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Full Length Article

Heterogeneous Expression of *Cymbidium longibracteatum* Magnesium Protoporphyrin IX Methyltransferase (*ClChlM*) Activates Chlorophyll Biosynthesis in Transgenic Tobacco

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Abstract

Magnesium protoporphyrin IX methyltransferase (ChlM) plays an important role in the regulation of chlorophyll biosynthesis and chloroplast development. In the present study, we isolated a ChlM gene, designated ClChlM, from *Cymbidium* [*Cymbidium longibracteatum* (Wu & Chen) Chen & Liu]. The open reading frame (ORF) sequence of ClChlM was 945 bp and encoded a putative protein of 314 amino acids. The deduced ClChlM contained the conserved SAM/SAH binding pocket and substrate binding sites. Subcellular localization analysis of ClChlM revealed that the protein was localized in the chloroplast. Ectopic overexpression of ClChlM in tobacco (*Nicotiana benthamiana* Domin) increased ALA-synthesizing capacity and chlorophyll content and widely upregulated the expression level of photosynthesis-related genes, such as *ClHemA*, *ClGSA*, *ClLhcb*, *ClCHLI*, and *ClCHLH*. In conclusion, these results demonstrate that ClChlM plays a crucial role in the regulation of chlorophyll biosynthesis in *C. longibracteatum* and will help in breeding for leaf colour variance in the future.

Keywords: *Cymbidium longibracteatum*; Mg protoporphyrin IX methyltransferase (ChlM); Gene clone; Subcellular localization; Functional verification

Introduction

As of great ornamental value, leaf colour is one of the most important traits for plants. Chlorophylls, carotenoids, and anthocyanins are the major pigments that determine leaf colour in plants (Li *et al.* 2016). Leaf colour variance is typically caused by the abnormal metabolism of pigments (Deng *et al.* 2014; Ding *et al.* 2019). According to the phenotype of the plant, leaf color variance can be classified as albinism, yellowness, light green, white emerald, green-white, yellow-green, green-yellow, stripe and evergreen (Afsar Awan *et al.* 1980). As a special plant material, leaf color mutants are crucial for the study of pigment metabolism. Recently, leaf color mutants have been widely studied in *Paeonia lactiflora* (Tang *et al.* 2020), *Oryza sativa* (Dong *et al.* 2013; Deng *et al.* 2017; Wang *et al.* 2017), *Zea mays* (Zhong *et al.* 2015), *Anthurium andraeanum* (Yang *et al.* 2015), *Lagerstroemia* (Li *et al.* 2015), and *Cymbidium* (Zhu *et al.* 2015; Jiang *et al.* 2018), and a great number of functional genes are identified. Chlorophyll, which is located in the chloroplast for photosynthesis, is the main pigment in the leaves of most plants (Czarnecki and Czarnecki 2012). The biosynthesis of chlorophyll begins with glutamyl-tRNA and proceeds through a 15-step enzymatic reaction that results in chlorophyll b (Nagata *et al.* 2005; Müller *et al.* 2014). The mutation of genes involved in chlorophyll metabolism can cause leaf color variation. For example, *OsChlH* loss-of-function results in the chlorine and lethal phenotype in rice (Jung *et al.* 2003), whereas NYC1 mutations cause the stay-green phenotype in *Arabidopsis* (Jia *et al.* 2015).

Magnesium protoporphyrin IX methyltransferase (ChlM) is one of the key enzymes for chlorophyll biosynthesis. ChlM catalyses methyl transfer from S-adenosylmethionine to magnesium protoporphyin IX, forming MgOME and S-adenosylcysteine (Shepherd *et al.* 2003; Shepherd and Hunter *et al.* 2004). In Arabidopsis, knock-out of AtChlM affects the formation of chlorophyll and subsequently the formation of chlorophyll, photosystem I and II, and cytochrome b6f (Pontier *et al.* 2007). In rice, OsChlM mutations cause the accumulation of magnesium protoporphyrin IX and decrease magnesium protoporphyrin IX monomethylester levels (Wang *et al.* 2017).

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Cymbidium is an economical genus of Orchidaceae cultivated in Southeast Asia (Kim and Chase 2017). Leaf variations in *Cymbidium* have ornamental value and have recently became of great interest. Using tissue culture-induced genetic mutation, we generated a leaf color variant with a yellowing rhizome and yellow leaves from wild-type *Cymbidium longibracteatum* 'Longchangsu' (Jiang *et al.* 2015). Previous comparative transcriptome analysis showed that the content of total chlorophyll significantly decreased in the leaf color variant and that a unigene encoding ChlM was differentially expressed between the two cultivars (Jiang *et al.* 2018). Here, we isolated the coding sequence (CDS) of *ClChlM* and performed functional analysis of ClChlM in transgenic tobacco.

Material and Methods

Plant material

Wild-type [*Cymbidium longibracteatum* (Wu & Chen) Chen & Liu] 'Longchangsu' was grown in the greenhouse at the Horticulture Institute of Sichuan Agricultural Sciences in Chengdu city (Jiang *et al.* 2015). Tobacco (*Nicotiana benthamiana* Domin) seeds were sown on sterilized Murashige and Skoog (MS) medium and grown in a climate chamber for genetic transformation. The growing conditions (16 h light/8 h dark) were maintained at 22°C.

RNA isolation and first-strand cDNA synthesis

Total RNA of 'Longchangsu' was extracted using the RNAprep Pure Plant Plus Kit (DP441, Tiangen Biotech Co., LTD, China). The quality of RNA was evaluated by NanoDrop 2000 (Thermo Scientific Inc., USA). Using the RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific Inc., USA), 1 μg of total RNA was employed for first-strand cDNA synthesis.

Isolation of *ClChlM* and sequence analysis

Based on the sequence of c19370_g1 from the previous RNA-Seq library (NCBI accession number: GSE100180), the specific primers were designed for open read frames (ORFs) of *ClChlM* amplification (Table S1). The amplification procedure was performed as follows: 94℃ for 4 min; 30 cycles of 94°C for 30 sec, 60°C for 30 sec, 72℃ for 1min, and extension at 72°C for 10 min.The amplicon was sub-cloned into the pEASY-Blunt cloning vector (TransGen Biotech, China) for sequencing.

Bioinformatic analysis

The molecular mass and theoretical isoelectric point of ClChlM were calculated by ExPASy (http://web.expasy.org). The localization of ClChlM was analysed by TargetP1.1 (http://www.cbs.dtu.dk/services/TargetP/). Multiple alignments of ChlM were performed with the online software MSA (https://www.ebi.ac.uk/Tools/msa/). The phylogenetic tree was performed by the online software iTOL (http://itol.embl.de/).

Subcellular location

The ORF sequence of the *ClChlM* gene without the termination codon was subcloned into the 5'-terminus of the green fluorescent protein (GFP) in the pJX002-GFP vector with double enzyme digestion of XhoI and SalI (TaKaRa, Japan). The recombination plasmid pJX002-ClChlM1-GFP was transformed into *A. tumefaciens* strain GV3101 and then infiltrated into tobacco leaves (Xiong *et al.* 2019). The location of the fusion protein was observed through fluorescence microscopy (Olympus BX51, Japan) 48 h after infiltration.

Tobacco transformation

To generate ClChlM-overexpressing tobacco transgenic lines, the ORF sequence of *ClChlM* was inserted into the plant binary expression vector pART-CAM to generate the vector pART-ClChlM. The vector was transformed into tobacco by *Agrobacterium*-mediated transformation (Li *et al.* 2020). Specific primer (Kan-F/Kan-R)-amplified PCR was used to detect positively transformed tobacco lines (Table S1).

Real-time quantitative PCR

The qRT-PCR was conducted in a 25 μL volume, including 12.5 μL SYBR buffer, 9.5 μL ddH_2O, 1 μL cDNA, 1 μL forward primer, 1 μL reverse primer. After 40 cyclys, melting curve was analyzed at 95°C for 15 s with a gradient increase from 60°C to 95°C.The EF1-α gene (GenBank Accession No. XM_009595030) was used as a reference (Huang *et al.* 2012). The target gene relative expression level was calculated as described (Jiang *et al.* 2018). For the determination of 5-aminolevulinic acid (ALA)-synthesizing capacity, the ALA-synthesizing capacity was detected using methods as previously described (Alawady and Grimm 2005). Tobacco leaves were cut into discs and incubated in phosphate buffer (20 mM, pH 7.5) with levulinic acid (40 mM, pH 6.9) for 4 h under light. Then, the supernatant was boiled for 10 min in ethyl acetoacetate. After mixing with an equal volume of Ehrlich's reagent, ALA derivatives were determined at 553 nm.

Determination of total chlorophyll content

To determine the content of chlorophyll a and chlorophyll b, leaves were ground to homogenate with 95% ethanol and diluted with acetone. Then, the extracting solution was detected using an ultraviolet spectrophotometer at 665 nm for chlorophyll a and at 649 nm for chlorophyll b (Dere *et al.* 2018).

Statistical analysis

Three duplicates of each experiment were performed. The statistical significance of the values was analyzed using the *t*-test.

Results

Cloning and characterization of *ClChIM*

Based on our previous transcriptome data, a unigene (c19370_g1) exhibited high similarity to the ChlM genes from other plants. A BLASTX search identified c19370_g1 containing a complete open read frame (ORF), which was further verified by PCR amplification and sequencing. The gene was termed ClChlM and deposited to NCBI under accession MG574594. The ORF of ClChlM was 1,143 bp, encoding 314 amino acids.

The molecular mass of ClChlM was 33.94 kDa, and the isoelectric point was 7.03. Conserved domain analysis showed that ClChlM contained SAM/SAH binding pocket and substrate binding sites, which were highly conserved among the ChlMs from other plants (Fig. 1). A phylogenetic tree was constructed using ClChlM and the other 20 ChlMs from different species. The phylogenetic tree was split into three branches. ClChlM was grouped into Clade III and highly relative to DcChlM (Fig. 2).

Subcellular localization of ClChlM

The online software TargetP 1.1 predicted that ClChlM should target chloroplasts. Transient transformation of *ClChlM-GFP* in tobacco leaves clearly showed the strong GFP fluorescence signal was observed in the chloroplast, which was coincident with the area of chloroplast autofluorescence (Fig. 3). These results implied that the ClChlM protein was localized in the chloroplasts.

Ectopic expression of *ClChlM* in tobacco

Four transgenic lines were obtained by amplification of the kanamycin fragment (Fig. 4A). qRT-PCR analysis revealed that three transgenic lines (L2, L3, and L6) exhibited high *ClChlM* expression levels compared with the transgenic lines (Fig. 4B). These three lines were used for further analysis.

Overexpression of *ClChlM* elevated ALA-synthesizing capacity and chlorophyll content

Chlorophylls are a group of cyclic tetrapyrrole pigments, and ALA is the precursor of tetrapyrrole biosynthesis. In the present study, we noticed that the ALA synthesis rate notably increased compared with CK (Fig. 5A). Chlorophyll determination showed that both chlorophylls a and chlorophyll b levels were significantly increased compared with that in CK (Fig. 5B–C).

Overexpression of *ClChlM* upregulated photosynthesis-related genes

To understand the change in photosynthesis-related genes in transgenic tobacco lines, qRT-PCR was used to reveal the differential expression profile of glutamyl-tRNA reductase (*ClHemA*), glutamate 1-semialdehyde aminotransferase (*ClGSA*), light-harvesting Chi-binding protein LHCB 2 of photosystem II (*ClLhcb*), *ClCHLI* and *ClCHLH* between transgenic lines and wild-type lines. The results showed that all genes were significantly upregulated in transgenic lines (Fig. 6).

Discussion

Chlorophyll is one of the major pigments that is crucial for photosynthesis. The obstruction of chlorophyll biosynthesis can cause leaf chlorosis in many plants such as Arabidopsis and rice (Pontier *et al*. 2007; Wang *et al*. 2017). ChlM is an essential enzyme that catalyzes the second important step in chlorophyll biosynthesis. In *C. longibracteatum*, we previous show the expression level of *ChlM* is different between "Longchangsu" and its leaf colour mutant (Jiang *et al*. 2018). In the present study, we isolate the ORF sequence of ClChlM. Overexpression of ClChlM in tobacco can notably increase the content of chlorophyll (Fig. 5), suggesting the important role of ClChlM in chlorophyll biosynthesis.

Sequences alignment analysis shows that ClChlM contains the conserved SAM/SAH binding pocket and substrate binding sites. The SAM/SAH binding pocket is a DXGCGXG motif that is crucial for SAM binding (Schubert *et al.* 2003). In *Arabidopsis*, three cysteine residues are crucial for the catalytion and redox-dependent activation of AtChlM (Richter *et al.* 2016). We found that the three residues are also highly conserved in ClChlM (Fig. 2), suggesting their putative role in the redox regulation in *Cymbidium*. Substrate binding sites are the sites for MgP binding (Karger *et al.* 2001). In 2014, Chen et al illustrate the molecular mechanism of ChlM based on the high resolution of crystal structure from *Synechocystis*. The crystal structures of SyChlM indicate that Tyr-15, Phe-16, Trp-24, Ile-27, Tyr-28, Val-36, Ile-40, Ile-138, His-139, Leu-174, Phe-219, and Tyr-220 are the core substrate binding sites for MgP (Chen *et al.* 2014). Here, we interestingly identified that all these amino acids are highly conserved in ClChlM (Fig. S1), indicating their essential role in maintaining the enzyme activity in *Cymbidium*.

Chloroplasts are organelles found in the cytoplasm of plant cells that conduct photosynthesis. As a key enzyme of chlorophyll biosynthesis, ClChlM subcellular localization clearly demonstrates that the protein is located in the chloroplasts, hinting at its crucial role in the regulation of photosynthesis. Recently, it has been suggested that ChlM can regulate protein-encoding photosynthesis at the posttranscriptional level (Czarnecki and Grimm 2012).

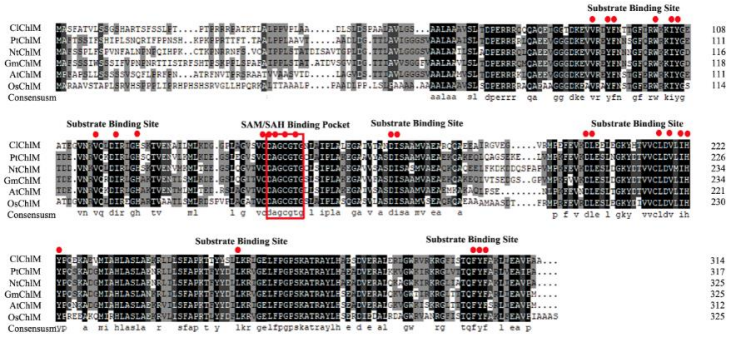

Fig. 1: Multiple sequence alignment of the ClChlMand ChlMs fromother five species. ClChlM: MG574594 for *C. longibracteatum*; PtChlM: XP_002318168 for *Populus trichocarpa*; NtChlM: NP_001313034 for *Nicotiana tabacum*; GmChlM: XP_003532350 for *Glycine max*; AtChlM: NP_849439 for *Arabidopsis thaliana*; OsChlM: XP_015641356 for *Oryza sativa*. The similar amino acid residues arerespectively represented by black and grey shadows. The red box showed SAM/SAH Binding Pocket, and the red circle showed Substrate Binding Site

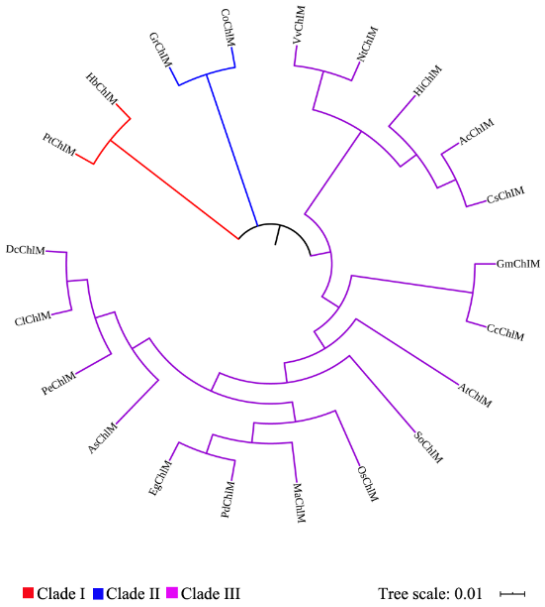

Fig. 2: Phylogenetic analysis of ChlM proteins from 21 plant species. ClChlM: MG574594 for *C. longibracteatum*; PtChlM: XP_002318168 for *Populus trichocarpa*; NtChlM: NP_001313034 for *Nicotiana tabacum*; GmChlM: XP_003532350 for *Glycine max*; AtChlM: NP_849439 for *Arabidopsis thaliana*; OsChlM: XP_015641356 for *Oryza sativa*; PdChlM: XP_008777131 for Phoenix dactylifera; EgChlM: XP_010909956 for *Elaeis guineensis*; AcChIM: PSR89413 for *Actinidia chinensis*; VvChIM: XP_002280872 for *Vitis vinifera*; GrChIM: XP_012467346 for *Gossypiumraimondii*; CsChIM: AVP39683 for *Camellia sinensis*; SoChIM: XP_021844757 for *Spinaciaoleracea*; HbChIM: XP_021673367 for *Heveabrasiliensis*; DcChlM: XP_020691545 for *Dendrobiumcatenatum*; PeChlM: XP_020570902 for *Phalaenopsisequestris*; AsChlM: PKA65777 for *Apostasiashenzhenica*; HiChlM: PIN15818 for *Handroanthusimpetiginosus*; MaChlM: XP_009418736 for *Musaacuminata*; CcChlM: XP_006436954 for *Citrusclementina*; CoChlM: OMO52366 for *Corchorusolitorius*

Lhcb is a light-harvesting antenna protein that is located on the thylakoid membrane of the chloroplast. Its function is to transfer the absorbed light energy to the action centre and start photosynthesis (Crepin and Caffari 2018). In barley, Gadjieva *et al.* found that the accumulation of MgPMe promotes *Lhcb* gene expression (Gadjieva *et al.* 2005). In this study, expression level of the *ClLhcb* gene was notably upregulated in overexpression transformation tobacco lines (Fig. 6). Additionally, compared with the control, the expression levels of several chlorophyll biosynthesis-related genes (*ClGSA*, *ClChlI*, *ClChlH*) were significantly induced, suggesting that chlorophyll biosynthesis is widely activated. This is consistent with the increase in ALA-synthesizing capacity and chlorophyll content in transgenic lines.

Conclusion

In the present study, we isolated a Mg protoporphyrin IX

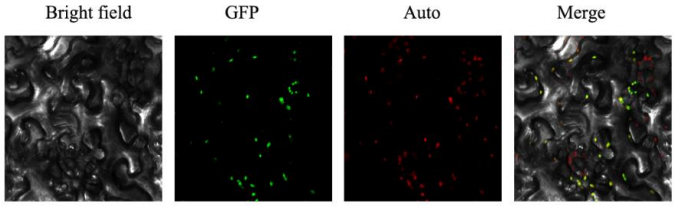

Fig. 3: Subcellular localization of ClChlM. The GFP-ClChlM vector was transiently expressed intobacco leaves, and the fluorescence was detected after 48 h

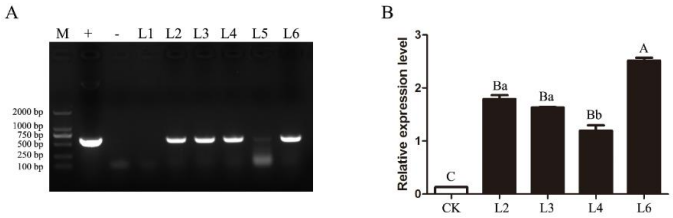

Fig. 4: Overexpression of ClChlM in tobacco. (A) Confirmation ofthe vector in tobacco resistant to kanamycin by PCR. (+), plasmid harboring 35S:ClChlM was used as the positive control; (-), ddH_2O was used as the negative control; L1-L6, six independent transgenic tobacco lines. (B) Confirmation of ClChlM expression in four positivetransgenic tobacco lines (Line 2, Line 3, Line 4, Line 6). CKrepresented transgenic tobacco expressing empty pBI-121 vector

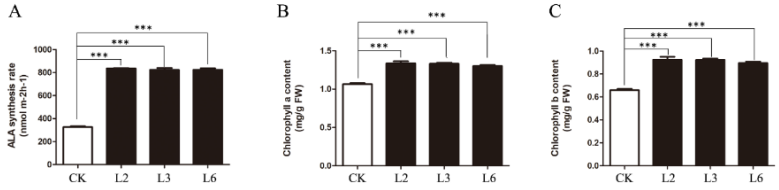

Fig. 5: Determenation of ALA synthesis rate (A)and chlorophyll content (B-C) in transgenic tobacco. The data represented the means of three biological replicates.*** indicated significant differences at $p < 0.001$

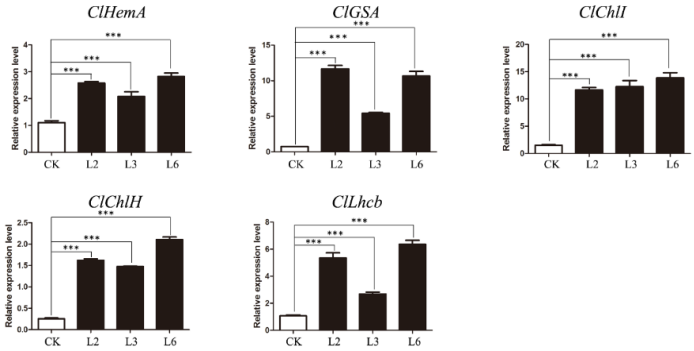

Fig. 6: Expression level of photosynthesis-related genes in CK and the transgenic lines. The data represented the means of three biological replicates.*** indicated significant differences at $p < 0.001$

methyltransferase encoding gene (*ClChlM*) in *C. longibracteatum*. The deduced ChlM contained conserved SAM/SAH binding pocket and substrate binding sites. Subcellular localization analysis of ClChlM showed protein localization in the chloroplast. Ectopic overexpression of ClChlM in tobacco elevated ALA-synthesizing capacity and chlorophyll content and widely upregulated the expression level of photosynthesis-related genes. These results showed that ClChlM plays a crucial role in the regulation of chlorophyll biosynthesis in *C. longibracteatum* and will be helpful in breeding leaf colour variance in the future.

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Author Contributions

YJ conceived and designed the experiments. , Y-QL , JJ and

H-YS performed the experiments. YJ analyzed the data. YJ, H-YS and J-R H wrote the paper. All authors have read and approved the manuscript in its final form.

Conflict of Interest

There is no conflict of interest among the authors and institutions where the research has been conducted

Data Availability Declaration

Primary and supplementary data reported in this article are available with the corresponding authors

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