WARM STRATIFICATION OF APPLE SEEDS LEADS TO ALTERATIONS IN BIOTIN-CONTAINING PROTEIN LEVELS

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Abstract

Warm stratification stimulates apple (*Malus domestica* Borkh.) seeds' ageing. In our work, warm stratification was used as a treatment leading to the controlled seeds' deterioration. We examined the levels of biotin-containing proteins (indicators of seeds' viability) and ubiquitin-marked proteins in the embryonic axes of aged seeds. Alterations in the level of biotin-containing proteins depended on the duration of warm stratification. No change in the content of ubiquitinated proteins was observed.

Key words: biotinylated proteins, seeds ageing, ubiquitin-marked proteins, warm stratification

Introduction

Seeds are the main mobile form of Spermatophyte, enabling dispersal in the environment. Seeds' longevity, defined as the total time span during which seeds remain viable, is a significant trait for ecology, agronomy, and economy (Walters et al., 2010, Morscher et al., 2015). Seed quality impacts germination and seedlings' development. Seed viability depends on plant species; e.g. long-term (more than 2,000 years) seed longevity has been described for the date palm (*Phoenix dactylifera* L.) (Sallon et al., 2008). However, prolonged seed storage leads to ageing; even favourable conditions (low humidity and low temperature) do not prevent viability loss (Walters et al., 2010, Morscher et al., 2015). Inappropriate storage conditions accelerate seed deterioration. During seeds' loss of viability, adverse metabolic changes, such as increased membrane permeability and genetic/proteomic damage, occur.

Seeds which tolerate dehydration (orthodox seeds) are more resistant than recalcitrant seeds (sensitive to desiccation) to adverse environmental factors, so they age slowly. Ageing of orthodox seeds can be artificially induced at high temperatures and high relative humidity. Accelerated ageing has been recognized as a good predictor of the loss of seeds' storability (Priestley, 1986). Alterations in the level of specific proteins could be used as markers of seed longevity (Rajjou et al., 2008, Sano et al., 2016). It has been proposed that a group of late embryogenesis abundant (LEA) proteins participate in modulating seed longevity (Sano et al.,

2016). As was demonstrated for pea (*Pisum sativum* L.) seeds, seed-specific biotinylated protein (SBP65, a member of the 3 LEA proteins) is accumulated at the end of the maturation state (Duval et al., 1994a). The decrease of SBP65 was observed during pea seeds' loss of dormancy, pointing to the involvement of these proteins in metabolic processes at early phases of germination (Duval et al., 1994b). Reduction of SBP65 levels was also demonstrated for the embryos of viviparous pea mutant (*vip-1*), which germinate precociously in the pods (Dehaye et al., 1997). It is suggested that this protein is engaged in maintaining seed longevity by scavenging free biotin, which is a cofactor of several carboxylases and decarboxylases (Sano et al., 2016). Nevertheless, there is no information about changes in the levels of biotin-containing proteins in seeds after artificially induced ageing.

In the literature, the mechanism of the degradation of biotin-containing proteins in plant cells is not well described. Proteolysis could be related to ubiquitination – labelling of proteins for degradation in proteasome (Stuttmann et al., 2009). There is no data concerning alteration in ubiquitin-marked protein content in seeds subjected to warm stratification.

Apple (*Malus domestica* Borkh.) seeds belongs to the orthodox type and are characterised by deep dormancy (Lewak, 2011) which can be broken by cold stratification (at 4°C in wetted sand) (Lewak, 2011, Dębska et al., 2013). On the contrary, embryos isolated from seeds subjected to long-term warm stratification (at 25°C in wetted sand) do not germinate well. Moreover, seedlings developed from those embryos have morphological anomalies (Dębska et al., 2013).

The aim of the work was to investigate the levels of biotin-containing proteins and ubiquitin-marked proteins isolated from embryonic axes of warm stratified apple seeds to evaluate loss of vigour during artificially induced ageing. The obtained data provide more insights in the relationship between seed longevity and storage conditions.

Material and methods

Apple (*Malus domestica* Borkh.) seeds were stratified at 32ºC for 7, 14, 21 and 40 days. Embryos isolated from stratified seeds were placed on glass Petri dishes containing filter paper wetted with distilled water. Embryos were cultured in a growth chamber at 20°C with 12/12 h (light/dark) photoperiod and light intensity 50 μmol PAR m^{-2} s^{-1}. After 24 h of the culture, embryonic axes were isolated and used for analysis.

Detection of biotin-containing proteins and ubiquitin-marked proteins

Isolation of biotin-containing proteins was done as described by Krasuska et al. (2016). Protein samples (3 μg) were placed on a nitrocellulose membrane. The blocking step was done using 5% (w/v) nonfat dry milk in Tris buffered saline with 0.05% (v/v) Tween-20 (TBST). After incubation the nitrocellulose membrane was washed with TBST and marked with alkaline phosphatase conjugated with streptavidin (S2890 Sigma-Aldrich) solution in 100 mM Tris-HCl pH 9.5 containing

100 mM NaCl and 5 mM $MgCl_2$. Visualization of the spots was performed using nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

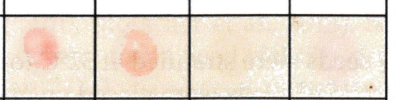
A similar procedure was done for ubiquitin-marked proteins. After insetting the protein probes on a nitrocellulose membrane and a blocking step, the nitrocellulose was washed with TBST. Then, the nitrocellulose membrane was incubated with a polyclonal anti-ubiquitin antibodies (AS08 307 Agrisera) solution in TBST (1:10,000) for 1 h. After another washing step with TBST, the nitrocellulose membrane was incubated with secondary antibodies (anti-Rabbit-Alkaline Phosphatase conjugated, A3687 Agrisera) 1:30,000. Visualisation of the protein spots was done as described above.

Both detection procedures were done in three independent experiments and the representative results are shown. After a dot blot test, the intensity of the spots was estimated using a densitometry analysis (ImageJ programme).

Results

Densitometry analysis indicated that the content of biotin-containing proteins in axes of apple embryos isolated from seeds stratified for 7 days was 148. A similar level of biotin-containing proteins in homogenates from embryonic axes isolated after 14 days of warm stratification was observed (Fig. 1). Prolonged warm stratification (21 and 40 days) decreased the content of these proteins. Moreover, no significant differences in the intensity of visualised protein spots for these probes were revealed (Fig. 1).

The level of ubiquitin-marked proteins isolated from embryonic axes of seeds stratified for 7, 14, 21 and 40 days was similar (Fig. 1).

	Biotin containing proteins				Ubiquitin marked proteins			
Days of warm stratyfication	7	14	21	40	7	14	21	40
Dot blot								
Densitometry analysis	148±5	145±12	118±5	120±7	235±10	228±15	240±15	248±13

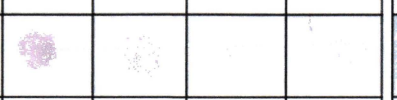
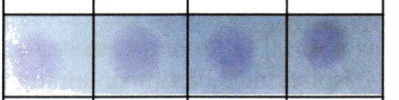
	Biotin containing proteins				Ubiquitin marked proteins			
Days of warm stratification	7	14	21	40	7	14	21	40
Dot blot								
Densitometry analysis	148±5	145±12	118±5	120±7	235±10	228±15	240±15	248±13

Figure 1. The levels of biotin-containing proteins and ubiquitin-marked proteins in axes of apple embryos isolated from seeds stratified at 32°C for 7, 14, 21 or 40 days. Densitometry analysis ± SD.

Discussion

The exposure of seeds to high temperature and high humidity is known as the accelerated ageing procedure and can be performed to indicate seeds' vigor and storability. A rapid deterioration process of seeds points to limited ability to germinate (McDonald, 1999). As was demonstrated for sunflower (*Helianthus annuus* L.) seeds, storage at 45°C and 100% RH reduced germination and led to the growth of atypical seedlings. Finally, such conditions resulted in the seeds' death (Corbineau et al., 1988). In apple seeds, warm stratification, opposite to cold stratification, does not alleviate dormancy (Lewak et al., 2011, Dębska et al., 2013). Furthermore, prolonged warm stratification leads to seeds' ageing and the formation of abnormal seedlings or even their death (Dębska et al., 2013).

Proteins and mRNA are stored in mature seeds as they are sufficient for the first phases of germination (Rajjou et al., 2004). Proteomic analyses done for Arabidopsis (*Arabidopsis thaliana* (L.) Heynh.) seeds subjected to controlled deterioration have revealed the linkage between loss in seed vigour and protein alterations in the dry seeds. During germination, the proteome of seeds with low vigor displayed some abnormalities (Rajjou et al., 2008). Rajjou et al. (2008) demonstrated a decreased level of the dehydrin/RAB (Responsive to abscisic acid) group of LEA proteins during seeds' ageing. The authors also proposed that this group of LEA is the most involved in seed longevity; h wever, they did not exclude other LEA proteins' participation in maintaining seed vigour. Our results indicated that biotin-containing protein levels in apple embryonic axes decrease as the warm stratification period is prolonged. This could point to a link between apple embryo vigour and the amount of such proteins. A lower abundance of biotin-containing proteins was observed during germination of apple embryos (Krasuska et al., 2016) and during pea seeds' loss of dormancy (Duval et al., 1994b), indicating the involvement of these proteins in metabolic pathways during early phases of seed germination. Moreover, the increased lev l of oxidation of pro ein homologous to SBP65 isolated from Arabidopsis deteriorated seeds has been demonstrated (Rajjou et al., 2008). It has been proposed that the survival of dry, aged Arabidopsis seeds can be altered due to the oxidation of biotin-containing proteins (Rajjou et al., 2008). The increase of protein oxidation is commonly linked to stress conditions and elevation of reactive oxygen species (ROS) levels, which are also high in aged tissues (Rajjou et al., 2008, Dębska et al., 2013). During warm stratification of apple seeds, the increase of ROS production and protein carbonylation (type of oxidation) was observed (D bska et al., 2013). Oxidation of proteins may stimulate their degradation. As we did not observe any changes in ubiquitin-marked proteins' levels, we suspect that the decrease of biotin-containing proteins' levels may be due to their oxidation and proteolysis.

In conclusion, the presented data indicate the relationship between the warm stratification-induced loss of viability of apple seeds and the decrease of seeds' biotin-containing proteins.

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