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Research article

Changes in the mitochondrial protein profile due to ROS eruption during ageing of elm (*Ulmus pumila* L.) seeds

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ABSTRACT

Reactive oxygen species (ROS)-related mitochondrial dysfunction is considered to play a vital role in seed deterioration. However, the detailed mechanisms remain largely unknown. To address this, a comparison of mitochondrial proteomes was performed, and we identified several proteins that changed in abundance with accompanying ROS eruption and mitochondrial aggregation and diffusion. These are involved in mitochondrial metabolisms, stress resistance, maintenance of structure and intracellular transport during seed aging. Reduction of ROS content by the mitochondrial-specific scavenger MitoTEMPO suppressed these changes, whereas pre-treatment of seeds with methyl viologen (MV) had the opposite effect. Furthermore, voltage-dependent anion channels (VDAC) were found to increase both in abundance and carbonylation level, accompanied by increased cytochrome *c* (cyt *c*) release from mitochondria to cytosol, indicating the profound effect of ROS and VDAC on mitochondria-dependent cell death. Carbonylation detection revealed the specific target proteins of oxidative modification in mitochondria during ageing. Notably, membrane proteins accounted for a large proportion of these targets. An *in vitro* assay demonstrated that the oxidative modification was concomitant with a change of VDAC function and a loss of activity in malate dehydrogenase. Our data suggested that ROS eruption induced alteration and modification of specific mitochondrial proteins that may be involved in the process of mitochondrial deterioration, which eventually led to loss of seed viability.

1. Introduction

The ability of seeds to germinate gradually declines during storage. Even under optimal storage conditions, deterioration can still occur and poses a huge obstacle to agricultural production and germplasm conservation. Seed deterioration is a complex biological process that is influenced by many factors. Reactive oxygen species (ROS) and free radical-mediated lipid peroxidation, protein oxidation and nucleic acid damage has long been identified as important causes leading to seed vigour loss during seed ageing (McDonald, 1999; Benamar et al., 2003; Xin et al., 2014). Hence, to uncover the detailed roles of ROS in seed viability loss it is important to determine the underlying mechanisms of seed ageing.

In the normal metabolism of oxygen, ROS are produced as a natural by-product that plays important roles in seed germination and dormancy release (Bailly et al, 2008). However, ROS levels should be tightly regulated by balancing production and scavenging; accumulation of ROS due to loss of antioxidant mechanisms can cause uncontrolled oxidative damage during seed imbibition (Choudhary and Agrawal, 2016). Bailly et al. (1996) showed that loss of viability in sunflower seeds was mainly associated with a loss of catalase (CAT) activity, and therefore an altered capacity of hydrogen peroxide detoxification that leads to lipid peroxidation in the early steps of seed imbibitions. Transcriptome reprogramming induced a decline in antioxidant capacity that ultimately led to loss

Abbreviations: CDT, controlled deterioration treatment; CMXRos, MitoTracker Red CMXRos; cyt *c*, cytochrome *c*; 2-DE, two-dimensional electrophoresis; ETC, electron transport chain; H_2DCFDA, 2′,7′-Dichlorodihydrofluorescein diacetate; H_2O_2, hydrogen peroxide; LSCM, Laser scanning confocal microscopy; MV, Methyl viologen; ROS, reactive oxygen species; SD, standard deviation; TCA, tricarboxylic acid; VDAC, voltage-dependent anion channel.

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of seed viability during pea seed ageing (Chen et al., 2013). Thus, as important scavengers of ROS, classical antioxidant systems play an important role in seed quality.

In non-photosynthesising plant cells, mitochondria are a major source of ROS generation. Therefore, mitochondrial proteins are usually the primary targets of oxidative damage under stress conditions (Das et al, 2001). Protein carbonylation, an irreversible and irreparable oxidative modification, is a main consequence of oxidative damage to proteins (Stadtman, 1992). The oxidized proteins tend to misfold, show lower function and greater susceptibility to proteolysis, which may severely affect the function of mitochondria. Many studies have shown that seed ageing is correlated with the dysfunction of mitochondria. For example, Amable and Obendorf (1986) proposed that mitochondria may be the site of a primary lesion during pea seed deterioration under high water content and high temperature conditions. In this situation, the loss of seed vigour is accompanied by a decline in cytochrome oxidase-dependent respiration, pyruvate metabolism through the TCA cycle and a concomitant decline in NADH levels. Mitochondrial membranes were identified as the primary target for ageing stress in pea seeds, and the decrease in membrane integrity strongly affected the phosphorylation efficiency of mitochondria (Benamar et al, 2003). Mitochondrial dysfunction that resulted from a retarded ASC-GSH cycle and elevated ROS accumulation was also found to arouse seed vigour loss in artificially aged soybean seeds (Xin et al, 2014). Our previous studies also demonstrated that controlled deterioration treatment (CDT) induces dynamic changes in mitochondrial physiology via increased ROS production, ultimately resulting in an irreversible loss of seed viability; the distinct spatial-temporal signature of ROS coincided with changes in ageing features and elm seed vigour during CDT (Wang et al, 2015). However, the detailed mechanisms underlying the relationship of mitochondrial ROS production, mitochondrial function and seed ageing are still not clear.

A proteomic approach has been used to provide insight into the mechanism of seed ageing in many studies. Rajjou et al (2008) revealed the essential mechanisms for seed vigour loss, such as translational capacity, mobilization of seed storage reserves and detoxification efficiency by proteomic analyses of aged Arabidopsis seeds. It has also shown that artificial and natural aging have similar characteristics. Xin et al. (2011) identified 40 proteins that changed in abundance during soybean seed ageing, indicating that artificial ageing affected the metabolism and energy production of soybean seeds. These proteome studies were generally conducted on whole cellular extracts after seed ageing. Mitochondria are a centre of energy and substance metabolism and their dysfunction is believed to play an important role in seed deterioration during ageing (McDonald, 1999; Wang et al., 2015). However, the detailed mechanism of mitochondrial dysfunction during seed ageing is still not clear. Yin et al. (2016) investigated mitochondrial proteomic alterations during the critical node of rice seed ageing, and found that the induction of the electron transport chain (ETC) alternative pathway was concomitant with decreases in the abundance of mitochondrial proteins associated with carbon, nitrogen and energy metabolism. However, the detailed mechanism by which ROS induces mitochondrial dysfunction has not been revealed. Oxidative damage of mitochondrial proteins has been investigated in fruit senescence by comparing the normal ageing groups with groups treated with oxidizing agents (Qin et al., 2009a; 2009b), and ageing related diseases in mammals (Stauch et al., 2015). However, the detailed mechanism of mitochondrial oxidative impairment in seed ageing is still not well known.

In this study, CDT was used to obtain aged samples within a short period to unravel the mechanisms of mitochondria-related seed vigour loss during storage. The link between mitochondrial morphological changes and ROS production was investigated using laser scanning confocal microscopy (LSCM) and several physiological methods. A comparative proteome approach was employed to screen alterations in mitochondrial protein expression profile during seed ageing. To gain a better understanding of the relationship between ROS production, mitochondrial proteomic changes and seed ageing, elm seeds were pre-treated with the mitochondria-specific ROS scavenger MitoTEMPO or methyl viologen (MV, an inhibitor of electron transfer that ultimately leads to ROS production) before ageing, followed by proteomic and other analyses. We identified a number of metabolism- and antioxidant-associated proteins as well as also some proteins that have not been previously reported to associate with mitochondrial dynamic changes during seed ageing. In addition, carbonylation detection was used to determine the targets of mitochondrial protein oxidative modification. Our data provide a great deal of information that enhances our understanding of ROS-provoked mitochondria-dependent cell death during seed ageing.

2. Methods

2.1. Materials and controlled deterioration treatment (CDT)

Elm seeds (*Ulmus pumilaL.*) were collected from the campus of Beijing Forestry University, China with an original germination rate of 98%. The germination tests were performed as previously described (Hu et al., 2012). Seeds were stored at −20 °C in tightly closed containers until required for analysis. CDT was performed as previously described (Wang et al., 2015). The seeds were equilibrated in sealed bottles with 37 °C saturated aqueous vapor for 1 d, and then began the CDT until viability loss (5 d) as judged by germination tests. Day 0 was served as control.

The elm seeds were treated with MitoTEMPO (Sigma) (20 nM) (Wang et al., 2015), MV (Sigma) (200 μM) or H_2O as a control as previously described. Concentration screening of MV was shown in Supplementary Fig. 1.

2.2. Determination of H_2O_2 content

10 seeds were grinded into powder using liquid nitrogen. Determination of H_2O_2 content was taken using a Hydrogen Peroxide Fluorescent Detection Kit (ARBOR ASSAYS, USA).

2.3. Confocal microscopy

Elm seeds rehydrated for 12 h were cut into pieces (40 μm in thickness) using a vibratome as the method described by Wang et al. (2015). Seed slices were stained with 100 μM 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA, H_2O_2 fluorescent dye) or 0.6 μM MitoTracker Red CMXRos (CMXRos; Invitrogen) respectively as previously described. LSCM observations were performed using a Leica TCS-SP5 laser scanning confocal microscope. H_2DCFDA signals were visualized by excitation at 488 nm and emission at 520–540 nm using a band-pass filter. CMXRos signals were visualized through 409 oil-immersion lenses, using 543 nm excitation from a He/Ne laser and a 580–620 nm band-pass filter.

2.4. Mitochondrial respiration assay

For mitochondria isolation, elm seeds without radicle protrusion were imbibed for 12 h, and then ground with pestle and mortar together in grinding medium (0.3 M sucrose, 50 mM Tris, pH 7.4, 50 mM KCl, 10 mM EDTA, 0.2% (w/v) BSA). The homogenate was squeezed through eight layers gauze and centrifuged at 3, 500 g for 15 min. The supernatant was centrifuged at 12, 000 g for