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Molecular mechanism of phosphorous signaling inducing anthocyanin accumulation in *Arabidopsis*

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ABSTRACT

Anthocyanins, flavonoid compounds derived from secondary metabolic pathways, play important roles in various biological processes. Phosphorus (P) is an essential macroelement for plant growth and development, and P-starvation usually results in anthocyanin accumulation. However, the molecular mechanism of P deficiency promotes anthocyanin biosynthesis has not been well characterized. Here, we provided evidence that the P signaling core protein PHOSPHATE STARVATION RESPONSE1 (PHR1) is physically associate with transcription factors (TFs) involved in anthocyanidin biosynthesis, including PRODUCTION OF ANTHOCYANIN PIGMENTS1 (PAP1/MYB75), MYB DOMAIN PROTEIN 113 (MYB113) and TRANSPARENT TESTA 8 (TT8). PHR1 and its homologies positively regulated anthocyanin accumulation in *Arabidopsis* seedlings under P-deficient conditions. Disruption of PHR1 simultaneously rendered seedlings hyposensitive to limiting P, whereas the overexpression of *PHR1* enhanced P- deficiency-induced anthocyanin accumulation. Genetic analysis demonstrated that *35S:PHR1-2HA-5* seedlings partially recovers the P deficiency insensitive phenotype of *myb-RNAi* and *tt8* mutants. In summary, our study indicated that protein complexes formed by PHR1 and MBW complex directly mediate the process of P-deficiency-induced anthocyanin accumulation, providing a new mechanistic understanding of how P-deficient signaling depends on the endogenous anthocyanin synthesis pathway to promote anthocyanin accumulation in *Arabidopsis*.

1. Introduction

Anthocyanins are a kind of natural pigments commonly found in plants (Liu et al., 2019). It has various roles to play in plant growth and adaptation, such as preventing plants from ultraviolet radiation, scavenging free radicals under adverse conditions, and acting as chelating agents for metals and metal-like substances in plants (Kim et al., 2017; Liang and He, 2018). Anthocyanins can also act as metabolic markers of nutrient deficiency, especially P deficiency. For example, plants can cope with low P stress by accumulating anthocyanins (Watanabe et al., 2013; He et al., 2020). The anthocyanin biosynthesis pathway is therefore essential to understand how plants adapt to environmental stresses.

In plants, the anthocyanin biosynthetic pathway takes phenylalanine as the direct precursor and is catalyzed by a series of enzymes, including phenylalanine ammoniate-lyase (PAL), chalconesynthase (CHS), chalcone isomerase (CHI), dihydroflavonol 4-reductase (DFR), anthocyanin synthase (ANS), leucoanthocyanidin dioxygenase (LDOX), and flavonoid 3-o-glucosyltransferase (UFGT; Besseau et al., 2007; Owens et al., 2008). During anthocyanin synthesis, the expression of these genes is mainly regulated by the MBW complex, which is composed of MYB protein, bHLH protein and WD40 repeat protein (Tohge et al., 2005; He et al., 2020). In this complex, the TF MYB75/PAP1 functions as a central regulator of anthocyanin biosynthesis in plants (He et al., 2020). For example, the accumulation of anthocyanin were strongly enhanced in *production of anthocyanin pigment 1-Dominant (pap1-D)* mutant plants,

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which is a overexpression plant of *PAP1*. (Borevitz et al., 2000). Previous studies had found that light, high/low temperature, plant hormones and other internal and external stimuli can affect anthocyanin synthesis and accumulation through the integration point of MYB75/PAP1 (Gonzalez et al., 2008; Bhargava et al., 2010; Qi et al., 2011; Xu et al., 2016). In addition, TFs AtMYB90/PAP2, AtMYB113 and AtMYB114 have also been reported to promote anthocyanin accumulation in *Arabidopsis* (Zimmermann et al., 2004; He et al., 2020). bHLH proteins can directly regulate the expression level of most anthocyanin synthase genes in plants (Gonzalez et al., 2008). For instance, TT8 determines seed coat coloring by regulating *DFR* expression, and the *tt8* mutant leads to the loss of anthocyanins and proanthocyanins, making the seed coat colorless in *A. thaliana* (Nesi et al., 2000). TTG1 is well studied in WD40 repeat protein family that lost of function leading to loss accumutaltion of anthocyanins. (Tohge et al., 2005; Nesi et al., 2001). MBW complex regulates anthocyanin accumulation mainly by interacting with promoters of targeted structural genes of flavonoids biosynthesis. For example, MYB75/PAP1, MYB90/PAP2, and bHLH TFs regulate the expression of structural genes association with anthocyanin syntheses, such as *PAL*, *CHS*, *DFR*, and *GST* in *Arabidopsis* (Borevitz et al., 2000; Zimmermann et al., 2004). Although anthocyanin biosynthesis has been well studied, the molecular mechanism of how plants respond to environmental stresses, such as P deficiency, via anthocyanin synthesis has been rarely reported.

P is one of the most important minerals for plant growth and crop productivity. Although P is abundant in soil, however, limited soluble inorganic phosphate is available and accessible to plants, resulting in P deficiency (Bowler et al., 2010). In response to P deficiency, several morphological features have evolved in land plants to enable them to grow and develop in P-stressed environments, such as slowing down taproot growth, forming additional lateral roots, and accumulating starch and anthocyanins (Yuan and Liu, 2008; Ham et al., 2018; Wang et al., 2019). The physiological responses of plants to P deficiency conditions are also finely regulated at the molecular level. PHR1, a MYB domain TF, is considered to be a central regulator of P signaling (Rubio, 2001). SPX proteins (SPX1/SPX2/SPX3/SPX4) containing a single SPX domain acts as a negative upstream regulator of PHR1 (Barabote et al., 2006; Duan et al., 2008). In response to low P signaling, SPX proteins regulates PHR1 to promote downstream *phosphorus-deficiency induction* (*PSI*) gene expression and fine-regulates the P-deficiency response (Wang et al., 2018). Several homologous of *PHR1* (*PHLs*) have been identified in different species (Wang et al., 2019), and PHR1 in combination with PHLs controls most transcriptional activation and inhibition responses to P deficiency (Sun et al., 2015). For example, the *phr1* mutation resulted in plant seedlings insensitive to low P signaling. On the contrary, overexpression of *PHR1* leaded to plant seedlings being hypersensitive to P deficiency signaling (Rubio, 2001; Bustos et al., 2010). PHR1 can also mediated the interaction between different signaling pathways by interacting with other vital proteins (Huang et al., 2018). Regarding P deficiency promoting anthocyanin synthesis in plants, previous studies have clearly shown that PHR1 regulates plant growth and development by regulating the transcription level of downstream genes and then promoting anthocyanin accumulation in *Arabidopsis* seedlings. For example, PHR1 protein can directly bind to the P1BS motif of PAP1 to up-regulate its transcription level (He et al., 2020). PHR1 positively regulated P starvation-induced anthocyanin accumulation through direct upregulation of structural genes *F3′H* and *LDOX* in *Arabidopsis* (Liu et al., 2022). In fact, direct interaction between PHR1 and other signaling pathways at the protein level has rarely been reported. For instance, previous research has demonstrated that Nitrogen-inducible GLK1 is involved in the remodeling of plants alter the root system architecture (RSA) under P starvation, and GLK1 had a positive role in phosphate-starvation response (PSR), and increased the binding ability of PHR1 to its target gene promoter through direct protein–protein interaction (Li et al., 2021). However, the molecular mechanism of how PHR1 or its homology regulates the interaction between P signaling and other regulatory factors of anthocyanin synthesis (such as MYB90, MYB113, MYB114, TT8, etc.) remains largely unknown.

In this study, we used molecular and genetic methods to reveal the biological functions of PHR1 in P deficiency-induced anthocyanin biosynthesis. We initially demonstrated that P deficiency could induce anthocyanin accumulation, and the progress depends on the essential proteins of anthocyanin synthesis. Next, we indicated that the P signaling core PHR1 TF interacts with the anthocyanin synthesis key protein. Further phenotypic analysis showed that PHR1/PHLs positively regulates P deficiency-induced anthocyanin accumulation. We also analyzed the possible genetic relationship between PHR1 and MYBs/TT8. Together, our research precisely revealed the molecular mechanism of P deficiency induced anthocyanin accumulation in plants, providing a new explanation for the interaction of endogenous and exogenous signals, and establish a theoretical basis for plant response to adversity stress.

2. Materials and methods

2.1. Materials and growth environment

All plants of *A. thaliana* used in the present study were in the Columbia (Col-0) genetic background. The *phr1* (SALK_067629C), *phl2* (SALK_114420C), *phl3* (SALK_010040C) and *phl3-40* (SALK_113627C) mutants was bought from the *Arabidopsis* Resource Center (http://abrc.osu.edu). The *myb-RNAi* (Multiple mutants of *myb75 myb90 myb113 myb114*), *tt8-1* and *pap1-D* mutants were provided by Prof. Hongquan Yang. The *phr1 phl2*, *phr1 phl3-40*, *phl2 phl3-40* double mutants and *phr1 phl2 phl3-40* triple mutants were generated by genetically crossing using standard techniques. To generate *35S:PHR1-2HA-5*, *35S:PHL2-2HA-3* and *35S:PHL3-2HA-7* transgenic plants, *PHR1*, *PHL2*, and *PHL3* full-length cDNA with the HA tag sequences were cloned in the sense orientation behind the CaMV 35S promoter into the binary vector pOCA30. Plants were cultivated at 22 °C in a growth chamber with a 16 h/8 h light/dark regime. Taq DNA polymerases and other common chemicals used in our study were provided by Takara Biotechnology (Dalian, China) and Shanghai Sangon (Shanghai, China), respectively.

2.2. Yeast two-hybrid assays

To create bait vectors containing the Gal4 DNA-BD (BD-PHR1, BD-PHL1, BD-PHL2, BD-PHL3 and BD-PHL4), the full-length CDS of PHR1/PHLs were fused to pGBKT7. To create prey vectors with the Gal4 AD (AD-MYB75, AD-MYB90, wAD-MYB113, AD-MYB114 and AD-TT8), We introduced the full-length CDS of MYB75, MYB90, MYB113, MYB114 and TT8 into pGADT7. Three truncated PHR1 sequences were fused to pGBKT7, and two truncated MYB75, MYB113, and TT8 sequences were ligated with pGADT7 to identify particular areas crucial for the interactions. We conducted two-hybrid tests as previously described by Hu et al. (2013). The corresponding plasmids linking pGBKT7 and pGADT7 were co-transformed into yeast *AH109* and screened on a plate containing SD-Leu-Trp. Colonies were further transferred to plates containing SD-Ade-Leu-Trp-His. The physical interaction strength was indicated by the ability of cells to grow on dropout medium lacking Leu, Trp, His and Ade for 4–6 days after plating. In this experiment, we performed at least four biological replicates and obtained consistent results. The primers used in cloning are listed in Table S1.

2.3. Bimolecular fluorescence complementation (BiFC) assay

To obtain PHR1-cYFP and PHR1$^{1-226}$-cYFP, the full-length cDNA or N-terminal 1–226 amino acid residue of PHR1 was cloned into pFGC-cYFP. To obtain GUS-cYFP and GUS-nYFP, the full-length cDNA residues of GUS were also cloned into pFGC-cYFP and pFGC-nYFP. The full-

length MYB75 and MYB113 were inserted into pFGC-nYFP to obtain MYB75-nYFP and MYB113-nYFP. The resulting plasmid were transformed into *Agrobacterium tumefaciens* strain *GV3101*. Then, the bacterial solution was centrifuged at 5000 g min^{-1} for 5 min, discarded supernatant, added into 5–10 mL tobacco injection, blown suspension and left for 30 min. Take different bacterial solution 1:1 (cYFP: nYFP) volume mixed evenly and injected into the leaves of well-growing tobacco (*N. benthamiana*) plants as described by Hu et al. (2019). The Infected tobaccos were placed in a dark and humid environment for 48 h, stained with 40, 6-diamidino-2-phenylindole (DAPI) dye. The YFP and DAPI fluorescence were observed by confocal laser-scanning microscope. The experiments were performed at least four times using different batches of wild tobacco plants; for each biological replicate, more than 12 tobacco plants were infiltrated and more than 600 cells were analyzed. The primers used for cloning are listed in Table S1.

2.4. RNA extraction, cDNA synthesis and qRT-PCR analysis

Total RNA was extracted from seeding (7 days) of the wild-type (WT) and/or mutants with 0.01 mMol/L^{-1} or 0.65 mMol/L^{-1} P treatment using the Trizol reagent provided by Invitrogen. Then quantitative real-time PCR (qRT-PCR) was performed according to the methods described by Hu et al. (2019). Briefly, 1.0 μg DNasetreated RNA was reverse-transcribed in a 20 μL reaction volume with oligo (dT) 18 primer using Moloney murine leukemia virus reverse transcriptase (Fermentas). Then, 1.0 μL cDNA was used for qRT-PCR with the SYBR Premix Ex Taq kit (Takara) on a Roche LightCycler 480 real-time PCR machine, according to the manufacturer's instructions. At least three biological replicates for each sample were used for qRT-PCR analysis. Relative expression levels were normalized to *ACTIN2* (*AT3G18780*; Inada et al., 2021; Kuběnová et al., 2021). The AT3G18780 gene encodes actin constitutively expressed in vegetative structures but not pollen (Inada et al., 2021; Kuběnová et al., 2021). The primers used for the qRT-PCR are listed in Supplemental Table S1.

2.5. Phenotypic analysis of plant response to low P

Seeds were sown on 1/2 MS medium with 0.65 mMol/L^{-1} (+P, control) and 0.01 mMol/L^{-1} (-P) KH_2PO_4. Cultured under long sunshine for about 7 days. Then, anthocyanin accumulation in seedlings and expression levels of anthocyanin synthase genes (*DFR, LDOX* and *UF3GT*) were detected by qRT-PCR to determine their phenotypes in response to low levels of P. More than three independent experiments were performed, and similar results were obtained.

2.6. Determination of anthocyanin content

Anthocyanin content determination was performed as previously described (Hong et al., 2009; Gou et al., 2011). Seedling samples were weighed (W g) and incubated overnight in 1 mL of HCl-methanol (the volume ratio of methyl alcohol to HCl was 99:1) under 4 °C conditions. Anthocyanin extract was then separated from plant tissue by centrifugation at 13000 rpm min^{-1} for 10 min. The absorbance (OD) of the anthocyanin extract at the wavelength of 530 nm and 600 nm was measured by spectrophotometer. Total anthocyanins where calculated by the formula (A530-A600)g^{-1} FW (Xie et al., 2016). We repeated the experiments at least three times.

2.7. Data analysis

Statistical analysis was performed by analysis of variance (ANOVA).

3. Results

3.1. P deficiency-induced anthocyanin accumulation was partially dependent on MYBs and TT8

Because P deficiency can promote anthocyanin accumulation in plants (Khan et al., 2016; He et al., 2020), we predicted that the process depends on the endogenous anthocyanin synthesis pathway. To test the prediction, we investigated the anthocyanin accumulation of anthocyanin synthesis deficient mutants *myb-RNAi*, *tt8* and anthocyanin synthesis acquired mutant (*pap1-D*) and WT on half-strength Murashige and Skoog (½ MS) medium with 0.65 mMol/L^{-1} (+P, control) and 0.01 mMol/L^{-1} (-P) KH_2PO_4. Consistent with previous reports, we found that P deficiency could enhances anthocyanin accumulation in WT seedlings (Fig. 1A and B). Similarly, P deficiency conditions increased significantly *pap1-D* anthocyanin synthesis (Fig. 1A and B). In contrast, there were insignificant difference of anthocyanin contents in the loss-of-function mutants *myb-RNAi* and *tt8* under control and P deficiency conditions (Fig. 1A and B). Using qRT-PCR analysis, we determined the expression levels of several anthocyanin biosynthesis genes in P deficiency-treated seedlings of these mutants, including *DFR*, *LDOX* and *UF3GT*. The transcript levels of *DFR*, *LDOX* and *UF3GT* were elevated in WT and *pap1-D* seedlings under P-deficiency condition, while *tt8* and *myb-RNAi* mutants have no significant differences (Fig. 1C).

3.2. PHR1 physically interacted with MYB75, MYB113 and TT8

Many studies have shown that the PHR1 transcription factor is a essential modulator of phosphorous signaling. Importantly, PHR1 also may function as a vital interaction node to integrate P deficiency signaling and other signaling pathways (Müller et al., 2015). To further explore the molecular mechanism of anthocyanin accumulation promoted by P deficiency, we identified possible physical interactions between P-deficiency signaling central proteins PHR1/PHLs (Bustos et al., 2010; Müller et al., 2015) and core components of the anthocyanin synthesis pathway (MYB75, MYB90, MYB113, MYB114 and TT8) by yeast two-hybrid analysis. The full-length sequences of anthocyanins synthesizing related proteins were fused to the Gal4 activation domain (AD) of the prey vector (AD-MYB75, AD-MYB90, AD-MYB113, AD-MYB114, AD-TT8). The full-length sequences of the P-deficiency signaling proteins were ligated with the Gal4 DNA-binding domain (BD) of the bait vector (BD-PHR1, BD-PHL1, BD-PHL2, BD-PHL3 and BD-PHL4). The PHR1 protein had a strong physical interaction with TT8 proteins but a weak physical interaction with MYB75, MYB90, MYB113 and MYB114 protein in the yeast two-hybrid system (Fig. 2A). PHL1 protein had a strong interaction with MYB75 and MYB90 proteins but a weak interaction with MYB113, MYB114 and TT8 proteins. Both PHL2 and PHL3 proteins strongly interact with MYB75 and MYB113 proteins. However PHL4 protein has no physical connection with MYB75, MYB90, MYB113, MYB114 or TT8 proteins.

To confirm that PHR1 interacts with MYB75, MYB113 and TT8 in plant cells, we carried out bimolecular fluorescence complementation (BiFC) assay. When PHR1-cYFP transiently was coexpressed with MYB75-nYFP or MYB113-nYFP in tobacco (*Nicotiana benthamiana*), YFP fluorescence was detected in the transformed cells' nucleus, as revealed by staining with 40, 6-diamidino-2-phenylindole (DAPI; Fig. 2B). No YFP signal was observed in the negative control assays in which GUS-cYFP was coexpressed with MYB75-nYFP or MYB113-nYFP. Similarly, YFP fluorescence could not be seen when PHR1-cYFP and GUS-nYFP were co-expressed (Fig. 2B).

3.3. Amino acid sequences of MYBs and TT8 interacting with PHR1

To identify the region of PHR1 that be essential for the interaction with MYB75, MYB90, MYB113, MYB114 and TT8, we fused three

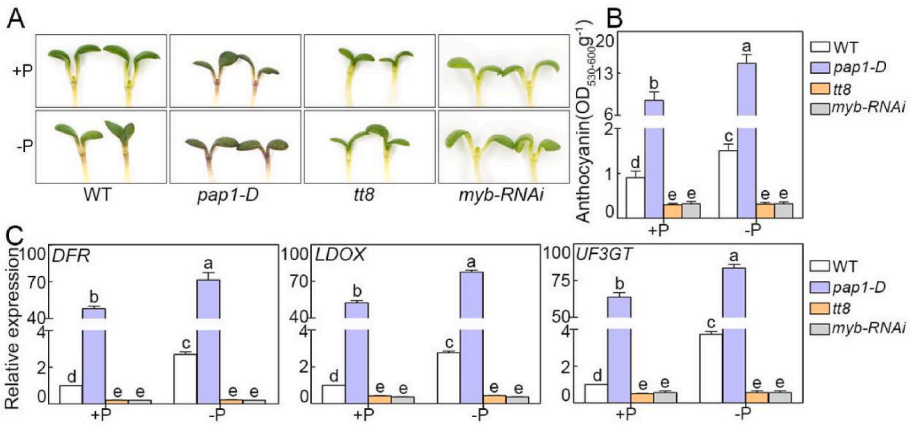

Fig. 1. P deficiency-induced anthocyanin accumulation was partially dependent on MYBs and TT8 proteins. A, Phenotypes of 7-d-old seedlings of WT, *pap1-D*, *myb-RNAi* and *tt8* grown on ½ MS medium containing 0.65 mMol/L^{-1} and 0.01 mMol/L^{-1} KH_2PO_4 (+P and -P). B, Anthocyanin content in 7-d-old seedlings of WT, *pap1-D*, *myb-RNAi* and *tt8* grown on ½ MS medium containing 0.65 mMol/L^{-1} and 0.01 mMol/L^{-1} KH_2PO_4 (+P and -P). C, qRT-PCR analysis the expression of *DFR, LDOX* and *UF3GT* in WT, *pap1-D*, *myb-RNAi* and *tt8*. The *ACTIN2* (*AT3G18780*) gene was used as a control. Different letters represent significant differences (ANOVA, $P < 0.05$).

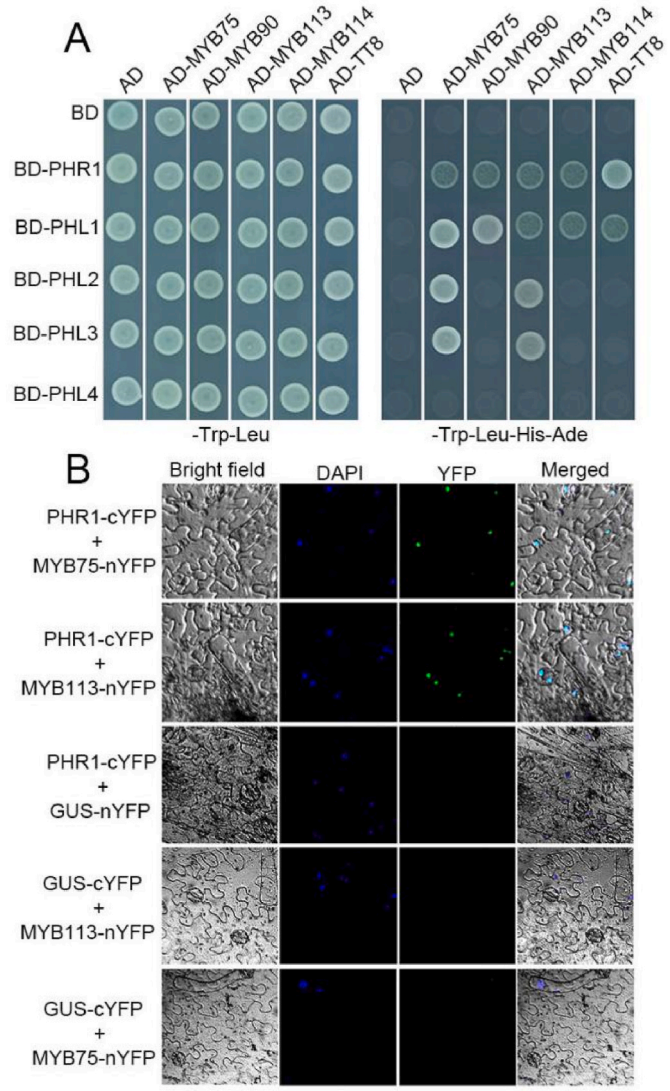

Fig. 2. PHR1 physically interacted with MYB75, MYB113 and TT8. A, Yeast two-hybrid analysis. Interaction of PHR1/PHLs with anthocyanin synthesis related proteins is indicated by the ability of yeast cells to grow on dropout medium that lacks Leu, Trp, His and Ade for 4 d after plating. BD and AD were negative controls. B, BiFC assays. When PHR1-cYFP and MYB75-nYFP, MYB113-nYFP were co-transformed into tobacco leaves, strong fluorescence was observed in the nucleus of tobacco, but no fluorescence signal was observed in the corresponding negative control. DAPI was used to stain the nucle.

truncated PHR1 variants to the Gal4 DNA-BD of the pGBKT7 vector as baits (BD-PHR1$^{1-226}$, BD-PHR1$^{219-410}$, BD-PHR1$^{293-410}$), and examined the interaction between these variants and MYB75, MYB90, MYB113, MYB114, TT8 by yeast two-hybrid analysis. Our segmentation basis is that the N-terminal amino acid residues 1–226 of PHR1 do not contain a special domain, while the intermediate amino acid residues 219–410 of PHR1 contain a MYB domain and the C-terminal amino acid residues 293–410 of PHR1 contain a CC domain. As shown in Fig. 3A, the N-terminal amino acid residues 1–226 of PHR1 (BD-PHR1$^{1-226}$) physically interacted with MYB75, MYB113 and TT8, but not with MYB90 and MYB114. Deleting the N-terminal amino acid residues 1–226 of PHR1 (BD-PHR1$^{219-410}$, BD-PHR1$^{293-410}$) completely abolished the PHR1-MYBs or PHR1-TT8 interaction (Fig. 3A). Thus, we chould see that the full length of PHR1 had a weak interaction with MYB75 and MYB113, but the N-terminal amino acid residues 1–226 of PHR1 had a stronger interaction with MYB75 and MYB113. This results indicated there may be a segment before segmentation which inhibits their interaction, or the full length autoactivation may be strong, resulting in an inconspicuous interaction between them.

To determine the MYB75, MYB113 and TT8 regions critical for the interaction with PHR1, the sequences of MYB75, MYB113 and TT8 were similarly truncated by us to obtain variants with the N-terminal domain or the C-terminal fragment (Fig. 3B). We fused the truncated MYB75, MYB113 and TT8 sequences to the prey vector's Gal4 AD (AD-MYB75 $^{(1-122)}$-N, AD-MYB113 $^{(1-123)}$-N, AD-TT8 $^{(1-358)}$-N, AD-MYB75 $^{(123-249)}$-C, AD-MYB113 $^{(124-247)}$-C and AD-TT8 $^{(359-519)}$-C) and examined the interaction between these variants and PHR1 by yeast two-hybrid analysis (Fig. 3B). As shown in Fig. 3B, the N-terminal domains of MYB75, MYB113 and TT8 physically interacted with PHR1. The C-terminal fragment of MYB75, MYB113 and TT8 did not physically interacted with PHR1.

We used BiFC assay to further confirm the interaction between PHR1's 1–226 amino acid sequence and MYBs and TT8 in plant cells. When PHR1$^{1-226}$-cYFP transiently was coexpressed with MYB75-nYFP or MYB113-nYFP in tobacco (*Nicotiana benthamiana*), strong YFP fluorescence was detected in the transformed cells' nucleus, as revealed by staining with 40, 6-diamidino-2-phenylindole (DAPI; Fig. 3C). In the corresponding negative control experiment, no YFP fluorescence signal was observed (Fig. 3C).

3.4. Disruption of PHR1, PHL2, and PHL3 attenuateed P deficiency-induced anthocyanin accumulation

Because PHR1 physically interacted with the MYB75, MYB113 and TT8. We hypothesized that PHR1 is involved in the P deficiency regulation of anthocyanin accumulation. To test this prediction, we evaluated the anthocyanin accumulation of the loss-of-function *phr1, phl2, phl3* and *phl3-40* single mutants on ½ MS supplemented with 0.65

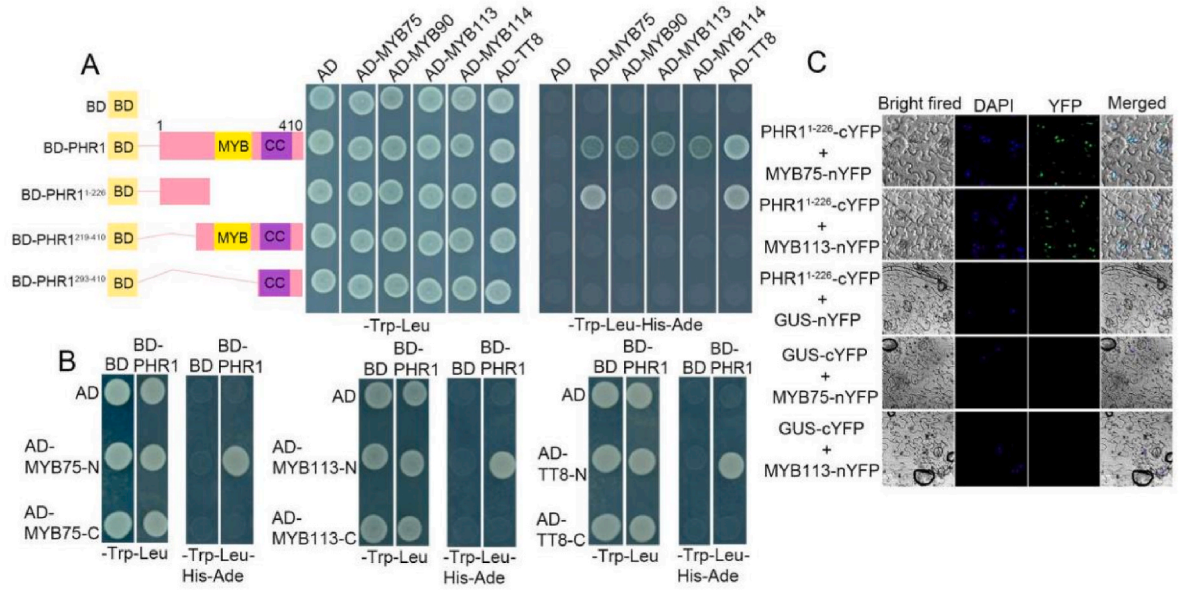

Fig. 3. The regions required for the interaction of PHR1 transcription factor with MYB75, MYB113 and TT8 proteins. A, Yeast two-hybrid analysis. The 1–226 amino acid residues of PHR1 interacted with MYB75, MYB113 and TT8 in full length. Left: Full length and segmentation diagram of PHR1 sequence. Right: Interaction strength. B, Yeast two-hybrid analysis. The N-terminal fragment of MYB75, MYB113 and TT8 interacted with PHR1. BD and AD were negative controls. C, BiFC assays. When PHR1$^{1-226}$-cYFP transiently was coexpressed with MYB75-nYFP or MYB113-nYFP in wild tobacco's leaf cells, strong YFP fluorescence was detected in the transformed cells' nucleus, but no fluorescence signal was observed in the corresponding negative control. DAP1 was used to stain the nuclei.

mMol/L^{-1} and 0.01 mMol/L^{-1} P. As shown in Fig. S1, Compared with normal phosphorous concentration (0.65 mMol/L^{-1}), WT showed more anthocyanin accumulation under low-P concentration (0.01 mMol/L^{-1}). Single mutant seedlings also showed more anthocyanin accumulation in response to low-P availability, but slightly lower than WT. We detected the expression levels of anthocyanin synthase genes (*DFR*, *LDOX* and *UF3GT*), and found that the expression levels of these genes were slightly higher in the mutants under low P concentration, but the difference was not significant (Fig. S1C). This finding showed that disruption of *phr1, phl2* and *phl3* alone had little effect on phosphorous-deficiency responses during anthocyanin accumulation.

Considering that PHR1 and its homologous proteins PHL2 and PHL3 play overlapping roles in P signaling pathways, we speculated that they might also have functional redundancy in anthocyanin accumulation.

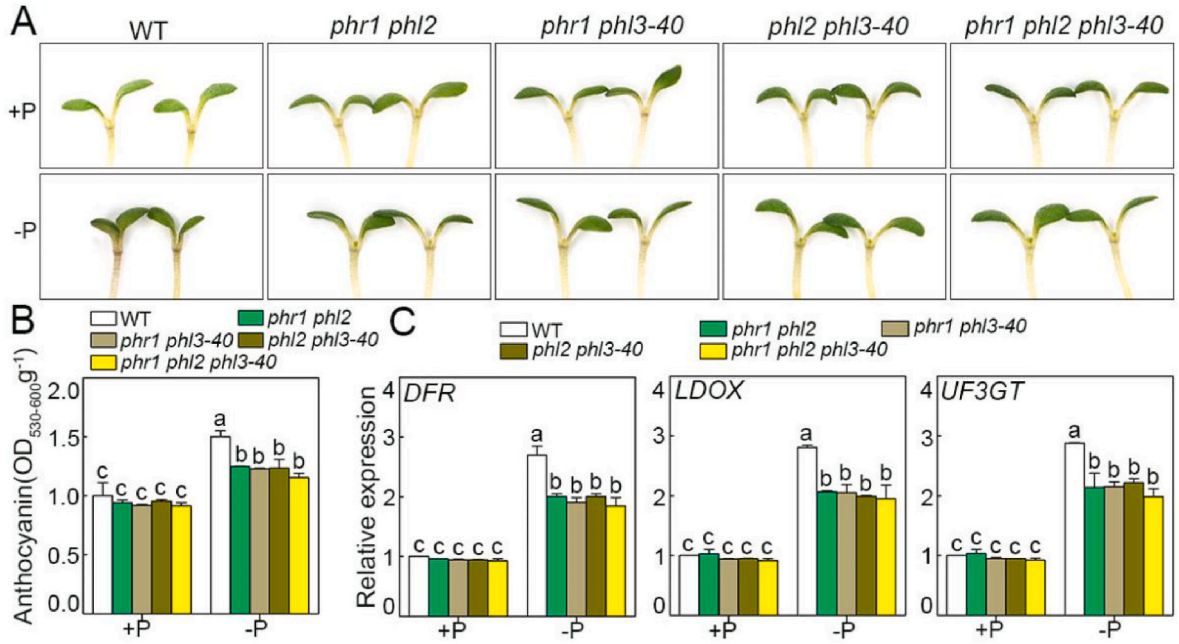

Fig. 4. The *phr1 phl2*, *phr1 phl3-40*, *phl2 phl3-40* and *phr1 phl2 phl3-40* multi-mutant seedlings showed reduced sensitivity to P deficiency-induced anthocyanin accumulation. A, Phenotypes of 7-d-old seedlings of WT, *phr1 phl2*, *phr1 phl3-40*, *phl2 phl3-40* and *phr1 phl2 phl3-40* grown on ½ MS medium containing 0.65 mMol/L^{-1} and 0.01 mMol/L^{-1} KH_2PO_4 (+P and -P). B, Anthocyanin content in 7-d-old seedlings of WT, *phr1 phl2*, *phr1 phl3-40*, *phl2 phl3-40* and *phr1 phl2 phl3-40* grown on ½ MS medium containing 0.65 mMol/L^{-1} and 0.01 mMol/L^{-1} KH_2PO_4 (+P and -P). C, qRT-PCR analysis of the expression of *DFR*, *LDOX*, *UF3GT* in the WT, *phr1 phl2*, *phr1 phl3-40*, *phl2 phl3-40* and *phr1 phl2 phl3-40* mutants. The *ACTIN2* (*AT3G18780*) gene was used as control. Different letters represent significant differences (ANOVA, $P < 0.05$).

To test this speculation, we constructed *phr1 phl2*, *phr1 phl3-40* and *phl2 phl3-40* double mutants and evaluated their performance in ½ MS medium containing 0.65 mMol/L^{-1} and 0.01 mMol/L^{-1} KH_2PO_4. Under low P conditions, the double mutant seedlings accumulated significantly lower anthocyanins than the WT seedlings (Fig. 4A and B). Further qRT-PCR analysis of the expression levels of anthocyanin synthase genes (*DFR*, *LDOX* and *UF3GT*) showed, consistent with the phenotype, the expression levels of *DFR*, *LDOX* and *UF3GT* were significantly lower in the double mutants than those in WT plants under low P conditions (Fig. 4C). We also constructed *phr1 phl2 phl3-40* triple mutant. As shown in Fig. 4A and B, the triple mutant was slightly less sensitive to anthocyanin accumulation than the double mutants, and the anthocyanin content and expression level of the anthocyanin synthase genes were much lower than WT (Fig. 4C). Taken together, our results showed that PHR1 and its homologs may positively regulate P deficiency-induced anthocyanin accumulation in *Arabidopsis* seedlings.

3.5. Overexpression of PHR1, PHL2 or PHL3 confered hypersensitive to P deficiency-induced anthocyanin accumulation

To further analyze the role of PHR1 and its homologs in anthocyanin biosynthesis, we generated transgenic plants overexpressing *PHR1 (35S:PHR1-2HA)*, *PHL2 (35S:PHL2-2HA)* and *PHL3 (35S:PHL3-2HA)* under the control of the CaMV 35S promoter, and several homozygous lines with relatively high expression levels were screened (*35S:PHR1-2HA-5, 35S:PHL2-2HA-3* and *35S:PHL3-2HA-7*). We investigated the accumulation of anthocyanins in these transgenic plants on ½ MS medium with 0.65 mMol/L^{-1} and 0.01 mMol/L^{-1} KH_2PO_4. As shown in Fig. 5A and B, anthocyanin accumulation in *35S:PHR1-2HA-5*, *35S:PHL2-2HA-3* and *35S:PHL3-2HA-7* seedlings with no significant difference from WT under normal P condition, but higher than WT under low P condition, showing purplish-red stem tips. The expression levels of anthocyanin synthase genes *DFR*, *LDOX* and *UF3GT* were also detected by us in *35S:PHR1-2HA-5, 35S:PHL2-2HA-3* and *35S:PHL3-2HA-7*. Compared with the WT, the expression levels of *DFR, LDOX* and *UF3GT* in *35S:PHR1-2HA-5, 35S:PHL2-2HA-3* and *35S:PHL3-2HA-7* were significantly higher than those in WT under low P conditions (Fig. 5C). Therefore, overexpression of *PHR1* and its homologs enhanced the response to P deficiency-induced anthocyanin accumulation in *Arabidopsis thaliana*, further supporting the view that PHR1 positively regulates P signaling to induce anthocyanin accumulation.

3.6. Overexpression of PHR1 partially recovered the deficiency of anthocyanin accumulation in myb-RNAi or tt8

Having ascertained that PHR1 interacts with MYB75, MYB113, TT8 and positively modulates anthocyanin accumulation induced, we asked whether the action of PHR1 in mediating anthocyanin accumulation depends on MYB75 and TT8. To test this possibility, we generated *myb-RNAi 35S:PHR1-2HA-5*, *tt8 35S:PHR1-2HA-5* plants by genetically crossing *35S:PHR1-2HA-5* with *myb-RNAi or tt8*, and analyzed anthocyanin accumulation in response to P-deficiency conditions. As shown in Fig. 6, the anthocyanin accumulation of *myb-RNAi 35S:PHR1-2HA-5, tt8 35S:PHR1-2HA-5* were between *35S:PHR1-2HA-5* and *myb-RNAi or tt8* under low P conditions (Fig. 6). These findings revealed that *35S:PHR1-2HA-5* plants could partially recovers the P deficiency insensitive phenotype of *myb-RNAi* or *tt8*. These results implied that PHR1 regulates P deficiency-induced anthocyanin synthesis partially through MYB75 and TT8 regulatory proteins.

4. Discussion

Due to the prevalent P shortage in agriculture and natural ecosystems, it is necessary to investigate the expression of genes related to many metabolic pathways of plants under P-starved conditions (Misson et al., 2005; Morcuende et al., 2007). Anthocyanin synthesis is one of the essential strategies for plants to cope with P deficiency (Diaz et al., 2006; Peng et al., 2008; Liang and He, 2018). Very recently, Liu et al. (2022) found that PHR1 positively regulates P starvation-induced anthocyanin accumulation through upregulation of genes that are involved in anthocyanin biosyntheses in *Arabidopsis*. However, the molecular mechanism of phosphorous signaling inducing anthocyanin accumulation in plants remains unclear. In this work, we demonstrated that low P availability could induce anthocyanin accumulation (Fig. 1), and then investigated how PHR1, an essential modulator of phosphate starvation signaling (Müller et al., 2015), regulates the endogenous anthocyanin synthesis pathway to promote anthocyanin accumulation in *Arabidopsis.* Below we discuss the results of our experimental investigations in

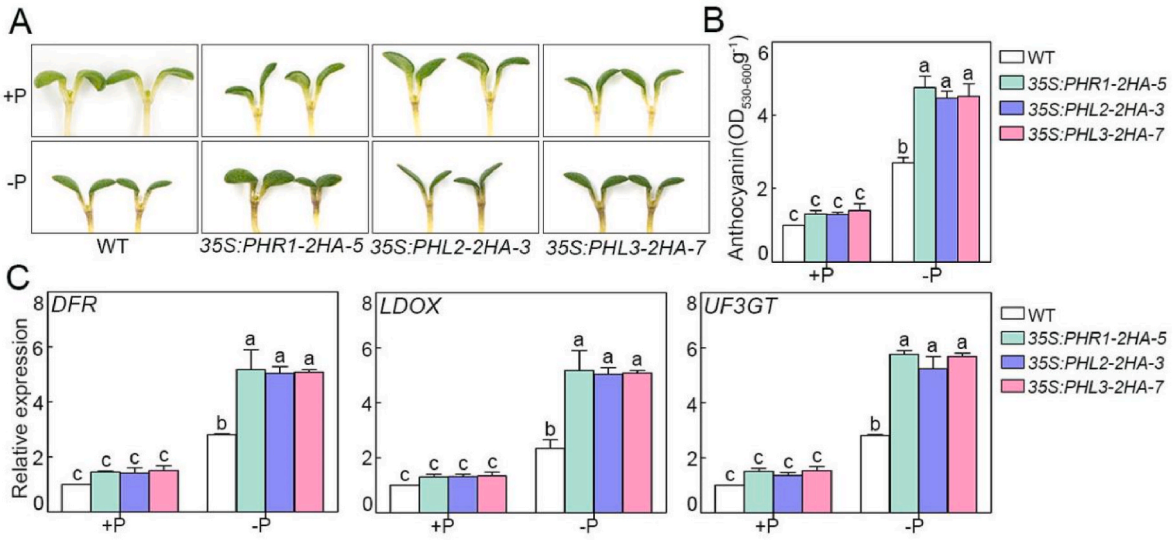

Fig. 5. Overexpression of *PHR1*, *PHL2* or *PHL3* confered hypersensitive to P deficiency-induced anthocyanin accumulation. A, Phenotypes of 7-d-old seedlings of WT, *35S:PHR1-2HA-5, 35S:PHL2-2HA-3 and 35S:PHL3-2HA-7* grown on ½ MS medium containing 0.65 mMol/L^{-1} and 0.01 mMol/L^{-1} KH_2PO_4 (+P and -P). B, Anthocyanin content in 7-d-old seedlings of WT, *35S:PHR1-2HA-5, 35S:PHL2-2HA-3 and 35S:PHL3-2HA-7* grown on ½ MS medium containing 0.65 mMol/L^{-1} and 0.01 mMol/L^{-1} KH_2PO_4 (+P and -P). C, qRT-PCR analysis of the expression of *DFR*, *LDOX* and *UF3GT* in the WT, *35S:PHR1-2HA-5, 35S:PHL2-2HA-3* and *35S:PHL3-2HA-7*. The *ACTIN2* (*AT3G18780*) gene was used as control. Different letters represent significant differences (ANOVA, $P < 0.05$).

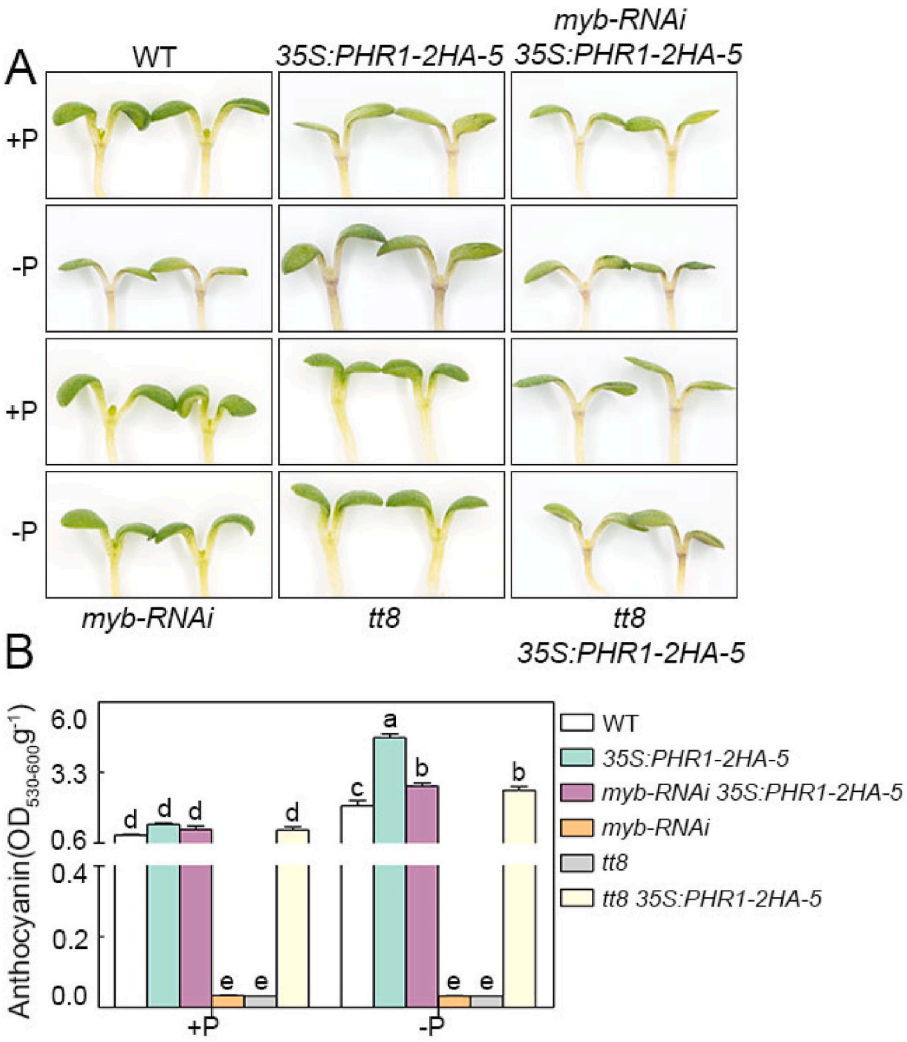

Fig. 6. Overexpression of *PHR1* partially restored the deficiency of anthocyanin accumulation in *myb-RNAi* or *tt8*. A, Phenotypes of 7-d-old seedlings of WT, *35S:PHR1-2HA-5*, *myb-RNAi 35S:PHR1-2HA-5, tt8 35S:PHR1-2HA-5, myb-RNAi* and *tt8* grown on ½ MS medium containing 0.65 mMol/L^{-1} and 0.01 mMol/L^{-1} KH_2PO_4 (+P and -P). B, Anthocyanin content in 7-d-old seedlings of WT, *35S:PHR1-2HA-5*, *myb-RNAi 35S:PHR1-2HA-5, tt8 35S:PHR1-2HA-5, myb-RNAi* and *tt8* grown on ½ MS medium containing 0.65 mMol/L^{-1} and 0.01 mMol/L^{-1} KH_2PO_4 (+P and -P). Different letters represent significant differences (ANOVA, $P < 0.05$).

Arabidopsis and consider how P signaling core protein with the anthocyanin synthesis related regulatory proteins in *Arabidopsis*.

Our results showed that P deficiency conditions resulted in significantly anthocyanin synthesis in WT and anthocyanin synthesis sensitive phenotype *pap1-D* (Fig. 1A and B). Previous studies had indicated that the anthocyanin biosynthesis pathway is catalyzed by a series of enzymes, among which DFR, LDOX and UF3GT play important roles (Besseau et al., 2007; Owens et al., 2008). Recently, it has been reported that PHR1 positively regulates phosphate starvation-induced anthocyanin accumulation by directly upregulating *LDOX* gene in *Arabidopsis* (Liu et al., 2022). Our results of qRT-PCR quantification revealed that the expression patterns of anthocyanin biosynthesis genes *DFR*, *LDOX* and *UF3G* in WT and *pap1-D* are related to anthocyanin accumulation in response to P limitation (Fig. 1C). These findings supported the previous views that P deficiency could enhances the anthocyanin accumulation via the endogenous anthocyanin synthesis pathway in *Arabidopsis* (Khan et al., 2016; He et al., 2020; Liu et al., 2022). On the contrary, there were no significantly difference in both anthocyanin content and the expression of *DFR, LDOX* and *UF3GT* among loss-of-function mutants *myb-RNAi* and *tt8* regardless of P availability (Fig. 1C). The results strongly suggested that MYBs and TT8 played important roles in P deficiency-induced anthocyanin accumulation in *Arabidopsis,* consisting with previous findings that the MYB-bHLH-WD40 (MBW) complex is the master and conservative regulator of anthocyanin synthesis (He et al., 2020; Meng et al., 2021). For example, MYB75 is a central regulator of anthocyanin synthesis and many endogenous and exogenous signals can regulate anthocyanin accumulation through it (Peng et al., 2008; He et al., 2020). In addition to MYB75 transcription factor, MYB90, MYB113, MYB114, TT8 may also involve in anthocyanin synthesis induced by low P.

The PHR1 TF is one of the most critical transcription factors in the plant P signaling pathway (Rubio, 2001; He et al., 2020; Liu et al., 2022). Our results pointed to a decisive regulatory role for PHR1 in P deficiency-mediated anthocyanin biosynthesis. First, we demonstrated that PHR1 and its homologous proteins interacted with regulatory proteins such as MYB75, MYB90, MYB113 and TT8 to form protein complexes (Fig. 2). By staging experiments, we showed that the 1–226 amino acid residues of PHR1 and the N-terminal regions of MYB75, MYB113 and TT8 were critical to the interaction (Fig. 3). However, we could see that the full length of PHR1 had a weak interaction with MYB75 and MYB113, but the N-terminal amino acid residues 1–226 of PHR1 had a stronger interaction with MYB75 and MYB113. This indicated that there may be a segment before segmentation which inhibited their interaction, or the full length autoactivation may be strong, resulting in an inconspicuous interaction between them.

It further verified that PHR1 interacted with regulatory proteins related to anthocyanin synthesis in low-P conditions. Further phenotypic analysis revealed that *phr1*-related mutants had lower sensitivity to deficient P than the WT (Fig. S1; Fig. 4). On the contrary, compared with the WT, overexpressed *PHR1* and its homologs showed a hypersensitive phenotype to deficient P, and anthocyanin content was significantly higher than that under normal P conditions (Fig. 5). The expression of

the anthocyanin synthase gene, including *DFR LDOX,* and *UF3GT,* also showed consistent results (Supplemental Fig. S1; Figs. 4 and 5). Based on these results, this study confirmed to the previous findings that PHR1 was a major regulator in low P responses (Rubio, 2001; Bustos et al., 2010; He et al., 2020; Liu et al., 2022). In line with previous studies (Rubio, 2001; Bustos et al., 2010; Sun et al., 2015; He et al., 2021), it is positively regulated P deficiency-induced anthocyanin accumulation and partially redundant with PHL2 and PHL3, as *phr1 phl2*, *phr1 phl3-40, phl2 phl3-40* double mutant seedlings and *phr1 phl2 phl3-40* triple mutant seedlings are less responsive than *phr1, phl2, phl3 and phl3-40 single mutant* seedlings to low P (Fig. S1; Fig. 4). By constructed *myb-RNAi 35S:PHR1-2HA-5* and *tt8 35S:PHR1-2HA-5,* we found that overexpression of *PHR1* could partially restoreed the insensitive phenotype of *myb-RNAi* and *tt8* to P deficiency during anthocyanin accumulation, indicating that PHR1 can regulates P deficiency-induced anthocyanin accumulation partially through MYBs and TT8 (Fig. 6). The results further revealed the genetic relationship between PHR1and MBW complex. Taking these results together, this study uncovered a direct link between P deficiency and flavonoid metabolism and provided a novel regulatory module, where PHR1-mediated P-deficiency introduced anthocyanin accumulation is largely dependent on MYBs and TT8.

The new regulatory pathways of anthocyanins and the interaction factors of each path to the synergistic regulation of anthocyanins are yet to be discovered. Currently, more and more regulatory factors of anthocyanin synthesis have been identified at the transcriptional level. For example, SPX4 could regulate P-dependent anthocyanin biosynthesis by interacting with PHR1 and PAP1 (Puga et al., 2014; He et al., 2020).

It remains unclear whether SPX4 directly interacts with MYB113 and TT8 to regulate anthocyanin synthesis. In addition to PHR1/PHLs proteins, other vital components of the P signaling pathway, such as SPX4, may also be essential for regulating anthocyanin accumulation. In addition, biochemical experiments will be conducted in the future to further analyze the regulatory interaction between PHR1 and the regulatory factors related to anthocyanin synthesis, so as to fully reveal the regulatory network of anthocyanin accumulation. The regulatory mechanisms of MYB, BHLH and WD40 transcription factors and their MBW complexes on gene expression were also need revealed.

5. Conclusion

Our study indicated that PHR1 and MBW complexes form protein complexes that directly mediates the process of P starvation-induced anthocyanin accumulation, providing a new mechanistic understanding of how P-deficient signaling depends on endogenous anthocyanin synthesis pathway to promote anthocyanin accumulation in *Arabidopsis*. This subject not only revealed the interaction between P signaling and anthocyanin synthesis signals at the protein level, but also provides a theoretical basis for the intrinsic connection between exogenous nutrient signals and endogenous anthocyanin synthesis signaling, which is of great significance for the in-depth understanding of environmental stress regulation of plant environmental adaptation.

Author contributions

YH and HL designed this study; HL and KH performed experiments; HL analyzed the data; HL and ZQZ interpreted the results and wrote the manuscript; All authors contributed critically to the drafts and gave final approval for publication.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2023.01.029.

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