RESEARCH PAPER

**PGL, encoding chlorophyllide a oxygenase 1, impacts leaf senescence and indirectly affects grain yield and quality in rice**

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Abstract

Chlorophyll (Chl) b is a ubiquitous accessory pigment in land plants, green algae, and prochlorophytes. This pigment is synthesized from Chl a by chlorophyllide a oxygenase and plays a key role in adaptation to various environments. This study characterizes a rice mutant, pale green leaf (*pgl*), and isolates the gene *PGL* by using a map-based cloning approach. *PGL*, encoding chlorophyllide a oxygenase 1, is mainly expressed in the chlorenchyma and activated in the light-dependent Chl synthesis process. Compared with wild-type plants, *pgl* exhibits a lower Chl content with a reduced and disorderly thylakoid ultrastructure, which decreases the photosynthesis rate and results in reduced grain yield and quality. In addition, *pgl* exhibits premature senescence in both natural and dark-induced conditions and more severe Chl degradation and reactive oxygen species accumulation than does the wild-type. Moreover, *pgl* is sensitive to heat stress.

Key words: Chlorophyll b, chlorophyllide a oxygenase, leaf senescence, pale green leaf, rice.

Introduction

Chlorophyll (Chl) is a green pigment found in cyanobacteria and the chloroplasts of algae and plants. Chl is an essential component of photosynthetic apparatuses and plays critical roles in plant development (Zhen et al., 2014). Aside from providing green color, Chl is also extremely important in photosynthesis because it plays essential roles in harvesting light energy and converting it to chemical energy (Fromme et al., 2003). Increasing the Chl content in rice is regarded as an important approach to enhancing the photosynthesis rate (Huang et al., 2013) to drive the accumulation of more photo-assimilates and ultimately increase crop yield (Bansal et al., 1999; Mitchell and Sheehy, 2006). Hence, breeders strive to develop plants with a long stay-green period to increase the yield potential of rice.

Two Chl species are present in higher plants: Chl a and Chl b. Most Chls are assembled with apoproteins to form...
Chl-protein complexes in plant leaves. Chl a is a component of both photosynthetic reaction centers and light-harvesting Chl-protein complexes (LHC), whereas Chl b only exists in LHC (Green and Durnford, 1996; Liu et al., 2004). The chemical structures of the two main Chl pigments differ in one side chain of the tetrapyrrole. Chl a has a methyl group, whereas Chl b carries a formyl group at the corresponding position in ring B. Moreover, their absorption spectra are different. Chl b containing-antenna complexes can harvest light energy at ~470 and 650 nm; light at this wavelength is not efficiently absorbed by Chl a. Therefore, organisms that use Chl b in their LHC can harvest a wider range of light energy than those that are limited to Chl a. Aside from its light-harvesting function, Chl b is also important for controlling photosynthetic antenna size by regulating the stability of the LHC (Bellemare et al., 1982). LHCCI levels increase when Chl b synthesis is activated by the Chl precursor and decrease in Chl b-less/deficient mutants (Paulsen et al., 1993; Espineda et al., 1999; Bailey et al., 2001). The binding of Chl b to the LHC proteins stabilizes the latter in the thylakoid membranes (Paulsen et al., 1993; Lindahl et al., 1995). By contrast, LHC proteins without Chl b binding are degraded by proteases (Hoober and Eggink, 2001).

Chl loss and disassembly of the photosynthetic apparatus are the most remarkable events in leaf senescence (Buchanan-Wollaston, 1997). A delay in Chl degradation can result in the stay-green phenotype, which shows a slower progression of senescence compared with a standard reference (Park et al., 2007). Senescence can be regarded as an oxidative process because of the overproduction of reactive oxygen species (ROS), such as hydrogen peroxide and superoxide radicals (Piquiry et al., 2000). ROS represent a ubiquitous signal and possible co-stressor of environment conditions including heat stress (Tomanek, 2014). The increased ROS levels under stresses can result in oxidative damage to cellular constituents and ultimately cell death (Mittler, 2002). Heat stress causes premature leaf senescence. Heat-induced leaf senescence is characterized by the loss of Chl and proteins, weakened metabolic activities, and oxidative damage (Wahid et al., 2007).

The Chl synthesis pathway has been well characterized, and the genes encoding enzymes involved in Chl synthesis have been isolated (Beale, 2005; Nagata et al., 2005). In the last step of Chl biosynthesis, Chl b is synthesized from Chl a through the oxidation of a methyl group on the D ring, which is catalyzed by chlorophyllide a oxygenase (CAO) (Tanaka et al., 1998; Espineda et al., 1999). The CAO gene was first identified in Chlamydomonas reinhardtii with six Chl b-less mutants (Tanaka et al., 1998). The gene’s sequence is highly conserved from cyanobacteria to higher plants (Tomitani et al., 1999; Mueller et al., 2012). CAO is a mononuclear iron-containing protein and has a [2Fe–2S] Rieske center and a tyrosine radical (Tanaka et al., 1998; Eggink et al., 2004). CAO contains three domains, which are sequentially named from the N terminus as the A, B and C domains (Nagata et al., 2004). The C domain is the conserved core domain of CAO, which contains a Rieske center and non-heme iron-binding motifs and catalyzes the conversion of Chl a to Chl b (Tomitani et al., 1999; Nagata et al., 2004). The B domain is less conserved and may function as a link that stabilizes CAO (Sakuraba et al., 2007). The A domain is thought to play a role in the regulation of the CAO protein level. Interestingly, this regulatory mechanism does not operate when the Chl b synthesizing activity is deficient (Yamasato et al., 2005, 2008).

Another study demonstrated that a sequence with ten amino acids is essential for CAO degradation. This sequence functions as the CAO degron for a chloroplast protease (Sakuraba et al., 2009). Chl b synthesis is known to be critical for LHC formation. However, if Chl b is over-produced, the accumulated free Chl b induces photodamage. Therefore, regulating CAO activity is important for Chl synthesis and chloroplast development.

Two homologous genes of CAO, OsCAO1 and OsCAO2, have been identified from the rice genome. These genes are highly homologous and positioned in tandem, which is most likely due to recent gene duplications (Lee et al., 2005). However, their expression patterns are entirely different. OsCAO1 is expressed in green tissues, with a higher expression level during the daytime and a lower expression level in the late afternoon and at night. In contrast, OsCAO2 functions in non-photosynthetic tissues, and its expression increases after dusk and decreases after the dark phase ends. These findings indicate that OsCAO1 and OsCAO2 play different roles in rice development. OsCAO1 plays a major role in Chl b synthesis and chloroplast development under the light, whereas OsCAO2 may function in the dark. A study of the two T-DNA mutants of OsCAO1 and OsCAO2 found that OsCAO2 knockout mutant leaves do not differ significantly from wild type leaves, whereas OsCAO1 knockout mutants have pale green leaves. These findings suggested that OsCAO2 cannot compensate for the loss of OsCAO1 (Lee et al., 2005).

This study performs a map-based cloning of the pale green leaf (pgl) locus in rice (Oryza sativa) and reveals that pgl harbors a single-base substitution in the coding region of OsCAO1, which results in a premature translational termination. On the basis of the expression analysis of PGL and related genes and the phenotypic characterization of pgl, the gene product of PGL is proposed to play important roles in Chl b and Chl a syntheses, Chl degradation, and leaf senescence in rice.

Materials and methods

Plant materials and growth conditions

The pgl mutant was derived from an M2 population of the japonica rice variety Yunjin (YY) by ethyl methane sulphonate (EMS) mutagenesis as previously described (Guo et al., 2005). The japonica variety YY and the indica variety TN1 were used to segregate the population construction. The plants grown under natural conditions were grown in a paddy field at the China National Rice Research Institute (CNRRI), Fuyang, Zhejiang Province, China and Lingshui, Hainan Province, China. The plants used in the heat stress and low light experiments were grown in a growth chamber. The chamber conditions for heat stress were as follows: the rice plants were treated at 42°C for 16 h during the daytime and 35°C for 8 h at night with a 32/25°C (day/night) temperature regime as a control. The chamber conditions for the low light experiment were as follows: the rice plants were grown under low (30 μmol m⁻² s⁻¹)
or moderate (150 μmol m⁻² s⁻¹) light conditions at 32°C, followed by 8 h dark at 28°C.

Chl content and rice quality determination
The total Chl in the leaves was extracted with 80% acetone. The extract was analyzed using a spectrophotometer (Shimadzu UV2400, Japan). The total Chl, Chl a, and Chl b contents were estimated with light absorption values of 470, 645 and 663 nm, respectively, according to Porra et al. (1994). The rice quality traits were measured as previously described (Su et al., 2011).

Transmission electron microscope (TEM) analysis
The leaf samples for the TEM analysis were harvested from 4-week-old plants, 20 d after flowering in the paddy. The detached leaves were soaked in a fixation buffer (2.5% glutaraldehyde in 100 mM phosphate buffer, pH 7.4). The polymerization and staining of the leaf samples were based on the method of Tanaka et al. (2003). The prepared samples were observed under a Hitachi H-7650 (Japan) TEM.

Map-based cloning of PGL
A mapping population was derived from a cross between pgl and TN1. A total of 2207 individuals with the mutant phenotype were used for mapping. The initial localization was determined with a total of 163 simple sequence repeat (SSR) markers scattered among all 12 chromosomes (www.gramene.org). Accordingly, 296 individuals were used for the primary mapping of PGL. For further mapping, new sequence tagged site (STS) and SSR markers between the two flanking markers were designed based on the differences between the genomic DNA sequences of japonica variety Nipponbare and indica variety 9311 (www.gramene.org/resources). All of the PCR products were separated on 4–5% agarose gels for visualization.

Rice transformation
For the complementation of the pgl mutation, a 6959 bp genomic DNA fragment containing the PGL coding region along with the upstream and downstream sequences was cloned into the binary vector pCAMBIA1300 to generate the transformation construct, pCAO1IF. The binary construct was introduced into the calli generated from the mature seed embryos of pgl through the Agrobacterium (EHA105)-mediated method. Both the sense and anti-sense of PGL coding sequences (CDS) were inserted into the binary vector pHQSN containing the 35S promoter (p35S::PGL and p35S::anti-PGL) and introduced into to overexpress and knock-down PGL, respectively, thereby allowing further exploration of the functions of PGL in rice. Rice transformation was performed as previously described (Li and Li, 2003).

Subcellular localization of PGL
To investigate the subcellular localization of PGL, the coding region sequence of PGL without the termination codon was cloned into the pCAMV35S-GFP binary vector to fuse PGL and the enhanced green fluorescent protein (eGFP). The fusion constructs (p35S::PGL-GFP and the control p35S::GFP) were transformed into tobacco (Nicotiana benthamiana) epidermal leaf cells using Agrobacterium-mediated injection. The cells were then examined under a confocal fluorescence microscope (Leica TCS SP5, Germany) after 48 h of incubation. The GFP and the Chl fluorescence were recorded at 522 nm, respectively. A truncated PGL was fused to the eGFP (p35S::OsCAO1::GFP) to test whether the premature termination of PGL in pgl affects the protein’s localization and was subsequently transformed into tobacco for GFP detection.

Analysis of reactive oxygen species
The accumulation of the superoxide anion was monitored with nitroblue tetrazolium (NBT) (0.5 mg ml⁻¹ in 10 mM potassium phosphate buffer, pH 7.6). Hydroxide was detected by 3,3′-diaminobenzidin (DAB, 1 mg ml⁻¹ in 50 mM Tris acetate buffer, pH 5.8). The staining and bleaching of the samples were performed as previously described (Wang et al., 2013).

RNA extraction and quantitative real-time PCR analysis
Total RNA was extracted from rice tissues using a Total RNA Extraction Kit (Axygen, cat NO, AP-MN-MS-RNA-250, USA). All of the samples were treated with DNase I (Promega; www.promega.com, USA). First-strand cDNA synthesis was primed with an oligo(dT) primer by using a ReverTra Ace qPCR-RT kit (Toyobo, Japan). Quantitative real-time PCR (qRT-PCR) was performed using a 2×SYBR Green PCR Master Mix (Applied Biosystems, USA) in an Applied Biosystems 7900HT Real-time PCR System. The mRNA expression of these genes were quantified, including OsCAO1, OsHEMA, OsCHLI, OsPORA, OsPORB, OsDVR, OsYGL1, OsLhcb1, OsLhcb4, OsNOL, OsNYC1, OsNYC3, OsNYC4, OsPAO, OsNCCR1, OsCatB, OsPOD1, OsPOD2, OsAPX1, OsAPX2, OsH36 and Actin1. The transcript data were normalized using Actin1 as an internal control. The error bars indicate the standard error of the mean. All of the experiments were performed in triplicate.

Results
The phenotype of pgl
A pgl mutant was identified in the mutagenized population to further understand the genetic mechanism of pigments. The pgl mutant exhibited pale-green leaves compared with the wild-type (WT) throughout the entire developmental process both in Fuyang, Zhejiang Province (120°00′E, 30°06′N) and Lingshui, Hainan Province (110°02′E, 18°03′N). pgl showed a more obvious phenotype at the tillering stage than at the seedling stage (Fig. 1A, C). The Chl content analysis indicated that all three Chl species contents were decreased in pgl compared with WT, especially Chl a and Chl b (Fig. 1B, D). In pgl, the ratio of Chl a/Chl b was dramatically increased compared with that of WT, reaching 21.4 and 27.7 at the seedling and tillering stages, respectively, because almost no Chl b could be detected in pgl. The ratio of Chl a/Chl b was 3.38 and 2.97 in WT rice at the seedling and tillering stage, respectively (Supplementary Table S1 available at JXB online).

Chl is one of the most important players in photosynthesis. The photosynthesis in the flag leaves at the heading stage was measured to investigate whether the massive decrease of the Chl content affected photosynthesis. As expected, the photosynthesis rate was significantly lower in pgl compared with WT (Fig. 1E). Light-harvesting chlorophyll-binding proteins make up light-harvesting complex II for light harvesting, which was closely related to photosynthesis rate (Caffarri et al., 2004). The expression levels of the photosynthesis-associated genes (i.e. Lhcb1 and Lhcb4) were also examined in the WT and pgl plants. The results showed that OsLhcb1 and OsLhcb4 were strongly down-regulated in pgl compared with the WT (Fig. 1F, G). The pgl phenotypic characterization suggests that PGL is essential for photosynthesis and Chl synthesis, especially Chl b synthesis.
pgl shows reduced grain yield and quality

A comparative analysis of the agronomic traits of WT and pgl was performed due to the substantially lower photosynthesis rate observed in pgl (Fig. 2; Supplementary Table S2). pgl plants demonstrated a lower tiller number and seed-setting rate than that of the WT plants (Fig. 2A–C). The seed-setting rate was only 43.2% in pgl, whereas it reached up to 71.0% in the WT. However, no significant difference between pgl and WT was found with respect to plant height, branch, grain numbers or grain weight (Supplementary Table S2). Consequently, the grain yield per plant for pgl was 8.92 g. This value was ~57% of the 15.60 g measured in WT (Fig. 2D; Supplementary Table S2).

The amount of photosynthesis occurring during the grain-filling stage is an important physiological factor that affects biomass and grain yield and further influences rice quality (Lin et al., 2002; Baig et al., 2005; Feng et al., 2013). Grain qualities including chalkiness degree (CD), gel consistency (GC), amylose content (AC) and gelatinization temperature (GT) in pgl and WT were further investigated (Fig. 2E–H; Supplementary Table S3). The CD in pgl was higher than that in WT (Fig. 2E, F). Moreover, the GC in pgl was lower than that in the WT (Fig. 2G). Nevertheless, no significant difference was found in the AC or GT between pgl and WT (Fig. 2H; Supplementary Table S3).

In summary, the PGL mutation resulted in the reduction of grain yield and quality in pgl.

Map-based cloning of PGL

A map-based cloning approach was used to isolate PGL and investigate the molecular basis of the pgl phenotype. Accordingly, 2207 plants with the pgl phenotype were selected from the population generated by crossing pgl with TN1 (i.e. 8943 plants). The ratio of individuals with a normal phenotype to those with a pgl phenotype showed a good fit to the expected value [i.e. 3:1 (χ² = 0.4929, P = 0.4826)]. The reciprocal crosses between pgl and indica varieties (i.e. ZF802, NJ06 and 9311) were performed to confirm the segregation ratio. All of the F₁ hybrids showed a normal leaf color. The segregation of the normal individuals to pgl phenotype plants in the F₂ population also showed a good fit to 3:1 (Supplementary Table S4). The results indicate that pgl is controlled by a single recessive gene. A bulked segregant analysis was employed to perform the preliminary mapping. A total of 163 pairs of SSR markers evenly distributed in the rice genome were selected for the analysis of the SSR polymorphisms between pgl and TN1. The identified polymorphic SSR markers were used to detect polymorphisms between the normal and mutant DNA pools, which were derived from the progeny of ‘pgl/TN1.’ Subsequently, PGL was mapped between SSR markers RM3451 and RM4771 on chromosome 10 (Fig. 3A).

A set of polymerase chain reaction (PCR)-based molecular markers was developed for fine mapping of PGL. PGL was further narrowed to an interval of ~35 kb between markers C4 and C6, which contains three predicted ORFs (Fig. 3A, B; Supplementary Table S5). The 35-kb genomic DNA segments from the WT and pgl plants were sequenced and...
PGL impacts leaf senescence and affects rice yield and quality

A mutation was identified in the predicted LOC_Os10g41780 gene in the pgl genome, which encodes chlorophyllide a oxygenase (CAO1). This gene had been previously reported by Lee et al. (2005). In the remainder of this paper, pgl will continue to be used as the mutant line name and PGL or OsCAO1 as the gene name.

A genetic complementation experiment was conducted to confirm that the PGL mutation was responsible for the mutant phenotype. Supplementary Table S6 illustrates the primers used for the vector construction in this study. A complementation vector containing the entire PGL coding region, a 2125-bp upstream region and a 1030-bp downstream sequence was inserted into the binary vector pCAMBIA1300 (pCAO1F, COM). The constructed and empty vectors were introduced into the mutant. The pale-green leaves were completely restored to normal color in the transgenic plants (Fig. 3C). The Chl contents were also restored to normal (Fig. 3D, E). These results demonstrated that the cloned candidate gene OsCAO1 was indeed responsible for the pgl phenotype.

PGL expression pattern and subcellular localization

qRT-PCR was performed using PGL-specific primers to determine the expression pattern of PGL in rice (Supplementary Table S7). PGL was expressed in the culm, sheath, blade and panicle, but not the root. The expression level was highest in the blade (15-fold higher than in the panicle). These results correlated well with the level of Chl synthesis in these organs (Fig. 4A), and were also consistent with those reported previously (Lee et al., 2005). Chl was synthesized in the leaf, culm, sheath, and panicle but not in the root. Furthermore, the leaf and the sheath are the major Chl synthesis organs, suggesting that PGL may be closely related to Chl synthesis and perhaps is one of the most important components in this pathway.
The localization of the homologous CAO of several organisms and the predication of bioinformatics suggested that OsCAO1 is most likely localized in the chloroplast. The transient expression of fluorescent protein in tobacco (*Nicotiana benthamiana*) was used to determine the subcellular localization of PGL and confirm this prediction. Subsequently, 35S::PGL-GFP and 35S::GFP were introduced into the epidermal cells of tobacco through *Agrobacterium* infection. GFP fluorescence was observed using a confocal laser scanning microscope. The GFP fluorescence signal was found only in the chloroplast in cells transformed with 35S::PGL-GFP (Fig. 4E–G). In contrast, the GFP fluorescence signal was present throughout the nucleus and the cytoplasm in the cells transformed with 35S::GFP; however, no signal overlapped between the GFP and the chloroplast (Fig. 4B–D). These results indicate that the OsCAO1 protein was localized to the chloroplast, providing further evidence for its role in Chl synthesis.

The truncated protein OsCAO1\(^{pgl}\) fused with GFP (35S::OsCAO1\(^{pgl}\)-GFP) was also transformed into tobacco to explore whether the premature translational termination of OsCAO1 in pgl affects OsCAO1 localization and results in the pale-green phenotype. The results showed that OsCAO1\(^{pgl}\) was also localized to the chloroplast (Fig. 4H–J). Since both OsCAO1 and OsCAO1\(^{pgl}\) were localized to the chloroplast, the chloroplast localization signal of OsCAO1 likely exists in the N-terminus.
The WT and *pgl* etiolated seedlings were compared during light-dependent leaf greening to further explore the contribution of *PGL* to chlorophyll synthesis. The WT etiolated seedlings quickly turned green after being exposed to light, whereas the *pgl* seedlings remained albino after 24 h of light exposure (Fig. 5B). Chl b synthesis was almost entirely inhibited in the *pgl* plants (Fig. 5C). Similarly, Chl a synthesis in the *pgl* plants lagged behind that in the WT plants (Fig. 5D). As a result, the *pgl* plants presented with a lower total Chl content than the WT plants and also had pale-green leaves (Fig. 5E). These results suggest that *PGL* is essential for light-dependent accumulation of high levels of Chl. The expression of the Chl synthesis-associated genes was also detected during greening (Fig. 5F–L). The *PGL* transcript levels were steadily up-regulated under lighting when 6-day-old WT etiolated seedlings were exposed to light for 24 h. In addition to *OsCAO1, OsHEMA, OsCHLH*, *OsDVR, OsPORB* and *OsYGL1* expression levels increased in WT plants after illumination, indicating that these genes are required for the light-dependent Chl synthesis during greening of etiolated plants. Only *OsPORA* showed no light-induced gene expression, indicating that it is not essential for this process. This is consistent with that of a previous study (Sakuraba et al., 2013). The expression of these genes was also measured in *pgl* plants. Compared with that observed in the WT plants, *OsHEMA* and *OsCHLH* expression levels were only mildly increased in the *pgl* plants, whereas the expression levels of *OsDVR, OsPORB* and *OsYGL1* that are critical for Chl synthesis, were suppressed in the *pgl* plants. A very weak increase in the *OsPORA* expression level in the *pgl* plants was also found. This increase may be related to the fact that *OsPORA* does not appear to be required for greening. These results indicate that *OsCAO1* is essential not only for Chl b synthesis but also for the accumulation of high levels of Chl a by regulating key genes in Chl synthesis.

**Leaf senescence exacerbated in *pgl***

The *pgl* plants exhibited a pale-green leaf in the paddy field and presented a severe withered phenotype in the leaf tip (Fig. 6A). Leaf variegation or necrotic lesions were reported to result from ROS accumulation and usually resulted in cell apoptosis (Jiang et al., 2011). The ROS production in the flag leaves of the WT and *pgl* plants was detected using NBT and DAB, respectively. More hydrogen peroxide accumulated in the *pgl* leaves compared with the WT leaves (Fig. 6B). The electrolyte leakage of the leaves was measured to examine whether cell death was induced in *pgl*. The electrolyte leakage in *pgl* was significantly higher than that in WT, suggesting that the *pgl* plants lost more membrane integrity during development compared with the WT plants (Fig. 6C). The expression levels of Chl degradation-associated genes (i.e. *OsNOL, OsNYC1, OsNYC3, OsNYC4, OsPAO* and *OsRCCR1*) and senescence-associated genes (i.e. *OsCatB, OsPOD1, OsPOD2, OsAPX1, OsAPX2* and *Osh36*) in mature leaves of the WT and *pgl* were compared in order to understand further the molecular basis for the early senescence phenotype (Fig. 6D). In *pgl, OsNYC1, OsPAO1*...
and OsRCCR1 expression levels were slightly down-regulated, whereas OsNOL, OsNYC3 and OsNYC4 expression was up-regulated compared with that of the WT plants. The OsNYC3 and OsNYC4 expression levels in the pgl plants reached up to 4-fold and 15-fold of those in the WT, respectively. The genes associated with ROS scavenging (i.e. OsCatB, OsPOD1, OsPOD2, OsAPX1 and OsAPX2) were all down-regulated in pgl compared with the level in WT (Fig. 6D). Moreover, Osh36, which is a senescence-inducible gene (Lee et al., 2001), quickly accumulated in the pgl plants, reaching up to 15 times higher than that in WT. This result suggests that premature senescence occurs in pgl.

The TEM analysis was conducted to reveal the chloroplast morphology and structure and to understand the senescence process better. The LHC complexes included Chl b and LHC proteins, which play important roles in the stabilization of the thylakoid membranes. Few cellular differences were observed between the WT and pgl plants during the seedling stage (Fig. 6E, I). Compared with that in the WT plants, the grana thylakoid (GTK) in the pgl plants had a disorderly arrangement in the chloroplast. Furthermore, thylakoid stacking was indistinct (Fig. 6F, J). These results suggest that OsCAO1 is essential for thylakoid development. Substantial differences in the leaf cells were found at the mature stage. Accordingly,
PGL impacts leaf senescence and affects rice yield and quality.

There was a large decrease in the number of chloroplasts in the pgl plants compared with that in the WT plants (Fig. 6G, K). Chloroplast degradation is one of the signs of senescence. Most chloroplasts in the pgl plants were degraded, whereas no obvious sign of chloroplast degradation was observed in the WT plants. Furthermore, the grana lamellae were arranged in an orderly manner and uniformly distributed (Fig. 6H, L). These results suggest that pgl exhibits a more serious senescence phenotype than WT under natural conditions in the paddy field.

Dark-induced senescence was also examined in addition to natural senescence. The detached leaves from the pgl and WT plants were kept in the dark at 28°C. Within five days, the leaves from the pgl plants turned completely yellow, and their Chl a and Chl b contents sharply decreased (Fig. 7A–C), whereas the leaves from the WT plants remained green and had high levels of Chl a and Chl b. The DAB and NBT stainings were much more pronounced in the pgl plants than that in the WT plants (Fig. 7D, E). Taken together, these results indicate that Chl b deficiency could speed up the aging process under dark-induced conditions.

**pgl sensitive to high temperature**

Temperature is one of the most important factors influencing the growth and development of plants. A continuously high temperature will accelerate rice aging. The pgl and WT plants

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**Fig. 6.** pgl leaves exhibit severe senescent phenotype under natural conditions. (A) Naturally senescent leaves (flag leaves) of WT and pgl 20 d after flowering. (B) Accumulation of superoxide anion radicals (O$_2^-$) in naturally senescent leaves, visualized by staining with NBT. (C) Electrolyte leakage in naturally senescent leaves. (D) Changes in transcript levels of senescence-associated genes in the leaves of WT and pgl at the mature stage. (E–L) Transmission electron microscopy (TEM) analysis of chloroplasts in pgl leaves. TEM analysis of the leaves of WT (E, F) and pgl (I, J) at the seedling stage. The samples were obtained at 20 d in the paddy field. TEM analysis of the flag leaves in the WT (G, H) and pgl (K, L) at the mature stage. The samples were obtained 20 d after flowering in the paddy field. OsNOL (LOC_Os03g45194) and OsNYC1 (LOC_Os01g12710), two short-chain dehydrogenase/reductases, represent Chl b reductases; OsNYC3, α/β hydrolase-fold family protein (LOC_Os06g24730); OsNYC4, THYLKOID FORMATION1, chloroplast precursor (LOC_Os07g37250); OsPAO, pheophoride a oxygenase (LOC_Os03g05310); OsRCCR1, red chlorophyll catabolite reductase (LOC_Os10g25030); OsCatB, catalase (LOC_Os06g51150); OsPOD1 (LOC_Os01g22370) and OsPOD2 (LOC_Os03g22010), two peroxidases; OsAPX1 (LOC_Os03g17690) and OsAPX2 (LOC_Os07g49400), two ascorbate peroxidases; OsH36, aminotransferase, senescence-induced protein (LOC_Os05g39770). GTK, grana thylakoid; PG, plastoglobule. Bar, 2 μm (E, I, G, K); bar, 0.5 μm (F, J, H, L). *, P<0.05, **, P<0.01 (Student's t-test).
results indicate that high temperature accelerates pgl aging. Accordingly, OsCAO1 or Chl b is suggested to play important roles in resisting high temperature stress.

**PGL affects the ROS scavenging system under low light**

Light is one of the important factors in plant growth. Low light affects the activity of the enzymes in the ROS scavenging system (Wang, 2014). To further explore the effects of ROS accumulation, we raised WT and pgl plants under different light conditions. The WT and pgl plants grew slowly under low light, and their leaves became slightly pale (Fig. 9A–D). In the WT plants, the total Chl and Chl b contents sharply decreased under the low light condition compared with normal light (Fig. 9E–G). In pgl, the total Chl content was also reduced under the low light condition compared with normal light (Fig. 9E), but low light had no obvious effect on the Chl b content (Fig. 9F). Subsequently, the Chl a/b ratio of the pgl plants was lower in low light than moderate light (Fig. 9G). Next, we assessed the expression levels of senescence-associated genes (i.e. OsCutB, OsPOD1, OsPOD2, OsAPX1, OsAPX2 and Osh36) in leaves at low and normal light conditions (Fig. 9H–M). After 14 days’ exposure to low light, the expression levels of all of the genes associated with ROS scavenging except OsAPX2 were slightly up-regulated in the WT plants. In the pgl plants, the expression of OsPOD2 was up-regulated under the low light compared with that detected under normal light conditions; the other genes were down-regulated under low light compared with normal light conditions. Since the ROS scavenging system is damaged in pgl, Osh36 had the highest expression level under low light conditions, suggesting that PGL also affects the ROS scavenging system under low light.

**Discussion**

**PGL essential for Chl synthesis in rice**

The role of CAO in Chl b synthesis in Arabidopsis thaliana has been reported previously (Espineda et al., 1999). Two homologous genes of CAO, OsCAO1 and OsCAO2, were found in the rice genome (Lee et al., 2005). OsCAO1 (i.e. PGL) is believed to catalyze the Chl a to Chl b conversion process in rice. The results of this study show that PGL encodes a chloroplast-localized protein involved in light-dependent Chl synthesis and is strongly expressed in the chlorenchyma including the culm, blade, sheath and panicle; its expression pattern correlated well with that of Chl synthesis. The premature termination of PGL resulted in the pgl phenotype and a lower Chl b content, which could be rescued by introducing a functional PGL fragment. The Chl content was substantially decreased in transgenic plants expressing anti-sense PGL, whereas the plants overexpressing PGL showed a slight increase in Chl b. Taken together, these results indicate that PGL is required for the Chl b biosynthesis in rice.

The mutation or inactivation of enzymes involved in the metabolic pathway of the Chl synthesis usually results in...
PGL impacts leaf senescence and affects rice yield and quality

The accumulation of their respective substrates in plants (Mock and Grimm, 1997; Wu et al., 2007). Chl b appears to be synthesized by the oxidation of the methyl group of Chl a to a formyl group. However, in the pgl plants, the Chl a content was decreased to 60% that of the WT plants. Chl a deficiency in the anti-sense transgenic plants and accumulation in PGL-overexpressed plants were also observed. The Chl synthesis-associated genes were further divided into two classes. Accordingly, the genes involved in the early steps (i.e. OsHEMA and OsCHLH) were up-regulated, and those involved in the later steps (i.e. OsDVR, OsPORB, OsYGL1 and OsCAO1 itself) were down-regulated in the pgl plants, suggesting that PGL may be involved in regulating Chl a synthesis in rice. Chl b deficiency promoted the expression of the Chl synthesis-related genes, especially to those involved in the early synthesis steps.

Chl b deficiency accelerates leaf senescence in rice

Both OsCAO1 over-expression and mutation in NYC1 (Chl b reductase) can cause Chl b accumulation in plants and lead to a delayed senescence phenotype (Kusaba et al., 2007; Sakuraba et al., 2012). In contrast, a shortage of Chl b can lead to premature aging. In our study, pgl plants exhibited a disorganized ultrastructure in the chloroplasts (Fig. 6E, L). Chl b existed in the light-harvesting Chl a/b-protein complex (LHCP). The LHCPs were localized to the thylakoid membrane. The LHCPs in PS II (LHC II) were encoded by the Lhcb gene families. Chl b is important for the stability of the LHCP (Bellemare et al., 1982). Lhcb1 and Lhcb4 are extremely important LHC II members, and the expressions of both were reduced in the pgl plants (Fig. 1F, G). LHC II is predominantly localized in the grana, which is the stacking region of the thylakoid membrane, and is thought to play an important role in grana formation (Allen and Forsberg, 2001). Moreover, the presence of more PGs in the pgl plants compared with the WT plants is a good indicator of senescence.

In addition, the pgl plants, more severe Chl degradation occurred than in the WT plants, a clear indication of senescence. The Chl content was rapidly degraded in pgl in dark-induced senescence. Compared with the expression levels in the WT plants, in the pgl plants, OsNYC1, OsPAO and OsRCCR expression levels were weakly down-regulated, but OsNYC3 and OsNYC4 expression levels were rapidly up-regulated more than 5-fold and 16-fold, respectively (Fig. 6D). Both OsNYC3 and OsNYC4 play a role in regulating the Chl-protein complex degradation during leaf senescence (Morita et al., 2009; Yamatani et al., 2013).

A greater accumulation of ROS in the pgl plants compared with the WT plants can also explain why the pgl plants exhibited early senescence. Under normal physiological conditions, cells control ROS levels by balancing the generation of ROS with their elimination by the ROS scavenging system. In pgl, the scavenging system is weakened. All of the genes involved...
in ROS elimination – OsCatB, OsPOD1, OsPOD2, OsAPX1 and OsAPX2 – were down-regulated in pgl. In both naturally and dark-induced senescent plants, two detected ROS species (hydrogen peroxide and superoxide anion radicals) accumulated in the pgl plants (Fig. 6B; Fig. 7B, C). Oxidative damage initiated by ROS is a major contributor to the functional decline that is characteristic of aging. Osh36, which is expressed exclusively during senescence, was used as a molecular mark for senescence, and was obviously increased in the pgl plants compared with the WT plants. Excessive ROS can induce apoptosis. The electrolyte leakage in the pgl plants was significantly higher than that in the WT plants (Fig. 6C). Furthermore, environmental stresses may induce the senescence process because of some sources of ROS formation. The pgl plants that were either germinated or transplanted at a high temperature exhibited premature aging and withering.
OsCAO1 disruption indirectly affects rice yield and quality

The leaf is the main photosynthetic apparatus and accounts for 90–95% of the dry matter in rice plants (Wu et al., 2007). In this study, the pgl plants exhibited pale-green leaves and reduced Chl content throughout their life cycle (Fig. 1A–D). The TEM analysis revealed that the thylakoids were abnormal in the pgl plants (Fig. 6E–L). The thylakoid is an important place for photosynthesis; thus, it is not surprising that the photosynthesis rate is decreased in pgl. The prediction that decreasing photosynthesis would lead to yield decreases seemed straightforward; however, the yield decline may be regulated by several factors including both the source and the sink. A previous study found that pgl had a lower source (photosynthesis) than WT (Fig. 1E). However, except for the seed-setting rate, the yield traits were unaffected by this mutation (Fig. 2C; Table S1). Two different aspects of rice growth contribute to this finding. First, decreased photosynthesis results in a lack of biomass production, which can lead to an inadequate supply of nutrients for the rice grain at the filling stage (Long et al., 2013). Second, the senescence in pgl at a later stage of rice growth was more severe than that in WT, which has a substantial influence on grain filling and further affected rice yield. In addition, abnormal grain filling causes insufficiently filled endosperm storage in the seeds and chalkiness (Lin et al., 2002), which could explain why the pgl plants had a higher CD than the WT plants. The GC is one of the key chemical characteristics of starch in the endosperm that plays an important role in the eating and cooking qualities of rice (Fan et al., 2005). In this study, the OsCAO1 mutation resulted in a large change in the GC of the rice grain. It was speculated that the photosynthetic assimilates from pgl leaves were limited during the grain-filling stage, thereby affecting the grain development.

PGL encodes a chlorophyllide a oxygenase (i.e. OsCAO1) with functions corresponding to AtCAO. The results of this study suggest that OsCAO1 is involved in several aspects of the metabolic process. OsCAO1 was considered the only enzyme responsible for Chl b formation in rice, similar to AtCAO in Arabidopsis thaliana. However, OsCAO1 affected Chl a synthesis by regulating the expression levels of the Chl synthesis-associated genes (e.g., OsDVR, OsPORA, OsPORB and OsYG1L). The evidence suggests that OsCAO1 also plays important roles in Chl degradation and ROS scavenging to regulate both natural and induced rice senescence.

**Supplementary data**

Supplementary data are available at *JXB* online.

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**Fig. S1.** The pathway of chlorophyll biosynthesis in plant.

**Table S1.** Pigment contents in leaves of wild-type and pgl.

**Table S2.** Grain yield traits in wild-type and pgl.

**Table S3.** Rice quality-related traits in wild-type and pgl.

**Table S4.** Segregation ratio of reciprocal crosses between pgl and indica varieties.

**Table S5.** Molecular markers (primers) used for mapping.

**Table S6.** Primers used for vector construction.

**Table S7.** Primers used for qRT-PCR.
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