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Research article

Photosynthetic and physiological analysis of the rice high-chlorophyll mutant (*Gc*)

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ABSTRACT

Chlorophyll (Chl) molecules are essential for harvesting light energy in photosynthesis. A rice high-chlorophyll mutant (*Gc*) with significantly increased Chl *b* was identified previously in Zhenshan 97B (*Oryza sativa indica*). However, the mechanism underlying this higher Chl *b* content and its effects on photosynthetic efficiency are still unclear. Immunoblot and blue native polyacrylamide gel electrophoresis (BN-PAGE) with a second dimension electrophoresis followed by the matrix-assisted laser-desorption/ionization time-of-flight (MALDI-TOF) analysis showed that a few core proteins of photosystem I (PSI) and photosystem II (PSII), and light-harvesting complex II (LHCII) proteins were overexpressed in the mutant plants. Remarkable differences in chloroplast ultrastructure were observed between the wild-type and mutant plants, with the latter having more highly stacked and larger grana. Chl florescence analysis demonstrated that *Gc* had markedly increased quantum efficiency of photosystem II (ΦPSII), photochemical quenching (qP), non-photochemical quenching (qN) and electron transport rate (ETR). This morphological and physiological adaptation might confer a higher photosynthetic capacity in *Gc* than the wild-type.

1. Introduction

Higher plants rely on chloroplasts to convert solar energy to chemical energy. The photosynthetic apparatus consists of a series of thylakoid-localized multi-subunit protein complexes. Photosystem I (PSI) and photosystem II (PSII) are each composed of a core reaction center surrounded by specific light-harvesting antenna complexes called LHCI and LHCII, respectively [1]. The LHC family is the most plentiful pigment–protein complex in the thylakoid membrane and binds roughly half of the total chlorophyll (Chl *a* and *b*) in chloroplasts [2]. Chl *a* is a component of both the photosynthetic reaction centers and the light-harvesting antenna, whereas Chl *b* is restricted to the antenna complexes [3]. The Chl molecules harvest light energy in the antenna systems and drive electron transfer in the reaction centers. Analysis of the complete genome of *Arabidopsis thaliana* shows that there are at least 27 genes encoding 15 enzymes involved in Chl biosynthesis from glutamyl-tRNA to Chl *b* [4].

Alterations in Chl biosynthesis and its regulation result in decreased Chl *b* in vivo [5,6]. Mutants defective in the Chl biosynthetic pathway have been identified in various plant species, and their chloroplast morphologies, physiological phenotypes, genetic characteristics and photosynthetic properties have been investigated systematically [7–11]. Impaired chlorophyllide esterification in Chl biosynthesis [11], abnormal development and differentiation of chloroplasts [12], disordered heme feedback [13], and damaged phytochrome regulation [14] all lead to diminished levels or absence of Chl *b*. The absence of Chl *b* has a profound effect on photosynthetic efficiency and capacity. Mutants with a greater Chl *a*/*b* ratio were often accompanied by altered LHC to compensate for changes in photosynthetic efficiency and capacity [15,16].

Recent studies of accumulating excess Chl *b* was correlated with overexpression of chlorophyllide *a* oxygenase (CAO) [17–19]. Besides CAO, other regulators involving in the biosynthetic regulation of Chl *b* were also reported. The RNA binding protein NAB1 of *Chlamydomonas reinhardtii* plays an important role in controlling

Abbreviations: Chl, chlorophyll; PSI, photosystem I; PSII, photosystem II; LHC, light-harvesting complex; F_0, minimum fluorescence yield; F_m, maximum fluorescence yield; ΦPSII, quantum efficiency of photosystem II; ETR, electron transport rate; qP, photochemical quenching; qN, non-photochemical quenching; PAR, photosynthetically active radiation; DM, dodecyl-β-D-maltoside; EDTA, ethylenediamine-tetraacetic acid.

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the expression of the PSII proteins at the posttranscriptional level in light-harvesting antenna [20]. Induction of *GLK* transcription factors in *A. thaliana* required for chloroplast development lead to increased levels of Chl [5]. It was generally accepted that the biosynthesis of Chl *b* is strictly regulated under different light conditions [18]. Overexpression of *Prochlorothrix CAO* lacking its regulatory A-domain in Arabidopsis resulted in over-accumulation of Chl *b*, which in turn making the transgenic plants photo-damaged under high light intensity [21]. However, we had reported a rice *Gc* mutant (*Oryza sativa indica*) with significantly higher level of Chl *b* relative to the wild-type. Its photosynthetic rate, biomass and grain yield also increased by 20%, 17% and 16% respectively [22]. The mechanism underlying this higher Chl *b* content and its effects on photosynthetic efficiency are still unclear, however.

In this study, we have investigated the Chl biosynthesis characteristics, Chl fluorescence, chloroplast ultrastructure, expression of genes and proteins responsible for Chl biosynthesis, chloroplast development and photosynthesis. These results indicated that *Gc* had a greater capacity for photosynthesis and many adaptive changes in photosystem proteins.

2. Results

2.1. Chl florescence

Chl *a* is distributed throughout the Chl binding subunits in both PSI and PSII, while Chl *b* is uniquely bound to Lhcbs. In order to investigate the possible effects of the increased Chl *b* in *Gc*, we compared the photosynthetic capacity of *Gc* and wild-type plants. Chl fluorescence induction experiments demonstrated that the ratio of variable fluorescence to maximum fluorescence (F_v/F_m), which reflects maximum photochemical efficiency of PSII, did not differ between the mutant and the wild-type plants (Table 1). However, the qP, which reflects the redox state of the Q_A electron acceptor of PSII, and qN, which reflects the capacity to dissipate excess light energy, were significantly higher in *Gc* compared with wild-type. Moreover, ETR and ΦPSII was significantly higher in *Gc* relative to wild-type. ETR is usually proportional to ΦPSII [23]. These results indicated that *Gc* leaf had a greater PSII capacity than its wild-type.

2.2. Expression of Chl biosynthesis, photosynthesis and chloroplast development genes

Deregulation of one or a few genes involved in Chl biosynthesis can lead to higher Chl content and affect the Chl *a*/*b* ratio [5,16]. Given that the level of Chl *b* is increased in *Gc* mutant, it is most likely that its Chl *a* or *b* biosynthetic enzymes are up-regulated. To test this, the expression of genes responsible for Chl biosynthesis was monitored by qRT-PCR with specific primer pairs (Table 2). However, the expression of *HEMA1*, *PORA*, *CHLI*, *CHLD*, *CHLH*, *CHLG* and *CAO*, which encode the enzymes for the Chl biosynthesis pathway was not significantly changed (Fig. 1), while the expression of plastid-encoded genes including *psaA*, *psbA*, *rbcL* and the nuclear *rbcS* gene were significantly up-regulated and even the *rbcL* mRNA levels were approximately 3-fold in *Gc* than in the wild-type. These results suggested that the up-regulated transcriptional level of photosynthesis and chloroplast development-related genes might contribute to the increased level of Chl *b* in *Gc*.

2.3. Thylakoid membrane proteins

In order to elucidate the molecular basis for the enhanced PSII photosynthetic capacity of *Gc* mutant, we analyzed the protein components of the thylakoid membrane. The protein complexes were extracted and subjected to SDS-PAGE. As shown in Fig. 2a, LHC proteins of around 26 kDa were up-regulated in the thylakoid membrane of *Gc*.

To further examine possible alterations in the composition of photosynthetic protein complexes in *Gc*, thylakoid membranes were solubilized with DM and subjected to BN-PAGE. The separated major bands: monomeric PSI and dimeric PSII (band I), $Cytb_6f$/ATPase/PSII monomer (band II), trimeric LHCII (band III), and monomeric LHCII (band IV) are shown in Fig. 2b. The LHCII trimer and LHCII monomer were overexpressed in *Gc* compared with the wild-type, consistent with the results from SDS-PAGE, while no significant difference in PSI monomer and PSII dimer, $Cytb_6f$/ATPase/PSII monomer were detected between the mutant and the wild-type in the BN-PAGE analysis.

In order to quantify the levels of individual proteins, the lanes of BN-PAGE were excised and subjected to a second dimensional SDS-urea-PAGE. Eleven differentially expressed proteins were identified by MALDI-TOF (Table 3). The data showed that two PSI reaction center subunits PsaA and PsaB, photosystem II 47 kDa protein, photosystem II 43 kDa protein and PsbA which belong to PSII were overexpressed in *Gc* mutant plants compared with the wild-type plants. And PSII light-harvesting proteins LHCb3, LHCb4, LHCb5 and LHCb6 were all highly overexpressed in *Gc* mutant. However, ATP synthase alpha and beta subunits were slightly repressed in the mutant plants in contrast to the wild-type plants (Table 3 and Fig. 2b).

The higher Chl content in the *Gc* mutant was accompanied by a decrease in the Chl *a*/*b* ratio from 3.5 to 1.9, a clear indication of a highly enhanced LHCII antenna system in the mutant. There are at least six Lhcb proteins that bind Chl *b* in higher plants (Lhcb1-6) [24]. We have shown by SDS-PAGE and 2D-SDS-urea-PAGE that LHC proteins were significantly overexpressed in *Gc* mutant compared with the wild-type (Fig. 2a and b). Immunoblot experiments were performed using antibodies raised against higher plant LHCI and LHCII proteins. It was revealed that the LHCII protein levels of LHCb3, LHCb4, LHCb5 and LHCb6 were significantly overexpressed, but the LHCI protein levels of Lhca1, Lhca2 and Lhca4 remained largely unaffected in *Gc* (Fig. 3a and b), consistent with the results of BN-PAGE and SDS-urea-PAGE. However, this altered LHCII composition in *Gc* did not prevent the formation of trimeric LHCII complexes as indicated by BN-PAGE (Fig. 2b). The relative level of the PSI core protein, PsaA, had been also quantified in the immunoblot analysis. The protein level of PsaA was increased, consistent with the results of qRT-PCR and BN-PAGE analysis. Moreover, the increased expression of Cyt f

Table 1
Chl fluorescence parameters of the wild-type (WT) and mutant plants (*Gc*). Data are from five replicates and presented as means ± SD. Significance comparisons were performed between WT and *Gc* under the same growth conditions. F_0, minimal Chl *a* fluorescence of dark-adapted leaves; F_v/F_m, ratio of variable fluorescence to the maximum fluorescence; qP, photochemical quenching; qN, non-photochemical quenching; ETR (electron transport rate), defined as photosynthetically active radiation (PAR) × 0.5 × 0.84 × ΦPSII (μmol photons m^{-2} s^{-1}); ΦPSII, actual PSII efficiency; data with different letters (a, b) are significantly different ($P < 0.05$) in the same column.

	F_0	F_v/F_m	qP	qN	ETR	ΦPSII
WT	0.22 ± 0.02 (a)	0.83 ± 0.01 (a)	0.91 ± 0.03 (a)	0.13 ± 0.02 (a)	67.66 ± 7.89 (a)	0.65 ± 0.04 (a)
Gc	0.23 ± 0.07 (a)	0.83 ± 0.02 (a)	0.95 ± 0.02 (b)	0.20 ± 0.04 (b)	75.51 ± 7.05 (b)	0.70 ± 0.02 (b)