Journal of Plant Physiology 221 (2018) 119–131

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Uncovering the basis of viability loss in desiccation sensitive *Trichilia dregeana* seeds using differential quantitative protein expression profiling by iTRAQ

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ARTICLE INFO

Keywords:
Desiccation
Hydrated storage
Proteomics
Recalcitrant seeds
Redox metabolism

ABSTRACT

Recalcitrant seeds, unlike orthodox types, are desiccation sensitive and hence, cannot be stored using conventional seed storage methods In this study, relative changes of protein expression in *T. dregeana* seeds during desiccation and hydrated storage (a short- to medium-term storage method) were analysed to understand the basis of their desiccation- and storage-induced viability loss. Isobaric Tags for Relative and Absolute Quantitation (iTRAQ) were used to compare (selected) protein expression levels across fresh, partially dehydrated and stored seeds. A total of 114 proteins were significantly differentially expressed in embryonic axes of fresh seeds and those seeds exposed to dehydration and hydrated storage (which exposed seeds to a mild dehydration stress). Proteins involved in protein synthesis were up-regulated in stored and dehydrated seeds, possibly in response to dehydration-induced repair processes and/or germinative development. A range of proteins related to antioxidant protection were variably up- and down-regulated in stored and dehydrated seeds, respectively. Additionally, a class I heat shock protein was down-regulated in dehydrated and stored seeds; no late embryogenesis abundant proteins were identified in both stored and dehydrated seeds; and storage and dehydration up-regulated proteins involved in the provision of energy for cell survival. The results suggest that dehydration- and storage-induced viability loss in recalcitrant seeds may be based on proteomic changes that lead to cellular redox imbalance and increased cell energy demands. This, together with the absence/down-regulation of proteins associated with desiccation tolerance in plant tissues may form part of the proteomic footprint for desiccation sensitivity in seeds.

1. Introduction

Recalcitrant seeds, unlike orthodox types, are desiccation and often chilling sensitive (Pammenter and Berjak, 1999; Roberts, 1973). This makes them unamenable to long-term germplasm conservation using conventional seed storage methods. They can, however, be stored in the short- to medium-term using hydrated storage, which involves maintaining seeds under conditions of reduced temperature and saturated relative humidity (Eggers et al., 2007). However, even under these conditions embryonic axes of recalcitrant seeds undergo germinative development and if additional water is not supplied to complete this process the seeds lose viability as a consequence of a mild desiccation stress (Berjak and Pammenter, 2000; Farrant et al., 1986; Pammenter et al., 1984).

Desiccation sensitivity in recalcitrant seeds has been attributed to the absence or poor expression of a range of mechanisms associated with desiccation tolerance (Pammenter and Berjak, 1999), which is a polygenic trait (Dussert et al., 2004). Examples of the mechanisms activated during drying in orthodox seeds include the active down-regulation/"switching off" of metabolism (Leprince et al., 2000), the production of late embryogenesis abundant (LEA) proteins (Farrant et al., 1993) and the up-regulation of "housekeeping" antioxidants that control reactive oxygen species (ROS) generation during water loss (Pukacka and Ratajczak, 2007), amongst others. For example, a number of enzymic and non-enzymic antioxidants involved in the quenching of ROS in seeds (Bailly, 2004; Kranner et al., 2006), appear to be either

Abbreviations: ANOVA, analysis of variance; APX, ascorbate peroxidase; DHAR, dehydroascorbate reductase; GR, glutathione reductase; H_2O_2, hydrogen peroxide; HPLC, high pressure liquid chromatography; iTRAQ, isobaric tags for relative and absolute quantitation; MDHAR, Monodehydroascorbate reductase; $\cdot O_2^-$, , Superoxide; ROS, reactive oxygen species; SOD, superoxide dismutase

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https://doi.org/10.1016/j.jplph.2017.12.011
Received 17 October 2017; Received in revised form 29 November 2017; Accepted 11 December 2017
Available online 16 December 2017
0176-1617/

inadequate or compromised during desiccation (Hendry et al., 1992; Varghese and Naithani, 2002) and hydrated storage (Tommasi et al., 2006) in recalcitrant seeds. However, there are also reports of antioxidants being enhanced during desiccation stress in recalcitrant seeds (e.g. components of the glutathione-ascorbate cycle in *A. saccharinum* [(Pukacka and Ratajczak, 2006).

Whilst aspects related to desiccation-induced oxidative stress (Varghese and Naithani, 2002), metabolic disruption (Roach et al., 2008) and ultrastructural damage (Berjak and Pammenter, 2000) have been relatively well researched in recalcitrant seeds, the proteomic basis of their desiccation sensitivity remains unclear and under-explored. This is largely because recalcitrant seeds contain many interfering compounds that present numerous challenges to proteomic studies (Garnczarska and Wojtyla, 2008; Parkhey et al., 2015). Despite these challenges, some proteomic studies have shown that desiccation induces a rapid accumulation of antioxidant enzymes and proteins in recalcitrant seeds (Chen et al., 2011). However, Bai et al. (2011) found that ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) enzyme activities are induced during the early stages of desiccation and then decline upon further dehydration, resulting in the inefficient removal of ROS.

Additionally, considerable interest has been directed towards the presence/absence of specific proteins known to play a role in desiccation tolerance (Battaglia et al., 2008; Tunnacliffe and Wise, 2007); for example, LEA proteins in *Castanospermum australe* (Delahaie et al., 2013) and *A. marina* (Farrant et al., 1996). In a comparative study of the heat-stable proteome of the recalcitrant seeds of *C. australe* and an orthodox legume *Medicago truncatula*, it was shown that for 12 LEA genes, polypeptides were either absent or strongly reduced in *C. australe* compared with *M. truncatula* (Delahaie et al., 2013). Though those authors showed non-seed specific dehydrins to accumulate at high levels in the cotyledons of recalcitrant *C. australe* compared with orthodox *M. truncatula* seeds, no dehydrins have been found in the recalcitrant seeds of *A. marina* (Farrant et al., 1996) and *T. dregeana* (Han et al., 1997). It has been speculated that the desiccation sensitivity of seeds is at least partially due to the insufficient accumulation and/or absence of certain dehydrins (Panza et al., 2007; Vertucci and Farrant, 1995). However, it should be noted that proteomic studies conducted on recalcitrant seeds to date are largely restricted to studies that have employed two dimensional electrophoresis (2-DE) with subsequent protein identification by mass spectrometry (MS/MS) (Bai et al., 2011; Chen et al., 2011; Delahaie et al., 2013; Parkhey et al., 2015). Although 2-DE protein separation can be used to produce insightful protein maps, there are several limitations to this approach related to technical reproducibility, correct spot matching and the low number of proteins identified (Balbuena et al., 2011). A further drawback of at least one of these key studies cited above (Delahaie et al., 2013) is that it only examined the heat-stable proteome extracted from the cotyledons, rather than the more metabolically active, developmentally important embryonic axis.

In light of the above, and in line with current proteomic research, the present study aimed to explore the total proteome extracted from the embryonic axes of the desiccation sensitive species *T. dregeana* using a high-throughput technique called Isobaric Tags for Relative and Absolute Quantification (iTRAQ), coupled to mass spectrometry. iTRAQ is a powerful gel-free proteomic method, considered to be one of the most robust techniques for differential quantitative proteomic analyses (Latterich et al., 2008; Wilm, 2009). The aim of this study was to functionally characterise proteins in terms of their involvement in cellular pathways in recalcitrant seeds, with a focus on antioxidant and catalytic activity that may influence their viability during partial dehydration and storage. This was done by identifying, quantifying, annotating and comparing proteins expressed in *T. dregeana* seeds exposed to partial dehydration and hydrated storage, both of which result in viability loss in recalcitrant seeds, with those expressed in freshly harvested (i.e. unstressed) seeds. The data presented allows for a more fundamental understanding of the proteomic basis of desiccation sensitivity in recalcitrant seeds.

2. Material and methods

2.1. Seed collection

Seeds of *T. dregeana* were obtained from mature and open capsules harvested directly from trees growing on the Westville campus (29°49.054′ S 30°56.521′ E) of the University of KwaZulu-Natal, Durban, South Africa. Seeds were collected over two seasons (April-June in 2012 and 2013).

2.2. Water content determination

Freshly harvested, dehydrated and stored seeds were sampled for embryonic axis WC at intervals that coincided with the viability assays. Immediately after excision, embryonic axis WC (n = 10) was measured gravimetrically using a 5-place balance (Mettler, Mt5, Germany). Axes were weighed before and after drying in an oven at 80 °C for 48 h. Water content was expressed on a dry mass basis (dmb; g H_2O per g dry matter [g g^{-1}]) as described in Varghese et al. (2011).

2.3. Hydrated storage treatment

T. dregeana seeds were prepared for, and subsequently stored hydrated at 16 °C (after Goveia et al., 2004). Hydrated storage is believed to maximise the storage life span of recalcitrant seeds by retaining water content at, or close to the same levels as those characterising the newly-harvested state (Pammenter et al., 1994). As recommended by those authors, water loss from the seeds investigated in the present study was avoided during hydrated storage by maintaining the seeds at high (c. 90–100%) relative humidity, achieved by maintaining a saturated atmosphere in the storage containers. Seeds that germinated in hydrated storage were regularly removed from the buckets and discarded to avoid fungal contamination. As alluded to in the Introduction, recalcitrant seeds progress towards germination in hydrated storage which progressively leads to a mild dehydration stress, which terminates in death if additional water is not supplied (Pammenter and Berjak, 1999). In the present study, after 12 months of storage > 50% of the seeds (n = 3) had to be removed from the buckets as they showed signs of germination. The remaining non-germinated seeds were assessed for viability (germinability) and used for protein analyses described below.

2.4. Desiccation treatment

Freshly harvested seeds were sown in commercial potting soil (Grovida, Durban, South Africa); prior to sowing, the soil was dried for 24 h to remove any excess moisture. Approximately 50 seeds per tray were randomly sown to a depth of 10 mm with the aril intact. The seeds were allowed to dry under glasshouse conditions at 25 °C for a period of 20 d. Seed germination was assessed as described below. Fourteen days after sowing, seeds subjected to 62% loss in axis water content which led to ± 50% viability loss, were used for the protein analyses described below.

2.5. Germination assessment

Seeds were retrieved from the storage (at monthly intervals) and desiccation (at two day intervals) treatments and assessed for germinability. The seeds (n = 15) were sown in commercial potting soil within seedling trays (five seeds per tray) and the soil was maintained at field capacity using deionised water for the duration of the trial. These studies were conducted within a glasshouse (26/18 °C, day/night; ambient