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Research article

Phosphoproteome and proteome analyses reveal low-phosphate mediated plasticity of root developmental and metabolic regulation in maize (*Zea mays* L.)

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ABSTRACT

Phosphate (Pi) deficiency has become a significant challenge to worldwide agriculture due to the depletion of accessible rock phosphate that is the major source of cheap Pi fertilizers. Previous research has identified a number of diverse adaptive responses to Pi starvation in the roots of higher plants. In this study, we found that accelerated axile root elongation of Pi-deprived maize plants resulted from enhanced cell proliferation. Comparative phosphoproteome and proteome profiles of maize axile roots were conducted in four stages in response to Pi deficiency by multiplex staining of high-resolution two dimensional gel separated proteins. Pro-Q DPS stained gels revealed that 6% of phosphoprotein spots displayed changes in phosphorylation state following low-Pi treatment. These proteins were involved in a large number of metabolic and cellular pathways including carbon metabolism and signal transduction. Changes in protein abundance of a number of enzymes indicated that low-Pi induced a number of carbon flux modifications in metabolic processes including sucrose breakdown and other downstream sugar metabolic pathways. A few key metabolic enzymes, including sucrose synthase (EC 2.4.1.13) and malate dehydrogenase (EC 1.1.1.37), and several signaling components involved in protein kinase or phosphatase cascades, auxin signaling and 14-3-3 proteins displayed low-Pi responsive changes in phosphorylation state or protein abundance. A variety of key enzymes and signaling components identified as potential targets for phosphorylation provide novel clues for comprehensive understanding of Pi regulation in plants. Protein phosphorylation, coordinating with changes in protein abundance, is required for maize root metabolic regulation and developmental acclimation to Pi starvation.

1. Introduction

Phosphorus (P), an essential macronutrient for all living organisms, not only constitutes a requisite structural component, but also plays important roles in cellular metabolism, energy conversion, signal transduction and protein activity regulation (Raghothama, 1999; Vance et al., 2003). Due to its relatively high immobility and uneven distribution in soil, phosphate (Pi) inadequacy is one of the major constraints for plant growth in natural ecosystems. A series of physiological and developmental strategies have been evolved in higher plants to facilitate adaptions to low-Pi environment. As the exclusive organ of plants for Pi acquisition from soil, root system exhibits great flexibility in response to Pi deficiency and modulates its structure and function at the metabolic, cellular and organ developmental level, for example,

Abbreviations: 2-DE, two-dimensional electrophoresis; ABP1, auxin binding protein 1; CBB, Coomassie Brilliant Blue; CHAPS, 3-((3-Cholamidopropyl) dimethylammonium)- 1-propanesulfonate; DTT, Dithiothreitol; ENO, enolase; F-6-P, Fructose 6-phosphate; FRK, Fructokinase; G-1-P, Glucose 1-phosphate; G-6-P, Glucose 6-phosphate; GPI, 6-phosphate isomerise; MDH, Malate dehydrogenase; MS, mass spectrometry; NAD-G3PDH, NAD dependent 3-phosphate glyceraldehyde dehydrogenase; NADP-G3PDH, NADP-dependent 3-phosphate glyceraldehyde dehydrogenase; P, phosphorus; PDC, Pyruvate dehydrogenase complex; PGM, Phosphoglucomutase; Pi, phosphate; PK, Pyruvate kinase; PMF, peptide mass fingerprint; PMSF, Phenylmethylsulfonyl fluoride; PP1, Protein phosphatase 1; PP2A, protein phosphatases 2A; PPDK, Pyruvate phosphate dikinase; PPi, Pyrophosphate; ProQ DPS, Pro-Q Diamond Phosphoprotein Gel Stain; PSR, phosphate starvation responsive; SUS2, Sucrose synthase 2; TCA, Trichloracetic acid; UGPase, UDP-glucose pyrophosphorylase.

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remodeling of root architecture and metabolic modifications to bypass Pi requirements (Raghothama, 1999; Vance et al., 2003).

Recent progresses shed light on complex regulation of P nutrition by dissecting genetic and molecular basis of low Pi responses in higher plants. A PHR1-centered signaling pathway involving transcription factor, microRNA and protein post-translational modification has been characterized in Arabidopsis (Bari et al., 2006). As a key transcriptional regulator of Pi starvation responsive (PSR) gene expression and root developmental modification, PHR1 is a target for SUMOylation directed by a small plant ubiquitin-like modifier (SUMO) E3 ligase SIZ1 (Miura et al., 2005). *miR*399, whose expression is directly regulated by PHR1, acts as a negative regulator for PHO2/UBC24 accumulation at the post-transcriptional level (Chiou et al., 2006). PHO2/UBC24 encoding an E2 ubiquitin-conjugating enzyme is essential for Pi-homeostasis regulation (Aung et al., 2006). In addition to PHR1, a number of transcription factors involved in low-Pi responses have been identified, such as WRKY75 (Devaiah et al., 2007a), ZAT6 (Devaiah et al., 2007b), and MYB62 (Devaiah et al., 2009) in Arabidopsis, *Os*PTF1 (Yi et al., 2005) and *Os*MYB2P-1 (Dai et al., 2012) in rice. These components, seemingly representing distinct Pi signaling pathways, are required for PSR gene expression and root remodeling. Transgenic maize lines overexpressing *Zm*PTF1, the homolog of *Os*PTF1 in maize, was reported to display improved low-Pi tolerance (Li et al., 2011).

As demonstrated by genome-wide expression studies in several species, Pi starvation responses require complex regulatory networks involving a large number of PSR genes. The transcriptome analyses in Arabidopsis, rice and bean revealed significant alternations in a large range of biochemical and signaling pathways and facilitated the detailed characterizations of P nutrition regulation (Wasaki et al., 2003; Misson et al., 2005; Hernandez et al., 2007). Microarray analyses using a low Pi tolerant maize genotype provided transcriptome profiling changes induced by Pi depletion in roots and highlighted its species-specific transcript responses (Calderon-Vazquez et al., 2008). Comparative proteomic analyses of maize roots provided a constructive view of maize inbred lines with variant low-Pi tolerance (Li et al., 2007a, 2008). Li et al. (2008) and some similar proteomic studies reported that the mRNA abundance of various genes is not always indicative of protein accumulation during Pi deprivation. This emphasizes the importance of post-transcriptional and post-translational mechanisms in the control of the plant Pi starvation response, and thus transcriptome profiling studies of plant stress responses need to be complemented with parallel post-transcriptional and post-translational modification analysis.

Reversible phosphorylation, a major post-translational modification in living organisms, acts as a key regulatory switch for activities of a large number of metabolic enzymes and signaling components (Huber and Hardin, 2004). Hence exploring the dynamic and large-scale phosphorylation profiling in response to cellular and environmental stimuli facilitate further understanding of metabolic and signaling networks. However, thus far little information is available on global phosphorylation modifications induced by Pi starvation. In our study multiplex staining of high-resolution 2-DE was performed for phosphoprotein and protein detection. Subsequently mass spectrometry was used for protein identification. This study represents a large-scale investigation for low-Pi responsive changes of phosphoproteome and proteome in maize root tip tissues.

2. Materials and methods

2.1. Plant growth conditions and treatments

The surface-sterilized seeds of maize inbred line Qi-319 were germinated in the dark at 28 °C for 4 days and then transferred to +P (1000 μM KH_2PO_4) nutrient solution. The compositions of the nutrient solution and the culture conditions were described previously (Li et al., 2007b). The endosperms of the 8-day-old seedlings were removed carefully, followed by culture in +P nutrient solution for 2 more days. Afterwards half of seedlings continued to grow in +P condition and the other were transferred into −P (5 μM KH_2PO_4) nutrient solution. The nutrient solution was replaced every 3 days. Biomass, total P concentration, and morphological parameters of root system were measured on the 1st, 3rd, 7th and 11th day after the onset of Pi-depletion as described previously. (Li et al., 2007b). Root tip fragments (1.5 cm) were sampled from axile roots for protein isolation at these four stages.

2.2. Histological analyses

Root tip fragments were fixed in formalin-acetic-alcohol solution, dehydrated in a graded ethanol/xylene series and embedded in paraffin. The 10 μm-thin sections were stained with hematoxylin according to the standard cytological procedures and imaged under a microscope (Olympus BX51).

2.3. Protein isolation

Proteins were extracted from root tip fragments via tricholoroacetic acid/acetone precipitation as described previously (Li et al., 2007a) and dissolved in protein solubilization buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 0.5% v/v carrier ampholyte pH 3–10, 40 mM DTT, 1 mM PMSF and phosphatase inhibitor cocktail (Merck & Co Inc). The protein samples were kept at −80 °C until used.

2.4. Two dimensional electrophoresis and gel staining

Two dimensional electrophoresis (2-DE) was performed using 17 cm immobilized pH 4 to 7 gradient (IPG) strips (Bio-Rad) in the first dimension, followed by 12% SDS-PAGE in the second dimension, as previously described (Li et al., 2007a). The gels were firstly stained with Pro-Q DPS (Invitrogen) for phosphoprotein detection according to the manufacturer's instruction and documented using Typhoon Trio scanner (GE Healthcare). Cy3 channel (532 nm excitation and 580 nm emission) was used and the resolution for scanning was 100 microns. Afterwards the gels were re-stained with Coomassie Brilliant Blue (CBB) G-250 for total protein detection and imaged using a GS-800 calibrated densitometer (Bio-Rad). PeppermintStic Phosphoprotein Molecular Weight Standards (Molecular Probes) were stained in parallel as a reference lane to validate the Pro-Q DPS specificity for phosphoprotein staining (Fig. S1). The phosphoprotein standards including both 23.6 kDa β-casein and 45 kDa ovalbumin were exclusively stained with Pro-Q DPS, whereas the remaining non-phosphorylated protein standards not (Fig. S1a). All of the standards were readily detected by the following CBB staining (Fig. S1b). The validated specific staining of phosphoproteins guaranteed the reliable phosphorylation detection for 2-DE based phosphoproteomic approach. The experiment was repeated three times with independent root samples as biological replicates.

2.5. Quantification of protein spots

The images of the gels stained with Pro-Q DPS and CBB were analyzed using PDQuest software, version 8.0.1 (Bio-Rad). Following background subtraction and spot detection, the spots were matched and normalized with the value of total density in gel image. The resulting relative spot intensities were used for statistical analyses. The statistical significance of quantitative data was