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Identification of the differentially accumulated proteins associated with low phosphorus tolerance in a Tibetan wild barley accession

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ABSTRACT

Low phosphorus (LP) in soil is a widely-occurred limiting factor for crop production in the world. In a previous study we identified a highly LP-tolerant Tibetan wild barley accession (XZ99). Here, a comparatively proteomic analysis was conducted using three barley genotypes differing in LP tolerance to reveal the mechanisms underlying the LP tolerance of XZ99. Totally, 31 differentially accumulated proteins were identified in the roots and leaves of the three genotypes using 2-dimensional gel electrophoresis coupled with mass spectrometry. They were involved in the various biological processes, including carbon and energy metabolism, signal transduction, cell growth and division, secondary metabolism, and stress defense. In comparison with XZ100 (LP sensitive) and ZD9 (LP moderately-tolerant), XZ99 had a more developed root system, which is mainly attributed to enhanced carbohydrate metabolizing proteins under LP conditions. The current results showed that Tibetan wild barley XZ99 and cultivated barley cultivar ZD9 differ in the mechanism of LP tolerance. The changes of the proteins associated with carbohydrate metabolism could account for the difference between the LP-tolerant and LP-sensitive genotypes. In addition, the mRNA expression levels of 9 LP responsive proteins were verified by qRT-PCR. The current results may open a new avenue of understanding the LP tolerance in plants on the proteomic basis.

1. Introduction

Phosphorus (P) is an important nutrient element for plant growth and development (Wu et al., 2013) and is absorbed by plants mainly in the form of inorganic phosphate (Pi). However, Pi in soil often exists in insoluble mineral forms that render it unavailable to plants. To maintain the normal phosphorus nutrition in plants, Pi fertilizers should be applied in massive amounts in most agricultural soils. Unfortunately, uptake and assimilation of Pi fertilizers in plants are quite inefficient, as a large proportion of applied Pi becomes immobile in soil (Bozzo and Plaxton, 2008). In addition, the resource of nonrenewable rock phosphate in the world could be depleted within the next century (Dawson and Hilton, 2011). Obviously, low phosphorus (LP) has been one of the major limiting factors affecting sustainable crop production, particularly in alkaline and acid soils (López-Bucio et al., 2000). It has been estimated that LP leads to reduced crop productivity in 40% of the world's arable land (Vance, 2001). Therefore, it is essential to develop high Pi-use efficient crop cultivars, so as to cope with declining Pi resource and LP availability in soil. A wide range of morphological and molecular mechanisms underlying LP tolerance in crops have been well documented (Raghothama and Karthikeyan, 2005). Nevertheless, the molecular mechanisms in plants are still largely unknown.

Proteome analysis by two-dimensional gel electrophoresis (2-DE) is a powerful approach in elucidating molecular mechanisms, and it has been used to study the responses of many plants to environmental stress (Alexova and Millar, 2013). Moreover, proteomics can be more efficient in identifying stress tolerant genes if it is applied to divergent tolerant and susceptible genotypes (Torabi et al., 2009). The proteins associated with abiotic stress tolerance have been identified using proteomic

Abbreviations: 2-DE, two-dimensional gel electrophoresis; 3-PGA, 3-phosphoglycerate; DHAP, dihydroxyacetone phosphate; DTT, dithiothreitol; E-4-P, erythrose 4-phosphate; EP, eppendorf; Fru1,6P2, fructose-1-6-biphosphate; Fru6P, fructose-6-phosphate; Glc6P, glucose-6-phosphate; GPT, glucose-6-phosphate/phosphate translocator; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; mRNA, messenger RNA; OPPP, oxidative pentose phosphate pathway; PEP, phosphoenolpyruvate; PPT, phosphoenolpyruvate/phosphate translocator; RPPP, reductive pentose phosphate pathway; Ru5P, ribulose-5-phosphate; RUBP, ribulose 1,5-bisphosphate carboxylase/oxygenase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TOF, time of flight; TP, triose phosphate; TPT, triose phosphate translocator.

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analysis in various tissues of some plant species, such as roots in maize (*Zea mays*) (Li et al., 2008), Arabidopsis (*Arabidopsis thaliana*) (Tran and Plaxton, 2008), and rice (*Oryza sativa*) (Torabi et al., 2009), roots, and leaves in soybean (*Glycine max*) (Chen et al., 2015), and leaves in rape (*Brassica napus*) (Yao et al., 2011).

Barley is one of the oldest cultivated crops in the world and now ranks the fourth largest among cereal crops after maize, rice, and wheat in terms of planting area. In comparison with wheat, barley is generally planted in the marginal areas with poor soil or harsh environments. Therefore, its ability of adaptation to abiotic stresses, including LP will be crucial to sustainable crop production (Saisho and Takeda, 2011). It is important to identify the beneficial genes and reveal the underlying molecular mechanism for successful development of barley cultivars with high P use efficiency. In the previous studies, we screened 104 Tibetan wild barley accessions and 20 cultivated barley genotypes, and found that some wild barley accessions had higher LP tolerance than the cultivars (Nadira et al., 2014a), and the main attributors of LP tolerance in Tibetan wild barley accession XZ99 were better root architecture, higher activities of carbohydrate metabolizing enzymes, and more exudation of organic acids (Nadira et al., 2014b). Therefore, it is imperative to reveal the proteins associated with LP tolerance through comparing the protein accumulation profiles of the genotypes differing in LP tolerance.

In the current study, we performed comparative proteome analysis using 2D electrophoresis and MALDI-TOF/TOF MS techniques, aiming at (i) identifying differentially accumulated proteins in leaves and roots and (ii) understanding the mechanisms or metabolic pathways underlying the differences in root morphology and LP tolerance among barley genotypes.

2. Materials and methods

2.1. Plant growth conditions and treatments

In this study, two Tibetan wild barley accessions XZ99 and XZ100, low P tolerant and sensitive, respectively, and one moderately-tolerant cultivar ZD9 were used (Nadira et al., 2014a). Seeds were disinfected with 3% H_2O_2 and germinated in a growth chamber (22/18° C, day/night). Seven-days-old uniform seedlings were transplanted into 5-L plastic containers with aerated basic nutrient solution (BNS) as described by Wu et al. (2003). Briefly, the following composition (mg l^{-1} water): $(NH4)_2SO_4$, 48.2; $MgSO_4$, 65.9; K_2SO_4, 15.9; KNO_3, 18.5; $Ca(NO_3)_2$, 59.9; KH_2PO_4, 24.8; Fe-citrate, 5; $MnCl_24H_2O$, 0.9; $ZnSO_47H_2O$, 0.11; $CuSO_45H_2O$, 0.04; HBO_3, 2.9; H_2MoO_4, 0.01 were used. The container was covered with a plate with 6 evenly spaced holes (two plants per hole). The pH of the hydroponic solution was adjusted to 5.8–6.0 with 1 N HCl or NaOH, as required. Half concentration solution was supplied to plants in the first week, and then changed into full concentration from the second week. After 10 days of seedling transplantation, plants were treated with (1) BNS containing 500 μM KH_2PO_4 (Control); (2) 10 μM KH_2PO_4 (LP). The modified nutrient solution was formulated as P-free by replacing all the phosphorus of BNS with nitrates or chlorides. To balance potassium in KH_2PO_4, the container at LP were supplemented with KNO_3 and KCl at the rate of 18.5 mg l^{-1} and 74.54 mg l^{-1} water, respectively. The experiments were designed as a randomized complete block design (RCBD) with three replicates of each treatment and conducted in a greenhouse of Zijingang Campus, Zhejiang University, Hangzhou, China. The nutrient solution was continuously aerated with pumps and renewed once a week.

2.2. Sampling

After 21 days of treatment, plants were collected and rinsed with tap water three times to remove surface ions, and then blotted dry with tissue paper. Fresh shoot and root weight of the plants from three independent replicates were measured. Root/shoot fresh weight ratio was calculated. After measurement of fresh shoot and root weight, plants were separated into roots and shoots, and dried at 105 °C for 3 h, followed by drying at 80 °C for 72 h, and then weighed. The shoot and root samples were ground to pass through a 0.42-mm screen (40-mesh) for phosphorus concentration determination. Meanwhile, leaves and roots were sampled from each treatment and control with three biological replicates, and stored at −80° C for use in RNA and protein extraction.

2.3. Phosphorus determination

For P determination, dry powdered tissues were digested with an acid mixture of HNO_3 (Nitric acid) (60%) and $HClO_4$ (Perchloric acid) (60%) (2.5:1) in an automatic distillation (FOSS, 4400 Kjeltic Auto Distillation) system. P concentrations in shoots and roots were estimated by the vanadate-molybdate yellow color method (Chapman and Pratt, 1961) using a spectrophotometer (SHIMADZU, UV-2410PC, Japan). Phosphorus accumulation was calculated from the following formula:

$$\text{P accumulation (mg plant}^{-1}) = \text{P concentration (mg g}^{-1}) \times \text{plant dry weight (g plant}^{-1})$$

2.4. Sugar extraction and analysis

Leaves and roots were homogenized to a very fine powder in liquid nitrogen and then sugar extraction was carried out (Yelle et al., 1991). Briefly, ethanol at 80% (v/v) containing mannitol as internal standard was added to each sample which was then kept at 80 °C in a water bath for 20 min before centrifugation for 15 min at 13,000*g*. After supernatant removal, the pellet was re-suspended and re-extracted twice in 80% ethanol to recover the residual soluble sugars. The combined supernatants constituted the soluble sugar fraction. The extracts were evaporated in a SpeedVac and re-suspended in water before injection into an HPLC system (712 WISP, Waters, Milford, MA, USA). An aqueous solution of 0.005% (w/v) Ca-EDTA (Sigma, St Louis, MO, USA) flowing at 0.5 mL min^{-1} was used as the mobile phase. The sugar column (Sugar-Pak 1, 6.5 × 300 mm, Waters) was used at 75 °C and eluted sugars were detected with a differential refractometer (Waters). Peaks were quantified using anhydrous sucrose, glucose (BDH Inc.), fructose (Fisher, Fair Lawn, NJ, USA), and mannitol (Sigma).

2.5. Protein extraction

Total protein extracts were carried out according to the phenol extraction method described by Carpentier et al. (2005) with some modifications. A portion (1 g) of leaf and root samples was ground to a fine powder form in liquid nitrogen using a mortar and pestle. The powder was suspended in 5 mL ice-cold extraction buffer [1.5% (w/v) polyvinylpyrrolidone (PVP), 0.7 M sucrose, 0.1 M KCl, 0.5 M Tris-HCl, pH 7.5, 250 mM Ethylenediaminetetraacetic acid (EDTA), complete protease inhibitor cocktail (Roche, Switzerland), 2% (v/v) β-mercaptoethanol and 0.5% (w/v) [(3-cholamidopropyl) dimethylammonium] −1-propane sulfonate (CHAPS)] and homogenized at 4 °C for 20 min. Then, 10 mL of Tris-HCl pH 7.5 saturated phenol was added and the resulting mixture was re-homogenized for 20 min at 4 °C. After centrifugation at 10,000*g* for 20 min at 4 °C,