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Proteomic analysis of soybean seedling leaf under waterlogging stress in a time-dependent manner

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ABSTRACT

Leaf is sensitive to environmental changes and exhibits specific responses to abiotic stress. To identify the response mechanism in soybean leaf under waterlogging stress, a gel-free/label-free proteomic technique combined with polyethylene glycol fractionation was used. Attenuated photosynthesis by waterlogging stress in the leaf of soybean seedlings was indicated from proteomic results. Defensive mechanisms such as reactive oxygen species (ROS) scavenging was also recognized. Cluster analysis revealed that proteins that exhibit characteristic dynamics in response to waterlogging were mainly related to photosynthesis. Among the identified photorespiration-related proteins, the protein abundance and enzyme activity of hydroxypyruvate reductase were transiently increased in control plants, but were clearly decreased in response to waterlogging stress. These results suggest that waterlogging directly impairs photosynthesis and photorespiration. Furthermore, hydroxypyruvate reductase may be a critical enzyme controlling the rate of photorespiration.

1. Introduction

Abiotic stresses negatively affect crop growth and productivity and constitute a major threat to the global food supply [1]. In particular, soil waterlogging influences the composition, metabolic activity, and productivity of most crop species [2]. For example, turbid flooding leads to anaerobic conditions [3], which activates glycolysis and fermentation pathways in plants [4]. The adverse effects of hypoxia and anoxia are well-documented on the vegetative growth of various crops, including wheat [5], rice [6], soybean [7], pea [8], lupine [9], barley [10], and chickpea [11]. In these species, the growth of shoots and roots was decreased, and nutrient uptake was inhibited in response to waterlogging stress [12], indicating that a reduced oxygen level severely affects plant growth and response activity.

Soybean is sensitive to various abiotic stresses, particularly waterlogging stress during the germination, early vegetative, and early reproductive stages [13,14]. Flooding decreases rates of photosynthesis and leaf expansion [15], leads to reduced gas exchange [16], and ultimately results in lower growth rates and reduced grain yields. The leaf yellowing and a reduction in leaf number were observed under waterlogging stress [17]. At the molecular level, the stress affects the levels of proteins in young seedlings involved in fermentation [18], ROS scavenging [19], glycolysis, and stress responses [20]. Several proteomic studies have examined the changes in the root system of soybean under waterlogging stress, revealing multiple pathways involved in stress perception and signal transduction [21].

Although the response was well-described in completely immersed organs such as hypocotyl and root in young seedlings [17–20], characterization of the systemic response mechanisms of soybean to waterlogging stress is still in its early stage. The leaf is an important plant organ due to its role in photosynthesis [22]. The biochemical changes in leaf cells results in the altered regulation of photosynthesis [23]. In regard to the effects of waterlogging on leaf of crop plants, the abundance of proteins related to energy metabolism [24–26], photosynthesis [24,26], and primary/secondary metabolism [25] was affected in maize, wheat, and tomato. Therefore, focusing on the leaf may allow the identification and characterization of the systemic stress tolerance mechanisms that are activated in response to flooding. In this study, a gel-free/label-free proteomic technique was used in combination with cluster analysis in order to identify the specific proteins that are changed in leaf to elucidate physiological circuitry of plants use against environmental stresses [21]. Pathways affected by the stress were analyzed by the function classification of the significantly changed leaf proteins and confirmed by enzyme activity analysis. It was suggested

Abbreviations: ROS, reactive oxygen species; LC, liquid chromatography; MS, mass spectrometry; PEG, polyethylene glycol

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that energy metabolism including photosynthesis and photorespiration along with ROS scavenging are regulated in leaf under waterlogging stress.

2. Materials and methods

2.1. Plant material and treatments

Soybean seeds (*Glycine* max L. cultivar Enrei) were sterilized in 3% sodium hypochlorite solution, sowed and kept on sand under white fluorescent light (600 μmol m^{-2} s, 12-h light period) in a growth chamber maintained at 25 °C. For morphological, proteomic, and enzymatic analyses, 6-day-old soybeans were submerged in water for 0, 2, 4, 6, and 8 days. Morphological parameters, including the fresh weight of roots and leaf, were measured. For all treatments for analyses, three independent experiments with plants sown on different days were performed as biological replicates (Fig. S1).

2.2. Protein extraction

A portion (500 mg) of collected root and leaf samples was ground in liquid nitrogen with a mortar and pestle. The obtained powder was transferred to a solution containing 10% trichloroacetic acid and 0.07%2-mercaptoethanol [27]. The resulting mixture was vortexed, sonicated for 10 min, and then incubated for 1 h at − 20 °C with vortexing every 15 min. The suspension was centrifuged at 9000 × *g* for 20 min at 4 °C, and the obtained pellet was washed twice with 0.07% 2-mercaptoethanol in acetone before being dried using a Speed-Vac concentrator (Savant Instruments, Hickville, NY, USA). The sample was resuspended in lysis buffer consisting of 7 M urea, 2 M thiourea, 5% CHAPS, and 2 mM tributylphosphine by vortexing for 1 h at 25 °C, and the resulting suspension was further centrifuged at 20,000 × *g* for 20 min at 25 °C. The supernatant was collected as crude extract. Protein concentrations were determined using the Bradford assay [28] with bovine serum albumin as the standard.

2.3. Polyethylene glycol fractionation

PEG fractionation of proteins was performed according to the method of Zhu et al. [29]. Briefly, a portion (200 mg) of the sample was homogenized in 5 mL extraction buffer consisting of 0.5 M Tri-HCl (pH 7.8), 2% Triton X-100, 20 mM $MgCl_2$, 2% 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA. The resulting slurry was sonicated for 5 min before being centrifuged at 12,000 × *g* for 15 min at 4 °C. The supernatant was collected and mixed with a 50% PEG (MW 4000) stock solution to give a final concentration of 8% PEG. The PEG-suspended solution was placed on ice for 30 min and was then centrifuged at 1500 × *g* for 10 min at 4 °C. The supernatant was collected and mixed with the 50% PEG stock solution to give a final concentration of 16% PEG. The resulting solution was placed on ice for 30 min and was then further centrifuged at 12,000 × *g* for 15 min at 4 °C. The supernatant was precipitated with four volumes of cold trichloroacetic acid/acetone at − 20 °C for 1 h. After centrifugation of the solution at 12,000 × *g* for 15 min at 4 °C, the resulting pellet was collected and mixed with lysis buffer, further centrifuged at 20,000 × *g* for 20 min at 25 °C, and the supernatant was collected as fractionated protein.

2.4. Protein enrichment and digestion for mass spectrometry analysis

Proteins (100 μg) were enriched with methanol and chloroform to remove any detergent from the sample solutions [30]. Briefly, 400 μL methanol was added and mixed with each sample before the further addition of 100 μL chloroform and 300 μL water. After mixing, the samples were centrifuged at 20,000 × *g* for 10 min to achieve phase separation. The upper aqueous phase was discarded and 300 μL methanol was slowly added to the lower phase. The samples were further centrifuged at 20,000 × *g* for 10 min, and the obtained pellets were dried, resuspended in 50 mM NH_4HCO_3, reduced with 50 mM dithiothreitol for 30 min at 56 °C, and then alkylated with 50 mM iodoacetamide for 30 min at 37 °C in the dark. Alkylated proteins were digested with trypsin and lysine endopeptidase (Wako, Osaka, Japan) at 1:100 enzyme/protein concentrations at 37 °C for 16 h. The resulting tryptic peptides were acidified with formic acid (pH < 3) and centrifuged at 20,000 × *g* for 10 min. The resulting supernatant was collected and analyzed by nanoliquid chromatography (LC)-mass spectrometry (MS)/MS.

2.5. Mass spectrometry analysis

Trypsinized peptides in 0.1% formic acid were loaded onto an Ultimate 3000 nanoLC system (Dionex, Germering, Germany) equipped with a C18 PepMap trap column (300 μm ID × 5 mm; Dionex) and were then separated by elution from the trap column using 0.1% formic acid in acetonitrile at a flow rate of 200 nL/min on a C18 Tip column (75 μm 1D × 120 mm; Nikkyo Technos, Tokyo, Japan) with a spray voltage of 1.8 kV. Peptide ions were analyzed on a nanospray LTQ Orbitrap MS (Thermo Fisher Scientific, San Jose, CA, USA) operated in data-dependent acquisition mode with Xcalibur software (version 2.1; Thermo Fisher Scientific). Full-scan mass spectra were acquired over 400–1500 *m/z* with a resolution of 30,000. A lock mass function was used to obtain high mass accuracy [31]. The ions $C_{24}H_{39}O_4^+$ (*m/z* 391.28429), $C_{14}H_{46}NO_7Si_7^+$ (*m/z* 536.16536), and $C_{16}H_{52}NO_8Si_8^+$ (*m/z* 610.18416) were used as lock mass standards. Values for the ion isolation window were set as follows: activation type was collision-induced dissociation, default charge state was 2, isolation width was 2.0 *m/z*, normalized collision energy was 35%, and activation time was 30,000. The values used for dynamic exclusion were as follows: repeat count was 2, repeat duration was 30 s, exclusion list size was 500, exclusion duration was 90 s, and exclusion mass width was ± 1.8 Da. The ten most intense precursor ions above the threshold value of 500 were selected for collision-induced fragmentation. The acquired MS spectra were used for protein identification.

2.6. Protein identification from mass spectrometry data

Identification of proteins was performed using the Mascot search engine (version 2.5.1; Matrix Science, London, UK) and Proteome Discoverer software (version 1.4.0.288; Thermo Fisher Scientific) against a soybean peptide database (54,175 sequences; Phytozome, version 9.1; http://www.phytozome.net/soybean) [32]. The parameters used in the Mascot searches were as follows: cysteine carbamidomethylation/methionine oxidation was set as a fixed modification/variable modification, trypsin was specified as the proteolytic enzyme, 1 missed cleavage was allowed, peptide mass tolerance was 10 ppm, fragment mass tolerance was 0.8 Da, and peptide charges were + 2, + 3, and + 4. An automatic decoy database search was performed as part of the search. Mascot results were filtered with the Mascot percolator to improve the accuracy and sensitivity of peptide identification [33]. False discovery rates for peptide identification of all searches were < 1.0%. Peptides with a percolator ion score of > 13 ($p < 0.05$) were used for protein identification.

2.7. Quantification of differentially abundant proteins using mass spectrometry data

The Mascot search results were exported in msf format for SIEVE analysis (version 2.2.49; Thermo Fisher Scientific) to compare the relative abundances of peptides and proteins between samples. For the analysis, the chromatographic peaks detected by MS were aligned and the peptide peaks were detected as a frame using a