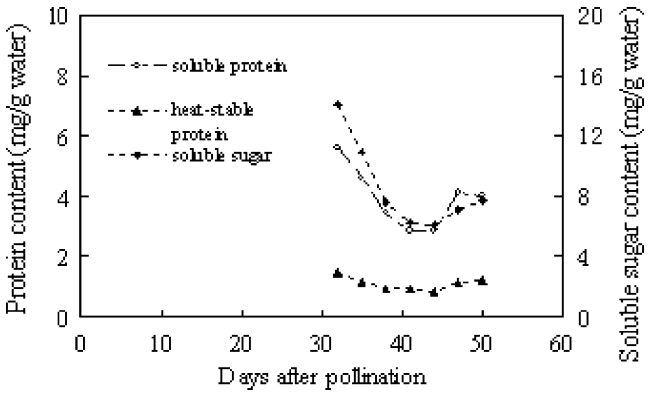

Fig. 6 The calculated protective molecule contents (on a moisture basis) at moisture content W_{50FS} in maize embryos at 32–50 DAP

lism; embryonic cells are ready for maturation drying. These are typical events and process happen in orthodox seeds. Similarly, degradation of mitochondria, ribosome, and chloroplast was observed in *Phaseolus lunatus* cells during seed maturation (Klein and Pollock 1968). As cessation of physiological activity is a prerequisite for desiccation tolerance in the cellular level, seed orthodox behavior is characterized by the arresting of embryonic cells in a dedifferentiated and metabolically inactive status.

Orthodox seeds usually accumulate huge amount of protective molecules, including soluble sugars and late embryogenesis abundant (Lea) proteins, in their late development, for examples, barley (Bartels et al. 1988), soybean (Rosenberg and Rinne 1988; Blackman et al. 1992), and wheat (Black et al. 1999). Most Lea proteins, as well as many other desiccation and cold acclimation-induced proteins, share the unique trait of boiling stability (Lin et al. 1990). The increase of freezing tolerance by sucrose incubation was also found to be accompanied by accumulation of protective molecules, such as boiling-stable proteins in carrot somatic embryos (Thierry et al. 1999) and asparagus embryogenic cell suspensions (Jitsuyama et al. 2002). These protective molecules may contribute to desiccation tolerance and cryotolerance by protecting membrane components (Crowe et al. 1987; Crowe et al. 1992) and improving vitrification (Sun and Leopold 1994; Buitink and Leprince 2004) although they alone may be insufficient to provide seeds with desiccation tolerance nor cryotolerance (Ooms et al. 1994; Steadman et al. 1996). Anyway, acquisition of desiccation tolerance and cryotolerance correlated with accumulate of soluble sugars and mature proteins during orthodox seed development, and decline of these protective molecules coincided with the loss of desiccation tolerance during germination (Rosenberg and Rinne 1989; Blackman et al. 1991). Previous studies had reported that expression of new proteins was involved in acquisition of desiccation tolerance in maize embryos (Bochicchio et al. 1988; Thomann et al. 1992). In this study, soluble sugars and soluble and heat-stable proteins all demonstrated a steady rise corresponding cryotolerance, suggesting that they may contribute to cryotolerance development. Moreover, the transition of maize embryos from cryosensitive to cryotolerance phase at 29 DAP and peaking of cryotolerance at 44 DAP both coincided with emergence of new proteins (Figs. 4 and 5). When this study was carried on, Huang (2008) studied proteome of desiccation tolerance during seed development using maize embryos from the same cultivar. By a combination of two-dimensional electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometer, she reported 111 proteins which were differentially expressed during desiccation tolerance development, among which 70 proteins were upregulated. We have special interest in the identity of the prominent 25-kDa protein upregulated in the later stages in our study. According to her work, 16 proteins in the range of 22–28 kDa upregulated and half of them were involved in stress response, including Ferritin, superoxide dismutase (Mn), maize 20S proteasome alpha subunit, 1-Cys peroxiredoxin antioxidant and TPA putative cystatin (Huang 2008). Furthermore, we calculated protective molecules content (on moisture basis) in dehydrated maize embryos at moisture content allowing 50% postthaw embryos to survive and found heat-stable protein content remained quite stable from 32 to 50 DAP (Fig. 6). Heat-stable proteins had more close correlation with cryotolerance in maize embryos than soluble sugar and soluble proteins and indicated their important role in the acquisition of cryotolerance.

Compared to accumulation of protective molecules, formation of reserve-related organelles in embryonic cells is more conspicuous in orthodox embryonic cells. Maize embryonic cells at late developmental stages contain great number of lipid bodies, starch grains, and protein bodies (Fig. 1e–h). Similar events were reported frequently, such as raped seeds (Leprince et al. 1990). On the contrary, recalcitrant seeds had only formation of nontypical protein bodies reported in *Durio zibethinus*, which never become fully filled, but contain only diffuse peripheral clumps of zibethinin protein (Brown et al. 2001). It is well known that these reserve materials will be mobilized and utilized during seed germination, but their role in acquisition of cryotolerance has not been elucidated yet. In cryobiology research, it has often been shown that isodiametric cells, with minimum vacuolation, are the most cryotolerant. Cells with these properties located in the central region of embryonic sections in this study. Then question arose: Cells outside the center are large in size and less isodiametric in shape, how did they increase cryotolerance? Are there different mechanisms for cryotolerance acquisition? As the close apposition between plasma membrane

and plasma membrane/intracellular membrane caused by cell shrinkage and cellular deformation is an causative factor for cryoinjury, which leads to lamellar-to-hexagonal II phase transitions and aparticulate domain production with fracture-jump lesions during freezing (Nagao et al. 2008), the ability to withstand the mechanical stresses associated with volume reduction is a major requirement for plant cells to tolerate desiccation (Iljin 1957), and most cells cannot tolerate >50% change in volume (Walters et al. 2002). We noticed that accumulation of lipid bodies, starch grains, and protein bodies was more evident in embryonic cells of large volume outside the center than those in the center (Fig. 1e–h). Protein bodies formed through storage proteins' accumulation primarily in the protein storage vacuoles of terminally differentiated cells of the embryos. This resulted in a general regression of all the cellular membranes, on the one hand. On the other hand, the replacement of cellular space by such hydrophobic or low-moisture materials will diminish cellular changes in volume during desiccation and freezing and prevent plasma membrane from approaching each other. Thus, the accumulation of lipid bodies, starch grains, and protein bodies may improve cellular stress tolerances through both methods. In addition, the compact alignment of lipid bodies along the plasma membrane is a progressive process that occurs during natural and artificial drying (Perdomo and Burris 1998; Cordova-Tellez and Burris 2002). This compact alignment of lipid bodies reduced hydrophilic surface of plasma and will limit the rate of water loss from plasma during desiccation and the rate of water imbibition into plasma during rehydration; both of them are critical to keeping membrane integrity. We suppose that huge accumulation of reserve materials is an important strategy for mature cells of large volume to increase desiccation tolerance and cryotolerance during seed maturation.

Acknowledgments This study was supported by the National Natural Science Foundation of China (No. 30571526) and the President's Foundation of the Chinese Academy of Sciences.

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

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