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Proline Accumulation Is Regulated by Transcription Factors Associated with Phosphate Starvation[1][OPEN]

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Pro accumulation in plants is a well-documented physiological response to osmotic stress caused by drought or salinity. In Arabidopsis (*Arabidopsis thaliana*), the stress and ABA-induced Δ1-PYRROLINE-5-CARBOXYLATE SYNTHETASE1 (*P5CS1*) gene was previously shown to control Pro biosynthesis in such adverse conditions. To identify regulatory factors that control the transcription of *P5CS1*, Y1H screens were performed with a genomic fragment of *P5CS1*, containing 1.2-kB promoter and 0.8-kb transcribed regions. The myeloblastosis (MYB)-type transcription factors PHOSPHATE STARVATION RESPONSE1 (PHR1) and PHR1-LIKE1 (PHL1) were identified to bind to *P5CS1* regulatory sequences in the first intron, which carries a conserved PHR1-binding site (P1BS) motif. Binding of PHR1 and PHL1 factors to P1BS was confirmed by Y1H, electrophoretic mobility assay and chromatin immunoprecipitation. Phosphate starvation led to gradual increase in Pro content in wild-type Arabidopsis plants as well as transcriptional activation of *P5CS1* and PRO DEHYDROGENASE2 genes. Induction of *P5CS1* transcription and Pro accumulation during phosphate deficiency was considerably reduced by *phr1* and *phl1* mutations and was impaired in the ABA-deficient *aba2-3* and ABA-insensitive *abi4-1* mutants. Growth and viability of *phr1phl1* double mutant was significantly reduced in phosphate-depleted medium, while growth was only marginally affected in the *aba2-3* mutants, suggesting that ABA is implicated in growth retardation in such nutritional stress. Our results reveal a previously unknown link between Pro metabolism and phosphate nutrition and show that Pro biosynthesis is target of cross talk between ABA signaling and regulation of phosphate homeostasis through PHR1- and PHL1-mediated transcriptional activation of the *P5CS1* gene.

Pro is known to accumulate to high levels in numerous plant species at low water potential caused by drought and salinity (Kemble and MacPherson, 1954; Szabados and Savouré, 2010; Verslues and Sharma, 2010). Furthermore, several reports describe Pro accumulation in response to other types of stress provoked by heavy metals (Schat et al., 1997; Jiang et al., 2012), oxidative agents (Yang et al., 2009; Ben Rejeb et al., 2015), or certain pathogens (Fabro et al., 2004; Senthil-Kumar and Mysore, 2012). Different protective functions were attributed to Pro, suggesting that it acts as osmoprotectant, stabilizing cellular structures and enzymes and scavenging reactive oxygen species (ROS), and maintains redox equilibrium in adverse conditions (Csonka, 1981; Delauney and Verma, 1993; Hoque et al., 2008; Székely et al., 2008; Szabados and Savouré, 2010; Verslues and Sharma, 2010; Sharma et al., 2011; Zouari et al., 2016). Besides the much-studied osmoprotective function, Pro has been implicated in the regulation of plant development, including flowering, pollen, embryo, and leaf development (Székely et al., 2008; Mattioli et al., 2009).

Pro content is regulated by the balance between its biosynthesis and degradation. The Glu-derived pathway is the most important for Pro biosynthesis in plants and is composed of two consecutive steps catalyzed by the bifunctional enzyme *Δ^1-pyrroline-carboxylate synthetase* (*P5CS*), that synthetizes Glu semialdehyde from Glu (Hu et al., 1992; Yoshiba et al., 1995; Funck et al., 2008). Glu semialdehyde is spontaneously converted to pyrroline-5-carboxylate (P5C) and is subsequently reduced to Pro by P5C reductase (P5CR; Delauney and Verma, 1990; Funck et al., 2012). The whole process is controlled by the first and rate-limiting step, mediated by the feedback regulated P5CS enzyme, which in Arabidopsis (*Arabidopsis thaliana*) is encoded by two genes, *P5CS1* (*AT2G39800*) and *P5CS2* (*AT3G55610*; Zhang et al., 1995; Strizhov et al., 1997; Székely et al., 2008; Szabados and Savouré, 2010). The production of Pro from Orn represents an alternative biosynthetic pathway and is mediated by Orn-δ-aminotransferase (δOAT, *AT5G46180*; Delauney et al., 1993). The importance of this pathway in Pro accumulation has, however, been questioned, as stress-induced Pro accumulation was not affected in knockout *oat* mutants (Funck et al., 2008). *P5CS2* is considered to be a housekeeping gene with constitutive expression throughout the plant, while the stress-induced *P5CS1* responds to hyperosmotic stress and is regulated by abscisic acid (ABA)-dependent and independent signals (Savouré et al., 1997; Strizhov et al., 1997; Székely et al., 2008;

[1] This work was supported by OTKA-NN110962, OTKA-K7623, OTKA-K109719, GINOP 2.3.2-15-2016-00001, and NTP-EFÖ-P-15-0157 grants.

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D.A. performed most of the experiments; G.V.H. and L.S. designed and supervised the experiments; G.S. provided technical assistance.

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www.plantphysiol.org/cgi/doi/10.1104/pp.17.00791

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Sharma and Verslues, 2010). While *P5CS2* can be activated by incompatible plant-pathogen interactions associated with hypersensitive response (Fabro et al., 2004), *P5CS1* induction was shown to depend on light (Abrahám et al., 2003) and respond to ROS signals (Ben Rejeb et al., 2015). Besides ABA and light, calcium and lipid signals were implicated in regulation of *P5CS* genes and Pro biosynthesis (Thiery et al., 2004; Parre et al., 2007). The *P5CS1* promoter contains sequence motifs that are conserved in related Brassicaceae species and can be binding sites for basic Leu zipper (bZIP), myeloblastosis (MYB), c-Myc (MYC), APETALA2/Ethylene-responsive element binding protein (AP2/EREBP), C2H2 Zn finger protein (C2H2_Zn) type transcription factors (Supplemental Fig. S1; Fichman et al., 2015). A recent chromatin immunoprecipitation (ChIP)-seq study suggests that several ABA-regulated transcription factors (TFs) can bind to the promoter region of the *P5CS1* gene (Supplemental Fig. S2; Song et al., 2016).

Pro degradation is an oxidative process, mediated by the rate-limiting Pro dehydrogenase (PDH) and P5C dehydrogenase (P5CDH) enzymes, both localized in the mitochondria, encoded by two and one genes, respectively (Kiyosue et al., 1996; Deuschle et al., 2001; Servet et al., 2012). Similar to the *P5CS* genes, the Arabidopsis *PDH1* and *PDH2* genes have remarkable differences in their transcriptional regulation (Funck et al., 2010). *PDH1* is induced by Pro or low osmolarity during stress release and was shown to be controlled by the bZIP transcription factors (Satoh et al., 2004; Weltmeier et al., 2006). Binding of S-type bZIP factors to the ACTCAT cis-acting element of the *PDH1* promoter was demonstrated and shown to be essential for hypo-osmolarity-dependent induction of this gene (Satoh et al., 2004; Weltmeier et al., 2006). In contrast, no transcription factors have been characterized that regulate *P5CS1*.

Phosphorus is an essential constituent of biomolecules, such as phospholipids, nucleic acids, and ATP, and is important for reversible protein modification. Soluble phosphate is limited in many soils due to insoluble complex formation with different metals or by microbial consumption converting an inorganic phosphate into an organic one, which is not available to plants. Phosphate deficiency affects 70% of cultivated land and seriously reduces crop yields, turning phosphate fertilization one of the essential elements of modern agriculture (Lynch, 2011; Herrera-Estrella and López-Arredondo, 2016). Phosphate deficiency generates a complex stress in plants, reduces shoot growth and root elongation, but enhances formation of lateral roots and root hairs, which facilitates phosphate acquisition (Lynch, 2011). Plants take up phosphorus as inorganic orthophosphate (Pi), mediated by high- and low-affinity phosphate transporters that are influenced by root system architecture, organic acid exudation, and soil microbes, mainly arbuscular mycorrhizal fungi (López-Arredondo et al., 2014). Physiological response to phosphate starvation includes changes in glycolysis and mitochondrial electron transport, excretion of several organic acids, enhancement of enzyme activities facilitating phosphate recycling and transport, anthocyanine accumulation, and leaf bleaching (Plaxton and Tran, 2011). Comprehensive metabolic profiling of phosphate-starved Arabidopsis plants revealed massive changes in primary and secondary metabolites, such as organic acids, sugars, glucosinolates, flavonoids, and amino acids, including Pro (Morcuende et al., 2007; Pant et al., 2015b; Valentinuzzi et al., 2015).

Regulation of phosphate homeostasis requires complex signaling network coordinating uptake, transport, and metabolism of this essential nutrient (Doerner, 2008; Rouached et al., 2010). Genome-wide transcript profiling allowed the identification of large sets of phosphate-regulated genes and defined the most important regulons responding to phosphate deprivation in shoots and roots (Morcuende et al., 2007; Müller et al., 2007; Bustos et al., 2010; Woo et al., 2012). The MYB-type PHOSPHATE STARVATION RESPONSE1 (PHR1) and PHR1-LIKE1 (PHL1) factors are the most important transcriptional regulators, which control the expression of target genes and define metabolic and developmental responses to phosphate deficiency (Rubio et al., 2001; Nilsson et al., 2007; Pant et al., 2015b). PHR1 was shown to be essential for adaptation to light stress and to maintain photosynthesis during Pi starvation (Nilsson et al., 2012). PHR1 was reported to regulate common transcriptional responses during phosphate starvation and hypoxia under light (Klecker et al., 2014). PHR1 is apparently a key regulator of metabolic changes during phosphorus limitation controlling amino acid pools and lipid remodeling, a dramatic response to phosphate deficiency (Pant et al., 2015a, 2015b). Starvation-regulated genes are enriched for the PHR1 binding site (P1BS) motif in their promoters, which binds both PHR1 and PHL1 factors. P1BS is important for high-level induction of PHR1 target genes during phosphate starvation (Rubio et al., 2001; Karthikeyan et al., 2009; Bustos et al., 2010). Interestingly, *PHR1* and *PHL1* genes are not induced by phosphate deprivation but are essential for transcriptional activation of the downstream target genes (Bustos et al., 2010).

In this study, we report the identification of PHR1 and PHL1 transcription factors as positive regulators of *P5CS1* transcription during phosphate starvation. We demonstrate that Pro accumulation is a consequence of phosphate starvation and is controlled by PHR1 and PHL1, which are essential for the enhanced expression of the *P5CS1* gene in such conditions. Our results suggest that ABA-dependent signals regulate the Pro biosynthetic pathway not only during salt and osmotic stress, but also in phosphate-starved plants. Our results reveal an important connection between Pro metabolism and phosphate nutrition and show that Pro accumulation is part of a large-scale metabolic response that is triggered by phosphate starvation.

RESULTS

Phosphate Starvation Response Factors Identified by a Yeast One-Hybrid (Y1H) Screen

To identify the transcription factors that bind to the regulatory region of *P5CS1* gene, Y1H screens were performed using the 1.95-kb-long genomic fragment of

P5CS1 as bait. The promoter and 5′ UTR region of the *P5CS1* gene contains most conserved cis elements, which were predicted as potential TF binding sites (Supplemental Figs. S1 and S2; Fichman et al., 2015; Song et al., 2016). Besides the 5′ regulatory region, introns have been reported to carry regulatory elements with capacity to enhance transcription in a number of genes (Lohmann et al., 2001; Casas-Mollano et al., 2006; Gallegos and Rose, 2015), including the high-affinity phosphate transporter *AtPht1;4* (Karthikeyan et al., 2009). Therefore, we decided to include a 1.2-kb 5′ region (promoter and 5′ UTR) and a 0.8-kb transcribed region (two exons and the first intron) in the bait genomic fragment (Fig. 1A). Eighty-six yeast colonies were identified that grew on selective medium and contained cDNA inserts of different lengths. cDNAs were rescued, and their nucleotide sequence was determined to identify the encoded proteins by sequence homology search. One yeast colony carried the full-length cDNA of the MYB-type transcription factor *PHR1*(*AT4G28610*), and four independent colonies contained cDNAs encoding the closely related *PHL1* (*AT5G29000*), known regulators of the Arabidopsis phosphate starvation response (Bustos et al., 2010). Analysis of the *P5CS1* bait sequence identified a conserved PHR1 binding site (P1BS; GAATATTC; Karthikeyan et al., 2009) in the first intron of the *P5CS1* gene (Fig. 1A; Supplemental Fig. S1), suggesting that this motif might be responsible for binding the identified TFs.

Binding of PHL1 and PHR1 to the bait was verified by retransformation of the bait-containing yeast strain with cloned factors, by electrophoretic mobility assay (EMSA) and in vivo ChIP assays. Bait-containing yeast cells were able to proliferate on selective medium when they were expressed either the PHR1 or PHL1 cDNAs, cloned in the pGAD424 vector, but failed to grow with the empty pGAD424 vector (Fig. 1B). Interaction of the identified PHR1 and PHL1 factors with *P5CS1* genomic

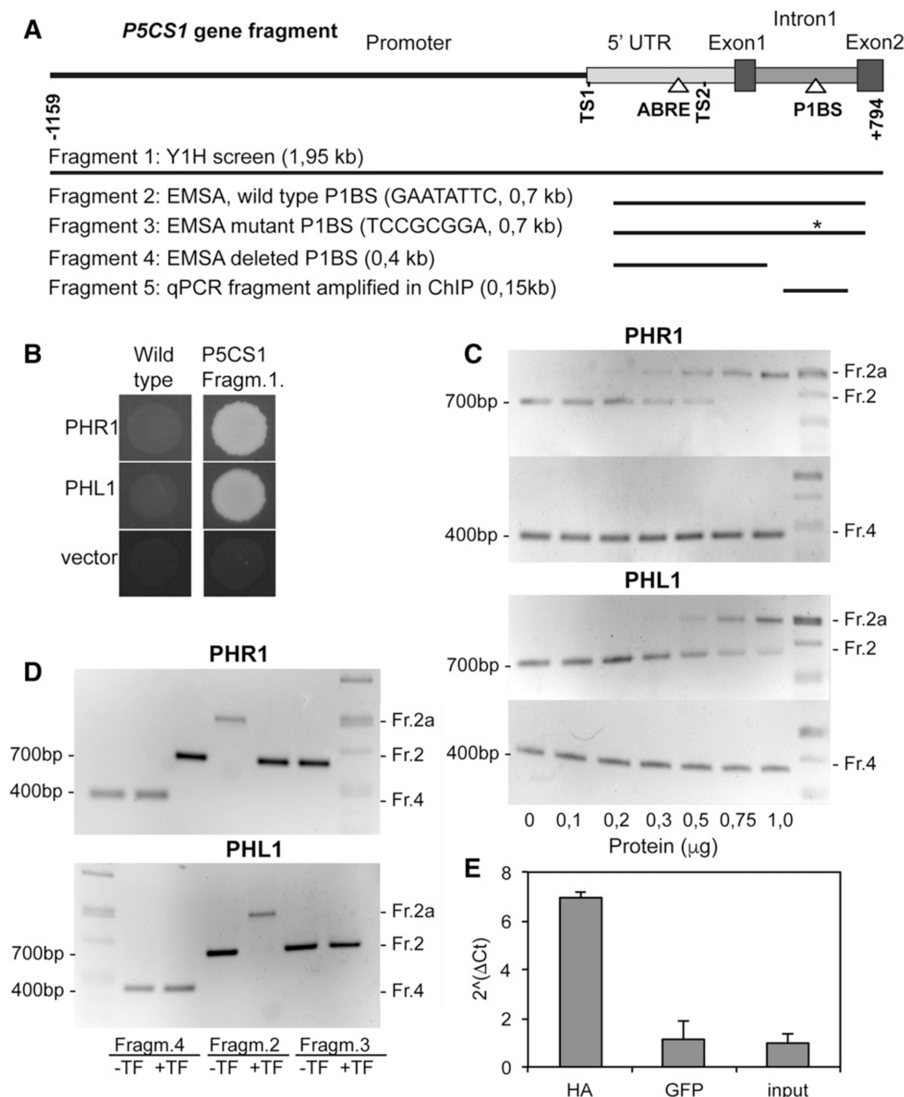

Figure 1. PHR1 and PHL1 factors bind to *P5CS1* regulatory sequences. A, Schematic map of the *P5CS1* regulatory region, including promoter (−1.2 kb), 5′ UTR, first and second exons, and the first intron, to +0.8 kb. Schematic map was adapted from a previous report (Fichman et al., 2015). Positions of promoter, 5′ UTR, exons, and first intron, and predicted ABRE and P1BS motifs are indicated. Fragments used for Y1H screen (Fragment 1), EMSA (Fragments 2–4), and ChIP amplification (Fragment 5) are shown. B, Y1H test of PHR1 and PHL1 factors and *P5CS1* genomic fragment as bait. C, Electrophoretic mobility assay (EMSA) of purified PHR1 and PHL1 factors with 0.7 kb and 0.4 kb genomic fragments of the *P5CS1* gene (Fragments 2 and 4). Note that increasing amount of PHR1 and PHL1 protein enhance the high mobility complex with the 0.7-kb fragment. D, EMSA with wild-type (Fragment 2), mutated (Fragment 3), and deletion derivative (Fragment 4) of the region containing the P1BS site with 1 μg purified PHR1 or PHL1 protein. Note, that electromobility shift can be observed only when Fragment 2 was used, which contained the wild-type P1BS sequence, but not with the mutated or deleted version (Fragments 3 and 4, respectively). E, ChIP assay. Normalized quantitative PCR data are shown, where the amount of P1BS-containing PCR product (Fragment 5) was related to PCR product obtained from a nonspecific intergenic region. HA, Samples precipitated with anti-HA beads; GFP, samples precipitated by anti-GFP beads; Input, qPCR data with samples without immunoprecipitation. Bars on diagrams indicate SE of three biological replicates.

sequences was subsequently tested by in vitro (EMSA) and in vivo (ChIP) protein-DNA binding assays. EMSA was performed with purified PHR1 and PHL1 proteins. A 700-bp *P5CS1* genomic fragment, containing the conserved P1BS motif, a 700-bp fragment with mutated P1BS motif (GAATATTC was changed to TCCGCGGA), and a 400-bp deletion derivative, missing the P1BS sequence, was incubated with purified PHR1 and PHL1 proteins and assayed with EMSA. Increasing amount of PHR1 and PHL1 proteins led to gradual enhancement in the electrophoretic mobility shift of the 700-bp genomic fragment on agarose gels. This gel shift was, however, not observed with the 400-bp fragment, which lacked the predicted P1BS site (Fig. 1, C and D). Electrophoretic mobility of the mutated 700-bp fragment, in which the P1BS motif was eliminated by point mutagenesis, was unchanged when it was coincubated with PHR1 or PHL1 proteins, suggesting that this sequence element was essential for protein binding (Fig. 1D). The EMSA assay therefore confirmed that the P1BS motif of the *P5CS1* first intron is essential and sufficient for binding of both PHR1 and PHL1 proteins.

To confirm the interaction of PHR1 factor with *P5CS1* genomic sequenced, chromatin immunoprecipitation was performed, using phosphate-starved Arabidopsis plants expressing the PHR1:HA gene fusion. Immunoprecipitation of the isolated chromatin was carried out with anti-HA microbeads, while Anti-GFP microbeads were employed as control. Quantitative PCR was employed to amplify the target DNA as well as nonspecific DNA fragments from unrelated chromosomal regions. After background subtraction and normalization to control PCR reactions, specific enhancement of HA-immunoprecipitated target DNA was detected when compared to mock samples (Fig. 1E). Experiments were repeated three times with similar results. ChIP experiments could therefore confirm that interaction of the PHR1 transcription factor and target DNA in the *P5CS1* gene occurs in plant cells.

Pro Accumulates during Phosphate Starvation

Pro accumulation during osmotic and salt stress is a well-documented metabolic response, which was shown to be controlled by both ABA-dependent and -independent regulatory pathways (Yoshiba et al., 1995; Savouré et al., 1997; Strizhov et al., 1997; Abrahám et al., 2003; Sharma and Verslues, 2010). Binding of the PHR1 and PHL1 transcription factors to *P5CS1* sequences suggested that Pro metabolism can also be influenced by phosphate levels. Pro contents and transcription of key genes in Pro metabolism were therefore tested under phosphate deficiency. Phosphate deprivation in our experimental system caused retardation of rosette growth, root elongation, accumulation of anthocyanine and hydrogen peroxide, enhanced lipid peroxidation, and more than one-thousand-fold induction of *induced by phosphate starvation1* (*IPS1*) gene (*AT3G09922*), known to be responsive to phosphate starvation (Supplemental Fig. S3; Martín et al., 2000). When wild-type Arabidopsis plants were cultured on medium lacking inorganic phosphate, Pro concentration started to increase after 7 d of starvation, and after 14 d it was seven times higher than in control, containing 2.5 mM phosphate (Fig. 2A). When culture medium was supplemented by additional phosphate (10 mM), Pro levels were not affected (Fig. 2A). Expression of genes that are known to control Pro metabolism was considerably altered by phosphate starvation. *P5CS1* expression increased 3- to 5-fold, while *PDH2* expression was enhanced by 4- to 6-fold in shoots and roots under phosphate starvation Figs. 2B and 3A). Expression of the other tested genes (*P5CS2*, *P5CR*, *PDH1*, *P5CDH*) was not or only slightly changed (Figs. 2B and 3B). Nevertheless, transcript levels of the induced *P5CS1* and *PDH2* genes were still lower than the related *P5CS2* or *PDH1* genes, respectively, which were not influenced by phosphate levels (Fig. 2B).

To study spatial and temporal changes in P5CS1 protein levels during phosphate starvation, fluorescence of the GFP-tagged P5CS1 was monitored in transgenic Arabidopsis plants harboring the genomic P5CS1-GFP gene fusion (Székely et al., 2008). GFP-derived fluorescence in roots of transgenic plants was weak and was detectable only close to the root tips of plants cultured on standard 1/2 Murashige and Skoog culture medium. GFP-derived fluorescence was, however, well visible in P5CS1-GFP plants on plates lacking phosphate. Enhanced fluorescence was detectable in root elongation zone as early as 4 d after transfer to phosphate-deprived medium, when other phenotypic alterations were not yet visible (Fig. 2C). Root proliferation is a characteristic developmental response of phosphate-starved plants, which facilitates phosphate uptake from Pi deficient soils (Lynch, 2011). GFP-derived fluorescence was strong in proliferating lateral roots also, which was typical in plants after 7 d or longer phosphate starvation (Fig. 2C; Supplemental Fig. S4). Intracellular localization of P5CS1-GFP fusion protein was similar in leaf cells in phosphate-starved and control plants (not shown). Western hybridization with anti-GFP antibody detected the P5CS1-GFP protein in phosphate-starved transgenic plants, but not in the plants grown on standard culture medium, containing 2.5 mM Pi. Similar western signal was obtained in plants that were treated by moderate salt stress, known to enhance *P5CS1* transcription (Fig. 2D; Strizhov et al., 1997). These results demonstrate that *P5CS1* is activated during phosphate starvation and suggest that the enhanced Pro biosynthesis leads to Pro accumulation under these conditions.

PHR1 and PHL1 Transcription Factors Regulate Pro Accumulation

To test the role of PHR1 and PHL1 factors in regulation of Pro metabolism, Pro accumulation in *phr1*, *phl1*, and *phr1phl1* double mutants were compared to wild-type plants under phosphate starvation. Pro levels in these mutants were similar to wild-type plants in

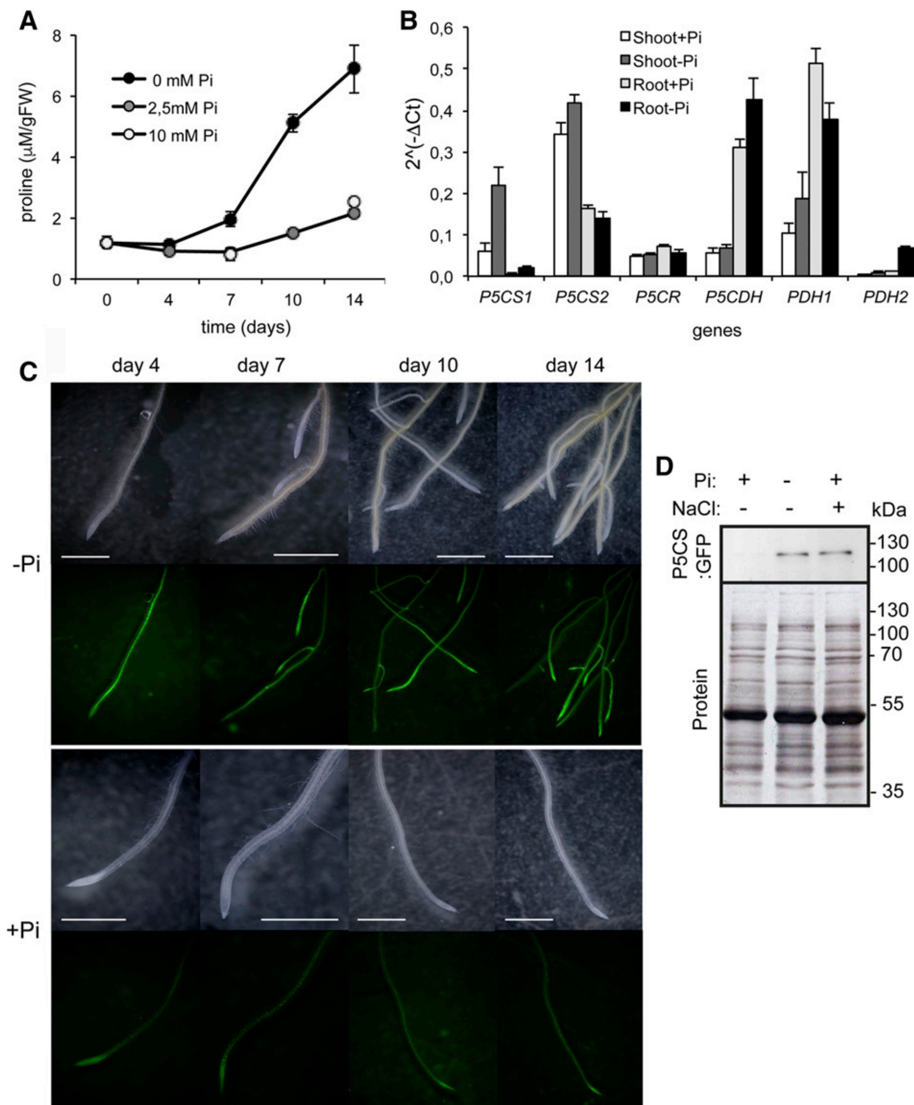

Figure 2. Phosphate starvation leads to Pro accumulation and *P5CS1* activation in Arabidopsis plants. A, Pro levels in wild-type Arabidopsis plants grown with or without phosphate for 14 d. B, Expression profiles of genes which control Pro biosynthesis (*P5CS1*, *P5CS2*, *P5CR*) or Pro catabolism (*P5CDH*, *PDH1*, *PDH2*) in wild-type Arabidopsis plants grown with or without phosphate for 14 d. Values were normalized to transcript levels of actin gene. C, GFP-derived fluorescence of P5CS1-GFP construct under phosphate starvation. Five-day-old seedlings expressing the genomic P5CS1-GFP fusion (Székely et al., 2008) were transferred to standard culture medium (+Pi) or medium deprived of phosphate (−Pi), and GFP-derived fluorescence was recorded in 3- to 4-d intervals. Note the enhanced GFP signals and proliferation of lateral roots in −Pi medium. D, Western detection of P5CS1-GFP protein in phosphate-starved (14 d on Pi-deficient medium) or salt-induced plants (14 d-old plants, treated with 75 mM NaCl for 24 h). Scale bar: 500 μm.

standard culture conditions but were 50% lower than in Col-0 plants during phosphate starvation (Fig. 3A). Salt and ABA are known to enhance free Pro content in most plants (Lehmann et al., 2010; Szabados and Savouré, 2010). Pro content was enhanced by salt and ABA treatments in *phr1* and *phl1* single mutants similar to wild-type plants but were significantly lower in the *phr1phl1* double mutant (Fig. 3A). These results suggest that PHR1 and PHL1 factors are important for Pro accumulation in phosphate-starved plants and can play a minor role in salt or ABA-induced Pro accumulation as well. To test the effect of *phr1* and *phl1* mutations on the expression of genes that control Pro biosynthesis and catabolism, transcript levels of *P5CS1*, *P5CS2*, *P5CR*, *PDH1*, *PDH2*, and *P5CDH* were monitored in phosphate-starved mutants. While *P5CS1* and *PDH2* were induced three to six times by phosphate deprivation in wild-type plants, activation of the *P5CS1* gene was minimal in *phr1* and *phr1phl1* mutants and was considerably reduced in *phl1*. Transcript levels of *PDH2* were similar to wild type plants in leaves of these mutants, and reduced in roots, while expression of the other prorelated genes was not altered during phosphate starvation (Fig. 3B). Our results are supported by gene expression data, available in supplementary files of transcript profiling experiments (Bustos et al., 2010). Although *P5CS1* and *P5CS2* transcripts were not distinguished in the Affymetrix 22.5K ATH1 chip commonly used in microarray-based transcript profiling, phosphate starvation considerably enhanced *P5CS1*/*P5CS2* and *PDH2* transcript levels and reduced *PDH1* expression but did not affect other genes in Pro metabolism (*P5CR*, *P5CDH*, *δOAT*; Supplemental Fig. S5; Bustos et al., 2010). When compared to wild-type plants, transcript levels of *P5CS1*/*P5CS2* and *PDH2* were clearly reduced in *phr1* and *phr1phl1* mutants (Supplemental Fig. S5). While *PHR1* and *PHL1* genes themselves are not induced in phosphate-starved plants, the encoded transcription factors are necessary for the activation of *P5CS1* and *PDH2* genes that are direct targets of PHR1 during

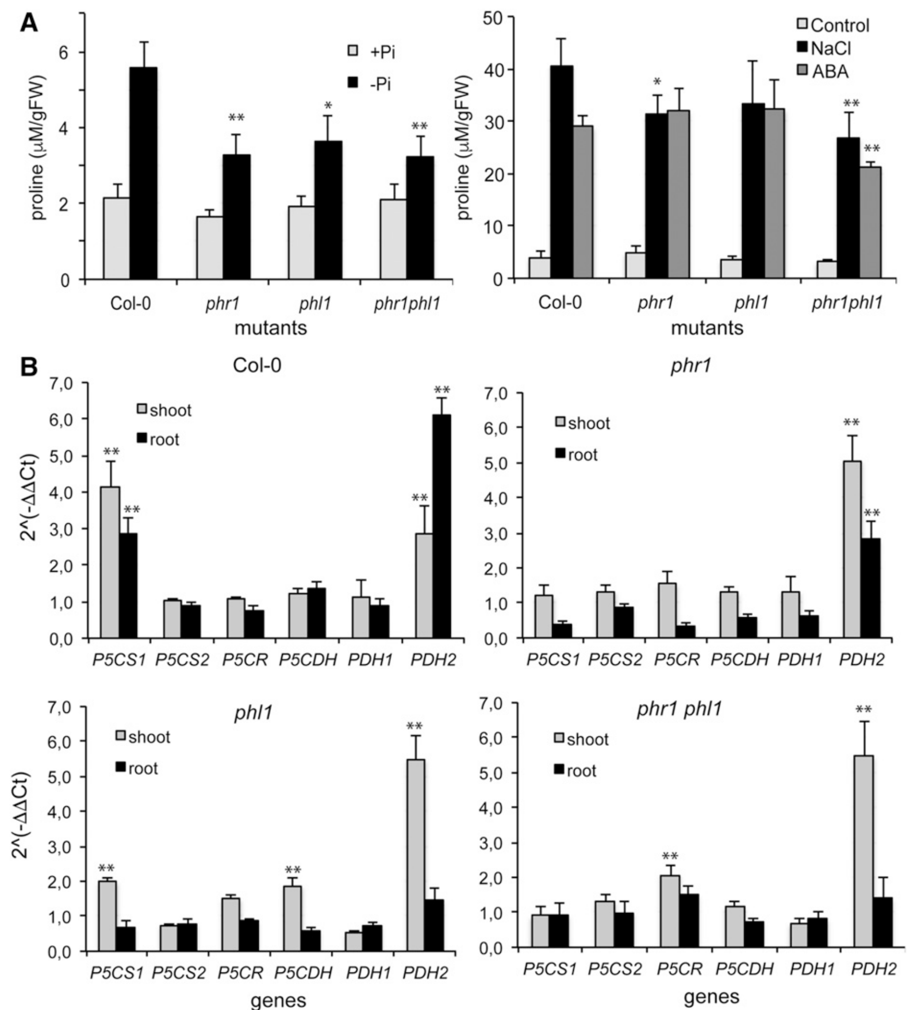

Figure 3. Pro metabolism in *phr1* and *phl1* mutants. A, Pro levels in wild type (Col-0) *phr1*, *phl1*, and *double phr1phl1* mutants subjected to phosphate starvation for 14 d or treated by 150 mM NaCl or 50 μM ABA for 3 d, after having grown on standard culture medium for 14 d. B, Transcript levels of genes controlling Pro metabolism in wild-type and mutant plants, growth with or without phosphate for 14 d. Relative transcript levels are shown, normalized to transcript data of plants grown on +Pi medium (2.5 mM Pi). Bars on diagrams indicate SE; * and ** show significant differences to Col-0 wild type (A) or to Pi+ medium (B) at $P < 0.05$ and $P < 0.005$, respectively (Student's *t* test).

phosphate deprivation (Fig. 3B; Supplemental Figs. S6 and S7; Bustos et al., 2010).

ABA Regulates Pro Accumulation during Phosphate Starvation

In our experimental system, phosphate deprivation reduced growth of wild-type Arabidopsis plants by nearly 50%. Rosette growth of *phr1, phl1*, and *phr1phl1* mutants was similar to wild-type plants in standard, phosphate-containing medium, while in the absence of phosphate, *phr1* and *phr1phl1* mutants were significantly smaller than wild-type (Fig. 4, A and B). Rosette growth of *aba2-3* mutant was, however, less reduced by phosphate starvation than wild-type plants or *prl1* and *phl1* mutants, as it was only 10% smaller in −Pi conditions than in standard medium (Fig. 4, A and B). Bleaching and leaf necrosis indicate an accumulation of ROS, oxidative damage, and cell death, which inversely correlates with plant viability (Giacomelli et al., 2007; Laloi and Havaux, 2015). During phosphate starvation, wild-type plants were smaller but did not produce bleached leaves, while 60% of *phr1phl1* double mutants and 70% of *aba2-3* mutants had necrotic leaves in such conditions (Fig. 4, A and C). These results suggest that ABA is implicated in the restriction of rosette growth and maintenance of viability in a phosphate-limiting environment.

Pro accumulation during salt and osmotic stress was shown to be controlled by both ABA-dependent and -independent pathways (Savouré et al., 1997; Strizhov et al., 1997; Sharma and Verslues, 2010). To investigate whether Pro accumulation is regulated by ABA-dependent signals in phosphate-starved plants, Pro content and transcript levels of Pro metabolic genes were tested in the *aba2-3* mutant, in which ABA biosynthesis is blocked (Léon-Kloosterziel et al., 1996) and in *abi4-1* and *abi5-1* mutants, in which key ABA signaling pathways are impaired (Finkelstein et al., 1998; Lopez-Molina and Chua, 2000). While phosphate deprivation enhanced free Pro content three to four times in wild-type plants and in the *abi5-1* mutant, it was only slightly increased in the phosphate-starved *aba2-3* in *abi4-1* mutants (Fig. 5A). When compared to wild-type plants, Pi starvation-dependent activation of *P5CS1* was reduced by 50% in *aba2-3* and in *abi4-1* mutants, while it was less affected

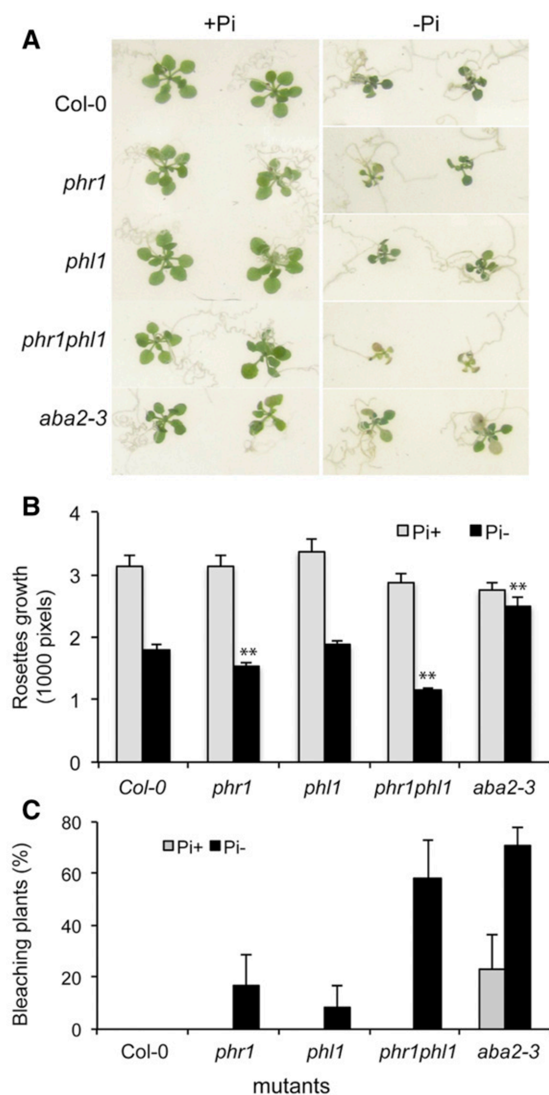

Figure 4. Growth and viability of *phr1, phl1*, and *aba2-3* mutants under phosphate starvation. A, Wild-type and mutant plants grown on standard (+Pi) and phosphate-deficient (−Pi) culture media for 14 d. B, Average rosette sizes of wild-type and mutant plants after 14 d of culture. C, Percentage of plants with bleaching leaves indicating cell death, after culture on phosphate containing and deficient media for 14 d. Note that wild-type plants had no leaves with necrotic symptoms. Bars on diagrams indicate SE; * and ** show significant differences to wild-type at $P < 0.05$ and $P < 0.005$, respectively (Student's *t* test).

in shoots and more reduced in roots of *abi5-1* (Fig. 5B). Expression of *PDH2* was not affected in shoots of these mutants, while in roots of *abi4-1* and *abi5-1* it was higher and lower than wild type, respectively (Fig. 5C). ABA biosynthesis is controlled by the drought-induced *nine-cis-epoxycarotenoid dioxygenase 3* (*NCED3*) gene, which encodes the rate-limiting 9-cis-epoxycarotenoid dioxygenase enzyme (Iuchi et al., 2001). *NCED3* expression was induced by phosphate starvation (Supplemental Fig. S7, A and B), suggesting that ABA biosynthesis is enhanced in such conditions. These results suggest that Pro accumulation and *P5CS1* activation during phosphate starvation is at least partially controlled by ABA-dependent signals.

To study whether damage of phosphate-starved plants is related to senescence, expression of known senescence-induced genes, the senescence-associated Cys proteases SAG12 (Lohman et al., 1994), the Gln synthetase (*GSR2*; Peterman and Goodman, 1991) and methallothionein1 (*MT1*; Zhou and Goldsbrough, 1994) was tested. While expression of the IPS1 marker gene

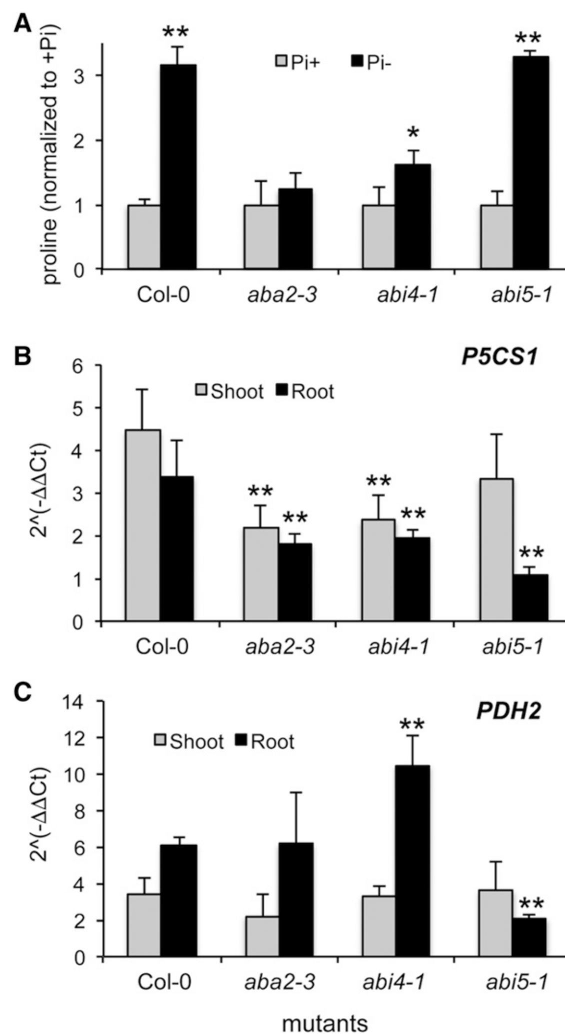

Figure 5. ABA regulates Pro accumulation during phosphate starvation. A, Pro levels of wild type (Col-0), an ABA-deficient mutant (*aba2-3*), and two ABA-insensitive mutants (*abi4-1, abi5-1*), which were cultured on media with or without phosphate (Pi+, Pi−) for 14 d. Normalized values are shown, where 1 corresponds to Pro levels of nonstarved plants. B and C, Expression of *P5CS1* (B) and *PDH2* (C) genes in shoots and roots of wild type (Col-0), *aba2-3, abi4-1*, and *abi5-1* mutants. Relative expression is shown, normalized to transcript data of plants grown on Pi-containing medium. Bars on diagrams indicate SE; * and ** show significant differences to not-treated (A) or wild-type (B and C) plants at $P < 0.05$ and $P < 0.005$, respectively (Student's *t* test).

was induced more than two thousand times by 14 d of phosphate deprivation, transcript levels of the senescence-related SAG12 were enhanced five times, *MT1A* and *GSR2* genes were only slightly induced in such conditions (Supplemental Fig. S7A). Expression data of microarray experiments showed that these genes are not or only slightly induced in the absence of phosphate (Supplemental Fig. S7B). Detrimental effects of phosphate starvation can therefore be associated with senescence-related processes.

Role of Pro Metabolism in Phosphate Starvation

Pro accumulation in the *p5cs1-1* mutant was completely abolished in phosphate-limiting conditions, suggesting that the *P5CS1* gene encodes the rate-limiting enzyme of Pro biosynthesis under such conditions (Fig. 6A). Pro concentration in the *pdh1-4* mutant was similar to that of wild-type plants, while it was 30% higher in the *pdh2-2* mutant under phosphate starvation (Fig. 6A). The function of Pro metabolism in phosphate starvation was subsequently tested by monitoring growth of the *p5cs1-1*, *pdh1-4*, and *pdh2-2* mutants in the presence or absence of inorganic phosphate. Rosette growth of these mutants was similar to wild type in both standard and phosphate-limiting conditions (Supplemental Fig. S8). Root growth of the mutants was similar to wild-type plants on Pi-containing medium, while in the absence of phosphate, *p5cs1-1* mutant roots were slightly but significantly shorter than wild type (Fig. 6B). In the *p5cs1-1* mutant transcriptional response of other Pro metabolic genes to phosphate starvation was similar to wild type, while in *pdh1-4 and pdh2-2* mutants, transcript levels of *PDH2* and *PDH1* genes were reduced, respectively (Supplemental Fig. S9). Exogenously supplied Pro (1 mM and 10 mM) reduced rosette and root growth of wild type and *phr1phl1* double mutants on phosphate-containing medium. In the absence of phosphate, size of *phr1phl1* plants was smaller than wild-type ones and was not influenced significantly by Pro (Supplemental Fig. S10). These results suggest that enhanced Pro biosynthesis is important to maintain root elongation during phosphate starvation but has no effect on rosette growth, while growth defects cannot be alleviated by externally supplied Pro.

DISCUSSION

Pro accumulation during osmotic and salt stress is a well-documented phenomenon in higher plants and is considered to be an important metabolic response to such conditions (Szabados and Savouré, 2010; Verslues and Sharma, 2010). Information on the effect of nutrients and particular nutrient starvation on Pro metabolism is, however, scarce. Our studies revealed that free Pro content is increased in Arabidopsis plants during phosphate starvation (Fig. 2). These results correlate with recent metabolomic data, revealing that Pro accumulation is one of the consequences of phosphorus deficiency in several plant species (Pant et al., 2015b; Valentinuzzi et al., 2015). Pro accumulation in phosphate-starved plants is driven by enhanced expression of *P5CS1*, encoding the key enzyme in the Pro biosynthetic pathway. Besides *P5CS1*, one of the Pro catabolic genes, *PDH2*, was induced by phosphate starvation (Figs. 2 and 3). Although transcripts of the Arabidopsis *P5CS1* and *P5CS2* genes cannot be distinguished in the most commonly used Affymetrix 22.5K ATH1 chip, microarray-based transcript profiling detected enhanced *P5CS1/2* and *PDH2* transcript levels in phosphate-starved plants (Supplemental Fig. S5; Morcuende et al., 2007; Müller et al., 2007; Bustos et al., 2010). Pro contents were reduced in the *p5cs1-1* mutant and enhanced in the *pdh2-2* mutant, suggesting that these two genes determine Pro levels in this type of stress (Fig. 6). The function of Pro in the adaptation to phosphate deficiency is, however, ambiguous, as plant growth was not or was only slightly affected in these mutants, and externally supplied Pro had no visible influence on plant growth on medium lacking phosphate (Fig. 6; Supplemental Fig. S10). By contrast, deficient Pro accumulation in *p5cs1* knockout mutants caused salt and drought hypersensitivity (Székely et al., 2008; Sharma et al., 2011), indicating that Pro is important for protection in such stresses. Elevated *P5CS1* and *PDH2* expression

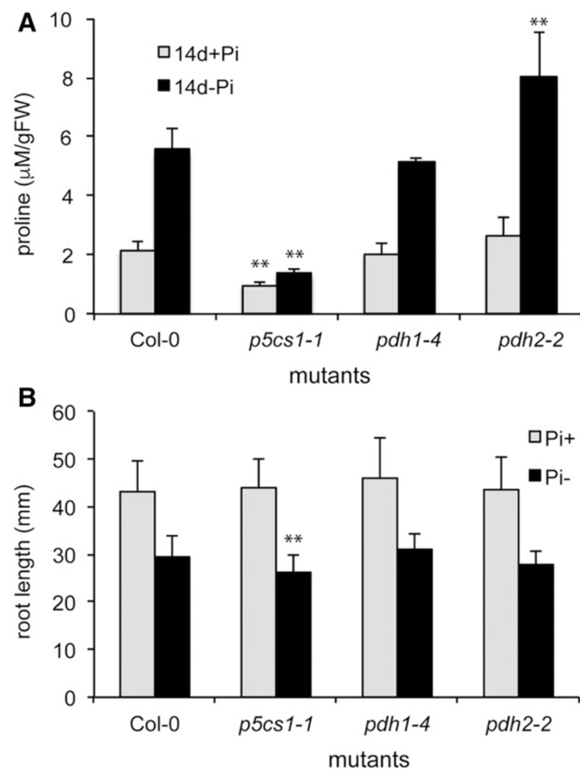

Figure 6. Effect of Pro accumulation on plant growth during phosphate starvation. A, Pro levels are shown in Col-0 wild type, *p5cs1-1*, *pdh1-4*, and *pdh2-2* mutants. B, Root elongation of wild-type and mutant plants grown in the presence or absence of 2.5 mM phosphate (+Pi and −Pi, respectively). Bars on diagrams indicate SE; ** show significant differences to wild type at $P < 0.05$ (Student's *t* test).

suggests that enhanced Pro turnover might take place in phosphate-starved plants. Such a scenario can be beneficial by regulating Nicotinamide adenine dinucleotide phosphate / Dihydronicotinamide-adenine dinucleotide phosphate (NADP+ / NADPH) ratio and cellular redox status during and after stress, consuming reducing power during Pro biosynthesis and/or supplying energy for mitochondrial electron transport through Pro oxidation (Kiyosue et al., 1996; Sharma et al., 2011; Servet et al., 2012; Bhaskara et al., 2015).

Both PHR1 and PHL1 factors were identified in our Y1H screen, using a 2-kb fragment of the *P5CS1* gene, where the conserved P1BS sequence element was identified in the first intron (Fig. 1; Supplemental Fig. S1). Sequence-specific binding of both PHR1 and PHL1 proteins to this motif could be demonstrated by EMSA, and in vivo binding of PHR1 was confirmed by ChIP assays. It is intriguing that the PHR1 and PHL1-binding P1BS motif was localized in the first intron of the *P5CS1* gene. Transcription-enhancing features of introns have been described in a number of genes, especially when they are close to the transcription initiation site (Lohmann et al., 2001; Casas-Mollano et al., 2006; Karthikeyan et al., 2009; Gallegos and Rose, 2015). For example, the transcription factors LEAFY and WUSCHEL cooperate in activating the expression of the *AGAMOUS* gene by recognizing specific binding sites in the first intron of *AGAMOUS* (Lohmann et al., 2001). Promoter analysis of the high-affinity phosphate transporter *AtPht1;4* gene has identified a P1BS motif in the first intron of the 5′ UTR, which was shown to be essential for high level of expression in roots during phosphate deprivation (Karthikeyan et al., 2009). *PDH2* is also induced by phosphate starvation (Fig. 3) and, similarly to *AtPht1;4* and *P5CS1*, has a conserved P1BS motif in its first intron (Supplemental Fig. S11). Earlier transcript profiling data suggest that *P5CS1* and *PDH2* genes can be regulated by PHR1 (Supplemental Figs. S5 and S6; Bustos et al., 2010). These data suggest that PHR1 binding motifs can be located in introns of several genes that can be important for transcriptional activation during phosphate deficiency. A recent ChIP-seq study revealed that ABA-induced transcription factors can bind to one or multiple sites of 5′ upstream region of the *P5CS1* gene, but none of these sequence motifs was located in introns (Supplemental Fig. S2; Song et al., 2016). PHR1 was recently reported to regulate epigenetic marks and DNA methylation near to cis-regulatory elements in the promoters of Pi-responsive genes (Yong-Villalobos et al., 2016). Methylation was, however, not predicted in the vicinity of P1BS motif in the intron of P5CS1 (http://neomorph.salk.edu/epigenome/epigenome.html); therefore, epigenetic regulation of this gene during phosphate starvation is unlikely.

Pro accumulation was attenuated in the *phr1phl1* mutant, when plants were exposed phosphate starvation as well as to salt or ABA treatments (Fig. 3). ABA was shown to regulate Pro accumulation and *P5CS1* activation during salt or osmotic stress (Savouré et al., 1997; Strizhov et al., 1997; Szabados and Savouré, 2010). Pro and *P5CS1* transcript levels were lower in the ABA deficient *aba2-3* and in the ABA insensitive *abi4-1* mutant during phosphate deprivation (Fig. 5). ABI4 is an AP2-type transcription factor that controls the expression of a large set of ABA-regulated genes and is implicated in sugar signaling (Finkelstein et al., 1998; Finkelstein, 2013).

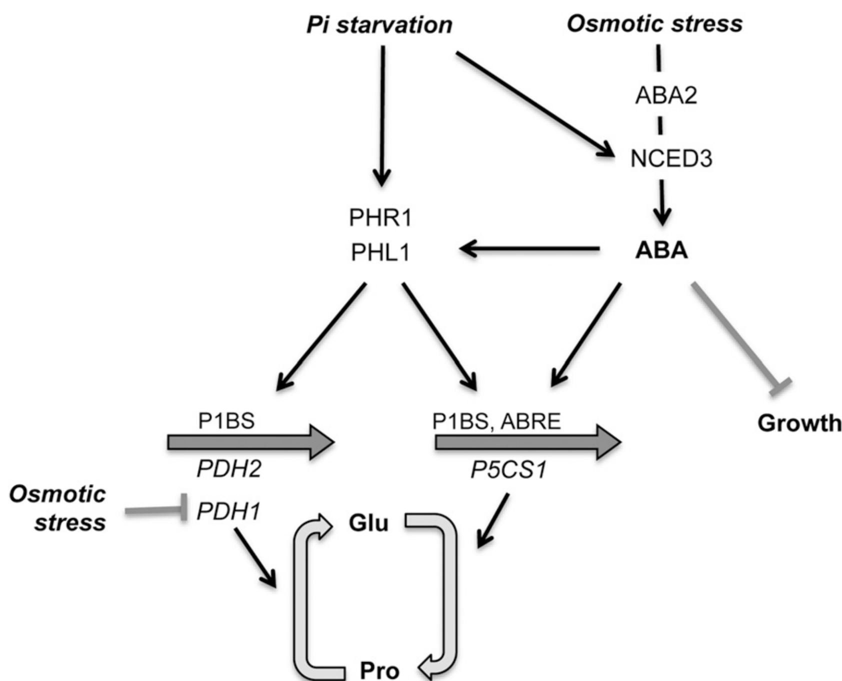

Figure 7. Regulation of Pro metabolism during osmotic stress and phosphate starvation. During osmotic stress, Pro accumulation takes place, controlled by the induction of *P5CS1* and repression of *PDH1* genes, respectively. *P5CS1* activation in this process is controlled by ABA signals, possibly through the ABRE cis-acting motif in the promoter. Phosphate starvation induces PHR1 and PHL1, which activates *P5CS1* through binding to its P1BS motif. *PDH2* is also induced by PHR1 and PHL1 and Pi deficiency. *NCED3* is induced during Pi deprivation, which can enhance ABA levels. ABA signals restrict plant growth and activate numerous stress-related genes, including *PHL1* and *P5CS1*.

These results suggest that ABA-dependent signals activate the Pro biosynthetic pathway not only during dehydration but also during phosphate insufficiency (Fig. 7). Connection between ABA regulation and phosphate starvation is, however, not limited to Pro metabolism. Mining of transcript profiling datasets revealed that a number of ABA-regulated target genes are also induced by phosphate deprivation, such as *RD29A*, *RAB18*, *RD20*, *RD22*, *P5CS1/2*, and *XERO2*, including *NCED3*, a key regulator of ABA biosynthesis (Supplemental Fig. S12; Bustos et al., 2010). Transcription of NCED3 was indeed enhanced by phosphate starvation in our conditions also (Supplemental Fig. S7). Several ABA signaling genes (e.g. *ABI1*, *ABI2*, *HAB1*, *OST1*, *ABF3*, *MYB2*, *MYC2*, *RAP2.12*) were induced by phosphate deprivation, which was attenuated in *phr1* and *phr1phl1* double mutants (Supplemental Fig. S12; Bustos et al., 2010), suggesting that a segment of the ABA regulon is controlled by the PHR1 and PHL1 transcription factors. Transcript profiling data revealed that *PHL1* can be induced by salinity, osmotic stress, and ABA as well (Supplemental Fig. S13; Kilian et al., 2007). On the other hand, a number of phosphate-responsive genes are also regulated by other stresses such as cold, drought, or salinity, some pathogens and hormones like ABA or ethylene (Woo et al., 2012), and senescence-induced genes can be upregulated by phosphate deprivation (Supplemental Fig. S7; Lohman et al., 1994). These results suggest that there is an intimate relationship among starvation, senescence-related pathways, and ABA signaling, which regulates responses to phosphate deficiency. ABA triggers defenses during drought or high-soil salinity and mediates growth inhibition (Finkelstein, 2013; Rowe et al., 2016). We found that on phosphate-deficient medium, rosette growth of the ABA deficient *aba2-3* mutant was less reduced than wild type, suggesting that ABA is implicated in growth inhibition in such nutritional stress. Enhanced leaf bleaching of the *aba2-3* mutant, however, indicates that ABA is needed to maintain viability during phosphate starvation. In contrast to *aba2-3*, both growth and viability was reduced in the *phr1phl1* double mutant under phosphate limitation (Fig. 4). Reduced Pro accumulation in these mutants is probably not responsible for compromised growth or leaf bleaching, as *p5cs1-1* mutants with low Pro levels had no similar symptoms (Figs. 5 and 6; Supplemental Fig. S8). Blocking of ABA biosynthesis was reported to release inhibition of root growth under moderate osmotic stress (Rowe et al., 2016), supporting our finding that ABA is implicated in growth control during stress. Growth restriction during osmotic stress can be mediated by growth-repressing DELLA proteins, which are stabilized by ABA, but are promoted to degradation by gibberellins (Achard et al., 2006; Golldack et al., 2013).

Our results reveal a previously unknown connection between phosphate and Pro metabolism. The *P5CS1* gene controls Pro biosynthesis and seem to be the target of cross talk between ABA signaling and regulation of phosphate homeostasis, controlled by the MYB-type transcription factors PHR1 and PHL1 (Fig. 7).

MATERIALS AND METHODS

Plant Material and Growth Conditions

All Arabidopsis (*Arabidopsis thaliana*) plants used in this study, including mutants were based on the Columbia 0 accession (Col-0). *p5cs1-1*, *pdh2-2*, and *pdh1-4* mutants were obtained from the SALK collection (SALK_058000, SALK_108179, SALK_119334, respectively; Székely et al., 2008). The *phr1*, *phl1*, and *phr1phl1* mutants were kindly provided by Dr. J. Paz-Ares (Centro Nacional de Biotecnologica, Madrid, Spain; Bustos et al., 2010). The *aba2-3*, *abi4-1*, and *abi5-1* lines are from the Arabidopsis Biological Resource Center stock (Arabidopsis Biological Resource Center stock numbers CS3834, CS8104, CS8105). Plants were grown as described earlier (Székely et al., 2008). Seeds were surface sterilized and germinated on medium solidified with 0.8% (w/v) phytoagar containing 5 mM KNO_3, 2.5 mM KH_2PO_4 (adjusted to pH 5.5 with KOH), 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 50 μM Fe-EDTA, 70 μM H_3BO_3, 14 μM $MnCl_2$, 0.5 μM $CuSO_4$, 1 μM $ZnSO_4$, 0.2 μM $NaMoO_4$, 10 μM NaCl, and 0.01 μM $CoCl_2$, 2.5 mM MES [2-(*N*-morpholino)-ethanesulfonic acid]-KOH (pH 5.5), 0.5% (w/v) Suc. Standard culture medium contained 2.5 mM KH_2PO_4 and was referred to +Pi medium. For −Pi medium, KH_2PO_4 was omitted (Ticconi et al., 2001). For phosphate starvation, 5-d-old seedlings, germinated on standard (+Pi) medium, were transferred to −Pi or +Pi medium and grown for 14 d. Salt and ABA treatments were applied by transferring 14-d-old in vitro grown plants to media containing 75 or 150 mM NaCl or 50 μM ABA for 1 to 3 d. Results shown were obtained with at least six technical and three biological replicates.

Real Time Quantitative RT-PCR

RNA isolation was performed as described (Gombos, 2017). First-strand cDNA synthesis of 2 μg of total RNA in a final volume of 20 μL was carried out with RevertAid M-MuLV Reverse Transcriptase (Fermentas), using random hexamers. Real-time PCR was carried out with the ABI 7900 Fast Real Time System (Applied Biosystems) with the following protocol: 45 cycles at 95°C for 15 s, followed by 60°C for 1 min. The specificity of the amplifications was verified at the end of the PCR run through use of the ABI SDS software. The normalized relative transcript levels were obtained by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). To reveal the possible gene expression changes in the Pro metabolism pathway, we examined the transcript abundance of the following genes: *P5CS1* (*AT2G39800*), *P5CS2* (*AT3G55610*), *P5CR* (*AT5G14800*), *P5CDH* (*AT5G62530*), *PDH1* (*AT3G30775*), *PDH2* (*AT5G38710*). The actin gene (*AT2G37620*) was used as an inner control, and *IPS1* (*AT3G09922*) was employed to check the stringency of the phosphate starvation. To reveal possible interactions among phosphate starvation, senescence, and ABA signals, expression of *SAG12* (*AT5G45890*), *MT1A* (*AT1G07600*), *GSR2* (*AT1G66200*), *NCED3* (*AT3G14440*), *PHR1* (*AT4G28610*), and *PHL1* (*At5G29000*) were tested in phosphate-starved and control plants. Primers used in this study are listed in Supplemental Figure S14.

Y1H Screening

The Y1H screen was performed principally as described (Ouwerkerk and Meijer, 2001). A 1.95-kb-long *P5CS1* genomic fragment was cloned into pHis3NB vector that has the His-3 reporter gene construct. The *HIS3* reporter construct was integrated at the nonessential *pyruvate decarboxylase 6* (*PDC6*) locus of Y187 yeast strain (Clontech) using the integrative vector, pINT1. The transformation was carried out as described (Gietz and Woods, 2002). To identify DNA binding proteins, two Arabidopsis cDNA libraries were used for Y1H screening. The pGAD10 expression library (MATCHMAKER cDNA Library, Clontech) was prepared from 3-week-old green vegetative tissues of Arabidopsis (Col-0). The pACT2 library was the Kim & Theologis lambda-ACT 2-hybrid library (https://www.arabidopsis.org/servlets/TairObject?type=library&id=23). Transformation of the yeast reporter strain with the two libraries generated 86 independent transformed colonies, which were plated on selective medium to permit proliferation of transformed yeast cells on high stringency conditions.

EMSA

The nonradioactive EMSA assay was based on protocols that used ethidium bromide staining to visualize gel mobility shifts (Ibarra et al., 2003; Förster-Fromme and Jendrossek, 2010). In order to achieve strong P1BS binding of the PHR1 and PHL1 factors, truncated proteins containing the C-terminal

DNA-binding sites were used (Bournier et al., 2013). Corresponding DNA fragments were PCR amplified (primers in Supplemental Data) inserted into the pET28a+ vector (Invitrogen) and transformed into *E. coli* BL21 DE3 Rosetta cells (New England Biolabs). Proteins were purified on His-Select Nickel affinity gel (Sigma). For EMSA, a 415-bp and 705-bp fragments of the *P5CS1* gene were generated by PCR and purified by EZ-10 Spin column PCR purification kit (Biobasic). Protein binding reactions were performed in a buffer containing 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4 (Alves and Cunha, 2012). The reaction was performed at room temperature for 30 min prior to load onto a 1.5% TAE agarose gel (pH 8.5). Separation and detection of the fragments was made as described (Alves and Cunha, 2012). Electrophoresis was run for 4 h at 12°C, and gels were stained with ethidium bromide (1.5 μg/mL, aqueous solution) for 40 min, eliminating the need of radiolabeling of the DNA fragment. Images were recorded with UVIDOC HD2 (Uvitech, Cambridge) system.

ChIP

ChIP was used to verify in planta the interaction of PHR1 protein and the P1BS site localized in *p5cs1* gene. The chromatin was isolated from transgenic plants expressing the epitope-tagged PHR1:HA protein, as described (Reimer and Turck, 2010). The immunoprecipitation was carried out with μMACS HA Isolation Kit (Miltenyi Biotec). Control ChIP experiment was carried out with Anti-GFP beads (Miltenyi Biotec), which does not bind HA-tagged proteins. The reverse cross linking and DNA purification was carried out by the ABCAM ChIP protocol (based on the description of Werner Aufsatz). Fragments of immunoprecipitated DNA were amplified by quantitative PCR using P5CS1 specific primers, flanking the P1BS motif in intron 1 (P5CS1-IPfw, P5CS1-IPrev, 133-bp fragment), and control primers amplifying a 178-bp fragment on chromosome 4 (13519698-13519876; Fig. 1; Supplemental Fig. S14). Results were calculated with the „background subtraction" method, as described (Haring et al., 2007).

Pro, Hydrogen Peroxide, and Malondialdehyde Determination

The ninhydrin-based colorimetric assay was used to determine the Pro level in Arabidopsis seedlings as described (Abrahám et al., 2010). The lipid peroxidation assay was carried out as reported (Heath and Packer, 1968), the hydrogen peroxide level was determined by the KI-method (Velikova et al., 2000).

Monitoring Expression of GFP-Tagged P5CS1 Gene

Gene fusions were previously made by inserting the eGFP reporter gene into the 3′ end of the *P5CS1* gene (Székely et al., 2008), and transgenic lines expressing the eGFP-tagged P5CS1 were employed to study spatial and kinetic regulation of the *P5CS1* gene. Fluorescence of the transgenic lines was monitored, and images were recorded with Olympus SZ12X stereo microscope.

Bioinformatic Analysis

Public transcriptomic data were compiled from AtGenExpress Visualization Tool (http://jsp.weigelworld.org/expviz/expviz.jsp; Kilian et al., 2007). Putative cis elements on P5CS1 genomic sequences were determined by AthaMap tool (http://www.athamap.de; Steffens et al., 2005), and Promomer tool (http://bar.utoronto.ca/ntools/cgi-bin/BAR_Promomer.cgi) as described (Fichman et al., 2015).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Sequence elements on the *P5CS1* promoter, 5′ UTR, exon 1, intron 1, and exon 2.

Supplemental Figure S2. Binding sites of 21 transcription factors on genomic regions of the *P5CS1* gene.

Supplemental Figure S3. Response of Arabidopsis plants to phosphate starvation.

Supplemental Figure S4. P5CS1-GFP fluorescence in root tips of transgenic Arabidopsis plants.

Supplemental Figure S5. Transcript profiles of Pro genes during phosphate starvation.

Supplemental Figure S6. Activation of Pro metabolic genes by PHR1.

Supplemental Figure S7. Expression of marker genes in phosphate-starved Arabidopsis plants.

Supplemental Figure S8. Growth *of p5cs1-1*, *pdh1-4*, and *pdh2-2* mutants on Pi+ and Pi− media.

Supplemental Figure S9. Expression of Pro metabolism genes in *p5cs1-1*, *pdh1-4*, and *pdh2-2* mutants.

Supplemental Figure S10. Effect of externally supplied Pro on plant growth.

Supplemental Figure S11. Sequence elements on the *PDH2* gene.

Supplemental Figure S12. Transcript profiles of selected ABA-related genes during phosphate starvation.

Supplemental Figure S13. Transcript profiles of *PHR1* and *PHL1* genes in response to salt and ABA.

Supplemental Figure S14. Primers used in this study and their nucleotide sequence.

ACKNOWLEDGMENTS

The authors are grateful to János Györgyey (BRC, Szeged) for financial and technical support, James Smart and Csaba Koncz for revision of the manuscript, Javier Paz-Ares (Centro Nacional de Biotecnologia, Madrid, Spain) for providing seeds of *phr1*, *phl1*, and *phr1phl1* mutants and PHR1.KA-expressing transgenic lines, and to Annemarie H. Meijer (Leiden University, Leiden, The Netherlands) for providing Y1H vectors.

Received June 13, 2017; accepted July 11, 2017; published August 1, 2017.

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