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Complex Assembly and Metabolic Profiling of *Arabidopsis thaliana* Plants Overexpressing Vitamin B_6 Biosynthesis Proteins

Jan Erik Leuendorf[a], Sonia Osorio[b], Agnieszka Szewczyk[c], Alisdair R. Fernie[b] and **Hanjo Hellmann[d,1]**

[a] Angewandte Genetik, Freie Universität Berlin, 14195 Berlin, Germany
[b] Max-Planck-Institut für Molekulare Pflanzenphysiologie, Potsdam-Golm 14476, Germany
[c] Pharmaceutical Faculty of the Collegium Medicum, Jagiellonian University, Krakow, Poland
[d] School of Biological Sciences, Washington State University, Pullman, USA

ABSTRACT In plants, vitamin B_6 biosynthesis requires the activity of PDX1 and PDX2 proteins. *Arabidopsis thaliana* encodes for three PDX1 proteins, named PDX1.1, 1.2, and 1.3, but only one PDX2. Here, we show *in planta* complex assembly of PDX proteins, based on split-YFP and FPLC assays, and can demonstrate their presence in higher complexes of around 750 kDa. Metabolic profiling of plants ectopically expressing the different PDX proteins indicates a negative influence of PDX1.2 on vitamin B_6 biosynthesis and a correlation between aberrant vitamin B6 content, *PDX1* gene expression, and light sensitivity specifically for PDX1.3. These findings provide first insights into *in planta* vitamin B_6 synthase complex assembly and new information on how the different PDX proteins affect plant metabolism.

Key words: Abiotic/environmental stress; metabolomics; metabolic regulation; molecular physiology.

INTRODUCTION

Vitamin B_6 is an important compound for all living organisms that serves, in its phosphorylated form, as an enzymatic cofactor for more than 100 biochemical reactions. It is mostly involved in amino acid metabolism, but also plays a role in storage carbohydrate degradation and fatty acid metabolism (Mooney et al., 2009). In addition, the compound is recognized as a potent antioxidant and is discussed to have numerous benefits for human health (Hellmann and Mooney, 2010).

Three B_6 vitamers—pyridoxine, pyridoxal, and pyridoxamine—have been described that differ only at their 4′ position in either an hydroxyl, an aldehyde, or an amino group, respectively (Mooney and Hellmann, 2010). Recently, the *de novo* biosynthetic pathway of vitamin B_6 was resolved in plants and fungi. It comprises two protein families, named Pyridoxine Biosynthesis 1 (PDX1) and PDX2 (Ehrenshaft et al., 1999; Wetzel et al., 2004; Tambasco-Studart et al., 2005, 2007). These proteins form a PLP synthase complex that directly synthesizes pyridoxal-5′-phosphate (PLP) from ribose-5-phosphate or ribulose-5-phosphate, in combination with either glyceraldehydes-3-phosphate or dihydroxyacetone phosphate and glutamine (Tambasco-Studart et al., 2005). While PDX2 acts as a glutaminase, which hydrolyze glutamine to glutamate in order to supply nitrogen for the PLP heterocycle, PDX1 arranges the final pyridine ring closure (Tambasco-Studart et al., 2005, 2007).

Crystallization studies in *Saccharomyces cerevisiae*, *Bacillus subtilis*, and *Geobacillus stearothermophilus* revealed that their PDX orthologs assemble to a synthase complex with a cogwheel-like structure (Strohmeier et al., 2006; Zein et al., 2006). Here, the core of the PLP synthase consists of 12 PDX1 proteins that interact in two hexameric layers joining face to face to form a dodecamer onto which 12 PDX2 monomers attach (Strohmeier et al., 2006; Zein et al., 2006; Neuwirth et al., 2009). Though crystallization studies are missing for plant PLP synthase complexes, it is likely that they assemble to a similar PLP synthase complex as described for *S. cerevisiae*, *B. subtilis*, or *G. stearothermophilus*.

The plant *Arabidopsis thaliana* encodes for three *PDX1* genes—*PDX1.1*, *1.2*, and *1.3*—but only for one *PDX2* (Tambasco-Studart et al., 2005; Wagner et al., 2006). For PDX1.2, the biological role remains to be shown. Evidently,

[1] To whom correspondence should be addressed. E-mail hellmann@wsu.edu, fax (001)-509-335-3184, tel. (001)-509-335-2762.

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based on *in vitro* experiments and protein–protein interaction data, the protein does not participate in the synthesis of vitB_6 (Tambasco-Studart et al., 2005; Wagner et al., 2006). Noteworthy, PDX1.2-like proteins are widely common in higher plants but appear to be absent in fungi (Leuendorf and Hellmann, unpublished data), making it likely that they represent plant-specific factors potentially affecting vitB_6 biosynthesis by assembling with other PDX1 proteins. In contrast, it has been established that PDX1.1, PDX1.3, and PDX2 catalyze the formation of PLP (Tambasco-Studart et al., 2005; Titiz et al., 2006). *Arabidopsis* plants affected in either PDX1.1 or PDX1.3 show severe developmental defects including shorter root growth, smaller rosette leaves, and delayed flowering time (Titiz et al., 2006; Wagner et al., 2006). Not surprisingly, loss of PDX2 or PDX1.1 and PDX1.3 is lethal and results in aborted embryogenesis as early as the globular stage (Tambasco-Studart et al., 2005, 2007). In addition, metabolic analyses of both leaves and roots of a *pdx1.3* loss-of-function mutant revealed broad changes in amino acid and fatty acid contents (Wagner et al., 2006; Lytovchenko et al., 2009). Surprisingly, many of these compounds were strongly increased, although vitB_6 levels were significantly reduced (Wagner et al., 2006; Leuendorf et al., 2008; Lytovchenko et al., 2009). Furthermore, *Arabidopsis PDX* genes are up-regulated by a variety of abiotic stress conditions, and *PDX* mutants react hypersensitively when exposed to high salt and mannitol concentrations, oxidative stress, high light, or UV-B light (Chen and Xiong, 2005; Titiz et al., 2006; Denslow et al., 2007; Gonzalez et al., 2007; Havaux et al., 2009), portraying the PDX family not only as key proteins for vitB_6 biosynthesis, but also as important factors for abiotic stress tolerance in plants.

Here, we describe the *in planta* complex assemblies of PDX proteins and their impact on metabolism in *Arabidopsis*. While ectopic expression of PDX1.1, PDX1.2, and PDX2, under the control of a 35S promoter and with an N-terminal myc-epitope, did not affect growth and development of the corresponding transgenic plants, ectopic expression of PDX1.3 yielded a high frequency of strong dwarf-like phenotypes. Correlating with these developmental defects are reduced *PDX1.3* expression and vitB_6 content levels in the respective plants. The myc-tagged PDX1.1 and PDX1.3 proteins are functional, as demonstrated by complementation of a *pdx1.3* loss-of-function mutant. Metabolic profiling of all transgenic plants revealed dramatic changes only in the plants with reduced *PDX1.3* levels. Interestingly, all PDX1 proteins interact with each other *in planta* and assemble into larger protein complexes of an estimated size similar to that of a PLP synthase.

RESULTS

Arabidopsis Plants Are Hypersensitive to Ectopic myc:PDX1.3 Expression

The current knowledge about protein–protein assemblies of PDX proteins from plants is very limited and only a few studies have investigated this aspect so far (Wagner et al., 2006; Leuendorf et al., 2008). Especially for PDX1.2, for which a biological role has not been assigned yet, it is of interest to understand whether the protein can assemble *in planta* with other PDX proteins and how its overexpression affects plant metabolism. To generate better knowledge about these questions, we introduced myc-epitope-tagged *PDX1* and *PDX2* expression constructs under the control of a cauliflower mosaic 35S promoter (further denoted as *P35S:myc:PDX*) into Col0 wild-type plants.

Nearly all plants overexpressing PDX1.1 developed like wild-type plants (Figure 1C); only in a very few cases were mild growth defects observed, most obviously being the appearance of slightly yellowish leaves (two out of 55, or 3.6%). Furthermore, we never observed any visible phenotypes for *P35S:myc:PDX1.2* or *P35S:myc:PDX2* plants (Figure 1C). Interestingly, a high frequency (56/200, or 28%) of the *P35S:myc:PDX1.3* plants showed a strongly stunted growth and exhibited bleached leaves (further denoted as either *P35S:myc:PDX1.3*^{stunted} or *P35S:myc:PDX1.3*^{normal}, respectively) (Figure 1A). They resembled the *GFP:*$_{PDX1.3}$ overexpressing plants we had described earlier that were affected in expression of both *PDX1.1* and *PDX1.3* as well as the transgene (Wagner et al., 2006). However, despite their strong developmental defects, and in contrast to the previously described co-suppressor lines, the current *P35S:myc:PDX1.3* plants were viable and produced offspring. In addition, the phenotype appeared to be more prominent in the shoot because primary root development was not affected (Supplemental Figure 1). Recent findings reported on the light sensitivity of *pdx1* mutants and demonstrated improved growth under low-light conditions (Titiz et al., 2006; Havaux et al., 2009). We also observed improved growth rates for *P35S:myc:PDX1.3*^{stunted} plants, when cultured under short-day conditions (Figure 1B). Although these mutant plants still had reduced rosette growth (Figure 1B and 1C), the overall development was improved, with larger and less bleached rosette leaves (compare Figure 1A and 1B).

Quantification of gene expression showed robustly increased expression levels of total *PDX1.1*, *PDX1.2*, and *PDX2* (endogenous plus the respective transgene), in *P35S:myc:PDX1.1*, *P35S:myc:PDX1.2*, and *P35S:myc:PDX2* plants, respectively (Figure 2A and 2B). However, we could not detect increased levels for total *PDX1.3* expression in either *P35S:myc:PDX1.3*^{normal} or *P35S:myc:PDX1.3*^{stunted} plants (Figure 2C and 2D). Rather, total *PDX1.3* expression levels were reduced in both lines to either 60 or 28%, respectively, when compared to wild-type plants. *PDX1.3* expression was also reduced in *P35S:myc:PDX1.1* (to around 45%) and *P35S:myc:PDX1.2* (to around 33%) plants (Figure 2A and 2B). However, *P35S:myc:PDX1.3*^{normal} plants also showed a decrease in *PDX1.1* expression (around 55% reduction), which, surprisingly, was even higher than that observed for PDX1.3 plants with a stunted phenotype (around 33% reduction) (Figure 2B and 2C). In contrast, *PDX2* expression was slightly