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ACQUISITION OF CRYOTOLERANCE IN MAIZE EMBRYOS DURING SEED DEVELOPMENT

Bin Wen* and Songquan Song

Xishuangbanna Tropical Botanical Garden, the Chinese Academy of Sciences, Mengla, Yunnan, China, 666303

*To whom correspondence should be addressed (E-mail: wenb@xtbg.org.cn).

Abstract

A desiccation-based cryopreservation protocol was employed to study the development of cryotolerance and desiccation tolerance in maize embryos from 23 to 50 days after pollination (DAP). Tolerances were acquired gradually and concomitantly. Maize embryos had low desiccation tolerance at 23 DAP when assessed by survival (embryo elongation) and emergence (root and shoot growth) after dehydration. Desiccation tolerance increased progressively, reached its maximum at 38 DAP, and remained constant afterwards. Cryotolerance, assessed by survival and emergence of post-thaw embryos, however, was nil until 26 DAP. Embryos at 29 DAP withstood cryoexposure within a very narrow moisture range only. Throughout development cryotolerance increased gradually, reached a maximum at 44 DAP and then remained at this level. The correlation between moisture content and cryopreservation success was notably influenced by the maize embryo's development stage. As seeds developed, the moisture content allowing 90% dehydrated embryos to survive and to emerge decreased, while the upper moisture content allowing 50% post-thaw embryos to survive and to emerge increased. Moisture contents of c. 14% allowed no less than 50% post-thaw embryos to emerge at the later development stages (e.g. c. 44 DAP); but no embryos within the same moisture range survived cryoexposure at 29 DAP, although they could withstand desiccation to this moisture level without impairment of survival and emergence. The relationship between cryotolerance and desiccation tolerance during maize seed development is discussed.

Keywords: cryopreservation; cryotolerance; desiccation tolerance; orthodox seeds; recalcitrant seeds; seed development; *Zea mays* L.

INTRODUCTION

Biological, mechanical and cost feasibility makes cryopreservation a suitable method for long-term preservation of plant germplasm (26). Compared to conventional seedbank storage, it has advantages such as reduced seed deterioration in storage, mechanical reliability, refrigeration-system stability, simplicity of procedure and higher cost effectiveness (26, 27, 32). Moreover, cryopreservation has been thought of as the most promising method for long-term storage of recalcitrant seeds (9,18,23), and even to date there is no alternative to cryopreservation for this purpose (4,5,12). Whilst orthodox seeds can be desiccated to low

moisture levels to enable cryopreservation, recalcitrant seeds are usually desiccation-sensitive at moisture levels that would ensure ice formation and cell death on cooling to ultra-low temperatures. After nearly 30 years of effort, only relatively few successes have been achieved in the cryopreservation of recalcitrant seeds and their embryos or embryo axes (11, 12). It can be inferred from this that cryotolerance is closely related to desiccation tolerance, but how closely they are related is still unknown. Since freezing and dehydration are fundamentally different stress vectors (10), it is relevant to investigate this relationship.

Although orthodox seeds are desiccation tolerant at maturity, they are not at early developmental stages (17); in other words, orthodox seeds undergo a transition from desiccation intolerance to desiccation tolerance during development. The questions we sought to answer were: do orthodox seeds have a similar developmental transition with regards to cryotolerance and, if so, when during development does this occur in relation to desiccation tolerance.

MATERIALS AND METHODS

Seed material

Zea mays L. cv. 'Nongda 108' seeds were bought from the Sinong Development Center of Maize Breeding, China Agricultural University. They were sown in the nursery garden of Xishuangbanna Tropical Botanical Garden in December 2004, and tended routinely during the following months. Female inflorescences were kept covered by envelopes to avoid natural pollination and controlled artificial pollination was conducted in March 2005. Seeds at different stages of maturity were collected as whole cobs at 3-day intervals from 23 to 50 days after pollination (DAP). After collecting, seeds were stored in polyethylene bags at 15°C until use within one week after collecting.

Moisture content and weight determination

Moisture content (% fresh weight) was determined gravimetrically on samples before and after drying for 17 ± 1 h in a ventilated oven at 103±2 °C, according to the ISTA recommendations for oily seeds (16). For seeds, 8 replicates of one individual seed were used; for embryos, 8 replicates of 5 individuals were used.

Fresh and dry weights of seeds at different stages of maturity were determined by sampling 8 replicates of 50 individuals; for embryos, 8 replicates of 10 individuals were used.

Desiccation treatment

Embryos were dehydrated by putting them in small containers (2 cm diameter) made of aluminum foil, and then the containers were put over activated silica gel in a closed desiccator for 0.5 h to18 h at 25-28°C, giving moisture levels between 73% and 4%. The endpoint of the desiccation treatment ranged from 9 h to 18 h, depending on seed maturity,

Embryo cryopreservation

Embryos were excised, avoiding any mechanical damage, and then desiccated, in batches of 120, over silica gel as described above. After dehydration, one half of the embryos at each moisture level were used for cryopreservation, the other half as the control. For cryopreservation, batches of 10 embryos were put in 2 ml-cryovials and then plunged in liquid nitrogen. After 3-day's cryostorage, the cryovials were removed and immersed for 60 to 90 s into 4 liters of 40°C deionized water for rapid thawing, prior to culturing. The control embryos were cultured directly after dehydration.

Embryo culture and viability evaluation

Embryos were cultured on moistened filter paper discs in clean Petri dishes (5cm diameter). All dishes were incubated at 25 ± 1°C in a temperature-controlled incubator, with an alternating photoperiod (14 h light / 10 h dark per day) with light (20 µmol m^{-2} s^{-1}) provided by white fluorescent tubes. Dishes were monitored and watered regularly for up to 3 weeks until all embryos germinated or rotted. Survival was scored as the percentage of embryos showing an apparent elongation of radicle or plumule after incubation, and emergence was scored as the percentage of embryos forming both root and shoot during this period. Both survival and emergence were expressed as means ± SD of 6 replicates of 10 embryos.

RESULTS

Seed development

Maize seeds are kernels composed of an embryo and endosperm. The embryos at 17 DAP were very small, but a drastic enlargement in size was observed in the next days. Seeds and embryos *in planta* grew from 23 to 44 DAP; seed and embryo dry weight increased from c. 73 mg and 4 mg respectively at 23 DAP to maxima of 262 mg and 39 mg respectively at 44 DAP. These changes represent c. 4- and 9-fold increases respectively in three weeks (Fig. 1a). Seed fresh weight reached a maximum at 38 DAP and that of the embryo at 44 DAP.

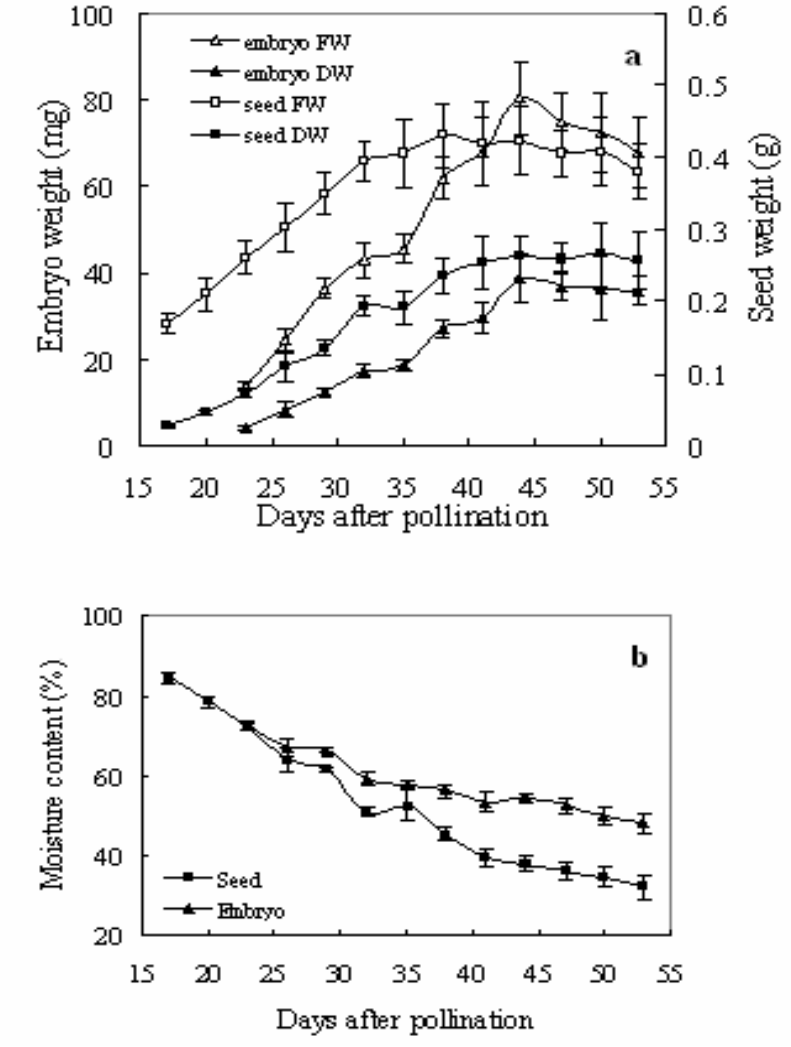

Figure 1. The changes in fresh weight (FW) and dry weight (DW) per seed and per embryo (a), and the changes in moisture contents (% FW) of seeds and embryos (b) of *Zea mays* during seed development. All values are expressed as means± SD of 8 replicates.

Later on, the fresh weight per seed and per embryo decreased slightly whilst dry weights essentially remained constant (Fig. 1a). Moisture content of seeds and embryos decreased throughout the whole development period investigated and reached 35% MC and 50% respectively by 53 DAP (Fig. 1b).

Development of desiccation tolerance in maize embryos

Desiccation tolerance, as measured by survival and emergence of desiccated embryos, progressively increased with seed development until 38 DAP (Fig. 2). Embryos acquired complete germinability at 17 DAP (data not shown), but those at early development stages had only low desiccation tolerance. For example, embryos at 23 DAP could tolerate 2 hours desiccation to 28% moisture content (MC) without damage to viability when measured as survival and emergence. However, 3 hours desiccation to 22% MC depressed survival to c. 40% and emergence to <20% and 5 hours desiccation to 15% MC reduced embryo viability to nil (Fig. 2a and c). At 26 DAP, embryos could tolerate 3 hours desiccation to 23% MC without reduction in survival and emergence; but 5 hours desiccation to 15% MC halved both survival and emergence and embryo viability was entirely lost after 7 hours desiccation to 10% MC. Embryo desiccation tolerance continued to increase gradually until 38 DAP (Fig. 2).

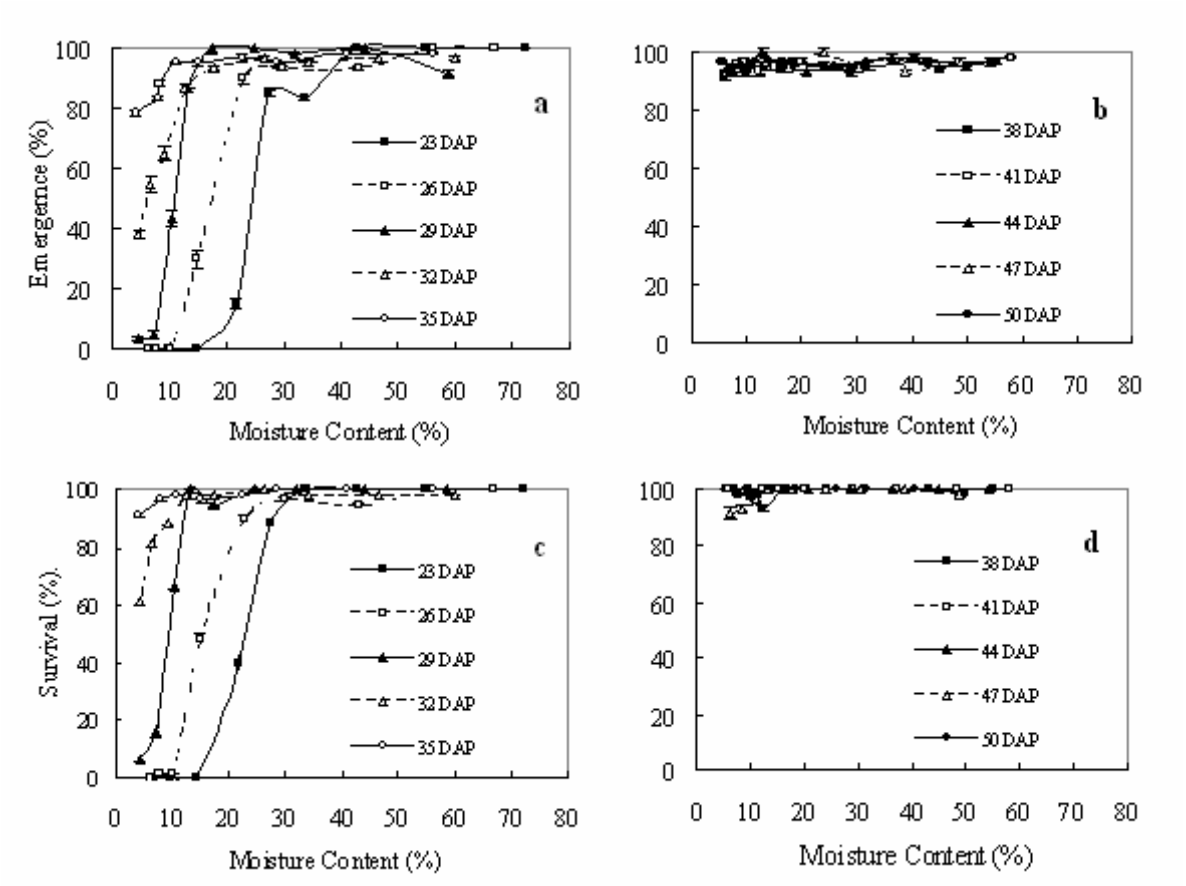

Figure 2. The changes in emergence (a, b) and survival (c, d) of *Zea mays* embryos at different developmental stages following dehydration to different moisture contents. Embryos excised from fresh seeds were immediately dehydrated over activated silica gel at 25-28℃ to the indicated moisture contents, and then grown on moistened filter paper discs. Those embryos forming both root and shoot were scored as 'emergence', and those embryos showing an apparent elongation of radicle or plumule were scored as 'survival'. All values are means ± SD of 6 replicates of 10 embryos.

The terms W_{90DS} and W_{90DE}, calculated by interpolation using survival curves and emergence curves in Fig. 2, were devised to describe the moisture contents at which 90% of embryos survived and emerged after dehydration, respectively. After 38 DAP, the maximum drying treatment (18 h) was taken as W_{90DS} and W_{90DE} as drying did not affect viability significantly. Both W_{90DS} and W_{90DE} were higher at the early development stages, decreased gradually with seed development and reached minima at 35 DAP (W_{90DS}) and 38 DAP (W_{90DE}). Both measures essentially remained constant during further development (Fig. 3a).

Development of cryotolerance in maize embryos

Cryotolerance, as assessed by survival and emergence of postthaw embryos, was nil in the early phases of seed development until 26 DAP (Fig. 4). Embryos acquired some cryotolerance at 29 DAP, with less than 50% survival and less than 30% emergence at a very narrow moisture range between 7% and 11% MC. Cryotolerance increased progressively with seed development, reached a maximum at 44 DAP and remained there. From 44 DAP onwards, embryos could withstand cryoexposure only if they were dehydrated to low moisture contents, i.e. 10%, prior to freezing (Fig. 4).

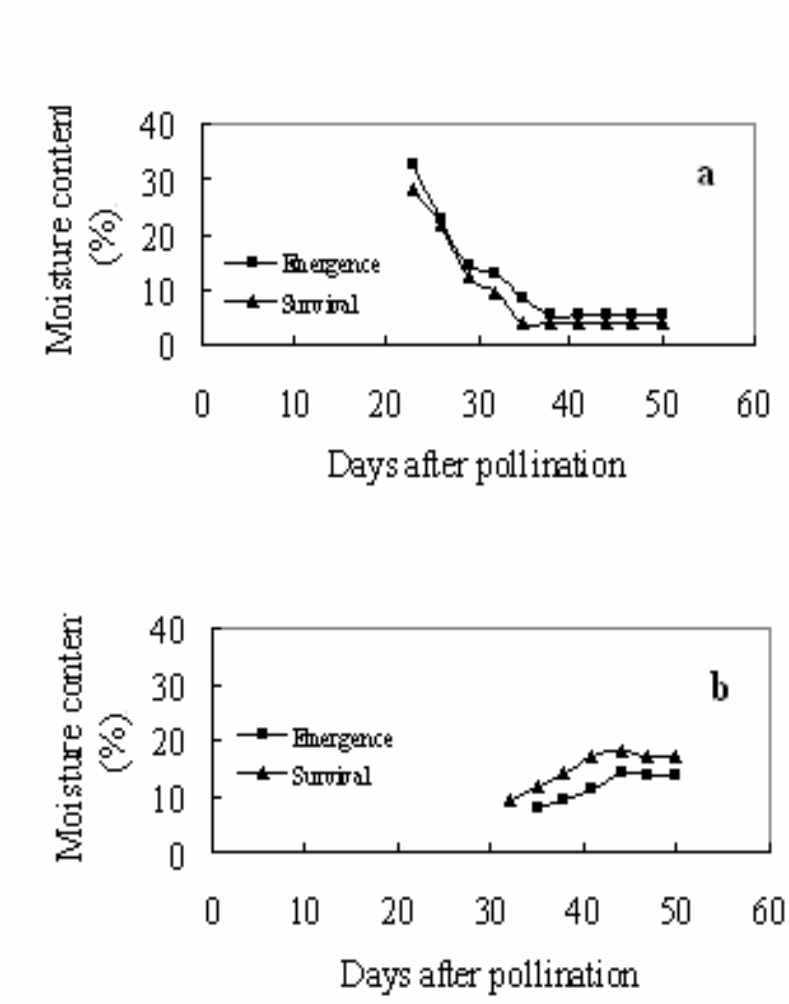

Figure 3. The changes in W_{90DS} and W_{90DE}, moisture contents allowing 90% *Zea mays* embryos to survive and to emerge at different developmental stages following dehydration to different moisture contents (a), and the changes in W_{50FS} and W_{50FE}, the maximum moisture contents allowing 50% post-thaw *Zea mays* embryos to survive and to emerge at different developmental stages following dehydration to different moisture contents prior to freezing (b).

Only a quarter of post-thaw embryos emerged, and a half survived over a very narrow moisture range at 29 DAP. The MC windows allowing 50% post-thaw embryos to survive and to emerge were calculated by interpolation using survival curves and emergence curves in Fig. 4, and the upper limit of these windows were expressed as W_{50FS} and W_{50FE}. These values indicated the highest moisture contents allowing 50% post-thaw embryos to survive and to emerge. Both W_{50FS} and W_{50FE} were low at this stage. But as seeds developed, they increased step by step, reached maxima for survival at 41 DAP and for emergence at 44 DAP and then remained unchanged later in development (Fig. 3b). Moisture contents of c. 14% enable no less than 50% post-thaw embryos to emerge at the later development stages; but no embryos at the same moisture range survived cryoexposure at 29 DAP, although they could withstand desiccation to this moisture range without impairment to their survival and emergence (Fig. 4).

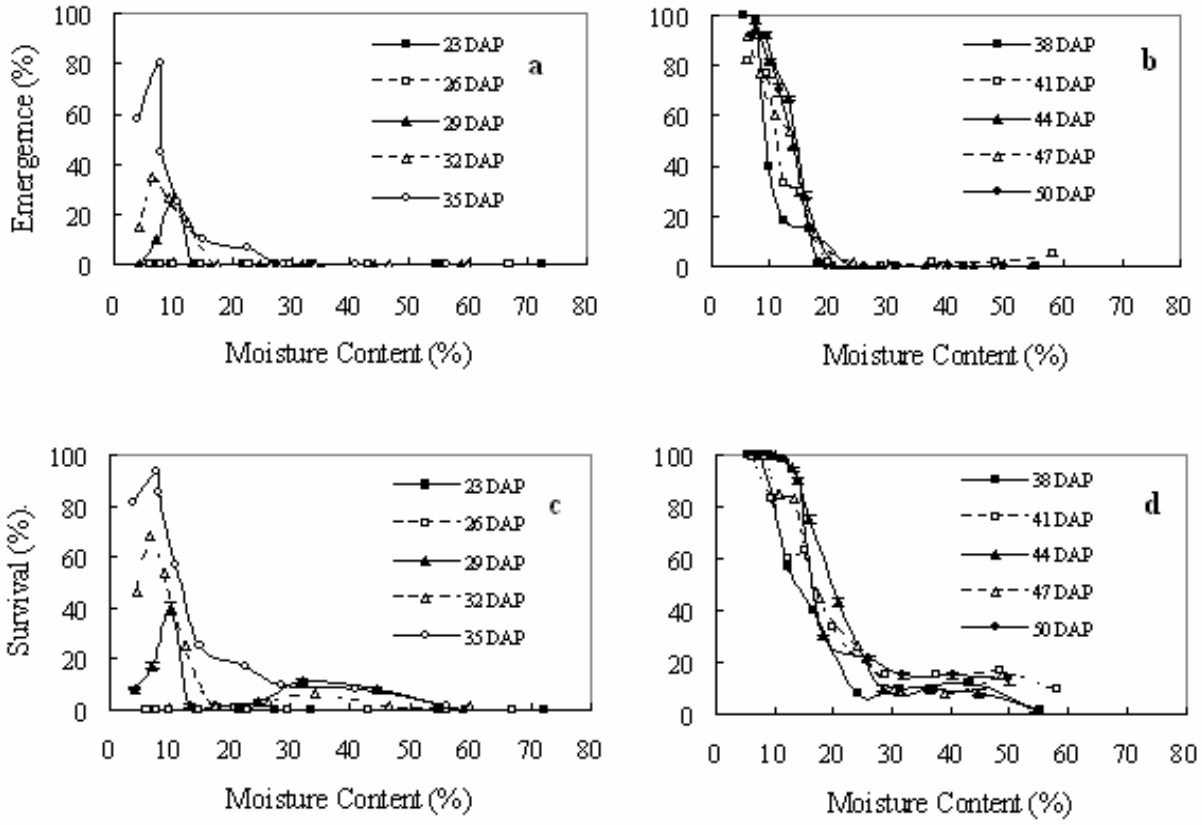

Figure 4. The changes in emergence (a, b) and survival (c, d) of postthaw *Zea mays* embryos at different developmental stages following dehydration to different moisture contents prior to freezing. Embryos excised from fresh seeds were immediately dehydrated over activated silica gel at 25-28℃ to the indicated moisture contents, cryostored for three days, and then thawed and cultured on moistened filter paper discs. Those embryos forming both root and shoot were scored as emergence, and those embryos showing an apparent elongation of radicle or plumule were scored as survival. All values are means ± SD of six replicates of 10 embryos.

DISCUSSION

Orthodox storage behavior of maize seeds has been established previously (e.g. 29), and mature maize seeds shown to be undamaged by exposure to liquid nitrogen (26). However, developing maize seeds may be desiccation sensitive and cryo-sensitive. There is a transition from desiccation intolerance to desiccation tolerance during orthodox seed development. In maize this transition has been reported as an abrupt change, occurring between 20 and 25 DAP (7) or between 25 and 28 DAP (33). These differences were probably due to the varieties used and diverse climates in the cultivation sites. By contrast, this study showed that the acquisition of desiccation tolerance in maize embryos was a gradual event, lasting from 23 to 38 DAP. These different results probably originate from the experimental methods used, for desiccation tolerance was regarded as the capability to withstand tolerance to a single level of drying. Moreover, slow-drying methods were employed in those studies, which are known to enhance seed maturation (1) and to induce desiccation tolerance of immature seeds (2). Differences in the drying methods employed have been used to explain the contrasting results about the acquisition of desiccation tolerance in soybean axes (28). Now it is more and more clear that desiccation tolerance is not an all-or-nothing phenomenon (3,19).

Compared to desiccation tolerance, much more remains unclear regarding cryotolerance. When and how seeds acquire cryotolerance is the most attractive question to answer. This study described in details the process of cryotolerance acquisition in developing maize embryos. It was a gradual event concomitant with the acquisition of desiccation tolerance. Cryotolerance was absent at the early developmental stages until 26 DAP, though embryos at these stages had some desiccation tolerance. Embryos acquired cryotolerance at 29 DAP, when desiccation tolerance reached a higher level, and then both cryotolerance and desiccation tolerance increased gradually in developing maize embryos. Embryos acquired maximum of desiccation tolerance at 38 DAP, and that of cryotolerance at 44 DAP. Afterwards, cryotolerance and desiccation tolerance remained constant essentially.

A double stress from desiccation and freezing is imposed on seeds during cryopreservation, the intensities of which depend on the moisture content level. Differential thermal analysis (DTA) demonstrated that the failure in cryopreservation of three recalcitrant-seed species (rambutan, durian and cempedak) could be attributed to the lack of a safe window between the threshold for desiccation sensitivity and critical moisture at which they survive freezing (15). It was presumed that cryopreservation of recalcitrant seeds may succeed through increased tolerance of the axes to desiccation, thereby lowering their critical moisture content, or by prevention of ice formation in moist tissues, thereby effectively raising their threshold moisture content (15). Development of cryotolerance in maize embryos indicated such changes in stress tolerance. Maize embryos at very early development stages (before 29 DAP) exhibited high recalcitrance, having too high a critical moisture content for desiccation sensitivity and relatively too low a threshold moisture content for cryopreservation with no safe window between them, resulting in cryopreservation failure at 23 and 26 DAP. As seeds developed, embryos at intermediate developmental stages, being equivalent to moderately and minimally recalcitrant seeds, survived to cryoexposure only within a safe window of moisture contents. This safe window was produced and widened both by a decrease in desiccation sensitivity and an increase in freezing tolerance. At the later developmental stages, desiccation did not cause damage to embryos any longer, so embryos could survive and develop after cryopreservation only if they were dehydrated to low enough moisture contents. Germinating orthodox seeds were used in cryopreservation research to simulate recalcitrant seeds because of the great similarity between them (8, 13). This study demonstrated the similarity in cryobehaviour between developing orthodox seeds and recalcitrant seeds.

Developing maize embryos suffered cryo-damage if they were plunged in liquid nitrogen at their initial moisture contents, which were always greater than or close to 50%, but could survive and emerge well when dried to moisture contents below 13% prior to freezing. Moisture content is the most important factor affecting the ability of germplasm to be stored in liquid nitrogen. Mazur (20) considered that lethal intracellular crystal formation might be the major factor causing cryopreservation failure. Therefore, all cryopreservation protocols are designed either to remove freezable water or to avoid ice formation through vitrification. But this correlation between moisture content and cryoinjury was notably influenced by the developmental stage. As seed development proceeds, the moisture contents allowing post-thaw embryos to survive and to emerge at a given percentage increases.

The development of cryotolerance was closely related to that of desiccation tolerance in maize embryos, for postthaw embryos survived and emerged only when desiccation tolerance rose to a higher level, and the further development in desiccation tolerance contributed to widening the safe moisture window for freezing. However, freezing and dehydration are fundamentally different stress vectors (10); the development of cryotolerance lagged apparently behind that of desiccation tolerance in this study. Moreover, desiccation tolerance is not the single contributor to cryotolerance, because when cryotolerance increased, embryos could survive to cryoexposure not only at lower moisture contents but also at higher moisture contents.

Neither the critical moisture content for drying, nor upper threshold moisture content for cryopreservation was constant for developing maize embryos. The cause of these changes in thresholds is thought-provoking. A number of processes or mechanisms, such as cellular de-differentiation, metabolic 'switch off', vitrification, protective molecule synthesis, and the presence and efficient operation of antioxidant system, have been suggested to confer, or contribute to, desiccation tolerance. These processes may confer protection against the consequences of water loss at different hydration levels, and the absence or ineffective action of one or more of them could determine the relative degree of desiccation sensitivity of seeds of individual species (6,14,21,22,24,25,31). Numerous proteins have been found, that are specifically expressed only at stages of development when maize embryos transited from desiccation intolerance to desiccation tolerance (7,30). The mechanisms involved in cryotolerance are so far unknown. It is of particularly interest to elucidate how *Zea mays* embryos with higher moisture contents at the later developmental stages are protected from freezing damage. The biochemical basis of cryotolerance in maize embryos is currently under investigation.

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