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# Identification of a Vinyl Reductase Gene for Chlorophyll Synthesis in *Arabidopsis thaliana* and Implications for the Evolution of Prochlorococcus Species

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**Chlorophyll metabolism has been extensively studied with various organisms, and almost all of the chlorophyll biosynthetic genes have been identified in higher plants. However, only the gene for 3,8-divinyl protochlorophyllide *a* 8-vinyl reductase (DVR), which is indispensable for monovinyl chlorophyll synthesis, has not been identified yet. In this study, we isolated an *Arabidopsis thaliana* mutant that accumulated divinyl chlorophyll instead of monovinyl chlorophyll by ethyl methanesulfonate mutagenesis. Map-based cloning of this mutant resulted in the identification of a gene (AT5G18660) that shows sequence similarity with isoflavone reductase genes. The mutant phenotype was complemented by the transformation with the wild-type gene. A recombinant protein encoded by AT5G18660 was expressed in *Escherichia coli* and found to catalyze the conversion of divinyl chlorophyllide to monovinyl chlorophyllide, thereby demonstrating that the gene encodes a functional DVR. DVR is encoded by a single copy gene in the *A. thaliana* genome. With the identification of DVR, finally all genes required for chlorophyll biosynthesis have been identified in higher plants. Analysis of the complete genome of *A. thaliana* showed that it has 15 enzymes encoded by 27 genes for chlorophyll biosynthesis from glutamyl-tRNA$^{glu}$ to chlorophyll *b*. Furthermore, identification of the *DVR* gene helped understanding the evolution of *Prochlorococcus marinus*, a marine cyanobacterium that is dominant in the open ocean and is uncommon in using divinyl chlorophylls. A *DVR* homolog was not found in the genome of *P. marinus* but found in the *Synechococcus* sp WH8102 genome, which is consistent with the distribution of divinyl chlorophyll in marine cyanobacteria of the genera Prochlorococcus and Synechococcus.**

## INTRODUCTION

Chlorophyll molecules universally exist in photosynthetic organisms and perform essential processes of harvesting light energy in the antenna systems and by driving electron transfer in the reaction centers (Fromme et al., 2003). According to the number of vinyl side chains, chlorophylls of oxygenic photosynthetic organisms are classified into two groups: 3,8-divinyl chlorophyll (divinyl chlorophyll) and 3-vinyl chlorophyll (monovinyl chlorophyll). Almost all of the oxygenic photosynthetic organisms contain monovinyl chlorophylls, regardless of the variation in their indigenous environments (Porra, 1997). The exceptions are species of *Prochlorococcus marinus*, marine picophytoplanktons that contain divinyl chlorophylls as their photosynthetic pigments (Chisholm et al., 1992). These organisms are presumably the most abundant photosynthetic organisms on the Earth; they account for a large fraction of the biomass, and they represent a substantial contribution to the global carbon cycle. The advantage for *Prochlorococcus* sp to have divinyl chlorophylls is that the absorbance maximum of the pigments in the Soret region red shifts by ~10 nm compared with monovinyl chlorophylls, enabling more efficient absorption of blue light that is enriched in deep water layers (Kirk, 1994).

Because monovinyl derivatives of chlorophyll molecules are exclusively synthesized from the divinyl derivatives, whether the organisms have monovinyl or divinyl chlorophyll should be determined by the activity of 3,8-divinyl protochlorophyllide *a* 8-vinyl reductase (DVR). If this enzyme is inactivated or absent in the cells, the organism should theoretically accumulate divinyl chlorophylls. Therefore, it is expected that the process for the divergence of chlorophyll types should be revealed by functional analysis of the *DVR* gene.

Chlorophyll metabolism has been extensively studied with various organisms by the biochemical (Pontoppidan and Kannangara, 1994) and genetic (Bollivar et al., 1994; Nakayashiki et al., 1995; Tanaka et al., 1998) methods. Because early enzymatic steps of chlorophyll biosynthesis from glutamyl tRNA to protoporphyrin IX are shared with the heme biosynthesis pathway, many essential data regarding the identity of the associated enzymes were obtained from studies with nonphotosynthetic organisms such as *Escherichia coli* (Narita et al., 1996). The later steps of chlorophyll biosynthesis are common with bacteriochlorophyll *a* biosynthesis (Porra, 1997; Suzuki et al., 1997). Directed mutational analysis with a photosynthetic bacterium, *Rhodobacter capsulatus*, provided the information for the genes involved with bacteriochlorophyll biosynthesis (Bollivar et al., 1994), and homologous genes have been isolated

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from oxygenic plants (Jensen et al., 1996). The 8-vinyl reductase was identified as bchJ in photosynthetic organisms by genetical methods (Suzuki and Bauer, 1995), but the homologous gene was not found in oxygenic plants. *DVR* is the only remaining gene that has not been identified for chlorophyll biosynthesis.

DVR performs reduction of 8-vinyl group on the tetrapyrrole to an ethyl group (Parham and Rebeiz, 1995) using NADPH as the reductant. This activity has been detected with isolated plastid membranes (Parham and Rebeiz, 1995) from cucumber (*Cucumis sativus*) and in solubilized crude extracts (Kolossov and Rebeiz, 2001). It has been proposed that 8-vinyl group reduction may occur at various steps of chlorophyll biosynthesis before and after the reduction of D pyrrole ring by protochlorophyllide oxidoreductase. As a result, it is likely that the monovinyl and divinyl chlorophyll biosynthesis reactions may operate in parallel rather than by the normally accepted single, linear pathway (Parham and Rebeiz, 1995). However, at this time, it is unclear whether different enzymes or a single enzyme are responsible for the reduction of the 8-vinyl group of various intermediate molecules of chlorophyll biosynthesis. Enzymatic studies with purified protein(s) would facilitate the identification of the exact substrate for DVR(s). A mutant accumulating only divinyl chlorophyll instead of monovinyl chlorophyll was previously reported in maize (*Zea mays*) (Bazzaz, 1981), and the mutant was capable of photosynthetic growth with divinyl chlorophylls. Although this finding suggests a single gene product exists for DVR, a mutation in the regulatory gene could not be excluded.

In this study, we isolated an *Arabidopsis thaliana* mutant that exhibits a predominant replacement of monovinyl chlorophyll with divinyl chlorophyll. The mutant plants grew photosynthetically under low-light conditions, and map-based cloning of the mutation resulted in the identification of a gene (AT5G18660) that has a sequence similarity to isoflavone reductases. The mutant phenotype was complemented by transformation with the wild-type gene. Recombinant proteins expressed in *E. coli* catalyzed the conversion of divinyl chlorophyllide to monovinyl chlorophyllide and therefore confirmed that the gene encodes a functional DVR. With the identification of *DVR* in *A. thaliana*, we now complete the identification of all the genes involved in chlorophyll biosynthesis in higher plants. We also discuss the evolution of *Prochlorococcus* sp in relation to their acquisition of divinyl-chlorophyll antenna systems.

## RESULTS

### Isolation of a Mutant That Accumulates Divinyl Chlorophylls

Figure 1 shows the last steps of chlorophyll biosynthesis. Because both divinyl and monovinyl types of protochlorophyllide and chlorophyllide can serve as substrates for protochlorophyllide oxidoreductase and chlorophyll synthetase, respectively, the final product of chlorophyll biosynthesis should be divinyl chlorophylls when the plants have a defect in DVR (Figure 1). To obtain divinyl chlorophyll-accumulating mutants, ethyl methanesulfonate–mutagenized seeds were germinated, and their pigment compositions were examined by HPLC according to the method of Zapata et al. (2000). Among 3000 seedlings examined, one line accumulated chlorophylls whose retention

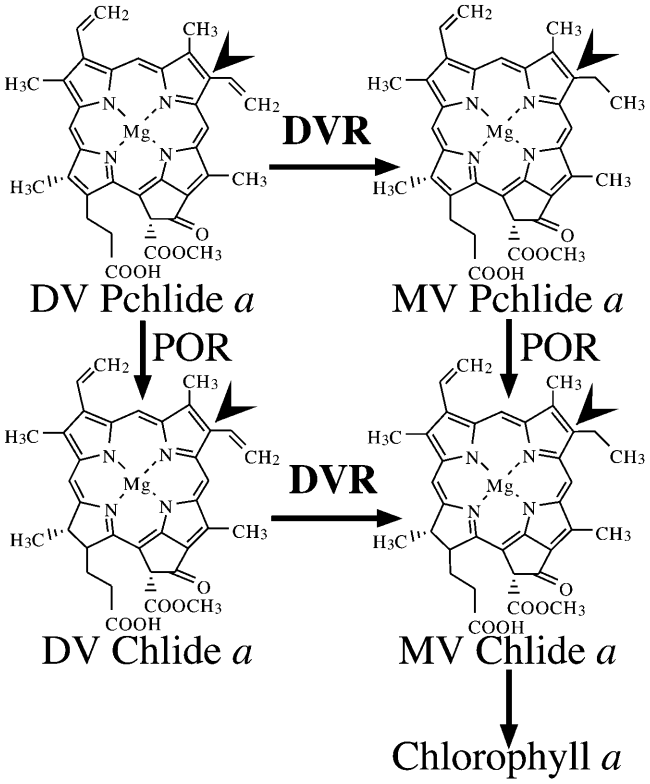



**Figure 1.** Activity of DVR.

DVR catalyzes the conversion of divinyl protochlorophyllide *a* or divinyl chlorophyllide to monovinyl protochlorophyllide *a* or monovinyl chlorophyllide by reduction of the vinyl group at position 8 of the macrocycle to an ethyl group. POR, light-dependent protochlorophyllide oxidoreductase.

times differed from those of monovinyl chlorophylls (Figure 2A) but were identical to those of divinyl chlorophyll *a* and *b* (Zapata et al., 2000). We subsequently determined the absorption spectra of these pigments. One pigment, whose retention time is 12 s fewer than that for monovinyl chlorophyll *a*, had absorbance peaks at 662 and 429 nm (Figure 2B). The Soret band peak shifted to the red by 10 nm compared with monovinyl chlorophyll *a*. Another pigment, whose retention time is close to that of monovinyl chlorophyll *b*, exhibited the same spectrum as monovinyl chlorophyll *b* except for a slight red shift of the Soret band. These chlorophyll spectra of the mutant were identical with those of divinyl chlorophyll *a* and *b* (Shedbalkar and Rebeiz, 1992). Based upon these results, we concluded that this mutant accumulates divinyl chlorophylls instead of monovinyl chlorophylls. However, a small amount of monovinyl chlorophyll (>4% of the total chlorophyll) still remained in this mutant. The mutant is pale green (Figure 2C), and the chlorophyll *a*/*b* ratios were between 6 and 10 depending on the developmental stage and growth conditions (the chlorophyll *a*/*b* ratios of the wild type were between 3 and 3.8). This mutant was capable of photosynthesizing and growing under low-light conditions (70 to 90 μmole $m^{-2}$ $s^{-1}$); however, it rapidly died under high-light conditions (1000 μmole $m^{-2}$ $s^{-1}$) (Figure 2D).

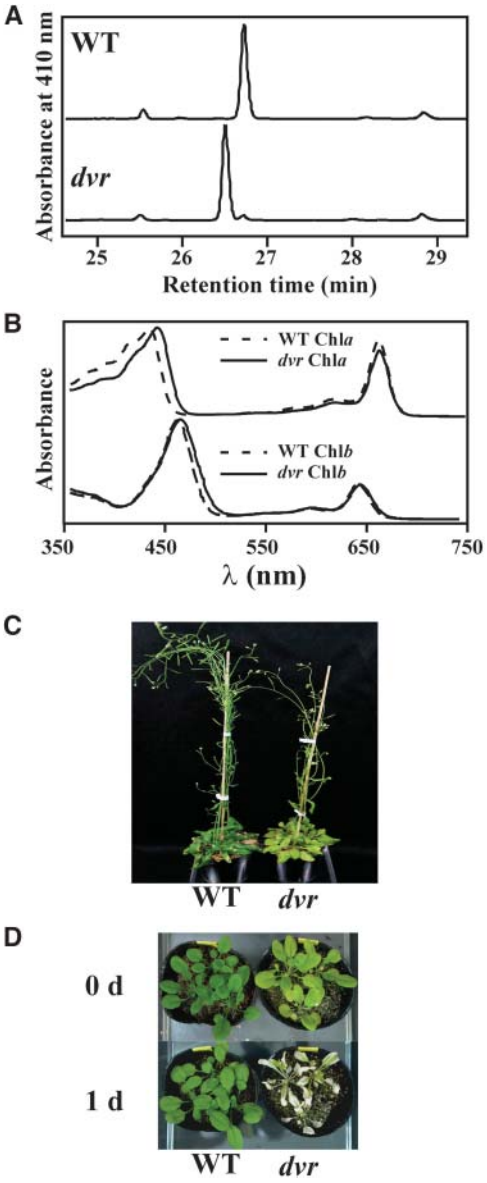


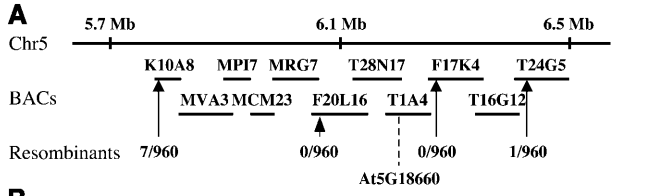

**Figure 2.** Accumulation of Divinyl Chlorophylls in a *dvr* Mutant of *A. thaliana*.

**(A)** HPLC chromatograms of *A. thaliana*.
**(B)** Absorption spectra of monovinyl chlorophyll and divinyl chlorophylls. The spectrum of chlorophylls extracted from the wild type and *dvr* mutant are indicated by the dashed and the solid lines, respectively.
**(C)** Phenotypes of the wild type and the *dvr* mutant. Photographs were taken 7 weeks after sowing.
**(D)** Low-light-grown seedlings were exposed to high light. 0 d, without high-light treatment; 1 d, high-light treatment for 1 d.

### Characterization of the *DVR* Gene

To obtain a fine mapping for the *DVR* gene locus, 960 divinyl chlorophyll-accumulating plants were selected from the F2 progeny of a cross of the *dvr* mutant and the Landsberg *erecta* ecotype. Using simple sequence length polymorphism and cleaved amplified polymorphic sequence makers, we mapped the *dvr* mutation to an interval between markers nga106 and CIW8. We identified 38 plants that exhibited a recombination in this interval. New single nucleotide polymorphism markers were generated from the sequence information from the Monsant Arabidopsis polymorphism collection database, and we confirmed that the *DVR* gene is located on the region covered by the BAC clones shown in Figure 3A. There were four open reading frames (ORFs) encoding putative reductases whose functions were not yet assigned. By sequencing these genes, we finally identified the mutation in AT5G18660 (Figure 3A). The *DVR* point mutation created a single base pair change from C to T at nucleotide 998 in the ORF, which resulted in an amino acid change from Pro to Leu (Figure 3B). Pigment analysis by HPLC showed the residual amount of monovinyl chlorophylls still remained in this mutant. The single amino acid exchange from Pro to Leu might not completely inactivate the protein function.

The *DVR* gene consists of an ORF of 1251 bp and encodes a protein of 417 amino acids. Computer analysis with TargetP (http://www.cbs.dtu.dk/services/TargetP/) predicted a chloroplast transit sequence of 49 amino acids in *DVR*. This is consistent with the fact that all of the enzymes for chlorophyll synthesis have a transit peptide in their sequence and function in chloroplasts. After removal of the transit peptide, the mature size was predicted to be 368 amino acids. BLAST analysis with the full-length *DVR* sequence indicates a significant homology of *DVR* to isoflavone reductases that catalyze the reduction of C-C double bonds. DVR has one putative transmembrene α-helix predicted by the SOSUI program (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html), indicating that the enzymes are located on the membranes. This is consistent with the report that DVR activity was found in membrane fractions.




B

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MSLCSSFNVFASYSPKPKTIFKDSKFISQFQVKSSPLASTFHTNESSTSLKYKRARLKPISS
LDSGISEIATSPSFRNKSPKDINVLVVGSTGYIGRFVVKEMIKRGFNVIAVAREKSGIRGKN
DKEETLKQLQGANVCFSDVTELDVLEKSIENLGFGVDVVVSCLASRNGGIKDSWKIDYEATK
NSLVAGKKFGAKHFVLLSAICVQKPLLEFQRAKLKFEAELMDLAEQQDSSFTYSIVRPTAFF
KSLGGQVEIVKDGKPYVMFGDGKLCACKPISEQDLAAFIADCVLEENKINQVLPIGGPGKAL
TPLEQGEILFKILGREPKFLKVPIEIMDFVIGVLDSIAKIFPSVGEAAEFGKIGRYYAAESM
LILDPETGEYSEEKTPSYGKDTLEDFFAKVIREGMAGQELGEQFF
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**Figure 3.** Positional Cloning of the *DVR* Locus.

**(A)** Fine mapping of the interval containing *DVR*. Number of recombinants are indicated with arrows for specific simple sequence length polymorphism and cleaved amplified polymorphic sequence markers across the interval.
**(B)** Deduced amino acid sequence of *DVR*. The cleavage site of the putative chloroplast-targeting sequence is indicated by an arrowhead. The single base pair point mutation is indicated by an asterisk. Putative transmembrane region predicted by the SOSUI program (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html) is underlined.

### Complementation Analysis

The identity of DVR was subsequently confirmed by complementing the *dvr* with a wild-type 1.2-kb genomic sequence corresponding to the ORF under the control of the 35S promoter of *Cauliflower mosaic virus*. The pale green mutant phenotype was rescued by the genomic DNA fragment in all of the mutants examined (Figure 4A), and the growth rate of the transgenic plants was nearly identical to that of wild-type plants. Furthermore, HPLC analyses and the absorbance spectra of extracted pigments from the plants confirmed that the transgenic plants accumulated monovinyl chlorophylls (Figure 4B).

### Enzymatic Assay of the Proteins

We expressed the *A. thaliana* DVR proteins in *E. coli* and examined whether the recombinant proteins exhibit DVR activity. Divinyl chlorophyllide *a* was chosen as a substrate because of previous biochemical studies that documented enzymatic specificity of DVR for this substrate (Kolossov and Rebeiz, 2001). The recombinant DVR protein was expressed in *E. coli*, and the cell extracts were incubated with divinyl chlorophyllide *a* in the presence of NADPH. Almost all of the divinyl chlorophyllide *a* was converted to monovinyl chlorophyllide *a* after a 10-min incubation (Figure 5), and this result thereby confirms that the *DVR* gene actually encodes 3,8-divinyl protochlorophyllide 8-vinyl reductase.

### Phylogenetic Analysis

Recently, whole genome sequences of *Prochlorococcus* sp and marine *Synechococcus* sp belonging to these two groups became available (Dufresne et al., 2003; Palenik et al., 2003; Rocap et al., 2003), and we examined whether these groups possess *DVR* homologs (Figure 6A). *Synechococcus* sp WH8102 contains a *DVR* gene, but three strains of *P. marinus* lack DVR

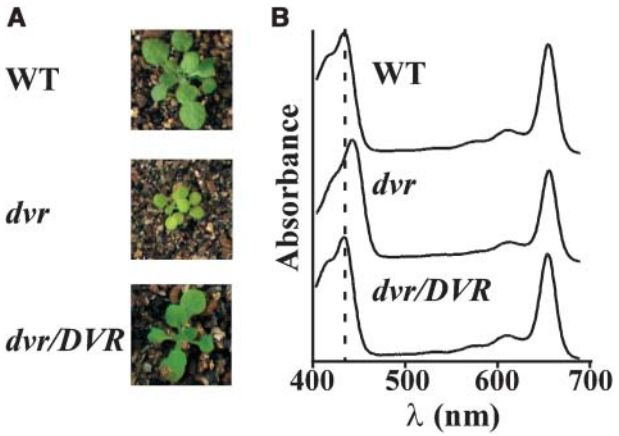


**Figure 4.** Complementation of the *dvr* Mutant by the Wild-Type *DVR* Gene.

**(A)** Phenotypes of the wild type, the *dvr* mutant, and the *dvr/DVR* plants. Photographs were taken 4 weeks after sowing.

**(B)** Absorption spectra of chlorophyll *a* extracted from the wild type, the *dvr* mutant, and the *dvr/DVR* plants.

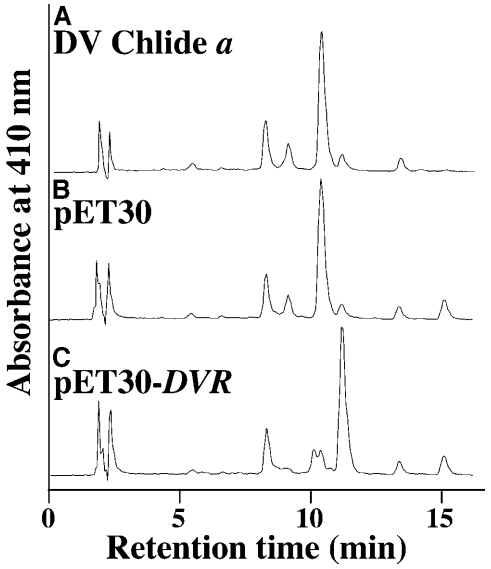


**Figure 5.** Assay of DVR.

Chlorophylls extracted from reaction mixtures were subjected to HPLC as described in Methods.

**(A)** Divinyl chlorophyllide prepared from purified divinyl chlorophyll *a*.

**(B)** Pigments extracted from mixtures reacted with control culture lysate.

**(C)** Pigments extracted from mixtures reacted with culture lysate containing DVR.

(Figure 6A). The physical arrangement of the genes around the *DVR* gene in *Synechococcus* sp WH8102 is shown in Figure 6B. Synteny in gene arrangement is well conserved in the genome of *P. marinus* MIT9313, which is phylogenetically most close to *Synechococcus* sp WH8102, except that the *DVR* gene was missing in that cluster. In addition to the *DVR* gene, some other genes were missing in the clusters of *P. marinus* sp strains SS120 and MED4, but the gene arrangements were partly conserved. This indicates that *Prochlorococcus* sp acquired divinyl chlorophylls by losing the *DVR* gene.

*DVR* genes were found in higher plants and green algae. However, complete genomic sequence data from the unicellular red alga *Cyanidioschyzon merolae*, which accumulates monovinyl chlorophylls, suggested that it lacks *DVR* homologs (Matsuzaki et al., 2004). These data suggest that another type of enzyme is involved in the reduction of the 8-divinyl group in this organism. In cyanobacteria, a *DVR* gene was found in *Synechococcus* sp WH1802 but was not found in other cyanobacterial lineages. In photosynthetic bacteria, *DVR* genes were found in genomes of *Chlorobium tepidum* and *Rhodobacter sphaeroides* but were not found in other photosynthetic bacteria.

## DISCUSSION

### *DVR* Encodes 3,8-Divinyl Protochlorophyllide *a* 8-Vinyl Reductase

It has remained unknown whether one or multiple vinyl reductases are responsible for the reduction of different precursors of

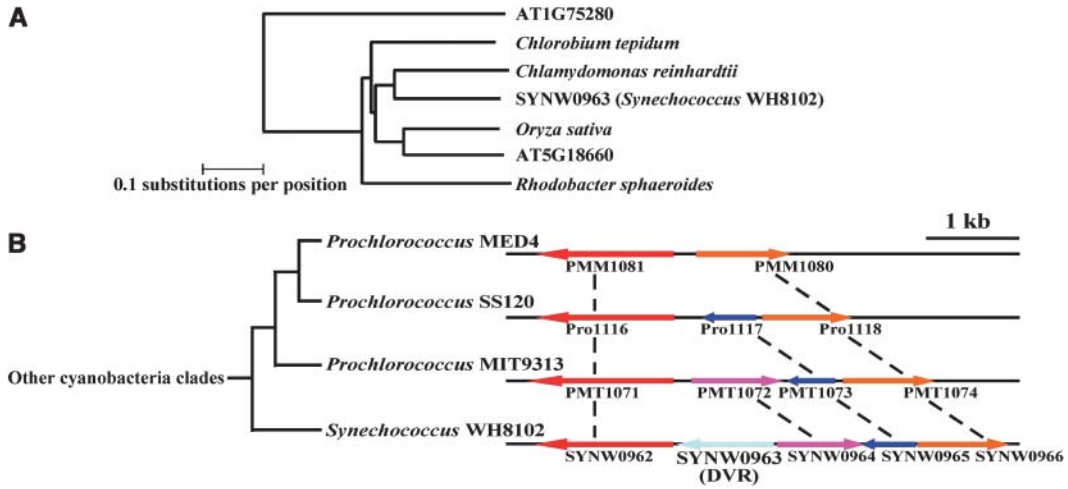


**Figure 6.** Phylogenetic Relationships of *DVR* Homologs.

**(A)** A phylogenetic tree constructed from *DVR* homologs. The rooted neighbor-joining tree was constructed using the isoflavone reductase-like protein (AT1G75280) from *A. thaliana* as an outgroup.

**(B)** Dendrogram depicting phylogenetic relationships of cyanobacteria taxa and ORF matching between *Synechococcus* sp WH8102 and *P. marinus*. The dendrogram was constructed from 16S rDNA sequences according to Ting et al. (2002) and only indicates phylogenetic relationships and does not show the proportion to the expected number of nucleotide substitutions. Dashed lines and same color of arrows indicate significant homology matches between specific ORFs. Arrows show orientation of ORFs. Names of genes are indicated under the arrows.

chlorophylls, such as protochlorophyllide or chlorophyllide. It was reported that divinyl chlorophyllide was efficiently converted to monovinyl chlorophyllide by membrane fraction from cucumber (Parham and Rebeiz, 1992), whereas divinyl protochlorophyllide was not. Another report indicated that the 8-vinyl group of protochlorophyllide was reduced by intact chloroplasts (Tripathy and Rebeiz, 1988). These reports indicated that there are multiple enzymes for the vinyl reduction. By contrast, data obtained from a maize mutant (Bazzaz and Brereton, 1982), which accumulated only divinyl chlorophylls, suggested that a single gene product is responsible for the reduction of the vinyl group of chlorophyllide and protochlorophyllide. However, the possibility that the maize gene encodes a regulator for the divinyl reduction cannot be excluded. In this study, mutant analyses and enzymatic assays with recombinant proteins clearly demonstrated that DVR catalyzes the reduction of the 8-vinyl group of chlorophyll molecules. Furthermore, DVR is encoded by a single-copy gene, and no other homologous genes were found in the *A. thaliana* genome. Considering the accumulation of monovinyl protochlorophyllide in greening tissues (Shioi and Takamiya, 1992), this enzyme might be responsible for the reduction of two different molecules in cells.

## Completion of Identification of the Genes for Chlorophyll Biosynthesis in Higher Plants

Chlorophyll *a* is synthesized from glutamyl-tRNA, and chlorophyll *b* is synthesized from chlorophyll *a* at the last step of chlorophyll biosynthesis (Beale, 1999). All of the genes for the enzymes responsible for chlorophyll biosynthesis in higher plants had been previously identified except for *DVR*. In this report, we succeeded to identify *DVR*, and as a result we have completed the identification of all chlorophyll biosynthetic genes in higher plants (Table 1). There are 15 enzymes and 27 genes required for chlorophyll biosynthesis from glutamyl-tRNA$^{glu}$ to chlorophyll *b* in *A. thaliana*. Among them, hydroxymethylbilane synthase, Mg-protoporphyrin IX monomethylester cyclase, geranylgeranyl reductase, and chlorophyllide *a* oxygenase conduct multistep reactions. Mg-chelatase is the only oligomeric enzyme, being comprised of three proteins, and all others are monomeric enzymes. Nine proteins are encoded by single-copy genes, and the others are encoded by gene families consisting of two to three members.

## Evolution of Divinyl Chlorophyll–Containing Prochlorococcus

Members of the genus Prochlorococcus are the most abundant photosynthetic organisms in the world's oceans (Partensky et al., 1999) and contribute significantly to the global carbon cycle. They have a wide geographical distribution in the oceans within 40°S to 40°N and have a vertical distribution in the water column, ranging from the surface to 100 to 200 m in depth (Partensky et al., 1999). *Prochlorococcus* sp are small unicellular organisms of cyanobacterial lineage, but their antenna systems are different from other cyanobacteria. They contain divinyl chlorophyll *a* and *b*, whereas most cyanobacteria contain monovinyl chlorophyll *a* and phycobilisomes but no chlorophyll *b*. *Prochlorococcus* sp seem to be able to grow photosynthetically over an irradiance range extending over more than three orders of size (Partensky et al., 1999).

**Table 1.** Genes Encoding Enzymes Involved in Chlorophyll Biosynthesis in the Arabidopsis Genome

| Enzyme Name | Gene Name | ORF Name from AGI Groups' Annotations |
|---|---|---|
| Glutamyl-tRNA reductase | *HEMA1* | AT1G58290 |
| | *HEMA2* | AT1G09940 |
| | *HEMA3* | AT2G31250 |
| Glutamate-1-semialdehyde 2,1-aminomutase | *GSA1* (*HEML1*) | AT5G63570 |
| | *GSA2* (*HEML2*) | AT3G48730 |
| 5-Aminolevulinate dehydratase | *HEMB1* | AT1G69740 |
| | *HEMB2* | AT1G44318 |
| Hydroxymethylbilane synthase (porphobilinogen deaminase) | *HEMC* | AT5G08280 |
| Uroporphyrinogen-III synthase | *HEMD* | AT2G26540 |
| Uroporphyrinogen III decarboxylase | *HEME1* | AT3G14930 |
| | *HEME2* | AT2G40490 |
| Coproporphyrinogen III oxidase | *HEMF1* | AT1G03475 |
| | *HEMF2* | AT4G03205 |
| Protoporphyrinogen oxidase | *HEMG1* | AT4G01690 |
| | *HEMG2* | AT5G14220 |
| Magnesium chelatase H subunit | *CHLH* | AT5G13630 |
| Magnesium chelatase I subunit | *CHL11* | AT4G18480 |
| | *CHL12* | AT5G45930 |
| Magnesium chelatase D subunit | *CHLD* | AT1G08520 |
| Magnesium proto IX methyltransferase | *CHLM* | AT4G25080 |
| Mg-protoporphyrin IX monomethylester cyclase | *CRD1* (*ACSF*) | AT3G56940 |
| 3,8-Divinyl protochlorophyllide *a* 8-vinyl reductase | *DVR* | AT5G18660 |
| Protochlorophyllide oxidoreductase | *PORA* | AT5G54190 |
| | *PORB* | AT4G27440 |
| | *PORC* | AT1G03630 |
| Chlorophyll synthase | *CHLG* | AT3G51820 |
| Chlorophyllide *a* oxygenase | *CAO* (*CHL*) | AT1G44446 |

We should note that angiosperms do not have light-independent protochlorophyllide oxidoreductase.

Phylogenetic analysis based on 16S rRNA has shown that *Prochlorococcus* sp are closely related to marine *Synechococcus* sp. These two groups, which belong to the marine picophytoplankton clade, simultaneously diverged, and it is possible that their distinct photosynthetic antenna systems also diverged at the same time. Complete genome sequences have been determined for three strains of Prochlorococcus and one for Synechococcus. Genomic analyses confirmed that the *DVR* gene is present in the *Synechococcus* sp WH8102 genome; however, it is lacking from three *Prochlorococcus* sp genomes. Interestingly, the physical arrangement of flanking genes around *DVR* in the *Synechococcus* sp WH8102 genome is well conserved in *P. marinus*, except the *DVR* gene is lacking (Figure 6). This observation strongly suggests that the progenitor of the genus Prochlorococcus lost the *DVR* gene and acquired divinyl chlorophyll during evolution. There are two hypotheses that may explain the exchange of chlorophyll types during the diversification of these two organisms. Firstly, it is possible that monovinyl chlorophyll was gradually exchanged and replaced by divinyl chlorophyll. In this case, it is suggested that the expression or activity of divinyl reductase gradually decreased during evolution. Secondly, it is possible that all of the monovinyl chlorophyll was simultaneously replaced by divinyl chlorophyll through a mutation or loss of the *DVR* gene. This simultaneous replacement is possible because mutants that accumulate divinyl chlorophyll have been shown to photosynthesize and grow.

There are two defined clades of *P. marinus*, which are adapted to either high- or low-light environments. According to the phylogenic tree constructed by 16S rDNA sequences, low-light-adapted *Prochlorococcus* sp appeared first, and high-light-adapted *Prochlorococcus* arose more recently (Moore et al., 1998). Our observations with the *dvr* mutant fit this hypothesis from the biochemical and physiological points of view. The *dvr* mutant can grow under low-light conditions but rapidly dies under high-light conditions (Figure 2D). These data indicate that divinyl chlorophyll cannot completely substitute for the monovinyl chlorophyll in the preexisting pigment system and that this substitution leads to photodamage under high-light conditions. It would be reasonable to speculate that replacement of monovinyl chlorophyll by divinyl chlorophyll by loss of *DVR* occurred on the lineage to Prochlorococcus in low-light conditions because this might have enabled these organisms to survive with divinyl chlorophyll. In addition, as described above, acquisition of divinyl chlorophyll would be especially beneficial under deep-layer water habitats. After the establishment of antenna systems with divinyl chlorophyll under low-light conditions, it would have been necessary for *Prochlorococcus* sp to adapt to high-light conditions during evolution.

We should note that the presence of a monovinyl chlorophyll *b*-like pigment was reported for *P. marinus* strain CCMP 1375 (Urbach et al., 1998). Considering the absence of *DVR* homologs in its genome as described above, a reexamination of the pigment composition of this strain may be necessary.

### Distribution of *DVR* among Various Photosynthetic Organisms

Phylogenetic trees of the genes for chlorophyll biosynthesis are reflected by the evolution of organisms; however, the *DVR* gene is an exception among chlorophyll biosynthesis genes. *DVR* was found in higher plants and green algae, but no homologs were found in the complete genome sequence of the unicellular red alga *C. merolae* that belong to the eukaryotic photosynthetic organisms. Among cyanobacteria, *Synechocystis* sp PCC6803, *Nostoc* sp PCC7120, and *Gloeobacter violaceus* PCC7421 do not contain *DVR* genes, although all of these organisms synthesize monovinyl chlorophyll *a*. The *bchJ* gene has been reported to encode the DVR activity in *R. capsulatus* (Bollivar et al., 1994). However, its homologs are not found in these genomes either. These observations imply that a third type of DVR protein that is not related to the *A. thaliana* DVR protein nor to the *R. capsulatus* BchJ protein may exist.

Some photosynthetic bacteria, including *R. sphaeroides* and *Chlorobium tepidum*, have *DVR* in addition to *bchJ*. Biochemical studies are required to clarify whether both DVR and BchJ

protein conduct the reduction of divinyl chlorophyll in these organisms.

## METHODS

### Plant Materials

*Arabidopsis thaliana* plants were grown at 23°C with a 16-h photoperiod (70 to 90 μmole $m^{-2}$ $s^{-1}$).

### Analysis of Pigments by HPLC

Chlorophylls and divinyl chlorophylls used for enzymatic assay were extracted from *A. thaliana* wild-type (ecotype Columbia) and *dvr* leaf tissue with 100% acetone, subjected to HPLC on a PREP-ODS column (250 mm long, 20 mm i.d.; Shimadzu, Kyoto, Japan), and eluted with methanol. Elution profiles were monitored by the absorbance at 650 nm. The chlorophyll composition of *A. thaliana* plants and reaction mixtures of the enzymatic assay were analyzed by HPLC on a Symmetry C8 column (150 mm long, 4.6 mm i.d.; Waters, Tokyo, Japan) according to the method of Zapata et al. (2000). Elution profiles were monitored by absorbance at 410 nm.

### Positional Cloning of *DVR*

The homozygous *dvr* mutant obtained by ethyl methanesulfonate treatment was crossed to wild-type Landsberg *erecta*. Genomic DNA from homozygous *dvr* F2 plants was extracted and used for initial mapping using simple sequence length polymorphism and cleaved amplified polymorphic sequence markers evenly distributed over the five chromosomes (http://www.arabidopsis.org/). PCRs were performed with 1 μL of genomic DNA in a 10-μL reaction mixture. The PCR conditions were 94°C for 5 s, 94°C for 30 s, 44 to 56°C (the annealing temperature was optimized for each specific pair of primers) for 30 s, 72°C for 1 min, and finally 72°C for 7 min. The entire PCR product then was separated on a 4% agarose gel. To narrow down the region of the *DVR* locus, new markers were designed based on the insertions/deletions information obtained from the Monsanto Web site (https://www.ncgr.org/cgi-bin/cereon/cereon_login.pl).

### Complementation of the *dvr* Mutant

For complementation of the *dvr* mutation, PCR fragments containing the AT5G18660 gene were amplified using genomic DNA extracted from wild-type Columbia and subcloned into pGreenII-0029 plasmid (Hellens et al., 2000). We incorporated the 35S promoter of *Cauliflower mosaic virus* and the *Tobacco mosaic virus* ω sequence (Y. Niwa, University of Shizuoka) into this vector for efficient overexpression of the transgene. Homozygous *dvr* plants were transformed with *Agrobacterium tumefaciens* strain C58 (GV2260). *A. thaliana* transformants were selected on plates containing half-strength MS medium, 0.7% agarose, and 50 μg $mL^{-1}$ kanamycin (Sigma-Aldrich, St. Louis, MO). The successfully grown transgenic plants were then transferred into soil pots and were subsequently grown with a 16-h photoperiod at 23°C.

### Assay of DVR

Divinyl chlorophyllide *a* that was used as substrate in enzymatic reactions was prepared from purified diviniyl chlorophyll *a* according to the method of Tsuchiya et al. (2003). For the production of DVR recombinant protein, the AT5G18660 gene was subcloned into the pET30 plasmid (Novagen, Madison, WI) between the *Bam*HI and *Not*I sites. A culture of *Escherichia coli* strain (BL21 DE3) containing either AT5G18660 or pET30 plasmid alone was grown overnight in 2 mL of LB broth containing 50 $\mu g^{-1}$ mL of kanamycin for positive and negative controls, respectively. Overnight cultures were subsequently used to inoculate 50 mL of LB broth containing 50 $\mu g^{-1}$ mL of kanamycin. To maximize protein production, isopropylthio-β-galactoside was added to the culture at a final concentration of 0.5 mM after 30 min at 30°C. After 7 h of incubation, the culture was harvested and resuspended with 2.4 mL of solution containing 6.7 $\mu g^{-1}$ mL of lysozyme and 3.3 $\mu g^{-1}$ mL of DNaseI in 50 mM Tris-HCl, pH 8.0. The culture lysate was stored at −20°C until use. DVR activity was assayed according to the method of Kolossov and Rebeiz (2001). The chlorophyll composition of reaction mixtures was analyzed by HPLC according to the method of Zapata et al. (2000).

### Retrieval of Sequences from Databases

The neighborhood search algorithm BLAST (Altschul et al., 1997) was employed for database searches through the National Center for Biotechnology Information, Cyanobase, Department of Energy Joint Genome Institute BLAST Web servers, and a server of the *Rhodobacter sphaeroides* genome project on the Web site of the University of Texas–Houston Health Science Center. The *DVR* homolog of *R. sphaeroides* was retrieved from the server of the *R. sphaeroides* genome project: ZP_00006667. Full-length protein sequences of putative *DVR* homologs from *Synechococcus* sp WH8102 and *Chlorobium tepidum* were obtained from the Cyanobase: SYNW0963 (accession number NP_897056) and CT1063 (accession number NP_661954). The *Chlamydomonas reinhardtii DVR* homolog (C_1330031) was obtained from the Department of Energy Joint Genome Institute BLAST Web server. The *DVR* homolog of rice (*Oryza sativa*) was obtained from the server of the National Center for Biotechnology Information using TBLASTN. *A. thaliana* isoflavone reductase-like protein (AT1G75280) was obtained from the Arabidopsis Information Resouce.

### Phylogenetic Analysis of *DVR* Homologs

The deduced amino acid sequences of *DVR* homologs were trimmed to the predicted isoflavone reductase consensus domain according to the pfam collection (Sonnhammer et al., 1998) and were aligned using the ClustalX algorithm with Gonnet residue weights (Thompson et al., 1997). A neighbor-joining tree (Saitou and Nei, 1987) was constructed based upon the multisequence alignment.

### ORF Matching between Synechococcus and Prochlorococcus ORFs

Predicted amino acid sequences of the genes around a putative *DVR* homolog (SYNW0963) from *Synechococcus* sp WH8102 were obtained from Cyanobase. These sequences were used for the BLAST searches (Altschul et al., 1997) against the protein data from three *Prochlorococcus marinus* genomes (MED4, MIT9313, and SS120). The threshold for reporting a match of a *Synehcococcus* sp WH8102 ORF to a specific *P. marinus* ORF was an expected value of $<E^{-06}$. *P. marinus* sequences with the highest scoring matches to individual *Synechococcus* sp WH8102 ORFs were isolated from the results of BLAST searches. Furthermore, these *P. marinus* sequences were verified by the BLAST searches against the protein data from the *Synechococcus* sp WH8102 genome. All of these sequences showed the expect value of $<E^{-10}$. The location of the *Synechococcus* sp WH8102 and *P. marinus* genes and the orientation of these genes were obtained from Cyanobase.

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