The role of recovery of mitochondrial structure and function in desiccation tolerance of pea seeds

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Mitochondrial repair is of fundamental importance for seed germination. When mature orthodox seeds are imbibed and germinated, they lose their desiccation tolerance in parallel. To gain a better understanding of this process, we studied the recovery of mitochondrial structure and function in pea (Pisum sativum cv. Jizhuang) seeds with different tolerance to desiccation. Mitochondria were isolated and purified from the embryo axes of control and imbibed–dehydrated pea seeds after (re-)imbibition for various times. Recovery of mitochondrial structure and function occurred both in control and imbibed–dehydrated seed embryo axes, but at different rates and to different maximum levels. The integrity of the outer mitochondrial membrane reached 96% in all treatments. However, only the seeds imbibed for 12 h and then dehydrated recovered the integrity of the inner mitochondrial membrane (IMM) and State 3 (respiratory state in which substrate and ADP are present) respiration (with NADH and succinate as substrate) to the control level after re-imbibition. With increasing imbibition time, the degree to which each parameter recovered decreased in parallel with the decrease in desiccation tolerance. The tolerance of imbibed seeds to desiccation increased and decreased when imbibed in CaCl2 and methylviologen solution, respectively, and the recovery of the IMM integrity similarly improved and weakened in these two treatments, respectively. Survival of seeds after imbibition–dehydration linearly increased with the increase in ability to recover the integrity of IMM and State 3 respiration, which indicates that recovery of mitochondrial structure and function during germination has an important role in seed desiccation tolerance.

Introduction

Desiccation tolerance generally refers to the ability of organisms to survive loss of 80 to 90% of protoplasmic water to reach \(<0.3 \text{ g w}^{-1} \text{ dry matter}\). Under these conditions, there is no free water in the living cells and the structure of macromolecules and membranes is protected by specific mechanisms (Hoekstra et al. 2001, Oliver et al. 2000). This phenomenon is widespread across the plant kingdom, including ferns, mosses, pollen, angiosperm resurrection plants and orthodox seeds. In orthodox seeds, desiccation tolerance is acquired during seed maturation approximately halfway through development. This trait ensures that seeds pass unharmed through maturation drying and retain viability in the dry state for long periods of time under natural conditions. 

Abbreviations – AOX, alternative oxidase; BSA, bovine serum albumin; CCO, cytochrome c oxidase; DTT, dithiothreitol; FCCP, p-trifluoromethoxyphenyhydrazone; IMM, inner mitochondrial membrane; MOPS, 3-(N-morpholino)propanesulphonic acid; MV, methylviologen; OMM, outer mitochondrial membrane; RCR, respiratory control ratio (State 3 rate/State 4 rate); ROS, reactive oxygen species; SHAM, salicylhydroxamic acid; State 3, respiratory state in which substrate and ADP are present; State 4, respiratory state in which all ADP has been converted into ATP; TX, Triton X-100.
conditions (Kermode and Finch-Savage 2002, Vertucci and Farrant 1995).

Bewley (1979) suggested that three protoplasmic properties are required for plant or plant tissue to establish desiccation tolerance: (1) minimize damage from desiccation and/or rehydration, (2) maintain cellular integrity in the dry state and (3) mobilize repair mechanisms on rehydration. These criteria highlight the importance of two basic cellular process, protection and repair, in desiccation tolerance (Oliver et al. 2005). Many specific protective mechanisms have been implicated in acquisition and maintenance of seed desiccation tolerance, including reduction of the degree of vacuolization, intracellular de-differentiation, ‘switching off’ metabolism, accumulation of protective molecules, such as late embryogenesis abundant (LEA) proteins, sucrose and certain oligosaccharides, as well as the presence and efficient operation of antioxidant systems (Berjak and Pammenter 2008, Pammenter and Berjak 1999). Although repair mechanisms have been identified in mosses and resurrection plants (Oliver 1996, Oliver et al. 2005, Proctor et al. 2007, Rascio and La Rocca 2005), their nature is still unclear in seed desiccation tolerance.

In seeds, the mitochondrion is the major organelle for energy supply and its function is tightly coupled to seed germination (Bewley 1997). During maturation drying, seed mitochondria disassemble because of desiccation stress and in electron micrographs of dry seeds the mitochondria appear with poorly differentiated structure (Ehrenshaft and Brambl 1990, Logan et al. 2001, Morohashi et al. 1981). A process of repair of mitochondrial structure and function is necessary for seed germination during the imbibition process (Bewley 1997). Many studies have shown that mitochondria in dry seeds recover the mature structure with well-defined cristae and a dense matrix during the germination process (Benamar et al. 2003, Ehrenshaft and Brambl 1990, Howell et al. 2006, Logan et al. 2001, Morohashi et al. 1981). These resurrected mitochondria then supply the energy and metabolic intermediates that seed germination requires.

Mitochondria are one of the targets for various stress damage, probably because of their large turnover of reactive oxygen species (ROS; Amirsadeghi et al. 2007, Macherel et al. 2007, Møller et al. 2007, Pastore et al. 2007). Prasad et al. (1994) indicated that the ability of maize seedlings to recover from chilling injury was, at least in part, because of the recovery of mitochondrial function. Severe seawater stress decreases durum wheat germination and delays seedling growth, and damages the mitochondrial succinate-dependent oxidative phosphorylation at the same time (Flagella et al. 2006). It is possible that loss of desiccation tolerance in seeds is because of desiccation damage to their mitochondria. Indeed, the respiration rate and activity of cytochrome c oxidase (CCO) and malate dehydrogenase in mitochondria from axes of recalcitrant Antirias toxicaria seeds and from embryos of orthodox maize seeds all decreased during dehydration (Song et al. 2009, Wu et al. 2009), but we do not have any information about their recovery during germination. In view of the importance of mitochondrial repair for seed germination, we here hypothesize that this process is also necessary for seed desiccation tolerance.

When mature seeds are imbibed and then dehydrated, survival decreases gradually with increasing imbibition time, that is, desiccation tolerance is lost during seed germination (Dasgupta et al. 1982, Koster and Leopold 1988, Reisdorph and Koster 1999, Senaratna and McKersie 1983). Thus germinating orthodox seeds resemble recalcitrant seeds, and this system has therefore been suggested to be a useful model for understanding the mechanisms of recalcitrance (Sun 1999). In nature, seeds may experience several imbibition-desiccation cycles during germination because of fluctuations in soil moisture. After the point at which the seeds have lost their desiccation tolerance, these cycles will decrease seed germination and seedling formation. Thus understanding desiccation tolerance will be important for optimizing not only storage of recalcitrant seeds, but also crop yield.

Desiccation tolerance can be improved in germinating seeds by treatment with certain exogenous chemicals, such as polyethylene glycol or abscisic acid (Bruggink and Vandertoorn 1995, Buitink et al. 2003). This method can be very useful for studying the mechanism of desiccation tolerance (Faria 2006). Production of ROS during dehydration is considered as one of the reasons causing desiccation damage (Bailly 2004). Thus the application of a ROS producer, such as methylviologen (MV) to desiccation-tolerant seeds would be expected to decrease or remove the tolerance. Ca2+ acting as a second messenger can improve the response of plant to many adverse stresses (Kudla et al. 2010, Lecourieux et al. 2006, Sanders et al. 2002). Ca2+ is also important for membrane structure (Møller 1983, White and Broadley 2003), but little is known about its effect on desiccation tolerance.

In this study, we have investigated the change of desiccation tolerance of pea (Pisum sativum cv. Jizhuang) seeds imbibed in distilled water for various times, and the effect of CaCl2 and MV, a producer of ROS (Halliwell and Gutteridge 2007) on desiccation tolerance of germinating seeds. Mitochondria were isolated and purified from the embryo axes of the untreated seeds and of a series of imbibed-dehydrated seeds.
(tolerant, partly tolerant and intolerant to desiccation) and used to monitor the recovery of mitochondrial structure and function during seed (re-)imbibition. A normal cumulative equation was applied to fit the time courses of mitochondrial parameters during (re-)imbibition. Our results indicated that the recovery of mitochondrial structure and function during germination is important in seed desiccation tolerance.

Materials and methods

Plant material

Pea (P. sativum cv. Jizhuang) seeds were obtained from Guangling Seed Company (Datong, Shanxi, China) in September 2008 and were kept in plastic bags at 5°C until used. The water content of the seeds was about 0.10 g H₂O g⁻¹ dry weight.

Seed imbibition, dehydration and survival

Seed imbibition and germination

Three replicates of 25 pea seeds each were placed on two layers of filter paper and imbibed in 40 ml distilled water or treatment solution (20 mM CaCl₂ or 2 mM MV both dissolved in distilled water) in closed 15-cm diameter Petri dishes at 20°C in darkness for up to 48 h. Radicle protrusion of 2 mm was used as the criterion of germination, and the number of germinated seeds was counted regularly throughout the test period.

Another sample of three replicates of 25 pea seeds each was imbibed in distilled water or treatment solution (20 mM CaCl₂ or 2 mM MV) to determine seed water uptake. These seeds were regularly removed from the Petri dishes to determine the seed weight, which was used to calculate the seed water content.

Dehydration and survival

The pea seeds were imbibed in distilled water or treatment solution (20 mM CaCl₂ or 2 mM MV) at 20°C in darkness for 12–48 h, and then dehydrated to a water content of about 0.10 g g⁻¹ by burying them in activated silica gel [1:8 (v:v) seeds: silica gel] within a closed plastic box for 24–36 h.

The untreated (control) and imbibed–dehydrated seeds were germinated on two layers of filter paper moistened with 40 ml of distilled water in Petri dishes (15-cm diameter) at 20°C in darkness for 7 days to measure seed survival. Likewise, seeds imbibed in CaCl₂ or MV for 25 h were tested for germination and survival. Seeds showing a normal epicotyl and primary radical were considered as being alive.

The control seeds (CK) imbibed in distilled water for 4, 12, 18, 24, 30 and 36 h, and the seeds imbibed in distilled water for 12, 25 or 36 h and dehydrated (H12D, H25D or H36D), as well as seeds imbibed in CaCl₂ or MV for 25 h and dehydrated (Ca25D or MV25D) were re-imbibed in distilled water for 4, 8, 12, 18, 24 and 30 h and then used for the isolation and purification of mitochondria (Fig. 1).

Determination of water content

The water content of seeds was determined according to the method of International Seed Testing Association (1999) and expressed on a dry mass basis (g H₂O g⁻¹ dry weight).

Transmission electron microscopy

Embryo axes of CK, H12D and H36D (labels see Fig. 1) seeds were used for fixation directly or after (re-)imbibition of the seeds for 24 h, respectively.

Fig. 1. Protocol used for the imbibition, dehydration and subsequent re-imbibition of pea seeds. CK, H12D, H25D, Ca25D, MV25D and H36D are dry seeds with a water content of about 0.10 g g⁻¹.
Mitochondria isolated and purified from embryo axes of the seeds (re-)imbibed for 24 h were also used. The embryo axes and purified mitochondria were fixed in 2.5% (v/v) glutaraldehyde, 100 mM sodium phosphate (pH 7.2) (for purified mitochondria, the solution also contained 0.4 M sucrose) for 2 h. The fixed embryo axes that had been rinsed (twice) and purified mitochondria that had been centrifuged (10,000 g for 10 min) and rinsed (twice) were post-fixed in 1% (v/v) osmium tetroxide, 100 mM sodium phosphate (pH 7.2) for 2 h, dehydrated in an ethanol series and then embedded in Epon resin. Ultra-thin sections were cut and stained with uranyl acetate followed by lead citrate before observation. The electron micrographs were recorded with a JEM-1230 transmission electron microscope (JEOL, Tokyo, Japan).

**Isolation and purification of mitochondria**

Mitochondria were prepared according to the method of Benamar et al. (2003) modified as follows: 200 embryo axes were homogenized using a precooled mortar and pestle in 20 ml of extraction buffer composed of 0.4 M sucrose, 20 mM 3-(N-morpholino)propanesulfonic acid (MOPS), 1 mM EDTA, 4 mM cysteine, 10 mM MgCl₂, 0.4% (w/v) bovine serum albumin (BSA) and 0.5% (w/v) insoluble polyvinylpyrrolidone, adjusted to pH 7.5 with KOH. After washing the mortar with 20 ml extraction buffer, the total 40 ml homogenate was filtered through four layers of gauze. The filtrate was centrifuged at 100 g for 5 min and 500 g for 10 min, and the supernatant was then centrifuged at 15,000 g for 20 min. The resulting pellet was suspended in washing buffer composed of 0.4 M mannitol, 1 mM EDTA, 0.2% (w/v) BSA, 10 mM MOPS–KOH, pH 7.2 and layered on to a discontinuous Percoll gradient: 3 ml of 25% Percoll containing 0.4 M mannitol, 5 mM MgCl₂, 0.2% BSA, 10 mM MOPS–KOH, pH 7.2, on top of 3 ml of 35% Percoll containing 0.4 M sucrose, 5 mM MgCl₂, 0.2% BSA, 10 mM MOPS–KOH, pH 7.2. After centrifugation at 10,000 g for 20 min, the mitochondrial band at the interface between 25 and 35% Percoll solution was collected and diluted eight times with washing buffer (without BSA), and then pelleted at 13,000 g for 15 min to remove the Percoll. This dilution and pelleting was repeated once. The pellets were finally resuspended in washing buffer (without BSA) and used for assay of enzyme activity and determination of oxygen consumption and protein content. All procedures were carried out at 4°C.

**Assay of CCO activity**

Activity of CCO (EC 1.11.1.5) was assayed using the method of Møller et al. (1987), which measures the oxidation of reduced cytochrome c at 550 nm. The latency of CCO activity was calculated as described by Møller et al. (1987).

\[
\text{Latency (\%)} = 100 \times \left( \frac{\text{rate} + \text{TX} - \text{rate} - \text{TX}}{\text{rate} + \text{TX}} \right)
\]

where (rate + TX) and (rate – TX) are the oxidation rate of reduced cytochrome c by CCO in the reaction medium with and without 0.05% (w/v) Triton X-100 (TX), respectively.

**Measurements of oxygen consumption**

Oxygen consumption of mitochondria was measured using an oxygen electrode system (Oxytherm, Hansatech Instruments, Pentney, King’s Lynn, Norfolk, UK). The reaction medium contained 0.4 M mannitol, 10 mM KH₂PO₄, 10 mM KCl, 5 mM MgCl₂ and 0.1% (w/v) BSA, 10 mM MOPS–KOH, pH 7.5, in a total volume of 1 ml. For substrate oxidation, first mitochondria (80–150 μg protein) and the substrate (1.5 mM NADH or 10 mM succinate) was added followed by two additions of ADP, the first time (50 μM) to activate and the second time (100 μM) to obtain States 3 and 4 respiration and to give the ADP/O ratio. A 1 mM EGTA was added before NADH in some control experiments to remove Ca²⁺ from the membranes, which might be sufficient to give maximal NADH oxidation activity (Møller 1983, Møller et al. 1981, Nash and Wiskich 1983). Uncoupler, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP; 2 μM) was used to uncouple NADH oxidation to obtain another measure of uninhibited respiration rate. For estimation of alternative oxidase (AOX) capacity, the cytochrome oxidase pathway during NADH oxidation was inhibited by potassium cyanide (1 mM), pyruvate (10 mM) and dithiothreitol (DTT; 10 mM) were added to activate AOX, and finally salicylhydroxamic acid (SHAM; 2 mM) was added to inhibit AOX. AOX capacity was calculated as the rate after addition of DTT and pyruvate minus the rate after SHAM addition (= residual respiration). The mitochondrial respiration was expressed as nmol O₂ mg protein⁻¹ min⁻¹.

**Determination of protein**

The concentration of protein in the purified mitochondria was determined according to Bradford (1976), using BSA as a standard.

**Statistical analysis**

A modified population based model (Eqn 2) was applied to fit the time courses of recovery of mitochondrial
structure and function. This model, derived from the model describing seed germination and dormancy release (Bradford 2002, Wang et al. 2009), assumes that recovery of the mitochondrial structure and function during (re-)imbibition follows a cumulative normal distribution:

\[ y = y_{\text{max}} \times \Phi\left(\frac{x - \mu}{\sigma}\right) \] (2)

where \( y \) is a parameter reflecting mitochondrial structure and function at a given imbibition or re-imbibition time \( x \), \( y_{\text{max}} \) is the maximum value of \( y \), \( \Phi \) is the normal probability integral and \( \mu \) and \( \sigma \) are mean and standard deviation of original distribution of \( y \).

**Results**

**Water uptake, germination and desiccation tolerance during imbibition**

Water uptake of pea seeds showed a triphasic behavior with a rapid initial water uptake at the first 10 h, followed by a slower uptake between 10 and 30 h and finally a slightly faster phase after 30 h (Fig. 2A). Water uptake in distilled water, \( \text{CaCl}_2 \) solution and MV solution was the same for the first 15 h and at 25 h, when seeds were dried, the water content differed by only 0.05 g g\(^{-1}\) between the three treatments (results not shown).

Seeds began to germinate at 16 h, and reached maximum germination (>95%) at about 36 h when imbibed in distilled water (Fig. 2B). Germination was delayed in the presence of \( \text{CaCl}_2 \) (important for structure) and MV (causes ROS formation), and the final germination percentage was also reduced by 10% in the presence of MV in the imbibition medium (Fig. 2B).
When the imbibed pea seeds were dehydrated, they maintained their maximum viability up to 15 h of imbibition, after which seed death occurred in a log cumulative normal distribution over imbibition time (Fig. 2C). No seeds survived after imbibition for 48 h followed by dehydration (Fig. 2C). At the same imbibition time followed by dehydration, the presence of CaCl₂ in the imbibition solution increased, and MV decreased, the survival percentage of seeds, as indicated by the shift in the T₅₀ value (Fig. 2C). Imbibition in CaCl₂ and MV for 25 h did not in itself affect seed viability, as seeds imbibed in these two solutions and then germinated without an intervening dehydration all survived (Fig. 2D).

Transmission electron micrographs of mitochondria

A morphological examination of mitochondria in situ and after isolation was performed using transmission electron microscopy (Fig. 3). In the control seeds (CK, Fig. 3A) and imbibed–dehydrated seeds (H12D and H36D, Fig. 3D, G), mitochondria had little discernible internal structure. After 24 h of re-imbibition, the mitochondrial structure was repaired, but only CK and H12D had fully differentiated mitochondria (Fig. 3B, C, E, F) and in H36D seeds the mitochondria could not recover the structure with well-defined cristae (Fig. 3H, I). The embryo axis cells in H36D seeds became plasmolyzed after dehydration (Fig. 3G insert),
and the plasmolyzed cells were still plasmolyzed after subsequent rehydration (Fig. 3H insert). This occurred despite the fact that the embryo axes were fully imbibed as seen from the high water content of the H36D axes (Fig. 3H insert) in comparison with CK and H12D axes after 24 h imbibition (Fig. 3B, E insert). Thus there may be desiccation damage to the plasma membrane in the H36D seeds.

**Purification of embryo axis mitochondria**

Mitochondria were isolated and purified from embryo axes of germinating pea seeds [0–36 h (re-)imbibition] pretreated in six different ways: control seeds (CK), seeds imbibed in distilled water for 12, 25 or 36 h and then dehydrated (H12D, H25D or H36D), or seeds imbibed in CaCl₂ or MV for 25 h and then dehydrated (Ca25D or MV25D) (Fig. 1).

Mitochondrial purity was monitored in crude and purified mitochondria by the specific activity of CCO, an enzyme found only in the inner mitochondrial membrane (IMM; Lardy and Ferguson 1969). The latency of CCO activity was used to monitor the integrity of the outer mitochondrial membrane (OMM), as an intact outer membrane does not permit the CCO substrate, reduced cytochrome c (12.5 kDa), to be oxidized by CCO (Neuburger 1985).

The CCO specific activity increased 2.2 to 6.5-fold from crude mitochondria to purified mitochondria (results not shown). This was mainly because of a large variation in the percentage of total CCO activity in the crude mitochondria recovered in the purified mitochondria. The recovery was linearly correlated with OMM integrity (Fig. S1A, supporting information). OMM integrity in purified mitochondria was linearly correlated with that of crude mitochondria (Fig. S1B) and it did not change as a result of purification (see x = y line in Fig. S1B). In summary, when the OMM of the crude mitochondria was relatively intact, a large percentage of the mitochondria was recovered in the purified fraction.

**Recovery of mitochondrial structure and function**

**CCO activity and integrity of OMM**

CCO activity was low in embryo axis mitochondria from CK seeds at 6 and 12 h of imbibition, but increased rapidly thereafter (Fig. 4A). CCO activity was higher in mitochondria from the imbibed–dehydrated seeds H12D, H25D and H36D than in mitochondria from CK seeds for the first 18 h of (re-)imbibition (Fig. 4A). However, the maximum CCO activity to be recovered was similar in CK, H12D and H25D seeds, and significantly lower in H36D seeds (Fig. 4A). Compared to H25D seeds, CaCl₂ treatment (Ca25D) increased CCO activity at all times of re-imbibition, while MV treatment (MV25D) had no effect (Fig. 4B).

In CK mitochondria, CCO latency increased very rapidly and reached 96% within 18 h of imbibition. In imbibed–dehydrated seeds, the final CCO latency was similar to that in CK seeds (Fig. 4C, D), whereas it took longer to recover in H36D seeds (Fig. 4C). Pretreatment of the seeds with CaCl₂ or with MV (followed by dehydration) had no effect on the recovery of CCO latency during germination (Fig. 4D).

**IMM integrity**

The functional intactness of the IMM was estimated by measuring the State 3 (respiratory state in which substrate and ADP are present)/State 4 (respiratory state in which all ADP has been converted into ATP) ratio, the respiratory control ratio (RCR), as this ratio will be relatively high when proton leakage across the IMM is low. A similar parameter is uncoupled rate ratio (FCCP/State 4).

Among seeds imbibed in H₂O, the recovery of IMM integrity as indicated by RCR and the uncoupled rate ratio, was more rapid in embryo axis mitochondria from the H12D and H25D seeds than in the CK mitochondria. However, only mitochondria from H12D seeds could recover IMM integrity to the same high level as the CK mitochondria (Fig. 5A, C). The degree of IMM integrity after 36 h re-imbibition was lower for H25D mitochondria and markedly lower for H36D mitochondria (Fig. 5A, C). CaCl₂ treatment (Ca25D) promoted, while MV treatment (MV25D) weakened, the recovery of the IMM integrity during re-imbibition compared to H₂O treatment (H25D; Fig. 5B, D).

**Respiration**

A high respiratory activity per milligram mitochondrial protein (relative to that of mitochondria purified from pea leaves – Nash and Wiskich 1983) indicates that the mitochondria are well able to contribute to seed metabolism.

The addition of Ca²⁺ to mitochondria oxidizing NADH in the standard medium did not change the rate of oxygen consumption measured. However, in the presence of EGTA the rate was reduced by 85% indicating that external NADH oxidation in pea seed mitochondria is dependent on Ca²⁺ (results not shown). The NADH oxidation rates presented in Fig. 6A, B are the maximum rates measured in the absence of EGTA.

State 3 respiration in CK mitochondria (NADH or succinate as substrate) was very low at 4 h re-imbibition, but increased rapidly to reach very high activities
after 36 h (Fig. 6A, C). In H12D, H25D and H36D mitochondria, State 3 respiration was higher than the respiration in control mitochondria at the start time, but only H12D mitochondria reached the same maximum level as the CK mitochondria after 30 h re-imbibition. For H25D and H36D mitochondria, the rates after 30 h were only 73 and 48% of CK mitochondria (NADH as substrate; Fig. 6A) or 81 and 40% (succinate as substrate; Fig. 6C) of CK mitochondria. State 3 respiration recovered better in mitochondria from Ca25D seed embryo axes than in mitochondria from H25D embryo axes (Fig. 6B, D). Although the State 3 respiration of MV25D mitochondria was a little higher than for H25D mitochondria early during re-imbibition, the maximum rate of respiration that the mitochondria could reach was similar (Fig. 6B, D). Recovery of AOX capacity was similar among CK, H12D and H36D mitochondria (Fig. 6E), but more rapid and reaching higher activities in H25D mitochondria (Fig. 6E). AOX capacity was higher in Ca25D and MV25D mitochondria than in control mitochondria (Fig. 6F). Generally, AOX capacity was only a small part of the State 3 activity (Fig. S2), but in CK mitochondria after 4 h re-imbibition they were almost the same size (Fig. S2A).

We calculated the ADP/O ratio during NADH oxidation and the values were all between 1.0 and 1.3 with no clear trend (results not shown).

Seed survival and mitochondrial recovery

According to the Eqn 2, the maximum integrity of IMM and oxygen consumption to be recovered during
Fig. 5. Recovery of integrity of IMM in purified mitochondria from pea embryo axes during seed (re-)imbibition. Mitochondria were isolated and purified from embryo axes of CK, H12D, H25D and H36D (A and C), and of CaD25 and MV25 (B and D) after (re-)imbibition, respectively. Uncoupled (FCCP), State 3 (St3) and State 4 (St4) rates of respiration were determined using NADH as substrate. The uncoupled rate ratio (FCCP/St4, A and B) and RCR (St3/St4, C and D) were calculated to indicate the integrity of IMM. The data are means ± standard error of mean (n = 3) and lines are regressed with Eqn 2.

(re-)imbibition was calculated and plotted against the percentage of seed survival (Fig. 7). Seed survival increased linearly with increase in the NADH- or succinate-dependent State 3 respiration, uncoupled rate ratio and RCR (Fig. 7A, B), whereas the pattern with AOX capacity was not clear (Fig. 7A).

Discussion

Orthodox seeds acquire desiccation tolerance during maturation and lose it during imbibition (Berjak and Pammenter 2008, Kermode and Finch-Savage 2002). In this, pea (P. sativum cv. Jizhuang) seeds gradually lost their desiccation tolerance after imbibition for longer than 15 h, and no seed survived (measured as the ability to germinate within 7 days) after imbibition for 48 h followed by desiccation (Fig. 2C). The desiccation tolerance of imbibed seeds was improved and impaired when imbibition took place in CaCl2 and MV solutions, respectively, instead of in distilled water (Fig. 2C). When seeds were imbibed with CaCl2, a small delay in germination was found in comparison with seeds imbibed in water. This may be because of the slight salt stress caused by the CaCl2 solution.

One of the key functions of mitochondria is to catalyze substrate oxidation and produce ATP for use in cellular metabolism. Mitochondria are affected by environmental stress, such as drought (Pastore et al. 2007), chilling (Prasad et al. 1994, Stupnikova et al. 2006), heavy metals like Cd2+ (Smiri et al. 2009) and ageing (Benamar et al. 2003). In orthodox seeds, mitochondria appear with poorly differentiated structure.
in the dry state. During germination, seed must initiate the process of recovery for mitochondrial intactness and function (Bewley 1997). This recovery requires the active participation of many physiological and biochemical processes, such as gene expression in both nucleus and mitochondria, protein synthesis, import and modification (Bewley 1997, Howell et al. 2006). If one of these processes is interrupted by desiccation, mitochondrial recovery may become impossible. Thus monitoring the recovery of mitochondrial functions
The seed survival related to the NADH-dependent St3 respiration and 6, 7, respectively. Linear models were applied to the relationships. Imbied–dehydrated seeds were estimated from the regressions in Figs 5A, AOX respiration (A), IMM integrity (B) to be recovered in the CK and maximum State 3 (St3) respiration with NADH or succinate as substrate $R^2$. The question is to what extent the purified mitochondria to produce ATP. However, it cannot be done in the homogenate or in the crude mitochondria, because an unknown proportion of these fractions is not mitochondria. The question is to what extent the purified mitochondria are representative of all the mitochondria present in the tissue before extraction? We observed a very good correlation between the latency of the purified mitochondria and the crude mitochondria (Fig. S1B). Yield calculated as total CCO activity in the purified mitochondria relative to the crude mitochondria also increases linearly with CCO latency (Fig. S1A). We conclude that the properties of purified mitochondria reflected those of the crude mitochondria reasonably well.

Although the importance of mitochondrial recovery for seed germination is well known (Benamar et al. 2003, Bewley 1997, Howell et al. 2006, Logan et al. 2001), we still do not know how to quantify this process, making it difficult to compare the effect of different treatments. We here attempt to apply a germination model (Bradford 2002, Wang et al. 2009) to quantify the mitochondrial recovery across a series of imbibition times, and clearly show the extent and the time of recovery of mitochondrial intactness and function in pea seed embryo axis during germination (Figs 4–6). Among the mitochondrial recoveries, OMM integrity recovered first (within 18 h, Fig. 4C), then CCO activity (Fig. 4A), IMM integrity (Fig. 5A, C), State 3 rate (Fig. 6A, C) and AOX capacity (Fig. 6E). These recoveries were impaired after loss of seed desiccation tolerance during imbibition. Seeds imbibed in distilled water for 12 h were desiccation tolerant (Fig. 2), and mitochondria in H12D seed embryo axis recovered as well or even better than CK mitochondria (Figs 3, 4A, C, 5A, C and 6A, C, E). In contrast, seeds imbibed in distilled water for 36 h lost their desiccation tolerance (Fig. 2), and recoveries in H36D mitochondria were slower and less complete than in CK mitochondria (Figs 3, 4A, C, 5A, C and 6A, C, E). When seed desiccation tolerance was increased by incubating the seeds in a CaCl$_2$ solution (Fig. 2D), the recovery of mitochondrial intactness and respiratory function was improved correspondingly (Figs 5B, D and 6B, D). The recovery of IMM integrity was clearly impaired in MV25D seed embryo axis (Fig. 5B, D), consistent with the observed decreased desiccation tolerance of the seeds (Fig. 2D). These results indicate that the recovery of mitochondrial intactness and respiratory function is important for seed desiccation tolerance.

The extent to which the mitochondrial intactness and function, especially the IMM integrity and State 3 respiration, could be recovered determined the survival of seeds (Fig. 7). It is perhaps not surprising that seed survival depends on the intactness of the embryo axis mitochondria measured as proton permeability (Fig. 7B), because this parameter determines the ability of the mitochondria to produce ATP. However, it

![Fig. 7. The relationships between survival percentage of seeds and recovery of structure and function of embryo axis mitochondria during imbibition and desiccation of seeds. The seed survival data include the survival of untreated seeds (CK), seeds imbibed in distilled water for 12, 25 and 36 h and dehydrated (H12D, H25D and H36D) and seeds imbibed in CaCl$_2$ and MV for 25 h and dehydrated (Ca25D and MV25D). The maximum State 3 (St3) respiration with NADH or succinate as substrate (A), AOX respiration (A), IMM integrity (B) to be recovered in the CK and imbibed–dehydrated seeds were estimated from the regressions in Figs 6 and 7, respectively. Linear models were applied to the relationships. The seed survival related to the NADH-dependent St3 respiration with $y = 229.1 + 3.854x$ (A, solid line; $R^2 = 0.80$); to the succinate-dependent St3 respiration with $y = 146.6 + 3.245x$ (A, dashed line; $R^2 = 0.91$); to the AOX capacity with two lines: $y = 11.2 + 1.754x$ (when $x \leq 50.20$) and $y = 151 + 1.038x$ (when $x \geq 50.20$) (A, dotted line; $R^2 = 0.74$); to the uncoupled rate ratio [FCCP/State 4 (St4)] with $y = 2.553 + 0.0323x$ (B, solid line $R^2 = 0.93$) and to the RCR (St3/St4) with $y = 1.578 + 0.0231x$ (B, dashed line; $R^2 = 0.80$).]
is interesting that seed survival is linearly correlated with the respiratory rate of isolated embryo axis mitochondria measured per milligram of mitochondrial protein (Fig. 7A). This implies that the mitochondrial recovery required for seed survival is affected by insertion of electron transport proteins into pre-existing mitochondria rather than by mitochondrial proliferation.

Cellular membranes are one of the major sites of desiccation injury (Leprince et al. 1993). Benamar et al. (2003) showed that mitochondrial membranes are the primary targets for ageing injuries and possible also for desiccation damage. However, they did not follow the germination process or the recovery for longer than 22 h, so it is not clear whether the incomplete recovery they observed after ageing was indeed incomplete or whether it was because of a delayed recovery. We monitored mitochondrial membrane integrity during the whole germination process, and found that IMM integrity and its recovery (Fig. 5) were more sensitive to desiccation than those of OMM (Fig. 4). OMM integrity reached 96% in all treatments although the recovery was slowed down in H36D mitochondria (Fig. 4C), while IMM integrity in H25D and H36D mitochondria never recovered to the same levels as in CK mitochondria even after 30 h (re-)imbibition (Fig. 5).

Ca²⁺ is very important for the structure and function of plant cells and plant membranes (White and Broadley 2003). It is also an important second messenger in plant cell in response to many abiotic stresses (Kudla et al. 2010, Lecourieux et al. 2006, Sanders et al. 2002). There is little information about the effect of Ca²⁺ on seed desiccation stress. We found that imbibition treatment with CaCl₂ markedly improved the subsequent desiccation tolerance of seeds (Fig. 2C) and the effect increased with increasing CaCl₂ concentrations (data not shown). A positive effect of CaCl₂ on seed desiccation tolerance has also been found in soybean (Glycine max) seeds (Song et al. 2003). The observed increase in desiccation tolerance by application of CaCl₂ (Fig. 2C) may result from the improved recovery of mitochondrial CCO activity (Fig. 4B), IMM integrity (Fig. 5B, D) and respiration (Fig. 6B, D).

Ca²⁺ has several roles in plant mitochondria. It is bound to the mitochondrial membranes where it probably contributes to stability and function (Møller 1983, Möller et al. 1981). Ca²⁺ is required for activity by a number of enzymes including the external NADH dehydrogenase in the respiratory chain in some species (Møller 2001, Möller et al. 1981, Pical et al. 1993). NADH oxidation by pea leaf mitochondria is Ca²⁺ dependent (Nash and Wiskich 1983), which we can confirm for pea embryo axis mitochondria (see section Results). Under the assay conditions used in this study, there was sufficient Ca²⁺ bound to the membranes to give maximum rates of NADH oxidation, similar to what has been observed previously, e.g. on Jerusalem artichoke mitochondria (Møller et al. 1981).

The improved recovery of CCO activity, IMM integrity and respiration in Ca25D seeds (Figs 4B, D, 5B, D and 6B, D) could be connected with the above direct effects of Ca²⁺ on the mitochondria. The effect could also be more indirect. Ca²⁺ can activate defense gene expression via signal transduction (Kudla et al. 2010, Lecourieux et al. 2006, Sanders et al. 2002) and CCO genes are induced during rice seed germination in the presence of Ca²⁺ (Howell et al. 2006). Howell et al (2007) indicated that oxygen deficit could decrease protein import into mitochondria. Recently, Kuhn et al. (2009) reported that Ca²⁺ is necessary for protein import into the IMM (Kuhn et al. 2009). We speculate that Ca²⁺ may also promote or maintain the protein import pathway, and in that way improve mitochondrial repair.

Oxidative stress leads to ROS accumulation and cellular damage (Bailly 2004, Möller et al. 2007). It has often been proposed that the loss of desiccation tolerance is because of ROS production (Finch-Savage et al. 1994, Hendry et al. 1992, Li and Sun 1999, Roach et al. 2010, Wu et al. 2009). In this study, we imbibed the seeds in a solution containing MV, a ROS producer (Halliwell and Gutteridge 2007), to test whether increased oxidative stress would affect the seed desiccation tolerance and the recovery of mitochondrial structure and function. The MV treatment markedly decreased the seed survival percentage (Fig. 2C) and the ability to recover IMM coupling during re-imbibition (Fig. 6B, D). MV treatment did not decrease the viability of imbibed seeds, but increased the sensitivity of the imbibed seeds to dehydration (Fig. 2D). We conclude that MV treatment does make pea seeds less desiccation tolerant and that this might be because of increased mitochondrial ROS production.

AOX activity is induced and increased in response to plant stress, such as cold and heat temperature (Armstrong et al. 2008, Fiorani et al. 2005, González-Meler et al. 1999, Rachmilevitch et al. 2007, Watanabe et al. 2008), drought (Bartoli et al. 2005, Ribas-Carbo et al. 2005) and pathogen attack (Maxwell et al. 1999, Simons et al. 1999). The capacity of the AOX pathway was low in mitochondria from CK pea seed embryo axes early in (re-)imbibition (germination) (Fig. 6E, F). Imbibition followed by desiccation had only a relatively small effect on AOX capacity except in H25D where the effect was larger (Fig. 6E). In the presence of CaCl₂ or MV in the imbibition solution, AOX capacity was further increased (Fig. 6F). As MV increases ROS production in the tissue, this could be the cause of the increased
AOX induction. Further studies of the effects of MV on mitochondrial ROS production and the consequences for mitochondrial structure and function are required.

In most of the mitochondria, AOX capacity was only 10–30% of the State 3 rate. Exceptions were CK and H36D early during (re-)imbibition where AOX was 60–100% of State 3 with NADH (and pyruvate) (Fig. S2). This indicates that the AOX does not protect the mitochondria against desiccation damage, but rather against damage during the early imbibition phase, when ROS is produced (Bailly 2004). However, the effect of AOX is not so strong that it can improve mitochondrial recovery.

Conclusions

The loss of desiccation tolerance during imbibition in pea seeds is correlated with a decrease in IMM integrity and cytochrome respiratory function during dehydration and in the subsequent recovery of these functions during re-imbibition. Ultimately, the mitochondria may be unable to provide the cell with sufficient energy and metabolic intermediates to maintain normal cellular physiology. The positive influence of the CaCl2 treatment on seed desiccation tolerance may well be due to the requirement for Ca2+ in membrane structure and function. In contrast, the negative effects of MV treatment on recovery may be due to ROS-induced damage.

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References


Supporting Information
Additional Supporting Information may be found in the online version of this article:

Fig. S1. Correlation of latency of crude mitochondrial CCO activity with (A) yield of purified mitochondria (PM) and (B) latency of PM CCO activity.

Fig. S2. Percentage of AOX to State 3 respiration in pea embryo axis mitochondria during (re-)imbibition.

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