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Coordinated transcriptional regulation of isopentenyl diphosphate biosynthetic pathway enzymes in plastids by phytochrome-interacting factor 5

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ABSTRACT

All isoprenoids are derived from a common C5 unit, isopentenyl diphosphate (IPP). In plants, IPP is synthesized via two distinct pathways; the cytosolic mevalonate pathway and the plastidial non-mevalonate (MEP) pathway. In this study, we used a co-expression analysis to identify transcription factors that coordinately regulate the expression of multiple genes encoding enzymes in the IPP biosynthetic pathway. Some candidates showed especially strong correlations with multiple genes encoding MEP-pathway enzymes. We report here that phytochrome-interacting factor 5 (PIF5), a basic-helix-loop-helix type transcription factor, functions as a positive regulator of the MEP pathway. Its overexpression in T87 suspension cultured cells resulted in increased accumulation of chlorophylls and carotenoids. Detailed analyses of carotenoids by HPLC indicated that some carotenoid biosynthetic pathways were concomitantly upregulated, possibly as a result of enhanced IPP metabolic flow. Our results also revealed other PIF family proteins that play different roles from that of PIF5 in IPP metabolism.

1. Introduction

Isoprenoids are, functionally and structurally, the most diverse group of natural products. More than 23,000 different isoprenoid compounds exist in nature. Like other families of natural products, the isoprenoids show a wide distribution and diversity in the plant kingdom. Isoprenoid compounds such as quinones, sterols, dolichols, chlorophylls, carotenoids, and phytohormones (abscisic acid, gibberellins, cytokinins, brassinosteroids, and strigolactones), play important roles in the cellular functions of plants. However, many plant isoprenoids are also important as valuable plant secondary metabolites, essential oils, drugs, dietary supplements, natural polymers, and agrochemicals, and are used in industrial, pharmacological, and agricultural applications. All of these plant isoprenoids are biosynthesized from isopentenyl diphosphate (IPP), which is derived from the mevalonate pathway in the cytosol/peroxisome/endoplasmic reticulum, and the non-mevalonate pathway (methyl-D-erythritol 5-phosphate (MEP) pathway) in plastids (Supplemental Fig. S1). Therefore, to establish metabolic engineering strategies to produce valuable plant isoprenoids, it is important to understand the mechanisms that regulate IPP biosynthesis.

In the plastid MEP pathway, there are at least three rate-limiting steps, which are catalyzed by 1-deoxy-D-xylulose 5-phosphate (DXP) synthase (DXS) [1], DXP reductoisomerase (DXR) [2], and 1-hydroxy-2-methyl-2-butenyl 4-diphosphate (HMBPP) reductoisomerase (HDR) [3]. In early studies, researchers attempted to metabolically engineer plastid isoprenoid pathways by overexpressing each of these rate-limiting enzymes. Constitutive overexpression of DXS in *Arabidopsis thaliana* achieved 1.4- and 1.3-fold increases in chlorophyll and carotenoid contents, respectively [1]. Like DXS, overexpression of DXR [3] or HDR [4] in *A. thaliana* also enhanced the metabolic flow of IPP, resulting in 1.25-fold or 1.6-fold increases in carotenoid contents. However, the effects of

Abbreviations: bHLH, basic helix-loop-helix; DXP, 1-deoxy-D-xylulose 5-phosphate; GGR, geranylgeranyl reductase; HMBPP, 1-hydroxy-2-methyl-2-butenyl 4-diphosphate; IPP, isopentyl diphosphate; PIF5, phytochrome-interacting factor 5; MEP, methyl-D-erythritol 5-phosphate; PIFs, phytochrome-interacting factors; PDS, phytoene desaturase.

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constitutive overexpression of these genes encoding MEP pathway enzymes on the levels of plastid isoprenoids were insignificant. This suggested that there is robust and redundant regulation of the metabolic flow of IPP through the MEP pathway, because of the physiological importance of plastid isoprenoids in photosynthesis and phytohormone-mediated regulation.

Another approach for metabolic engineering *in planta* is to manipulate transcription factors that regulate the expression of multiple genes encoding enzymes in a particular metabolic pathway [5,6]. However, little is known about the transcriptional regulation mechanism of the MEP pathway in plants. In this study, we used a transcriptome co-expression analysis to identify candidate transcription factors that regulate the expression of multiple enzymes in the MEP pathway of *A. thaliana*.

2. Methods

2.1. Materials

The Arabidopsis full-length cDNA clones for phytochrome-interacting factors (PIFs) *PIF3* (AT1G09530) and *PIF5* (AT3G59060), in which a 4-base deletion in the coding region was occurred (PIF5ΔC), was developed by the plant genome project of RIKEN Genomic Sciences Center [7,8]. Arabidopsis T87 suspension-cultured cells [9] were provided by the RIKEN Bioresource Center through the National Bio-Resource Project of the MEXT, Japan. The Gateway plant binary vector pGWB2 [10] was provided by Dr. T. Nakagawa (Shimane University, Japan).

2.2. Co-expression analyses

Co-expression analyses based on correlation coefficients between all combinations of Arabidopsis genes obtained from the ATTED-II database (http://atted.jp/) [11] and preparation of a gene list of 2239 Arabidopsis putative TAFs were essentially as described previously [12]. The cut-off value for Pearson's correlation coefficient was 0.60. The set of genes encoding enzymes involved in isoprenoid biosynthesis was prepared based on the list in Lange et al. [13].

2.3. Overexpression of transcription factors in T87 cells

Total RNAs were extracted from *A. thaliana* (Col-0) seedlings grown on MS plates under long-day conditions at 22 °C and from T87 cells using an Extract-A-Plant RNA Isolation Kit (Clontech, Mountain View, CA, USA). The total RNAs were used to synthesize first-strand cDNAs with a PrimeScript II 1st strand cDNA synthase Kit (Takara Bio, Ohtsu, Japan) using an oligo dT primer. *PIF1*, *PIF4,* and *PIF5* genes were amplified by PCR with KOD-Plus-DNA polymerase (Toyobo, Osaka, Japan), using first-strand cDNAs as templates and appropriate primers (Supplemental Table S1). Each cDNA was purified and subcloned into the Gateway entry vector pENTR/D-TOPO (Life Technologies, Carlsbad, CA, USA), and then transferred into a Gateway destination vector pGWB2 harboring the CaMV35S promoter for constitutive expression in plants [10], using LR Clonase II enzyme mix (Life Technologies).

T87 suspension cultured cells were maintained under continuous light at 22 °C on a rotary shaker (120 rpm) in T87 medium, a modified JPL medium [9]. Stable transformation of T87 cultured cells was achieved by co-cultivation with *Agrobacterium tumefaciens* GV3101(pMP90) carrying the resulting constructs, pGWB2-*PIF1*, pGWB2-*PIF4*, or pGWB2-*PIF5*, as described previously [14]. To obtain transgenic suspension cultured cells, resulting transgenic calli were re-suspended in T87 medium supplemented with 200 mg/l Claforan and 10 mg/l hygromycin or 5 mg/l kanamycin. Cell lines overexpressing PIF3 and PIF5ΔC and the vector control line based on pGWB2 were generated according to Ogawa et al. [15].

2.4. Semi-quantitative reverse transcription-PCR (RT-PCR)

Total RNAs extracted from T87 cultured cells, 8 days after subculture to antibiotic-free T87 medium, were treated with recombinant DNase I (Roche, Basel, Switzerland) and purified. Transcript levels of each target gene in T87 cells were quantified by semi-quantitative RT-PCR with Ex-Taq DNA polymerase (Takara Bio), in which the first-strand cDNAs, transcribed using a PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio) with oligo dT primers, were used as templates. The standard PCR program was as follows: 22–30 cycles of 96 °C for 30 s, 55–61.5 °C for 30 s and 72 °C for 1 min, with 10 min final extension at 72 °C. The gene-specific primer sequences, annealing temperatures, and PCR cycles are provided in Supplemental Table S2 and S3. The resulting PCR products were subjected to electrophoresis on 2% agarose gels, and the band intensities were measured by ImageJ software (http://rsb.info.nih.gov/ij/index.html). The value of band intensity for each gene was normalized to that of β-tubulin *TUB4* (At5g44340). The transcript level of each gene is shown relative to that in the vector control line (set to 1).

2.5. Quantification of chlorophylls and carotenoids

T87 cultured cells, grown in antibiotic-free T87 medium for 7 or 8 days after subculture, were ground into a powder with a mortar and pestle. To extract compounds from approximately 30 mg ground cells, 300 µl methanol was added. For quantification by HPLC, 1.5 µg astaxanthin was added as the internal standard. The suspension was vortexed for 5 min at 4 °C, and then 300 µl 50 mM Tris–HCl (pH 7.5) containing 1 M NaCl was added, followed by further mixing for 5 min at 4 °C. After adding 800 µl chloroform and vortexing for 10 min at 4 °C, the mixture was centrifuged at 5000 rpm for 5 min at 4 °C to obtain a clear partition of the hypophase, which was filtered through a 0.2-µm chromafil filter (Macherey-Nagel, Düren, Germany), dried by centrifugal evaporation, and then stored at −80 °C until analysis.

Total chlorophylls and carotenoids were quantified by absorption photometry as described previously [16]. Samples for absorption photometry were prepared by dissolving the residue in 500 µl acetone. Separation and quantification of carotenoids by HPLC were performed as described elsewhere [17]. Samples for HPLC were prepared by dissolving the dried residue in 100 µl ethyl acetate. Chromatography was carried out on a system consisting of a pump (models 302 and 305) and a sample injector (model 231) (Gilson, Middleton, WI, USA) with an SPD-M20A diode array detector (Shimadzu, Kyoto, Japan), monitoring continuously from 300 to 800 nm. Data were collected and analyzed using LCsolution software (Shimadzu). The column temperature was maintained at 30 °C by a CTO-10ASvp column oven (Shimadzu). We used a Develosil C30 UG-5 (250 × 4.6 mm) reverse-phase column coupled to a C30 UG guard column (Nomura Chemical Co., Seto, Japan). The mobile phase consisted of 99% methanol containing 0.01% ammonium acetate (A) and 95% *tert*-methyl butyl ether containing 4% methanol and 0.01% ammonium acetate (B). The following gradient was used: 100% A, 0% B isocratic for 12 min, 84.2% A, 15.8% B isocratic for 12 min, then a linear gradient to 31.6% A, 68.4% B by 55 min. A conditioning phase (55–75 min) was used to return the column to the initial concentration of A. In all phases, flow rates of 1 ml/min were used. All carotenoids were quantified against known amounts of authentic standards. The level of each carotenoid in the vector control was set to 1.

3. Results and discussion

3.1. Screening of transcription factors by in silico co-expression analysis

To analyze the coordinated transcriptional regulation of genes encoding isoprenoid biosynthetic enzymes, we conducted co-expression analyses based on ATTED-II for the 68 genes related to isoprenoid biosynthesis (listed in Supplemental Table S4). Co-expression relationships were visualized as a graph, in which a line connected a pair of genes with a high correlation coefficient (>0.6 in this case). This analysis revealed two discrete clusters; a large and complex co-expression network module consisting of plastid isoprenoid biosynthetic enzymes, including MEP-pathway enzymes, and a small cluster of non-plastid enzymes including MVA-pathway enzymes (Fig. 1). This co-expression analysis led to the hypothesis that the MEP pathway and other plastid isoprenoid biosynthetic pathways might be regulated coordinately by several common transcription factors. To screen for potential transcription factors regulating isoprenoid metabolism in plastids, we conducted a secondary co-expression analysis between another two sets of genes; the plastid-related gene cluster, and transcription factor genes in *A. thaliana*. We selected transcription factors correlated with multiple plastid-related cluster genes, especially MEP pathway genes, as candidates (Table 1).

To functionally analyze these candidates, we created transgenic *Arabidopsis* T87 lines overexpressing each candidate gene, and then analyzed the transcript levels of MEP pathway genes. Among them, PIF5 was identified as a strong candidate for a transcriptional regulator of the MEP pathway, based on the magnitude of the effect on transcript levels of MEP pathway genes, and the correlation between its transcript level and those of MEP pathway genes.

PIF5 is a basic helix-loop-helix (bHLH) transcription factor that participates in signaling in response to light. It specifically interacts with the photoactivated form of phytochromes, the photoreceptors that perceive and respond to red to far-red regions [18–20], especially in shade-avoidance syndrome [21–23]. As with other bHLHs, PIF5 is thought to function in a homodimer form, in which the C-terminal helix is the key motif for dimerization, DNA binding, and transcription activity [18,24].

3.2. PIF5-overexpressing T87 cell lines

In the PIF5-overexpressing T87 cell lines (PIF5-OX), the transcript levels of MEP pathway genes, particularly *DXR*, 2-*C*-methyl-D-erythritol 4-phosphate cytidyltransferase (*MCT*),

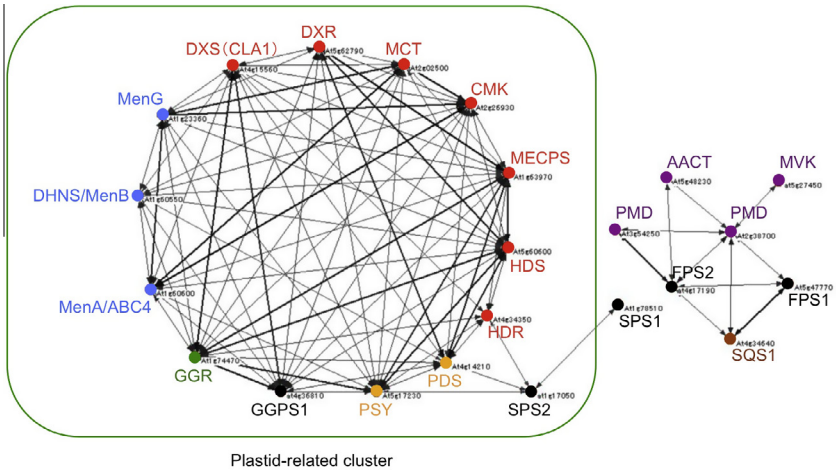

Fig. 1. Co-expression analysis among isoprenoid biosynthetic pathway genes. Red points indicate genes encoding MEP pathway enzymes. Similarly, orange: carotenoid biosynthetic enzymes; green: chlorophyll biosynthetic enzyme; blue: phylloquinone biosynthetic enzymes; purple: MVA pathway enzymes; brown: sterol biosynthetic enzymes; black: prenyltransferases. AGI codes of enzymes are as follows: MenA/ABC4 (At1g60600), DHNS/MenB (At1g60550), MenG (At1g23360), AACT (At5g48230), MVK (At5g27450), PMD (At3g54250), PMD (At2g38700), SQS1 (At4g34640), GGPS1 (At4g36810), SPS1 (At1g78510), SPS2 (At1g17050), FPS1 (At5g47770), FPS2 (At4g17190).

Table 1
Correlations between transcription factor genes and isoprenoid biosynthesis genes.

Isoprenoid biosynthetic gene	Transcription factor gene						
	COL6 (At1g68520)	*BEE2* (At4g36540)	*GLK1* (At2g20570)	*C2H2-type Zinc finger* (At5g54630)	*ARR14* (At2g01760)	*PIF5* (At3g59060)	*COL4* (At5g24930)
DXS (CLA1)	0.647	0.668					
DXR							0.626
MCT	0.644	0.657	0.628	0.609	0.644		
CMK	0.663	0.656	0.670	0.608	0.624		
MECPS	0.633		0.631			0.633	0.644
HDS	0.710		0.690	0.642		0.704	0.649
HDR	0.605					0.774	0.628
GGR	0.706	0.643	0.663	0.609	0.625	0.668	0.681
PSY	0.687	0.708				0.631	0.650
PDS	0.600					0.621	

Notes: AGI codes of isoprenoid biosynthetic pathway genes listed are as follows: *DXS* (*CLA1*), 1-deoxy-D-xylulose 5-phosphate synthase (At4g15560); *DXR*, 1-deoxy-D-xylulose 5-phosphate reductoisomerase (At5g62790); *MCT*, 2-*C*-methyl-D-erythritol 4-phosphate cytidyltransferase (At2g02500); *CMK*, 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol kinase (At2g26930); *MECPS*, 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate synthase (At1g63970); *HDS*, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-phosphate synthase (At5g60600); *HDR*, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-phosphate reductase (At4g34350); *GGR*, geranylgeranyl reductase (At1g74470); *PSY*, phytoene synthase (At5g17230); *PDS*, phytoene desaturase (At4g14210). Only values for high Pearson's correlation coefficients (>0.6) were indicated.

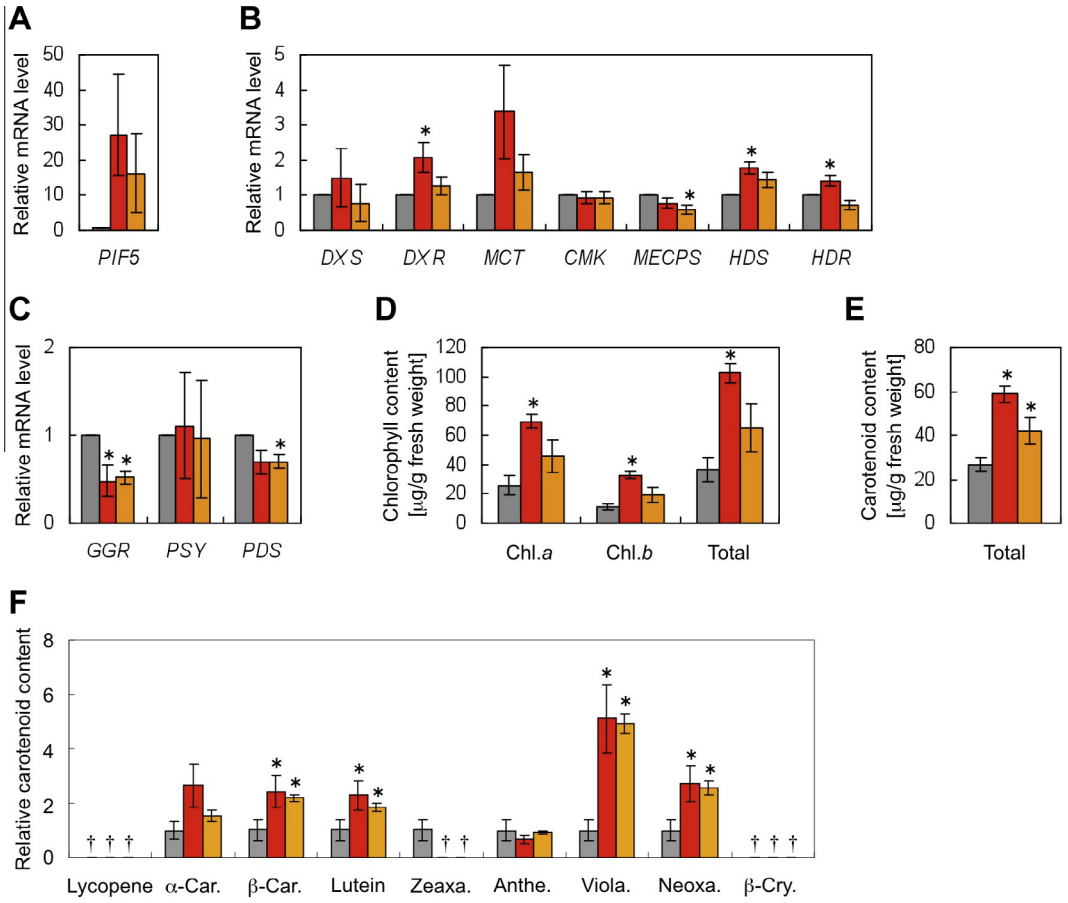

Fig. 2. Expression analysis and pigment quantification in PIF5-OX. Relative transcript levels of *PIF5* (A), MEP pathway genes (B), and chlorophyll and carotenoid biosynthetic enzymes (C). Total chlorophylls content as determined by absorption photometry (D). Carotenoids content as determined by absorption photometry (E) and HPLC (F). Gray bar: a vector control line; Red and tangerine bars: PIF5-OX line 1 and 2, respectively. In all cases, mean values +/− SD ($n = 3$) are shown. Asterisks indicate significant difference (Student's *t*-test $P < 0.05$); daggers, not detected.

HMBPP synthase (*HDS*), and *HDR*, were significantly increased, accompanied by approximately 11-fold to 17-fold increases in *PIF5* transcript levels (Fig. 2A and B). To determine whether there was an increased metabolic flow of IPP in the plastids, we quantified total chlorophylls and total carotenoids. The total chlorophyll content was increased by up to 2.8-fold and that of total carotenoids was increased by up to 2.2-fold, despite the significant decrease in geranylgeranyl reductase (*GGR*) and phytoene desaturase (*PDS*) transcript levels (Fig. 2C–E). GGR and PDS are key enzymes in chlorophyll and carotenoid metabolic pathways, respectively (Supplemental Fig. S1). The HPLC analysis revealed increased levels of several carotenoids, including those produced in the branched pathway from lycopene to α-carotene and β-carotene, in the PIF5-OX (Fig. 2F). These findings strongly suggested that there was a greater supply of the upstream common precursor for these metabolic pathways. Together, these results indicated that PIF5 positively regulates the transcription of MEP pathway genes and functions as an IPP-metabolism enhancer.

3.3. *PIF5ΔC-overexpressing T87 cell lines*

The DNA termed *PIF5ΔC* has a 4-bp frameshift deletion in the coding region, resulting in a truncated gene product lacking 144 amino acids from the C-terminal. PIF5ΔC was predicted to show defective transcription activity because it lacks the helix responsible for dimerization. Unexpectedly, PIF5ΔC-overexpressing T87 cell lines (PIF5ΔC-OX) were similar to PIF5-OX lines in terms of transcription of MEP pathway genes (Fig. 3A and B). Cells overexpressing PIF5ΔC also showed increased accumulation of chlorophylls and carotenoids, but to a lower extent than that in PIF5-OX cells (Fig. 3D–F). Conversely, the transcript levels of *GGR* and *PDS* were not significantly affected (Fig. 3C). This result, combined with the fact that there was lower accumulation of chlorophylls and carotenoids in PIF5ΔC-OX than in PIF5-OX lines, suggested that the down-regulation of *GGR* and *PDS* in PIF5-OX was caused by feedback regulation of other transcription factors in response to the enhanced accumulation of carotenoids and chlorophylls.

Based on these facts, we concluded that the essentially non-active PIF5ΔC weakly enhanced IPP metabolism. PIF5 has an active phytochrome B binding motif (APB) in its N-terminus, and the amount of PIF5 protein is regulated by the binding of phytochrome B (phyB) through ubiquitination and subsequent degradation by the 26S proteasome [21,25]. PIFΔC with an intact APB domain could not function as an active transcription factor, but could function as a scapegoat protein for endogenous PIF5 to bind phyB. This hypothesis was also supported by the fact that the up-regulation of *PIL1* and *HFR1*, direct target genes of PIF5 [22], were observed to a lesser extent in PIF5ΔC-OX lines than PIF5-OX lines (Supplemental Fig. S2).

3.4. *Cell lines overexpressing other PIF family transcription factors*

The APB motif in other PIF family can bind phyB, though the interaction is weak [20]. If the APB domain is sufficient to enhance IPP metabolism, then other members of the PIF family could show the same effects as PIF5 and PIF5ΔC. To test this hypothesis, we

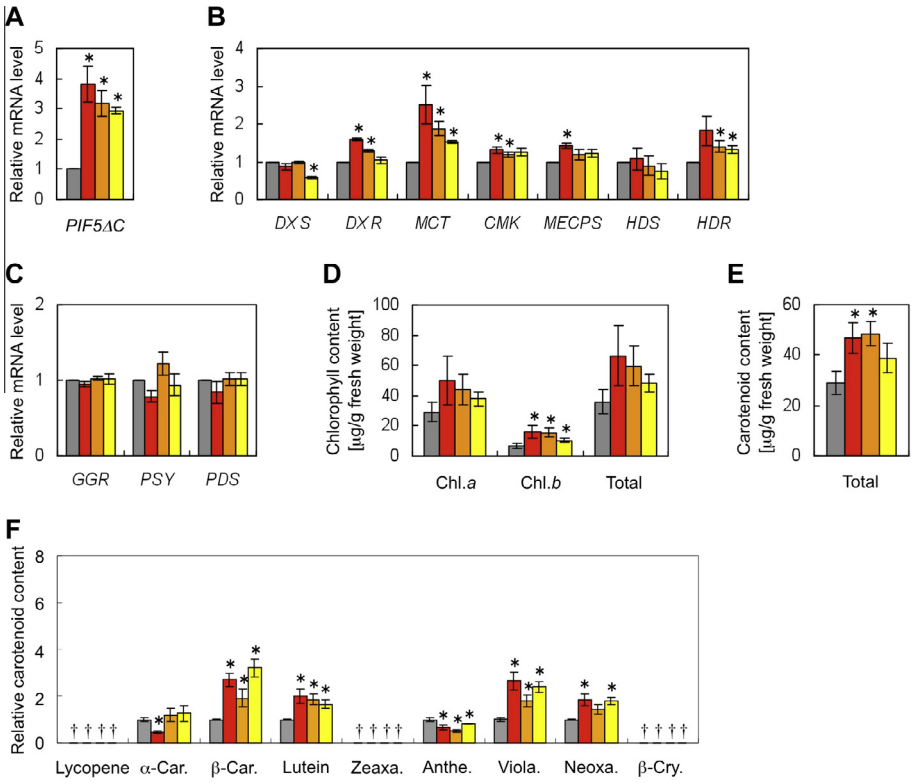

Fig. 3. Expression analysis and pigment quantification in PIF5ΔC-OX. Relative transcript levels of *PIF5* (*PIF5ΔC*) (A), MEP pathway genes (B), and chlorophyll and carotenoid biosynthetic enzymes (C). Total chlorophylls content as determined by absorption photometry (D). Carotenoids content as determined by absorption photometry (E) and HPLC (F). Gray bar: a vector control line; Red, tangerine and yellow bars: PIF5ΔC-OX line 1, 2 and 3, respectively. In all cases, mean values +/− SD ($n = 3$) are shown. Asterisks indicate significant difference (Student's *t*-test $P < 0.05$); daggers, not detected.

prepared T87 cell lines overexpressing *PIF1*, *PIF3*, and *PIF4* (PIF1-OX, PIF3-OX, and PIF4-OX, respectively). Expression analyses of PIF1-OX and PIF4-OX revealed similar patterns of gene transcription to that in PIF5-OX; that is, up-regulation of *MCT* and *HDS* and down-regulation of *GGR* (Supplemental Figs. S3 and S4), but to a lower extent than those in PIF5-OX and PIF5ΔC-OX. These findings suggested that phytochrome-mediated degradation of endogenous PIF5 was inhibited in these transgenic cell lines, although PIF1-OX and PIF4-OX did not show significant increases chlorophylls and carotenoids (Supplemental Figs. S3 and S4). By contrast, PIF3-OX showed significant decreases in chlorophylls and carotenoids (0.38 and 0.57 their respective levels in the vector control line) (Fig. 4). Thus, there was an inverse correlation between chlorophyll/carotenoid accumulation and *PIF3* transcript levels. However, there were no significant changes in the transcript levels of MEP pathway genes that were up-regulated in PIF5-OX (*DXR*, *MCT*, *HDS*, and *HDR*) (Fig. 4). These results showed that PIF3 could not regulate MEP pathway genes, but negatively

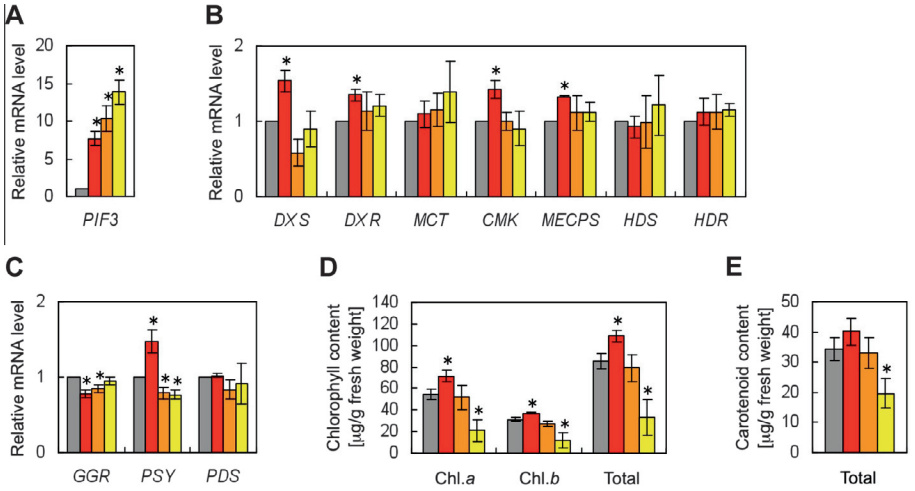

Fig. 4. Expression analysis and pigment quantification in PIF3-OX. Relative transcript levels of *PIF3* (A), MEP pathway genes (B), and chlorophyll and carotenoid biosynthetic enzymes (C). Total chlorophylls content as determined by absorption photometry (D). Carotenoids content as determined by absorption photometry (E). Gray bar: a vector control line; Red, tangerine and yellow bars: PIF3-OX line 1, 2 and 3, respectively. In all cases, mean values +/− SD ($n = 3$) are shown. Asterisks indicate significant difference (Student's *t*-test $P < 0.05$); daggers, not detected.

regulated isoprenoid metabolism in plastids. To summarize, overexpression of the APB domain containing PIF proteins, PIF1, PIF3 and PIF4, is not sufficient to enhance isoprenoid metabolism in T87 cells.

Among the members of the PIF family (PIF1, PIF3, PIF4, PIF5 and PIF6 [18]), PIF3 is considered to be a negative regulator of chlorophyll biosynthesis, since PIF3 down-regulated the expression of *HEMA1* and *GUN5*, both of which are involved in tetrapyrrole formation in the chlorophyll biosynthetic pathway [26,27]. These findings are consistent with our result that the amount of total chlorophylls was decreased in PIF3-OX, despite the fact that there were no significant changes in the transcript levels of MEP pathway genes (Fig. 4). Another study investigating the correlation between PIF family members and isoprenoid biosynthesis [28] showed that PIF1 negatively regulated phytoene synthase (*PSY*) expression and carotenoid biosynthesis, and that other PIF family including PIF5 also involved in the negative regulation. These results contradict those of the present study; we observed that PIF5 positively regulated carotenoid biosynthesis, while in PIF1-OX, there were no significant changes in the mRNA level of *PSY* or the amount of total carotenoids (Fig. 2 and Supplemental Fig. S3). This difference may be at least partly because of differences in the plant samples used. Toledo-Ortiz et al. [28] targeted the metabolic transition of *A. thaliana* seedlings in the de-etiolation stage. In light of these facts, their results can support our hypothesis that the appropriate PIF functions according to the situation; for example, carotenoid biosynthesis is mainly regulated by PIF1 in dark conditions but by PIF5 in light conditions.

3.5. Conclusions and perspectives

We identified transcription factors that coordinately regulate the expressions of many genes encoding IPP biosynthetic pathway enzymes. PIF5 was one of the most potent regulators of plastid isoprenoid biosynthesis. PIF5 binds the G-box (5′-CACGTG-3′) *cis*-element in promoter regions of target genes [29]. However, among all of the genes encoding MEP pathway enzymes, only MECPS has a G-box consensus motif in the 2-kbp region upstream of its coding sequence. Therefore, PIF5 might not directly regulate the expression of MEP pathway genes. Although the precise mechanism by which PIF5 regulates isoprenoid biosynthesis is yet to be elucidated, this is the first report of increased accumulation of plastid isoprenoids, accompanied by up-regulation of MEP pathway genes, as a result of overexpression of transcription factors. Our results suggest that IPP metabolism is a critical pathway for plants, and that it is strictly regulated by the balance of redundant and/or antagonistic effects of multiple transcription factors in response to various stimuli, as shown in recent report on the intricate co-expression network of isoprenid pathway genes [30]. Thus, a multidimensional perspective is required for detailed analyses of the regulation of IPP metabolism. In that sense, it appears that co-expression analysis is a useful tool to discover transcription factors that comprehensively regulate IPP metabolism.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.040.

References

[1] J.M. Estévez, A. Cantero, A. Reindl, S. Reichler, P. León, 1-Deoxy-D-xylulose-5-phosphate synthase, a limiting enzyme for plastidic isoprenoid biosynthesis in plants, J. Biol. Chem. 276 (2001) 22901–22909.
[2] S.S. Mahmoud, R.B. Croteau, Metabolic engineering of essential oil yield and composition in mint by altering expression of deoxyxylulose phosphate reductoisomerase and menthofuran synthase, Proc. Natl. Acad. Sci. 98 (2001) 8915–8920.
[3] P. Botella-Pavía, Ó. Besumbes, M.A. Phillips, L. Carretero-Paulet, A. Boronat, M. Rodríguez-Concepción, Regulation of carotenoid biosynthesis in plants: evidence for a key role of hydroxymethylbutenyl diphosphate reductase in controlling the supply of plastidial isoprenoid precursors, Plant J. 40 (2004) 188–199.
[4] L. Carretero-Paulet, A. Cairó, P. Botella-Pavía, O. Besumbes, N. Campos, A. Boronat, M. Rodríguez-Concepción, Enhanced flux through the methylerythritol 4-phosphate pathway in Arabidopsis plants overexpressing deoxyxylulose 5-phosphate reductoisomerase, Plant Mol. Biol. 62 (2006) 683–695.
[5] E. Grotewold, Transcription factors for predictive plant metabolic engineering: are we there yet?, Curr Opin. Biotechnol. 19 (2008) 138–144.
[6] P. Broun, Transcription factors as tools for metabolic engineering in plants, Curr. Opin. Plant Biol. 7 (2004) 202–209.
[7] M. Seki, P. Carninci, Y. Nishiyama, Y. Hayashizaki, K. Shinozaki, High-efficiency cloning of Arabidopsis full-length cDNA by biotinylated CAP trapper, Plant J. 15 (1998) 707–720.
[8] M. Seki, M. Narusaka, A. Kamiya, J. Ishida, M. Satou, T. Sakurai, M. Nakajima, A. Enju, K. Akiyama, Y. Oono, M. Muramatsu, Y. Hayashizaki, J. Kawai, P. Carninci, M. Itoh, Y. Ishii, T. Arakawa, K. Shibata, A. Shinagawa, K. Shinozaki, Functional annotation of a full-length Arabidopsis cDNA collection, Science 296 (2002) 141–145.
[9] M. Axelos, C. Curie, L. Mazzolini, C. Bardet, B. Lescure, A protocol for transient gene-expression in Arabidopsis-Thaliana protoplasts isolated from cell-suspension cultures, Plant Physiol. Biochem. 30 (1992) 123–128.
[10] T. Nakagawa, T. Kurose, T. Hino, K. Tanaka, M. Kawamukai, Y. Niwa, K. Toyooka, K. Matsuoka, T. Jinbo, T. Kimura, Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation, J. Biosci. Bioeng. 104 (2007) 34–41.
[11] T. Obayashi, K. Kinoshita, K. Nakai, M. Shibaoka, S. Hayashi, M. Saeki, D. Shibata, K. Saito, H. Ohta, ATTED-II: a database of co-expressed genes and cis elements for identifying co-regulated gene groups in Arabidopsis, Nucleic Acids Res. 35 (2007) D863–869.
[12] M.Y. Hirai, K. Sugiyama, Y. Sawada, T. Tohge, T. Obayashi, A. Suzuki, R. Araki, N. Sakurai, H. Suzuki, K. Aoki, H. Goda, O.I. Nishizawa, D. Shibata, K. Saito, Omics-based identification of Arabidopsis Myb transcription factors regulating aliphatic glucosinolate biosynthesis, Proc. Natl. Acad. Sci. USA 104 (2007) 6478–6483.
[13] B. Lange, M. Ghassemian, Genome organization in *Arabidopsis thaliana*: a survey for genes involved in isoprenoid and chlorophyll metabolism, Plant Mol. Biol. 51 (2003) 925–948.
[14] H. Suzuki, S. Takahashi, R. Watanabe, Y. Fukushima, N. Fujita, A. Noguchi, R. Yokoyama, K. Nishitani, T. Nishino, T. Nakayama, An isoflavone conjugate-hydrolyzing beta-glucosidase from the roots of soybean (Glycine max) seedlings: purification, gene cloning, phylogenetics, and cellular localization, J. Biol. Chem. 281 (2006) 30251–30259.
[15] Y. Ogawa, T. Dansako, K. Yano, N. Sakurai, H. Suzuki, K. Aoki, M. Noji, K. Saito, D. Shibata, Efficient and high-throughput vector construction and Agrobacterium-mediated transformation of *Arabidopsis thaliana* suspension-cultured cells for functional genomics, Plant Cell Physiol. 49 (2008) 242–250.
[16] H.K. Lichtenthaler, Chlorophylls and carotenoids: pigments of photosynthetic biomembranes, in: R.D. Lester Packer (Ed.), Methods in Enzymology, Academic Press, 1987, pp. 350–382.
[17] P.D. Fraser, P.M. Bramley, The biosynthesis and nutritional uses of carotenoids, Prog. Lipid Res. 43 (2004) 228–265.
[18] A. Castillon, H. Shen, E. Huq, Phytochrome interacting factors: central players in phytochrome-mediated light signaling networks, Trends Plant Sci. 12 (2007) 514–521.
[19] T. Yamashino, A. Matsushika, T. Fujimori, S. Sato, T. Kato, S. Tabata, T. Mizuno, A Link between circadian-controlled bHLH factors and the APRR1/TOC1 quintet in *Arabidopsis thaliana*, Plant Cell Physiol. 44 (2003) 619–629.
[20] R. Khanna, E. Huq, E.A. Kikis, B. Al-Sady, C. Lanzatella, P.H. Quail, A novel molecular recognition motif necessary for targeting photoactivated phytochrome signaling to specific basic Helix-Loop-Helix transcription factors, Plant Cell 16 (2004) 3033–3044.
[21] S. Lorrain, T. Allen, P.D. Duek, G.C. Whitelam, C. Fankhauser, Phytochrome-mediated inhibition of shade avoidance involves degradation of growth-promoting bHLH transcription factors, Plant J. 53 (2008) 312–323.
[22] P. Hornitschek, S. Lorrain, V. Zoete, O. Michielin, C. Fankhauser, Inhibition of the shade avoidance response by formation of non-DNA binding bHLH heterodimers, EMBO J. 28 (2009) 3893–3902.
[23] P. Leivar, P.H. Quail, PIFs: pivotal components in a cellular signaling hub, Trends Plant Sci. 16 (2011) 19–28.
[24] G. Toledo-Ortiz, E. Huq, P.H. Quail, The Arabidopsis basic/helix-loop-helix transcription factor family, Plant Cell 15 (2003) 1749–1770.

[25] Y. Shen, R. Khanna, C.M. Carle, P.H. Quail, Phytochrome induces rapid PIF5 phosphorylation and degradation in response to red-light activation, Plant Physiol. 145 (2007) 1043–1051.
[26] P.G. Stephenson, C. Fankhauser, M.J. Terry, PIF3 is a repressor of chloroplast development, Proc. Natl. Acad. Sci. USA 106 (2009) 7654–7659.
[27] J. Shin, K. Kim, H. Kang, I.S. Zulfugarov, G. Bae, C.H. Lee, D. Lee, G. Choi, Phytochromes promote seedling light responses by inhibiting four negatively-acting phytochrome-interacting factors, Proc. Natl. Acad. Sci. USA 106 (2009) 7660–7665.
[28] G. Toledo-Ortiz, E. Huq, M. Rodriguez-Concepcion, Direct regulation of phytoene synthase gene expression and carotenoid biosynthesis by phytochrome-interacting factors, Proc. Natl. Acad. Sci. USA 107 (2010) 11626–11631.
[29] P. Hornitschek, S. Lorrain, V. Zoete, O. Michielin, C. Fankhauser, Inhibition of the shade avoidance response by formation of non-DNA binding bHLH heterodimers, EMBO J. 28 (2009) 3893–3902.
[30] E. Vranová, D. Coman, W. Gruissem, Structure and dynamics of the isoprenoid pathway network, Mol. Plant 5 (2012) 318–333.