

Research Article

PROTEOMIC ANALYSIS INDICATES ACTIVATION OF REACTIVE OXYGEN SPECIES SIGNALING DURING SEED GERMINATION AND SEEDLINGS GROWTH IN *HORDEUM VULGARE* (BARLEY)Seo Hyun Lee^{1#}, Ravi Gupta^{1#}, Yu Ji Kim¹, Cheol Woo Min¹, So Wun Kim¹, Woo Duck Seo², Sun Tae Kim^{1*}¹Department of Plant Bioscience, Life and Industry Convergence Research Institute, Pusan National University, Miryang 627-707 Republic of Korea²Division of Crop Foundation, National Institute of Crop Science, Rural Development Administration, Jeollabuk-do 565-851 Republic of Korea

Abstract: Seed germination occur as a result of cross-talk between embryo and embryo-surrounding tissues, however, the underlying mechanisms, especially during initial germination stage, are still not fully understood. Here, we utilized a gel based proteomics approach to investigate the changes in proteome during seed germination and early stages of seedlings growth. Proteins from different stages of germination and growing seedlings were isolated using Tris-Mg/NP-40 buffer and resolved using high-resolution two-dimensional gel electrophoresis. A total of 63 differentially modulated spots were identified in 2 to 4 days germinated seeds compared to that of 1 day germinated seeds. Among those proteins, 21 and 42 proteins showed increased and decreased abundances respectively. Gene ontology analysis revealed that proteins involved in methionine metabolism, amino acid biosynthesis, energy metabolism, and defense response were up regulated; while more than half of down regulated proteins were associated with the metabolic processes, and cell division. H₂O₂ concentration was more than two fold higher in 4 days germinating seeds in comparison with the 1 day germinating seeds, suggesting the involvement of ROS signaling during seed germination. Taken together, these results enriched our knowledge of the biological process in seed germination and seedlings growth in barley.

Keywords: Barley; Proteomics; Seed germination; Reactive oxygen species; Hydrogen peroxide

Note : Coloured Figures and Supplementary Information available on Journal Website in "Archives" Section

Introduction

Seed germination is one of the most crucial events in the plant's life cycle as it gives birth to the new generation (Bewley, 1997). Mature seeds are highly dehydrated with negligible to nil metabolic activities (Gupta *et al.*, 2016b). Therefore, seed germination always begins with the uptake of water by the dry seeds and end-up

with the radicle emergence (He and Yang, 2013). Successful seed germination is governed by both internal and external factors. Internal factors mainly include seed age and seed physiology while external factors include storage, and environmental conditions (Bewley, 1997). There are three phases in the process of seed germination where the first phase includes rapid water uptake, second include metabolic activation and the last phase include radicle protrusion (Bewley, 1997). Of these, the second phase is considered as the most crucial stage during which seeds regains the metabolic activities to produce energy, required for seed coat breakage and radicle emergence. In most cases, these metabolic

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activities include degradation of starch and lipids by the action of amylase and lipases (Barros *et al.*, 2010; Nonogaki, 2006).

Physiologically, seed germination is a complex process which is tightly regulated by a series of signaling and gene expression events (He and Yang, 2013). To understand the physiology of seed germination, several lines of studies have been conducted so far, which with no doubt, have increased our understanding of seed germination process (Gupta *et al.*, 2016; He and Yang, 2013; Galland *et al.*, 2012). Analysis of seed proteome of model plants like in rice, Arabidopsis and soybean has shown degradation of storage proteins and several other proteins related to the seed maturation and desiccation during germination (Fu *et al.*, 2005; Yang *et al.*, 2007; He and Yang, 2013; Han *et al.*, 2013). Although, these studies have contributed significantly in understanding the process of seed germination in model plants, information regarding the non-model crops like barley, is still missing. Therefore, here we utilized a proteomics approach in order to understand the process of seed germination and early seedlings growth in barley. Barley seeds were germinated for 1-4 days and total proteins were used for the proteome analysis using a 2DE MS-based approach. Differentially modulated proteins spots were identified and functionally validated by gene ontology (GO) program.

Material and Methods

Seed imbibition and germination

For germination, barley (*Hordeum vulgare*) seeds were washed with deionized water twice and imbibed at 4°C in dark for three days. The imbibed seeds were germinated in 65% humidity at 25°C in 16/8 h day/night cycle. The germinating seeds and seedlings were harvested in liquid nitrogen after 1, 2, 3 and 4 days after germination and stored at -80 °C until further processing.

Quantification of hydrogen peroxide

H_2O_2 concentration in the germinating barley seeds was quantified using the OxiSelect Hydrogen Peroxide/Peroxidase Assay Kit (Cell Biolabs, San Diego, CA, USA) as per the manufacturer's protocol. H_2O_2 concentrations

obtained were expressed as nmol of H_2O_2 /mg protein. This kit offers a sensitive quantitative colorimetric assay for hydrogen peroxide or peroxidase quantification. The probe in the kit reacts with H_2O_2 in presence of peroxidase in a 1:1 stoichiometry to produce a bright pink colored product which is read by a standard colorimetric microplate reader. Absorbance values are proportional to the H_2O_2 or peroxidase levels in the samples.

Protein extraction and 2-DE analysis

Total proteins were extracted using phenol precipitation method as described previously (Hurkman and Tanaka, 1986). In brief, 1 g of germinated barley seedlings was ground into a fine powder using liquid nitrogen and proteins were extracted in 5 ml protein extraction buffer containing 0.5 M Tris-HCl, pH 8.3, 2% v/v NP-40 and 20 mM MgCl₂. After centrifugation at 12000 rpm for 15 min at 4°C, an equal volume of Tris-saturated phenol (pH 7.6) was added to the supernatant and centrifuged again at 3000 × g for 15 min. The supernatant was collected and precipitated with 4 volume of methanol contain 0.1 M ammonium acetate at -20°C overnight. Precipitated proteins were centrifuged at 3000 × g for 10 min, and protein pellet was washed twice with methanol contain 0.1 M ammonium acetate and 80% acetone, respectively. The protein pellets were finally stored in 80% acetone at -20°C until analysis.

For 2D analysis, protein pellets were dissolved in rehydration buffer containing 7 M Urea, 2 M thiourea, 4% v/v CHAPS, 2 M DTT, and 0.5% v/v IPG buffer pH 4-7 (GE Healthcare, WI, USA) and protein concentration was determined using 2D-Quant kit (GE Healthcare, WI, USA)(Gupta *et al.*, 2015). An equalamount of protein samples (500 µg) were loaded on 24 cm IPG strip (immobilized pH 4-7; GE HealthCare, WI, USA). Isoelectric focusing was performed at 50 V for 4 h, 100 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 2000 V for 1 h, 4000 V for 2 h, 8000 V for 5 h, 8000 V for 9 h, and 50 V for 6 h by IPG phor II platform (GE Healthcare, WI, USA). Each equilibrated strip was then placed onto a vertical slab gel (13% SDS-PAGE). Electrophoresis was performed at 2Wper gel, 500 V, and 300 mA for

30 min, followed by 16W per gel, 700 V, and 300 mA. Gels were stained with colloidal CBB G-250 staining solution. The 2D gel images were scanned with a transmissive scanner (Power Look 1120) with a 32-bit pixel depth and 300 dpi resolution. Protein spots on 2D gels were detected and processed during Image Master 2D Platinum software 6.0 (GE Healthcare, WI, USA). The intensity of each spot was determined from three biological replicates. The significantly changed ($p < 0.05$) spots were selected for further identification.

In-gel digestion and Mass spectrometer analysis

Trypsin digestion and MALDI-TOF/TOF-MS (Applied Biosystems, Framingham, MA, USA) analysis of 2D protein spots were performed as described previously (Kim *et al.*, 2004; Gupta *et al.*, 2016a). Data were subjected to a Mass Standard Kit (Applied Biosystems) for the 4700 Proteomics Analyzer (calibration mixture 1). Acquired MS/MS spectra were searched against the NCBI database (version 20111208; 9760158 sequences) by Protein Pilot v.3.0 (with MASCOT as the database search engine). Peptide mass tolerance and fragment mass identifications had statistically significant search scores (greater than 95% confidence, equivalent to MASCOT expect value ($p < 0.05$)), which were consistent with the experimental *pI* and MW of protein, and accounted for the majority of ions present in the mass spectra.

Statistical analysis and functional annotation of the identified proteins

Identified proteins were functionally annotated using Gene Ontology (GO) database (<http://www.geneontology.org>). For hierarchical clustering, Multi-experiment viewer (MeV) program was used and the clustering was carried out using log₂-transformed intensity values of the differentially expressed proteins.

Results and Discussion

Hydrogen peroxide accumulation accompanied with seeds germination

Seed germination takes place when quiescent dry seeds are released from the dormancy. This initial

stage of germination is believed, to begin with, imbibition and ends with the water uptake (Bewley, 1997). During the germination process, dynamic changes of proteins and metabolites have been observed in plants (Gallardo *et al.*, 2001). Here, we employed proteomics tools to investigate the protein profile changes to understand the molecular mechanism of the seed germination and seedlings growth processes. Barley seeds were imbibed in distilled water in a dark growth chamber to break its dormancy. Seed samples were harvested at 1, 2, 3 and 4 days post germination (dpG) to study the protein changes in the early germination process. Fig. 1A shows the growth and morphology of the germinated barley seedlings. Upon imbibition, metabolic activity occurs in the embryo and aleuronic layer which helps the seeds to release from the dormancy. The previous study showed that the application of methyl viologen (as a generator of ROS including OH⁻) helps the seeds to overcome the dormancy (Whitaker *et al.*, 2010). These ROS radicals are generated from the protein glycation or non-enzymatic lipid auto-oxidation that occurs in the embryo and aleuronic layer upon imbibition. Here, we observed an accumulation of hydrogen peroxide during early seed germination and seedlings growth. As showed in Fig. 1B, accompanied with seedling growth, the hydrogen peroxide level at four days germinated seeds is dramatically increased up to 2.5 times comparing to that of one day germinated seeds. This result revealed the importance of hydrogen peroxide in breaking seed dormancy and early stages of seedling growth. Hydrogen peroxide also acts as signaling molecules and its accumulation indicates functioning of hydrogen peroxide signaling during initial stages of seedling growth.

Identification of Differentially Expressed Proteins by 2DE and MS

To analyze the protein expression profiles of growing seedlings, a 2-DE MS based approached was used. Total proteins were isolated from 1-4 days old seedlings and were resolved on high-resolution 2DE gels (Fig. 2). A total 94 significantly modulated proteins (fold change ≥ 2 , $p < 0.05$), which were reproducibly detected on three independent replicates, were identified by the

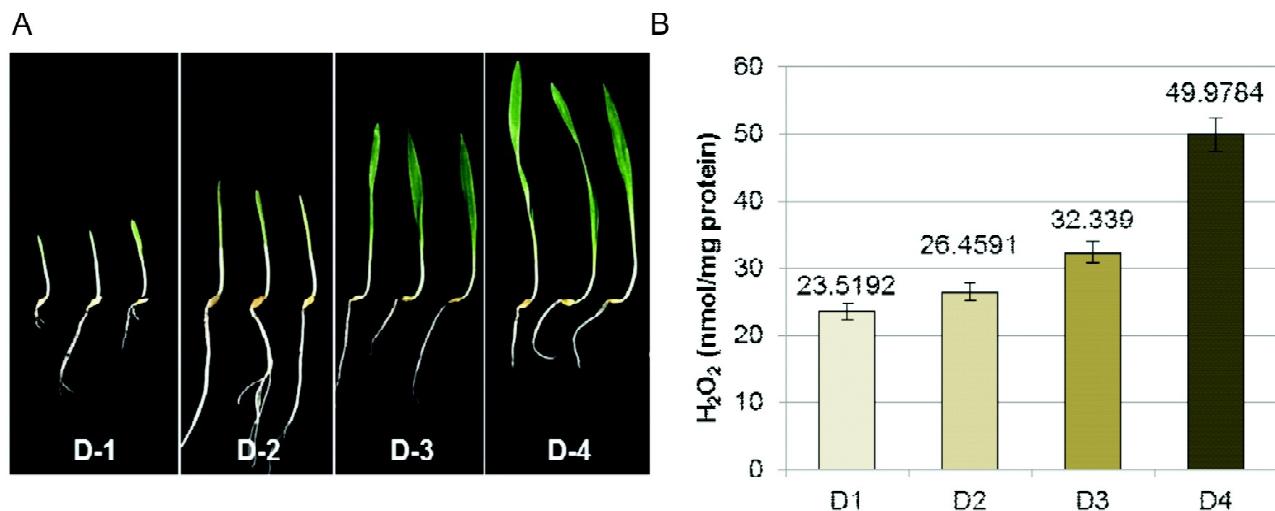


Figure 1: Hydrogen peroxide accumulation accompanied with seed germination processes. A, Phenotype of germinating seeds and seedlings.B, Hydrogen peroxide quantification in the germinating seeds at different stages. The average level of H_2O_2 is labeled on top of each bar.

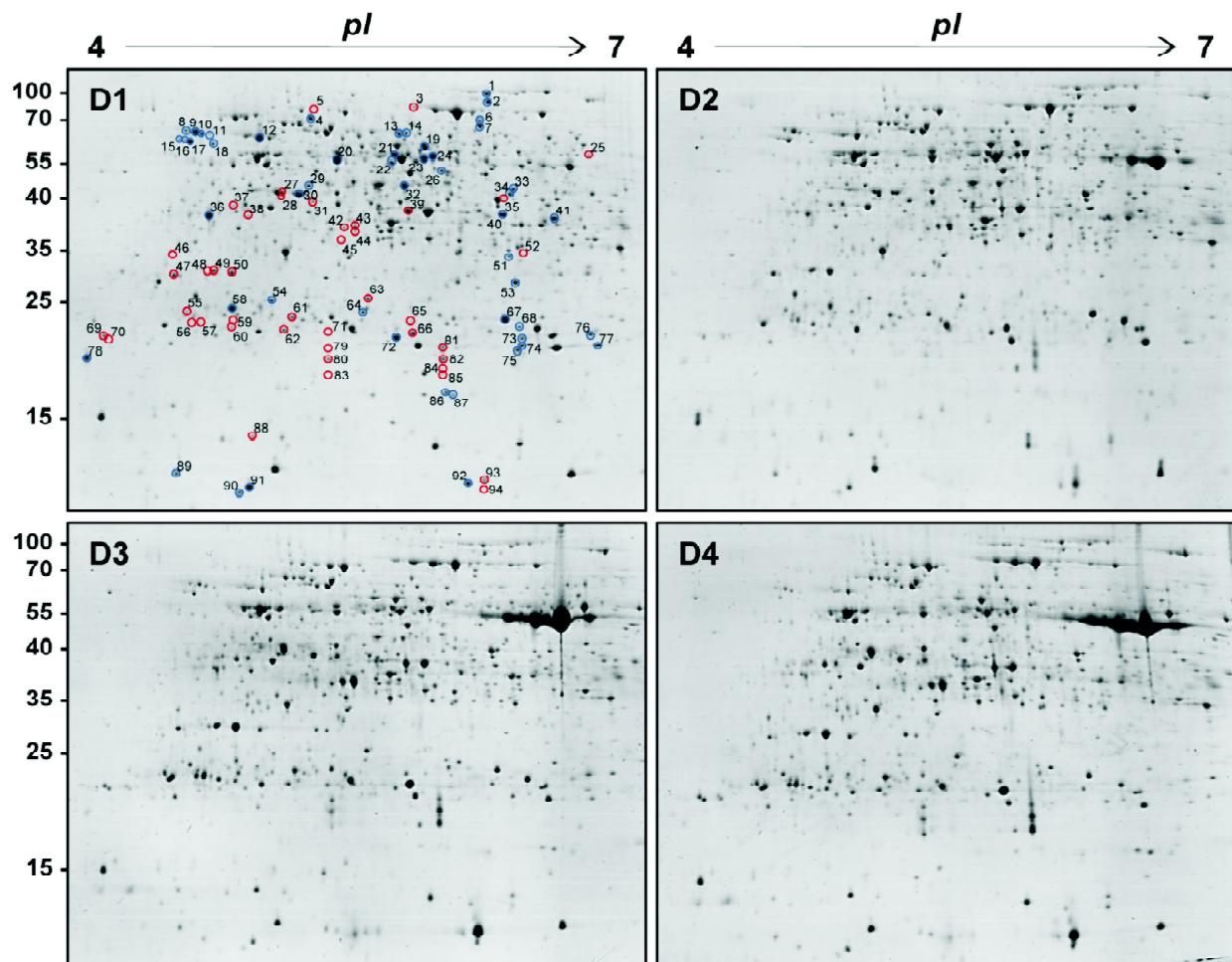


Figure 2: Differential expression of protein spots during seed germination stages. Same amount of total seed proteins (500 μg) were resolved on 2-DE gels and stained with colloidal CBB. Differentially expressed protein spots are encircled with red and blue colors.

ImageMaster2DPlatinum software (Fig. 2 and Fig. S1). Out of these differentially modulated proteins, 63 were successfully identified by MS with high confidence. These 63 proteins included 21 and 42 proteins with increased and decreased abundances respectively (summarized in Supplementary Table S1). To clearly observe the changes in protein spots, a close-up view of identified spots were depicted from the three biological replicates of each sample (Fig. 3). Among the 21 protein spots showing increased abundance, three (spots 52, 55 and 94) were induced 2 dpg, and two (spots 48 and 83) were observed 3dpg (Fig. 3A). Of proteins showing decreased abundance, three (spots 78, 86 and 87) were undetectable at 2 dpg and three (spots 64,

74 and 75) were degraded after 3 dpg (Fig. 3A).

Hierarchical clustering and Gene Ontology analysis of the identified proteins

In order to visualize the protein expression profiles and to group the proteins with similar abundance patterns, hierarchical clustering was performed using log₂ intensity values of differentially expressed spots. Hierarchical clustering clearly separated all the proteins into two major groups with proteins showing increased and decreased abundance as compared to the 1dpg (Fig. 4A). GO-term enrichment analysis was carried out to functional annotate the differentially modulated proteins based on its biological functional category (Fig. 4B and 4C).

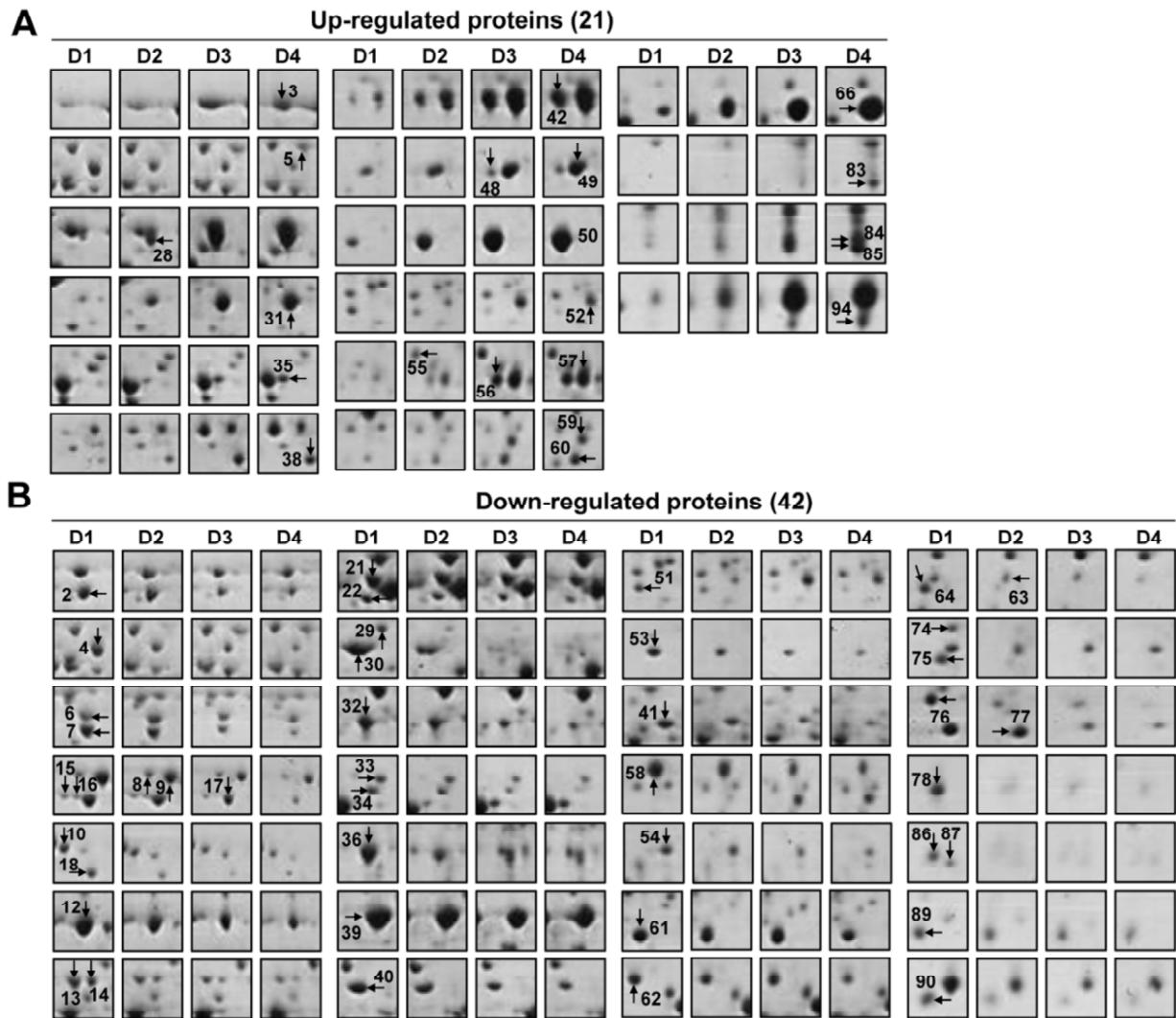


Figure 3: Close-up view of 2-DE gel maps to show differentially regulated protein spots. The up-regulated (A) and down-regulated proteins (B) were grouped. Numbers of protein spots are corresponding to Figure 2.

Proteins with increased abundance were mostly related with biosynthetic process (21.4%), photosynthesis (14.3%), defense response (14.3%), glycolysis (14.3%), protein polymerization (14.3%), proteolysis (7.1%), metabolic process (7.1%), and pentose-phosphate shunt non-oxidative branch (7.1%) (Fig. 4B) which proteins showing decreased abundance were mostly related to the metabolic process (53.8%), cell (19.2%), biosynthetic process (7.7%), transcription (7.7%), translation (7.7%), and defense response (3.8%) (Fig. 4C).

Functional groups associated with the seed germination and seedlings growth

Translation and transcription regulation during seed germination

In rice, seed germination and seedlings growth are accompanied by various physiological and molecular changes (He and Yang, 2013). The translation, but not transcription, was required during the germinating process (Sano *et al.*, 2012). Here, we found that proteins related to the transcription and translation showed decreased abundance during early germination (Fig. 4C), suggesting in barley the onset mRNA regulation may not occur during the early germination procedure.

Metabolic regulation during seed germination

Previously, it was reported that the reprogramming of the metabolism is one of the key events during the seed germination in rice, barley, tomato, soybean and other plants (Wang *et al.*, 2015; Han *et al.*, 2013; Kim *et al.*, 2009; Wang

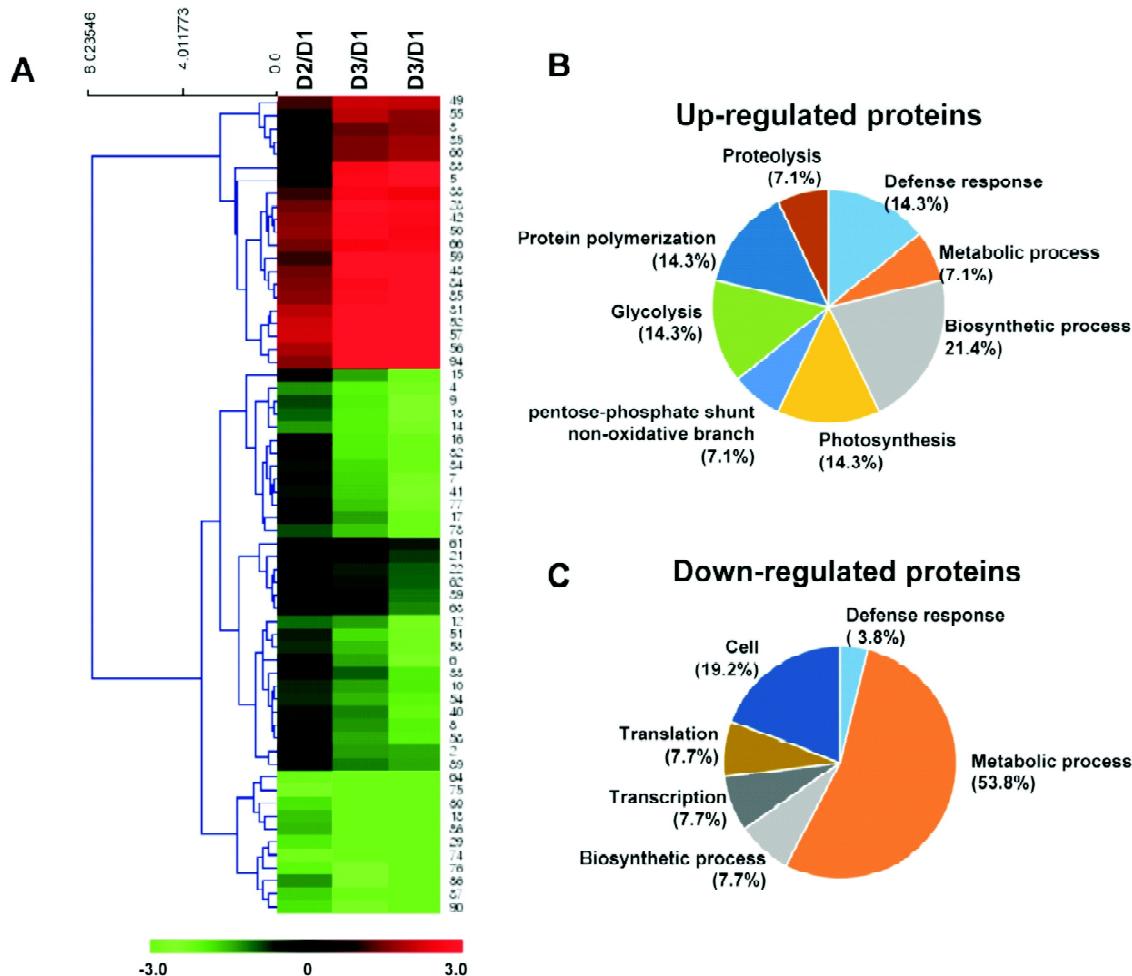


Figure 4: Hierarchical clustering and GO enrichment analysis of differentially modulated proteins. A, Hierarchical clustering of differentially expressed protein spots were generated based on the log₂ expression ratio comparing with expression level at D1. B and C, GO enrichment of proteins up-regulated (B) or down-regulated (C) during seed germination process.

et al., 2008; Sheoran et al., 2005; Yang et al., 2007). In this work, it was shown that the metabolism related proteins were highly enriched, suggesting key changes in the metabolic during seed germination and early stages of barley growth. Moreover, decreased proteins have a much bigger number than that of increased proteins, indicating reconstruction of metabolite mechanisms were initiated during germination process. It was also observed that several proteins involved in photosynthesis and energy metabolism process have been significantly induced after germination, including photosystem II stability/assembly factor HCF13 (spot 38), ribose5P_isomerase (spot 55), chlorophyll a-b binding protein (spot 57), and finger and CHY zinc finger domain-containing protein 1 (spot 50). The previous study of RING finger proteins revealed that over expression of RING finger proteins in *Arabidopsis* improve seeds germination rate under salt and PEG treatment, suggesting its pivotal roles in the germination process (Lim et al., 2013).

GO enrichment analysis showed that proteins related to the glycolysis and pentose-phosphate pathway were specifically accumulated during seed germination. For instance, the glycolysis-related proteins, fructose-bisphosphate aldolase (spot 42) and phosphoglycerate kinase (spot 29), were the most highly expressed proteins during seed germination and seedling growth. It was known that the glycolysis cycle plays an important role in providing energy for seed germination, which generated from endosaccharides. Previous work also exhibited that the most prominently expressed up-regulated proteins during rice germination process belonging to glycolysis cycle (Kim et al., 2009).

Proteins involved in proteolysis and lipid breakdown

Among proteins showing increased abundance, a group of proteins was involved in proteolysis, protein polymerization, and photosynthesis. It was known that seed storage proteins, such as soybean gycinin and α -conglycinin, are degraded by proteases during seed germination (Kim et al., 2011). The degraded storage proteins are believed

as the main source of amino acids, which support the new protein synthesis during germination. Moreover, these free amino acids also undergo further lysis to produce energy via glycolytic and TCA cycles, which is required to sustain the initial growth of seedlings. In this study, we identified plenty of proteins which were degraded during barley seed germination seedlings growth, suggesting an active proteolysis which is involved in this degradation process. Similarly, a lipase (spot 22) was also identified showing decreased abundance at 2, 3 and 4 dpg as compared to 1 dpg, suggesting that its requirement during early stages of seed germination. This result is consistent with the results obtained during rice germination process, which occurs in phase II and III (He and Yang, 2013), suggesting the similar protein degradation is essential in barley germinating regulation.

Oxidative stress related proteins

Here, we identified 5 isoforms of thioredoxin (spots 8, 9, 10, 40 and 89), 2 isoforms of 1-Cys peroxiredoxin (spots 74 and 76) and an ascorbate peroxidase. These proteins are involved in the detoxification of reactive oxygen species (ROS), which are formed as a byproduct of metabolism. Surprisingly, all of these proteins showed decreased abundance from seed germination to seedlings growth. Interestingly, during this period, metabolism is increased which contribute to the higher production of the ROS. These results suggested an accumulation of ROS during seedlings growth as a result of higher production of ROS and down-regulation of ROS detoxifying enzymes. These results further supported the H_2O_2 quantification results where a higher content of the H_2O_2 was found during seedlings growth. An increased level of ROS was earlier reported in the rice embryo during germination, which is supposed to cause protein oxidation, especially the carbonylation (Zhang et al., 2016).

Conclusions

Seed germination and seedlings growth are the two most important aspects of plant development. In this study, proteome changes during germination and growth of the barley seedlings were evaluated using a 2DE approach.

Our results revealed that metabolism related proteins are the most abundant category that changes during seed germination and seedlings growth. Meanwhile, proteins involved in photosynthesis and energy metabolism process were also highly induced, so as proteins belonging to glycolysis and pentose-phosphate pathway. These results together indicate that during early seed germination process, energy supply is very important. Furthermore, our results show the accumulation of ROS that indicate the involvement of ROS signaling during early growth of barley seedlings. Taken together, our results provide new evidence to understand plant development process, and may be helpful in the generation of next generation transgenic plants.

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Abbreviations

ROS-reactive oxygen species; 2DE-two-dimensional gel electrophoresis; GO-gene ontology; dpg-days post germination

Conflict of Interest

The authors do not have any conflict of interest with the contents of this manuscript.

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