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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.