JOURNAL OF PROTEOMICS 106 (2014) 1–16

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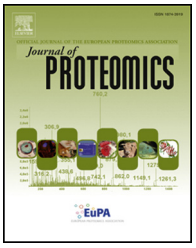

Analysis of initial changes in the proteins of soybean root tip under flooding stress using gel-free and gel-based proteomic techniques

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ARTICLE INFO

Article history:
Received 15 February 2014
Accepted 2 April 2014

Keywords:
Soybean
Flooding stress
Root tips
Proteomics
Protein–protein interaction

ABSTRACT

Flooding has a severe negative effect on soybean cultivation in the early stages of growth. To obtain a better understanding of the response mechanisms of soybean to flooding stress, initial changes in root tip proteins under flooding were analyzed using two proteomic techniques. Two-day-old soybeans were treated with flooding for 3, 6, 12, and 24 h. The weight of soybeans increased during the first 3 h of flooding, but root elongation was not observed. Using gel-based and gel-free proteomic techniques, 115 proteins were identified in root tips, of which 9 proteins were commonly detected by both methods. The 71 proteins identified by the gel-free proteomics were analyzed by a hierarchical clustering method based on induction levels during the flooding, and the proteins were divided into 5 clusters. Additional interaction analysis of the proteins revealed that ten proteins belonging to cluster I formed the center of a protein interaction network. mRNA expression analysis of these ten proteins showed that citrate lyase and heat shock protein 70 were down-regulated, whereas calreticulin was up-regulated in initial phase of flooding. These results suggest that flooding stress to soybean induces calcium-related signal transduction, which might play important roles in the early responses to flooding.

Biological significance
Flooding has a severe negative effect on soybean cultivation, particularly in the early stages of growth. To better understand the response mechanisms of soybean to the early stages of flooding stress, two proteomic techniques were used. Two-day-old soybeans were treated without or with flooding for 3, 6, 12, and 24 h. The fresh weight of soybeans increased during the first 3 h of flooding stress, but the growth then slowed and no root elongation was observed. Using gel-based and gel-free proteomic techniques, 115 proteins were identified in root tips, of which 9 proteins were commonly detected by both methods. The 71 proteins identified by the gel-free proteomics were analyzed by a hierarchical clustering method based on induction levels during the flooding stress, and 5 protein clusters were

Abbreviations: IEF, isoelectric focusing; 2-DE, two-dimensional gel electrophoresis; CBB, Coomassie brilliant blue; LC, liquid chromatography; MS, mass spectrometry; qRT-PCR, quantitative reverse transcription polymerase chain reaction

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http://dx.doi.org/10.1016/j.jprot.2014.04.004
1874-3919/

recognized. Protein interaction analysis revealed that ten proteins belonging to cluster I formed the center of a protein interaction network. mRNA expression analysis of these ten proteins showed that citrate lyase and heat shock protein 70 were down-regulated in response to flooding stress, whereas calreticulin was up-regulated. These results suggest that flooding stress to soybean induces calcium-related signal transduction, which might play important roles in the early responses to flooding.

1. Introduction

Flooding is a major abiotic stress that negatively impacts the productivity of arable farmland and is responsible for major economic losses in the agricultural industry [1]. Flooding leads to decreased oxygen concentrations in the soil, thereby restricting mitochondrial oxidative phosphorylation [2]. Under such conditions, plants experience energy deficiency and loss of cellular function, which adversely affect growth [3]. Among the major agricultural crops, soybean is particularly sensitive to flooding stress, as the plant growth and grain yields are markedly reduced in flooded soil [4]. Hashiguchi et al. [5] reported that the elongation of soybean roots under flooding stress is suppressed in the first 24 h and is significantly retarded after 48 h. These findings indicate that plant responses in the initial stages during flooding are the most critical for soybean growth and survival.

The plant root system is important for nutrient uptake from the surrounding soil. Tip of the root system is a functionally important region for development. The root tip contains the apical meristem, which is comprised of actively dividing cells that can develop into primary and lateral roots [6]. In soybean, the root tip is characterized by an open meristem and quiescent center, which is located below the meristem and is mainly composed of a pool of stem cells that are important for root development [7]. The soybean root tip also contains the elongation region [6]. The root tip plays key roles in the development of root structure and responses to environmental stresses, particularly flooding [8]. From these reasons, the study of soybean root tip proteins is expected to provide insight in plant responses to guard against flooding stress.

To better understand the underlying mechanisms of soybean responses to flooding stress, proteomic techniques have been applied. For example, proteomic analysis of soybean seedling roots and hypocotyls under flooding stress revealed that proteins related to protein destination and storage, defense, and energy metabolism were differentially expressed [9]. Among the identified proteins, the levels of alcohol dehydrogenase, which is involved in anaerobic metabolism [10], and cytosolic ascorbate peroxidase, which functions in active oxygen scavenging [11], were increased and decreased, respectively, in response to flooding stress. Additionally, it has been reported that a number of proteins related to folding and synthesis of protein are dephosphorylated under flooding conditions, and changes in translational and post-translational control affected the balance of proteins related to several metabolic pathways, including carbohydrate metabolism [12]. Changes in the level of protein phosphorylation also influence the regulation of energy and production-related metabolic pathways [13]. Although many types of proteins which responded to flooding stress were reported, the relationships and interactions between these proteins have not been characterized completely.

In this study, to understand the initial responses of soybean to flooding stress, two-dimensional gel electrophoresis (2-DE)-based and gel-free proteomic techniques were applied. In addition, protein–protein interactions of differentially expressed proteins were analyzed using goodness-of-fit-test based on S-system differential equations. Furthermore, the genes corresponding to differentially expressed proteins were analyzed using quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

2. Materials and methods

2.1. Plant material

Seeds of soybean (*Glycine max* L. cultivar Enrei) were sterilized with 1% sodium hypochlorite solution, rinsed in water, and sown on 500 mL silica sand moistened with 125 mL water in a plastic case (180 mm × 140 mm × 45 mm). Soybeans were grown in a growth chamber illuminated with white fluorescent light (160 μmol m^{-2} s^{-1}, 16 h light period/day) at 25 °C and 70% relative humidity. Two-day-old soybeans were treated without (control) or with flooding for 3, 6, 12, and 24 h. For sampling, 5 mm-long root tips were collected and immediately frozen in liquid nitrogen. Three independent experiments were performed as biological replicates for the gel-based proteomics, gel-free proteomics, and qRT-PCR analyses (Fig. 1).

2.2. Protein extraction

A portion (500 mg) of root tip sample was ground to powder in liquid nitrogen using a mortar and pestle. The powder was transferred into a solution of 10% trichloroacetic acid and 0.07% 2-mercaptoethanol in acetone and was then vortexed. The resulting suspension was sonicated for 10 min and then incubated for 60 min at −20 °C. After incubation, the suspension was centrifuged at 9000 ×*g* for 20 min at 4 °C. The resulting supernatant was discarded, and the pellet was washed twice with 0.07% 2-mercaptoethanol in acetone. The final pellet was dried using a Speed-Vac concentrator (Savant Instruments, Hicksville, NY, USA) and resuspended by vortexing for 60 min at 25 °C in lysis buffer consisting of 8 M urea, 2 M thiourea, 5% CHAPS, and 2 mM tributylphosphine. The suspension was centrifuged at 20,000 ×*g* for 20 min at 25 °C and the supernatant was collected as protein extract for use in 2D gel-based proteomics and gel-free proteomics