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Comparative proteomic analysis provides insight into the biological role of protein phosphatase inhibitor-2 from *Arabidopsis*

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ABSTRACT

Protein phosphatase inhibitor-2 (PPI-2) is a conserved eukaryotic effector protein that inhibits type one protein phosphatases (TOPP). A transfer-DNA knockdown of AtPPI-2 resulted in stunted growth in both vegetative and reproductive phases of *Arabidopsis* development. At the cellular level, AtPPI-2 knockdown had 35 to 40% smaller cells in developing roots and leaves. This developmental phenotype was rescued by transgenic expression of the AtPPI-2 cDNA behind a constitutive promoter. Comparative proteomics of developing leaves of wild type (WT) and AtPPI-2 mutant revealed reduced levels of proteins associated with chloroplast development, ribosome biogenesis, transport, and cell cycle regulation processes. Decreased abundance of several ribosomal proteins, a DEAD box RNA helicase family protein (AtRH3), Clp protease (ClpP3) and proteins associated with cell division suggests a bottleneck in chloroplast ribosomal biogenesis and cell cycle regulation in AtPPI-2 mutant plants. In contrast, eight out of nine *Arabidopsis* TOPP isoforms were increased at the transcript level in AtPPI-2 leaves compared to WT. A protein-protein interaction network revealed that >75% of the differentially accumulated proteins have at least secondary and/or tertiary connections with AtPPI-2. Collectively, these data reveal a potential basis for the growth defects of AtPPI-2 and support the presumed role of AtPPI-2 as a master regulator for TOPPs, which regulate diverse growth and developmental processes.

Biological significance: Comparative label-free proteomics was used to characterize an AtPPI-2 T-DNA knockdown mutant. The complex, reduced growth phenotype supports the notion that AtPPI-2 is a global regulator of TOPPs, and possibly other proteins. Comparative proteomics revealed a range of differences in protein abundance from various cellular processes such as chloroplast development, ribosome biogenesis, and transporter activity in the AtPPI-2 mutant relative to WT *Arabidopsis*. Collectively the results of proteomic analysis and the protein-protein network suggest that AtPPI-2 is involved in a wide range of biological processes either directly or indirectly including plastid biogenesis, translational mechanisms, and cell cycle regulation. The proposed protein interaction network comprises a testable model underlying changes in protein abundance in the AtPPI-2 mutant, and provides a better framework for future studies.

1. Introduction

Among the large, diverse families of phospho-protein phosphatases in eukaryotes, type one protein phosphatases (TOPPs) comprise a highly conserved clade [1]. A total of nine TOPP genes are expressed in *Arabidopsis thaliana* [2,3]. Expression of these genes is ubiquitous as they have been detected in all tissues studied. Transient expression of *Arabidopsis* TOPPs in *Vicia faba* guard cells as C-terminal GFP fusions resulted in localization to both the nucleus and cytoplasm [4]. Similarly, co-expression of TOPP2 in Vicia epidermal cells using particle bombardment also displayed dual expression in the nucleus and cytoplasm [4,5]. TOPPs are involved in a diverse range of plant developmental processes including cell division, blue light signaling, cell differentiation, and embryo development [6]. Using affinity chromatography and other molecular approaches to identify clients, several TOPP interacting proteins have been identified including nuclear inhibitor of protein phosphatase

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1, suppressor of DIS2, GL2 expression modulators, protein phosphatase inhibitor-2 (PPI-2), and inhibitor-3 (Inh3) in *Arabidopsis* [4,5]. Similarly, in rice a salt sensitive 1 protein was identified as potential interactor with TOPP by yeast two-hybrid screening [7].

Among the various TOPP interacting proteins, PPI-2 is arguably the most ancient, and was first identified from a mammalian system as a heat-stable protein capable of inhibiting TOPP activity [8]. Although mammalian PPI-2 was identified three decades ago, the plant PPI-2 ortholog was only recently identified as a nuclear-localized protein and confirmed as a potent inhibitor of TOPP activity [5]. *Arabidopsis* PPI-2 was shown to be phosphorylated in vivo and several in vivo phosphoproteomic studies provide evidence for multisite phosphorylation (P3DB, http://www.p3db.org/). Six in vivo phosphorylation sites were confirmed using an in vitro reconstitution assay with three different protein kinases suggesting AtPPI-2 may functions as a general TOPP inhibitor that occupies a point of intersection between multiple kinase-mediated signaling pathways [9]. Among the in vivo phosphorylation sites, Ser45 which flanks the PxTPY motif was phosphorylated by at least two kinases while the Ser140 residue within the protein phosphatase (PP)-binding RKxHY motif was phosphorylated by three different kinases [9]. These two Ser residues are conserved in plants [5,9]. Results of biochemical analysis showed AtPPI-2 is capable of inhibiting activity of all nine *Arabidopsis* TOPP isoforms via the primary interaction motif RVxF [5]. In addition, it has been demonstrated that phosphorylated forms of AtPPI-2 had higher phosphatase-inhibitory activity in comparison with the non-phosphorylated form [9].

Although in mammalian systems PPI-2 is involved in cell cycle regulation [10] little is known about the physiological function of PPI-2 in plant cells. It was recently reported that in addition to the known interaction with TOPPs, AtPPI-2 also physically interacts with SnRK2 family kinases and the ABA receptor PYL [11]. Furthermore, transgenic expression of a reporter gene (GUS) driven by an AtPPI-2 promoter revealed strong expression of the reporter gene in all organs of *Arabidopsis* seedlings [11]. Subcellular localization showed that AtPPI-2 is targeted to the nucleus, however, tandem affinity purification revealed several chloroplastic and/or cytosolic interacting proteins in addition to TOPPs [5]. The results of these studies suggest a potential extra-nuclear role for this protein inhibitor [11].

In the present study, we characterize an AtPPI-2 T-DNA knockdown mutant. The complex, reduced growth phenotype supports the notion that AtPPI-2 is a global regulator of TOPPs, and possibly other proteins [11]. As a stunted growth phenotype can be attributed to multiple cellular processes or pathways we turned to a multidisciplinary experimental approach to determine the molecular and biochemical impact of a PPI-2 knockdown. Proteomics has advantages over other systems approaches in that the data directly reflect the final product of gene regulation, and ultimately the phenotype. Ultimately, a proteomics-driven strategy was selected to compare WT and PPI-2 knockdown plants in order to understand the cause of the growth phenotype. Comparative, label-free proteomics revealed a range of differences in protein abundance from various cellular processes including chloroplast development, ribosome biogenesis, and transporter activity in the AtPPI-2 mutant relative to WT *Arabidopsis*. Many of the differentially abundant proteins are predicted to be chloroplast-localized and ribosomes-associated. It seems unlikely that these proteins are direct targets of AtPPI-2 though plastid development could be a target of the TOPP proteins that AtPPI-2 is known to effect.

2. Materials and methods

2.1. Plant materials, growth conditions and RT-PCR analysis

The T-DNA insertion line (Salk_110571C) in *Arabidopsis* (Columbia-0) was obtained from ABRC. These seeds were further selected by growing on 0.5× MS medium (Sigma, St Louis, USA) containing 50 mg/L Kanamycin. Five to ten T_1 plants that carried Kanamycin resistance were transferred to soil and allowed to self-fertilize to produce T_2 plants. For physiological and proteomic analysis, these T_2 seedlings were grown on 0.5× MS medium, pH 5.7, 1% (w/v) sucrose supplemented with 1% (w/v) phytoagar (RPI Corporation, IL USA) unless otherwise indicated. For soil growth, seeds were sown in a 1:1 mixture of water-saturated vermiculite and peat moss-enriched soil in a controlled growth chamber (14-h-light/10-h-dark cycle, 23 °C d/20 °C night, 50% humidity, and light intensity of 200 μmol m^{-2} s^{-1}). For RT-PCR analysis, total RNA was isolated with the RNeasy Plant Mini Kit (Qiagen). The first strand was synthesized from equal amounts of total RNA with SuperScript III Reverse Transcriptase (Invitrogen). A total of three cycles 25, 30 and 35 were tested for the primer pairs. Twenty five cycles was insufficient to detect most of the transcripts; 30 and 35 cycles were optimal for transcript detection. Primers for genomic PCR, RT-PCR analysis and various complementations are listed in Supplemental Table S1.

2.2. Analysis of cell sizes

To analyze the cell size of the wild type and AtPPI-2 mutant plants, seedlings were grown in half MS media under continuous light for 2 weeks, and microscopic analysis of the root and leaf epidermal cells were conducted using an Olympus IX70 microscope equipped with an ORCA-AG digital camera (Japan). A minimum of ten roots and leaves were used to determine the cell sizes of wild type and AtPPI-2 mutant plants. The relevant parameter was measured on digital images using ImageJ [12].

2.3. Protein extraction from WT and AtPPI-2 mutant leaves

Leaves from wild type and AtPPI-2 mutant plants were frozen in liquid nitrogen and ground in a mortar to obtain a fine powder. Aliquots (500 mg) of the powder were subjected to phenol protein extraction as described by [13]. Protein concentration was determined using the BCA Protein Kit (Thermo Fisher Scientific, Houston, TX) using BSA as a standard. Protein extracts were prepared in three biological replicates. Gel electrophoresis was performed under denaturing conditions in 13% polyacrylamide gels using 20 mA per gel. Gels were stained with colloidal Coomassie blue stain under standard conditions.

2.4. Protein digestion and mass spectrometry analyses

Before protein digestion, gel lanes for each biological replicate were sliced into 10 equal-sized segments, diced into approximately 1 mm cubes with a scalpel, and transferred into a 96 well filtration plate (Multi Screen Solvinert Plates, Millipore). Tryptic digestion was carried out according to [14]. Each trypsin-digested and dried sample was reconstituted in 0.1% (v/v) formic acid and analyzed by nanospray-liquid chromatography-tandem mass spectrometry (nLC-ESI-MS/MS) performed with a LTQ Orbitrap XL mass spectrometer (Thermo Fisher, San Jose) as described previously [9].

For in-solution trypsin digestion, total proteins (100 μg) were digested with trypsin (1:50, w/v) for 20 h as described previously [15]. Prior to MS analysis, freeze dried-peptides were dissolved by adding 20 μL of 0.1% formic acid. Five microliters of each sample were analyzed using an EASY-nLC 1000 liquid chromatography (LC) system attached to an Orbitrap Elite mass spectrometer (Thermo Fisher, San Jose, CA). The chromatographic system was composed of ProteoPer II PicoFrit (150 μm id., 30 μm tip) analytical column (15 cm, New Objective, Woburn, MA) packed with of 5 μm, 300 Å Magic C18AQ media (Michrom Bioresources). The gradient was performed at a 1.2 μL min^{-1} flow rate using an acetonitrile gradient (1%–35% solvent B for 85 min; solvent A = 0.1% formic acid in mass spectrometry-grade water, solvent B = 0.1% formic acid in acetonitrile). Precursor masses were scanned with the analyzer set to FTMS; mass range, normal; resolution, 60,000; scan type, positive mode; data type, centroid; and a scan range of 400–2000 *m/z*. The 15 most abundant ions from

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