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Gel-free/label-free proteomic analysis of root tip of soybean over time under flooding and drought stresses

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ABSTRACT

Growth in the early stage of soybean is markedly inhibited under flooding and drought stresses. To explore the responsive mechanisms of soybean, temporal protein profiles of root tip under flooding and drought stresses were analyzed using gel-free/label-free proteomic technique. Root tip was analyzed because it was the most sensitive organ against flooding, and it was beneficial to root penetration under drought. UDP glucose: glycoprotein glucosyltransferase was decreased and increased in soybean root under flooding and drought, respectively. Temporal protein profiles indicated that fermentation and protein synthesis/degradation were essential in root tip under flooding and drought, respectively. *In silico* protein–protein interaction analysis revealed that the inductive and suppressive interactions between *S*-adenosylmethionine synthetase family protein and B-S glucosidase 44 under flooding and drought, respectively, which are related to carbohydrate metabolism. Furthermore, biotin/lipoyl attachment domain containing protein and Class II aminoacyl tRNA/biotin synthetases superfamily protein were repressed in the root tip during time-course stresses. These results suggest that biotin and biotinylation might be involved in energy management to cope with flooding and drought in early stage of soybean-root tip.

1. Introduction

Climate changes affect the magnitude and frequency of hydrological fluctuations, resulting in devastating events such as flooding and drought [1]. Crop growth and productivity are adversely affected by flooding [2] and drought [3]. For example, in rape seed, the low oxygen concentrations reduce energy metabolism during maturation, leading to affecting lipid synthesis and starch synthesis [4]. In tomato leaf, ion leakage, lipid peroxidation, and *in vivo* hydrogen peroxide are increased under waterlogging stress, while chlorophyll content is decreased [5]. In wheat, severe drought stress reduces shoot/root biomass and the rates of photosynthesis and root respiration [6]. It was also reported that several physiological parameters are decreased and hydrogen peroxide production is induced in peanut under drought stress [7]. Because these observations clearly indicate that flooding and drought negatively influence crop growth, clarification of plant-response mechanisms to these stresses may be helpful to improve soybean tolerance.

Soybean, which is an important legume crop and a major source of protein and oil for human and animal [8], is particularly sensitive to flooding [9] and drought [10]. Under flooding conditions, soybean displays inhibited nutrient uptake [11], reduced nitrogen fixation [12], and suppressed growth [13], whereas severe drought leads to reducing photosynthesis [14], leaf water potential [15], and total seed yield [16]. Although the growth rate of soybean was inhibited under both flooding and drought stresses, there were much difference of morphological responses between these two stresses, especially the physiological differences observed in the root compared to the hypocotyl and leaf [17]. Moreover, flooding markedly suppressed the growth of root tip [18], whereas drought reduced the root diameter [19].

The responsive mechanisms underlying flooding and drought have been characterized in some crops like rice [20], wheat [21,22], and soybean [23,24]. In rice, the ethylene production and energy metabolism were modulated to adapt to flooding and drought stresses [20]. In wheat, proteins related to glycolysis and cell wall structure were decreased; while defense related proteins were increased under flooding [21]; and the metabolism related to gliadins synthesis was enhanced under drought [22]. Recently, the global responsive mechanisms of soybean under flooding and drought were summarized, suggesting that energy consumption and plant defenses were essential metabolic regulations to against flooding; while osmotic adjustment and programmed cell death played roles in drought [25].

Abbreviations: LC, liquid chromatography; MS, mass spectrometry; qRT-PCR, quantitative reverse transcription polymerase chain reaction; ADH, alcohol dehydrogenase; PDC, pyruvate dehydrogenase; UGGT, UDPglucose: glycoprotein glucosyltransferase; PCA, principal component analysis.

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Under flooding, proteins related to glycolysis and fermentation were mainly changed indicating an alternative pathway of energy generation [23]. Under drought, the content of proline was increased but then decreased during drought recovery confirming that the osmotic adjustment was the major mechanism responsive to drought [26]. Besides, the quenching capacity of antioxidant of soybean was affected under flooding and drought. Kausar et al. [17] reported that the biophoton emissions were increased and decreased under flooding and drought resulting from different levels of reactive oxygen species in soybean cell, which was consistent with that differentially redox signaling was involved under different stresses [19]. Taken together, these results indicated that complex responsive mechanisms were involved in flooding and drought, including the specific mechanisms under different stresses and the common metabolisms with differentially control levels. Hence, there is a need to carry out the systemic experiments to obtain the responsive mechanisms in details in soybean.

The temporal profiles of flooding-responsive proteins were analyzed by cluster analysis in soybean during the initial stages of flooding stress and most change proteins were involved in calcium-related signal transduction [27]. Cluster analysis was also used to examine the expression profiles of nuclear proteins in chickpea and it revealed that regulatory and functional proteins were activated under dehydration conditions [28]. These studies [27,28] suggested that determination of the temporal protein profiles combined with cluster analysis is a useful approach to identify the mechanisms in response to flooding and drought stresses in soybean. In the present study, the sensitive organ under flooding and drought was investigated and the common proteins identified under both stresses were illustrated to improve soybean tolerance through the multifunctional protein. Proteins were analyzed using nanoliquid chromatography (LC) mass spectrometry (MS)/MS, and quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed to confirm the mRNA expression level of target genes. Based on the time-dependent changes of protein abundance, analyses of cluster and *in silico* protein-protein interactions were additionally performed.

2. Materials and methods

2.1. Experimental design and statistical analysis

Soybean seeds (*Glycine max* L. cultivar Enrei) were sterilized, grown, and treated as the same methods described by Oh and Komatsu [19]. Organ-specific experiment was performed and followed by mRNA expression analysis; and for time-dependent experiment, the obtained proteomic data were analyzed by cluster analysis and *in silico* protein–protein interaction analysis (Supplemental Fig. 1). For sampling, the 5 mm length of root tip was collected, and remaining part was used as root. For cotyledon, the 5 mm length from the front part was collected (Supplemental Fig. 1A). For time-dependent experiments, the root tip of 2-, 3-, and 4-day-old soybeans treated without or with flooding and drought for 1 and 2 days were collected as samples (Supplemental Fig. 1B). Three independent experiments were performed as biological replicates.

Statistical significance of the results was estimated with Student's *t*-test when only two groups were compared and calculations were performed by GraphPad Prism (version 6.0; GraphPad software, La Jolla, CA, USA). Statistical significance was valued with one-way ANOVA followed by Duncan's multiple comparison when multiple groups were compared and calculations were performed by SPSS software (version 22.0; IBM, Armonk, NY, USA). $p < 0.05$ was considered as statistically significant.

2.2. Protein extraction for proteomic analysis

A portion (500 mg) of collected samples was ground to powder in liquid nitrogen using a mortar and pestle. The powder was added to a solution of 10% trichloroacetic acid and 0.07% 2-mercaptoethanol in acetone, and the resulting suspension was mixed thoroughly by vortexing. The mixture was sonicated for 10 min and incubated for 1 h at −20 °C. After centrifugation at 9,000×*g* for 20 min at 4 °C, the supernatant was removed and the remaining pellet was washed twice with 0.07% 2-mercaptoethonal in acetone. The pellet was dried using a Speed-Vac concentrator (Savant Instruments, Hicksville, NY, USA) and resuspended in lysis buffer containing 8 M urea, 2 M thiourea, 5% CHAPS, and 2 mM tributylphosphine by vigorous vortexing for 1 h at 25 °C. The suspension was centrifuged at 20,000×*g* for 20 min at 25 °C, and the resulting supernatant was collected as protein extract. Protein concentration was determined using the Bradford method [29] with bovine serum albumin as the standard.

2.3. Protein identification and quantitation through mass spectrometry

Extracted proteins (100 μg) in lysis buffer were purified, digested, acidified, and desalted as described by Nanjo et al. [30]. The purified peptides (2 μL) were separated using an Ultimate 3000 nanoLC system (Dionex, Germering, Germany), and the peptide ions were detected using a nanospray LTQ Orbitrap Discovery MS (Thermo Fisher Scientific, San Jose, CA, USA) in data-dependent acquisition mode with the installed Xcalibur software (version 2.1; Thermo Fisher Scientific). Data acquisition through mass spectrometry analysis was performed as described by Komatsu et al. [31], and a lock mass function was used to obtain high mass accuracy [32]. The top ten most intense precursor ions were selected for collision-induced fragmentation in the linear ion trap at a normalized collision energy of 35%. Dynamic exclusion was employed within 90 sec to prevent the repetitive selection of peptides [33].

Identification of proteins was performed using Mascot search engine (version 2.5.1; Matrix Science, London, UK) with a soybean peptide database (55,787 sequences) constructed from the soybean genome database (Phytozome version 9.1, http://www/phytozome.net/soybean) [34]. Proteome Discoverer software (version 1.4.0.288; Thermo Fisher Scientific) was used to process the acquired raw data files. The parameters of Mascot searches were the same as described by Komatsu et al. [31] except the peptide mass tolerance was set at 10 ppm and fragment mass tolerance was set at 0.8 Da. Mascot results were filtered with the Percolator function to improve the accuracy and sensitivity of peptide identification. For all searches, false discovery rates for peptides identification were less than 0.01. Peptides with more than 13 ($p < 0.05$) percolator ion score were used for protein identification. The acquired Mascot results were exported into SIEVE software (version 2.1.377; Thermo Fisher Scientific) for quantitation analysis, which was carried out as described by Komatsu et al. [31]. To estimate the variability of the observed proteomic data, the principle component analysis (PCA) was performed using SIEVE software. Significantly changed proteins were selected based on more than 2 matched peptides and a *p*-value of less than 0.05. The functional analysis of identified proteins was performed using MapMan bin codes (http://mapman.gabipd.org/) [35].

2.4. RNA extraction and quantitative reverse transcription polymerase chain reaction

A portion (100 mg) of collected samples was ground to powder in liquid nitrogen using a sterilized mortar and pestle, and total RNA was then extracted using an RNeasy Plant Mini kit (Qiagen, Valencia, CA, USA). Extracted RNA was reverse transcribed to cDNA using iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. qRT-PCR was performed using SsoAdvanced SYBR Green Supermix (Bio-Rad) on a MyiQ Single-Color Real-Time PCR Detection system (Bio-Rad). The PCR conditions were as follows: 95 °C for 30 sec, followed by 45 cycles of 95 °C for 10 sec and 60 °C for 30 sec. Gene expression was normalized using the 18S rRNA gene (X02623.1) as an internal control. The qRT-PCR primers