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Phytochrome-interacting factors PIF4 and PIF5 negatively regulate anthocyanin biosynthesis under red light in *Arabidopsis* seedlings

Zhongjuan Liu[a,b,1], Yongqiang Zhang[b,1], Jianfeng Wang[b], Ping Li[b], Chengzhou Zhao[b], Yadi Chen[b], Yurong Bi[b,*]

[a] College of Earth and Environmental Sciences, Lanzhou University, Lanzhou, Gansu 73000, People's Republic of China
[b] Key Laboratory of Cell Activities and Stress Adaptations, Ministry of Education, School of Life Sciences, Lanzhou University, Lanzhou, Gansu 730000, People's Republic of China

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ABSTRACT

Light is an important environmental factor inducing anthocyanin accumulation in plants. Phytochrome-interacting factors (PIFs) have been shown to be a family of bHLH transcription factors involved in light signaling in *Arabidopsis*. Red light effectively increased anthocyanin accumulation in wild-type Col-0, whereas the effects were enhanced in *pif4* and *pif5* mutants but impaired in overexpression lines *PIF4OX* and *PIF5OX*, indicating that PIF4 and PIF5 are both negative regulators for red light-induced anthocyanin accumulation. Consistently, transcript levels of several genes involved in anthocyanin biosynthesis and regulatory pathway, including *CHS*, *F3′H*, *DFR*, *LDOX*, *PAP1* and *TT8*, were significantly enhanced in mutants *pif4* and *pif5* but decreased in *PIF4OX* and *PIF5OX* compared to in Col-0, indicating that PIF4 and PIF5 are transcriptional repressor of these gene. Transient expression assays revealed that PIF4 and PIF5 could repress red light-induced promoter activities of *F3′H* and *DFR* in *Arabidopsis* protoplasts. Furthermore, chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR) test and electrophoretic mobility shift assay (EMSA) showed that PIF5 could directly bind to G-box motifs present in the promoter of *DFR*. Taken together, these results suggest that PIF4 and PIF5 negatively regulate red light-induced anthocyanin accumulation through transcriptional repression of the anthocyanin biosynthetic genes in *Arabidopsis*.

1. Introduction

The purple natural pigments found in many plant species, anthocyanins, belong to a class of plant secondary metabolites known as flavonoids, which play important roles in many plant functions such as protection of photosynthetic apparatus and free radical scavenging [1,2]. The basal levels of anthocyanins in many tissues can be induced significantly in response to a variety of external stimuli, including low temperature, drought, ultraviolet (UV) radiation, high intensity light, and nutrient depletion [2,3]. From phenylalanine, the anthocyanin biosynthetic pathway is catalyzed by numerous enzymes [1,3,4], which have been identified through the isolation of *transparent testa* (*tt*) mutants in the model plant *Arabidopsis* [5], sequentially including chalcone synthase (CHS), chalcone isomerase (CHI), dihydroflavanol reductase (DFR), flavanone 3-hydroxylase (F3H), flavonoid 3′-hydroxylase (F3′H), leucoanthocyanidin dioxygenase (LDOX) and UDP-flavonoid 3-glucosyl transferase (UF3GT) [1,3,6,7]. For investigating how anthocyanin biosynthesis is regulated in plants, many transcriptional regulators have been identified, including members of the MYB, bHLH and WD-repeat families, which generally interact with each other to form MBW (MYB-bHLH-WD40) regulatory complex [1,7,8]. For instance, Transparent Testa 2 (TT2, also known as MYB123) interacts with the cofactors TT8 (bHLH42) and Transparent Testa Glabra 1 (TTG1, a WD40-repeat protein) to form a ternary MBW complex that regulates the expression of the *DFR* [9]. Production of Anthocyanin Pigment 1 (PAP1, also known as MYB75), a member of the R2R3-MYB transcription factor family, could dimerize with bHLH factors, such as TT8 or Glabra 3 (GL3), to regulate

Abbreviations: Bc, constant blue light; CHI, chalcone isomerase; ChIP-qPCR, chromatin immunoprecipitation-quantitative PCR; CHS, chalcone synthase; COP1, constitutively photomorphogenic 1; PIFs, phytochrome interacting factor; DFR, dihydroflavanol reductase; EGL3, enhancer of GL3; EMSA, electrophoretic mobility shift assay; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; FRc, constant far red light; GL3, glabra 3; HY5, long hypocotyl 5; LDOX, leucoanthocyanidin dioxygenase; MBW, MYB-bHLH-WD40; PAP1, production of anthocyanin pigment 1; phyB, phytochrome B; qRT-PCR, quantitative reverse transcription PCR; Rc, constant red light; TT8, transparent testa 8; TTG1, transparent testa glabra 1; UF3GT, UDP-flavonoid 3-glucosyl transferase; WLc, constant white light.

* Corresponding author. Tel.: +86 931 8912560; fax: +86 931 8912561.
E-mail address: yrbi@lzu.edu.cn (Y. Bi).

[1] These authors contributed equally to this work.

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their downstream transcriptions including genes related to anthocyanin biosynthesis [10,11]. Also, another bHLH factor, enhancer of GL3 (EGL3), like GL3, could interact with TTG1, PAP1, and PAP2 in the process of regulating anthocyanin production [12].

Light is one of the most important environmental factors in the regulation of anthocyanin accumulation in plants, which not only provides the source of energy for plant life, but also acts as a signal affecting plant growth and development throughout its entire life cycle [13]. Since its spectrum, intensity and direction may vary significantly between locations, plants have developed sophisticated photoreceptor systems that allow them to adapt to variable light conditions [14]. As cryptochromes (CRYs) are the receptors determining responses to blue light, phytochromes (phys) regulate various processes in response to red and far red light, including seed germination, seedling development, chloroplast development, and flowering [14]. In *Arabidopsis*, there are five phys, namely phyA-E, that possess overlapping but distinct functions [14,15]. PhyA acts as a photoreceptor for the very low fluence response (VLFR) and the far-red high irradiance response (FR-HIR), whereas the other four phytochromes act as photoreceptors for the red light LFR, and phyB is the major phytochrome responsible for regulating various light responses [14]. After light absorption, phytochromes rapidly enter into the nucleus, where they control the responses to light through at least two pathways. First, they act to destabilize the Suppressor of PhyA-105 (SPA) and exclude an E3-ubiquitin ligase, Constitutively Photomorphogenic 1 (COP1), from the nucleus, thus preventing the degradation of positive signaling factors such as Long Hypocotyl 5 (HY5) and its homolog HYH, and allowing photomorphogenesis to occur [13,16]. Second, phytochromes directly bind to Phytochrome-interacting factors (PIFs), a family of bHLH proteins, and target them for degradation, thus relieving the repression of light responses [15,17].

The first of these bHLH proteins identified as a phytochrome-interacting protein was PIF3 [18]. Subsequently, several other PIFs, including PIF1, PIF4 and PIF5, were also found and shown to be able to interact with phytochromes and participate in many light responses [15,19]. Nevertheless, these proteins vary in their abilities to regulate different light responses. For example, PIF1 negatively regulates seed germination and chlorophyll biosynthesis [20,21]; PIF3 negatively regulates chloroplast development and chlorophyll biosynthesis [22,23], whereas it positively regulates anthocyanin accumulation [24,25]; PIF4 and PIF5 negatively regulate phytochrome-mediated inhibition of shade avoidance [26] and dark-induced senescence [27]. In addition, PIF1, PIF3, PIF4 and PIF5 all positively regulate hypocotyl elongation in an additive manner: single *pif* mutant had only weak effect, whereas a constitutive photomorphogenetic phenotype was found in the seedlings of the quadruple mutant *pif1pif3pif4pif5* (*pifq*) [23,28]. As transcription factors, PIFs usually bind sequence-specifically to the core DNA G-box motif (CACGTG) of their target promoters [15], suggesting a direct signaling pathway from photoactivated phytochromes to downstream genes.

Anthocyanin accumulation in developing plant seedlings is induced by CRYs in blue light [29] and by phyA in far-red light [30], and the regulation generally occurs at the transcription level for the anthocyanin biosynthetic genes, including *CHS*, *CHI* and *DFR* [6,24]. Besides the participation of MBW complex in the regulation of anthocyanin biosynthetic genes, HY5 is another key factor involved in regulation of anthocyanin biosynthesis. ChIP (chromatin immunoprecipitation)-chip assay found that HY5 could directly bind to the promoters of many genes involved in anthocyanin biosynthesis including *CHS* and *F3H* [16]. Furthermore, PAP1 and PAP2 are degraded in darkness depending on the presence of COP1 [31]. In addition, PIF3 has been proved to positively regulate anthocyanin biosynthesis in a HY5-dependent manner under far-red light [24], but whether and how other PIFs such as PIF4 and PIF5 are involved in anthocyanin biosynthesis under red light still remain unclear. In this study, red light-induced anthocyanin accumulation was first found to be enhanced in mutants *pif4* and *pif5* compared to their wild-type, whereas overexpression lines *PIF4OX* and *PIF5OX* accumulated little anthocyanins under the same conditions, and further studies indicated that both PIF4 and PIF5 are transcriptional repressors of red light-induced up-regulation of anthocyanin biosynthetic genes. These results lead us to conclude that PIFs, especially PIF4 and PIF5, are negative regulators of anthocyanin accumulation under red light.

2. Materials and methods

2.1. Plant materials and growth conditions

The *pif1-1* (*pif1*), *pif3-3* (*pif3*), *pif4-2* (*pif4*), *pif5-2* (*pif5*), *pif4pif5*, *pifq*, *PIF4OX* and *PIF5OX* were kindly provided by Prof. Peter H. Quail at University of California. Transgenic line *35S:PIF5-HA* and mutant *hy5-215* (*hy5*) were gifts from Prof. Julin N. Maloof at University of California and Prof. Xing-Wang Deng at Yale University, respectively. Mutant *phyB-9* was obtained from the Arabidopsis Biological Resource Center (ABRC). All the materials are in the *Arabidopsis thaliana* L. Columbia (Col-0) ecotype background. Seeds were surface-sterilized with 20% (v/v) bleach solution for 10 min, rinsed with sterile water for three times, and then sown on medium containing 1/2 Murashige and Skoog (MS) salt and 0.68% phytoblend agar. Seeds on agar plates were cold-treated at 4 °C for 3 days in the dark and then transferred to a three color chamber (Percival) at 22 °C with indicated light conditions for growth. Unless otherwise noted specially, the intensity of red light used in the experiments is 10 μmol m^{-2} s^{-1}.

2.2. Anthocyanin measurement

Anthocyanin content of seedlings was determined using the protocol of Vandenbussche et al. [6] with some minor modifications. In brief, about 10 mg seedlings were harvested in 1.5 mL tubes. Extraction of anthocyanins was performed in 300 μL of methanol containing 1% HCl for 24 h at 4 °C. Then chlorophyll was extracted by adding 200 μL of chloroform and 200 μL of water and mixing. Subsequently, the samples were centrifuged for 10 min at 10,000 × *g* at 4 °C. The upper phase was taken and the absorbance at 530 nm (OD_{530}) was measured using a spectrophotometer. The anthocyanin levels were expressed as OD_{530} per gram fresh weight (OD_{530}/g F.W.).

2.3. Quantitative reverse transcription PCR analysis (qRT-PCR)

Seedlings were harvested in liquid nitrogen, ground, and RNA was extracted using RNAiso Plus reagent (TaKaRa). The complementary DNA (cDNA) was synthesized using a oligo$(dT)_{18}$ primer and moloney murine leukemia virus (M-MuLV) reverse transcriptase (Fermentas) at 42 °C for 60 min. Then the first-strand cDNA was synthesized from 1 μg of total RNA using PrimeScript RT reagent Kit containing gDNA eraser (TaKaRa). PCR reactions were performed using the CFX96 Real Time System (Bio-Rad) with SYBR Premix Ex Taq II Kit (TaKaRa), according to the procedure described by the manufacture. The raw data were analysed with CFX Manager Software (version 1.1), and expression was normalized to *Actin2* to minimize variation in cDNA template levels. Relative expression levels were calculated using the comparative threshold (Ct value) method. Fold changes ($2^{-\Delta\Delta Ct}$) were expressed relative to the control. Mean values were obtained from three biological replicates. Primer sequences used for qRT-PCR can be found in Supplementary Table S1.