Application of the *Synechococcus nirA* Promoter To Establish an Inducible Expression System for Engineering the *Synechocystis* Tocopherol Pathway

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Tocopherols are important antioxidants in lipophilic environments. They are synthesized by plants and some photosynthetic bacteria. Recent efforts to analyze and engineer tocopherol biosynthesis led to the identification of *Synechocystis* sp. strain PCC 6803 as a well-characterized model system. To facilitate the identification of the rate-limiting step(s) in the tocopherol biosynthetic pathway through the modulation of transgene expression, we established an inducible expression system in *Synechocystis* sp. strain PCC 6803. The *nirA* promoter from *Synechococcus* sp. strain PCC 7942, which is repressed by ammonium and induced by nitrite (S.-I. Maeda et al., J. Bacteriol. 180:4080–4088, 1998), was chosen to drive the expression of Arabidopsis thaliana *p*-hydroxyphenylpyruvate dioxygenase. The enzyme catalyzes the formation of homogentisic acid from *p*-hydroxyphenylpyruvate. Expression of this gene under inducing conditions resulted in up to a fivefold increase in total tocopherol levels with up to 20% of tocopherols being accumulated as tocotrienols. The culture supernatant of these cultures exhibited a brown coloration, a finding indicative of homogentisic acid excretion. Enzyme assays, functional complementation, reverse transcription-PCR, and Western blot analysis confirmed transgene expression under inducing conditions only. These data demonstrate that the *nirA* promoter can be used to control transgene expression in *Synechocystis* and that homogentisic acid is a limiting factor for tocopherol synthesis in *Synechocystis* sp. strain PCC 6803.

Vitamin E is a collective term that refers to the biological activity of a group of eight natural amphipathic compounds: α-, β-, γ-, and δ-tocopherol and α-, β-, γ-, and δ-tocotrienol (Fig. 1). Tocotrienols can be distinguished from their corresponding tocopherols by the presence of three isolated double bonds in their prenyl side chains. These compounds are synthesized by plants and certain photosynthetic bacteria and are well recognized as effective oxygen radical scavengers in lipophilic environments such as oils and the lipid bilayer of biological membranes (3, 4). In photosynthetic organisms, tocopherols are suggested to function as membrane-associated antioxidants and as constituents of the chloroplast membranes (17, 18, 27, 41). α-Tocopherol is an essential component in the mammanian diet and has the highest vitamin E activity among the isomers described above (3). Because of these health benefits and biological functions, there is considerable interest in engineering tocopherol biosynthesis to increase tocopherol levels and optimize their composition in agricultural crops.

The regulation and rate-limiting reactions in tocopherol biosynthesis are currently poorly understood. The first committed reaction of tocopherol biosynthesis is the prenylation of homogentisic acid (HGA), derived from the shikimate pathway, with phytodiphosphatase (PDP) derived from the 2C-methyl-D-erythritol 4-phosphate (MEP)-pathway, for the formation of 2-methyl-6-phytylbenzoquinol (Fig. 1). Tocotrienols are synthesized when geranylgeranyl diphosphate replaces PDP in the prenylation reaction of HGA, resulting in the formation of 2-methyl-6-geranyltocopherol. Therefore, the availability of HGA has an impact on tocopherol and tocotrienol biosynthesis. The cyanobacterium *Synechocystis* sp. strain PCC 6803 has been used as a model organism for elucidating the mechanisms of critical biological processes such as photosynthesis (14, 16), metabolic engineering of fatty acid saturation, and zeaxanthin biosynthesis (2, 21). A common strategy to define the rate-limiting steps of a biosynthetic pathway experimentally is to modulate expression of specific pathway enzymes and then monitor the corresponding changes of intermediates and end products. In this setting, it is advantageous to use inducible promoters for the controlled expression of target genes.

Cyanobacteria are prokaryotic organisms that perform oxygen photosynthesis through the operation of a photosynthetic system very much like that present in the chloroplasts of higher plants. They preferentially use inorganic carbon, nitrogen, and mineral salts for growth. Nitrate and ammonium are excellent nitrogen sources for cyanobacteria (11). Nitrate is taken up into the cells and reduced to ammonium by sequential action of nitrate reductase and nitrite reductase. The genes encoding the nitrate transporter (*ntrABCD*) (29), nitrate reductase (*narB*) (32), and nitrite reductase (*nirA*) (22) form the *nir* operon (*nirA-nrtABCD-narB*) in *Synechococcus* sp. strain PCC 7942 (38). In all cyanobacteria tested to date, transcription of

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the nir operon is repressed by ammonium (20, 38). In contrast, the presence of nitrate in the culture medium enhances mRNA levels of the nir operon (12, 23, 24). This is further evidenced by discovering the requirement of the nitrate-promoted NtcB transcription factor for regulation of nir operon transcription in cyanobacteria (1, 12, 13, 19, 24, 38, 39). We hypothesized that the nirA promoter of Synechococcus can be used as a tight inducible system for controlling transgene expression in Synechocystis. Furthermore, by substitution of ammonium with nitrate for activation of the promoter this system would produce a minimal perturbation on the cellular processes in Synechocystis. Several studies have provided evidence that p-hydroxyphenylpyruvate dioxygenase (Hpd) limits tocopherol biosynthesis in plants (10, 15, 31, 42, 43). We reasoned, therefore, that transgenic expression of Arabidopsis thaliana hpd (hpda) under nirA promoter control would be an excellent model for developing a controlled Synechocystis expression system and to demonstrate tocopherol pathway engineering in Synechocystis.

**MATERIALS AND METHODS**

**Construction of a nirAP-based expression plasmid.** Custom multiple cloning sites (MCS) were made by annealing the primers MCS2 (5'-AAGCGCTGACAATTGTCGACCAGGCGAGTCTAGGCACTGCTGACGGAATGTTAACTAGATGGAAGGGCGGCAAGTTCGGTGGCGGTGCCTGCTGAGGCTTCG) and MCS2-rev.complp (5'-AGAGCTCTAGGCGAGTGTCAGATGCGGCCGCTCTAGAACA) into the EcoRV site of pBluescript SK(+) (Stratagene, La Jolla, CA), yielding the plasmid pCER7.

A 166-bp fragment upstream of the nirA operon was amplified by PCR from the genomic DNA of Synechococcus sp. strain PCC 7942 by using the primer pair nirA1 forward (5'-TAGGCCCTCCTCTCTAGGATCAAAAAAGT-3') and nirA3 (5'-CTTGAGGATTATATCTCGCTGCTAAGACACTCA-3'). Underlined bases in the primer sequence indicate Stul and NdeI restriction sites that were added at the 5' and 3' termini of the nirA promoter element, respectively. The resulting PCR fragment was digested with Stul and NdeI, gel purified, and ligated into Stul- and NdeI-digested pCER7, resulting in the formation of pCER11. Promoter sequence integrity was confirmed by DNA sequence analysis. The amplified region possesses an NtcB-binding site (an inverted repeat with a LysR motif, TGCANTGCA), an NtcA-binding site (a palindromic structure with a conserved sequence signature, GTAN8TAC), and a TAN3T sequence fitting the −10 box of the Escherichia coli 

**FIG. 1.** Schematic drawing of the tocopherol biosynthetic pathway. Abbreviations: DMAPP, dimethylallyldiphosphate; ChlP, geranylgeranyl-diphosphate reductase; Hpd, p-hydroxyphenylpyruvate dioxygenase; IPP, isopentenyldiphosphate; MEP, methylenetolyl phosphate; TyrA, bi-functional chorismate mutase and prephenate dehydrogenase; Vte1, tocopherol cyclase; Vte2, homogentisate phytyltransferase; Vte3, 2-methyl-6-phytylbenzoquinol methyltransferase; Vte4, γ-tocopherol methyltransferase.
Strain PCC 6803 (ATCC 27184) was obtained from the American Type Culture Collection. Complete segregation of the mutant genome (data not shown). The nptII gene, which confers resistance to gentamicin, serves as the selection marker. Bracketing nptII and the selection marker are two transcrip-tional terminators (TT) from the plasmid, pHP45a. The origin of replication (oriV) and replication proteins (repABC), as well as the origin of transfer (oriT) are derived from the broad-host-range plasmid RSF1010 (34).

Cloned into XbaI- and NotI-digested pMON36546, resulting in the formation of a replication origin, plasmid pMON36547. Generation of Synechocystis sp. strain PCC 6803 ΔrepABC. The open reading frame slr0090 had been identified to encode the Synechocystis sp. strain PCC 6803 hpd gene (6). To create a hpd mutant, a knockout construct, pMON29153, was generated. This construct harbors the Synechocystis hpd interrupted by insertion of the nptII gene. Plasmid pMON29153 was constructed by digesting pMON29138 (43) with BstXl, filling in the sticky ends using Klenow fragment, and inserting the nptII expression cassette (blunted EcoRl fragment) from pUC4K (40). The resulting plasmid contained the nptII cassette inserted 647 bp downstream of the ATG start codon. This recombinant vector was transformed into wild-type (WT) Synechocystis sp. strain PCC 6803 by using the transformation procedure described by Williams (44). Homologous recombination led to the replacement of the ATG start codon. This recombinant vector was transformed into the slr0090 mutant, a knockout construct, pMON29153, was transformed into the slr0090 mutant host, and the selection marker was two transcrip-tional terminators (TT) from the plasmid, pHP45a. The origin of replication (oriV) and replication proteins (repABC), as well as the origin of transfer (oriT) are derived from the broad-host-range plasmid RSF1010 (34).

FIG. 2. Plasmid map of the inducible cyanobacterial expression vector pCER20. The expression of target gene(s) is under the transcrip-tional control of the nitrite reductase promoter, nirAp. The aacC gene, which confers resistance to gentamicin, serves as the selection marker. Bracketing nirAp and the selection marker are two transcrip-tional terminators (TT) from the plasmid, pHP45a. The origin of replication (oriV) and replication proteins (repABC), as well as the origin of transfer (oriT) are derived from the broad-host-range plasmid RSF1010 (34).

Cloned into XbaI- and NotI-digested pMON36546, resulting in the formation of plasmid pMON36547. Generation of Synechocystis sp. strain PCC 6803 ΔrepABC. The open reading frame slr0090 had been identified to encode the Synechocystis sp. strain PCC 6803 hpd gene (6). To create a hpd mutant, a knockout construct, pMON29153, was generated. This construct harbors the Synechocystis hpd interrupted by insertion of the nptII gene. Plasmid pMON29153 was constructed by digesting pMON29138 (43) with BstXl, filling in the sticky ends using Klenow fragment, and inserting the nptII expression cassette (blunted EcoRl fragment) from pUC4K (40). The resulting plasmid contained the nptII cassette inserted 647 bp downstream of the ATG start codon. This recombinant vector was transformed into wild-type (WT) Synechocystis sp. strain PCC 6803 by using the transformation procedure described by Williams (44). Homologous recombination led to the replacement of the ATG start codon. This recombinant vector was transformed into the slr0090 mutant, a knockout construct, pMON29153, was transformed into the slr0090 mutant host, and the selection marker was two transcrip-tional terminators (TT) from the plasmid, pHP45a. The origin of replication (oriV) and replication proteins (repABC), as well as the origin of transfer (oriT) are derived from the broad-host-range plasmid RSF1010 (34).

Strains, growth conditions, and cell sample preparation. Synechocystis sp. strain PCC 6803 (ATCC 27184) was obtained from the American Type Culture Collection. WT and recombinant cells of Synechocystis sp. strain PCC 6803 were cultivated photoautotrophically at 30°C under continuous illumination provided by fluorescent lamps (70 μE m⁻² s⁻¹). Liquid cultures were shaken at 225 rpm on a rotary shaker. The basal medium (BG11) was a nitrogen-free medium composed of a 1:1 (vol/vol) mixture of 150 mM NaCl, CoCl₂, and ferric citrate, respectively. Ammonium-containing medium (BG11NH₄⁺) and nitrate-containing medium (BG11NO₃⁻) were prepared by addition of 17.6 mM NH₄Cl or 17.6 mM NaNO₃, respectively, to the basal medium. Both media were buffered with 10 mM N-Tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)-NaOH (pH 8.0). For growth on solid media, BG11NH₄⁺ medium containing 1.5% (wt/vol) agar (Difco) was used. Plasmids replicating autonomously in Synechocystis were transformed into Synechocystis sp. strain PCC 6803 via triparental mating (9), and transformants were selected on medium supplemented with kanamycin at 25 μg ml⁻¹ and/or gentamicin at 10 μg ml⁻¹. Conjuncted colonies were spread on a 0.45-μm-pore-size cellulose nitrate membrane filter (Whatman) and plated on nonselective solid ammonium medium. Colonies were incubated as described above, and transferred to selective ammonium medium plates. Resistant colonies were used to inoculate 2 ml of liquid BG11NH₄⁺ medium supplemented with gentamicin (and/or kanamycin) and incubated for 2 days. These cultures served as precultures for the final 150-ml liquid cultures. Cell density in the 150-ml cultures was monitored spectrophotometrically (SpectraMax; Molecular De-vices) at 730 nm (A₇₃₀). Cells were harvested when the A₇₃₀ of the cell culture reached 0.4 to 0.5 by 10 min of centrifugation at 25°C and 3,500 × g. The cell pellet was washed with 20 ml of BG11 and resuspended in 150 ml of fresh nitrate (BG11NO₃⁻) or ammonium (BG11NH₄⁺) medium. For promoter activation, cells were subsequently grown under the light and temperature conditions described above. Cell samples were harvested at various time intervals to measure tocopherol and tocotrienol content, gene expression, and enzyme activity.

Analysis of hpd₆ transcription by reverse transcription-PCR (RT-PCR). Nitrate (BG11NO₃⁻) or ammonium (BG11NH₄⁺) medium-grown Synechocystis har-vested from three representative cultures was ground in liquid nitrogen. Total RNA was isolated as previously described (26), and any contaminating DNA was removed by treatment with RNase-free DNase (Promega, Madison, WI). Three micrograms of total RNA for each sample was reverse transcribed to generate cDNA in two 50-μl reactions by using an Omniscript RT kit according to the manufacturer's recommendations (QIAGEN, Inc., Valencia, CA). An aliquot of cDNA corresponding to 200 ng of total RNA was subjected to 25 cycles of PCR with primers 5’-TTCCTTCGTGCGCTTCTATCG-3’ (forward) and 5’-ACTCTTTGATCTGATCATCGC-3’ (reverse), resulting in the amplification of a 600-bp fragment internal to the hpd₆ gene. The reaction was done under the following thermocycle conditions: 5 min of incubation at 95°C, followed by 25 cycles of 1 min at 95°C, 1 min of annealing at 56°C, and a 2-min extension at 72°C. The amount of amplified DNA was estimated by DNA gel electrophoresis with ethidium bromide staining.

Tocopherol and tocotrienol analyses. Tocopherols for WT and recombinant Synechocystis strains were measured by a normal-phase high-pressure liquid chromatography (HPLC) as described previously (33), but lipophilized cell pellets were used instead of fresh harvested cells. Tocotrienol content was analyzed by using the same procedure with tocotrienol standards purchased from Calbiochem (La Jolla, CA). Tocopherol content was normalized to the dry cell mass.

Protein analysis. The presence of Hpd₆ protein in recombinant strains was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis with peptide-directed antibodies obtained by immunization of a rabbit with a synthetic peptide (CRTLREMRKRSSIGG). Peptide synthesis and antibody generation were performed by Sigma-Genosys, St. Louis, MO. Synechocystis sp. strain PCC 6803 cell extracts were prepared by six passages through a French press (Simmon-Spectronic Instruments) and mixed with cells suspended in 50 mM Tris-HCl (pH 7.6) containing 5 mM dithiothreitol, 3 mM dichlorophenolindophenol (Sigma), 250 mM reduced glutathione (Sigma), 100 U of catalase (Sigma), 100 μg of RNase (Sigma), and 250 μg of protein extract in a total volume of 500 μl. The reaction was incubated for 30 min at 30°C and terminated by the addition of 150 μl of 20% (wt/vol) perchloric acid. Precipitated protein was removed by 5 min of centrif-
FIG. 3. Complementation of Synechocystis sp. strain PCC 6803 Δsr0090 with Arabidopsis hpd driven by the nirA promoter. Total tocopherol content was normalized to dry cell mass. Cells used for tocopherol analysis were harvested after an incubation period of 10 days. Tocopherol levels shown for strains Synechocystis Δsr0090 and Synechocystis Δsr0090(pMON36546) were below the limit of detection (<5 ng/mg). Abbreviations: Δsr0090, Synechocystis hpd insertion mutant; pMON36546, empty vector control; pMON36547, nirAp:hpdΔ.

RESULTS AND DISCUSSION

Functional complementation of Synechocystis sp. strain PCC 6803 Δsr0090 with hpdΔ driven by the nirA promoter. A targeted mutation in Synechocystis sp. strain PCC 6803 was created by homologous recombination (see Materials and Methods), and the resulting mutant, Synechocystis sp. strain PCC 6803 Δsr0090, was analyzed for changes in tocopherol content compared to WT cultures. Tocopherol levels in the mutant were below the limit of detection (<5 ng/mg dry cell mass) (Fig. 3) (6), indicating an essential role of Hpd in tocopherol biosynthesis. This result is consistent with the loss of Hpd activity in this mutant (data not shown). The mutant cell growth rates were comparable to that of WT cells, indicating that photosynthesis was not affected (6). Subsequently, the mutant was used to test the functionality of nirAp:hpdΔ in complementation experiments.

For complementation, Synechocystis sp. strain PCC 6803 Δsr0090 was transformed with pMON36547. This plasmid harbored a nirAp:hpdΔ expression cassette. As shown in Fig. 3, complemented strains grown on BG110NO3− medium contained ~3.5-fold more tocopherol than WT cells grown on the same medium. As expected, Synechocystis sp. strain PCC 6803 Δsr0090(pMON36547) failed to accumulate tocopherol when grown in BG110NH4+ medium (Fig. 3). To confirm hpdΔ expression in BG110NO3−-grown cells, RT-PCR amplifications were performed. The hpdΔ transcript was detected in Synechocystis sp. strain PCC 6803 Δsr0090(pMON36547) at 2 h after nitrate induction and continued to accumulate at 8 and 16 h postinduction (Fig. 4). In contrast, the transcript was not detected in Synechocystis sp. strain PCC 6803 Δsr0090-(pMON36547) cultivated in BG110NH4+ medium. These results support the induction of nirAp by nitrate in Synechocystis sp. strain PCC 6803 and the functionality of hpdΔ, complementing the Δsr0090 mutation.

Nitrate-responsive expression of hpdΔ under nirA promoter control in Synechocystis sp. strain PCC 6803. To further demonstrate nitrate-dependent activation of the Synechococcus nirA promoter and assess its efficacy in induction of heterologous gene expression in Synechocystis, the expression vector pMON36547 and the empty vector control pMON36546 were conjugated into WT Synechocystis sp. strain PCC 6803. In order to analyze gene expression and tocopherol levels, transgenic and WT cells were grown in parallel and sampled at 2, 4, 10, and 12 days after inoculation. As shown in Fig. 5, utilization of different nitrogen sources in the culture media resulted in substantial differences in HpdΔ polypeptide level and enzyme activity. Using anti-HpdΔ peptide antibody, a single immunoreactive band was detected in an extract of Synechocystis sp. strain PCC 6803(pMON36547) expressing hpdΔ when grown in BG110NO3− medium. Extracts of this recombinant strain grown in BG110NH4+ did not contain detectable immunore-
active proteins against anti-Hpd\(_{\alpha}\) peptide antibody (Fig. 5A). These results confirmed the induction of hpd\(_{\alpha}\) in BG11\(_{\alpha}\)NO\(_{3}^{-}\) and its repression by ammonium. The immunoreactive band was also not observed in extracts of WT cells growing in either BG11\(_{\alpha}\)NH\(_{4}^{+}\) or BG11\(_{\alpha}\)NO\(_{3}^{-}\) medium. This result was consistent with the lack of cross-reactivity with the E. coli-expressed Synechocystis Hpd. Hpd activity in E. coli expressing hpd\(_{\alpha}\) was typically 30 to 50 nmol min\(^{-1}\) mg of protein\(^{-1}\).

To evaluate whether the increase in polypeptide levels corresponds with increased enzyme activity, Hpd enzyme assays were carried out with crude extracts of the Synechocystis strains. As shown in Fig. 5B, the extract derived from nitrate-induced Synechocystis sp. strain PCC 6803 (pMON36547) had ~5-fold increased Hpd activity compared to extracts derived from the vector control [Synechocystis sp. strain PCC 6803 (pMON36546)] or WT cell extracts. Such an increase in Hpd activity was not observed with cell extracts derived from Synechocystis sp. strain PCC 6803 (pMON36547) incubated in ammonium-containing medium. In addition, the culture supernatant of hpd\(_{\alpha}\)-expressing Synechocystis sp. strain PCC 6803 (pMON3654) incubated in BG11\(_{\alpha}\)NO\(_{3}^{-}\) medium turned a dark brown color after 10 days of incubation (Fig. 6). A similar phenomenon was observed in recombinant E. coli cultures expressing Arabidopsis or Synechocystis hpd (data not shown) and has been reported in other systems as well (7, 8). The brown color is thought to be the result of HGA accumulation and excretion into the culture medium (7). In aqueous medium HGA can be oxidized and polymerized to form a brown melanin-like pigment. Western blotting experiments, enzyme assay data, and color changes of the culture supernatant are consistent with transgenic hpd\(_{\alpha}\) expression in Synechocystis sp. strain PCC 6803 (pMON36547) incubated in BG11\(_{\alpha}\)NO\(_{3}^{-}\). Similarly, Western blot and enzyme assay data obtained with crude extracts of the same strain after incubation in BG11\(_{\alpha}\)NH\(_{4}^{+}\) did not provide any evidence for hpd\(_{\alpha}\) expression, suggesting that the nirA promoter is inactive under such growth conditions.

**Transgenic expression of hpd\(_{\alpha}\) led to increased tocopherol levels.** To determine whether the tocopherol content and composition had been affected by transgenic expression of hpd\(_{\alpha}\), aliquots of the cell culture were taken at various time points for tocopherol analysis. Cultures of Synechocystis sp. strain PCC 6803 harboring pMON36547 that were incubated for 12 days in BG11\(_{\alpha}\)NO\(_{3}^{-}\) had fivefold-increased tocopherol levels compared to the WT control, whereas cells from the same strain incubated in BG11\(_{\alpha}\)NH\(_{4}^{+}\) and cultures from the vector control had tocopherol levels comparable to the WT control (Fig. 7). Thus, tocopherol accumulation showed a positive correlation with the Hpd\(_{\alpha}\) polypeptide level and enzyme activity. These results confirm functionality of the nirA promoter for regulation of transgene expression via nitrate availability, demonstrate its utilization for engineering of the tocopherol metabolic pathway in Synechocystis, and support the hypothesis that Hpd catalyzes a rate-limited reaction in tocopherol biosynthesis (10, 15, 31, 42, 43).

More interestingly, Synechocystis cells expressing hpd\(_{\alpha}\) accumulated up to 20% of their total tocopherols as tocotrienols.

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**FIG. 5.** Induction of nirA in BG11\(_{\alpha}\)NO\(_{3}^{-}\) media. (A) Immunoblot analysis of Hpd\(_{\alpha}\) peptide in Synechocystis sp. strain PCC 6803 harboring pMON36547 (nirA::hpd\(_{\alpha}\)) or pMON36546 (empty vector control) incubated for 4 days in BG11\(_{\alpha}\)NO\(_{3}^{-}\) medium (lane 1, 4dNO\(_{3}^{-}\)) or for 2 days in BG11\(_{\alpha}\)NO\(_{3}^{-}\) or BG11\(_{\alpha}\)NH\(_{4}^{+}\) medium (remaining lanes). (B) Hpd enzyme activity detected in the corresponding crude cell extracts. Hpd enzyme activity in *E. coli* expressing hpd\(_{\alpha}\) was 48.8 kDa for Hpd\(_{\alpha}\) peptide antibody (Fig. 5A). These results confirmed the induction of hpd\(_{\alpha}\) in BG11\(_{\alpha}\)NO\(_{3}^{-}\) and its repression by ammonium. The immunoreactive band was also not observed in extracts of WT cells growing in either BG11\(_{\alpha}\)NH\(_{4}^{+}\) or BG11\(_{\alpha}\)NO\(_{3}^{-}\) medium. This result was consistent with the lack of cross-reactivity with the E. coli-expressed Synechocystis Hpd. Hpd activity in *E. coli* expressing hpd\(_{\alpha}\) was typically 30 to 50 nmol min\(^{-1}\) mg of protein\(^{-1}\).

**FIG. 6.** Synechocystis culture supernatants. Culture supernatants shown above were collected by 25 min of centrifugation at 4,000 × g after 10 days of incubation at 30°C. The culture supernatant of *Synechocystis* sp. strain PCC 6803 (pMON36547) incubated in BG11\(_{\alpha}\)NO\(_{3}^{-}\) medium exhibits a characteristic brown color that is typical for bacteria excreting HGA (7).
FIG. 7. Tocopherol content and composition in Synechocystis sp. strain PCC 6803 cultures grown in BG11 0NH4 and BG11 0NO3 media. Tocopherol and tocotrienol contents of Synechocystis cultures are normalized to dry cell mass. Cells used for this experiment were harvested after an incubation period of 12 days.

(FIG. 7). None of the WT or vector control Synechocystis samples contained detectable tocotrienol levels. As described above, the tocotrienol precursor, 2-methyl-6-geranylglycerol-benzoquinone, is formed via the condensation of HGA and geranylgeranyl diphasate catalyzed by the homogentisate phytyltransferase (Vte2), whereas tocopherol synthesis utilizes HGA and PDP. The formation of tocotrienols by Synechocystis Vte2 is consistent with data provided by Collakova and Della-Penna (5) and suggests that 2-methyl-6-phytylbenzoquinol methyltransferase (Vte3), tocopherol cyclase (Vte1), and γ-methyltransferase (Vte4) in Synechocystis sp. strain PCC 6803 can utilize tocotrienol precursors as substrates as well. In addition, the formation of tocotrienols and accumulation of HGA provide evidence for a limitation of endogenous PDP, suggesting that experiments to engineer further tocopherol increases in Synechocystis should focus on this part of the pathway.

Conclusions. A 166-bp DNA fragment from the 5′ untranslated region of the nirA operon was isolated from the genome of Synechococcus sp. strain PCC 7942. Sequence analysis revealed the presence of the consensus sequence (GTANpTAC) for cyanobacterial NtcA-binding sites, an L1-like inverted repeat containing a LysR motif (TN11p), and the putative −10 element, showing a typical structure for a nitrogen-regulated promoter in cyanobacteria (12, 24, 38). By monitoring the expression level of hpdAt and its impact on tocopherol levels in Synechocystis sp. strain PCC 6803 or Synechocystis sp. strain PCC 6803ΔhpdAt0090 harboring pMON36547, a nirAp:hdpa expression construct, we were able to show that the nirA promoter is active in BG11 0NO3 medium and inactive in BG11 0NH4 medium. More importantly, the results presented here are the first, to our knowledge, to demonstrate that the nitrate-inducible nirA promoter can be used to drive and regulate the transcription of transgenes in Synechocystis sp. strain PCC 6803 and that this regulation can lead to changes in metabolite flux for production of target chemicals, such as tocopherols. Therefore, the nirA promoter system provides a suitable tool for metabolic engineering in Synechocystis. Its ability to be inactivated in ammonium-containing medium makes it particularly suitable for the expression of potentially toxic genes or for engineering metabolic pathways that may produce toxic products or intermediates. In the present study, we obtained evidence that the upregulated hpdAt expression results in an increased synthesis of HGA, tocopherols, and tocotrienols in Synechocystis in vivo, indicating that Hpd plays an important role in tocopherol production and that additional enzymes such as geranylgeranyl diphasate reductase may be required to overcome additional constraints for further enhancement of the tocopherol accumulation in this strain. The results obtained from this photosynthetic model system provide useful information for tocopherol metabolic engineering in other organisms.

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