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In-depth proteomic analysis of *Glycine max* seeds during controlled deterioration treatment reveals a shift in seed metabolism

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ABSTRACT

Seed aging is one of the major events, affecting the overall quality of agricultural seeds. To analyze the effect of seed aging, soybean seeds were exposed to controlled deterioration treatment (CDT) for 3 and 7 days, followed by their physiological, biochemical, and proteomic analyses. Seed proteins were subjected to protamine sulfate precipitation for the enrichment of low-abundance proteins and utilized for proteome analysis. A total of 14 differential proteins were identified on 2-DE, whereas label-free quantification resulted in the identification of 1626 non-redundant proteins. Of these identified proteins, 146 showed significant changes in protein abundance, where 5 and 141 had increased and decreased abundances, respectively while 352 proteins were completely degraded during CDT. Gene ontology and KEGG analyses suggested the association of differential proteins with primary metabolism, ROS detoxification, translation elongation and initiation, protein folding, and proteolysis, where most, if not all, had decreased abundance during CDT. Western blotting confirmed reduced level of antioxidant enzymes (DHAR, APx1, MDAR, and SOD) upon CDT. This in-depth integrated study reveals a major downshift in seed metabolism upon CDT. Reported data here serve as a resource for its exploitation to metabolic engineering of seeds for multiple purposes, including increased seed viability, vigor, and quality.

Biological significance: Controlled deterioration treatment (CDT) is one of the major events that negatively affects the quality and nutrient composition of agricultural seeds. However, the molecular mechanism of CDT is largely unknown. A combination of gel-based and gel-free proteomic approach was utilized to investigate the effects of CDT in soybean seeds. Moreover, we utilized protamine sulfate precipitation method for enrichment of low-abundance proteins, which are generally masked due to the presence of high-abundance seed storage proteins. Reported data here serve as resource for its exploitation to metabolic engineering of seeds for multiple purposes, including increased seed viability, vigor, and quality.

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1. Introduction

Owing to the unique nutrient composition, soybean seeds are one of the most important agricultural commodities worldwide. Soybean seeds are a rich source of proteins, oils, anthocyanins, and isoflavones [1–3]. Therefore, humans have developed a variety of soybean products for consumption, including infant formulas, flours, protein isolates and concentrates, and textured fibers. Soy proteins are also considered as meat substitutes and can be obtained from cheese, drinks, miso, tempeh, tofu, and salami. Soybean seeds have numerous health benefits such as in the prevention of cancer, diabetes, and obesity, lowering of plasma cholesterol, and protection against bowel and kidney disease [4,5]. Therefore, past decades has seen a gradual increase in soybean consumption, and where Mexico, Indonesia, Malaysia, Taiwan, and South Korea, are the top importers of soybean seeds.

Various biochemical changes occur in the seeds during transportation and post-harvest storage, which negatively affects their quality. It has been observed that the post-harvest storage accelerates the process of seed aging in *Arabidopsis thaliana* [6], *Brassica napus* [7], and *Oryza sativa* [8]. Seed aging is an inevitable process that deteriorates the seed quality. In an agricultural context, aged seeds show reduced germination, increased the time from sowing to germination, reduced the seedling relative growth rates [9,10]. It was observed that the orthodox seeds, stored at low temperature and moisture content retain their

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viability for a longer time than those stored under high temperature and humidity conditions [11]. In addition to storage conditions, loss of seed quality can occur during the pre-harvest period by field weathering. Pre-mature soybean seeds have high moisture content (50–55%) which decreases to 14% during seed maturation. However, weather conditions during pre-harvest period strongly influence the quality of the mature seeds [12]. These studies suggest that abnormal environmental conditions strongly affect the basic seed metabolism during the pre-harvest period in the pre-matured seed.

A recent proteomic study proposed the mechanism of soybean seed deterioration during pre-harvest storage while identifying 42 protein spots mainly associated with photosynthesis, protein folding and assembly, protein biosynthesis, primary metabolism, signal transduction, and transcription regulation [13]. Further, their results showed that accumulation of reactive oxygen species (ROS) was highly increased, while nitric oxide (NO) production was down-regulated as compared with control in developmental stage seeds. In rice, several proteins related to the seed aging have been identified and characterized [8]. Lipoxygenase enzyme activity was found to be decreased and that resulted in the reduction of beta-carotenoids during post-harvest storage of golden rice cultivar which contains a high amount of the vitamin A precursor beta-carotenoid [8]. Despite these studies, not much effort has directed at the level of the proteome to understand the complex process of seed aging. This is especially true in the matured soybean seeds and the focus of our research.

Seeds are rich in seed storage proteins (SSPs) that constitute up to 70 to 80% of the total seed protein content [15]. In the case of soybean, β-conglycinin and glycinin are major SPs that together account for >70% of the total protein content [16–18]. Glycinin is a hexamer of 360 kDa and accounts for 60% of the total SSPs, while β-conglycinin is a trimer of 180 kDa that contributes 40% to the total SSPs concentration in soybean seeds. Due to the presence of these SSPs, it is very difficult to identify and/or characterize the low-abundance regulatory proteins. Therefore, previous attempts on analysis of seed physiology including the mechanism of seed aging ended up largely with the identification of highly abundant SPs.

Availability of appropriate methods for extraction of low-abundance proteins (LAPs) is now providing a platform for the identification of proteins involved in the signal perception and transduction during environmental perturbations [17]. In the present study, fully matured soybean seeds were used as source material for controlled deterioration treatment (CDT) for their physiological, biochemical, and in-depth proteomics studies. To enrich LAPs, the extracted total seed proteins were subjected to protamine sulfate precipitation (PSP) method which leads to the specific depletion of SSPs [19,20]. Fractionated protein samples thus prepared were utilized for identification of differential proteins using multiple quantitative proteomics approaches, two/one-dimensional electrophoresis (2-DE/1-DE) coupled with matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/TOF-MS) and label-free quantification.

2. Materials and methods

2.1. Plant materials

Soybean seeds (cv. Daewon) were grown in the experimental fields of National Institute of Crop Science (NICS), Rural Development Administration (RDA) at Miryang, Korea in June. The soil was supplemented with a standard RDA N-P-K fertilizer (N-P-K = 3-3-3.3 kg/10 acres). Seeds were harvested in October (average temperature 23.5 ± 3.5 °C, average day length 12 h 17 min) [21].

2.2. Controlled deterioration treatment and germination test

CDT and seed germination test were carried out as reported previously [22]. For CDT, 25 g of soybean seeds were incubated at 99% relative humidity and 42 °C for 3 (D3) and 7 (D7) days. This whole procedure was carried out inside a 25 L sealed plastic chamber where seeds were kept in the Petri plates, placed over a perforated plastic plate inside the chamber with 200 mL of water at the bottom of the plastic chamber to maintain the humidity. CDT treatment was performed with a control treatment in three independent replicates. Therefore, our experimental design included 9 seed lots (3 treatments × 3 replicates) which were subsequently used to perform proteomics and biochemical analyses.

For determining the germination rate and viability of CDT seeds, a total of 20 seeds were subsequently planted in five separate pots (4 seeds/pot) in three biological replicates (total 60 seeds/treatment) and allowed to germinate in standard soil in a growth room (16/8 h day/night, 25 °C and 70% RH).

2.3. Lipid peroxidation and hydrogen peroxide assays

Lipid peroxidation assays were carried out using OxySelect™ TBARS Assay Kit (STA-330, Cell Biolabs, INC, USA) according to the manufacturer's protocol. Briefly, 0.1 g of seed powder was homogenized in PBS (phosphate-buffered saline) containing 1× butylated hydroxytoluene (BHT) solution (final conc. 0.05%), to prevent further oxidation of lipids during sample processing and centrifuged at 15,922 ×*g* for 5 min at 4 °C to collect the supernatant. The collected supernatant was mixed with equal volume of sodium dodecyl sulfate (SDS) lysis buffer followed by addition of 1× thiobarbituric acid (TBA) reagent (conc. 5.2 mg/mL) to each sample. Samples were incubated at 95 °C for 45 to 60 min and then cooled to the room temperature in an ice bath for 5 min followed by centrifugation at 1000 ×*g* for 15 min. The absorbance of the supernatant was measured at 532 nm. The concentration of malondialdehyde (MDA) was calculated using the standard curve, prepared from known concentrations of MDA.

Quantification of H_2O_2 was carried out using the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (A2218, Thermo Fisher Scientific, USA) according to the manufacturer's protocol. For detection of the (H_2O_2) activity, 10-acetyl-3,7-dihyfroxyphenoxnazine with horseradish peroxidase (HRP) was used to detect the released H_2O_2 from CDT samples. In brief, 0.1 g of the sample was homogenized in 1× reaction buffer (50 mM sodium phosphate pH 7.4, provided in the kit) and centrifuged at 15,922 ×*g* for 5 min at 4 °C. The supernatant was mixed with equal volume of working reagent which contained 100 μM of 10-acetyl-3,7-dihyfroxyphenoxnazine and 0.2 U/mL of HRP. The mixture was incubated for 30 min at room temperature. The fluorescence of mixture was measured with a fluorescence microplate reader using excitation and emission wavelengths of 571 nm to 585 nm, respectively. The concentration of H_2O_2 in the seed samples was calculated using the standard curve, prepared from known concentrations of H_2O_2.

2.4. Protein preparation using protamine sulfate precipitation method and Western blot analysis

Protein extraction was performed as described previously [19,20]. Finely ground soybean seed powders (1 g) were homogenized in 10 mL of Tris-Mg/NP-40 buffer [0.5 M Tris-HCl (pH 8.3), 2% (v/v) NP-40, 20 mM $MgCl_2$] and centrifuged at 15,922 ×*g* for 10 min at 4 °C. The collected supernatant was incubated on ice for 30 min with 0.1% (w/v) protamine sulfate (PS) solution. The extract was again centrifuged at 15,922 ×*g* for 10 min at 4 °C in order to divide the PS-supernatant (PSS) and PS-pellet (PSP) fractions, as described earlier [18,20]. Pellet fraction was dissolved in equal volume of Tris-Mg/NP-40 buffer and proteins were precipitated from both fractions using trichloroacetic acid/acetone precipitation method. Finally, washed pellets were dissolved in 80% acetone containing 0.07% β–mercaptoethanol and stored −20 °C until further analysis. Antibody generation [18] and Western blot analysis [19] was also performed as described previously [18,19]. Primary antibodies used for Western blot included anti-rice dehydroascorbate reductase (DHAR), anti-rice ascorbate peroxidase1

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