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ORIGINAL ARTICLE

Cytological and physiological changes related to cryotolerance in recalcitrant *Livistona chinensis* embryos during seed development

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Abstract Cytological and biochemical changes in recalcitrant *Livistona chinensis* embryos following the acquisition and loss of cryotolerance to liquid nitrogen during seed development were studied. The embryonic cells were always hydrated and contained fully functional organelles throughout seed development. However, the central cells in the root-epicotyl end of the embryo exhibited partial dedifferentiation during the middle developmental stages, although extensive reduction of mitochondria and vacuolation and intensive accumulation of starch grains, lipid, and protein bodies were not observed. Total soluble sugar content rose then decreased on a fresh weight and water weight basis, while soluble and heat-stable proteins increased in number and content then decreased, as seeds matured. These cytological and biochemical features differ from those of orthodox seeds, providing a physiological basis for the recalcitrant behavior of *L. chinensis* seeds. The changes were closely correlated with acquisition and loss of cryotolerance in *L. chinensis* embryos and are presumed to contribute to cryotolerance, which would account for the cryotolerance variation in *L. chinensis* embryos. Cryotolerance is suggested to be a complex, multifaceted process, and accumulation of soluble sugars and soluble and heat-stable proteins alone is not enough to increase cryotolerance per se without acting in combination with a decrease of cellular metabolic activity.

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Keywords Chinese fan palm · Cryopreservation · Cryotolerance · Desiccation tolerance · *Livistona chinensis* · Recalcitrant seeds · Seed development

Abbreviations

WAF	Weeks after flowering
W_{50FS}	The upper limit of water content allowing 50% post-thaw embryos to survive

Introduction

Recalcitrant seeds differ from orthodox seeds in lacking tolerance to desiccation and consequently to freezing, thus causing considerable problems for agroforestry practice and germplasm conservation of species with recalcitrant seeds. Elucidation of the seed recalcitrance mechanism will help to find means of overcoming the phenomenon for these purposes. In this respect, comparative and developmental studies have an important role, and work by Nkang and Chandler (1986), Hong and Ellis (1990), Farrant et al. (1993a, 1993b, 1997), Still et al. (1994), and Walters et al. (2001), for example, have increased our knowledge of recalcitrant seed biology.

Subcellular organization and metabolic activity are strongly correlated with the degree of desiccation tolerance seeds attain during development (Farrant et al. 1997). Orthodox seeds always intensively accumulate protective molecules and transit to a metabolism-inactive state during late embryogenesis (reviewed by Vertucci and Farrant 1995; Pammenter and Berjak 1999); in contrast, recalcitrant seeds usually maintain fully functional organelles and retain active metabolism (for examples see Dodd et al. 1989a; Farrant et al. 1992a, 1993b; Farrant and Walters 1998;

Brown et al. 2001; Bhattacharya et al. 2002). Furthermore, recalcitrant seeds initiate germination-related events before dispersal in some species, such as *Avicennia marina* (Berjak et al. 1984). However, these cytological and biochemical studies were mostly focused on desiccation tolerance and have rarely related to cryotolerance.

Although always sensitive to cryoexposure, recalcitrant *Livistona chinensis* embryos have a acquisition of cryotolerance at the early developmental stage followed by a loss of cryotolerance prior to shedding of the seed from the parent plant, with peak cryotolerance achieved midway through seed development from 27 to 36 weeks after flowering (Wen and Song 2007b). Such a loss of cryotolerance was also detected in seeds of tea (Kim et al. 2002) and Alexander palm (Shao et al. 2009). These findings raise the following questions. Are these changes in cryotolerance correlated with cytological and biochemical events and processes? How do these cytological and biochemical features compare with what is known of orthodox seeds? What is the physiological basis of cryotolerance development? In particular, what events and processes are related to the loss of cryotolerance in the final stage of recalcitrant seed development? The acquisition and loss of cryotolerance in *L. chinensis* embryos has been described previously (Wen and Song 2007b), but further cytological and biochemical studies are required to investigate the physiological basis for cryotolerance development in recalcitrant *L. chinensis* embryos on the one hand, and to provide a comparison to cryotolerance development in orthodox maize embryos (Wen and Song 2007a; Wen et al. 2009) on the other hand.

Materials and methods

Plant material

Fruits of Chinese fan palm (*L. chinensis* [Jacq.] R.Br.) were manually collected between 4 and 45 weeks after flowering (WAF) from trees growing in Menglun, Mengla, Xishuangbanna, China, in 2004. The flowering date, developmental stages at which fruit were harvested, isolation of the embryos and determination of water content have been described previously (Wen and Song 2007b).

Ultrastructural examination

Ultrastructural changes occurring during seed development were investigated using transmission electron microscopy. Ten fresh embryos at each developmental stage were dissected and 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, dehydrated in a graded acetone series, and embedded in epoxy resin. First, 1 μm semithin sections were cut and checked with a light microscope, and then 50–70 nm ultrathin sections were cut with a LKB-V ultramicrotome. The ultrathin sections were double stained with uranyl acetate and lead citrate, and observed and photographed with a JEM-1011 transmission electron microscope (Wen et al. 2009).

Sample preparation for biochemical analyses

Three hundred to 500 fresh embryos at each developmental stage were isolated and immediately plunged into liquid nitrogen. After isolation of all embryos, they were ground with a mortar and pestle in liquid nitrogen, and the resultant powder stored at −80°C prior to extraction of soluble sugars and proteins.

Extraction and determination of soluble sugars

Extraction of soluble sugars followed the method of Wen et al. (2009). About 0.1 g (fresh weight) of the powdered sample was homogenized in a mortar and pestle in 5 mL deionized water containing a few drops of ether. The homogenate was washed with 80°C deionized water into a beaker and diluted to a total volume of 30 mL. The mixture was heated in a water bath at 80°C for 30 min. After cooling, proteins were removed by dropwise addition of saturated neutral lead acetate solution until no further white precipitate formed. After centrifugation at 5,000×*g* for 15 min, the supernatant was transferred to a tube containing 1.0 g sodium oxalate powder to remove the lead acetate and centrifuged again at 5,000×*g* for 15 min. The clear supernatant was used to determine the soluble sugar content.

Soluble sugar content was measured using a modified anthrone reagent method (Fairbairn 1953). One gram of anthrone was dissolved in 1 L 72% (*v*/*v*) cool sulfuric acid, and a calibration curve was constructed using analytical-grade glucose. After oscillation, a 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 and the optical density at 620 nm was determined with a DU-800 spectrophotometer (Beckman Coulter, USA). Six determinations were recorded for each sample.

Extraction of soluble and heat-stable proteins

The method described by Thierry et al. (1999) was used to extract soluble and heat-stable proteins. About 0.2 g (fresh weight) of the powdered sample was vigorously homogenized in 3.0 mL extraction buffer (50 mM Tris–HCl [pH 7.0], 10 mM NaCl, 1 mM DTT, and 2 mM PMSF) in a chilled pestle and mortar on ice. The homogenate was centrifuged twice at 15,000×*g* at 4°C for 15 min each. Half of the supernatant was used to analyze the soluble protein

content. The remaining supernatant was boiled at 95°C for 10 min in a water bath, cooled in tap water for 10 min, then centrifuged at 15,000×*g* at 4°C for 15 min to remove precipitated proteins. The supernatant was used to analyze the heat-stable protein content.

Determination of protein content

The protein content of each sample was assayed using the Coomassie Brilliant Blue method (Bradford 1976). Four determinations were performed for each sample. A protein standard curve was constructed using bovine serum albumin (BioRad, Fraction V) dilutions prepared in the respective sample solution.

SDS polyacrylamide gel electrophoresis of soluble and heat-stable proteins

Soluble and heat-stable proteins were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Ten micrograms of soluble proteins or 7.5 μg of heat-stable proteins were loaded in each lane of 10% or 12.5% SDS gels, respectively, electrophoresed at 200 V with a Bio-Rad Mini-PROTEAN II system according to the manufacturer's instructions, and stained overnight with colloidal Coomassie Blue G-250.

Results

As development of the fruits, seeds and embryos of *L. chinensis* has been described in detail in a previous study (Wen and Song 2007b), only a brief summary is presented here. The main change in fruit and seed size took place during the first 13 WAF, but accumulation of dry matter was very slow in the same period. During subsequent development, the dry weight of fruits, seeds, and embryos increased by 1.9 times, 2.5 times, and 3.53 times, respectively, from 15 to 27 WAF, and remained constant after this period. From 15 to 45 WAF, the water content of the fruits, seeds, and embryos decreased by 17%, 46%, and 18%, respectively, but the embryo water content was still as high as approximately 70% (on a fresh weight basis) at maturity. The osseous endocarp thickened to form a hard layer covering the seed at about 21 WAF. Some seeds were found to have germinated on the mother plants after 36 WAF, with the radicle penetrating the testa and epicarp.

L. chinensis embryos acquired desiccation tolerance and cryotolerance gradually during development. Desiccation tolerance peaked at 25–33 WAF. While embryos could not be cryopreserved until 21 WAF, they survived cryoexposure with a peak emergence of 2.5% within a very narrow moisture window around 9% at 23 WAF, and subsequently cryotolerance increased progressively and reached a maximum at 27–36 WAF. After 36 WAF, desiccation tolerance and cryotolerance decreased with seed maturation (for details, see Wen and Song 2007b).

Ultrastructural observation of cells in the developing embryos

The palm embryos consist of a large single cotyledon, a very short root-hypocotyl axis, and an epicotyl. The cotyledon end of *L. chinensis* embryo forms a haustorium, while the root-epicotyl end develops into the root and stem after germination. As the haustorium always fails to develop after freezing, it is not a good index of cryotolerance, so the root-epicotyl end was chosen for cytological observation in this study. Electron microscopy revealed the embryonic tissue to be a mosaic of cells at a variety of developmental stages, differing from one another in size and intracellular structure, indicating a strategy of continuous development without arrest of differentiation and maturation. Generally, peripheral cells were larger and highly vacuolated with one large central vacuole restricting the cytoplasm to a thin parietal layer, while central cells had a high nuclear to cytoplasm ratio, and their nucleus was central with small vacuoles distributed around it. Both types of cells lacked storage reserves and exhibited an active state. Substantial changes were observed during seed development.

Cells at 19 WAF (Fig. 1a–d) commonly contained well-differentiated mitochondria (Fig. 1b, d), abundant endoplasmic reticulum (Fig. 1b, d), Golgi bodies (Fig. 1d), and sporadic lipid bodies (Fig. 1c), indicative of active metabolism. The regularly shaped nuclei possessed compact nucleoli (Fig. 1c). The large central vacuoles of the peripheral cells contain scattered clumps of electron-dense material, while the small vacuoles of the central cells were electron-lucent (Fig. 1a, b).

Cytoplasm of samples at 30 WAF (Fig. 1e–h) contained fine granular material, possibly of a proteinaceous nature. Lipid bodies increased in frequency but were still few in number (Fig. 1g, h). Mitochondria (Fig. 1h) and endoplasmic reticulum (Fig. 1h) can be found in some cells, but their numbers had decreased, especially for endoplasmic reticulum. Vacuoles were electron-dense with a lot of content, presumably proteins (Fig. 1f). These vacuoles also had an increasing granularity (Fig. 1h). These all indicated a relatively low level of metabolism. In the central region there was a mass of less-differentiated cells, relatively uniform in appearance, small in size, with an isodiametric and globular shape and few organelles (Fig. 1g).

At 39 WAF (Fig. 1i–l), embryonic cells showed increased mitochondria numbers (Fig. 1l), meanwhile vacuoles had more electron-lucent area (Fig. 1i, k), so presumably they had resumed active metabolism. The mass of cells in the central region had lost their uniform appearance (Fig. 1k). They developed numerous vacuoles; some were filled with clumps of electron-dense material,

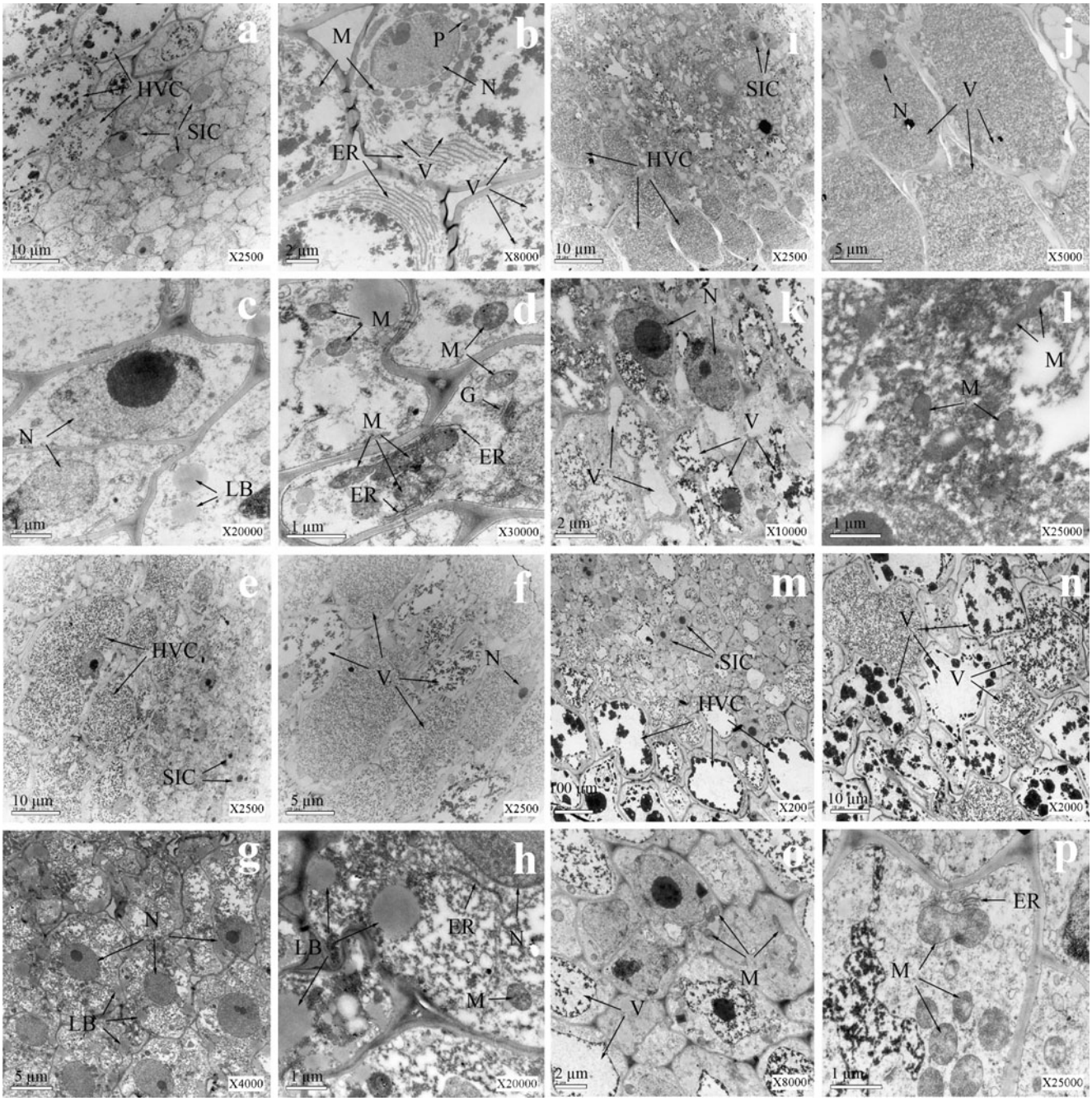

Fig. 1 Electron micrographs of sections from the root-epicotyl end of *Livistona chinensis* embryos during seed development. **a**–**d** at 19 weeks after flowering (WAF), **e**–**h** at 30 WAF, **i**–**l** at 39 WAF, **m**–**p** at 45 WAF. **b**, **f**, **j** and **n** are peripheral sections, **c**, **g**, **k** and **o** are central sections, **a**, **e**, **i** and **m** for overview, **d**, **h**, **l** and **p** for organelles in detail. HVC = highly vacuolated cells, SIC = small isodiametric cells, N = nucleus, M = mitochondria, V = vacuole, ER = endoplasmic reticulum, G =Golgi bodies; LB = lipid body, P = plastids

whereas some were electron-lucent. The central vacuoles of the peripheral cells were still full of granular material (presumably proteins, Fig. 1j) while the central cells contained large vacuoles with scattered dense deposits; these might be tannin (Fig. 1k).

Cells at 45 WAF (Fig. 1m–p) were characterized by aggregates of dense material in vacuoles, which might be tannin deposits. In addition, mitochondria (Fig. 1p) and vacuoles (Fig. 1m–o) further increased in size and number. These mitochondria were well-differentiated and active (Fig. 1p). The endoplasmic reticulum was again visible, which were very short ER profiles (Fig. 1p). It appears that the vacuolar protein content has been/is being depleted (Fig. 1n).

Over the course of seed development, intensive accumulation of insoluble reserves was absent, i.e., starch grains and typical protein bodies were not observed. The only common reserve-related organelles were vacuoles containing scattered clumps of electron-dense material, possibly proteins, which commonly appeared in embryonic cells during the middle developmental stages (Fig. 1e–h).

Changes in soluble sugar content during seed development

Clear changes in soluble sugar content of *L. chinensis* embryos occurred during seed development (Fig. 2). Expressed on a dry weight basis, total soluble sugar content was 330–410 mg/g within 23 WAF, and 400–450 mg/g after 25 WAF. On a fresh weight basis, and especially based on the water weight of the embryo, this value gradually increased, then remained at high levels in the middle stages and finally decreased in the final stage. When soluble sugar content is expressed on the basis of *water weight* of the embryo, i.e., weight of sugar per gram water, it is actually the concentration of soluble sugars in aqueous solutions. This is an important index to measure the vitrification capability of aqueous solutions in cryopreservation. For example, soluble sugar content increased from 65 mg/g to 140 mg/g on a water weight basis from 15 WAF to 21 WAF, remained at about 200 mg/g at 25–36 WAF, and decreased to about 160 mg/g again after 39 WAF (Fig. 2). When expressed on the basis of fresh or water weight, the variation of total soluble sugar content in *L. chinensis* embryos correlated well with cryotolerance development in the same period.

Analysis of soluble and heat-stable proteins in embryos during seed development

Whether expressed on the basis of dry weight, fresh weight, or water weight of the embryos, the total soluble protein content in *L. chinensis* embryos progressively increased and then gradually decreased during seed development; this pattern was especially clear when expressed on the basis of water weight (Fig. 3a). For example, soluble protein content increased steadily from 14 to 65 mg/g on a water basis from 15 WAF to 25 WAF, remained above 75 mg/g between 27 WAF and 33 WAF, and then began to decrease after 36 WAF, falling below 55 mg/g after 39 WAF. At the same time, the composition of the soluble proteins also changed. A conspicuous change in protein profiles took place between 19 and 21 WAF (Fig. 4), coinciding with the transition of *L. chinensis* embryos from cryosensitive to cryotolerant. In embryos before 19 WAF, most of the proteins were of high molecular weight (98–32 kDa). During subsequent development, the number and content of soluble low-molecular-weight proteins, especially those between 30 and 19 kDa, accumulated greatly. But after 36 WAF, bands for these proteins were distinctly weaker on SDS-PAGE gels (Fig. 4).

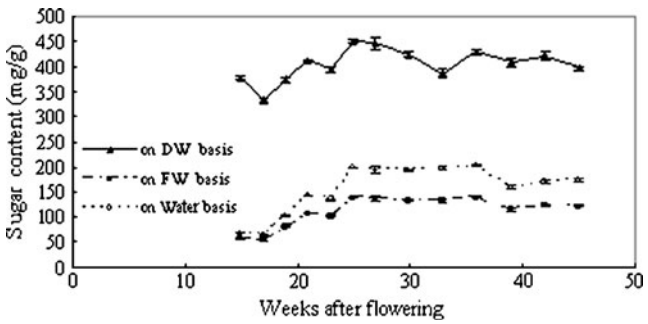

Fig. 2 Changes in soluble sugar content in *L. chinensis* embryos during seed development. All values are expressed as the mean±SD of six replicates

Similar trends were observed for heat-stable proteins in the embryo. The total content of heat-stable proteins increased and then decreased during seed development (Fig. 3b). For example, heat-stable protein content increased tenfold (from 2.7 to 30 mg/g on a water weight basis) from 15 WAF to 23 WAF. The content was highest at 25–33 WAF, varying between 34 and 42 mg/g, and then decreased below 27 mg/g after 36 WAF. Heat-stable proteins in *L. chinensis* embryos are mainly of <35 kDa molecular weight. Bands for these proteins were rare up to 19 WAF. However, after 21 WAF, numerous new protein bands appeared when heat-stable proteins were much more abundant, but these bands became less obvious in the final stages (Fig. 5). The decline in content of soluble and heat-stable proteins late in development indicated a change of protein metabolism in *L. chinensis*, in accordance with cytological observations.

In a previous study, the upper limit of water content allowing 50% of post-thaw embryos to survive (W_{50FS}) was used to describe the change in cryotolerance of *L. chinensis* embryos during development. To quantitatively analyze the correlative relationship between protective molecules, including soluble sugars, soluble and heat-stable proteins, and cryotolerance, their contents were co-plotted against the corresponding W_{50FS}, but failed to find clear trend (data not shown). Furthermore, based on their initial values, I calculated contents of these protective molecules when the embryos were dehydrated to water content equal to W_{50FS}. The calculated protective molecule contents (on a water weight basis) at the W_{50FS} water content were quite constant for all developmental stages since 27 WAF onwards, they were 12.34±2.45, 4.12±0.56, and 1.98±0.23 mg/g water, respectively, though these values much higher at 23 and 25 WAF (Fig. 6).

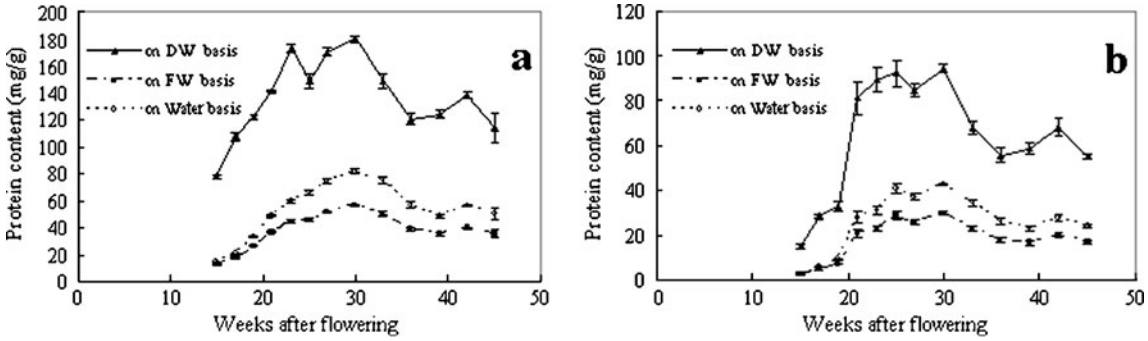

Fig. 3 Changes in content of soluble proteins (**a**) and heat-stable proteins (**b**) in *L. chinensis* embryos during seed development. All values are expressed as the mean±SD of four replicates

Discussion

In accordance with their inherent sensitivity to freezing, *L. chinensis* embryonic cells showed a subcellular organization typical of hydrated, metabolically active tissue, containing organelles which appeared fully functional and metabolically active throughout development, indicating ongoing metabolism; reserve-related organelles were rare, and extensive formation of starch grains and typical protein bodies was absent. Following the development of cryotolerance obvious changes were detected by cytological and biochemical approaches. Concomitant with acquisition and loss of cryotolerance during seed development, *L. chinensis* embryos underwent correlative physiological changes, demonstrated by redifferentiation following dedifferentiation in subcellular organization (Fig. 1), an increase followed by a decline in total soluble sugar content (Fig. 2), and a decrease following an increase in number and content of soluble and heat-stable proteins (Figs. 3, 4, and 5) in embryonic cells as seeds matured. There was a transient stage exhibiting partial intracellular dedifferentiation and obvious accumulation of protective sugars and proteins, corresponding with the peak of cryotolerance in the middle stages of *L. chinensis* seed development. Thus the events and processes involved in cryotolerance development, such as intracellular dedifferentiation, 'switching off' of active metabolism and accumulation of protective sugars and proteins, are not limited to orthodox seeds, but also may occur in recalcitrant seeds, although not as adequately as in orthodox seeds such as maize embryos (Wen et al. 2009). On the one hand, these cytological and biochemical features differed in extent from those occurring in maize embryos (Wen et al. 2009), providing evidence for recalcitrant behavior in this seed species; on the other hand, these cytological and biochemical changes were closely correlated with acquisition and loss of cryotolerance in *L. chinensis* embryos, providing a good explanation for their cryotolerance variation. These changes were all presumed to contribute to cryotolerance. As in maize, it is hard to discern from these studies which change is more critical because of the quantitative nature of cryotolerance. The acquisition and loss of cryotolerance in seeds may be a multifactorial result of these cytological and biochemical changes.

Cytological studies revealed that seed recalcitrance is characterized by actively metabolic tissues. For example, radicle meristem cells from *Aesculus hippocastanum* seeds maintain abundant mitochondria and conspicuous endoplasmic reticulum profiles at seed maturity (Farrant and Walters 1998). Embryonic cells from mature *Podocarpus henkelii* seeds are fully hydrated, contain numerous mitochondria and all organelles appear fully functional (Dodd et al. 1989a), and seed germination increases the degree of vacuolation, endoplasmic reticulum, polysomes,

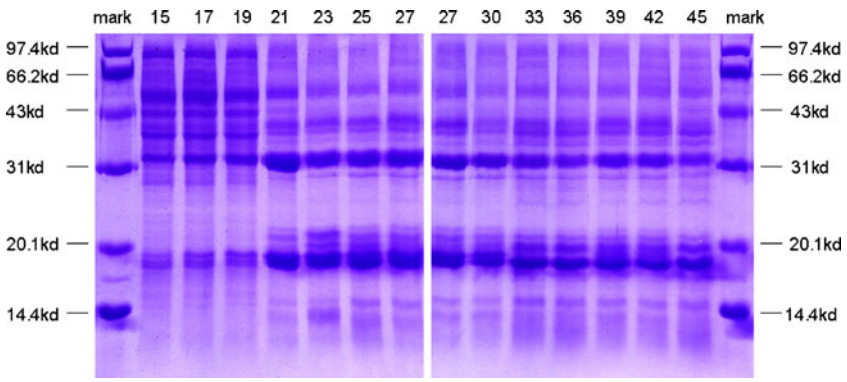

Fig. 4 SDS-PAGE profile of soluble proteins in *L. chinensis* embryos during seed development from 15 to 45 weeks after flowering. Aliquots of the protein extract were electrophoresed on a 12% constant acrylamide gel with 10 μg of proteins loaded in each lane. Molecular mass markers and their molecular masses are shown on both sides

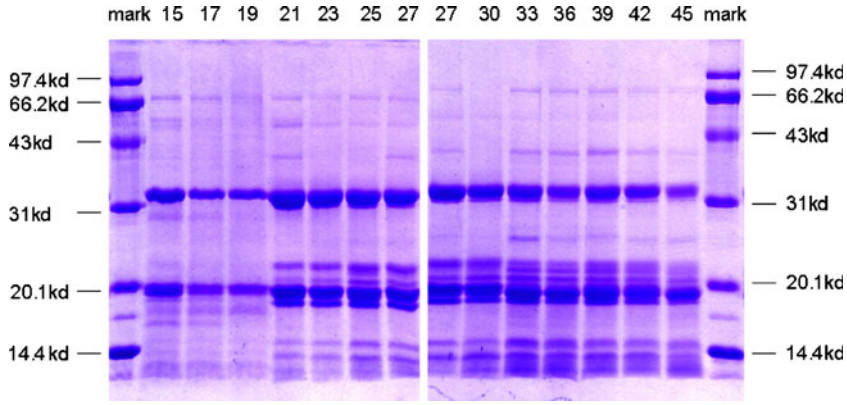

Fig. 5 SDS-PAGE profile of heat-stable proteins in *L. chinensis* embryos during seed development from 15 to 45 weeks after flowering. Aliquots of the protein extract were electrophoresed on a 12% constant acrylamide gel with 7.5 μg of proteins loaded in each lane. Molecular mass markers and their molecular masses are shown on both sides

and Golgi bodies (Dodd et al. 1989b). The same cytological traits were found in orthodox seeds losing desiccation tolerance during imbibition and germination, such as imbibed maize embryos (Crèvecoeur et al. 1976) and oat embryos (Sargent et al. 1981). Imbibition itself does not result in failure of cryopreservation, as extensive cryodamage became notable in hydrated maize embryos only after mitochondria developed cristae (Isaacs and Mycock 1999). Therefore, loss of desiccation tolerance and cryotolerance is related to germination-associated metabolic events. Berjak et al. (1984) studied newly shed *Avicennia marina* seeds and found that root primordial cells exhibited increased polysome levels, enhanced mitochondrial activity, and heightened vacuolation upon initial dehydration. These subcellular events are analogous to those occurring in orthodox seeds after germination is initiated. This is the basis for the hypothesis that recalcitrant seeds are equivalent to germinating seeds. Recalcitrant seeds may have initiated germination on the parent trees or shortly after shedding, and the ongoing germination process contributes to their increased desiccation sensitivity during hydrated storage (Pammenter et al. 1984; Farrant et al. 1986; Pammenter et al. 1994). However, this does not exclude a relatively quiescent phase occurring just

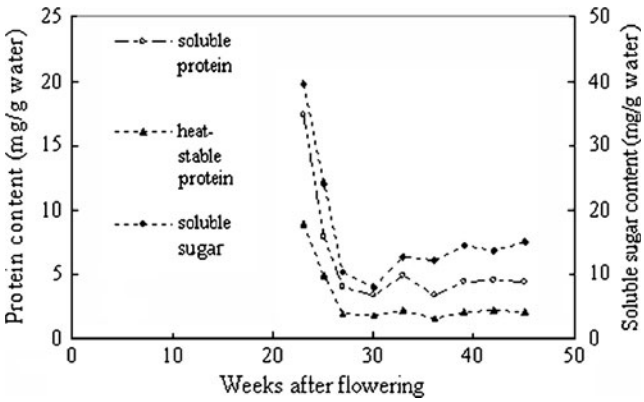

Fig. 6 The protective molecule content (on a water weight basis) estimated at a water content of W_{50FS} (the upper limit of water content allowing 50% of post-thaw embryos to survive) in *L. chinensis* embryos at 23–45 weeks after flowering

before germination, even though the seeds pass from development to germination without maturation drying. The present study followed embryonic cells in the root-epicotyl end and found that extensive mitochondrial degradation and reduced vacuolation, and intensive accumulation of starch grains, lipid and protein bodies, which are exhibited by orthodox maize embryonic cells (Wen et al. 2009), did not occur in recalcitrant *L. chinensis* embryonic cells. Only a partial dedifferentiation occurred to cells in the central region of the root-epicotyl end around 30 WAF, when these cells were relatively uniform in appearance, were of smaller size and contained fewer organelles, and were arrested in a less-differentiated state. At the cellular level seed recalcitrance is characterized by ongoing cellular differentiation and maturation, and fully functional subcellular organization.

Soluble sugars may contribute to desiccation tolerance and cryotolerance by protecting membrane components and improving vitrification (Crowe et al. 1992; Sun and Leopold 1994), but there is conflicting data for their efficiency (Chen and Burris 1990; Leprince et al. 1990; Ooms et al. 1994; Steaman et al. 1996; Chabrillange et al. 2000; Aberlenc-Bertossi et al. 2003). The same is true for soluble and heat-stable proteins, including LEAs (Farrant et al. 1992b, 1996; Finch-Savage and Blake 1994; Han et al. 1997; Lin et al. 1990). In this study, the contents of soluble sugars and soluble and heat-stable proteins changed with variation of cryotolerance in *L. chinensis* embryos, having good correlation between each other. However, it is hard to establish a quantitative relationship between cryotolerance and the contents of these protective molecules, i.e., soluble sugars and soluble and heat-stable proteins, thus none of them can be an indicator of cryotolerance. That embryos before 27 WAF had a much higher calculated protective molecule content (Fig. 6), but poor cryotolerance (Wen and Song 2007b) which may mean that accumulation of protective molecules alone is not enough to improve cryotolerance without appropriate ultrastructural changes, suggesting that cryotolerance development is actually a multi-factor consequence.

The precise characteristics of seed recalcitrance appear to be species specific (Berjak and Pammenter 2004). The loss of desiccation tolerance and cryotolerance in recalcitrant seeds before seed shedding, together with the concurrent decline of soluble sugars and stress-related proteins, might be a phenomenon unique to those seeds of tropical origin and be related to both germination initiation and reserve mobilization during the final developmental stages. The importance of developmental status for seed cryopreservation was suggested (Goveia et al. 2004), and cryotolerance loss at later seed development stages was reported in embryos/embryonic axes of tea (Kim et al. 2002), Chinese fan palm (Wen and Song 2007b), and Alexander palm (Shao et al. 2009). Such cryotolerance loss may also exist in seeds of jackfruit (Thammasiri 1999; Krishnapillay 2000) and cacao (Pence 1991) as similar protocols were reported to achieve successful cryopreservation of immature embryonic axes but cryopreservation of mature embryonic axes failed. However, the underlying mechanism responsible for this loss has not been elucidated yet. A previous study (Wen and Song 2007b) reported that both the critical water content for drying and the upper threshold water content for freezing changed with the acquisition and loss of cryotolerance in *L. chinensis* embryos during seed development, for which the cytological and biochemical changes observed in this study provide a partial explanation.

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

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