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Proteomic analysis of soybean root including hypocotyl during recovery from drought stress

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ABSTRACT

Soybean is a nutritionally important crop that exhibits reductions in growth and yield under drought stress. To investigate soybean responses during post-drought recovery, a gel-free proteomic technique was used. Two-day-old soybeans were treated with drought stress for 4 days and recovered for 4 days. Root including hypocotyl was collected during the drought treatment and recovery stage. Seedling growth was suppressed by drought stress, but recovered following stress removal. The malondialdehyde content increased under drought stress, but decreased during the recovery stage. A total of 792 and 888 proteins were identified from the control and recovering seedlings, respectively. The identified proteins were related to functional categories of stress, hormone metabolism, cell wall, secondary metabolism, and fermentation. Cluster analysis indicated that abundances of peroxidase and aldehyde dehydrogenase were highly changed in the seedlings during the post-drought recovery. The activity of peroxidase decreased under drought conditions, but increased during recovery. In contrast, the activity of aldehyde dehydrogenase was increased in response to drought stress, but decreased during the recovery stage. These results suggest that peroxidase and aldehyde dehydrogenase play key roles in post-drought recovery in soybean by scavenging toxic reactive oxygen species and reducing the load of harmful aldehydes.

Biological significance: Post-drought recovery response mechanisms in soybean root including hypocotyl were analyzed using gel-free proteomic technique. A total of 643 common proteins between control and drought-stressed soybeans changed significantly in abundance over time. The proteins that changed during post-drought recovery were assigned to protein, stress, hormone metabolism, secondary metabolism, cell wall, redox, and glycolysis categories. The analysis revealed that peroxidase and aldehyde dehydrogenase were increased in protein abundance under drought stress. The enzyme activity of peroxidase decreased under drought but increased during recovery. The activity of aldehyde dehydrogenase was increased under drought stress but decreased during recovery stage. Peroxidase and aldehyde dehydrogenase reduce the toxic reactive oxygen species and aldehydes from the plant, respectively, and help to recover from drought stress. The study provides information about post-drought recovery mechanism in soybean.

1. Introduction

Due to climatic changes, drought has become a more frequent and severe abiotic stress for a number of agriculturally important crops, such as rice, wheat, maize, cotton, tea, sorghum, and soybean [1]. The physiological effects induced by drought stress include decreased photosynthetic activity [2], increased oxidative stress, altered cell wall elasticity [3], abscisic acid accumulation, and toxic metabolite generation [4]. In soybean, exposure to drought inhibits root and shoot growth, decreases pod and nodule number, carbon/nitrogen content, and grain yields at different developmental stages [5]. Drought stress led to significant reduction in seed yields (24%–50%) in soybean crops from distinct geographical regions [6,7]. In addition, drought stress induces the accumulation of reactive oxygen species (ROS) and lipid peroxidation activity [8]. Losses in soybean yields attributable to drought stress are a major point of concern for the agriculture sector.

The drought response mechanisms of plants vary between species, though several common features have been identified. Plants exhibit either drought escape or drought tolerance mechanisms, with tolerance mechanisms being further classified into drought avoidance and drought tolerance [9,10]. Plant roots are able to sense decreases in soil water content [11]. Under these conditions, fine roots are formed by

Abbreviations: LC, liquid chromatography; MS, mass spectrometry; ROS, reactive oxygen species; SOD, superoxide dismutase; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase.

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plants and are capable of penetrating smaller soil pores and thereby increase the exploratory capabilities of the root system [12]. In soybean, drought stress also has detrimental impacts on symbiotic nitrogen fixation [13] and reduces calcium content of seed during seed formation [14]. In response to water deficiency, abscisic acid produced in the roots is transported to the leaves where it accumulates due to the closure of stomata resulting from decreased transpiration [15]. Although soybean exhibits several physiological responses to reduce water deficiency, methods to increase the drought-tolerance of plants are needed to minimize agricultural yield losses caused by drought.

The drought response mechanisms in rice [16], wheat [17], maize [18], barley [19], and soybean [8,20] have been analyzed using proteomic techniques. The inhibition of photosynthesis caused by hypersensitive early stomatal closure and the less-efficient synthesis of detoxification-related proteins increased the drought tolerance of maize [18]. Studies in wheat have indicated that differential responses to drought are due to change pattern of protein between wild and modern genotypes of wheat [17]. In barley, chloroplast metabolism and energy-related proteins have an important function in drought adaptation [19]. Yamaguchi et al. [21] reported that the abundance of soybean proteins involved in isoflavonoid biosynthesis, lignin biosynthesis, and ferritin proteins are involved in growth inhibition of the root elongation region. Protein abundances of energy-related and ROS-scavengers were increased in soybean root under drought stress [8,20]. These findings indicated that increased energy demand and reduced oxidative damage were the main responses to drought stress in soybean.

Proteomic studies on post-drought recovery have provided valuable information on the mechanisms that occur in plants to recover from drought stress. In rice, superoxide dismutase (SOD), actin depolymerizing factor, and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) activase were increased in leaves under drought stress conditions, but the level of SOD continued to increase during the recovery period [22]. Several oxidative stress-related proteins, including SOD, oxidoreductase, and aldehyde reductase, were increased in the root of *Vigna radiata* in response to drought stress and recovery [23]. Post-drought recovery in *Camellia sinensis* was enhanced by the foliar spray of potassium, calcium, manganese, and boron, which led to the increased activities of SOD, catalase, peroxidase, and glutathione reductase [24]. Stem hydraulic conductance rapidly returned to the control level after the post-drought irrigation of *Eucalyptus pauciflora*, suggesting that this species utilizes an active mechanism of repair [25]. Taken together, the findings from these reports indicate that plant species utilize different post-drought recovery mechanisms. However, the mechanisms by which drought-sensitive soybean deals with drought stress and recovers, have not been revealed. In the present study, the temporal protein profiles of root including hypocotyl were analyzed using gel-free proteomic technique to unravel the mechanisms involved in post-drought recovery in soybean.

2. Materials and methods

2.1. Plant material and treatments

Soybean (*Glycine max* L. cv Enrei) seeds were sterilized in a 2% sodium hypochlorite solution, thoroughly rinsed with water, and then sown 2 cm below the surface of 450 mL quartz sand wetted with 100 mL water in seedling cases ($145 \times 55 \times 95$ mm^3). Ten seeds were sown in each seedling case, which were then incubated at 25 °C in a growth chamber (Sanyo, Tokyo, Japan) under white fluorescent light (160 μmol m^{-2} s^{-1}, 16 h light period/day). Soybeans were grown for 2-days, stressed with drought by withholding the water supply for 4 days, and recovered from drought by re-watering for 4 days. The roots including hypocotyl were collected on the days 2 (pre-stress), 6 (at the end of 4-day stress), 8 (2-day recovered), and 10 (4-day recovered) (Supplemental Fig. 1). A control group of seedlings that received normal watering was also included and sampled on the same days as the treatment group. The average seedling growth was estimated. All experiments were repeated as three independent biological replicates. Biological replicate means that sowing dates were different with same growth conditions. For further confirmation of root proteins, samples of the root tip, elongation region of the root, lateral roots, and hypocotyl were separately collected and analyzed.

2.2. Protein extraction

A portion (500 mg) of the collected the root samples, which consisted of the root including the hypocotyl, root tips, elongation region of the root, lateral roots, and hypocotyl was ground to powder separately in liquid nitrogen using a mortar and pestle. The powder was transferred to an acetone solution containing 10% trichloroacetic acid and 0.07% 2-mercaptoethanol, and the resulting mixture was vortexed and then sonicated for 10 min. The suspension was incubated for 1 h at −20 °C with vortexing every 15 min and was then centrifuged at 9000 ×*g* at 4 °C for 20 min. The supernatant was discarded and the pellet was washed twice with 0.07% 2-mercaptoethanol in acetone. The pellet was dried using a Speed-Vac concentrator (Savant Instruments, Hicksville, NY, USA) and was then 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. The suspension was centrifuged at 20,000 ×*g* for 20 min at 25 °C, and the supernatant was collected as protein extract. Protein concentrations were determined using the Bradford assay [26] with bovine serum albumin as the standard.

2.3. Protein purification and digestion for mass spectrometry analysis

Protein extracts (150 μg) were precipitated with methanol and chloroform to remove any detergent from the sample solutions, as previously described [27]. Briefly, 150 μL sample was mixed with 600 μL methanol, and the resulting suspension was further mixed with 150 μL chloroform and 450 μL water. The sample was centrifuged at 20,000 ×*g* for 10 min to achieve phase separation. The upper aqueous phase was discarded and 450 μL methanol was added slowly to the lower phase. The samples were further centrifuged at 20,000 ×*g* for 10 min, and the obtained pellets were dried at room temperature. The dried samples were reduced with 50 mM dithiothreitol for 30 min at 56 °C, followed by alkylation with 50 mM iodoacetamide for 30 min at 37 °C in the dark. Alkylated proteins were digested with trypsin and lysyl endopeptidase at 1:100 enzyme/protein concentrations at 37 °C for 16 h. The resulting tryptic peptides were acidified with 20% formic acid and analyzed by nano-liquid chromatography (LC)-mass spectrometry (MS).

2.4. Nanoliquid chromatography-tandem mass spectrometry analysis

A nanospray LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was operated in data-dependent acquisition mode with the installed XCalibur software (version 2.0.7, Thermo Fisher Scientific). Peptides in 0.1% formic acid were loaded onto a C18 PepMap trap column (300 μm ID × 5 mm; Dionex, Germering, Germany) of an Ultimate 3000 NanoLC system. The peptides were eluted from the trap column with a linear acetonitrile gradient (8%–30% over 120 min) in 0.1% formic acid at a flow rate of 200 nL/min. The peptides eluted from the trap column were separated and sprayed onto a C18 capillary tip column (75 μm ID × 120 mm; Nikkyo Technos, Tokyo, Japan) at a spray voltage of 1.5 kV. Full-scan mass spectra were acquired in the nanospray LTQ Orbitrap MS system over 400–1500 *m/z* with a resolution of 30,000. A lock mass function was used for high mass accuracy [28]. The six 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 s to prevent the repetitive selection of peptides, as previously described [29].