Circadian Clock PRR Proteins Promote Anthocyanin Synthesis in Arabidopsis Seedlings Postprint

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Abstract

The circadian clock is a conserved endogenous regulatory mechanism that drives and maintains rhythmic expression of plant physiological traits. The PRR (PSEUDO-RESPONSE REGULATOR) protein family constitutes an important component of the central oscillator of the circadian clock, regulating various life processes such as seed germination, hypocotyl elongation, and flowering in plants. Anthocyanins are plant secondary metabolites that play important roles in plant reproduction, growth and development, and resistance to abiotic stress. This study used Arabidopsis thaliana as a research model to investigate the regulatory function and molecular mechanism of circadian clock PRR proteins in anthocyanin biosynthesis. The results showed that: (1) In PRR gene single and multiple mutant seedlings, anthocyanin accumulation was significantly reduced, and the expression of certain anthocyanin synthesis-related genes was significantly decreased. (2) Conversely, in PRR5 overexpression seedlings, anthocyanin accumulation and the expression of certain anthocyanin synthesis-related genes were significantly increased. (3) Protein interaction experiments demonstrated that the PRR5 protein can interact with anthocyanin regulatory proteins such as MYB75, TT8, MYB90, and MYB113 to form complexes. (4) Genetic analysis revealed that PRR5-induced anthocyanin synthesis in Arabidopsis seedlings depends on MYB family anthocyanin regulatory proteins. In summary, circadian clock PRR proteins may promote anthocyanin synthesis and accumulation in Arabidopsis seedlings through interactions between PRR5 and MYB75, TT8, and other proteins.

Full Text

Preamble

Circadian Clock PRR Proteins Stimulate Anthocyanin Synthesis in Arabidopsis thaliana Seedlings

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Abstract

The circadian clock is a conserved endogenous regulatory mechanism that drives and maintains rhythmic expression of plant physiological traits. The PRR (PSEUDO-RESPONSE REGULATOR) protein family constitutes a critical component of the circadian clock central oscillator, regulating diverse life processes including seed germination, hypocotyl elongation, and flowering. Anthocyanins are plant secondary metabolites that play important roles in plant reproduction, growth, development, and stress resistance. This study investigates the regulatory function and molecular mechanism of circadian clock PRR proteins in anthocyanin biosynthesis using Arabidopsis thaliana as a model system. The results demonstrate: (1) Anthocyanin accumulation and expression of certain anthocyanin synthesis-related genes were significantly reduced in both single and multiple PRR gene mutant seedlings. (2) Conversely, anthocyanin accumulation and expression of certain anthocyanin synthesis-related genes were significantly elevated in PRR5-overexpressing seedlings. (3) Protein-protein interaction experiments revealed that PRR5 physically interacts with anthocyanin regulatory proteins including MYB75, TT8, MYB90, and MYB113 to form protein complexes. (4) Genetic analysis demonstrated that PRR5-induced anthocyanin synthesis in Arabidopsis seedlings depends on MYB family anthocyanin regulatory proteins. In conclusion, circadian clock PRR proteins likely promote anthocyanin synthesis and accumulation in Arabidopsis seedlings through interactions between PRR5 and MYB75, TT8, and other regulatory proteins.

Keywords: Arabidopsis thaliana, circadian clock, PRR protein, anthocyanin, MBW complex

Introduction

Anthocyanins, also known as anthocyanidin glycosides, are plant secondary metabolites widely distributed in flowers, fruits, and leaves (Liang et al., 2018; Song et al., 2021). Six major anthocyanidins—pelargonidin, cyanidin, peonidin, petunidin, delphinidin, and malvidin—undergo glycosylation, methylation, and

acylation modifications to generate over 600 anthocyanin variants, conferring diverse colors to plants (Li et al., 2018; Wang et al., 2018; Gardeli et al., 2019; Sun et al., 2021). Anthocyanins enhance plant resistance to various environmental stresses and play crucial roles in plant adaptation (Hatier et al., 2013; Fan et al., 2016; Li et al., 2018). Additionally, anthocyanins possess significant ornamental, nutritional, and medicinal value (Davies et al., 2012; Peiffer et al., 2016; Nomi et al., 2019). Structural genes encoding enzymes in the anthocyanin biosynthetic pathway are classified into two categories: early biosynthetic genes such as *CHS*, *CHI*, and *F3H*, and late biosynthetic genes including *DFR*, *ANS*, and *UFGT* (Deng & Lu, 2017; Chen et al., 2020). These structural genes are precisely regulated by multiple transcription factors, with the most extensively studied and critical being the MYB (Myeloblastosis), bHLH (basic Helix-Loop-Helix), and WDR (also called WD40) families (Tanaka et al., 2008). MYB75/PAP1 is a key MYB transcription factor regulating anthocyanin synthesis in Arabidopsis, and MYB75-overexpressing transgenic plants accumulate substantial anthocyanins in roots, stems, leaves, and flowers (Rinaldo et al., 2015; Shin et al., 2015). Additionally, MYB family members MYB90/PAP2, MYB113, and MYB114 positively regulate anthocyanin biosynthesis in Arabidopsis (Gonzalez et al., 2008; Maier et al., 2013; Shi & Xie, 2014). bHLH proteins including TT8, GL3, EGL3, and MYC1 participate in anthocyanin synthesis regulation by activating expression of structural genes such as *CHS* and *DFR* (Martinez-Garcia et al., 2000). WDR proteins such as TTG1 are also essential for inducing stable anthocyanin accumulation (Payne et al., 2000). In addition to their individual functions in promoting anthocyanin synthesis, these three transcription factor families can form ternary MBW complexes that directly regulate structural gene expression to promote anthocyanin synthesis (Liang et al., 2018). Accumulating evidence indicates that microRNAs also participate directly or indirectly in regulating anthocyanin synthesis in plants (Varsha et al., 2019; He et al., 2019; Maria et al., 2019). Given the biological functions of anthocyanins and their application prospects in breeding, food, and medicine, in-depth investigation of anthocyanin biosynthesis and regulatory signaling holds significant applied value and scientific importance.

Recent research has made important advances in understanding how exogenous environmental signals and endogenous phytohormone signals regulate anthocyanin biosynthesis and accumulation. For example, both light intensity and quality play critical roles in anthocyanin synthesis (Guo et al., 2008; Maier & Hoecker, 2015), low temperature promotes anthocyanin biosynthesis (Mori et al., 2007; Zhang et al., 2011), and phytohormones including abscisic acid, jasmonic acid, and auxin affect anthocyanin synthesis and accumulation by regulating expression of anthocyanin synthesis-related genes (Ji et al., 2015; Xie et al., 2016; Chen et al., 2019; Chen et al., 2020). The plant circadian clock system receives temporal information about dynamic changes in environmental factors such as light, temperature, and nutrients through the core oscillator (input pathway), generates endogenous circadian rhythms in plants (central oscillator), and subsequently regulates numerous plant developmental processes including flowering,

biotic and abiotic stress responses, and hormone metabolism (output pathway) (Wei et al., 2018). PRR5, PRR7, and PRR9 in the PRR family are key components of the early loop of the central oscillator and play important roles in regulating plant growth, development, and stress responses (Sanchez & Kay, 2016). Both the circadian clock and anthocyanins function importantly in plant stress responses (Wei et al., 2018; Song et al., 2021), yet no studies have reported on circadian clock signaling regulation of anthocyanin synthesis. Recent research demonstrated that circadian clock PRR proteins promote ABA signaling to inhibit seed germination and post-germination growth (Yang et al., 2021), while ABA signaling can induce anthocyanin synthesis and accumulation in Arabidopsis seedlings (Chen et al., 2020). This raises the question: are circadian clock PRR proteins directly involved in anthocyanin synthesis?

Using Arabidopsis as experimental material, this study employs molecular biology and genetics approaches to investigate the biological function and molecular mechanism by which circadian clock PRR proteins promote anthocyanin synthesis in Arabidopsis seedlings. These findings are significant for discovering novel biological functions of PRR proteins and deepening our understanding of how circadian clock signaling regulates environmental adaptation in plant seedlings.

Materials and Methods

Plant Materials and Growth Conditions

All wild-type (WT), mutant, and overexpressing Arabidopsis plants used in this study were in the Columbia (Col-0) genetic background. Mutant seeds *prr5-1* (SALK_{006280}), *prr5-2* (SALK_{135000C}), *prr7-1* (SALK_{091569C}), and *prr7-2* (SALK_{030430C}) were obtained from the Arabidopsis Biological Resource Center at Ohio State University. The *prr5 prr7* double mutant was generated by genetic crossing of *prr5-1* and *prr7-2*. The *prr5 prr9* and *prr5 prr7 prr9* mutants were provided by Dr. Lei Wang from the Institute of Botany, Chinese Academy of Sciences, and *myb-RNAi* was provided by Professor Hongquan Yang. To generate *35S:PRR5-FLAG* transgenic plants, full-length *PRR5* cDNA fused with a 2FLAG tag sequence was cloned into the pOCA30 vector driven by the CaMV 35S promoter using *Sma*I and *Xba*I restriction sites. T3 generation homozygous PRR5-overexpressing plants were obtained through *Agrobacterium*-mediated transformation of wild-type (Col-0) Arabidopsis (Yang et al., 2021). Appropriate amounts of Arabidopsis mutant, overexpressing, and wild-type seeds were surface-sterilized by soaking in 20% commercial bleach (Liby White Clothing Stain Remover) for 8 minutes, then rinsed 3–5 times with sterile water. Seeds were sown on half-strength Murashige and Skoog (1/2 MS) solid medium containing 1% (w/v) sucrose (pH 5.8). After stratification at 4°C for 24 hours, plates were transferred to a growth chamber under long-day conditions (22°C constant temperature, 16 h white light/8 h dark). Approximately 8-day-old seedlings were transplanted to moist soil, covered with plastic wrap, and maintained under long-day conditions. The wrap was removed after 2–3 days.

Anthocyanin Content Measurement

Six-day-old Arabidopsis seedlings grown under long-day conditions (including Col-0, *prr5-1*, *prr5-2*, *prr7-1*, *prr7-2*, *prr5 prr7*, *prr5 prr9*, *prr5 prr7 prr9*, and *35S:PRR5-FLAG*) were harvested at Zeitgeber time 10 (ZT 10, the 10th hour of the light period) and weighed as W(g). One milliliter of anthocyanin extraction solution (methanol:hydrochloric acid, 99:1 v/v) was added to each sample. Samples were shaken in darkness at 4°C for 24 hours, then centrifuged at 13,000 rpm for 10 minutes. The supernatant was collected and measured spectrophotometrically at 530 nm and 657 nm (A530 and A657) using the extraction solution as a blank. Relative anthocyanin content was calculated using the formula (A530 - 0.25 × A657) g^{-1} fresh weight (Chen et al., 2020). All experiments were performed with at least three biological replicates.

RNA Extraction and RT-qPCR

Six-day-old Arabidopsis seedlings grown under long-day conditions were harvested at ZT 10. Total RNA was extracted using TRIzol reagent and reverse-transcribed into cDNA for RT-qPCR analysis (Han et al., 2020). Primers used for RT-qPCR are listed in Table 1 , with *ACTIN2* serving as the internal reference gene. All experiments were performed with at least three biological replicates.

Yeast Two-Hybrid Assay (Y2H)

The yeast two-hybrid system (Hu et al., 2019) was used to screen for anthocyanin synthesis regulatory proteins that potentially interact with circadian clock PRR proteins. Full-length coding sequences of *PRR5*, *PRR7*, and *PRR9* were cloned into the pGBKT7 vector to generate constructs BD-PRR5, BD-PRR7, and BD-PRR9. Truncated constructs BD-PRR5$^{1-180}$, BD-PRR5$^{172-558}$, and BD-PRR5$^{502-558}$ were also generated (Yang et al., 2021). Full-length coding sequences of anthocyanin synthesis regulatory proteins MYB75, MYB90, MYB113, MYB114, and TT8 were cloned into the pGADT7 vector to generate AD-MYB75, AD-MYB90, AD-MYB113, AD-MYB114, and AD-TT8 constructs. Truncated constructs AD-MYB75-N (amino acids 1–122), AD-MYB75-C (amino acids 123–249), AD-TT8-N (amino acids 1–358), and AD-TT8-C (amino acids 359–519) were also prepared (Xie et al., 2016; Chen et al., 2020). Primers used for generating these constructs are listed in Table 2 .

Bimolecular Fluorescence Complementation (BiFC) Assays

Coding sequences of *PRR5*, *PRR5*$^{1-180}$, and *GUS* were fused with the C-terminal yellow fluorescent protein (c-YFP) fragment in pFGC-cYFP (Kim et al., 2008) to generate PRR5-cYFP, PRR5$^{1-180}$-cYFP, and GUS-cYFP constructs. Coding sequences of *MYB75*, *MYB90*, *MYB113*, *TT8*, and *GUS* were fused with the N-terminal YFP fragment in pFGC-nYFP (Kim et al., 2008) to generate MYB75-nYFP, MYB90-nYFP, MYB113-nYFP, TT8-nYFP,

and GUS-nYFP constructs. The resulting plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101. Different bacterial cultures (OD_{600} = 1.0) were mixed at a 1:1 volume ratio and infiltrated into healthy leaves of *Nicotiana benthamiana*. After incubation in a dark, humid environment for 48 hours, YFP and DAPI fluorescence were observed using a confocal laser microscope (Olympus, Tokyo, Japan) (Yang et al., 2021). Primers used for BiFC construct generation are listed in Table 2.

Gene IDs

The Arabidopsis gene IDs used in this study are as follows: *PRR5*, AT5G24470; *PRR7*, AT5G02810; *PRR9*, AT2G46790; *MYB75*, AT1G56650; *MYB90*, AT1G66390; *MYB113*, AT1G66370; *MYB114*, AT1G66380; *TT8*, AT4G09820; *DFR*, AT5G42800; *LDOX*, AT4G22880; *UF3GT*, AT5G54060. All gene sequence information is available from The Arabidopsis Information Resource (TAIR) database.

Results and Analysis

PRR5 and PRR7 Mutants Exhibit Reduced Anthocyanin Content

To investigate whether circadian clock PRR proteins regulate anthocyanin synthesis, we examined six-day-old wild-type (WT), *prr5-1*, *prr5-2*, *prr7-1*, and *prr7-2* mutant seedlings grown on 1/2 MS medium. Compared with wild-type, anthocyanin accumulation was visibly reduced in all mutant seedlings, as evidenced by lighter-colored shoot apices (Figure 1 [Figure 1: see original paper]A). Quantitative anthocyanin measurements confirmed these phenotypic observations, showing that wild-type seedlings had the highest anthocyanin content, while *prr5-1*, *prr5-2*, *prr7-1*, and *prr7-2* mutants had significantly lower anthocyanin levels. Notably, *prr5-1* and *prr5-2* mutants showed lower anthocyanin content than *prr7-1* and *prr7-2* mutants (Figure 1B). These results indicate that loss of PRR5 and PRR7 function reduces anthocyanin content, suggesting that PRR5 and PRR7 promote anthocyanin accumulation in seedlings. To further validate these findings, we examined expression of structural genes *DFR*, *UF3GT*, and *LDOX*, which encode key enzymes in the anthocyanin biosynthetic pathway and directly activate anthocyanin biosynthesis (Tanaka et al., 2008). RT-qPCR analysis of total RNA extracted from WT, *prr5-1*, *prr5-2*, *prr7-1*, and *prr7-2* seedlings revealed significantly reduced expression of *DFR*, *UF3GT*, and *LDOX* in all mutants (Figure 1C–E). These results demonstrate that PRR5 and PRR7 promote anthocyanin accumulation by inducing expression of structural genes such as *DFR* in Arabidopsis seedlings.

PRR5, PRR7, and PRR9 Synergistically Promote Anthocyanin Accumulation

To determine whether PRR5, PRR7, and PRR9 proteins coordinately regulate anthocyanin biosynthesis, we examined anthocyanin accumulation pheno-

types in multiple mutants including *prr5 prr7*, *prr5 prr9*, and *prr5 prr7 prr9*. Compared with wild-type, anthocyanin accumulation was markedly lower in all double and triple mutants, with *prr5 prr7 prr9* triple mutants showing the lowest anthocyanin levels and *prr5 prr7* double mutants accumulating less anthocyanin than *prr5 prr9* double mutants (Figure 2 [Figure 2: see original paper]A). Quantitative anthocyanin measurements confirmed these phenotypic observations (Figure 2B). Compared with single mutants (*prr5-1*, *prr5-2*, *prr7-1*, *prr7-2*) (Figure 1A,B), *prr5 prr7* and *prr5 prr7 prr9* seedlings showed significantly reduced anthocyanin content, while *prr5 prr9* double mutants had slightly lower anthocyanin levels than *prr5-1* and *prr5-2* single mutants (Figure 2A,B). Consistently, expression analysis of structural genes *DFR*, *UF3GT*, and *LDOX* revealed significantly lower expression in double and triple mutants compared with wild-type, with the lowest expression in *prr5 prr7 prr9* triple mutants and lower expression in *prr5 prr7* than in *prr5 prr9* double mutants (Figure 2C–E). These results demonstrate that PRR5, PRR7, and PRR9 synergistically promote anthocyanin accumulation in seedlings, with *prr5 prr7 prr9* triple mutants exhibiting the most severe reduction in anthocyanin accumulation.

Overexpression of PRR5 Increases Anthocyanin Content

To further confirm the biological function of PRR proteins in regulating anthocyanin synthesis, we generated CaMV35S promoter-driven PRR5-overexpressing transgenic plants (*35S:PRR5-FLAG*) (Yang et al., 2021) and selected three homozygous lines with high expression levels (*35S:PRR5-FLAG-9*, *35S:PRR5-FLAG-10*, and *35S:PRR5-FLAG-15*) for anthocyanin content analysis. As shown in Figure 3 [Figure 3: see original paper]A and B, anthocyanin accumulation was significantly higher in all three overexpression lines compared with wild-type, manifesting as darker purple-red shoot apices. Consistently, expression of *DFR*, *UF3GT*, and *LDOX* was significantly higher in PRR5-overexpressing seedlings than in wild-type (Figure 3C–E). These results further confirm that circadian clock PRR proteins promote anthocyanin accumulation in plant seedlings.

MYB75 and TT8 Proteins Interact with PRR5

To explore the molecular mechanism by which circadian clock PRR proteins regulate anthocyanin synthesis in Arabidopsis seedlings, we used the yeast two-hybrid system to screen for anthocyanin synthesis-related proteins that interact with PRR proteins. We fused PRR5, PRR7, and PRR9 with the Gal4 DNA-binding domain (BD-PRR5, etc.) as baits, and fused MYB75, MYB90, MYB113, MYB114, and TT8 with the Gal4 DNA-activation domain (AD-MYB75, etc.) as prey. Yeast two-hybrid assays revealed strong interactions between PRR5 and both MYB75 and TT8 proteins, while interactions between PRR5 and MYB90 or MYB113 were relatively weak (Figure 4 [Figure 4: see original paper]).

To further verify these interactions, we performed bimolecular fluorescence

complementation (BiFC) assays in plant cells. PRR5 was fused with the C-terminal YFP fragment driven by the CaMV35S promoter (PRR5-cYFP), while MYB75, MYB90, MYB113, and TT8 were fused with the N-terminal YFP fragment (MYB75-nYFP, MYB90-nYFP, MYB113-nYFP, and TT8-nYFP). Co-infiltration of PRR5-cYFP with MYB75-nYFP, MYB90-nYFP, MYB113-nYFP, or TT8-nYFP into tobacco leaves yielded strong fluorescent signals in the nucleus, whereas no fluorescence was observed in control combinations (Figure 5 [Figure 5: see original paper]). These results demonstrate that PRR5 interacts with anthocyanin synthesis-related proteins MYB75, MYB90, MYB113, and TT8 to form complexes in plant cell nuclei.

Interaction Domains Between MYB75, TT8, and PRR5 Proteins

To identify the regions required for interaction between PRR5 and MYB75, MYB90, MYB113, or TT8, truncated PRR5 sequences were fused to the Gal4 DNA-binding domain and tested in yeast two-hybrid assays. As shown in Figure 6 [Figure 6: see original paper]A, neither the PR domain (BD-PRR5$^{1-180}$) nor the CCT domain (BD-PRR5$^{502-558}$) of PRR5 interacted with MYB75, MYB90, MYB113, or TT8, whereas the C-terminal fragment of PRR5 (BD-PRR5$^{172-558}$) did interact with these proteins. These results indicate that the C-terminal amino acid domain of PRR5 mediates its interaction with MYB75, MYB90, MYB113, and TT8. We also truncated MYB75 and TT8 proteins, which showed strong interaction with PRR5 in yeast, into N-terminal and C-terminal fragments fused to the Gal4 activation domain. As shown in Figure 6B–C, deletion of the N-terminal regions of MYB75 and TT8 abolished their interaction with PRR5. These results demonstrate that the N-terminal amino acid domains of MYB75 and TT8 mediate their interaction with PRR5.

PRR5 Promotes Anthocyanin Synthesis in a MYB-Dependent Manner

Our previous results showed that PRR proteins interact with anthocyanin synthesis-related proteins MYB75, MYB90, MYB113, and TT8 to induce anthocyanin synthesis. To further investigate whether PRR5 promotes anthocyanin synthesis in a MYB-dependent manner, we generated *myb-RNAi 35S:PRR5-FLAG* hybrid lines through genetic crossing and analyzed anthocyanin accumulation phenotypes in F3 generation homozygous plants. As shown in Figure 7 [Figure 7: see original paper], anthocyanin accumulation in *myb-RNAi 35S:PRR5-FLAG* hybrid seedlings was significantly reduced, similar to the *myb-RNAi* mutant phenotype, showing lighter-colored shoot apices (Figure 7A). Quantitative anthocyanin measurements confirmed these observations (Figure 7B). These results demonstrate that PRR5 promotes anthocyanin synthesis in a MYB-dependent manner.

Discussion and Conclusion

The circadian clock system enables plants to perceive and anticipate periodic environmental changes, coordinating the dynamic balance between metabolic homeostasis, growth, development, and defense responses to ensure that critical developmental processes occur at appropriate times (Greenham & McClung, 2015; Wei et al., 2018). The circadian clock PRR protein family plays important roles in regulating plant growth, development, and stress responses, influencing seedling photomorphogenesis and enhancing Arabidopsis tolerance to cold, drought, and salinity (Kreps et al., 2002; Kaczorowski & Quail, 2003; Keily et al., 2013; Liu et al., 2013). Recent studies have shown that circadian clock PRR proteins promote ABA signaling to inhibit seed germination and post-germination growth during daytime (Yang et al., 2021), while ABA signaling can induce anthocyanin synthesis and accumulation in Arabidopsis seedlings (Chen et al., 2020). Anthocyanins play crucial roles in plant resistance to various stresses, and the MBW complex formed by MYB, bHLH, and WDR transcription factors represents the most critical transcriptional regulator of anthocyanin biosynthesis (Liang et al., 2018; Li et al., 2018). Exogenous environmental signals such as light and temperature, as well as endogenous phytohormone signals including abscisic acid and jasmonic acid, affect anthocyanin biosynthesis and accumulation by regulating expression of anthocyanin synthesis-related genes (Mori et al., 2007; Guo et al., 2008; Xie et al., 2016; Chen et al., 2020).

This study demonstrates that anthocyanin content is reduced in Arabidopsis seedlings carrying mutations in circadian clock *PRR* genes. Analysis of double and triple mutant phenotypes revealed that PRR5, PRR7, and PRR9 synergistically promote anthocyanin accumulation. RT-qPCR analysis of these mutants showed significantly reduced expression of key structural genes in the anthocyanin biosynthetic pathway (*DFR*, *UF3GT*, and *LDOX*), consistent with the anthocyanin accumulation phenotypes. Furthermore, analysis of PRR5-overexpressing transgenic plants revealed significantly higher anthocyanin content than wild-type. Therefore, we propose that circadian clock PRR proteins promote anthocyanin synthesis in seedlings by regulating expression of certain anthocyanin biosynthetic structural genes. Yeast two-hybrid and BiFC assays demonstrated that PRR5 physically interacts with anthocyanin regulatory proteins MYB75, MYB90, MYB113, and TT8. The Arabidopsis PRR protein family belongs to the CCT (CONSTANS/CONSTANS-LIKE/TOC1) superfamily, possessing an N-terminal PRR/RLD (RECEIVER-LIKE DOMAIN) and a C-terminal CCT domain (Farre and Liu, 2013). Domain mapping experiments revealed that neither the N-terminal PR domain nor the C-terminal CCT domain of PRR5 interacts with MYB75 and other regulatory proteins; instead, the C-terminal region containing the CCT domain mediates these interactions. We also confirmed that the N-terminal domains of MYB75 and TT8, which strongly interact with PRR5, mediate their binding to PRR5. These findings suggest that circadian clock PRR5 protein can interact with anthocyanin regulatory proteins MYB75, MYB90, MYB113, and TT8 to form complexes. Further analysis of

myb-RNAi 35S:PRR5-FLAG hybrid lines showed that anthocyanin content in these seedlings was similar to that of *myb-RNAi* mutants, demonstrating that PRR5 promotes anthocyanin synthesis in a MYB-dependent manner.

In summary, this study reveals that Arabidopsis circadian clock PRR proteins promote anthocyanin accumulation in seedlings, likely through interaction between PRR5 and the MBW complex to regulate anthocyanin synthesis. Our findings establish a connection between circadian clock signaling and anthocyanin synthesis and provide initial insights into the regulatory mechanism by which circadian clock PRR proteins promote anthocyanin synthesis in Arabidopsis seedlings, which is important for understanding how circadian clock signaling regulates plant environmental adaptation. Seed germination and seedling establishment are critical developmental stages in the plant life cycle and represent periods of high sensitivity to environmental conditions, during which seedlings are vulnerable to death under adverse conditions. Arabidopsis seeds are light-requiring and germinate primarily near the soil surface, exposing seedlings to potential stresses such as high light and drought during daytime. Anthocyanins serve as natural photoprotectants and enhance drought resistance (Guo et al., 2008; Ahmed et al., 2014). We propose that circadian clock PRR proteins promote anthocyanin synthesis and accumulation in seedlings to protect against potential daytime stresses such as high light and drought, while simultaneously inhibiting seed germination and hypocotyl elongation during daytime (Li et al., 2020; Yang et al., 2021) to coordinate the dynamic balance between defense responses and growth, enabling plants to safely complete critical developmental transitions. However, the detailed molecular mechanism by which PRR proteins regulate anthocyanin synthesis through the MBW complex requires further investigation, and whether the biological function of circadian clock PRR proteins in promoting seedling anthocyanin synthesis is conserved across different species needs additional study, which would be important for crop improvement and production. Furthermore, whether other circadian clock system proteins participate in anthocyanin synthesis regulation remains to be explored.

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Note: Figure translations are in progress. See original paper for figures.