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Short title

PHR1 and PHL1 controls proline biosynthesis.

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Title

Proline accumulation is regulated by transcription factors associated with phosphate starvation

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Short summary

Proline accumulation and activation of the *P5CS1* gene is an ABA-dependent molecular response to phosphate starvation in Arabidopsis, and is controlled by the PHR1 and PHL1 transcription factors.

Key Words

Arabidopsis, phosphate starvation, P5CS1, proline, PHR1, PHL1, ABA

Author contribution

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

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Abstract

Proline accumulation in plants is a well-documented physiological response to osmotic stress caused by drought or salinity. In *Arabidopsis thaliana* the stress and ABA-induced Δ1-PYRROLINE-5-CARBOXYLATE SYNTHETASE 1 (*P5CS1*) gene was previously shown to control proline biosynthesis in such adverse conditions. To identify regulatory factors which control the transcription of *P5CS1*, yeast one hybrid (Y1H) screens were performed with a genomic fragment of *P5CS1*, containing 1.2 kB promoter and 0.8 kB transcribed regions. The MYB-type transcription factors PHOSPHATE STARVATION RESPONSE 1 (PHR1) and PHR1-LIKE 1 (PHL1) were identified to bind to *P5CS1* regulatory sequences in the first intron, which carry a conserved PHR1-binding site (P1BS) motif. PHR1 and PHL1 binding to P1BS was confirmed by Y1H, electrophoretic mobility assay (EMSA) and chromatin immune precipitation (ChIP). Phosphate starvation led to gradual increase in proline content in wild type Arabidopsis plants as well as transcriptional activation of *P5CS1* and PROLINE DEHYDROGENASE 2 (*PDH2*) genes. Induction of *P5CS1* transcription and proline 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 proline metabolism and phosphate nutrition, and show that proline biosynthesis is target of crosstalk between ABA signaling and regulation of phosphate homeostasis through PHR1 and PHL1-mediated transcriptional activation of the *P5CS1* gene.

Key words

Arabidopsis, phosphate starvation, P5CS1, proline, PHR1, PHL1, abscisic acid

Introduction

Proline 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 Savoure, 2010; Verslues and Sharma, 2010). Furthermore, several reports describe proline accumulation in response to other types of stress provoked by heavy metals (Schat, 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 proline, suggesting that it acts as osmoprotectant, stabilizing cellular structures and enzymes, scavenging reactive oxygen species (ROS), and maintain redox equilibrium in adverse conditions (Csonka, 1981; Delauney, 1993; Hoque et al., 2008; Székely et al., 2008; Szabados and Savoure, 2010; Verslues and Sharma, 2010; Sharma et al., 2011; Zouari et al., 2016). Besides the much-studied osmoprotective function, proline has been implicated in the regulation of plant development including flowering, pollen, embryo and leaf development (Székely et al., 2008; Mattioli et al., 2009).

Proline content is regulated by the balance between its biosynthesis and degradation. The glutamate-derived pathway is the most important for proline biosynthesis in plants, and is composed of two consecutive steps catalyzed by the bifunctional enzyme Δ^1-pyrroline-carboxylate synthetase (P5CS), that synthetizes glutamate semialdehyde (GSA) from glutamate (Hu et al., 1992; Yoshiba et al., 1995; Funck et al., 2008). GSA is spontaneously converted to pyrroline-5-carboxylate (P5C) and is subsequently reduced to proline 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 feed-back regulated P5CS enzyme, which in Arabidopsis is encoded by two genes, *P5CS1 (AT2G39800)* and *P5CS2 (AT3G55610)* (Zhang et al., 1995; Strizhov et al., 1997; Székely et al., 2008; Szabados and Savoure, 2010). The production of proline from ornithine represents an alternative biosynthetic pathway and is mediated by ornithine-□-aminotransferase (□OAT, *AT5G46180*) (Delauney et al., 1993). The importance of this pathway in proline accumulation has however been questioned, as stress-induced proline 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 ABA-dependent and independent signals (Savouré et al., 1997; Strizhov et al., 1997; Székely et al., 2008) (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 (Abraham 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 proline 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 bZIP, MYB, MYC, AP2/ERBP, C2H2_Zn type transcription factors (Figure S1) (Fichman et al., 2015). A recent ChIP-seq study suggest that several ABA-regulated TFs can bind to the promoter region of the *P5CS1* gene (Figure S2) (Song et al., 2016).

Proline degradation is an oxidative process, mediated by the rate limiting proline 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 proline or low osmolarity during stress release and was shown to be controlled by the basic leucine zipper (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 which regulates *P5CS1*.

Phosphorus is an essential constituent of biomolecules such as phospholipids, nucleic acids, 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 inorganic phosphate into 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 Lopez-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 which are influenced by root system architecture, organic acid exudation and soil microbes, mainly arbuscular mycorrhizal fungi (Lopez-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 proline (Morcuende et al., 2007; Pant et al., 2015; 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 define the most important regulons responding to phosphate deprivation in shoots and roots (Morcuende et al., 2007; Muller et al., 2007; Bustos et al., 2010; Woo et al., 2012). The MYB-type PHOSPHATE STARVATION RESPONSE 1 (PHR1) and PHR1-LIKE 1 (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., 2015). 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., 2015; Pant et al., 2015). 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 proline 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 proline biosynthetic pathway not only during salt and osmotic stress, but also in phosphate-starved plants. Our results reveals an important connection between proline metabolism and phosphate nutrition and shows that proline 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 screen

To identify the transcription factors that bind to the regulatory region of *P5CS1* gene, yeast one-hybrid 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 (Figure S1,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 (Figure 1A). 86 yeast colonies were identified which 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 Phosphate Starvation Response 1 (*PHR1, AT4G28610*), and four independent colonies contained cDNAs encoding the closely-related PHR1-like 1 factor (*PHL1, AT5G29000*), known regulators of the *Arabidopsis thaliana* 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 (Figure 1A, S1), suggesting that this motif might be responsible for binding the identified TFs.

Binding of PHL1 and PHR1 to the bait was verified by re-transformation of the bait-containing yeast strain with cloned factors, by electrophoretic mobility shift assay (EMSA) and in vivo chromatin immunoprecipitation (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 (Figure 1B). Interaction of the identified PHR1 and PHL1 factors with *P5CS1* genomic 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 700bp *P5CS1* genomic fragment, containing the conserved P1BS motif, a 700bp fragment with mutated P1BS motif (GAATATTC was changed to TCCGCGGA) and a 400bp deletion derivative, missing the P1BS sequence, was incubated with purified PHR1 and PHL1 proteins and assayed with EMSA. Increasing

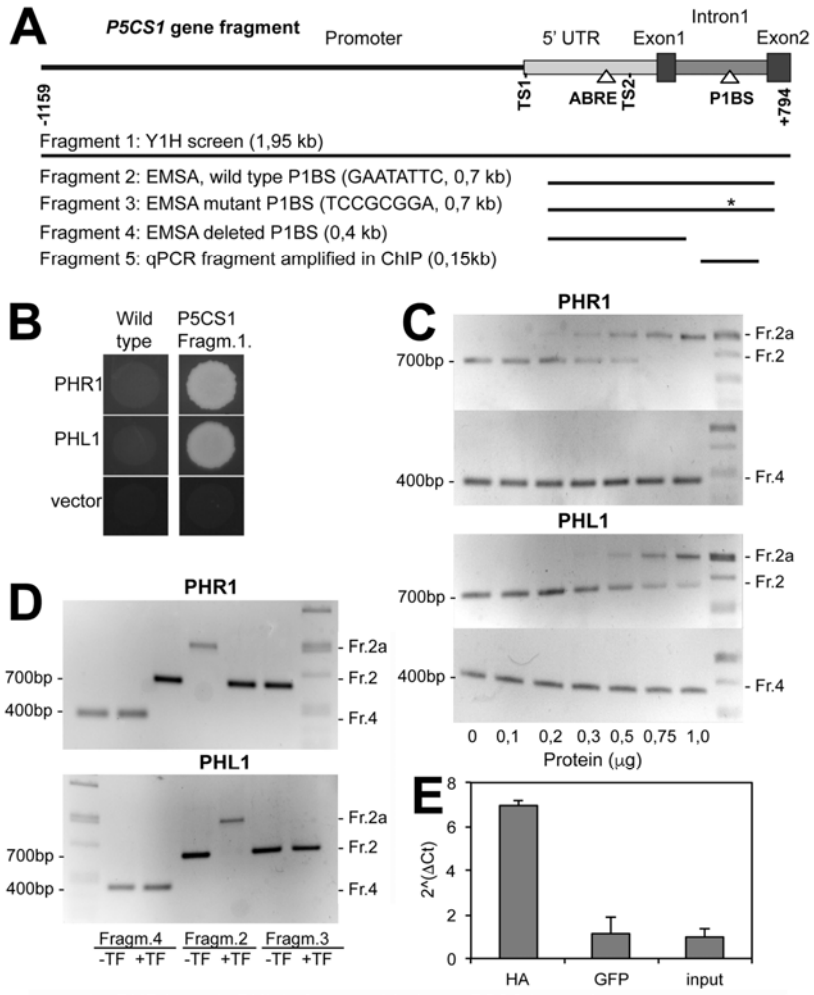

Figure 1. PHR1 and PHL1 factors bind to *P5CS1* regulatory sequences. A) Schematic map of the *P5CS1* regulatory region, including promoter (-1.2 kb), 5' UTR, first and second exons and the first intron, to +0.8 kb. Schematic map was adapted from a previous report (Fichman et al., 2015). Positions of promoter, 5'UTR, exons and 1st intron, and predicted ABRE and P1BS motifs are indicated. Fragments used for yeast one hybrid screen (Y1H, 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 mg 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) Chromatin Immunoprecipitation (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 non-specific 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 standard error of three biological replicates.

amount of PHR1 and PHL1 proteins led to gradual enhancement in the electrophoretic mobility shift of the 700bp genomic fragment on agarose gels. This gel shift was however not observed with the 400bp fragment, which lacked the predicted P1BS site (Figure 1C,D). Electrophoretic mobility of the mutated 700bp 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 (Figure 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 non-specific DNA fragments from unrelated chromosomal regions. After background subtraction and normalisation to control PCR reactions, specific enhancement of HA-immunoprecipitated target DNA was detected when compared to mock samples (Figure 1E). Experiments were repeated three times with similar results. ChIP experiment could therefore confirm that interaction of the PHR1 transcription factor and target DNA in the *P5CS1* gene occurs in plant cells.

Proline accumulates during phosphate starvation

Proline 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; Abraham et al., 2003; Sharma and Verslues, 2010). Binding of the PHR1 and PHL1 transcription factors to *P5CS1* sequences suggested that proline metabolism can also be influenced by phosphate levels. Proline contents and transcription of key genes in proline 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 thousand fold induction of *IPS1* gene (*AT3G09922*), known to be responsive to phosphate starvation (Figure S3) (Martin et al., 2000). When wild type Arabidopsis plants were cultured on medium lacking inorganic phosphate, proline concentration started to increase after 7 days of starvation and after 14 days it was 7 times higher than in control, containing 2.5 mM phosphate (Figure 2A). When culture medium was supplemented by additional phosphate (10 mM), proline levels were not affected (Figure 2A). Expression of genes which are known to control proline 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 Figure

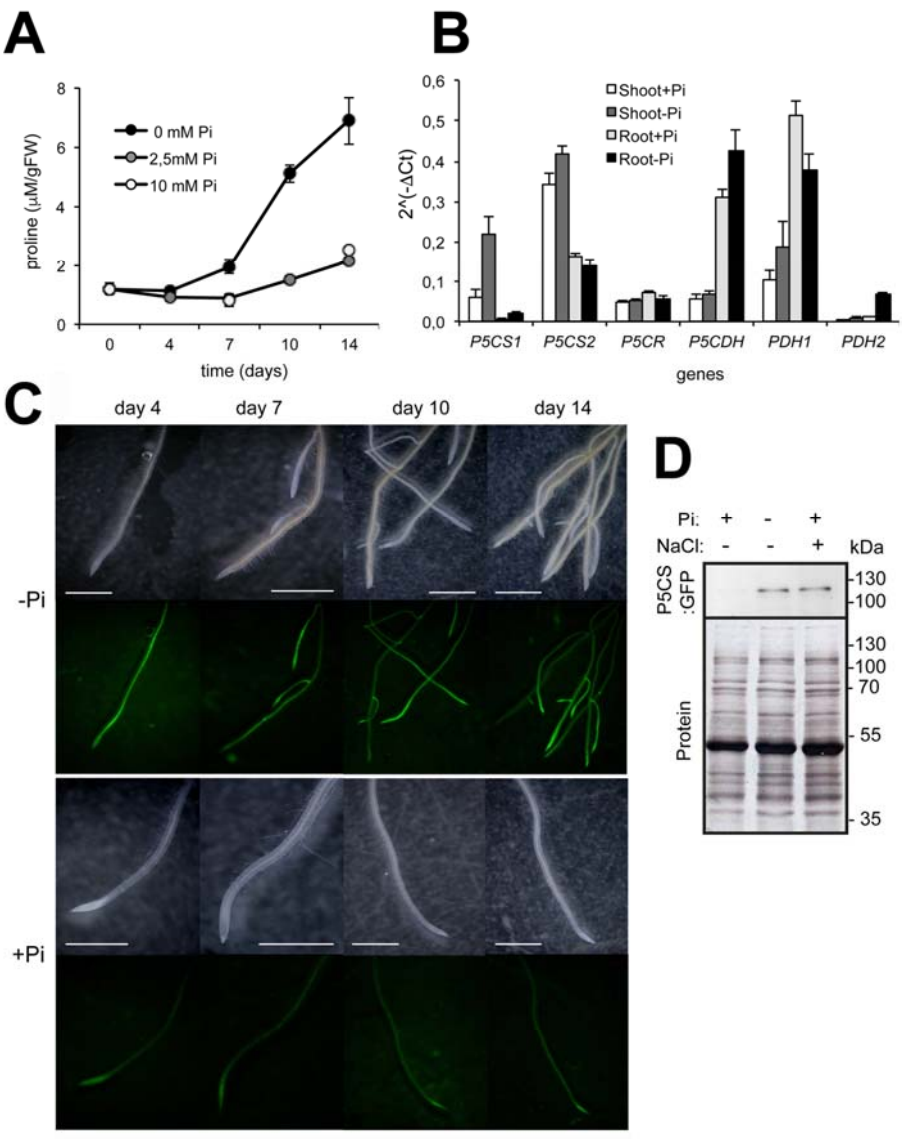

Figure 2. Phosphate starvation leads to proline accumulation and *P5CS1* activation in Arabidopsis plants. A) Proline levels in wild type Arabidopsis plants grown with or without phosphate for 14 days. B) Expression profiles of genes which control proline biosynthesis (*P5CS1, P5CS2, P5CR*), or proline catabolism (*P5CDH, PDH1, PDH2*) in wild type Arabidopsis plants grown with or without phosphate for 14 days. Values were normalized to transcript levels of actin gene. C) GFP-derived fluorescence of P5CS1-GFP construct under phosphate starvation. 5 days-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 3-4 day 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 days on Pi deficient medium) or salt-induced plants (14 days-old plants, treated with 75mM NaCl for 24 hours). Scale bar: 500µm.

2B,3A). Expression of the other tested genes (*P5CS2, P5CR, PDH1, P5CDH*) was not or only slightly changed (Figure 2B,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 (Figure 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 1/2MS 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 days after transfer to phosphate-deprived medium, when other phenotypic alterations were not yet visible (Figure 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 days or longer phosphate starvation (Figure 2C, 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 2.5mM Pi. Similar Western signal was obtained in plants which were treated by moderate salt stress, known to enhance *P5CS1* transcription (Figure 2D) (Strizhov et al., 1997). These results demonstrate that *P5CS1* is activated during phosphate starvation, and suggest that the enhanced proline biosynthesis leads to proline accumulation under these conditions.

PHR1 and PHL1 transcription factors regulate proline accumulation.

To test the role of PHR1 and PHL1 factors in regulation of proline metabolism, proline accumulation in *phr1, phl1* and *phr1phl1* double mutants were compared to wild type plants under phosphate starvation. Proline levels in these mutants were similar to wild type plants in standard culture conditions, but were 50% lower than in Col-0 plants during phosphate starvation (Figure 3A). Salt and ABA are known to enhance free proline content in most plants (Lehmann et al., 2010; Szabados and Savoure, 2010). Proline 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 (Figure 3A). These results suggest that PHR1 and PHL1 factors are important for proline accumulation in phosphate-starved plants, and can play a minor role in salt or ABA-induced proline accumulation as well. To test the effect of *phr1* and *phl1* mutations on the expression of genes which control proline 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 3 to 6 times by

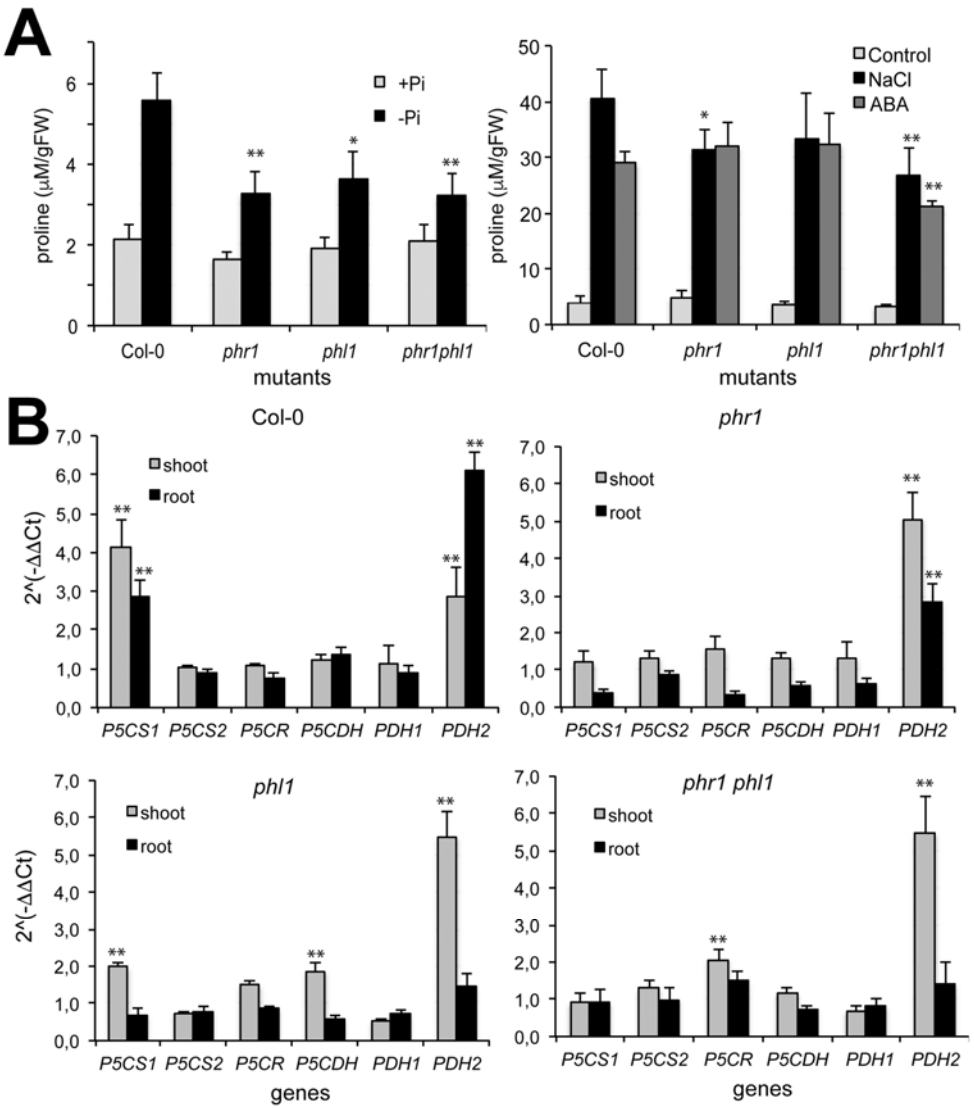

Figure 3. Proline metabolism in *phr1* and *phl1* mutants. A) Proline levels in wild type (Col-0) *phr1, phl1* and *double phr1phl1* mutants subjected to phosphate starvation for 14 days or treated by 150 mM NaCl or 50 µM ABA for three days, after having grown on standard culture medium for 14 days. B) Transcript levels of genes controlling proline metabolism in wild type and mutant plants, growth with or without phosphate for 14 days. Relative transcript levels are shown, normalized to transcript data of plants grown on +Pi medium (2,5 mM Pi). Bars on diagrams indicate standard error, * 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 t-test).

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 pro-related genes was not altered during phosphate starvation (Figure 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, reduced *PDH1* expression but did not affect other genes in proline metabolism (*P5CR, P5CDH, OAT*) (Figure S5) (Bustos et al., 2010). When compared to wild type plants, transcript levels of *P5CS1/P5CS2* and *PDH2* were clearly reduced in *phr1* and *phr1,phl1* mutants (Figure 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 which are direct targets of PHR1 during phosphate deprivation (Figure 3B, S6, S7) (Bustos et al., 2010).

Abscisic acid regulates proline 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 (Figure 4A,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 (Figure 4A,B). Bleaching and leaf necrosis indicate an accumulation of reactive oxygen species, oxidative damage and cell death, which inversely correlates with plant viability (Giacomelli et al., 2007; Laloi and Havaux, 2015). During phosphate strarvation 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 (Figure 4A,C). These results suggest, that ABA is implicated in the restriction of rosette growth and maintenance of viability in a phosphate-limiting enviroment.

Proline 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 proline accumulation is regulated by ABA-dependent signals in phosphate-starved plants, proline content and transcript levels of proline metabolic genes were tested in the *aba2-3* mutant, in which ABA biosynthesis is blocked (Leon-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

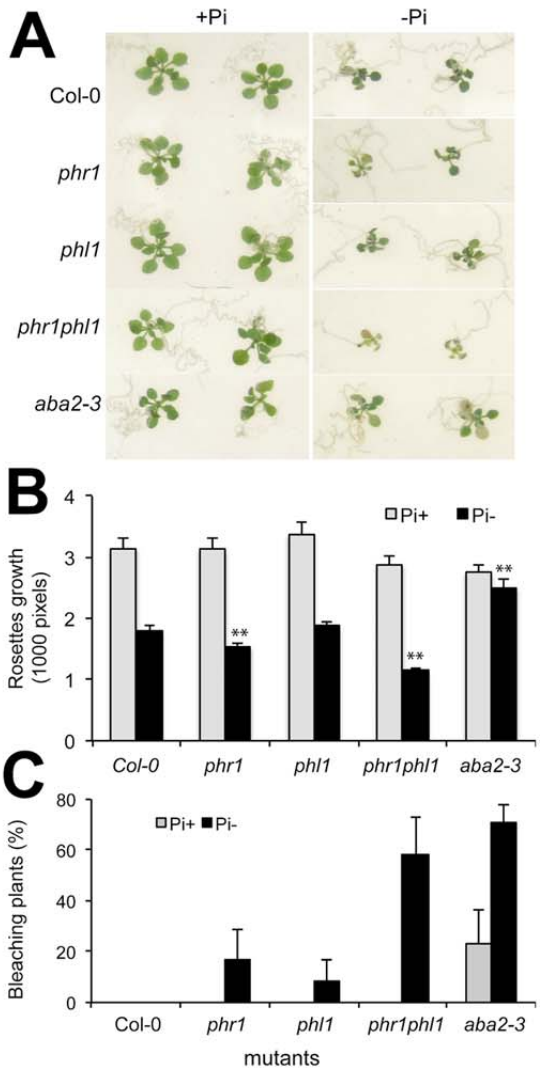

Figure 4. Growth and viability *of phr1, phl*1 and *aba2-3* mutants under phosphate starvation. A) Wild type and mutant plants grown on standard (+Pi) and phosphate deficient (-Pi) culture media for 14 days. B) Average rosette sizes of wild type and mutant plants after 14 days of culture. C) Percentage of plants with bleaching leaves indicating cell death, after culture on phosphate containing and deficient media for 14 days. Note, that wild type plants had no leaves with necrotic symptoms. Bars on diagrams indicate standard error, * and ** show significant differences to wild type at $p<0.05$ and $p<0.005$, respectively (Student t-test).

phosphate deprivation enhanced free proline content 3 to 4 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 (Figure 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 in shoots and

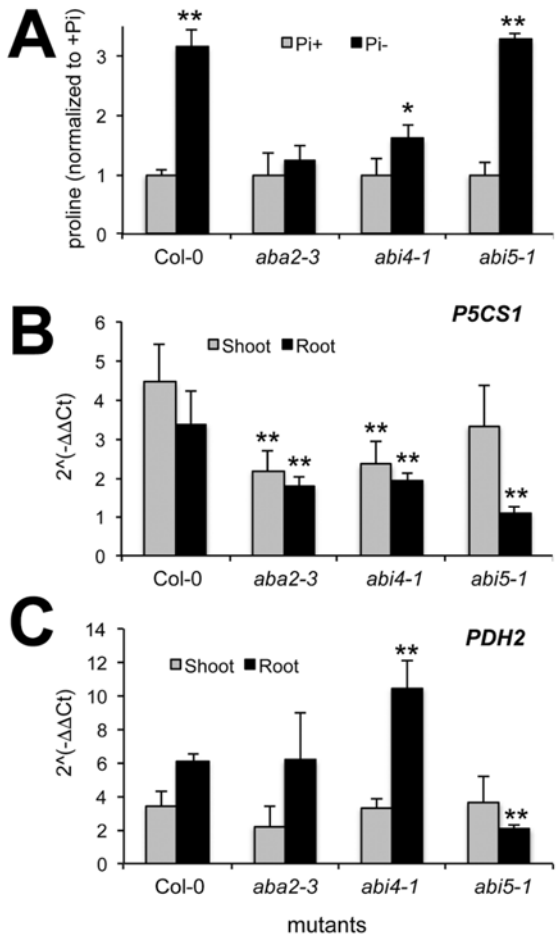

Figure 5. Abscisic acid regulates proline accumulation during phosphate starvation. A) Proline levels of wild type (Col-0), an ABA-deficient mutant (*aba2-3*) and two ABA insensitive mutants (*abi4-1, abi5-1*), which were cultured on media with or without phosphate (Pi+, Pi-) for 14 days. Normalized values are shown, where 1 corresponds to proline levels of non-starved plants. B,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 standard error, * and ** show significant differences to not-treated (A) or wild type (B,C) plants at $p<0.05$ and $p<0.005$, respectively (Student t-test).

more reduced in roots of *abi5-1* (Figure 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 (figure 5C). ABA biosynthesis is controlled by the drought-induced *NCED3* gene, which encodes the rate limiting 9-cis-epoxycarotenoid dioxygenase enzyme (Iuchi et al., 2001).

316 *NCED3* expression was induced by phosphate starvation (Figure S7A,B), suggesting that ABA
317 biosynthesis is enhanced in such conditions. These results suggest that proline accumulation
318 and *P5CS1* activation during phosphate starvation is at least partially controlled by ABA-
319 dependent signals.

320 To study whether damage of phosphate-starved plants is related to senescence,
321 expression of known senescence induced genes, the senescence-associated cysteine
322 proteases SAG12 (Lohman et al., 1994), the glutamine synthetase (*GSR2*) (Peterman and
323 Goodman, 1991) and methallothionein 1 (*MT1*) (Zhou and Goldsbrough, 1994), was tested.
324 While expression of the IPS1 marker gene was induced more than two thousand times by 14
325 days of phosphate deprivation, transcript levels of the senescence-related SAG12 were
326 enhanced five times, *MT1A* and *GSR2* genes were only slightly induced in such conditions
327 (Figure S7A). Expression data of microarray experiments showed, that these genes are not or
328 only slightly induced in the absence of phosphate (Figure S7B). Detrimental effects of
329 phosphate starvation can therefore be associated with senescence-related processes.

330

331 *Role of proline metabolism in phosphate starvation.*

332 Proline accumulation in the *p5cs1-1* mutant was completely abolished in phosphate
333 limiting conditions, suggesting that the *P5CS1* gene encodes the rate-limiting enzyme of proline
334 biosynthesis under such conditions (Figure 6A). Proline concentration in the *pdh1-4* mutant was
335 similar to wild type plants, while it was 30% higher in the *pdh2-2* mutant under phosphate
336 starvation (Figure 6A). The function of proline metabolism in phosphate starvation was
337 subsequently tested by monitoring growth of the *p5cs1-1, pdh1-4* and *pdh2-2* mutants in the
338 presence or absence of inorganic phosphate. Rosette growth of these mutants was similar to
339 wild type in both standard and phosphate limiting conditions (Figure S8). Root growth of the
340 mutants was similar to wild type plants on Pi containing medium, while in the absence of
341 phosphate, *p5cs1-1* mutant roots were slightly but significantly shorter than wild type (Figure
342 6B). In the *p5cs1-1* mutant transcriptional response of other proline metabolic genes to
343 phosphate starvation was similar to wild type, while in *pdh1-4 and pdh2-2* mutants transcript
344 levels of *PDH2* and *PDH1* genes were reduced, respectively (Figure S9). Exogenously supplied
345 proline (1 mM and 10 mM) reduced rosette and root growth of wild type and *phr1phl1* double
346 mutants on phosphate-containing medium. In the absence of phosphate, size of *phr1phl1* plants
347 was smaller than wild type ones, and was not influenced significantly by proline (Figure S10).

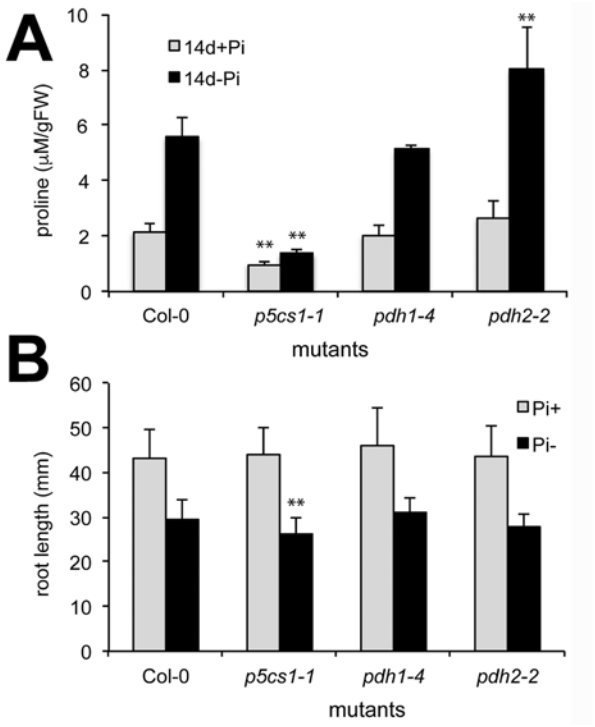

Figure 6. Effect of proline accumulation on plant growth during phosphate starvation. A) Proline levels are shown in Col-0 wild type, *p5cs1-1, pdh1-4* and *pdh2-2* mutants. B) Root elongation of wild type and mutant plants grown in the presence or absence of 2,5 mM phosphate (+Pi and –Pi, respectively). Bars on diagrams indicate standard error, ** show significant differences to wild type at $p<0.05$ (Student t-test).

These results suggest, that enhanced proline 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 proline.

Discussion

Proline accumulation during osmotic and salt stress is a well-documented phenomenon in higher plants and is considered as an important metabolic response to such conditions (Szabados and Savoure, 2010; Verslues and Sharma, 2010). Information on the effect of nutrients and particular nutrient starvation on proline metabolism is however scarce. Our studies revealed that free proline content is increased in Arabidopsis plants during phosphate starvation (Figure 2). These results correlate with recent metabolomic data, revealing that proline accumulation is one of the consequences of phosphorus deficiency in several plant species (Pant et al., 2015; Valentinuzzi et al., 2015). Proline accumulation in phosphate-starved plants is driven by enhanced expression of *P5CS1*, encoding the key enzyme in the proline biosynthetic pathway. Besides *P5CS1*, one of the proline catabolic genes, *PDH2* was induced by phosphate starvation (Figure 2,3). 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 (Figure S5) (Morcuende et al., 2007; Muller et al., 2007; Bustos et al., 2010). Proline contents were reduced in the *p5cs1-1* mutant and enhanced in the *pdh2-2* mutant, suggesting that these two genes determine proline levels in this type of stress (Figure 6). The function of proline 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 proline had no visible influence on plant growth on medium lacking phosphate (Figure 6, S10). By contrast, deficient proline accumulation in *p5cs1* knockout mutants caused salt and drought hypersensitivity (Székely et al., 2008; Sharma et al., 2011), indicating that proline is important for protection in such stresses. Elevated *P5CS1* and *PDH2* expression suggests that enhanced proline turnover might take place in phosphate-starved plants. Such scenario can be beneficial by regulating NADP/NADPH ratio and cellular redox status during and after stress, consuming reducing power during proline biosynthesis and/or supplying energy for mitochondrial electron transport through proline 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 yeast one hybrid (Y1H) screen, using a 2 kb fragment of the *P5CS1* gene, where the conserved P1BS sequence element was identified in the first intron (Figure 1,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

386 confirmed by ChIP assays. It is intriguing, that the PHR1 and PHL1-binding P1BS motif was
387 localized in the first intron of the *P5CS1* gene. Transcription-enhancing features of introns have
388 been described in a number of genes, specially when they are close to the transcription initiation
389 site (Lohmann et al., 2001; Casas-Mollano et al., 2006; Karthikeyan et al., 2009; Gallegos and
390 Rose, 2015). For example, the transcription factors LEAFY and WUSCHEL cooperate in
391 activating the expression of *AGAMOUS* gene by recognizing specific binding sites in the first
392 intron of *AG* (Lohmann et al., 2001). Promoter analysis of the high-affinity phosphate transporter
393 *AtPht1;4* gene has identified a P1BS motif in the first intron of the 5' UTR region, which was
394 shown to be essential for high level of expression in roots during phosphate deprivation
395 (Karthikeyan et al., 2009). *PDH2* is also induced by phosphate starvation (Figure 3), and
396 similarly to *AtPht1;4* and *P5CS1*, has a conserved P1BS motif in its first intron (Figure S11).
397 Earlier transcript profiling data suggest, that *P5CS1* and *PDH2* genes can be regulated by
398 PHR1 (Figure S5,S6) (Bustos et al., 2010). These data suggest, that PHR1 binding motifs can
399 be located in introns of several genes, which can be important for transcriptional activation
400 during phosphate deficiency. A recent ChIP-seq study revealed that ABA-induced transcription
401 factors can bind to one or multiple sites of 5' upstream region of the *P5CS1* gene, but none of
402 these sequence motifs was located in introns (Figure S2) (Song et al., 2016). PHR1 was
403 recently reported to regulate epigenetic marks and DNA methylation near to cis regulatory
404 elements in the promoters of Pi responsive genes (Yong-Villalobos et al., 2016). Methylation
405 was however not predicted in the vicinity of P1BS motif in the intron of P5CS1
406 (http://neomorph.salk.edu/epigenome/epigenome.html), therefore epigenetic regulation of this
407 gene during phosphate starvation is unlikely.
408 Proline accumulation was attenuated in the *phr1phl1* mutant, when plants were exposed
409 phosphate starvation as well as to salt or ABA treatments (Figure 3). ABA was shown to
410 regulate proline accumulation and *P5CS1* activation during salt or osmotic stress (Savouré et
411 al., 1997; Strizhov et al., 1997; Szabados and Savoure, 2010). Proline and *P5CS1* transcript
412 levels were lower in the ABA deficient *aba2-3* and in the ABA insensitive *abi4-1* mutant during
413 phosphate deprivation (Figure 5). ABI4 is an AP2-type transcription factor which controls the
414 expression of large set of ABA-regulated genes and is implicated in sugar signaling (Finkelstein
415 et al., 1998; Finkelstein, 2013). These results suggest, that ABA-dependent signals activate the
416 proline biosynthetic pathway not only during dehydration but also during phosphate insufficiency
417 (Figure 7). Connection between ABA regulation and phosphate starvation is however not limited
418 to proline metabolism. Mining of transcript profiling datasets revealed, that a number of ABA-
419 regulated target genes are also induced by phosphate deprivation such as *RD29A, RAB18,*

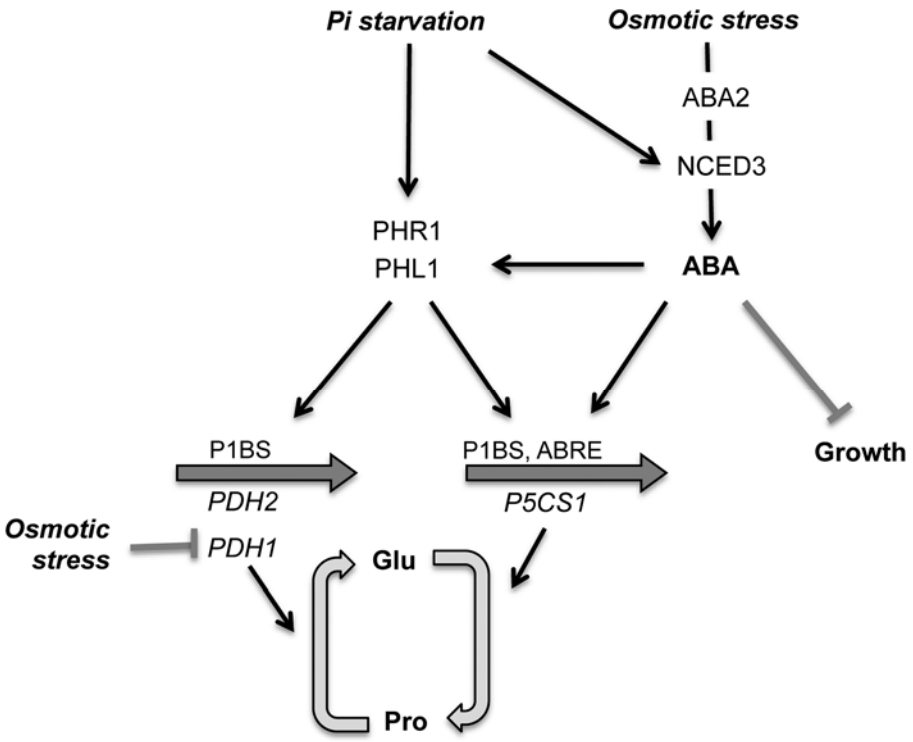

Figure 7. Regulation of proline metabolism during osmotic stress and phosphate starvation. During osmotic stress, proline accumulation takes place, controlled by the induction of *P5CS1* and repression of *PDH1* genes, respectively. *P5CS1* activation in this process is controlled by ABA signals, possibly 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, incuding *PHL1* and *P5CS1*.

RD20, RD22, P5CS1/2, XERO2, including *NCED3,* a key regulator of ABA biosynthesis (Figure S12) (Bustos et al., 2010). Transcription of NCED3 was indeed enhanced by phosphate starvation in our conditions also (Figure S7). Several ABA signaling genes (eg. *ABI1, ABI2, HAB1, OST1, ABF3, MYB2, MYC2, RAP2.12*) were induced by phosphate deprivation, which was attenuated in *phr1* and *phr1phl1* double mutants (Figure 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 (Figure 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 (Figure S7) (Lohman et al., 1994). These

results suggest, that there is an intimate relationship between starvation, senescence-related pathways and ABA signaling, which regulates responses to phosphate deficiency. ABA triggers defenses during drought or high soil salinity and mediate 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 (Figure 4). Reduced proline accumulation in these mutants is probably not responsible for compromised growth or leaf bleaching, as *p5cs1-1* mutants with low proline levels had no similar symptoms (Figure 5, 6, 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 proline metabolism. The *P5CS1* gene controls proline biosynthesis and seem to be the target of crosstalk between ABA sigaling and regulation of phosphate homeostasis, controlled by the MYB-type transcription factors PHR1 and PHL1 (Figure 7).

Materials and Methods

Plant material and growth conditions

All Arabidopsis 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 ABRC stock (ABRC 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

463 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
464 $MnCl_2$, 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
465 MES [2-(*N*-morpholino)-ethanesulfonic acid]-KOH (pH 5.5), 0.5% (w/v) Sucrose. Standard
466 culture medium contained 2.5 mM KH_2PO_4 and was referred to +Pi medium. For -Pi medium,
467 KH_2PO_4 was omitted (Ticconi et al., 2001). For phosphate starvation, 5 day-old seedlings,
468 germinated on standard (+Pi) medium, were transferred to –Pi or +Pi medium and grown for 14
469 days. Salt and ABA treatments were applied by transferring 14 days-old in vitro grown plants to
470 media containing 75 or 150 mM NaCl or 50 µM ABA for 1 to 3 days. Results shown were
471 obtained with at least 6 technical and 3 biological replicates.
472
473 *Real Time Quantitative RT-PCR*
474 RNA isolation was performed as described (Gombos, 2016). First-strand cDNA
475 synthesis of 2 µg of total RNA in a final volume of 20 µL was carried out with RevertAid M-MuLV
476 Reverse Transcriptase (Fermentas), using random hexamers. Real-time PCR was carried out
477 with the ABI 7900 Fast Real Time System (Applied Biosystems) with the following protocol: 45
478 cycles at 95 °C for 15 s, followed by 60 °C for 1 min. The specificity of the amplifications was
479 verified at the end of the PCR run through use of the ABI SDS software. The normalized relative
480 transcript levels were obtained by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). To reveal the
481 possible gene expression changes in the proline metabolism pathway we examined the
482 transcript abundance of the following genes: *P5CS1 (AT2G39800), P5CS2 (AT3G55610),*
483 *P5CR (AT5G14800), P5CDH (AT5G62530), PDH1 (AT3G30775), PDH2 (AT5G38710).* The
484 actin gene (*AT2G37620*) was used as an inner control and *IPS1* (*AT3G09922*) was employed to
485 check the stringency of the phosphate starvation. To reveal possible interactions between
486 phosphate starvation, senescence and ABA signals, expression of *SAG12 (AT5G45890), MT1A*
487 *(AT1G07600), GSR2 (AT1G66200), NCED3 (AT3G14440), PHR1 (AT4G28610)* and *PHL1*
488 *(At5G29000)* were tested in phosphate-starved and control plants. Primers used in this study
489 are listed in Figure S14.
490
491 *Yeast One-hybrid screening*
492 The yeast one-hybrid screen was performed principally as described (Ouwerkerk and
493 Meijer, 2001). A 1.95 kbp long *P5CS1* genomic fragment was cloned into pHis3NB vector
494 which has the His3 reporter gene construct. The *HIS3* reporter construct was integrated at the
495 nonessential *PDC6* locus of Y187 yeast strain (Clontech) using the integrative vector, pINT1.
496 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 weeks 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.

Electrophoretic mobility shift assay

The non-radioactive EMSA assay was based on protocols which used ethidium bromide staining to visualize gel mobility shifts (Ibarra et al., 2003; Forster-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 Supplement) 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 electromobility shift assay (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, 2012). The reaction was perfomed 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, 2012). Electrophoresis was run for 4 h at 12^{o}C, and gels were stained with ethidium bromide (1,5 µg/ml, aquaeus solution) for 40 min, eliminating the need of radiolabelling of the DNA fragment. Images were recorded with UVIDOC HD2 (Uvitech, Cambridge) system.

Chromatin Immunoprecipitation

Chromatin Immunoprecipitation (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 crosslinking 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) (Figure 1, Figure S14). Results were calculated with the „background subtraction" method, as described (Haring et al., 2007).

Proline, hydrogen-peroxide and malondialdehyde determination

The ninhydrin-based colorimetric assay was used to determine the proline level in Arabidopsis seedlings as described (Abraham 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 figures

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

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

Figure S3: Response of Arabidopsis plants to phosphate starvation.

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

Figure S5: Transcript profiles of proline genes during phosphate starvation.

Figure S5: Growth *of phr1, phl1, phr1phl2* and *aba2-3* mutants in the absence of Pi.

Figure S6: Activation of proline metabolic genes by PHR1.

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

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

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

Figure S10: Effect of externally supplied proline on plant growth.

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

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

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

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

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583 **Figures**

584 Figure 1. PHR1 and PHL1 factors bind to *P5CS1* regulatory sequences. A) Schematic map of
585 the *P5CS1* regulatory region, including promoter (-1.2 kb), 5' UTR, first and second exons and
586 the first intron, to +0.8 kb. Schematic map was adapted from a previous report (Fichman et al.,
587 2015). Positions of promoter, 5'UTR, exons and 1st intron, and predicted ABRE and P1BS
588 motifs are indicated. Fragments used for yeast one hybrid screen (Y1H, fragment 1), EMSA
589 (fragments 2-4) and ChIP amplification (fragment 5) are shown. B) Y1H test of PHR1 and PHL1
590 factors and *P5CS1* genomic fragment as bait. C) Electrophoretic mobility assay (EMSA) of
591 purified PHR1 and PHL1 factors with 0.7 kb and 0.4 kb genomic fragments of the *P5CS1* gene
592 (Fragments 2 and 4). Note, that increasing amount of PHR1 and PHL1 protein enhance the high
593 mobility complex with the 0.7 kb fragment. D) EMSA with wild type (Fragment 2), mutated
594 (Fragment 3) and deletion derivative (Fragment 4) of the region containing the P1BS site with 1
595 □g purified PHR1 or PHL1 protein. Note, that electromobility shift can be observed only when
596 Fragment 2 was used, which contained the wild type P1BS sequence, but not with the mutated
597 or deleted version (Fragments 3 and 4, respectively). E) Chromatin Immunoprecipitation (ChIP)
598 assay. Normalized quantitative PCR data are shown, where the amount of P1BS-containing
599 PCR product (fragment 5) was related to PCR product obtained from a non-specific intergenic
600 region. HA: samples precipitated with anti-HA beads. GFP: samples precipitated by anti-GFP
601 beads. Input: qPCR data with samples without immunoprecipitation. Bars on diagrams indicate
602 standard error of three biological replicates.

603 Figure 2. Phosphate starvation leads to proline accumulation and *P5CS1* activation in
604 Arabidopsis plants. A) Proline levels in wild type Arabidopsis plants grown with or without
605 phosphate for 14 days. B) Expression profiles of genes which control proline biosynthesis
606 (*P5CS1, P5CS2, P5CR*), or proline catabolism (*P5CDH, PDH1, PDH2*) in wild type Arabidopsis
607 plants grown with or without phosphate for 14 days. Values were normalized to transcript levels
608 of actin gene. C) GFP-derived fluorescence of P5CS1-GFP construct under phosphate
609 starvation. 5 days-old seedlings expressing the genomic P5CS1-GFP fusion (Székely et al.,
610 2008) were transferred to standard culture medium (+Pi), or medium deprived of phosphate (-
611 Pi), and GFP-derived fluorescence was recorded in 3-4 day intervals. Note the enhanced GFP
612 signals and proliferation of lateral roots in –Pi medium. D) Western detection of P5CS1-GFP
613 protein in phosphate-starved (14 days on Pi deficient medium) or salt-induced plants (14 days-
614 old plants, treated with 75mM NaCl for 24 hours). Scale bar: 500μm.

Figure 3. Proline metabolism in *phr1* and *phl1* mutants. A) Proline levels in wild type (Col-0) *phr1, phl1* and *double phr1phl1* mutants subjected to phosphate starvation for 14 days or treated by 150 mM NaCl or 50 μM ABA for three days, after having grown on standard culture medium for 14 days. B) Transcript levels of genes controlling proline metabolism in wild type and mutant plants, growth with or without phosphate for 14 days. Relative transcript levels are shown, normalized to transcript data of plants grown on +Pi medium (2,5 mM Pi). Bars on diagrams indicate standard error, * 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 t-test).

Figure 4. Growth and viability *of phr1, phl*1 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 days. B) Average rosette sizes of wild type and mutant plants after 14 days of culture. C) Percentage of plants with bleaching leaves indicating cell death, after culture on phosphate containing and deficient media for 14 days. Note, that wild type plants had no leaves with necrotic symptoms. Bars on diagrams indicate standard error, * and ** show significant differences to wild type at $p<0.05$ and $p<0.005$, respectively (Student t-test).

Figure 5. Abscisic acid regulates proline accumulation during phosphate starvation. A) Proline 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 days. Normalized values are shown, where 1 corresponds to proline levels of non-starved plants. B,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 standard error, * and ** show significant differences to not-treated (A) or wild type (B,C) plants at $p<0.05$ and $p<0.005$, respectively (Student t-test).

Figure 6. Effect of proline accumulation on plant growth during phosphate starvation. A) Proline 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 standard error, ** show significant differences to wild type at $p<0.05$ (Student t-test).

Figure 7. Regulation of proline metabolism during osmotic stress and phosphate starvation. During osmotic stress, proline 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, incuding *PHL1* and *P5CS1*.

653

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