Effects of Dehydration Stress on Germination Behavior and Mitochondrial Small HSP in Imbibed *Beta vulgaris* L. seeds *

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Abstract: Availability of water is the determinant of seed germination. Air drought caused by dry paper towels was used to mimic dehydration stress. The rate of germination and seedling growth, and recovery of growth activity in sugar beet (*Beta vulgaris* L.) seeds imbibed decreased with dehydration stress. Activities of cytochrome c oxidase (CCO) and malate dehydrogenase (MDH) in mitochondria increased at the initial phase of dehydration stress, and then decreased. Low molecular weight heat shock protein (lmw HSP) 22 in mitochondria decreased with dehydration stress, and lost until 20 d of dehydration stress. It is concluded that mitochondrial lmw HSP 22 have a positive correlation with desiccation tolerance of seeds and seedlings.

Keywords: activities of CCO and MDH; *Beta vulgaris* L. cv. Loke; dehydration stress; germination rate; growth recovery; lmw HSP 22
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Seed germination incorporates those events that commence with the uptake of water by the quiescent seed and terminate with the elongation of the embryonic axis. Seeds of many species are non-dormant when shed from the parent plant and can germinate over a wide temperature range. It seems that in these cases the major, perhaps the only, determinant of germination is the availability of water, since water influences the progress and kinetics of most biologically significant reactions.

Plants respond to stress at the cellular level by synthesizing a range of specific "stress" proteins which include heat shock proteins (HSPs) and late embryogenesis abundant (LEA) proteins. Many HSPs are expressed constitutively in the absence of any stress, and are essential for normal cell function, but may be up-regulated by a variety of stress treatments that cause protein unfolding. HSPs can be broadly classified into four main groups according to their molecular mass. The four classes are the HSP 90s, HSP 70s, cpn 60s and a fourth group called the low molecular weight (lmw) HSPs. This fourth class encompasses proteins whose molecular masses are between 15 000 and 30 000[1]. The lmw HSPs have been identified in many organisms but are particularly prevalent in plants[1]. Many stress proteins have recently been found to be chaperones, a class of proteins involved in the folding of newly synthesized proteins[2]. The chaperones have been proposed to function during stress in the binding of partially denatured proteins, thus preventing their degradation, and in the refolding of these proteins into their native structure in an ATP-dependent manner after relief of stress.

Lund et al.[3] have found that levels of HSP 70 and chaperonin (cpn) 60 do not change to any extent during stress treatments. In contrast, levels of mitochondrial HSP 22 increase dramatically during stress and decrease after the stress is relieved. The 23 000 light—stress-regulated HSP of *Chenopodium rubrum* L. is located in the mitochondria. Mitochondria may be a major target for the protective effects of HSP against oxidative stress. The repairs of membrane, mitochondria, DNA and protein synthesis using extant mRNAs could be earlier events during seed germination.

In this paper, we have studied the effect of dehydration stress on germination rate of sugar beet seeds and growth recovery after dehydration stress, and the changes in mitochondrial lmw HSP 22.

1 Materials and methods

Sugar beet (*Beta vulgaris* L. cv. Loke) seeds

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(supplied by Novartis Seeds AB, Landskrona, Sweden) were imbibed for 72 h at 7 ℃, and then germinated under dehydration stress caused by dry paper towels within closed plastic box for 0, 4, 8, 12, 16, and 20 d, respectively.

1.1 Determination of water content

Water content of seeds and/or seedlings germinated for different time under dehydration stress were determined gravimetrically (80 ℃ for 48 h). 200 seeds and/or seedlings were sampled each time for these determinations. Water contents are expressed on a fresh weight basis (FWB).

1.2 Test of growth recovery

The growth recovery tests were performed in special plastic box at 14 ℃ in the dark. A cover paper and a pleated paper were put into the plastic box, 40 mL of deionised water was carefully added along the side of the paper and left for 2 h before sowing. The pleated paper has 50 fold; 2 seeds and/or seedlings germinated for different time under dehydration stress were sown from the edge of the pleated paper per fold, i. e. 100 seeds and/or seedlings per box. The cover paper was folded over the pleated paper after sowing. The lid was put on the box, and then the box was sealed with the Parafilm and incubated in darkness at 14 ℃. Germination percentage and seedling length were determined after 5 d of sowing. Seeds showing radicle emergence were counted as germinated.

1.3 Isolation of mitochondria

All procedures were carried out between 0 and 4 ℃. 100 seeds and/or seedlings were homogenized by moving the piston up and down 30 times in a precooled glass-teflon homogenizer in 35 mL ice-cold extraction medium containing 0.3 mol/L sucrose and 10 mmol/L MOPS (pH 7.2). The brei was squeezed through a 300 μm mesh nylon cloth. The filtrate was centrifuged at 1 000 g for 10 min. The supernatant was centrifuged at 41 400 g for 30 min. The pellet was resuspended in extraction medium, and centrifuged at 1 480 g for 10 min, and then the supernatant was centrifuged at 41 400 g for 30 min. The pellet was resuspended in a small volume of extraction medium. The volume of the resultant samples (washed mitochondria) was measured, and DMSO was added to the sample to make the final concentration 5%. The samples were frozen in liquid nitrogen and were stored in −80 ℃.

1.4 Enzyme assays

CCO (EC 1.3.9.1) activity was measured as in Rasmusson and Møller[4]. NAD^+-dependent MDH (EC 1.1.1.37) was measured as in Møller et al[5]. Protein was determined by the method of Lowry et al. (1951), with standard curves prepared using bovine serum albumin.

1.5 SDS-PAGE and immunoblots

Samples (washed mitochondria) were mixed with a sample buffer[6] and heated at 95 ℃ for 4 min. Discontinuous SDS-polyacrylamide gels (12% separation gel, 4% stacking gel) were prepared and run according to the method of Laemmli[6] using a Mini-Protein Ⅱ Electrophoresis Cell (Bio-Rad).

Proteins were transferred from the polyacrylamide gel to a PVDF membrane (Amersham Life Science Ltd) according to Bio-Rad's manual (Mini Trans-Blot Electrophoretic Transfer Cell Instruction Manual, Bio-Rad). The transfer buffer contained 25 mmol/L Tris, 192 mmol/L glycine, $\varphi = 20\%$ methanol, pH 8.3. The proteins were transferred at 100 V (constant voltage) for 1 h.

Blots were rinsed in water and blocked for at least 1 h at the room temperature in 20 mmol/L Tris, 137 mmol/L sodium chloride, pH 7.6 (TBS) containing $\rho = 5\%$ dried milk powder plus $\varphi = 0.1\%$ Tween 20. After incubation with primary antibody and with secondary antibody, proteins were detected using ECL Plus Western blotting detection system (Amersham Life Science Ltd).

The HSP 22 antibody from *Zea mays* L. was a gift from Thomas E. Elthon (School of Biological Sciences and the Center for Biotechnology, University of Nebraska, Nebraska, USA). Anti-mouse Ig antibodies conjugated with peroxidase was purchased from Amersham Life Science Ltd.

2 Results

2.1 Changes in water content and rate of germination and growth

Sugar beet seeds, which are fruits in fact, were harvested, stored and sowed in production. Water content of dried sugar beet seeds was 8.7% (FWB, similarly hereinafter). Their water content increased to 52.7% at 72 h of imbibition. Water content in imbibed seeds progressively declined during dehydration stress; when seeds were dehydrated for 16 and 20 d, for example, water content of seeds and seedlings were decreased by 15.9% and 20.6%, respectively (Figure 1).

The rate of germination and seedling growth in imbibed seeds increased at the initial phase of dehydra-

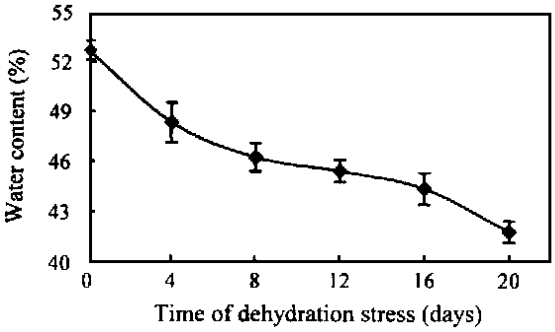

Fig 1　Changes in water content of imbibed sugar beet seeds during dehydration stress.

Seeds were first imbibed for 72 h, and then germinated under dehydration stress as indicated time at 7 ℃

tion stress, and then decreased (Figure 2). The germination rate of seeds, however, dramatically declined after 8 d of dehydration stress; for example, germination rate of seeds was only increased by 6% from 8 to 12 d of dehydration stress; 7%, from 12 to 16 d; and 1% from 16 to 20 d (Figure 2).

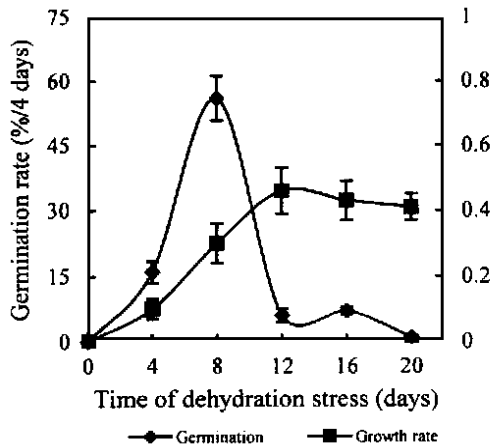

Fig 2　Effects of dehydration stress on rate of germination and seedling growth in imbibed sugar beet seeds

Seeds were first imbibed for 72 h, and then germinated as Material and methods at 7 ℃. Seeds showing radicle emergence were counted as germinated. Seedling length was measured from the tip of radicle to the tip of the primary leaf. It should be noted that non-germinated seeds are not included in the measurements of seedling length.

2.2　Growth recovery

Seeds and/or seedling suffered from dehydration stress for indicated time were conducted growth recovery at the normal conditions (see Material and methods). The recovery of growth activities of seeds and/or seedlings, which were measured as increases in germination and seedling length after 5 d of recovery growth of 100 seeds and/or seedlings, decreased with dehydration stress (Figure 3). When water content of seeds and/or seedlings was decreased from 48.3% at the 4 d of dehydration stress to 45.4% at the 12 d, growth of seedling was almost similar; whereas, their water content was decreased to 44.4%, growth of seedlings significantly declined (Figure 3).

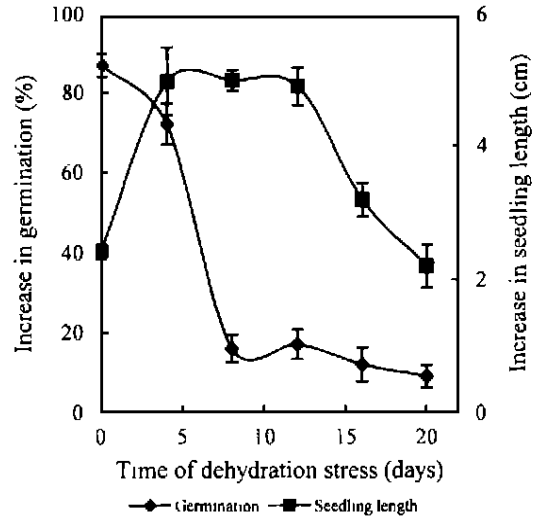

Fig 3　Growth recovery of sugar beet seeds and seedlings suffered from dehydration stress for indicated time

The imbibition of seeds, dehydration stress of seeds and seedlings, and the measurements of germination and seedling length as in Figure 2.

2.3　Changes in activities of CCO and MDH

Specific activities of CCO and MDH in mitochondria markedly increased at the initial phase of dehydration stress; however, the activities of CCO and MDH decreased as water content of seeds and seedlings declined from 44.4% to 41.8% (Figure 4). Figure 4 also showed that specific activity of MDH was higher than that of CCO in the mitochondria. The changes in total activities of CCO and MDH in mitochondria with dehydration stress were similar to those in their specific activity, and total activity of MDH were also higher than that of CCO (Figure 4).

2.4　SDS-PAGE and immunoblot analysis of mitochondria HSP 22

Coomassie blue-stained SDS-PAGE gel showed that proteins in mitochondria markedly changed with dehydration stress of imbibed seeds. Mitochondrial proteins with approximate M_w of 37 000, 33 000, 22 000 and 18 400 slightly increased from 52.7% water content at 0 d of dehydration stress to 48.3% at 4 d; and then progressively declined (Figure 5).

Antibodies against HSP 22 from Zea mays L. could recognize the proteins from mitochondria of sugar beet seeds and seedlings, and the HSP 22 of mitochondria gradually declined with dehydration stress, and

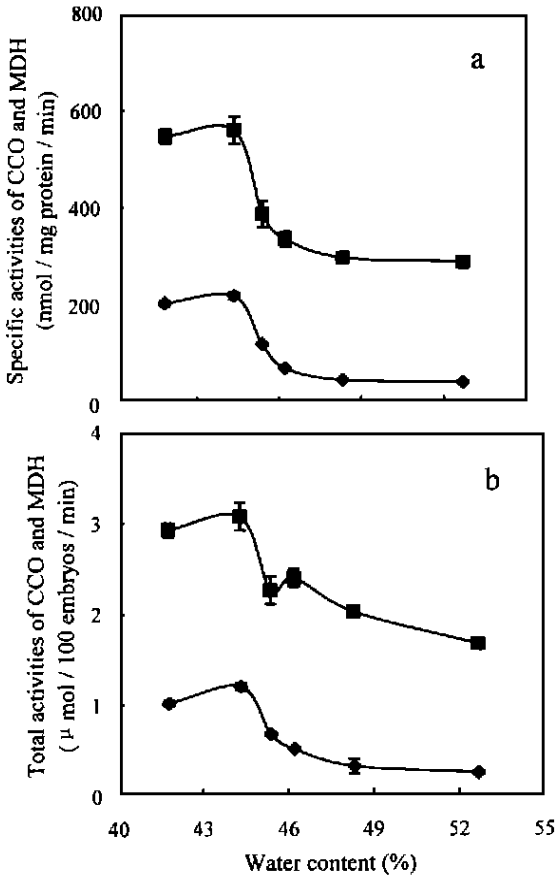

Fig 4 Changes in specific (a) and total (b) activities of CCO and MDH in mitochondria from seeds and seedling suffered from dehydration stress

The imbibition of seeds, dehydration stress of seeds and seedlings as in Figure 2.

lost until 20 d (Figure 5).

3 Discussion

Air drought caused by dry paper towels could mimic dehydration stress and enable imbibed seeds to dehydrate gradually (Figure 1), which made it convenient to study the effect of dehydration stress on germination behavior of seeds. Imbibed sugar beet seeds could germinate under gentle dehydration stress, but the rate of germination and seedling growth gradually decreased with dehydration stress (Figure 2). The general consequences of reduced water potential (ψ) for seed germination are that both the rate and the final percentage of germination are lowered as ψ decreases.

The recovery of growth activities of seeds and seedlings progressively decreased with dehydration stress (Figure 3), showed that seeds and seedlings have been damaged by dehydration stress.

CCO, which is a marker enzyme of mitochondria (Struglics et al., 1993), is the terminal complex of the electron transport chain. The reaction catalyzed by CCO is the four-electron reduction of O_2. MDH catalyses the oxidation of malate to oxaloacetate which is a necessary substrate for citric acid cycle. Activities of CCO and MDH markedly increased at the initial phase of dehydration stress; whereas, activities of CCO and MDH started to decrease as water content of seeds and seedlings declined from 44.4% to 41.8% (Figure 4). These results showed that activities of CCO and MDH were strongly correlated with desiccation tolerance of imbibed seeds and seedlings, and that the function of mitochondria was impaired by dehydration stress.

HSPs are thought to act as molecular chaperones and to prevent further aggregation of partially folded/denatured proteins or to promote the correct folding of the target protein. Work by Lee et al. (1995) has shown that two cytosolic lmw HSPs from pea can enhance refolding and prevent aggregation of artificially denatured proteins in an ATP-independent manner. The lmw HSP 22 in mitochondria from sugar beet seeds could be a constitutive protein (Figure 5). Work by Bettey and Finch Savage[7] has showed that HSP 17.6 accumulated in the latter stages of Brassica seed development and was present in mature dry seeds and in imbibed seeds. Mitochondrial proteins with approximate M_w of 37 000, 33 000, 22 000 and 18 400 slightly increased from 52.7% water content at 0 d of dehydration stress to 48.3% at 4 d; and then progressively declined (Figure 5). lmw HSP 22 of mitochondria, however, gradually declined with dehydration stress, and lost until 20 d (Figure 5). Results showed that change in lmw HSP 22 in mitochondria (Figure 5) was in close relationship with desiccation tolerance of imbibed seeds (Figure 2), recovery of growth activity (Figure 3) and activities of CCO and MDH in mitochondria (Figure 4). The mitochondrial lmw HSP could protect NADH: ubiquinone oxidoreductase (complex I), and consequently electron transport from complex I to cytochrom c: O_2 oxidoreductase (complex Ⅳ) in submitochondrial vesicles; and addition of purified lmw HSP to submitochondrial vesicles lacking this HSP increased complex I electron transport rates 100% in submitochondrial vesicles assayed at high temperature[8].

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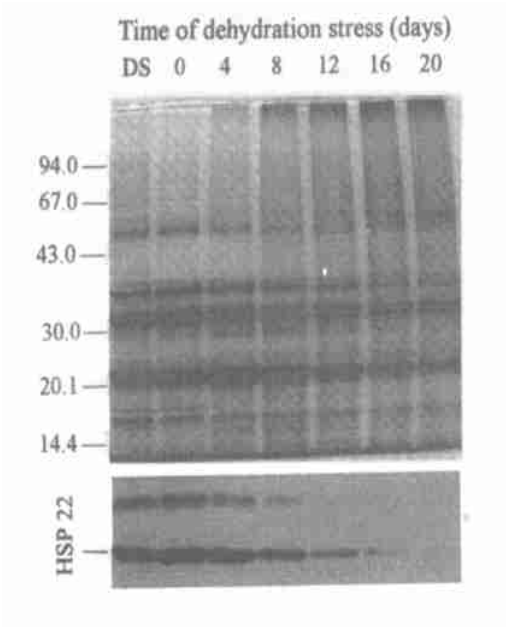

Fig 5　SDS-PAGE and immunoblot analysis of mitochondria HSP 22

Seeds were imbibed for 72 h, and then germinated under dehydration stress for the indicated time and the mitochondria were immediately isolated. The top panel is a Coomassie blue-stained SDS-PAGE gel loaded with 20 μg of mitochondrial protein per lane. Approximate molecular mass markers are on the left. The bottom panel is immunoblot of similar gels probed with the HSP 22 monoclonal antibody from T. E. Elthon. DS, dry seed.

to Professor N. W. Pammenter and Professor P. Berjak for reviewing the manuscript.

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脱水胁迫对吸胀甜菜种子的萌发行为和线粒体小分子量热休克蛋白的影响

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摘　要: 水分的可利用性是种子萌发的决定因子. 由干燥纸巾引起的空气干旱被用来模拟脱水胁迫. 吸胀的甜菜(*Beta vulgaris* L.)种子的萌发速率、幼苗生长速率和生长活性的恢复随着脱水时间的延长而下降. 线粒体细胞色素 c 氧化酶 (CCO) 和苹果酸脱氢酶 (MDH) 的活性在脱水胁迫的初期增加, 然后下降. 线粒体小相对分子质量热休克蛋白 (lmw HSP) 22 的含量随着脱水胁迫下降, 到脱水胁迫 20 d 丧失. 可以认为线粒体 lmw HSP 22 与种子和幼苗的脱水耐性呈正相关.

关键词: CCO 和 MDH 活性; 甜菜(*Beta vulgaris* L.); 脱水胁迫; 萌发速率; 生长恢复; 线粒体小相对分子质量热休克蛋白 (lmw HSP) 22

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