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Evolution of a new chlorophyll metabolic pathway driven by the dynamic changes in enzyme promiscuous activity

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Evolution of a new chlorophyll metabolic pathway

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Abbreviations:

CAO, chlorophyllide *a* oxygenase; Chlide, chlorophyllide; CBR, chlorophyll *b* reductase; DV,

divinyl; DVR, 3,8-divinyl chlorophyllide reductase; *E. coli*, *Escherichia coli*; Fd, ferredoxin; FNR, ferredoxin-$NADP^+$ (oxido)reductase; HCAR, 7-hydroxymethyl chlorophyll *a* reductase; HMChl *a*, 7-hydroxymethyl chlorophyll *a*; HMChlide *a*, 7-hydroxymethyl chlorophyllide *a*; MV, monovinyl; PChlide, protochlorophyllide; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase.

Abstract

Organisms generate an enormous number of metabolites; however, the mechanisms by which a new metabolic pathway is acquired are unknown. To elucidate the importance of promiscuous enzyme activity for pathway evolution, the catalytic and substrate specificity of Chl biosynthetic enzymes were examined. In green plants, Chl *a* and Chl *b* are interconverted by the Chl cycle: Chl *a* is hydroxylated to 7-hydroxymethyl chlorophyll *a* followed by the conversion to Chl *b* and both reactions are catalyzed by chlorophyllide *a* oxygenase. Chl *b* is reduced to 7-hydroxymethyl chlorophyll *a* by Chl *b* reductase and then converted to Chl *a* by 7-hydroxymethyl chlorophyll *a* reductase (HCAR). A phylogenetic analysis indicated that HCAR evolved from cyanobacterial 3,8-divinyl chlorophyllide reductase (DVR), which is responsible for the reduction of an 8-vinyl group in the Chl biosynthetic pathway. In addition to vinyl reductase activity, cyanobacterial DVR also has Chl *b* reductase, HCAR activities; consequently, three of the four reactions of the Chl cycle already existed in cyanobacteria, the progenitor of the chloroplast. During the evolution of cyanobacterial DVR to HCAR, the HCAR activity, a promiscuous reaction of cyanobacterial DVR, became the primary reaction. Moreover, the primary reaction (vinyl reductase activity) and some disadvantageous reactions were lost, but the neutral promiscuous reaction (NADH dehydrogenase) was retained in both DVR and HCAR. We also show that a portion of the Chl *c* biosynthetic pathway already

existed in cyanobacteria. We discuss the importance of dynamic changes in promiscuous activity and of the latent pathways for metabolic evolution.

Keywords: Chlorophyll biosynthesis, Divinyl chlorophyll, Pathway evolution, Promiscuous activity, *Synechocystis*.

Introduction

Metabolism plays an essential role in biological activities by producing various molecules, reducing power and energy. Several hundred thousand secondary metabolites are produced in plants and are involved in protection against environmental stress, such as pathogens, herbivory and ultraviolet radiation (Dixon 2001; Neilson et al. 2013). Indeed, organisms have developed metabolic pathways to produce an enormous number of metabolites (Kliebenstein and Osbourn 2012; Petersen et al. 2009). Although the mechanisms by which a new metabolic pathway is acquired remain unknown, several hypotheses on the evolution of metabolic pathways have been proposed (Fani and Fondi 2009), such as the retrograde hypothesis (Horowitz 1945), the Granick hypothesis and patchwork hypothesis (Jensen 1976).

The patchwork hypothesis is based on gene duplication and the assumption of a broad substrate specificity of the ancestral enzyme and the catalysis of many different but similar reactions. In fact, it has been revealed that, in addition to the primary activity, enzymes have promiscuous and minor activities that are not involved in any cellular processes (Copley 2003; Khersonsky and Tawfik 2010; O'Brien and Herschlag 1998; Rison and Thornton 2002). Such promiscuous enzymes can catalyze the same reaction using a similar substrate and can also catalyze multiple chemical transformations that are classified as different reactions. This idea is supported by the analysis of enzyme superfamilies, showing that 20%

are able to catalyze the reaction of a different EC class (Galperin and Koonin 2012; Schmidt et al. 2003). During evolution, one of these promiscuous activities may become valuable under certain environmental conditions, and selection will favor an increase in the level of this activity. This hypothesis based on enzyme promiscuity may solve Ohno's dilemma that a duplicated gene must be free of inactivating lesions long enough to acquire a new function (Bergthorsson et al. 2007). Nonetheless, the manner in which the primary reaction is substituted by the promiscuous reaction and which environmental and cellular states affect the evolutionary fate of a promiscuous activity are almost completely unknown.

Chl has an essential role in photosynthesis by harvesting light energy and driving electron transfer (Nelson and Yocum 2006). Anoxygenic photosynthetic bacteria contain bacteriochlorophyll *a*, *b*, *c*, *d*, *e* and *g* (Chew and Bryant 2007), and oxygenic phototrophs use Chl *a*, *b*, *c*, *d* and *f* (Chen and Blankenship 2011) for photosynthesis. Contributing to the photosynthetic characteristics of each organism, these Chl species have been acquired by different lineages by adding several steps to the latter part of the core pathway of Chl biosynthesis. For example, the interconversion pathway of Chl *a* and Chl *b,* designated as the Chl cycle (Ito et al. 1996), was acquired by the lineage of green plants (Meguro et al. 2011) via the addition of reactions at the last step of the core pathway, whereas Chl *c* species appeared after secondary endosymbiosis (Sanchez-Puerta et al. 2007) via the addition of several steps at divinyl (DV)-protochlorophyllide (PChlide) *a*. Chl metabolism is a good

model to study pathway evolution because the Chl biosynthetic pathway has been extensively studied and all the enzymes responsible for Chl *a* and Chl *b* biosynthesis in vascular plants and cyanobacteria have been identified (Beale 2005; Kato et al. 2010; Nagata et al. 2005). This knowledge enables the investigation of the evolutionary process of the Chl biosynthetic pathway based on enzymatic and phylogenetic analyses.

Accordingly, to elucidate the dynamic changes in promiscuous activity during enzyme evolution and to evaluate the contribution of promiscuous activity to pathway evolution, we examined the catalytic and substrate specificity of the enzymes of the later stage of Chl biosynthesis, which contribute to the diversity of Chl species. We found that cyanobacterial 3,8-divinyl chlorophyllide reductase (DVR), a Chl biosynthetic enzyme, has many promiscuous activities. One of these promiscuous reactions (7-hydroxymethyl chlorophyll *a* reductase activity) has become the primary reaction of the enzyme and participates in the Chl cycle in green plants; other promiscuous activities have been lost or retained during evolution. We propose that enzymes retain a broad catalytic and substrate specificity if these reactions are not disadvantageous and that this broad specificity of the enzyme is a driving force of pathway evolution.

Results

Reductant and Substrate Specificity of DVRs

Figure 1 shows the Chl biosynthetic pathways of oxygenic photosynthetic organisms. At the later steps of Chl biosynthesis, DV-chlorophyllide (Chlide) *a* is converted to monovinyl (MV)-Chlide *a* by DVR. Two different DVRs have been identified in oxygenic photosynthetic organisms, one of which was initially identified in *Arabidopsis* (Nagata et al. 2005) and is found among most eukaryotic photosynthetic organisms and in a small group of the genus *Synechococcus*. Enzymatic experiments have clarified that *Arabidopsis* DVR uses NADPH as reductant. The other group was identified in *Synechocystis* sp. PCC 6803 (referred to hereafter as *Synechocystis*) (Slr1923) (Islam et al. 2008; Ito et al. 2008) and green sulfur bacteria (BciB) (Liu and Bryant 2011; Saunders et al. 2013), and its ortholog is found among most cyanobacteria. To examine the properties of these two DVRs, we prepared recombinant proteins of *Arabidopsis* DVR (AT5G18660) and cyanobacterial DVR (Slr1923) and determined DVR activity in the presence of NADPH or reduced ferredoxin (Fd) prepared with NADPH, ferredoxin-$NADP^+$ (oxido)reductase (FNR) and Fd (Fig. 2). *Arabidopsis* DVR showed high activity when NADPH was used as the reductant (sample 5), whereas recombinant Slr1923 required Fd (sample 4). Based on these enzymatic properties, we refer to the *Arabidopsis*-type DVR as N-DVR (NADPH-dependent DVR) and to the Slr1923-type as F-DVR (Fd-dependent DVR).

We next examined the substrate specificity of *Arabidopsis* N-DVR and *Synechocystis* F-DVR (Fig. 2). As we reported previously (Nagata et al. 2005), *Arabidopsis* N-DVR

catalyzes a reaction using DV-Chlide *a* (sample 5), but the activity is very low when DV-Chl *a* is used as a substrate (sample 9). Although we detected no activity when DV-PChlide was used (sample 13), it was reported that *Arabidopsis* DVR reduced a small amount of DV-PChlide after a 10-h incubation (Wang et al. 2013), a discrepancy that may be due to the different experimental conditions. In contrast, cyanobacterial F-DVR exhibited a broad substrate specificity and converted DV-PChlide *a* (sample 12), DV-Chlide *a* (sample 4) and DV-Chl *a* (sample 8) to the respective monovinyl molecules. These results indicate that F-DVR has a broad substrate specificity, whereas N-DVR has a high specificity for DV-Chlide *a*.

A Portion of the Reactions of the Chl Cycle Evolved from a Promiscuous Activity of F-DVR

Eukaryotic green plants contain Chl *b* as a photosynthetic pigment, which is synthesized from Chl *a* via 7-hydroxymethyl chlorophyll (HMChl) *a* by Chlide *a* oxygenase (CAO) (Tanaka et al. 1998; Tomitani et al. 1999). Chl *b* is reduced to HMChl *a* by Chl *b* reductase (CBR) (Ito et al. 1996; Kusaba et al. 2007) and then converted to Chl *a* by HMChl *a* reductase (HCAR) (Meguro et al. 2011). In this process, HCAR catalyzes the dehydroxylation of the $–CH_2OH$ at the C7 position to $–CH_3$. This inter-conversion pathway of Chl *a* and Chl *b* is referred to as the Chl cycle, which plays a crucial role in Chl

degradation during senescence and in light environment acclimation (Tanaka and Tanaka 2011). The Chl cycle exists in the chloroplasts of green algae and vascular plants, but HCAR of the Chl cycle shows high sequence identity to cyanobacterial F-DVR (Supplementary Fig. S1). A phylogenetic analysis showed that HCAR branched off within the F-DVR cluster (Fig. 3), indicating that HCAR evolved from F-DVR (Meguro et al. 2011). However, the catalytic reactions of these two enzymes are largely different. Thus, to elucidate how F-DVR has evolved to HCAR, we examined the enzymatic activity of F-DVR and HCAR, including their promiscuous activities. When 7-hydroxymethyl chlorophyllide (HMChlide) *a* was incubated with F-DVR, Chlide *a* was generated (Fig. 4A, sample 4, 4B), indicating that F-DVR has HCAR activity as a promiscuous activity. However, this promiscuous activity (HCAR activity) was extremely low compared to the primary catalytic activity (DVR activity) and to the HCAR activity of *Arabidopsis* HCAR. To verify the promiscuous activity of F-DVR, a lysate of *Escherichia coli* (*E. coli*) expressing recombinant F-DVR was used for the enzymatic analysis (Fig. 4C, sample 4). Interestingly, in addition to Chlide *a*, a low but significant level of Chlide *b* was found in the reaction mixture (Fig. 4A, sample 4 and 4C, sample 4), indicating that F-DVR can convert HMChlide *a* to Chlide *b*, the reverse reaction of Chl *b* reductase. When HMChlide *a* was incubated with F-DVR without a reductant, the level of Chlide *a* became very low, and Chlide *b* increased (Fig. 4C, sample 8). This result is reasonable because the conversion of HMChlide *a* to Chlide *a* is a reductive

reaction and the conversion of HMChlide *a* to Chlide *b* is an oxidative reaction. When Chlide *b* was incubated with the *E. coli* lysate containing F-DVR, HMChlide *a* and Chlide *a* accumulated (Fig. 4C, sample 6). The extracts from *E. coli* had no promotive effect on F-DVR activities (Supplementary Fig. S2). F-DVR activities might be partly lost during purification. In contrast, F-DVR did not catalyze the conversion of Chlide *a* to HMChlide *a* in the Chl cycle (Supplementary Fig. S3). These observations indicate that F-DVR can catalyze the interconversion of Chlide *b* and HMChlide *a* and the conversion of HMChlide *a* to Chlide *a*; therefore, three of the four reactions of the Chl cycle can be catalyzed by cyanobacterial F-DVR (Table 1). In contrast, HCAR has no DVR activity (Supplementary Fig. S4, sample 1). This result is supported by the previous observation that the *Arabidopsis dvr* mutant accumulated only DV-Chl, in spite of the presence of HCAR (Nagata et al. 2005). HCAR has also no catalytic activity to convert HMChlide *a* to Chlide *b* or Chlide *b* to HMChlide *a* (Supplementary Fig. S4, sample 2, 3). Thus, HCAR evolved from F-DVR via an increase in its promiscuous HCAR activity and a decrease in its DVR and other promiscuous activities.

During the course of these experiments, we identified an NADH dehydrogenase activity of F-DVR when ferricyanide was used as an oxidant. The *K*m values of purified F-DVR and HCAR for the oxidation of NADH were calculated from double reciprocal plots of three independent experiments as 127±35 and 148±70 μM, respectively. However,

NADPH dehydrogenase activity was not detected under any other conditions tested. This promiscuous activity has not changed much during the evolution of F-DVR to HCAR. The deduced amino acid sequence of F-DVR shows homology to that of FpoF protein of the NDH1 complex, which is involved in the oxidation of $F_{420}H_2$ (Islam et al. 2008). Thus, it might be possible that F-DVR can utilize NADH instead of $F_{420}H_2$ (Prommeenate et al. 2004). However, *Synechocystis* F-DVR did not use NADH as a reductant for the reduction of DV-Chlide *a* (Supplementary Fig. S5).

The Chl c_2 to Chl c_1 Conversion Was Pre-existent in Cyanobacteria as Promiscuous Activity of F-DVR

Chromists and dinoflagellates use Chl *c* as a major light-harvesting pigment; however, the enzymatic mechanism of Chl *c* biosynthesis remains completely unknown, and no experimental evidence has been reported for the pathway of Chl *c* biosynthesis (Green 2011). The possible pathway is that Chl c_2 is synthesized from DV-PChlide *a* by the dehydrogenation of 17-propionate to an acrylic moiety and that Chl c_1 is synthesized by the reduction of the 8-vinyl group of Chl c_2 or Chl c_1 is synthesized from MV-PChlide by the dehydrogenase (Fig. 1), which suggests the involvement of DVR in Chl *c* biosynthesis. Either F-DVR or N-DVR is found in most photosynthetic organisms, yet both F-DVR (Supplementary Fig. S1 and Fig. 3) and N-DVR exist in the genome of diatoms that contain

Chl *a* and Chl c_1. Thus, to investigate the function of these two DVRs, *Phaeodactylum tricornutum* N-DVR was expressed in *E. coli*, and its catalytic properties were examined. Diatom N-DVR could not convert DV-PChlide *a* to MV-PChlide *a* (Supplementary Fig. S6, sample 3), as observed with the N-DVRs of other organisms. Interestingly, diatom N-DVR can efficiently utilize both DV-Chl and DV-Chlide (Supplementary Fig. S6, sample 1, 2), different from other N-DVRs (Fig. 2, sample 5, 9), though an in vitro experiment showed that diatom N-DVR does not convert Chl c_2 to Chl c_1 (Fig. 5, sample 3). These results indicate that diatom N-DVR has evolved to function in Chl *a* biosynthesis and suggest that diatom F-DVR is responsible for the biosynthesis of Chl c_1 from Chl c_2. To examine this possibility, we attempted to measure diatom F-DVR activity. However, active diatom F-DVR could not be expressed in *E. coli* despite extensive efforts. Therefore, we used cyanobacterial F-DVR instead of diatom F-DVR to examine whether the enzyme can convert Chl c_2 to Chl c_1: cyanobacterial F-DVR was incubated with Chl c_2, and the reaction mixture was subjected to HPLC. Chl c_2 was efficiently converted to Chl c_1 (Fig. 5, sample 4), suggesting that diatom F-DVR can also convert Chl c_2 to Chl c_1. These results suggest that Chl c_2 to Chl c_1 conversion already existed in cyanobacteria due to the broad substrate specificity of F-DVR.

Discussion

Pre-existence of the Partial Chl Cycle and Chl c_1 Biosynthesis Pathway as Promiscuous Reactions in Cyanobacteria

Chl *b* is a photosynthetic pigment of the peripheral antenna systems of green algae and plants, and Chl *a* and Chl *b* is interconverted by the Chl cycle. HCAR in the Chl cycle shows a high sequence homology to F-DVR, and our phylogenetic tree clearly shows that HCAR evolved from F-DVR. However, the catalytic properties of these enzymes are largely different. Cyanobacterial F-DVR has a broad substrate specificity; in addition to a DVR activity (reduction of $–CH=CH_2$ to $–CH_2CH_3$), the enzyme also has HCAR activity (dehydroxylation of $–CH_2OH$ to $-CH_3$), though the activity is low (Fig. 4). Interestingly, F-DVR has other catalytic activities, such as the interconversion of HMChlide *a* and Chlide *b* and conversion of HMChlide *a* to Chlide *a* (Fig. 4), indicating that the Chl cycle partially exists in cyanobacteria. It cannot be excluded that CAO and F-DVR tentatively formed the Chl cycle in the early phase of evolution, with F-DVR then being substituted by CBR and HCAR to generate the present Chl cycle.

Most photosynthetic organisms contain either N-DVR or F-DVR. One exception is the diatom, which contains both N-DVR and F-DVR. Based on the enzymatic experiments using diatom N-DVR and cyanobacterial F-DVR, Chl biosynthesis in diatoms can be proposed as follows. First, an unidentified dehydrogenase converts DV-PChlide *a* to Chl c_2, and an 8-vinyl group of Chl c_2 is reduced to Chl c_1 by F-DVR. An alternative pathway is the

reduction of DV-PChlide *a* to MV-PChlide *a* by F-DVR followed by the conversion to Chl c_1. For the synthesis of Chl *a*, DV-PChlide is converted to DV-Chlide *a* by PChlide oxidoreductase, and a vinyl group of DV-Chlide *a* or DV-Chl *a* is then reduced to MV-Chlide *a* or MV-Chl *a* by N-DVR. Arguably, it is reasonable for diatoms to possess two different DVRs because these organisms must differently regulate Chl c_1 and Chl *a* synthesis. Chl c_1 synthesis might be regulated by F-DVR and Chl *a* by N-DVR, enabling the fine regulation of the Chl c_1/Chl c_2 ratio, regardless of the Chl *a* synthesis rate.

Our enzymatic experiments of the Chl cycle and Chl *c* biosynthesis indicated that 3 of the 4 reactions of the Chl cycle and Chl c_2 to Chl c_1 conversion activity already exist in cyanobacteria, the progenitor of the chloroplast. It should be noted that, although these metabolic pathways potentially existed in the progenitor, the pathways are not functional due to a lack of substrate molecules. However, the pre-existence of a latent metabolic pathway is a strong driving force of metabolic evolution. When the progenitor acquired Chl *b*, the Chl cycle was immediately formed because of the presence of F-DVR. When the unidentified dehydrogenase was obtained, both Chl c_1 and Chl c_2 could be synthesized using the pre-existing F-DVR. This idea is consistent with the report that, when *Coptis japonica* scoulerine-9-O-methyltransferase was introduced to *Eschscholzia californica*, the reaction product of this enzyme was synthesized and was further converted to novel molecules by endogenous enzymes (Takemura et al. 2010), indicating a latent metabolic pathway.

Catalytic Promiscuity and Enzyme Evolution

It has been suggested that promiscuous enzyme activity plays an essential role in pathway evolution (Copley 2012; James and Tawfik 2001; Nam et al. 2012). Indeed, a promiscuous activity becomes a major activity when the activity plays an important role in a new environment during evolution (Deng et al. 2010; Hackenberg et al. 2011). In contrast, an enzyme may lose its major activity when the activity is not beneficial or becomes disadvantageous. In addition, some neutral promiscuous activities may be retained for other reasons during the long evolutionary process. Although this idea appears reasonable and can explain how a new pathway appears, there is no report clearly demonstrating the fate of promiscuous enzyme activity during evolution. Nonetheless, our detailed enzymatic study of cyanobacterial F-DVR and *Arabidopsis* HCAR partly elucidated the dynamic changes in individual promiscuous activities during evolution (Fig. 6).

In addition to DVR activity, cyanobacterial F-DVR has many promiscuous activities as discussed previously. Except for the major (DVR) and NADH dehydrogenase activities, most of the activities are neutral for cyanobacteria, as the cells lack the substrates, such as Chl *b*, Chl c_2 and HMChl *a*, for these activities. The presence of some of these activities (HCAR, Chl c_1 synthesis and NADH dehydrogenase activities) in both chloroplasts and cyanobacteria indicates the presence of these promiscuous activities in the common

progenitor. Other promiscuous activities might also exist in the progenitor, although we have no direct evidence for it. Therefore, the question arises as to why has cyanobacterial F-DVR retained so many nonfunctional activities over such long evolutionary time. One possible explanation is that the increase in catalytic specificity resulted in a decrease in the catalytic rate of the primary activity (van Loo et al. 2010), which is well characterized for Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco). Rubisco catalyzes the incorporation of CO_2 into ribulose 1,5-bisphosphate, the first step in the production of carbohydrates by plants. However, Rubisco cannot completely discriminate between O_2 and CO_2 due to their electrostatic similarity, resulting in the unwanted oxygenation of ribulose 1,5-bisphosphate (photorespiration). Enzymatic studies of various Rubisco enzymes have shown that higher specificities for CO_2 over O_2 are accompanied by lower carboxylation rates (Whitney et al. 2011). Alkaline phosphatases and arylsulfatases are evolutionarily related, and *E. coli* alkaline phosphatase was found to have a low level of sulfatase activity (O'Brien and Herschlag 1999; O'Brien and Herschlag 1998), and amino acid substitution of the phosphatase decreased both the sulfatase and phosphatase activities. These results indicate that an increase in catalytic specificity is occasionally accompanied by a decrease in the catalytic rate of the primary reaction, a situation that can enable the promiscuous activity to remain over a long period of time.

In the next evolutionary stage, the DVR activity of F-DVR disappeared, and the HCAR

activity increased, resulting in the appearance of HCAR. However, it is unclear why HCAR needed to lose its DVR activity. Modern green plants employ N-DVR for the reduction of the 8-vinyl group. Additionally, it is well known that Chl biosynthesis must be strictly regulated to avoid photodamage. However, Chl biosynthesis would not be finely controlled if two different DVR enzymes were present because DVR and HCAR are regulated differently: N-DVR must be functional during Chl synthesis in the greening phase, and HCAR functions mainly during senescence. Thus, the DVR activity of HCAR was disadvantageous for the plant, and HCAR had to lose its DVR activity, a notion that is supported by the fact that most photosynthetic organisms contain only one type of DVR. The minor promiscuous activity of F-DVR, the interconversion of Chl *b* and HMChl *a*, has also disappeared (Supplementary Fig. S4). CBR is responsible for Chl *b* to HMChl *a* conversion in modern green plants, and the interconversion of Chl *b* and HMChl *a* by HCAR would disturb the Chl cycle. Furthermore, it should be noted that all the enzymes of the Chl biosynthetic pathway are irreversible, which might be important for the fine regulation of Chl biosynthesis. In this sense, the involvement of a reversible enzyme (F-DVR) in the Chl cycle must be avoided. The question arises as to why F-DVR has not evolved to CBR. One possible reason for it is that F-DVR could not acquire a sufficient catalytic activity compared to NYC1 and NOL. Another reason is that present CBR (NOL and NYC1) have other important function besides CBR activities such as the direct interaction with

light-harvesting Chl-protein complex (Shimoda et al. 2012) which could not be achieved by F-DVR.

Conversely, NADH dehydrogenase activity has been retained during the evolution from F-DVR to HCAR, though it is not clear whether the NADH dehydrogenase activity of F-DVR and HCAR participate in some cellular process. Furthermore, the NADH dehydrogenase activity of these enzymes is not related to Chl metabolism, which might be the reason why the NADH dehydrogenase activity has been retained over such long evolutionary time during which a new Chl metabolic pathway appeared. In the present study, we showed that F-DVR has many promiscuous activities involving different chemical reactions and different substrates. Accordingly, it is reasonable to assume that F-DVR has additional promiscuous activities that have not yet been identified. A large number of promiscuous reactions form many latent metabolic pathways, some of which might have become functional during evolution.

In summary, enzymes have many promiscuous activities, and some neutral activities are retained to maintain a high activity of the primary reaction. However, when a promiscuous activity becomes disadvantageous during evolution, the enzyme loses this activity; in contrast, when a promiscuous activity becomes important under certain environmental or cellular conditions, this activity will become the primary activity. These dynamic changes in primary and promiscuous activities drive pathway evolution.

Materials and Methods

Expression and Purification of Recombinant DVR

The coding regions of F-DVR derived from *Synechocystis* (Slr1923) and N-DVR derived from *Phaeodactylum tricornutum* (XP_002184654) were cloned into pET-30a(+) (Novagen) using the *Nde*I and *Xho*I sites. The coding region of N-DVR from *Arabidopsis* (AT5G18660) was cloned into pCold ProS2 (Takara) at the *Nde*I and *EcoR*I sites. The primers used in the construction are listed in Supplementary Table S1. The expression plasmids were introduced into *E. coli* BL21 (DE3). DVR cloned into pET-30(a)+ was expressed as reported previously (Shimoda et al. 2012). DVR cloned into pCold ProS2 was expressed at 15°C for 24 h with 0.4 mM isopropyl-β-thiogalactopyranoside. During the induction of *Synechocystis* F-DVR and *Arabidopsis* HCAR, 100 μM ammonium ferric citrate was added into the medium. After incubation, the culture was harvested by centrifugation, and the collected cells were resuspended in buffer (25 mM Tris-HCl, pH 7.5 and 150 mM NaCl) and disrupted by sonication. The recombinant protein contained in the soluble fraction was applied to a nickel column (HisTrap HP, GE Healthcare), and the recombinant protein was eluted with a buffer containing 500 mM imidazole. The imidazole was removed from the purified DVR protein using a desalting column (HiTrap Desalting, GE Healthcare). *Arabidopsis* N-DVR was further purified using an ion exchange column: the recombinant protein was applied to an

ion exchange column (HiTrap DEAE FF, GE Healthcare) equilibrated with 25 mM Tris-HCl (pH 7.5) buffer. The column was eluted with a linear gradient of the buffer containing 0.5 M NaCl for 5 min at a flow rate of 1 mL min^{-1}. The column chromatography for protein purification was performed using an ÄKTAprime plus system (GE Healthcare). When the *E. coli* lysate was used as the source of the enzyme, 30 mL of the culture was resuspended in 1 mL of BugBuster Protein Extraction Reagents (Novagen) containing 1 μL benzonase. HCAR was prepared as reported previously (Shimoda et al. 2012). The purified protein and *E. coli* lysate were analyzed by SDS-PAGE (Supplementary Fig. S7) as reported previously (Meguro et al. 2011).

Preparation of Chl Derivatives

DV-Chl was extracted from a slr1923-deficient *Synechocystis* mutant (Ito et al. 2008). HMChl *a* was obtained via the reduction of Chl *b* with $NaBH_4$, as reported previously (Shimoda et al. 2012). Chlide was prepared from Chl through hydrolysis with recombinant chlorophyllase (Tsuchiya et al. 1999), as reported previously (Shimoda et al. 2012). PChlide was synthesized by the chemical oxidation of Chlide with 2,3-dichloro-5,6-dicyanobenzoquinone (Shedbalkar et al. 1991). Chlide was solubilized in 200 μL diethyl ether, and 1 μL of 100 mM 2,3-dichloro-5,6-dicyanobenzoquinone solubilized in acetone was added. After 5 min, the reaction was stopped by the addition of 1

mL of water. The diethyl ether phase was washed with water several times to remove the unreacted 2,3-dichloro-5,6-dicyanobenzoquinone, and the diethyl ether was evaporated using nitrogen gas. Chl c_2 was prepared from dinoflagellates (a kind gift from Prof. T. Horiguchi, Hokkaido University). A Chl c_2 and Chl c_1 mixture was prepared from *Phaeodactylum tricornutum*, and Chl *c* was extracted with acetone from the harvested cells. Hexane was added to the acetone mixture to remove the hydrophobic Chl *a*. The Chl *c* contained in the aqueous phase was transferred to diethyl ether, and the diethyl ether was evaporated using nitrogen gas.

Enzyme Assay

Purified recombinant DVR (10 μg) or a culture lysate containing expressed DVR (10 μL) was suspended in 50 μL reaction buffer (25 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% Triton X-100 and 1 mM NADPH). For the analysis of *Synechocystis* F-DVR and HCAR, 1 μL of spinach FNR (0.1 mg mL^{-1}, Sigma-Aldrich) and 1 μL of spinach Fd (1 mg mL^{-1}, Sigma-Aldrich) were added to the reaction buffer. The pigments were solubilized in acetone, and 1 μL of the solution was added to the reaction buffer. PChlide (150 pmol), Chl c_2 (300 pmol) and other Chl derivatives (500 pmol) were used for every reaction. The reaction mixtures were incubated for 15 min at 25°C and stopped by the addition of 200 μL of acetone. The pigments were analyzed by HPLC using a C8 column, as reported previously

(Shimoda et al. 2012). When the culture lysate containing *Synechocystis* F-DVR was incubated with HMChlide *a* or Chlide *b*, the concentration of NADPH, FNR and Fd was four times higher than the standard conditions, and the reaction mixtures were incubated for 60 min.

Sequence Analysis and Phylogenetic Analysis of DVR

A database search was performed using the National Center for Biotechnology Information database. The amino acid sequences were aligned using the ClustalW program (Thompson et al. 1994) in the BioEdit program (http://www.mbio.ncsu.edu/bioedit/bioedit.html). For the phylogenetic analysis, the protein sequences were aligned using MEGA 5 software (Tamura et al. 2011), and the maximum likelihood bootstrap method (1,000 replicates).

NADH Dehydrogenase Activity Assay

The NADH dehydrogenase activities of *Synechocystis* F-DVR and HCAR were measured in the presence of 1 mM potassium ferricyanide using 10 μg mL^{-1} purified *Synechocystis* F-DVR or HCAR. The decrease in absorbance at 340 nm was monitored with a spectrophotometer (U-3310, Hitachi).

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Disclosures

Conflicts of interest: No conflicts of interest declared.

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Table 1. Summary of various DVR properties.

	Reductant		Vinyl group			Hydroxymethyl group		Formyl group
			Reduction			Reduction	Oxidation	Reduction
	NADPH	Fd	DV-Chl(ide) *a*	DV-PChlide *a*	Chl c_2	HMChlide *a*		Chlide *b*
SyF-DVR	X	O	O	O	O	O	O	O
AtN-DVR	O	n.d.	O	X	n.d.	n.d.	n.d.	n.d.
PhaeN-DVR	O	n.d.	O	X	X	n.d.	n.d.	n.d.
HCAR	X[a]	O	X	n. d.	n.d.	O	X	X

The properties of purified recombinant *Synechocystis* F-DVR (SyF-DVR), *Arabidopsis* N-DVR (AtN-DVR), *Phaeodactylum* N-DVR (PhaeN-DVR) and *Arabidopsis* HCAR are summarized.

[a], (Meguro et al. 2011). n.d., not determined.

Legends to figures

Figure 1. Chl metabolic pathway.

The core pathway of Chl synthesis shared (black arrows), the Chl cycle in green plants (light blue arrows) and Chl *c* synthesis pathway in diatom (red arrows) are shown. Dashed circles indicate the reaction site. 8-vinyl reductase and 17^1 oxidase are hypothetical enzymes. POR; PChlide oxidoreductase, Chl syn; Chl synthase.

Figure 2. Substrate specificity of DVR.

Chl derivatives were incubated with purified recombinant *Synechocystis* F-DVR (SyF-DVR) or *Arabidopsis* N-DVR (AtN-DVR), and the pigment compositions were analyzed using HPLC. 1, MV-Chlide *a*. 2, DV-Chlide *a*. 3, DV-Chlide *a* incubated with SyF-DVR and NADPH. 4, DV-Chlide *a* incubated with SyF-DVR, NADPH, FNR and Fd. 5, DV-Chlide *a* incubated with AtN-DVR and NADPH. 6, MV-Chl *a*. 7, DV-Chl *a*. 8, DV-Chl *a* incubated with SyF-DVR, NADPH, FNR and Fd. 9, DV-Chl *a* incubated with AtN-DVR, NADPH. 10, MV-PChlide *a*. 11, DV-PChlide *a*. 12, DV-PChlide *a* incubated with SyF-DVR, NADPH, FNR and Fd. 13, DV-PChlide *a* incubated with AtN-DVR, NADPH.

Figure 3. Phylogenetic tree of F-DVR and HCAR.

Phylogenetic maximum likelihood tree was constructed with the translated sequence of HCAR and DVR. The bootstrap values for each clade are indicated on each node, and the scale bar indicates the number of amino acid substitutions per site. The F subunit of *Methanosaeta* $F_{420}H_2$ dehydrogenase was used as an outgroup. *Arabidopsis thaliana*

(accession No. NP_171956); *Chlamydomonas reinhardtii* (accession No. XP_001699546); *Chloroherpeton thalassium* ATCC 35110 (accession No. YP_001996119); *Gloeobacter violaceus* PCC 7421 (accession No. NP_923824); *Methanosaeta thermophila*_PT (accession No. YP_842613); *Phaeodactylum tricornutum* CCAP 1055/1 (accession No. XP_002186085); *Physcomitrella patens* subsp. patens (accession No. XP_001770443); *Rhodopseudomonas palustris* BisA53 (accession No. YP_780232) *Synechocystis* sp. PCC 6803 (accession No. NP_441896); *Thalassiosira pseudonana* CCMP1335 (accession No. XP_002288079).

Figure 4. Various activities of *Synechocystis* F-DVR.

A, Various activities of *Synechocystis* F-DVR (SyF-DVR) analyzed using purified recombinant SyF-DVR. After incubation, the pigment compositions were analyzed by HPLC. The absorption spectra corresponding to peak 1 and peak 2 are shown in B. 1, Chlide *a*. 2, Chlide *b*. 3, HMChlide *a*. 4, HMChlide *a* and SyF-DVR incubated with NADPH, FNR and Fd. 5, Chlide *b* and SyF-DVR incubated with NADPH, FNR and Fd. 6, HMChlide *a* and SyF-DVR incubated without any reductant. B, Analysis of pigments accumulating after incubation of HMChlide *a* with SyF-DVR. Absorption spectra corresponding to peak 1 and peak 2 in A and peak 3 and peak 4 in C were recorded with a photodiode array detector. C, Various activities of SyF-DVR analyzed using a culture lysate of *E. coli* containing SyF-DVR. After incubation, the pigment compositions were analyzed by HPLC. A culture lysate of cells containing the empty pET vector was used for the negative control. 1, Chlide *a*. 2, Chlide *b*. 3, HMChlide *a*. 4, HMChlide *a* and the culture lysate containing SyF-DVR incubated with NADPH, FNR and Fd. 5, HMChlide *a* and the culture lysate containing empty vector incubated with NADPH, FNR and Fd. 6, Chlide *b* and the culture lysate containing SyF-DVR

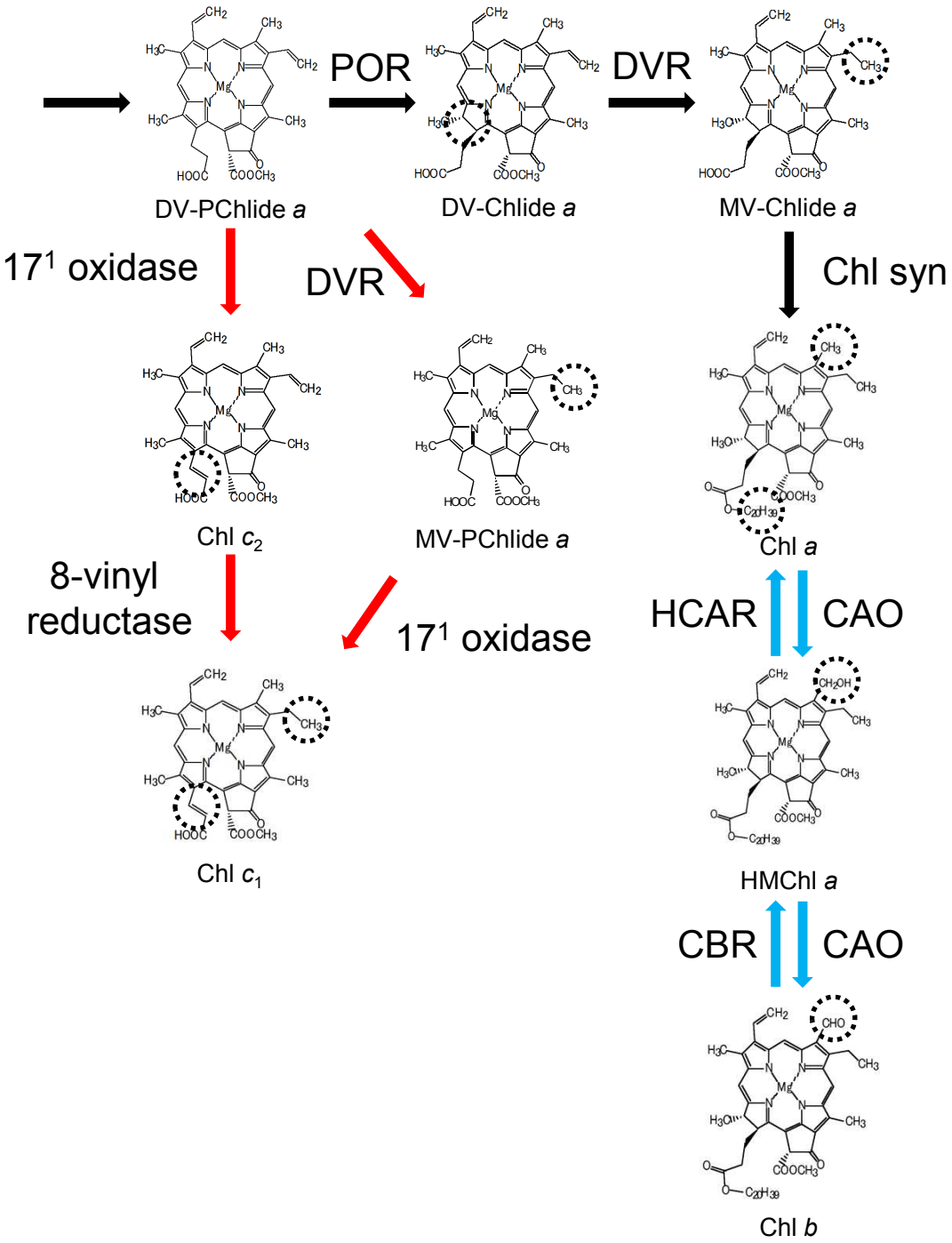
incubated with NADPH, FNR and Fd. 7, Chlide *b* and the culture lysate containing empty vector incubated with NADPH, FNR and Fd. 8, HMChlide *a* and the culture lysate containing SyF-DVR incubated without any reductant. 9, Chlide *b* and the culture lysate containing SyF-DVR incubated without any reductant.

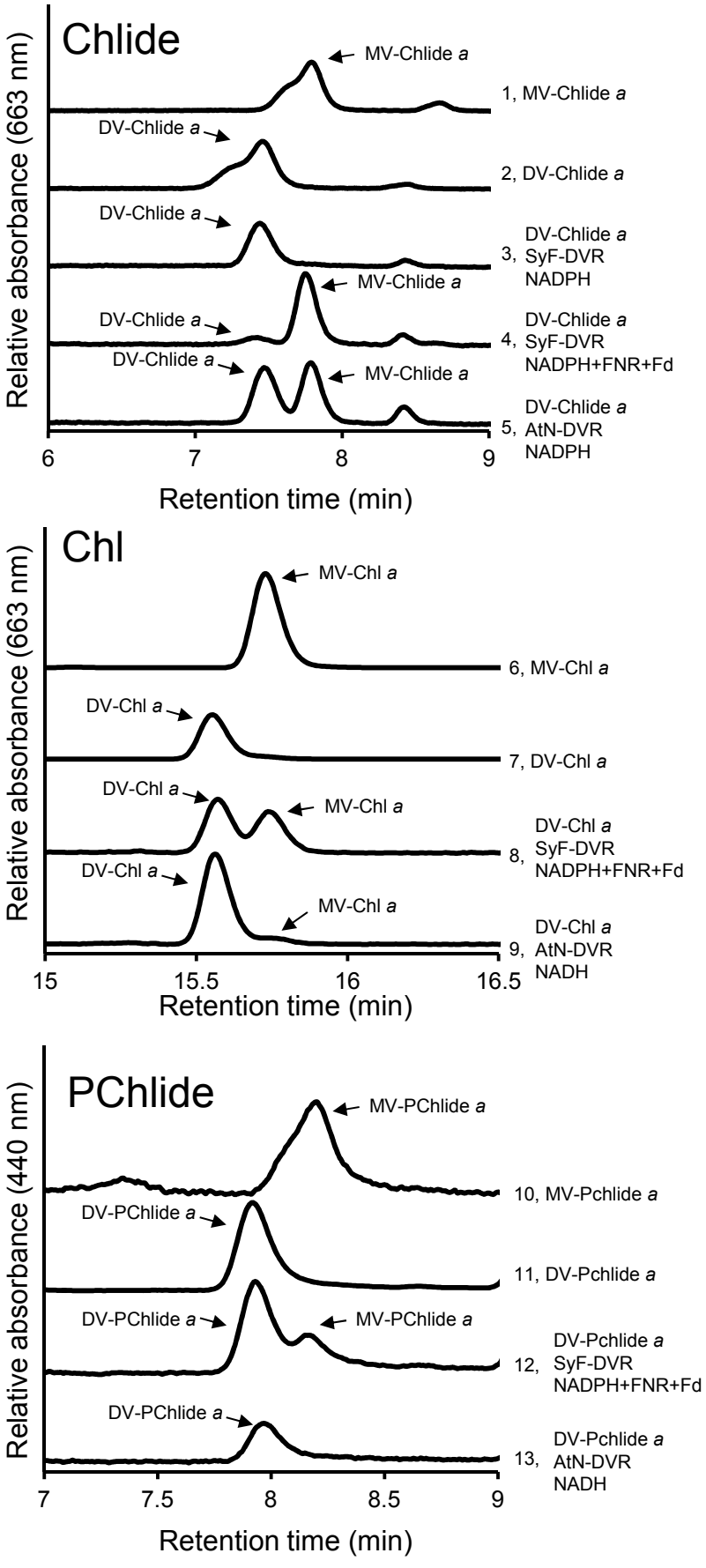
Figure 5. Chl c_2 reduction by DVR.

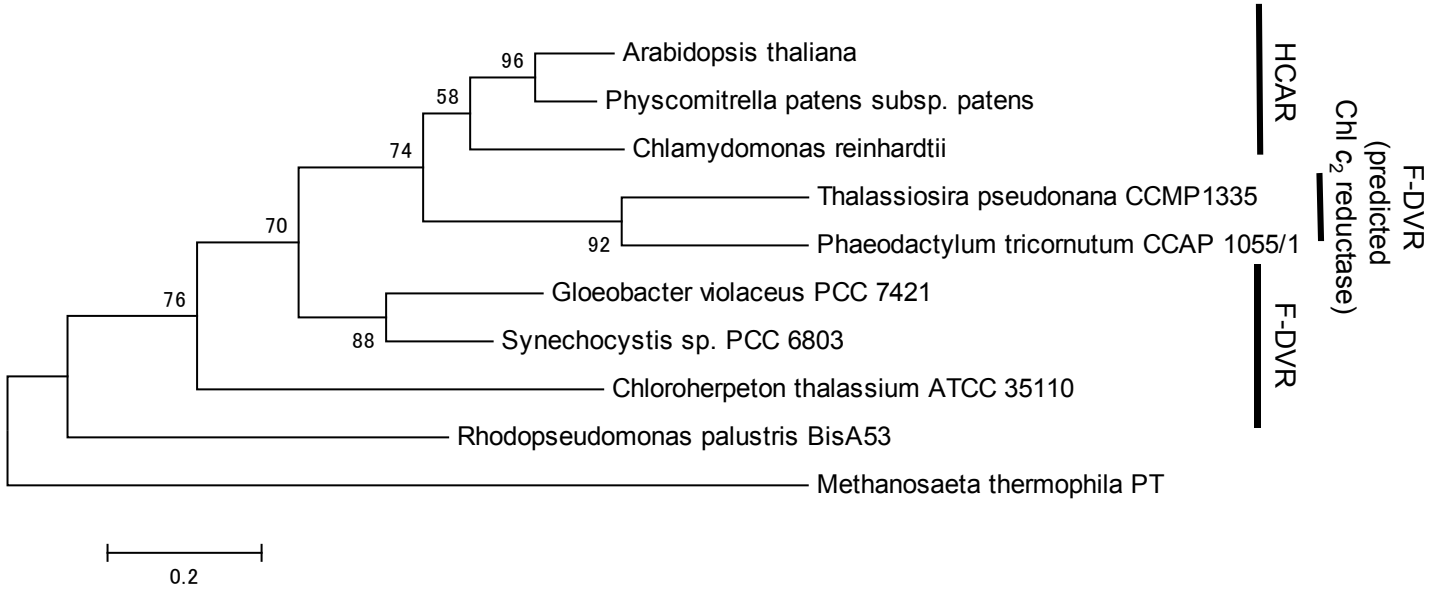
Chl c_2 prepared from dinoflagellates was incubated with *Synechocystis* F-DVR (SyF-DVR) or *Phaeodactylum* N-DVR (PhaeN-DVR). The pigment compositions were analyzed by HPLC. 1, Chl c_2. 2, Chl c_2 and Chl c_1 prepared from *Phaeodactylum*. 3, Chl c_2 incubated with PhaeN-DVR, NADPH. 4, Chl c_2 incubated with SyF-DVR, NADPH, FNR and Fd.

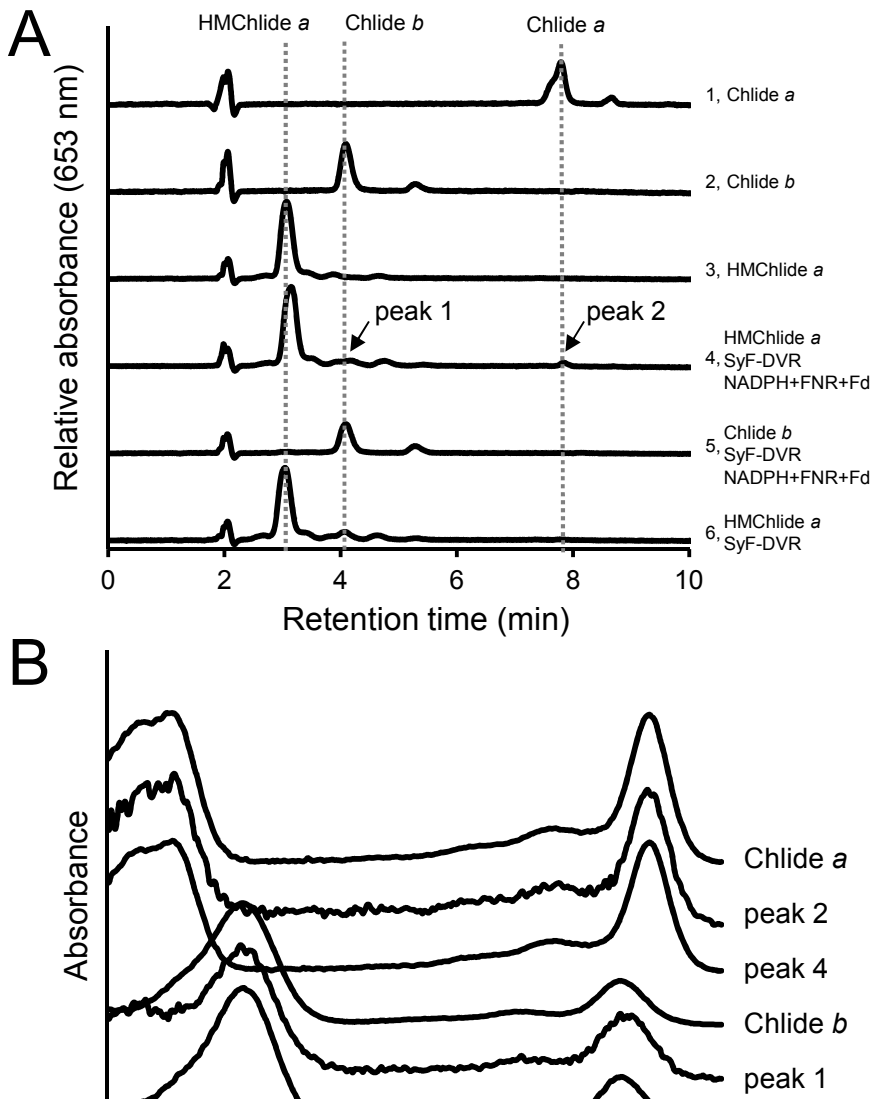
Figure 6. Schematic representation of the changes in the primary and promiscuous activities during evolution of F-DVR to HCAR.

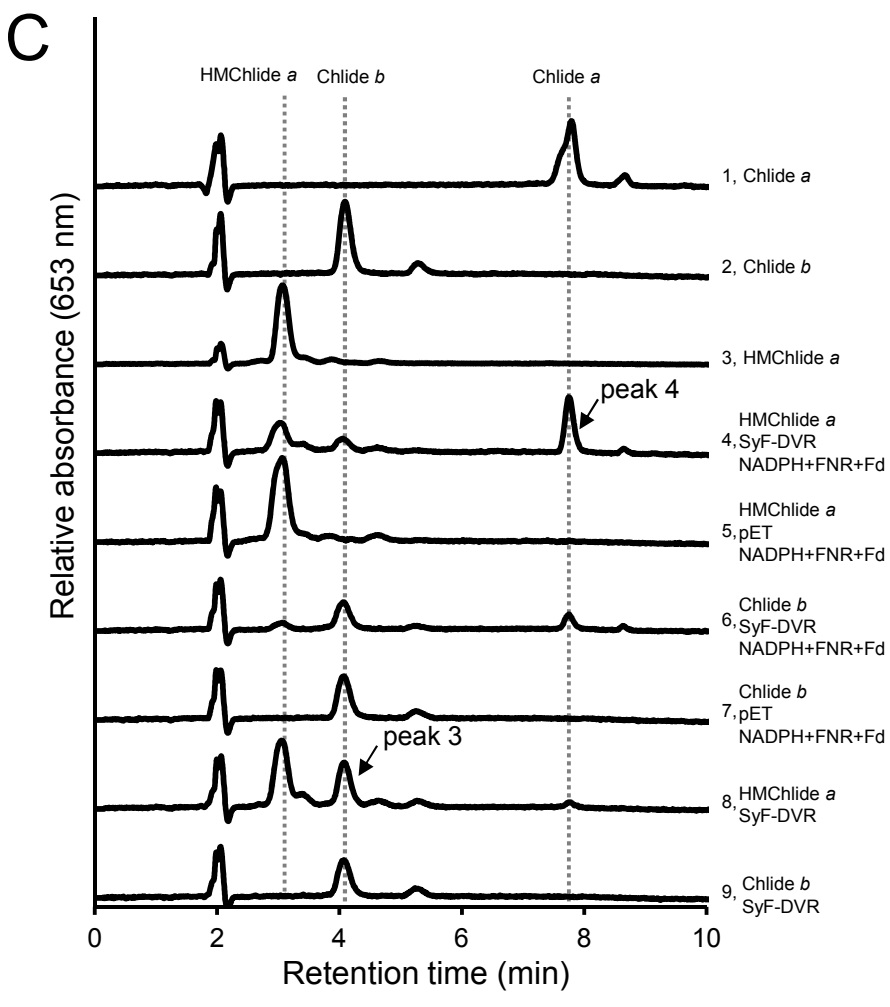
The size of the arrow represents the level of enzymatic activity.

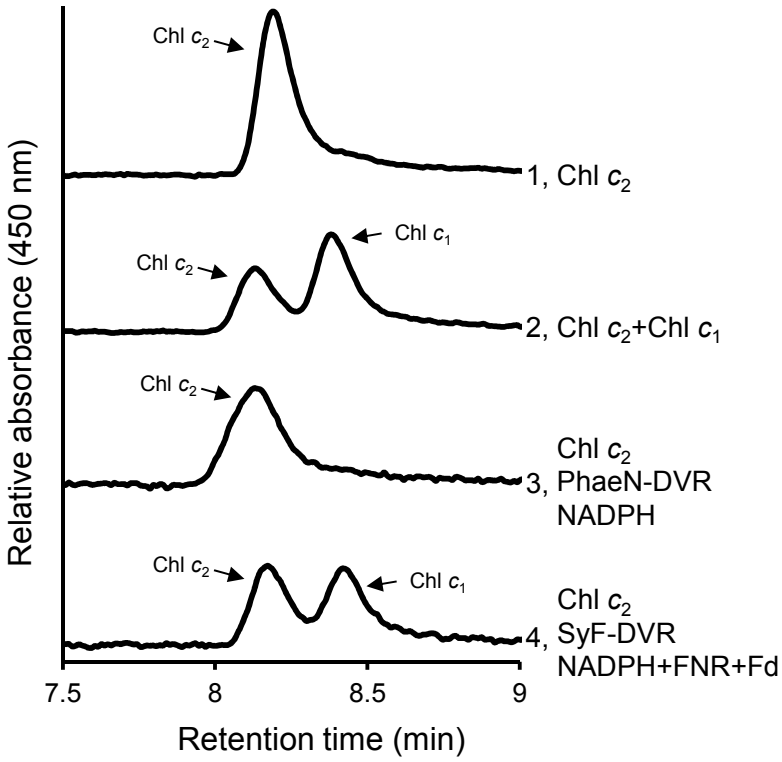

POR
DVR
DV-PChlide a
DV-Chlide a
MV-Chlide a
17¹ oxidase
DVR
Chl syn
Chl c2
MV-PChlide a
Chl a
8-vinyl reductase
17¹ oxidase
HCAR
CAO
Chl c1
HMChl a
CBR
CAO
Chl b

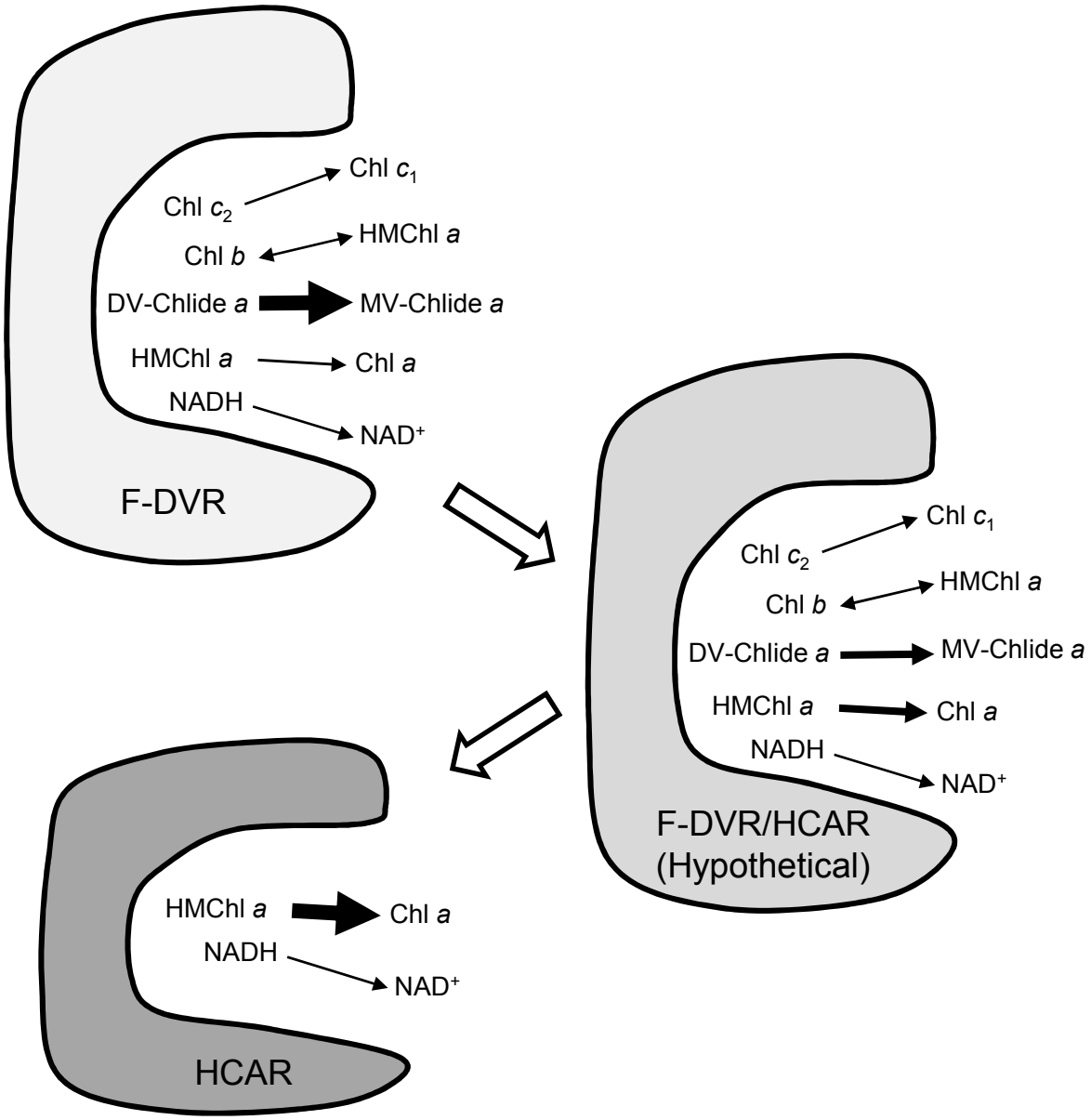

Chlide
Relative absorbance (663 nm)
MV-Chlide a
1, MV-Chlide a
DV-Chlide a
2, DV-Chlide a
DV-Chlide a
3, DV-Chlide a SyF-DVR NADPH
MV-Chlide a
DV-Chlide a
4, DV-Chlide a SyF-DVR NADPH+FNR+Fd
DV-Chlide a
MV-Chlide a
5, DV-Chlide a AtN-DVR NADPH
6
7
8
9
Retention time (min)
Chl
Relative absorbance (663 nm)
MV-Chl a
6, MV-Chl a
DV-Chl a
7, DV-Chl a
DV-Chl a
MV-Chl a
8, DV-Chl a SyF-DVR NADPH+FNR+Fd
DV-Chl a
MV-Chl a
9, DV-Chl a AtN-DVR NADH
15
15.5
16
16.5
Retention time (min)
PChlide
Relative absorbance (440 nm)
MV-PChlide a
10, MV-Pchlide a
DV-PChlide a
11, DV-Pchlide a
DV-PChlide a
MV-PChlide a
12, DV-Pchlide a SyF-DVR NADPH+FNR+Fd
DV-PChlide a
13, DV-Pchlide a AtN-DVR NADH
7
7.5
8
8.5
9
Retention time (min)


Arabidopsis thaliana
Physcomitrella patens subsp. patens
Chlamydomonas reinhardtii
Thalassiosira pseudonana CCMP1335
Phaeodactylum tricornutum CCAP 1055/1
Gloeobacter violaceus PCC 7421
Synechocystis sp. PCC 6803
Chloroherpeton thalassium ATCC 35110
Rhodopseudomonas palustris BisA53
Methanosaeta thermophila PT
96
58
74
70
92
76
88
0.2
HCAR
F-DVR
F-DVR (predicted Chl c2 reductase)


A
HMChlide a
Chlide b
Chlide a
Relative absorbance (653 nm)
1, Chlide a
2, Chlide b
3, HMChlide a
peak 1
peak 2
4, HMChlide a SyF-DVR NADPH+FNR+Fd
5, Chlide b SyF-DVR NADPH+FNR+Fd
6, HMChlide a SyF-DVR
0
2
4
6
8
10
Retention time (min)
B
Absorbance
Chlide a
peak 2
peak 4
Chlide b
peak 1
peak 3
400
500
600
700
Wavelength (nm)


C
HMChlide a
Chlide b
Chlide a
Relative absorbance (653 nm)
1, Chlide a
2, Chlide b
3, HMChlide a
peak 4
4, HMChlide a SyF-DVR NADPH+FNR+Fd
5, HMChlide a pET NADPH+FNR+Fd
6, Chlide b SyF-DVR NADPH+FNR+Fd
7, Chlide b pET NADPH+FNR+Fd
peak 3
8, HMChlide a SyF-DVR
9, Chlide b SyF-DVR
0
2
4
6
8
10
Retention time (min)


Chl c_2
1, Chl c_2
Chl c_1
Chl c_2
2, Chl c_2+Chl c_1
Chl c_2
Chl c_2
3, PhaeN-DVR
NADPH
Chl c_2
Chl c_1
Chl c_2
4, SyF-DVR
NADPH+FNR+Fd
Relative absorbance (450 nm)
7.5
8
8.5
9
Retention time (min)


Chl c_1
Chl c_2
HMChl *a*
Chl *b*
DV-Chlide *a*
MV-Chlide *a*
HMChl *a*
Chl *a*
NADH
NAD^+
F-DVR
Chl c_1
Chl c_2
HMChl *a*
Chl *b*
DV-Chlide *a*
MV-Chlide *a*
HMChl *a*
Chl *a*
NADH
NAD^+
F-DVR/HCAR
(Hypothetical)
HMChl *a*
Chl *a*
NADH
NAD^+
HCAR