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Dissecting root proteome of transgenic rice cultivars unravels metabolic alterations and accumulation of novel stress responsive proteins under drought stress

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ABSTRACT

Generation of drought tolerant rice plants by overexpressing *Arabidopsis DREB1A* is a significant development for abiotic stress research. However, the metabolic network regulated in the drought tolerant transgenic rice plants is poorly understood. In this research study, we have demonstrated the comparative proteome analysis between the roots of wild type and transgenic *DREB1A* overexpressing homozygous plants under drought stress condition. After 7 d of dehydration stress at reproductive stage, the plants were re-watered for 24 h. The roots were collected separately from wild type and transgenic plants grown under water, drought stress and re-watering conditions and total proteins were analyzed by two-dimensional gel electrophoresis (2-DE) coupled with mass spectrometry (MS). Among the large number of differentially accumulated spots, 30, 27 and 20 spots were successfully identified as differentially expressed proteins in three different conditions respectively. The major class of identified proteins belongs to carbohydrate and energy metabolism category while stress and defense related proteins are especially up-accumulated under drought stress in both the plants. A novel protein, R40C1 was reported to be up-accumulated in roots of transgenic plants which may play an important role in generation of drought tolerant plants. Protein–protein interaction helps to identify the network of drought stress signaling pathways.

1. Introduction

Food security is a major challenge in developing countries. To alleviate this problem, crop yield must to be increased. Globally, 40% of the areas need to be cultivated by crops to meet the food demand in future where drought might be a major constrain among abiotic stresses [1]. Rice, a staple food crop for half of the world's population, is cultivated mainly in the irrigated and rain-fed low land. Rain-fed low land is the major harvesting area in South and Southeast Asia which accounts for 90% of the globally cultivated rain-fed land. In Asia, about 50% of cultivated land is rain-fed although majority of grain yield comes from the irrigated land [2]. The cultivation in rain-fed land depends on rainfall. Inadequate rainfall causes drought. Recent climatic changes such as uncertainty of rainfall, improper intensity and distribution of water worsen the drought condition and severely affect rice production in rain-fed lowland. In India, about 7.3 million ha of low land rice out of 20.4 million ha rain-fed rice areas are estimated to be drought-prone [3]. Severe affect of water stress in rice growth is commonly reflected from reduction of leaf area in vegetative stage [4]. The significant reduction during flowering stage is exerted by thwarted panicle exertion, perturbation of anthesis and spikelet desiccation, abnormal pollination and loss of viable pollen grain has been reported due to water scarcity during flowering [5]. Improvement of drought resistance has major impact on rice biomass production.

The complex signaling responses by plants during drought stress are indicated by morphological, biochemical and physiological alteration including higher transpiration rate and wilting, reduced

Abbreviations: ABA, abscisic acid; SnRK2, SNF-related protein kinase 2; DREB, dehydration-responsive element-binding; ROS, reactive oxygen species; 2DE, two dimensional gel electrophoresis; WT, wild type; IPG, immobilized pH gradient; GDP, Guanosine diphosphate; MALDI-TOF MS, matrix assisted laser desorption ionization-time of flight mass spectrometry.

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photosynthesis rate and mineral uptake. Plant adopts several lines of defense mechanisms to avoid or tolerate water stress. The different signaling molecules functioning downstream of abscisic acid (ABA), such as SNRK2 and AREB/ABFs act as signal transducers in the leaf which ultimately facilitates drought tolerance reducing the amount of carbon fixation and impeding plant growth [6]. ABA-independent signaling mechanism by activation of *NAC* or *DREB* gene is another way of molecular defense against drought [7]. The generation of reactive oxygen species (ROS) during stress can damage the structures of proteins, lipid and cell membrane integrity ultimately destroying the plant cell [8]. The higher amount of ROS scavenging enzymes in plants increases the resistance to cope up with that particular stress condition. The relative abundance of hydrophobic amino acids such as leucine and alanine in drought tolerant plants are very significant to maintain the structural stabilization of protein [9]. Therefore, understanding the underlying molecular signaling pathways and interaction among signaling molecules in response to drought stress are very critical for development of drought tolerant plants. Over the last decades, a number of transgenic plants overexpressing different drought responsive genes or transcription factors have been generated which exhibited variable degrees of resistance under water stress [10–13].

Comparative proteomics approaches from stress tolerant and susceptible plants unravel the relative protein abundance which helps to understand the differential gene expression during cellular stress [14]. The proteomics studies of different species under drought stress have been extensively studied to date [15–19]. However, the molecular regulation of different transcription factors and interaction with different signaling proteins to alter the metabolic pathways remains unclear. The altered gene expression or necessary changes due to integration and overexpression of dehydration responsive transgene is poorly understood till date. For example, transgenic rice plants overexpressing the transcription factor gene(s) finely adjust or increase the expression level of several downstream dehydration-responsive target genes that helps to improve drought resistance [20]. The altered level of expression of several protein families such as biosynthesis enzymes associated with carbohydrate or energy metabolism, water-channel proteins, carriers of photosynthetic electron transport chain, ROS scavenging enzymes, cytoskeleton stabilizing, heat shock proteins have been well elucidated from the root proteome profiles under different abiotic stresses [19]. However, no such regulatory network of protein expression from transgenic plants under drought stress is reported till date. The comparative proteomics approach of transgenic drought tolerant rice roots can explore the novel insights about the proteome profile of transgenic roots under water stress. The study of proteome profile from transgenic roots may facilitate to acquire knowledge about downstream proteins associated with the transgene overexpression under drought stress or changes due to transgene integration.

The rice plants overexpressing *AtDREB*1A gene under the control of dehydration stress inducible *rd*29A promoter showed a significant level of dehydration tolerance over wild type plants [11]. Root acts as primary organ to perceive the dehydration signal from changes in the soil condition. In present study, roots of transgenic and wild type (WT) rice plants are considered for comparative proteomics analysis. The plants were exposed to drought stress for 7 d at flowering initiation period. The retrieval of natural watery condition was also followed after resupplying of water. The roots of wild type and transgenic plants from drought stress and re-watering conditions were taken into account for proteomics study. Major differentially up-accumulated proteins in transgenic rice roots were separated and identified by two-dimensional gel electrophoresis (2-DE) and matrix assisted laser desorption ionization-time of flight-tandem mass spectrometry (MALDI-TOF-MS/MS) analysis respectively.

2. Materials and methods

2.1. Plant materials and growth and drought stress induction

Rice (*Oryza sativa* L. Subspecies *indica*) cv. BR 29 and *AtDREB*1A overexpressing homozygous transgenic BR-29 lines were selected as WT drought sensitive and transgenic drought tolerant lines respectively. The plants were grown till flowering initiation stage in green house at day/night temperature regime of 30/25 °C under natural illuminating condition and relative humidity of 70–80%. The plants were grown in fertilizer enriched (N:P:K = 80:40:40 kg/ha) paddy field soil. At booting stage, six sets of plants (3 plants from wild type and transgenic lines for each condition viz. water, stressed and re-watering) at 3 d before heading stage were selected for drought stress. The standing water was removed carefully from each pot and water supply was stopped for 7 d. The re-watering was implied in one set of plants for 24 h and visual phenotypic changes were observed. The plants were then taken out from pots and roots were washed carefully with double distilled water and wiped with blotting paper. The root morphology was observed and samples of roots were taken in three replicates from control, drought stressed and re-watering condition for proteomics and Real time PCR analysis. The root samples were stored in RNA later solution for RNA isolation purpose and kept at −80 °C.

2.2. Protein extraction

2 g of root tissues were grinded to fine powder with liquid nitrogen using mortar and pestle at 4 °C. Total protein was extracted following phenol extraction method [21]. The grinded powder of root tissues was homogenized with 10 ml of ice-cold extraction buffer [0.5 M Tris–HCl (pH 7.5), 30% sucrose, 50 mM EDTA-Na, 2% SDS, 2% 2-mercaptoethanol (2-ME), and 2% polyvinylprrolidone (PVP), dithiothreitol (DTT), PMSF] followed by equal volume (10 ml) of Tris saturated phenol (pH 8.0). The homogenate was centrifuged at 8000 × *g* for 30 min. The upper aqueous phase was collected and phenol phase was extracted twice with equal volume of cold buffer. The aliquots from each extraction were mixed and total protein was precipitated from collected aqueous phase by mixing with cold methanol solution containing 100 mM ammonium acetate. The mixing solution was kept at −20 °C for overnight. The content was centrifuged at 8000 × *g* for 20 min. Protein pellet was rinsed with cold methanol and washed with acetone twice. The pellet was air dried and kept at −80 °C until use.

2.3. Two-dimension polyacrylamide gel electrophoresis (2-DE)

For 2-DE, the protein pellet was resuspended in 2-DE sample buffer consisting of 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 20 mM DTT and 1% (v/v) Bio-Lyte pH 3–10 (Bio-Rad, Hercules, CA, USA). The concentration of total protein content was measured for each sample following the method described by Bradford [22], using bovine serum albumin (BSA) as standard. The one dimension protein separation using iso electric focusing (IEF) was followed according to protocol mentioned in Bio-Rad IEF manual. 700 μg of total protein sample corresponding to volume of 300 μl was used for rehydration of immobilized pH gradient (4–7, linear gradient) 17 cm strips (Bio-Rad, Hercules, CA, USA). IEF was carried out with the IEF Cell (Bio-Rad) using following condition: 250 V linear for 30 min, 10,000 V linear for 4 h, 10,000 V for 43,000 Vh, 1000 V for 5 min. The working temperature at 50 °C and electrical current at 50 mA per strip was maintained. The focused strips were equilibrated twice in equilibration buffer I (50 mM Tris–HCl, pH 8.8, 6 M urea, 20% [v/v] glycerol and 2% [w/v] sodium dodecyl sulfate [SDS] and 100 mg DTT) and equilibrium buffer II (250 mg iodoacetamide instead of DTT) respectively, for 15 min each. The equilibrated strips