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REVIEW

MPEC: an important gene in the chlorophyll biosynthesis pathway in photosynthetic organisms

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Abstract

One of the least understood enzymatic steps in chlorophyll biosynthesis is the formation of isocyclic ring, which is catalyzed by the Mg-protoporphyrin IX monomethyl ester (MgPME) cyclase that is involved in the conversion of MgPME to protochlorophyllide. Several genes encoding part of this enzyme have been identified and functional analysis of them has been performed. The enzyme plays important roles in higher plants and photosynthetic bacteria. The review focuses on the current knowledge of MgPME cyclase coding genes, with emphasis on their organization, expression pattern, and functional analysis obtained from mutants.

Additional key words: isocyclic ring; MgPME cyclase.

Introduction

Since chlorophyll (Chl) biosynthesis has been the subject of a broad and intensive research effort, its pathways are well established and the enzymatic properties of individual enzymes are well understood (Wettstein *et al*. 1995, Eckhardt *et al*. 2004, Bollivar 2006, Vavillin and Vermaas 2002, Kovacevic *et al*. 2007). The entire process involves about 20 different enzymatic steps (Reinbothe and Reinbothe 1996, Beale 1999). One of the least understood enzymatic steps is formation of the isocyclic ring. In Chl biosynthetic pathway, isocyclic ring formation occurs after insertion of Mg^{2+} into the macrocycle of protoporphyrin IX and the esterification of the 6-propionate to form Mg-protoporphyrin IX monomethyl ester (MgPME) which is then converted to protochlorophyllide (Pchlide) in at least three steps by MgPME cyclase (Fuesler *et al*. 1984, Wong *et al*. 1985, Porra *et al.* 1995, Bollivar and Beale 1996). Biochemical and genetic data revealed that MgPME cyclase is an essential enzyme in Chl/bacteriochlorophyll (Bchl) biosynthesis pathway, since plants or phototrophs were lethal if the MgPME cyclase coding genes were knocked out completely from their genomes.

Because few reviews give information about this enzyme, we first discuss the properties of the MgPME cyclase. The subsequent sections deal with identification and isolation of the MgPME cyclase genes (called *MPEC* genes in this article) that encode the putative enzyme. Then, we discuss their expression pattern responding to different environment factors. Mutants often play important roles in exploring the gene function, so in the last section we highlight the recent results obtained by mutant analysis. In this way, we summarize and update the knowledge of the *MPEC* genes and focus on their expression and functional analysis.

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Abbreviations: ALA – δ-aminolevulinic acid; Bchl – bacteriochlorophyll; Chl – chlorophyll; Chlide *a* – chlorophyllide *a*; LHC1 – light-harvesting complex 1; LHC2, light-harvesting complex 2; MgP – Mg-protoporphyrin IX; MgPME – Mg-protoporphyrin IX monomethyl ester; Pchlide – protochlorophyllide; PS – photosystem; SAM – *S*-adenosyl-L– methionine; WT – wild type.
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The general pathway of Chl synthesis

The process of tetrapyrrole synthesis from glutamate to the final product Chl is very complex, and numerous enzymes involved in the process have been identified in higher plants (Porra 1997, Suzuki *et al.* 1997, Papenbrock and Grimm 2001). In photosynthetic eukaryotes, Chl synthesis begins with a common precursor, δ-aminolevulinic acid (ALA), which is formed from glutamate through the activity of three enzymes, glutamyl-tRNA synthetase, glutamyl-tRNA reductase, and glutamate-1-semialdehyde aminotransferase (Ilag *et al.* 1994, McCormac *et al.* 2001). The formation of ALA is the rate-controlling point of the whole pathway. Eight molecules of ALA are subsequently condensed to the first porphyrin, uroprophyrinogen III, which is further oxidatively converted to protoporphyrin IX through three enzymatic steps (Beale 1999). The early parts of Chl pathway are shared with the biosynthesis of cobalamins, sirohemes, and hemes (Meskauskiene *et al.* 2001). The chelation of magnesium onto protoporphyrin IX results in the formation of MgP (Papenbrock *et al.* 2000), which is methylated to form MgPME by a methyl transferase (Alawady and Grimm 2005). MgPME is the substrate for MgPME cyclase responsible for the formation of the isocyclic ring of Pchlide *a* (Chereskin *et al.* 1982, Wong and Castelfranco 1985). Late steps in Chl synthesis include the light-dependent conversion of Pchlide *a* to chlorophyllide (Chlide *a*), the formation of Chl *a*, and final production of complete Chl *b* (Fujita 1996, Reinbothe *et al*. 1996, Heyes and Hunter 2005, Pattanayak *et al*. 2005, Rudiger *et al*. 2005). A brief outline of the Chl biosynthetic steps is presented in Fig. 1 with the enzymes involved in some of the biochemical steps.

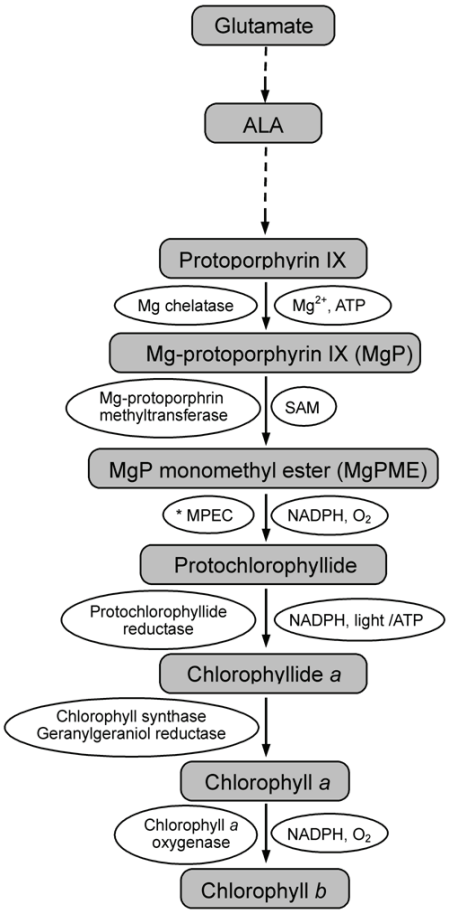

Fig. 1. A simplified scheme of the chlorophyll biosynthesis pathway of higher plant.

Properties of the MgPME cyclase

The net synthesis of Pchlide from MgPME *in vitro* has been studied in some details by Mattheis and Rebeiz (1977). It was deduced that the MgPME cyclase might be a collection or a complex of several enzymes because several intermediates are involved in the reaction (Walker *et al*. 1991). The enzyme was extracted from chloroplast extracts of cucumber cotyledons, wheat leaves, and the green alga *Chlamydomonas reinhardtii* (Chereskin *et al.* 1982, Nasrulhaq-Boyce *et al*. 1987, Bollivar and Beale 1995). Much of the early work was done with cell extract of cucumber cotyledons which was fractionated into two components, membrane-bound and soluble fractions. The soluble component had a molecular mass greater than 30 kDa, while the membrane-bound component was more labile than the soluble component (Wong and Castelfranco 1984, Walker *et al*. 1991). Recombination of the two fractions was required to restore the cyclase activity, and neither fraction alone was sufficient to catalyze the conversion of MgPME, but the details were still poorly understood. The cyclase activity was strongly inhibited by chelators of iron (such as CN^- and N_3^-) in the reconstituted system, but was inhibited slightly or not at all by the same reagents in intact developing chloroplasts. The cyclase system may involve a membrane-bound haemoprotein that is not accessible in intact chloroplasts and that contains tervalent iron, whereas in the reconstituted system the tervalent iron is inhibited much more strongly by CN^- and N_3^- (Whyte and Castelfranco 1993). Biochemical characterization established that MgPME is converted into Pchlide through the 6-β-hydroxy analogue and the 6-β-oxo analogue, and that the latter is then cyclized with the formation of a new carbon-carbon bond between the active methylene of the 6-β-oxo-methylpropionate side chain and the γ-meso carbon of the macrocycle (Wong *et al*. 1985, Walker *et al*. 1988). In addition, the cofactors are NADPH and molecular oxygen (Walker *et al.* 1991, Gough *et al*. 2000).

MPEC genes exist widely in photosynthetic organisms

Although the study of the MgPME cyclase has a long history, the corresponding genes coding the enzyme have not been identified until recently. The first identification of the plant *MPEC* gene was performed by differential hybridization screening of a *Pharbitis nil* cDNA library. A cDNA clone corresponding to an mRNA in which abundance increased during the dark was isolated and designated *PNZIP*. The full-length sequence of this gene was 1 402 bp and the predicted amino acid sequence contained 370 residues with a predicted molecular mass of approximately 43 kDa. It is not similar to any other gene with a known function in the database (Zheng *et al.* 1998). After that, several homologous cDNA clones of *PNZIP* have been cloned from different plants and photosynthetic bacteria (Table 1). A phylogenic tree using the MEGA 3.1 (Molecular Evolutionary Genetics Analysis) tool indicated that the *MPEC* genes could be roughly divided into four groups: monocotyledons, dicotyledons, algae, and cyanobacteria (Fig. 3). Sequence comparison analysis showed that they share very high similarities in both nucleotide sequences and amino acid sequences (Fig. 2). The conserved regions include a leucine zipper domain and two copies of EX_n DEXRH motif. Leucine zipper proteins without a basic domain are present in both animal and plant kingdoms. They act as negative transcriptional regulators by forming non-functional heterodimeric complexes with those transcription factors containing the basic domain (Kageyama and Pastan 1989, de Vetten *et al.* 1992, Ron and Habener 1992, Bange *et al.* 1994, Sun *et al.* 1996). The two copies of motif EX_nEXRH could provide the necessary ligands to bind a binuclear-iron cluster, which suggest the involvement of iron in the enzymatic transformation of the side chain of MgPME (Chereskin and Castelfranco 1982, Spiller *et al.* 1982, Wallar and Lipscomb 1996). These findings also suggest that *MPEC* genes exist widely throughout evolution of photosynthetic organisms, being present in photosynthetic bacteria, algae, and higher plants.

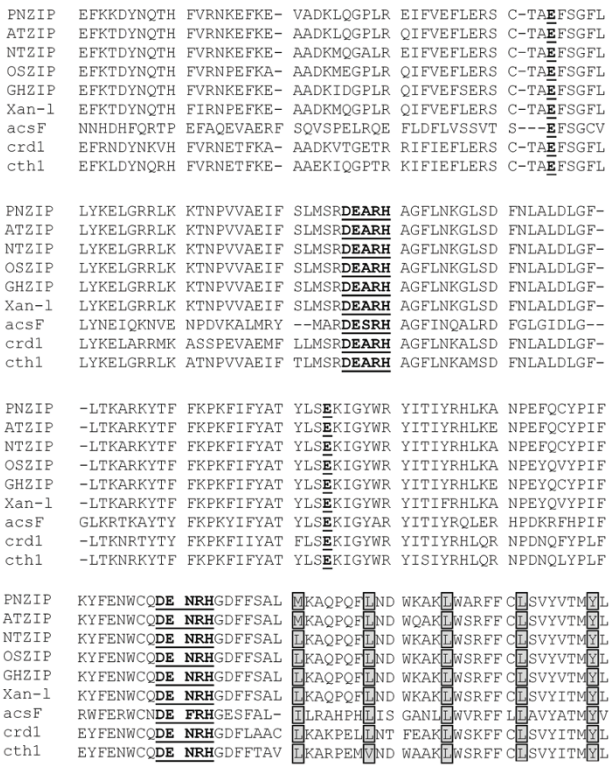

Fig. 2. Comparison and alignment of partial MPEC amino acids from different organisms. xan-l is the abbreviation of xantha-l; the leucine residues in the leucine zipper domain are boxed and shaded, and the $[EX_nEXRH]_2$ motifs are underlined and bold typed.

Table 1. *MPEC* genes isolated from green algae, photosynthetic bacterium, and higher plants.

MPEC gene	Organism	GenBank accession No.	Reference
Crd1	*Chlamydomonas reinhardtii*	AF237671	Moseley *et al.* 2000
Cth1	*Chlamydomonas reinhardtii*	AF337037	Moseley *et al.* 2002
acsF	*Rubrivivax gelatinosus*	AY057871	Pinta *et al.* 2002
PNZIP	*Pharbitis nil*	U37437	Zheng *et al.* 1998
BNZIP	*Brassica napus*	AY322556	Liu *et al.* 2004
CSZIP	*Cucumis sativus*	AY221169	Liu *et al.* 2004
NTZIP	*Nicotiana tabacum*	AY221168	Liu *et al.* 2004
OSZIP	*Oryza sativa*	AP000815	Liu *et al.* 2004
RDZIP	*Rosa davurica*	AY322555	Liu *et al.* 2004
SBZIP	*Salix babylonica*	AY322554	Liu *et al.* 2004
SOZIP	*Spinacia oleracea*	AY322553	Liu *et al.* 2004
TAZIP	*Triticum aestivum*	AY322552	Liu *et al.* 2004
TRZIP	*Trifolium repens*	AY322557	Liu *et al.* 2004
ATZIP/CHL27	*Arabidopsis thaliana*	U38232/AF236101	Zheng *et al.* 2002/ Tottey *et al.* 2003
Xantha-l	*Hordeum vulgare*	AY887063	Rzeznicka *et al.* 2005

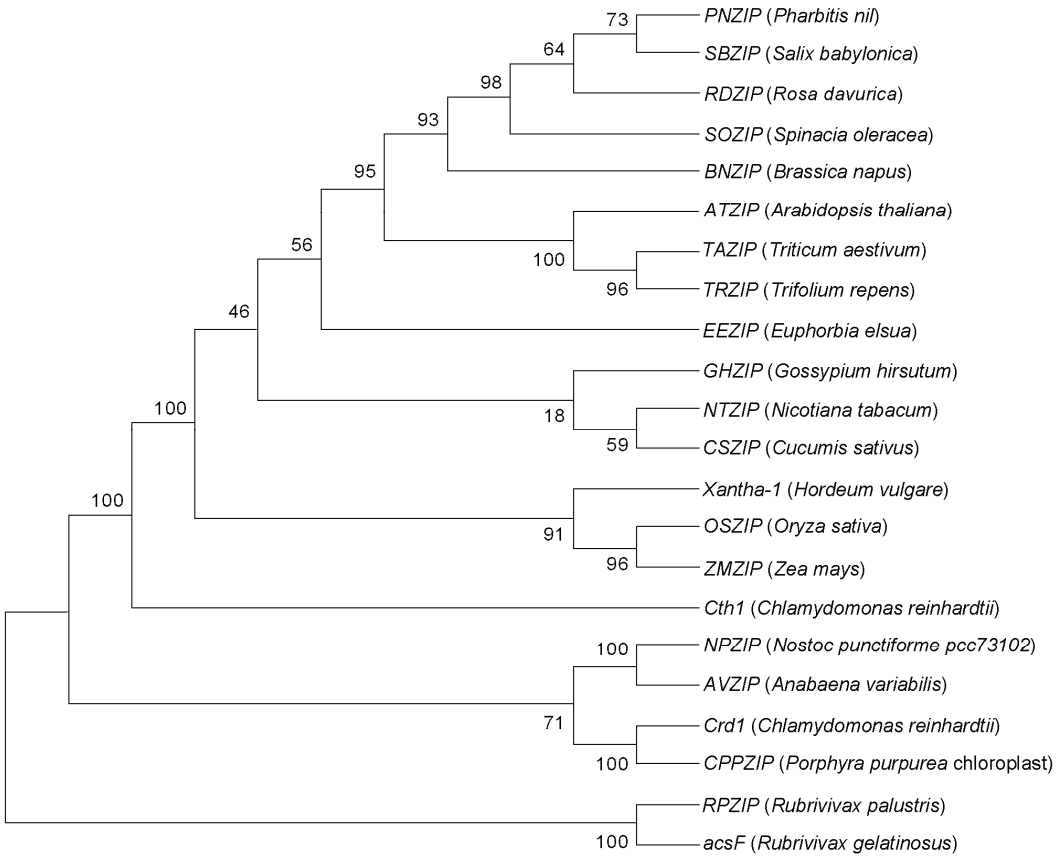

Fig. 3. Phylogenetic tree based on the deduced amino acid sequences of *MPEC* genes using MEGA 3.1. AVZIP, *Anabaena variabilis* (ZP-00158688); CPPZIP, *Porphyra purpurea* chloroplast (U38804); EEZIP, *Euphorbia elsua* (AF417577); GHZIP, *Gossypium hirsutum* (AAR20445); NPZIP, *Nostoc punctiforme pcc73102* (ZP00107241); RPZIP, *Rubrivivax palustris* (AF195122); ZMZIP, *Zea mays* (AY108897). Information about *Crd1, Cth1, acsF, PNZIP, BNZIP, CSZIP, NTZIP, OSZIP, RDZIP, SBZIP, SOZIP, TAZIP, TRZIP, ATZIP/CHL27,* and *Xantha-l* is shown in Table 1.

MPEC gene organization and expression

In *Ch. reinhardtii*, *Crd1* and *Cth1* are localized in chloroplast membranes. Studies of the enzyme from *Arabidopsis* suggest that the enzyme is membrane associated. It is present in both the thylakoid and envelope membranes (Tottey *et al.* 2003). Southern blot analysis showed that *PNZIP* is present as single copy in *Pharbitis nil* genomes. A similar DNA gel-blot hybridization analysis of the *Arabidopsis* genome using the *ATZIP* cDNA or the tobacco genome using the *NTZIP* cDNA as probe revealed similar results, suggesting that the *MPEC* gene probably represents a single locus also in the genome of other plants (Zheng *et al*. 2002, Liu *et al.* 2004). The highest content of *MPEC* mRNA was found in photosynthetic tissues but was virtually undetectable in non-photosynthetic tissues (Fig. 4*A* – Zheng *et al*. 1998, 2002, Liu *et al*. 2004). Consistent with these results, the *PNZIP* promoter is a strong promoter expressed specifically in photosynthetically active mesophyll cells (Yang *et al.* 2003, Xue *et al.* 2005).

Photosynthesis depends on photon energy which regulates the expression of many photosynthetic genes and the activity of the gene products. So it is not surprising that the expression of *PNZIP* exhibits a rhythmic pattern of mRNA accumulation with a circadian periodicity of approximately 24 h when plants are exposed to continuous darkness (Fig. 4*D*), suggesting that its expression is under the control of an endogenous clock (Thimm *et al*. 2004). In addition, either a far-red (FR) radiation treatment at the end of day or exposure to red (R) radiation in the middle of night would reduce its mRNA contents. Thus the *PNZIP* gene is also regulated by phytochrome during the dark (Fig. 4*E*,*F*). Moreover, in tobacco, Northern hybridization analysis showed that *NTZIP* mRNA contents are induced by radiant energy and low temperature, but are repressed by strong irradiance (Fig. 4*B*,*C*). Transgenic tobaccos constitutively expressing antisense RNA to *NTZIP* display chlorosis and a lack of ability to turn green under normal growth conditions, proposing that *NTZIP* and its homologues are involved in Chl biosynthesis.

Exploring *MPEC* genes with mutants

Since *PNZIP* cDNA was initially isolated by a differential hybridization screening because of its increased mRNA contents during the dark, the early study mainly focused on the light and dark regulation of *PNZIP* gene expression and the possible role of the phytochrome in this process. *PNZIP* is regulated by both an endogenous clock and the phytochrome, but the respective study has not been correlated with Chl biosynthesis. Current understanding of the *MPEC* genes was achieved by using genetic studies of photosynthetic bacteria and plant mutants. Each study has provided significant information in the investigation of coding genes of this important enzyme. In the following sections we discuss the latest findings regarding *MPEC* genes obtained from studies on mutants.

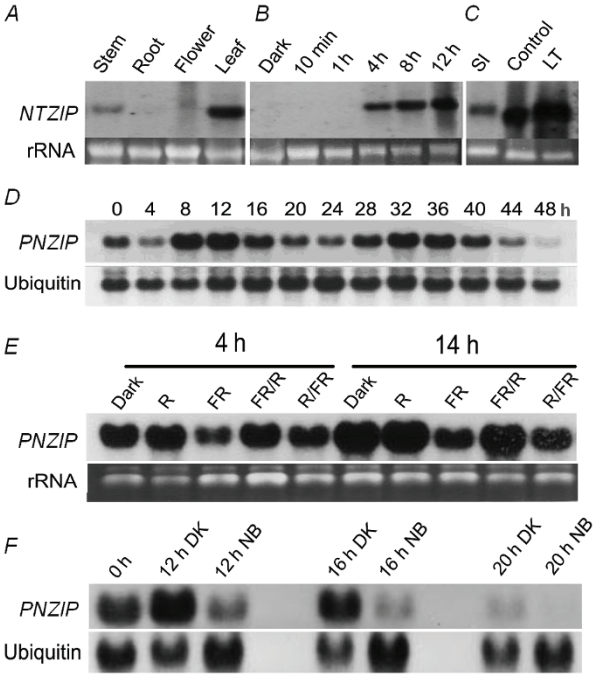

Fig. 4. Expression pattern of *MPEC* genes in different plants: (*A*) Northern blot analysis of *NTZIP* mRNA accumulation in different organs. (*B*) Northern blot analysis of *NTZIP* mRNA accumulation during dark and light treatment. (*C*) Northern blot analysis of *NTZIP* mRNA accumulation at low temperature (LT) and after strong irradiation (SI). (*D*) Northern blot analysis of *PNZIP* mRNA contents during an extended dark treatment. (*E*) Effects of end-of-day treatments of *P. nil* seedlings with red (R) and far-red (FR) radiation on flowering and *PNZIP* mRNA accumulation during the dark. Seedlings were grown in continuous radiation for 6 d, and just before the transition to darkness at 0 h they were exposed for 10 min to R, FR, FR, and then R, or R and afterwards FR (R/FR). (*F*) Northern blot analysis of *PNZIP* mRNA accumulation during continuous darkness or following an NB (night break) treatment. Seedlings were grown in continuous irradiation for 6 d and then treated with three durations of darkness (DK 12, 16, or 20 h) or were interrupted at the 8th h of dark treatment by a 10-min NB with RR.

The *Crd1* gene of *Ch. reinhardtii* is a homologous gene to *PNZIP*, which was first identified for genes in response to copper deficiency. Experimental evidence showed that the *crd1* strains failed to accumulate photosystem (PS) 1 and light-harvesting complex (LHC) 1 during hypoxia or copper deficiency, and they contained reduced amounts of LHC2, while the abundance of the D1 protein of PS2 was only slightly reduced and the accumulations of other photosynthetic complexes were normal, revealing that *Crd1* was required for the maintenance of PS1 and LHCs during hypoxia or Cu deficiency (Moseley *et al*. 2000). The initial observation led to the identification of another locus, *Cth1* (*copper target homolog 1*), which is similar but not identical to *Crd1*. *Crd1* abundance was increased in Cu or O_2 deficient cells (–Cu/–O_2), while *Cth1* accumulated in Cu-sufficient, oxygenated cells. Thus the tightly coordinated isoenzymes *Cth1* and *Crd1* brought up dynamic changes in the interactions between PS1 and associated LHCs (Moseley *et al*. 2002).

Studies on *Cth1* and *Crd1* have not lead to its MgPME cyclase function. The best characterized gene to date is the *acsF* (previously *orf358*) in the purple bacterium *Rubrivivax gelatinosus*. This locus was isolated by interposon mutagenesis of an open reading frame in the region of the genome. A strain in which the *acsF* gene was disrupted could not synthesise Bchl *a* but accumulated MgPME under high oxygenation, indicating that *acsF* was involved in the cyclase reaction only under aerobic conditions in purple bacterium. However, the mutant lacking the *acsF* gene exhibited a phenotype similar to the wild type (WT) under low-oxygenation conditions. Pinta *et al.* (2002) therefore proposed that two different mechanisms for MgPME cyclization might coexist in *R. gelatinosus*. Ouchane *et al.* (2004) further confirmed this proposal by analyzing the Bchl accumulation in the WT and three mutant *R. gelatinosus* strains (*bchE, acsF*, and *bchE-acsF* double mutant) grown under low or high aeration. The *bchE* gene is involved in the MgPME conversion (Bollivar *et al.* 1994). Mutants lacking the *acsF* gene produce Bchl *a* under low oxygenation and anaerobiosis and accumulate MgPME under high oxygenation. The *bchE* mutants exhibited normal Bchl *a* accumulation only under high oxygenation and accumulated MgPME under low oxygenation and anaerobiosis. However, the double knockout mutants accumulated MgPME under both conditions. Because no obvious sequence similarity was found between *acsF* and *bchE* genes, it was concluded that both *acsF* and *bchE* were involved in MgPME cyclization and catalyzed the same step of Bchl biosynthesis in the pathway. Nevertheless, *acsF* acted strictly under high oxygenation, whereas *bchE* was active only when the oxygen tension dropped. This result also explained the presence of Bchl under either aerobiosis or anaerobiosis in *R. gelatinosus* and in

other facultative aerobic photosynthetic bacteria. In addition, Ouchane *et al.* (2004) suggested two models of the cyclization mechanism. Enzymes of the aerobic class require oxygen as a substrate and act through a binuclear iron centre; while enzymes of the anaerobic class use water as substrate requiring SAM and act through a 4Fe-4S cluster. BchE also requires vitamin B12 (Porra *et al*. 1995, Gough *et al.* 2000, Sofia *et al*. 2001, Ouchane *et al.* 2004). The conversion of MgPME to Pchlide *via* two different mechanisms is shown in Fig. 5.

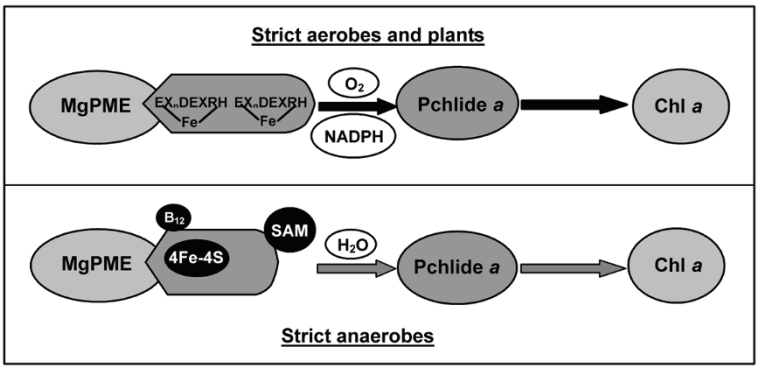

Fig. 5. Schematic representation of the two classes of enzymes catalyzing the same reaction under different oxygen conditions (aerobiosis *versus* anaerobiosis).

When mutants were not obtainable or poor available at the seedling stage, information about the gene function could be obtained by over-expressing them or by expressing antisense genes using either the constitutive promoters such as cauliflower mosaic virus CaMV 35S promoter or tissue specific promoters. Tottey *et al*. (2003) provided an example of this approach, in which *Arabidopsis* plants were transformed with the antisense *CHL27* gene (the same gene as *ATZIP* mentioned above). The T1 generation of the transformed antisense plants displays four phenotypic lines: one line dies early after transfer to soil; the second line shows severe chlorosis at a later developmental stage; the third line displays a variegated phenotype pattern; and the fourth line is phenotypically identical to WT plants. The pigment contents were analyzed by HPLC showing that the chl27-as lines accumulated MgMPE, whereas the major compound in WT plant extracts was Pchlide. This demonstrates that the chl27-as lines are blocked at the cyclase step and that the *CHL27* gene is required for the synthesis of Pchlide from MgMPE in *Arabidopsis* (Tottey *et al*. 2003). The homologous gene in barley has also been recently identified and mapped to the *xantha-l* locus (Rzeznicka *et al.* 2005). The cyclase activity was measured in barley chloroplast extracts of three mutants, *xantha-l35*, *viridis-k23*, and *viridis-k170*. All three mutants produce reduced amounts of Pchlide and increased MgPME accumulation when fed with ALA in the dark, indicating that these genes are involved in the isocyclic ring formation. *In vitro* assay for the aerobic cyclase activity was performed with *xantha-l* and *viridis-k* mutants and WT-plants. Pellet (P) and supernatant (S) fractions were prepared in parallel with WT and mutant chloroplast extracts, respectively. As expected, reconstitutions of the WT P and S were active and extracts from mutants showed no cyclase activity. Combinations of WT P and mutant S were also active, while combinations of WT S and mutant P were all inactive. These data indicated that the cyclase component in the S fraction was not affected in the mutants, but a cyclase component in the P fraction was inactive in both *xantha-l* and *viridis-k* mutants. A multitude of evidence has suggested that *xantha-l* and *viridis-k* are non-allelic genes, and that the aerobic cyclase is composed of at least three gene products, a soluble protein, a membrane-bound component encoded by *CRD1/CTH1/acsF/CHL27/Xantha-l*, and another membrane-bound component encoded by *Viridis-K* (Rzeznicka *et al.* 2005).

Conclusions

The Chl/Bchl biosynthesis is almost completely biochemically and genetically elucidated. However, cyclization of MgPME to Pchlide remains an area of relative ignorance and there have been considerable problems with attempts to connect genetic and biochemical data. The recent application of genetics in the bacterium *R. gelatinosus* and the higher plant *Arabidopsis* has led to a better understanding of this enzyme. Based on the current studies, the information of the cyclase enzyme is divided into two major areas: (*1*) biosynthesis in photosynthetic tissues, which is under the control of their promoters and environmental signals, primarily radiant energy, temperature, and oxygen conditions; (*2*) the enzyme is composed of at least three gene products, one soluble protein and two membrane-bound proteins, and all three proteins are required to restore the aerobic cyclase activity. Although our understanding of the mechanism of the cyclase has improved, much remains to be learned about the environmental and developmental regulation of the *MPEC* genes. It is also important to elucidate the specific proteins that govern the assembly of MPEC complexes, or those that bind to it. To our

knowledge, all the genes that have been cloned to date encode a membrane-bound cyclase subunit. There is now the possibility to begin exploring the other polypeptide components possibly involved in this cyclic reaction and to identify the unknown soluble component of this enzymatic step.

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