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A Chlorophyll-Deficient Rice Mutant with Impaired Chlorophyllide Esterification in Chlorophyll Biosynthesis

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ABSTRACT

Chlorophyll synthase catalyzes esterification of chlorophyllide to complete the last step of chlorophyll biosynthesis. Although the chlorophyll synthases and the corresponding genes from various organisms have been well characterized, chlorophyll synthase mutants have not yet been reported in higher plants. In this study, a rice (Oryza Sativa L.) chlorophyll-deficient mutant, yellow-green leaf 1 (ygl1), was isolated, which showed yellow-green leaves in young plants with decreased chlorophyll synthesis, increased level of tetrapyrrole intermediates, and delayed chloroplast development. Genetic analysis demonstrated that the phenotype of ygl1 was caused by a recessive mutation in a nuclear gene. The ygl1 locus was mapped to chromosome 5 and isolated by map-based cloning. Sequence analysis revealed that it encodes the chlorophyll synthase and its identity was verified by transgenic complementation. A missense mutation was found in a highly conserved residue of YGL1 in the ygl1 mutant, resulting in reduction of the enzymatic activity. YGL1 is constitutively expressed in all tissues, and its expression is not significantly affected in the ygl1 mutant. Interestingly, the mRNA expression of the cab1R gene encoding the Chl a/b-binding protein was severely suppressed in the ygl1 mutant. Moreover, the expression of some nuclear genes associated with chlorophyll biosynthesis or chloroplast development was also affected in ygl1 seedlings. These results indicate that the expression of nuclear genes encoding various chloroplast proteins might be feedback regulated by the level of chlorophyll or chlorophyll precursors.
INTRODUCTION

Chlorophyll (Chl) molecules universally exist in photosynthetic organisms. They perform essential processes of harvesting light energy in the antenna systems and driving electron transfer in the reaction centers (Fromme et al., 2003). Their metabolism has been extensively studied in various organisms by both biochemical (Pontoppidan et al., 1994) and genetic approaches (Bollivar et al., 1994a; Nakayashiki et al., 1995; Tanaka et al., 1998). Early enzymatic steps of chlorophyll biosynthesis in converting 5-aminolevulinate acid (ALA) to protoporphyrin IX (Proto IX) are shared with the heme biosynthesis pathway. Many essential data regarding the identity of the associated enzymes were obtained from studies on 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 abundant information on the genes involved in bacteriochlorophyll biosynthesis (Bollivar et al., 1994b), and homologous genes have been isolated from oxygenic plants (Jensen et al., 1996). With the recent identification of 3,8-divinyl protochlorophyllide a 8-vinyl reductase (DVR), all genes required for chlorophyll biosynthesis have been identified in higher plants (Nagata et al., 2005). Analysis of the complete genome of Arabidopsis thaliana show that there are at least 27 genes encoding 15 enzymes involved in chlorophyll biosynthesis from glutamyl-tRNA to Chl b (Nagata et al., 2005).

Chlorophyll synthase is believed to be bound to the thylakoid membranes and to catalyze prenylation of chlorophyllide (Chlide) with geranylgeranyl diphosphate (GGPP) or phytyl diphosphate (PhyPP), the last step of chlorophyll biosynthesis (Rüdiger, 1980; Soll and Schultz,
This step is essential for the accumulation of Chl a (Eichacker et al., 1990, 1992) and is likely essential for stable assembly of other thylakoid membrane components (Paulsen et al., 1990; Rüdiger, 1992, 1993). Detailed investigations of the properties of chlorophyll synthase became feasible after demonstrating that the bacteriochlorophyll synthase gene (*bchG*) of *R. capsulatus* encodes bacteriochlorophyll synthase (Bollivar, 1994b). The recombinant enzyme, produced by expression of *R. capsulatus* bchG in *E. coli*, specifically accepted bacteriochlorophyllide but not Chlide, while expression of the chlorophyll synthase gene (*CHLG*) from *Synechococcus sp. PCC 6803* yielded a chlorophyll synthase that accepted Chlide but not bacteriochlorophyllide. Both enzymes exhibited a marked preference for PhyPP over GGPP (Oster et al., 1997a), however, *A. thaliana* chlorophyll synthase preferred GGPP as the substrate (Oster et al., 1997b). The G4 gene (later named *CHLG*) of *A. thaliana* encoding chlorophyll synthase had previously been isolated and heterologously expressed in *E. coli* (Gaubier et al., 1995; Oster et al., 1997a). Further characterization of the heterologously expressed chlorophyll synthase from oat (*Avena sativa*) revealed the importance of Cys and Arg residues in the enzyme and a requirement for Mg$^{2+}$ ions for its activity (Schmid et al., 2001). Random sequence analysis of expressed sequence tagged (EST) cDNAs from rice yielded a putative chlorophyll synthase homolog (Lopez et al., 1996; Scolnik et al., 1996), however, the biochemical properties and physiological functions remain unknown.

Mutants deficient in chlorophyll synthesis have been identified in a number of multicellular plant species (Killough et al., 1993; Falbel et al., 1996a, 1996b). Their genetic characteristics, microstructures, absorption spectra, fluorescence and physiological properties have been studied systematically (Killough et al., 1993; Falbel et al., 1996a, 1996b; Havaux et al., 1997). However, mutants in the chlorophyll synthase have not been reported. In this study, we isolated a rice chlorophyll-deficient mutant, *ygl1*. The mutant plant exhibits a yellow-green leaf phenotype, decreased level of chlorophyll and delayed chloroplast development. Map-based cloning of the mutation resulted in the identification of the *YGL1* gene, which has a sequence similarity to the chlorophyll synthase gene. The *ygl1* mutant carries a missense mutation (C to T, at residue 592), resulting in an amino acid change (Pro-198 to Ser) in the active region of the enzyme. The mutant phenotype was complemented by transformation with the wild-type gene. Esterification activity of the mutant recombinant protein expressed in *E. coli* was reduced compared to that of wild-type.
This study reports the identification of the first mutant of the last step of chlorophyll biosynthesis in higher plants.

RESULTS

The ygl1 mutant has Reduced Chlorophyll Accumulation and Delayed Chloroplast Development

The ygl1 mutant was a spontaneous mutant isolated from indica rice cultivar, Zhenhui 249, which exhibited a yellow-green leaf phenotype. The ygl1 mutant was slightly smaller than wild-type throughout the developmental stage (Fig. 1 A, B and C) and exhibited reduced levels of Chl a and b as well as carotenoid (Car) content (Table 1). Leaves of the ygl1 mutant had 20 to 70% reduction of Chl, and 30 to 40% reduction of carotenoid levels compared to those in wild-type at different stages, with the most significant differences detectable in 4-week-old plants. The Chl a:b ratio appeared highest at the seedling stage, due likely to the potential of Chl b synthesis in suffering a more severe decline than Chl a. The Chl a:b ratio then declined to eventually reach the wild-type level. Together this suggests that the ygl1 mutant exhibited delayed greening during photomorphogenesis because of slow rates of chlorophyll accumulation. Eventually, mutant plants accumulated substantial quantities of chlorophyll, reaching almost the wild-type levels and becoming slightly yellow with the maturation of leaves.

To investigate how the ygl1 mutation affects chloroplast development, we compared the ultrastructures of plastids in the ygl1 mutant and wild-type plants at different developmental stages using transmission electron microscopy (TEM). Granal stacks in the ygl1 mutant appeared less dense (Fig. 1E) and lacked granal membranes compared to those of wild-type (Fig. 1D) in developing leaves. Granal development in the ygl1 mutant was slower than that of wild-type, and granal membranes in the ygl1 mutant increased when the leaf became mature (data not shown).

The ygl1 Locus Maps to a Putative Gene Encoding Chlorophyll Synthase on Chromosome 5

For genetic analysis of the ygl1 mutant, four F2 populations were constructed from the crosses between the ygl1 mutant and four cultivars, PA64, W002, USSR5 and 02428, All F1 plants from the four crosses displayed wild-type phenotype, and their F2 progenies all showed a segregation ratio of 3:1 (green: yellow-green plants, $\chi^2 < \chi^2_{0.05}=3.84$; $P>0.05$, Table II). Therefore, the yellow-green leaf phenotype in the ygl1 mutant was controlled by a single recessive nuclear gene.
To map the \textit{ygl1} locus, an F$_2$ mapping population was generated from a cross between the \textit{ygl1} mutant with the cultivar PA64. The \textit{ygl1} gene was mapped to an interval between markers RM516 and RM164 on chromosome 5 (Fig. 2A). Comparison of the chromosomal locations and leaf color phenotypes indicated that \textit{ygl1} was a novel gene and different from the previously identified genes related to leaf color alteration (Nagato et al., 1998). To narrow down the search for a candidate gene affected in \textit{ygl1} mutant plants, a larger F$_2$ mapping population consisting of more than 12,000 plants of which 2741 segregants showed the \textit{ygl1} mutant phenotype were used for fine-mapping. Three SSR markers and seven cleaved amplified polymorphic sequence (CAPS) markers were developed (Table III) between markers RM516 and RM164. The \textit{ygl1} locus was mapped to an 11-kb DNA region between the two cleaved amplified polymorphic sequence (CAPS) markers P23 and P8 on a single BAC, AC136221 (Fig. 2B and 2C). Within this region, two open reading frames (ORF) were predicted using the program FGENESH 2.2 (www.softberry.com). The first ORF encoded a putative chlorophyll synthase that shows a high similarity to the oat chlorophyll synthase gene, an enzyme required for Chl a biosynthesis (Schmid et al., 2001). The second ORF was the \textit{Osem} gene, encoding a protein similar to embryonic abundant protein. To define the molecular lesions of the \textit{ygl1} mutant, both candidate ORFs were amplified by RT-PCR from the \textit{ygl1} mutant and wild type plants, respectively, and sequenced. Comparison of the sequences revealed that only the first ORF was altered, exhibiting a single nucleotide mutation at codon 592 (T$\rightarrow$C) in the eighth exon, which resulted in an amino acid change from Pro-198 to Ser (Fig. 3A). Therefore, we tentatively designated the first ORF as the \textit{YGL1} gene.

Searching the rice genome database revealed that \textit{YGL1} is a single copy gene with a 1,131-bp ORF. The coding region of \textit{YGL1} gene is comprised of 15 exons and encodes a 376 amino-acid protein with the molecular mass of approximately 41kDa. \textit{YGL1} contains an apparent chloroplast-targeting sequence of 47 amino acids at its N-terminus. Multiple amino acid sequence alignments showed that \textit{YGL1} had a significant similarity to the representatives from particular classes of chlorophyll and bacterchlorophyll synthases from different organisms (Fig. 3A). For example, \textit{YGL1} is 88.39% identical to chlorophyll synthase from \textit{A. sativa} (Schmid et al., 2001), 74.68% identical to the \textit{A. thaliana} G4 gene product (Oster et al., 1997b), and 53.94% identical to the \textit{Synechocystis sp. PCC 6803} enzyme (Kaneko et al., 1995). Moreover, \textit{YGL1} is also

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Gene} & \textbf{Organism} & \textbf{Identity} & \textbf{Reference} \\
\hline
\textit{YGL1} & \textit{A. sativa} & 88.39\% & Schmid et al., 2001 \\
\hline
\textit{Osem} & \textit{A. sativa} & 88.39\% & Schmid et al., 2001 \\
\hline
\textit{YGL1} & \textit{A. thaliana} & 74.68\% & Oster et al., 1997b \\
\hline
\textit{YGL1} & \textit{Synechocystis sp. PCC 6803} & 53.94\% & Kaneko et al., 1995 \\
\hline
\end{tabular}
\caption{Similarity of \textit{YGL1} to other chlorophyll synthases.}
\end{table}
homologous (23 to 30%) to various bacteriochlorophyll synthases (Bollivar et al., 1994a, 1994b; Addlesee et al., 2000; Xiong et al., 1998; Lopez et al., 1996).

We then analyzed the possible phylogenetic relationships between YGL1 and its related proteins from higher plants and cyanobacteria (Fig. 3B). The result indicated that rice YGL1 was more closely related to chlorophyll synthase from monocotyledon plants oat (A. sativa) than to those of other species. Not surprisingly, YGL1 has a phylogenetically much closer relationship to chlorophyll synthases of the higher plant species than to bacteria proteins. In addition, it is interesting to note that bacteriochlorophyll synthases lack a motif (WAGHDF-197) that exists only in the chlorophyll synthase (Supplementary Fig. 1). Analysis with the transmembrane calculation programs (Nilsson et al., 2002) revealed that the ygl1 mutation site occurred at or close to the end of a transmembrane helix (data not shown).

The identity of ygl1 was subsequently confirmed by genetic complementation experiments (Fig. 4A). The color of leaves, the levels of Chl and the ratio of Chl a:b were all restored to levels of wild-type plants upon transformation with the YGL1 gene (Fig. 4B and 4C). Therefore, this confirms that observed abnormal phenotypes of the ygl1 mutant plants resulted from mutation of the YGL1 gene.

**YGL1 mRNA Expression Level Is Not Affected by the ygl1 Mutation of YGL1**

We compared the level of YGL1 transcript in ygl1 mutant and wild-type plants using RT-PCR. Figure 5A showed that YGL1 mRNA was expressed at similar levels in root, leaf sheaths, leaves and young panicles in both the ygl1 mutant and wild-type. We also examined the effect of light and dark growth conditions on the expression of YGL1. No change in transcript levels was observed when ygl1 or wild-type plants were grown under light or dark conditions (Fig. 5B). Furthermore, no significant differences of YGL1 mRNA levels were observed in the mutant compared to wild-type from early to mature stages (Fig. 5C). These results indicate that the missense mutation of ygl1 does not affect its own mRNA expression.

We next addressed the question of whether the ygl1 mutation affected the transcript of other genes associated with chlorophyll biosynthesis, chloroplast development or photosynthesis. Analysis of mRNA levels using real-time PCR showed that the expression of genes involved in chlorophyll biosynthesis, such as glutamyl tRNA reductase (HEMA1), was reduced by about 40%, and chlorophyllide a oxygenase (CAO1) and NADPH:protochlorophyllide oxidoreductase
(PORA) were slightly reduced in ygl1 mutant seedlings compared with wild-type (Fig. 6).

Interestingly, the expression of cab1R, which encodes the light-harvesting Chl a/b binding protein of PS II (Matsuoka, 1990), was severely suppressed, whereas another cab gene, cab2R, showed only slightly decreased mRNA levels in the ygl1 mutant. The expression levels of plastid genes, psaA and psbA encoding two reaction center polypeptides and rbcL encoding the large subunit of ribulose-1, 5-bisphosphate carboxylase (Rubiso), were not significantly reduced in the ygl1 mutant. However, the expression of the nuclear rbcS gene encoding the small subunit of Rubiso (Kyozuka et al., 1993) was slightly decreased in the ygl1 mutant (Fig. 6). Taken together, it is likely that that the ygl1 mutation affected the transcript of most nuclear genes, such as cab1R, HEMA1, CAO1 etc., but not the expression of plastid-encoded genes including psaA, psbA and rbcL in the ygl1 mutant.

Single Amino Acid Change Causes a Reduction in Chlorophyll Synthase Activity

We then examined whether the Pro-198 to Ser amino acid substitution in ygl1 impaired enzymatic function of the ygl1 mutant. An in vitro assay was used to compare the esterification activity of the recombinant chlorophyll synthase enzymes produced from E. coli using Chlide a along with two different substrates, GGPP and PhyPP (Oster et al., 1997a). The result showed that the esterification activity of the recombinant mutant ygl1 exhibited approximately 35.22% and 21.75% esterification of Chlide a with GGPP and PhyPP, respectively, compared to wild-type recombinant enzymes YGL1, whose activity was set at 100% (Fig. 7A).

The rate of chlorophyll accumulation was next compared in the ygl1 mutant and wild-type seedlings. The seedlings were grown in darkness for one week, chlorophyll content and Chl a:b ratio were measured after exposure to white light at various times. The results showed that the rate of chlorophyll accumulation was slower in the ygl1 mutant than in the wild-type (Fig. 7B). The ratio of Chl a:b was lower in the ygl1 mutant than in wild-type initially, and it became higher after two days. Notably, the peak value at two days in ygl1 mutant was substantially more than the peak value of wild-type at five hours (Fig. 7C). One possible explanation is that Chl a synthesis becomes limiting and Chl a preferentially assembles reaction centers (RCs) in the ygl1 mutant; once the RCs were no longer incorporating the majority of Chl a, Chl b was produced in significant amounts only when there was leftover Chl a (Falbel et al., 1996a, 1996b). These data
suggest that aberration of chlorophyll synthase function results in decrease of chlorophyll a
synthesis in the ygl1 mutant.

Reduced Activity of Chlorophyll Synthase Results in Accumulation of Intermediates of
Chlorophyll Biosynthesis

When angiosperm plants were grown in dark conditions, protochlorophylide (Pchlide)
accumulated instead of chlorophyll and plants had an etiolated phenotype (Schoch et al., 1977,
1978). Mock and Grimm (1997) characterized transgenic plants with deficiencies in
coproporphyrinogen oxidase and uroporphyrinogen decarboxylase, two preceding enzymes in the
metabolic pathway of chlorophyll synthesis. These plants accumulated their respective substrates,
uroporphyrin(ogen) and coproporphyrin(ogen), in young leaves up to several hundred-fold times
the levels in control plants and exhibited necrotic lesions. Since the ygl1 mutant had deficient
chlorophyll synthase activity, Chlide was predicted to accumulate in ygl1 mutant plants. Not
surprisingly, compared to wild-type plants, ygl1 mutants accumulated higher level of Chlide and
other intermediates, including ALA (Fig. 8A), Proto IX, Mg-protoporphyrin IX (Mg-Proto IX),
and Pchlide, in leaves of seedlings (Fig. 8B). Together these results showed that chlorophyll
synthase play a critical role in chlorophyll biosynthesis.

DISCUSSION

Chlorophyll synthase has been the subject of thorough investigation (for reviews, see Willows,
2003; Suzuk, 1997). However, to our knowledge, no corresponding chlorophyll synthase mutant
has been found in higher plants. In this study, we have identified and functionally characterized
the chlorophyll synthase mutant, ygl1, of rice at the molecular level.

Lopez et al. (1996) described bacteriochlorophyll and chlorophyll synthases as belonging to the
family of polyprenyltransferases, and suggested a homologous region (domain II) to be the
binding site of the polyprenyl PP. Multi-alignment analysis showed that this region spans residues
140 to 162 in the YGL1 sequence (Fig. 3A). Oster found that the esterification activity of oat
chlorophyll synthase was lost when the residue of His-197 (His-195 in YGL1) was mutated,
presumably due to His-197 possible overlap with the Mg$^{2+}$ binding site from Chlide (Personal
communication). A point mutation (Pro-198 to Ser) in YGL1 was found at the highly conserved
Pro-198, which compromised the esterification activity of chlorophyll synthase (Fig. 7A).
Analysis of YGL1 derived amino acid sequence showed that Pro-198 was in proximity to a motif (WAGHDF-197) specifically found only in chlorophyll synthases, but not in bacteriochlorophyll synthases (Supplementary Fig. 1), which differ, in part, based on preference of substrates, either Chlide (targeted by chlorophyll synthases) or bacteriochlorophyllide (for bacteriochlorophyll synthases) (Oster et al., 1997a). Therefore, the importance of the essential Pro-198 residue in YGL1 could be attributed to its location in or proximity to the binding site of Chlide. Future studies to pursue this possibility would help further elucidate the substrate specificity and targeting mechanisms between the synthase family members.

In this study, the ygl1 mutant seedlings displayed a yellow-green phenotype and became green with leaf chlorophyll accumulation at the mature stage (Table I). The carotenoid content was significantly lower in the mutant plants compared to wild-type, even in older leaves in which the chlorophyll content was the same as wild-type (Table I). This result might be related to the parallel degradation of pigments and pigment binding proteins of the photosynthetic apparatus (Cunningham FX et al. 1998; Papenblock et al., 2000).

Mutation of the YGL1 gene reduced chlorophyll levels, and resulted in a yellow-green phenotype more or less specific to younger plants. Why the ygl1 mutation affects chlorophyll biosynthesis most dramatically in the early developmental stage but is restored in later stages is not yet completely understood. One possible explanation is that there might be other chlorophyll synthase homologs with redundant functional activities in later stages. However, no other rice chlorophyll synthase genes were identified from a survey of rice genome database (International Rice Genome Sequencing Project, 2005). These results were consistent with those of Gaubier et al. (1995) and Schmid et al. (2001), which showed that the chlorophyll synthase sequence represented a single-copy gene in A. thaliana and A. sativa by Southern and Northern blot analysis. Since we did not find significant differences in transcription level of the YGL1 gene at the different development stages, one possibility is that the enzyme is regulated at the translational level. This hypothesis remains to be tested directly.

Moreover, the delayed chloroplast development might lead to a slow accumulation of chlorophyll in ygl1 mutant seedling leaves. Chlorophyll synthase was proposed to localize to the thylakoid membranes where esterification of Chlide a with phytol or earlier alcohol precursors take place (Rüdiger, 1980; Soll and Schultz, 1981; Block et al., 1980). Soll et al. (1983) showed...
that the chlorophyll synthase in chloroplasts is more stable than those from etioplasts. This implied that Chl a biosynthesis catalyzed by chlorophyll synthase was associated with chloroplast development (Biswal et al., 2003; El-Saht, 2000). Therefore, chlorophyll deficiency caused by the ygl1 mutant might be due to delayed formation of thylakoid membranes, and the underdeveloped chloroplast led to the decrease of chlorophyll accumulation in ygl1 seedlings stage.

Previous reports indicated that the transcript of the *A. thaliana* G4 gene was detected only in green or greening tissues, and its expression was not strictly light-dependent, while oat CHLG gene was constitutively expressed (Gaubier et al., 1995; Schmid et al., 2001). Our experiments showed that rice YGL1 was constitutively expressed, which is consistent with trends observed for oat CHLG (Schmid et al., 2001). Another notable observation was the effect of the YGL1 mutation on the mRNA expression of some genes associated with chlorophyll biosynthesis or chloroplast development. Among the genes examined, we found that the expression of most nuclear genes, including *cab1R*, *HEMA1*, *CAO1* and *PORA*, were reduced at different levels, whereas the plastid-coded genes, such as *psaA*, *psbA* and *rbcL*, were not significantly influenced in ygl1 seedlings (Fig. 6). This suggests, then, that the expression of the plastid-encoded genes might be regulated at the level of translation rather than transcription.

In the ygl1 mutant, the transcript level of *cab1R* gene was severely impaired and markedly different from that of *cab2R*, which was only slightly decreased at the young seedling stage (Fig. 6), indicating that both are differentially regulated (Matsuoka, 1990). Although the expression of the nuclear multi-gene (*cab*) family was a marker for chloroplast development and tightly controlled by both light and plastid signals, including a circadian clock, hormones, and sucrose levels (Karlin-Neumann et al., 1988; Flores et al., 1986; Millar et al., 1996; Dijkwel et al., 1997), it was not clear whether the expression of the *cab* gene might be an indirect consequence of chlorophyll deficiency, or, even more plausibly, of the feedback regulation by plastid signals from the accumulation of higher levels of chlorophyll precursors in the ygl1 mutant. Elucidation of the mechanism would provide greater understanding towards cellular signaling and feedback mechanisms between nucleus and plastids.

*CAO* was previously considered to be the only enzyme responsible for Chl b synthesis. Recombinant CAO had been shown to convert Chlide a into Chlide b, most likely by a two-step oxygenation (Oster et al. 2000), with chlorophyll synthase adding a hydrophobic phytol tail to
produce Chl a and Chl b (Oster et al. 1997). The ratio of Chl a:b was reported to correlate with
*CAO* mRNA levels in *A. thaliana* (Harper et al., 2004) and *Dunaliella salina* (Masuda et al.,
2002; 2003b). However, our studies show that *CAO* mRNA was slightly decreased and the Chl
a:b ratio was largely increased (from 3.63 to 7.91, Table I) in the *ygl1* mutant, suggesting that
*CAO* activity might be regulated at the post-transcriptional level (Tanaka et al., 2001).

Chl a is required for the formation of photosynthetic reaction centers and light-harvesting
complexes (LHC), and Chl b is exclusively located in the light-harvesting pigment protein
complexes of PS I and PS II. An appropriate ratio of Chl a:b is critical in the regulation of
photosynthetic antenna size (Jansson, 1994; Oster et al., 2000; Tatsuru et al., 2003). However,
partial block in Chl a biosynthesis caused a decrease of the chlorophyll content and an increase in
the ratio of Chl a:b in young leaves of the *ygl1* mutant (Falbel et al., 1996a), indicating that the
total number of photosystems decreased and light-harvesting antenna complexes might be lower
than that of wild-type. Further characterization of the *YGL1* gene could provide deeper insight
into understanding the relationship between the biosynthesis of Chl a and Chl b, and between the
biosynthesis of chlorophyll, carotenoids and proteins, the regulation of photochemical reactions,
as well as the assembly of the thylakoid membranes and chloroplast development.

**MATERIALS AND METHODS**

**Plant Materials**

The rice (*Oryza sativa*) yellow green leaf mutant (*ygl1*) was isolated from the *indica* cultivar
Zhenhui249. The *ygl1* was crossed with an *indica* rice cultivar, PA64, to construct the F2 mapping
population. PA64 has a major genetic background of *indica* and minor gene flows from *javanica*
(Bao et al, 2005).

**Genetic Analysis and Marker Development**

Genomic DNA was extracted and analyzed for co-segregation using available SSR (McCouch
et al., 2002) from F2 plants. New SSR markers were developed based on the Nipponbare genome
sequence information from NCBI database and by searching for simple repeat sequences with the
SSRIT program (Temnykh et al., 2001). CAPS markers were developed on comparisons of
original or CAPS length by using SNP2CAPS soft (Thiel et al., 2004) between the *indica* var.
PA64s (Yu et al., unpublished data) and *indica* var. 9311 according to the data published in
http://www.ncbi.nlm.nih.gov.
Sequence Analysis

The full-length CHLG protein sequences were retrieved from GenBank and used for phylogenetic analysis according to the methods described by Li et al. (2003). The signal peptide was predicted with SignalP version 2.0 (Nielsen et al., 1998). Phylogenetic analysis and Multiple sequence alignment were conducted by using DNAMAN version 6.0 (Lynnon Biosoft). The residue-specific hydropathy index was predicted by using the transmembrane calculation programs (Nilsson et al., 2002).

Complementation of the ygl1 Mutant

As Agrobacterium-mediated transformations are difficult to perform in indica rice, the ygl1 gene was also transferred to Wuyunjing 8 (spp. japonica) by five rounds of backcrosses with Wuyunjing 8 and self crossed for five generations. We obtained an isogenic line with ygl1 allele in japonica genetic background and named it as ygl2, which was used as transforming material.

For complementation of the ygl1 mutation, a full-length cDNA fragment encoding YGL1 was amplified by RT-PCR using the primer 5'-AATGCAGAGTCTCCAATGGCCACCTC-3' and 5'-GGACTAGTGCTTTCATCAGTGCTGTT-3' from the wild type. The primers incorporated a PstI site at the N-terminal end and a SpeI site at the C-terminal end of the ORF. PCR products were cloned into the pMD18-T vector (TaKaRa). Then the YGL1 cDNA fragment from wild type was digested with PstI and SpeI and ligated into the PstI and SpeI sites of a binary vector pCUbi1390 (Lu, unpublished data) harboring a hygromycin-resistant gene. The resulting pCUYGL1 plasmid, which contained the YGL1 coding sequence driven by the ubiquitin promoter, was transformed into Agrobacterium tumefaciens strain EHA105 by electroporation, and transformed to japonica rice ygl2 for complementation testing according to a published method (Hiei et al., 1994).

Analysis of RT-PCR and Real-time PCR

Total RNA was extracted from leaves, leaf sheaths, young panicles and roots according to the method described by Wadsworth et al. (1988). cDNA synthesis was performed using 5μg total RNA for each sample. RNA was treated in 1X buffer with 5U of DNase I (MBI Fermentas) added to the reaction and incubated for 30min at 37°C. The reaction was stopped by adding 1μL of 25mM EDTA, followed by 10min incubated at 65°C. For reverse transcription (RT)–PCR, first-strand cDNA was reverse transcribed from total RNA with oligo (dT) and AMV reverse transcription.
transcriptase (TaKaRa). Amplification of ygl1 and YGL1 cDNA (GenBank accession: EF432576) was carried out with specific primers (forward primer, 5'-CAGTCTCCAATGGCCACCT-3'; reverse primer 5'-TGCTTTTCATCAGTGGCTGTG-3'). PCR products were cloned into the pMD18-T vector (TaKaRa). The level of gene expression was analyzed by real time PCR, and included HEMA1 (GenBank accession: J013000F15) for glutamyl-tRNA reductase, PORA (GenBank accession: NM 197169) for protochlorophyllide oxidoreductase, CAO1 (GenBank accession: J013116K15) for chlorophyllide a oxygenase, cab1R (X13908) and cab2R (X13909) for chlorophyll a/b binding protein from rice, psaA (AAS46121) and psbA (GenBank accession: AAS46104) for two reaction center polypeptides, rbcL (Hirai et al., 1985; GenBank accession: AAS46127) for large subunit of ribulose-1, 5-bisphosphate carboxylase, and rbcS (GenBank accession: X07515). The sequences of the PCR primers are as follows: HEMA1, 5' CGCTATTTCTGATGCTATGGGT 3', 5' TCTTGGGTGATGAT TGTTTGG 3'; PORA, 5' TGTACTGGAGCTGGAACAACAA 3', 5' GAGCACAGCAAAATCCTAGACG 3'; CAO1, 5' GATCCATACCCGATCGACAT 3', 5' CGAGAGACATCCGGTAGAGC 3'; cab1R, 5' AGATGGGTTTAGTGCGACGAG 3', 5' TTTGGGATCGAGGGAGTATTT 3'; cab2R, 5' TGTCTCCATGTTCCGGCTCT 3', 5' GCTACGGTCCCCACTTCACT 3'; psaA, 5' GCGAGCAAATAAAACACCTTC 3', 5' GTACCAGCTTAACGTGGGGAG 3'; psbA, 5' CCCTCATTAGCAGATTGTTTTT 3', 5' ATGATTGTATTCCAGCGAGC 3'; rbcL, 5' CCTGGCAGCTTTCCGGCAGTAA 3', 5' ACAACGGGGCTCGATGTGATA 3'; rbcS, 5' TTCGCTGAGTTTTGGCTATTT 3', 5' GGACTTGGAGCCCTGGAAGG 3'. Actin (GenBank accession: X15865) was used for normalization as a control. Primers for Actin: 5' AGGAAGGCTGGAAGGGACC3', 5' CGGGAAATTGTGAGGGACAT 3'. PCR was carried out in a total volume of 25μL containing 0.2μM of each primer and 1X SYBR green PCR master mix (PE Applied Biosystems). Reactions were amplified in a BIO-RAD iCycler as follows: 95°C for 10 min, then 40 cycles of 95°C for 20 sec, 60°C for 20 sec, 72°C for 30 sec. The 2^ΔΔCT method was used to calculate relative changes in gene expression as described (Livak et al., 2001).

**Recombinant Enzymes Activity Assays**

Both YGL1 and ygl1 full-length cDNAs were isolated by RT-PCR from the total RNA from ygl1 and wild-type leaves with the RT-PCR system (TaKaRa) using primer 1
(5’CGCGGATCCCAGTCTCCAATGGCCACCT3’) and primer 2 (5’CCCAAGCTTTGCTTTCA
TCAGTGGCTGGT3’). The primers incorporated a BamHI site at the N-terminal end and a
HindIII site at the C-terminal end of the ORF. The PCR products were inserted into pMD18-T
vectors and sequenced to obtain the correct clones, pMDYGL1 and pMDygl1. The pMDYGL1
and pMDygl1 plasmids were then digested and cloned into the corresponding site of the bacterial
expression vector pET28-a(+) (Novagen) to generate pETYGL1 and pETYgl1, sequenced to
confirm YGL1 and ygl1 sequences, respectively, then introduced into E. coli BL21 for protein
expression. Protein expression and recombinant enzyme activity assays was according to the
method as described by Schmid et al. (2001).

**Pigment and Chlorophyll Precursor Determination**

Total chlorophyll and carotenoids were determined with DU 800 UV/Vis Spectrophotometers
(Beckman Coulter, Inc.) according to the method of Arnon (1949). Determination of ALA content
was based on Richard methods (1975). The precursors, including Proto IX, Mg-Proto IX, Pchlide
and Chlide, were assayed as described by Santiago-Ong (2001) and Masuda (2003a). Leaves
(approximately 30mg fresh weight) of wild-type and ygl1 mutant were cut and homogenized in 5
mL 9:1 acetone:0.1 m NH4OH, and centrifuged at 3,000g for 10 min. The supernatants were
combined and washed successively with an equal volume of hexane three times prior to
spectrophotometric analysis. Chlorophyll precursors in the acetone phase were quantified with a
Hitachi F-4500 fluorescence spectrophotometer (Tokyo, Japan) using Ex400:Em632 for
Protoporphyrin IX, Ex440:Em633 for protochlorophyllide, Ex440:Em672 for chlorophyllide and
Ex420:Em595 for Mg-protoporphyrin.

**Transmission Electron Microscopy (TEM) analysis**

Wild-type and ygl1 mutant leaf samples were harvested from one week and one-month-old
plants grown in a greenhouse at medium light intensity (~150μmol photons m⁻² s⁻¹). Leaf sections
were fixed in a solution of 2% glutaraldehyde and further fixed in 1% OsO4. Tissues were stained
with uranyl acetate, dehydrated in ethanol, and embedded in Spurr’s medium prior to thin
sectioning. Samples were stained again and examined with a JEOL (Tokyo) 100 CX electron
microscope.

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FIGURE LEGENDS

Figure 1. Phenotypic characterization of the rice ygl1 mutants. A, 4-week-old plants. B, 10-week-old plants. C, 15-week-old plants. D, Chloroplasts of the first leaf from top to base have abundant, well-ordered stacks in 4-week-old wild-type. E, Chloroplasts of the first leaf in 4-week-old ygl1 mutant have few or no membrane stacks and only occasional long, parallel and unstacked membranes in worse order than in wild-type. Examples of chloroplast (Cp), plastoglobule (Pg) and mitochondrion (Mt). Bar equals 0.5 μm.

Figure 2. Map-based cloning of the ygl1 locus. The map was constructed based on the publicly available sequence of rice chromosome 5. Seven CAPS markers (P5, P8, P11, P20, P23, P25 and P26) were produced during this study, while three SSR markers RM516, RM5454 and RM3838 were obtained from the public database, and SSR markers y1, y5 and y22 were developed in the work. A, The ygl1 locus was mapped to a region between markers RM516 and RM5454 on the long arm of rice chromosome 5 (Chr.5) with 252 recessive individuals. B, Fine mapping of the ygl1 locus between y1 and RM3838 from a segregating population of 2741 recessive individuals. Two BAC contigs (AC144742 and AC136221) cover the ygl1 locus. C, The ygl1 gene was narrowed down to an 11-kb genomic DNA region between the CAPS markers P23 and P8, and co-segregated with P25 and P26.

Figure 3. Sequence analysis of YGL1 homologs. A, Alignment of the derived amino acid sequence of O.sativa with published chlorophyll and bacteriochlorophyll synthase sequences.
Identical residues are boxed in black, similar residues are highlighted in grey. The Pro-198 to Ser change is indicated with an asterisk at the mutation site of ygl1. Domain II, suggested to be the binding site of the polyprenyl PP, is underlined. GenBank accession numbers for the respective protein sequences are *Oryza sativa* (OsYGL1, ABO31092); *Avena sativa* (AsCHLG, AJ277210); *Arabidopsis thaliana* (AtCHLG, At3G51820); *Synechooystis sp. PCC 6803* (SCHLG, BA000022); *Rhodobacter capsulacus* (RcbchG, CAA77532); *Rhodobacter sphaeroides* (RsbchG, CP000143); *Heliobacillus mobilis* (HmbchG, AAC84024); and *Chloroflexus aurantiacus* (CabchG, AAG15227). B, A phylogenetic tree representing alignment of YGL1 proteins. The Rooted tree using percentage identities is based on a multiple sequence alignment generated with the program DNAMAN. Scale represents percentage substitution per site.

**Figure 4.** Complementation of the *ygl1* mutant by wild-type gene. The *japonica* rice *ygl2* with *ygl1* allele was used as transforming material (see MATERIALS and METHODS). A, Phenotypes of the wild-type, the *ygl2* mutant, and the transgenic plant, *ygl2* (*ygl1*)/YGL1. Photographs were taken two weeks after sowing. B, Total Chl levels of wild-type, *ygl2* mutant, and *ygl2*(*ygl1*)/YGL1. Chl was extracted from the second leaf of 2-week-old plants. C, Chl a:b ratio calculated from (B). Error bars represent standard deviation (±SD), and representative data from three independent experiments are presented.

**Figure 5.** Expression analysis of YGL1 by RT-PCR. Total RNA was extracted from root (R), leaf sheath (S), leaf (L) and young panicle (P) of wild-type and *ygl1* mutant. A, Expression patterns of YGL1 in root (R), leaf sheath (S), leaf (L) and young panicle (P) of wild-type and *ygl1* mutant. B, *YGL1* expression in wild-type and the *ygl1* mutant leaves of 2-week-old plants grown in dark or under light.. C, *YGL1* expression in wild-type and *ygl1* mutant leaves of 4, 10 and 15-week-old plants. RT-PCR was repeated three times, and representative results (25 cycles) are shown. *Actin* was amplified as a control.

**Figure 6.** Expression analysis of genes associated with chlorophyll biosynthesis, photosynthesis or chloroplast development by real time PCR. Total RNA was extracted from leaves of 4-week-
old (4w) plants. *Actin* was amplified as a control. Error bars represent standard deviation (±SD) and representative data from three independent experiments are presented.

**Figure 7.** Activity of recombinant proteins and time course of Chl accumulation. A, Activity of recombinant YGL1 and ygl1 proteins in *E.coli*. Total enzyme activity was determined using the extracts from the induced bacterial cells with Chlde a plus PhyPP or Chlde a plus GGPP. Equal amounts of protein were used. B, Time course of chlorophyll accumulation. C, Time course of Chl a:b ratio based on the results in B. The seedlings were grown in darkness for one week, chlorophyll content and Chl a:b ratio was measured after exposure to white light for various times as indicated. Error bars represent standard deviation (±SD) from three independent experiments are presented.

**Figure 8.** Analysis of chlorophyll intermediates in wild-type and *ygl1* mutant. Chlorophyll intermediates were measured in second leaf from 2-week-old wild-type and *ygl1* mutants. A, Levels of ALA. B, Relative fluorescence of Proto IX, Mg-proto IX, Pchlide and Chlide. Error bars represent standard deviation (±SD) and representative data from three independent experiments are presented.

**Supplementary Figure 1.** Clustal W alignment of amino acid sequences surrounding the substituted residue of YGL1 paralogs and bacterial chlorophyll synthase orthologs. Accession number from expasy server (http://www.expasy.ch/); synthase type B and C indicate bacteriochlorophyll and chlorophyll synthase, respectively. Motif only in chlorophyll synthase is underlined; the arrow indicates the Pro-198 to Ser change in the *ygl1* mutant.
Table I. Pigment contents in leaves of wild type and ygl1 mutant, in mg.g⁻¹ fresh weight

| Growth stage   | Total Chl | Chl a:b ratio | Car   |
|----------------|-----------|---------------|-------|
| 4-week-old     | WT        | 4.84±0.16     | 3.63±0.01 | 0.54±0.04 |
|                | ygl1      | 1.53±0.01     | 7.91±0.01 | 0.35±0.01  |
| 10-week-old    | WT        | 3.55±0.29     | 3.46±0.02 | 0.67±0.04  |
|                | ygl1      | 2.60±0.03     | 4.38±0.08 | 0.40±0.00  |
| 15-week-old    | WT        | 3.32±0.08     | 3.13±0.04 | 0.57±0.04  |
|                | ygl1      | 2.97±0.06     | 3.35±0.01 | 0.40±0.01  |

*Chl and Car were measured in acetone extracts from 2nd leaf of different growth stages from top. Values shown are the mean standard deviation (±SD) from five independent determinations.
Table II. Segregation of F2 populations from four crosses

| Cross               | ygl1/PA64 | ygl1/W002 | ygl1/USSR5 | ygl1/02428 |
|---------------------|-----------|-----------|------------|------------|
| Numbers of Green plants\(^b\) | 263       | 152       | 810        | 155        |
| Numbers of Yellow plants          | 89        | 43        | 251        | 48         |
| Total numbers             | 352       | 195       | 1061       | 203        |
| \(\chi^2\)               | 0.50      | 0.75      | 0.95       | 0.13       |
| \(P\)                     | 0.49      | 0.41      | 0.34       | 0.72       |

\(^a\)The female partner for the cross. \(^b\)Green plants and yellow plants were determined by visual inspection.

\(^c\)\(P>0.05\) considered as significant.
Table III. The PCR-based molecular markers designed for fine mapping

| Markers | Primer pairs | Fragment size (bp) | Restriction enzyme | Originated BAC |
|---------|--------------|--------------------|--------------------|----------------|
| y1      | 5’GCCTGTTGAAAGCGTCGTA3’ 5’AGGGTGCTGAGTCACAATAGGT3’ | 116 |  | AC144742 |
| y5      | 5’CCCAAAACTAATTTCTCTCT3’ 5’ACATCTGTAACCAATCTCTCC3’ | 100 |  | AC136221 |
| y22     | 5’CTGCCCTTGAATAATGACG3’ 5’CGACTGATCGATCTCC3’ | 177 |  | AC104281 |
| P5      | 5’GGCTAGTTATGGGGTGGTA3’ 5’CCCTTTTCAAATCTAGGGA3’ | 709 | XbaI | AC136221 |
| P8      | 5’CGTGACTAGTTGAGCAGCTT3’ 5’GGTACAGCCAGCCAGGAG3’ | 922 | TaqI | AC136221 |
| P11     | 5’TGTAGCGCCGCATGTGTTA3’ 5’CCGGTGAGTTGCAGAT3’ | 934 | HapII | AC136221 |
| P20     | 5’GCAGTTATGGAAGTCTACG3’ 5’ATTACATCTAACGTGCAAGTC3’ | 685 | NsiI | AC136221 |
| P23     | 5’CAACCAACTCAAGCTCTTT3’ 5’ATATTTCCTCCCTACCCA3’ | 197 | TaqI | AC136221 |
| P25     | 5’GGATAGGCTCCAAACAAC3’ 5’GGGAAATCACAAGAAAGCA3’ | 834 | StyI | AC136221 |
| P26     | 5’CTCTTCACCTAAAGGTCCACA3’ 5’TCTTCAGCATTTGGAGCA3’ | 740 | MfeI | AC136221 |
Figure 4

B

C

mg g⁻¹ FW

WT

ygl2

ygl2(ygl1)/YGL1

Chl

0.00

2.00

4.00

6.00

8.00

WT

ygl2

ygl2(ygl1)/YGL1

Chl a:b

ratio

0.00

2.00

4.00

6.00

8.00
Figure 5

A  
\[
\begin{array}{cccc|cccc}
R & S & L & P & R & S & L & P \\
ygl1 & WT & & & WT & & & \\
\end{array}
\]

B  
\[
\begin{array}{cccc|cccc}
\text{dark} & \text{light} & \text{ygl1} & WT & \text{ygl1} & WT & \\
\end{array}
\]

C  
\[
\begin{array}{cccc|cccc}
4w & 10w & 15w & \text{ygl1} & WT & \text{ygl1} & WT & \text{ygl1} & WT \\
\end{array}
\]
Figure 6

[Bar chart showing relative expression of various genes in WT and ygl11 strains]
Figure 8

A

B

WT

ygl1

ALA

µg.mg⁻¹ FW

0.00

1.00

2.00

3.00

4.00

5.00

ProtoIX

Mg-ProtoIX

Pchlide

Chide

relative fluorescence

0.00

10.00

20.00

30.00

40.00

WT

yglI

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