

Chlorophyll biosynthesis and transcriptome profiles of chlorophyll *b*-deficient type 2b rice (*Oryza sativa* L.)

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Abstract

Photosynthetic and transcriptomic characteristics of a chlorophyll (Chl) *b*-deficient mutant type 2b rice (ch14) were investigated in this study. The ultrastructure of chloroplast in ch14 demonstrated irregular chloroplast enhancement (loss of starch granules, indistinct membranes, and thinner grana). Ch14 had significantly lower carotenoid, Chl *a*, Chl *b*, and total Chl contents, but a higher ratio of Chl *a* to Chl *b* than a wide-type rice. 3,594 genes were differentially expressed in ch14, among which 309 transcription factors were related to Chl degradation and biosynthesis, chloroplast formations, and the photosynthesis capacity. *PsbR*, *GSA-AT*, *PBGD*, *PPOX*, *MgMT*, and *POR* genes were down-regulated, reducing Chl content and photosynthetic capacity in the ch14. This study suggests that Chl degradation may be attributed to abnormal chloroplast development and down-regulation of gene expression in the common pathway and Mg branch and the rise in Chl *a* to Chl *b* ratio may be involved in the alternative Chl *b* degradation pathway.

Keywords: chl *b*-deficient mutant; chlorophyll; gene expression; photosynthesis; transcriptome profiles

Introduction

Global agriculture has been facing a serious challenge, with a required increase in foodstuffs of 25-110% by 2050 to meet the increasing demands of the growing population (Ray *et al.*, 2013; Hunter *et al.*, 2017). Among the agriculture products, rice (*Oryza sativa* L.) is one of the most important cereal crops, which accounts for 89% of total food production and consumption in Asia (Evans *et al.*, 1998). Thus, rice yield improvements have become a preferred solution for meeting the world's food supply (Milovanovic and Smutka, 2017).

Photosynthesis is an essential biological mechanism in higher plants. In this process, light energy absorbed by chloroplast in the plant cell is converted into approximately 90% biomass (Demirbaş, 2001; Murchie *et al.*, 2009). Therefore, crop yields are boosted by improving photosynthesis (Yang *et al.*, 2015; Flood *et al.*, 2016). To maintain photosynthetic machinery, work efficiently, chlorophylls (Chl) including Chl *a* and

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Chl *b* are indispensable. They absorb and transmit light energy to help plants develop through photosynthesis (Yang *et al.*, 2015). Chl *b*, which is produced from Chl *a* by Chl *b* oxygenase (*CAO*), accounts for approximately one-third of 10⁹ tons of total Chl in nature each year (Tomitani *et al.*, 1999; Morita *et al.*, 2005). Based on the systemic transition, Chl *b* absorbs blue and orange lights (peaks at 453 nm and 652 nm, respectively), meanwhile Chl *a* absorbs red and blue lights (peak at 430 nm and 665 nm, respectively) (Ito *et al.*, 1996; Masuda and Fujita, 2008). As a consequence, Chl *b* acts as an accessory pigment, that assists in photosynthesis by directing light to Chl *a* (Tanaka *et al.*, 1998). Chl *b* is located in antenna complexes, whereas Chl *a* is found in both photosynthesis reaction centers and light-harvesting antennas. Moreover, the biogenesis of Chl *b* is closely related to antenna size, which is a significant issue in photosynthesis effectiveness (Oster *et al.*, 2000). Recognition and regulating Chl *b* biogenesis are thus an effective approach to enhance agricultural yield.

The ratio of Chl *a* to Chl *b* (Chl *a/b*) also affects plant photosynthetic capacity (Ito *et al.*, 1996). In this regard, the conversion of Chl *a* to Chl *b*, as well as Chl *b* degradation, could play a significant role in the variation of the Chl *a/b*. As a result, the mechanism underlying Chl *a/b* variation must be explained by understanding the Chl biosynthesis and degradation pathway, which comprise over 15 reactions (Masuda and Fujita, 2008). Studies have reported that a massive increase in Chl content, including Chl *a* and Chl *b*, dramatically improves crop productivity (Long *et al.*, 2006; Gupta, 2013). However, the biogenesis of the photosynthetic machinery is still unclear. Thus, understanding the biosynthesis and degradation of Chl may be an effective solution to enhance biomass production and crop yields (Huang *et al.*, 2013).

Chl-deficient mutants have become a useful tool to investigate the Chl mechanism, chloroplast development, and molecular biology of photosynthesis (Bujaldon *et al.*, 2017; Liu *et al.*, 2018). Therefore, they have been employed for studying Chl biosynthesis pathway and photosynthetic machinery in various crops such as *Hordeum vulgare* (Lokstein *et al.*, 1993), *Zea mays* (Hopkins *et al.*, 1980), *Triticum aestivum* (Zhao *et al.*, 2011), *Pisum sativum* (Dutta *et al.*, 2009), *Oryza sativa* (Wu *et al.*, 2007), *Glycine max* (Kato and Palmer, 2004), *Beta vulgaris* (Allen *et al.*, 1988), and *Ipomoea batatas* (Zhu *et al.*, 2019). Several rice mutants have been generated for studying Chl biosynthesis (Terao *et al.*, 1985; Cha *et al.*, 2002; Jung *et al.*, 2003). A series of 16 distinct Chl-deficient rice mutants is divided into two types (which depend on Chl *a/b*): Chl *b*-lacking rice (type 1) with Chl *a/b* = ∞ (no Chl *b*); and Chl-deficient type 2 including Chl *b*-deficient type 2a (Chl *a/b* ~ 10) and type 2b (Chl *a/b* ~ 15) (Terao *et al.*, 1985). Several of them have been used for studying Chl biosynthesis, but the Chl biosynthesis in rice has not been fully understood. Therefore, further studies on Chl biosynthesis in rice are still required. However, no study has reported the Chl biosynthesis and degradation in Chl *b*-deficient type 2b rice (ch14).

This study examined the Chl biosynthesis and degradation in ch14 rice. The content of Chl *a*, Chl *b*, total Chl, and carotenoid (Car) in leaves of ch14 and wide-type rice (Norin No.8, wt) was determined. Next-generation sequencing (NGS) was also employed to determine the transcriptome profiles and photosynthetic machinery of ch14. Transcription factors (TFs) and differential expression genes (DEGs) relative to Chl degradation and biosynthesis in ch14 and wt rice were compared.

Materials and Methods

Plant samples and growth conditions

Seeds of wt and ch14 were graciously received from Dr. Tomio Terao (National Institute of Agrobiological Resources, Tsukuba Science City, Japan). The seeds were planted in a growth chamber (Firstek Scientific Co Ltd, New Taipei City, Taiwan), and the seedling was subsequently practiced for 6 weeks at 25 °C, and relative humidity of ≥ 80%, under a 12/12 photoperiod with the light intensity of 2000 μmol s⁻¹ m⁻². The leaves of these plants were then harvested, frozen in liquid nitrogen, and stored at -80 °C for further experiments.

Assessment of pigment contents

Approximately 0.1 g of wt and mutant leaf tissues were cut into pieces and extracted using 80% (v/v) acetone. The absorbance of the resulting extracts was then measured at 663.6 (OD_{663.6}), 646.6 (OD_{646.6}), and 440.5 (OD_{440.5}) nm using a Hitachi U2800 UV-Visible spectrophotometer (Tokyo, Japan) to determine pigment contents (total Chl, Chl *a*, Chl *b*, and Car) using the method described by Yang *et al.* (1998). The pigment contents were calculated (on the basis of fresh weight), as follows:

$$\text{Chl } a = 12.25\text{OD}_{663.6} - 2.55\text{OD}_{646.6} \quad (1)$$

$$\text{Chl } b = 20.31\text{OD}_{646.6} - 4.91\text{OD}_{663.6} \quad (2)$$

$$\text{Total Chl} = 17.76\text{OD}_{646.6} + 7.34\text{OD}_{663.6} \quad (3)$$

$$\text{Car} = 4.69\text{OD}_{440.5} - 4.91\text{Total Chl} \quad (4)$$

To validate the Chl content, SPAD values were determined *in situ* using a chlorophyll meter (SPAD-502Plus, Konica Minolta, Osaka, Japan).

Ultrastructure microscope

Leaf tissues of wt and mutant rice were prepared by the method of Spurr (1969). The leaves were cut into small sections (approximately 0.5 × 0.5 × 0.5 mm). The tissues were placed in 2.5% glutaraldehyde for 24 h at 4 °C, and then immersed in 1% OsO₄ for 2 h. Subsequently, the tissues were cut into 70 nm thin sections by using an ultramicrotome (Leica EM UC6, Wetzlar, Germany), stained with uranyl acetate (1%, w/v) and lead citrate (1%, w/v), and then observed using a Phillip Tecnai 12 transmission electron microscope (JEOL Ltd., Tokyo, Japan).

cDNA libraries construction and transcriptome sequencing

RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) was employed to extract total RNA from the leaves of wt and mutant plants. The purity and quantity of RNA samples were evaluated using 1% formaldehyde agarose gel electrophoresis and determined with a NanoDrop™ 2000 spectrophotometer (NanoDrop, Wilmington, USA). RNA concentration and integrity were determined using an Agilent 2100 Bio-analyzer (Agilent RNA 6000 Nano Kit; Agilent Technologies, Santa Clara, USA). Briefly, two sets of paired-end cDNA libraries were constructed from wt and mutant rice samples. The constructed cDNA libraries were used for transcriptome sequencing using the BGISEQ-500 platform (Beijing Genomics Institute, Shenzhen, Guangdong, China). SOAPnuke v1.5.2 (<https://github.com/BGI-flexlab/SOAPnuke>) was used to remove unknown nucleotides, low-quality sequences, and adapter sequences. Transcriptome sequencing and cDNA library construction were conducted independently by Tri-I Biotech (New Taipei City, Taiwan).

Analysis of transcriptome profiles

The clean reads were mapped to the *O. sativa* 'Nipponbare' reference genome (<http://rapdb.dna.affrc.go.jp/>) using HISAT2 v2.0.4 (<http://www.ccb.jhu.edu/software/hisat>) relying on hierarchical indexing for the spliced alignment of transcripts in HISAT (Hierarchical Indexing for Spliced Alignment of Transcripts). The clean reads were linked to the reference using Bowtie2 v2.2.5 (<http://bowtie-bio.sourceforge.net/Bowtie2/index.shtml>), and then calculated gene expression levels using RSEM v1.2.12 (<http://deweylab.biostat.wisc.edu/RSEM>). Generation of samples, hierarchical clustering and pearson correlation analyses were respectively conducted using the ggplot2, hclust, and cor functions of R. Furthermore, DEGs was identified using PossionDis, which is based on Poisson distribution with default parameters of a FDR ≤ 0.001 and fold change ≥ 2.00 (Audic and Claverie 1997), and subsequently subjected to KEGG pathway and Gene Ontology (GO) analyses for determinations of functional enrichment using the phyper function of R.

Quantitative RT-qPCR

Quantitative RT-qPCR was conducted to analyse the basic expression levels of DEGs involved in Chl biosynthesis and degradation pathways. Total RNA (μg) obtained from the leaves of wt and mutant plants was used to synthesize cDNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostic Systems, Branchburg, USA) and oligo (dT) primers. The sequences of primer sets used for amplification (Table S1 in the Supplementary Material) were designed using a Primer Premiere 6 software (Premiere Biosoft, Palo Alto, USA). RT-qPCR was carried out using the StepOne Plus Real-Time PCR system (Applied Biosystems, Life Technologies Inc., Foster City, USA) with Roche FastStar Universal SYBR Green Master reagent (Roche Diagnostic Systems, Branchburg, USA). Relative gene expression levels were then calculated using $2^{-\Delta\Delta C_t}$ comparative Ct method (Livak and Schmittgen, 2001).

Statistical analysis

The relative gene expression, SPAD values, and pigment contents were statistically analysed using the least significant difference (LSD) *t*-test at a *p*-value ≤ 0.05 . These analyses were conducted using the SAS v8.0 statistical package (SAS Institute, Cary, North Carolina, USA).

Results

Characterization of Chl b-deficient type 2b rice

Figure 1 shows the leaf color and characteristics of wt and ch14 grown under controlled conditions for 6 weeks. Dwarf and unhealthy plantlets were observed in ch14 (~25 cm in height) with green leaves, whereas normal plantlets were observed in wt (~40 cm in height) with dark green leaves (Figure 1A). The level of Chl *a* and Chl *b* in ch14 was significantly lower than those observed in wt (Figure 1B). Consequently, the total Chl content of ch14 (1.71 mg g^{-1}) was about two-fold less than that of wt (3.25 mg g^{-1}). The ch14 leaves had a Chl *a/b* of 15.7, which is nearly 4.8-fold higher than wt leaves (Figure 1B). Similarly, Car content in ch14 (0.28 mg g^{-1}) was found to be nearly two-fold less than that in wt (0.51 mg g^{-1}). Moreover, soil-plant analysis development (SPAD) values were used to validate the Chl accumulation in the leaves of ch14 and wt. The SPAD value of ch14 (15.29) was approximately 55.4% of wt rice (28.72) (Figure 1C), which were consistent with Chl contents and indicative of irregular Chl biosynthesis and degradation in ch14. Moreover, ultrastructure analysis revealed that wt leaves presented regular chloroplasts with distinct membranes, stroma lamellae, small starch granules, one or two plastoglobuli, and thick stacked grana (Figure 2A). By contrast, the chloroplasts in ch14 leaves exhibited irregular development with indistinct membranes, indistinct or absent stromal lamellae, no starch granules, many plastoglobuli, and thinner stacked grana (Figure 2B).

Genome mapping and gene expression analysis

In this work, wt and ch14 seedling samples were sequenced using the BGISEQ-500 platform. The raw sequencing reads were filtered by removing reads with a high content of unknown bases, adaptor sequences, and low-quality reads. The resulting 25.31-29.4 million clean reads were subsequently mapped to the rice reference genome (*Oryza sativa* 'Nipponbare') using HISAT, with match ratios of 96.49%-96.85% (Table S2 in the Supplementary Material). 87.64% and 89.19% sequences in the ch14 and wt libraries were mapped to the reference transcript, respectively. The uniquely mapped reads in wt (88.31%) and ch14 (88.94) libraries were linked to a single locus in the reference genome (Table S3 in the Supplementary Material). In addition, 90.21% of the expressed genes were shared between the wt and ch14 genomes (Figure S1 in the Supplementary Material).

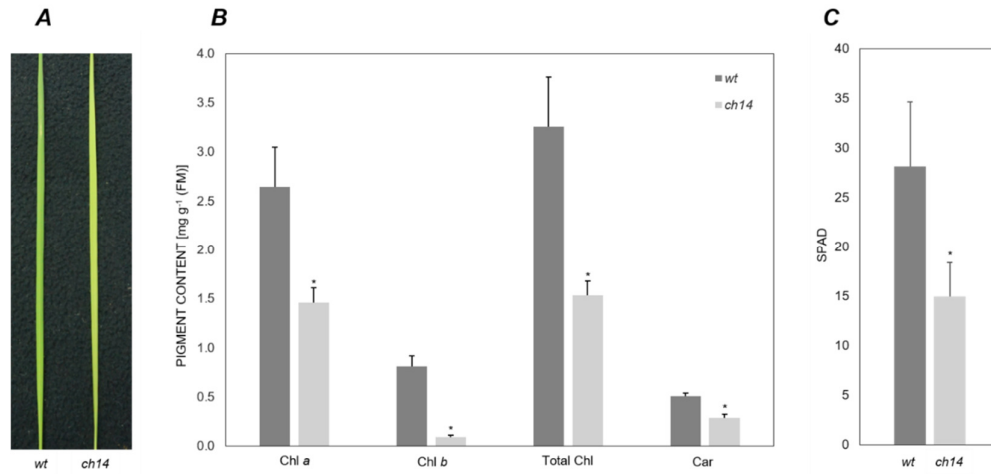


Figure 1. (A) Leaf coloration, (B) pigment contents, and (C) SPAD values of wt and ch14 rice. Asterisks (*) demonstrate significant differences ($p \leq 0.05$)

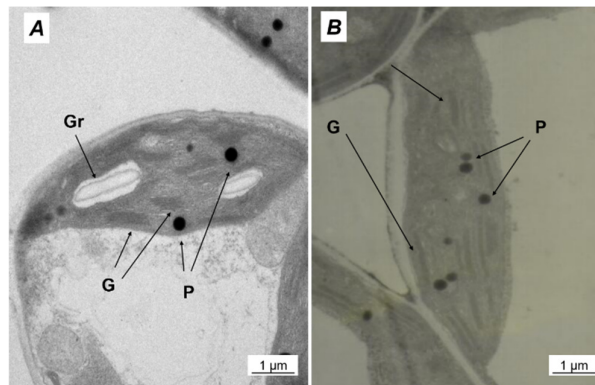


Figure 2. Chloroplast ultrastructure of (A) wt and (B) ch14 rice. G: grana; Gr: granules; P: plastoglobuli

Detection and annotation of DEGs

Functional annotation was conducted to acquire information with respect to protein function, pathway involvement, and GO. Sequence orientation-driven genes were aligned based on the KEGG database. GO is a global criterion functional identification scheme for genes in which biological processes, cellular components, and molecular functions are assigned to transcripts. The distribution of detected genes among the GO categories is shown in Figure 3. Total of 6,948 genes were mapped, with 2,991, 2,426, and 1,531 genes being assigned to “cellular component”, “molecular function”, and “biological process”, respectively. Figures 4A and 4B show DEGs in wt and ch14. This study accordingly identified 2,105 and 1,489 DEGs being up- and down-regulated in ch14 leaves, respectively; whereas 23,015 genes exhibited no significant differences in expression between wt and ch14 leaves (Figure 4B).

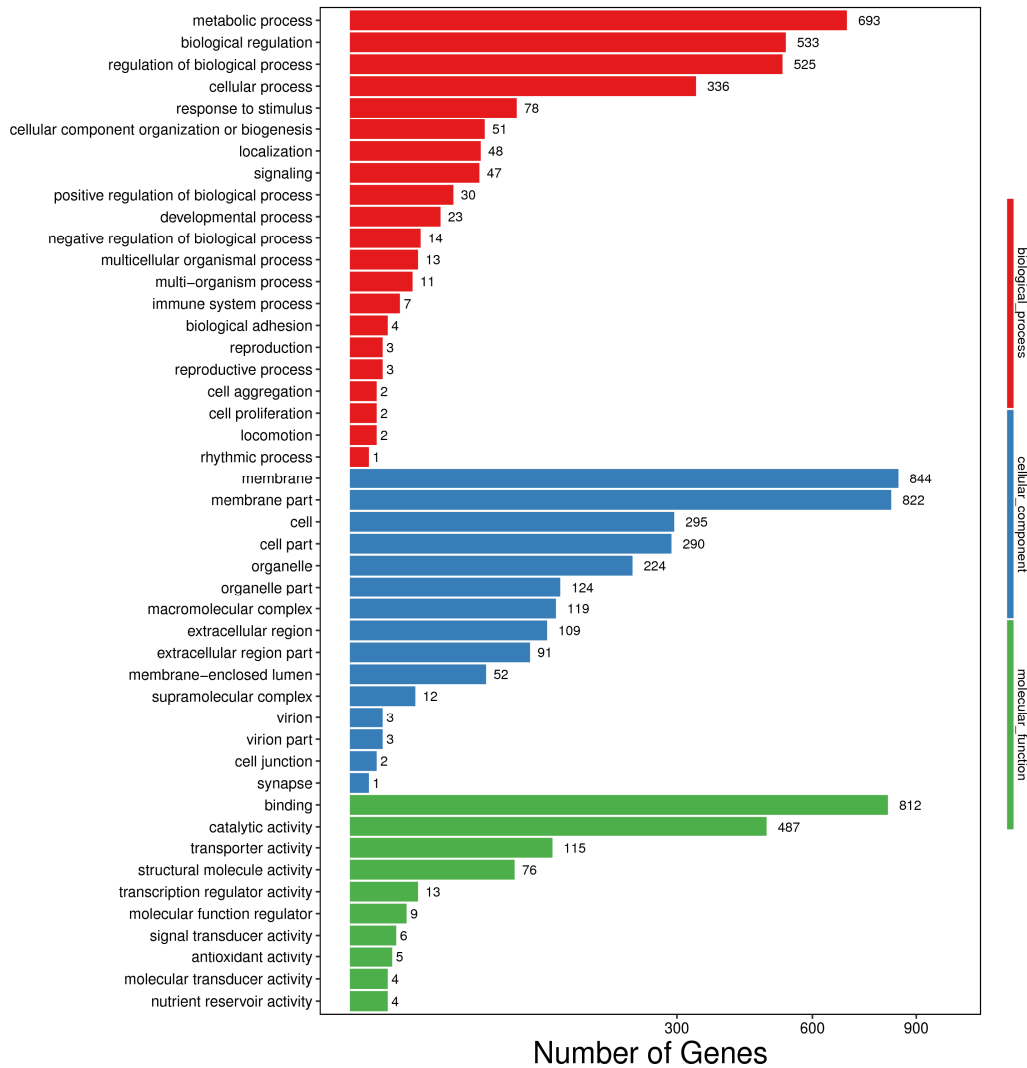


Figure 3. Gene ontology classification of DEGs in wt and ch14

Expression profiles of DEGs

Based on KEGG pathway annotation, DEGs linked to chloroplast production and cell division were discovered. Chloroplast photosystem II protein genes (*PsbR* with Osa_4342395) were found to be down-regulated in ch14 (by 3.81-fold). Furthermore, the *Lhcb1* gene (Osa_4324705) encoding a photosystem II 10 kDa protein was up-regulated (1.54-fold) (Table 1). A total of 5 and 24 DEGs relative to Chl degradation and biosynthesis were respectively detected based on KEGG pathway annotation. Figure 5 shows the expression levels of these DEGs. In ch14, *GSA-AT* (Osa_4346136), *UROS* (Osa_4328432), and *PPOX* (Osa_4327918) were down-regulated (-1.34, -1.08, -1.08, -1.08, respectively) in common pathway, whereas *MgMT* (Osa_4340015) and *POR* (Osa_4337415, and Osa_4349004) were down-regulated (-1.51, and -1.25, respectively) in Mg branch as compared to the wt (Figure 5). These results indicated that the processes of Chl biosynthesis and degradation may be detrimentally affected in the ch14.

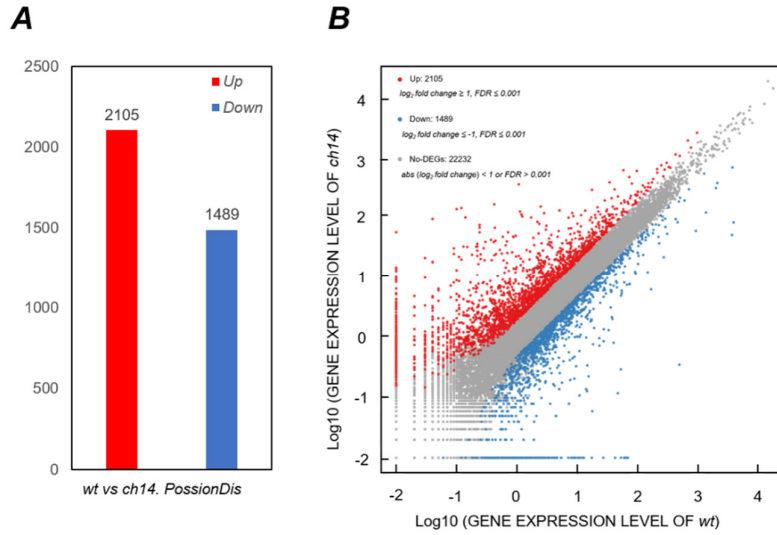


Figure 4. DEGs in wt and ch14 rice. (A) Comparison of down- and up-regulated DEGs. (B) gene classification based on differences in expression level

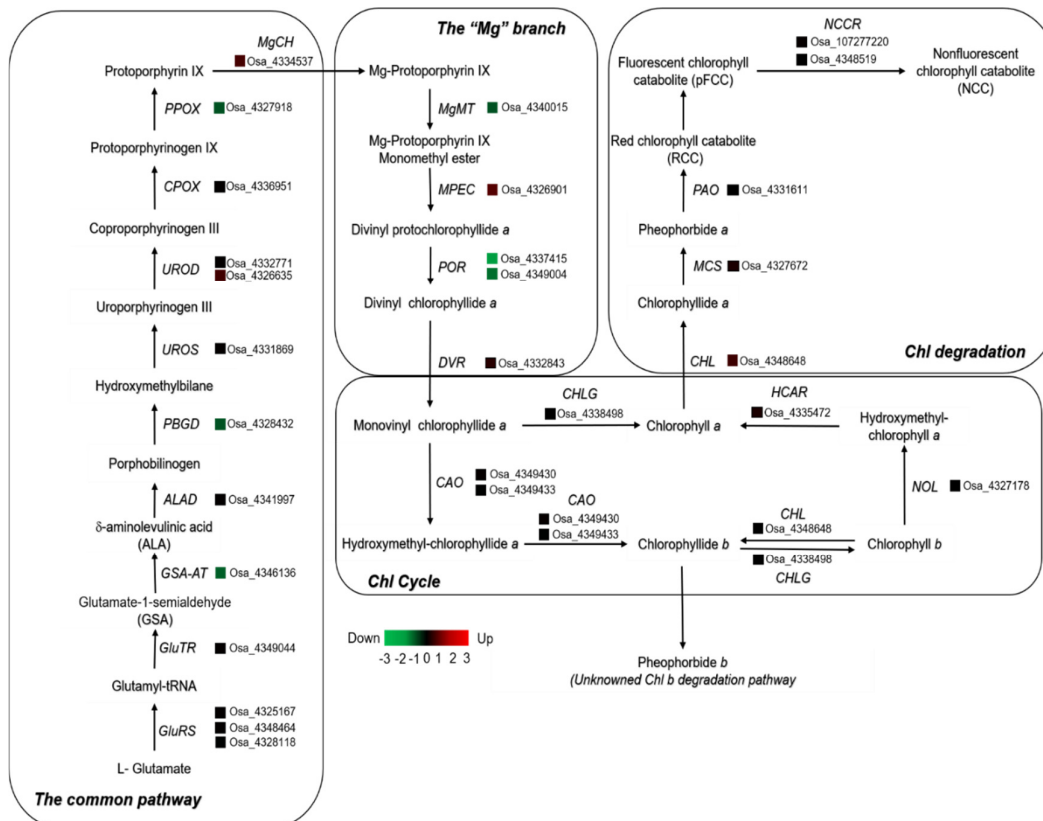


Figure 5. Expression profiles of DEGs involved in Chl degradation and biosynthesis. The expression levels of the ch14 are compared with those of wt rice. Another Chl *b* degradation pathway is suggested as an alternative degradation mechanism involving pheophorbide *b*.

Table 1. Unigenes distribution amongst KEGG pathways

Functions	Gene ID	TF family	Log2 fold change	Expression in ch14 compared to wt	Annotation
Chlorophyll biosynthesis	Osa_4346136	<i>GSA-AT</i>	-1.34	Down-regulated	Glutamyl-tRNA synthetase
	Osa_4328432	<i>PBGD</i>	-1.08	Down-regulated	Hydroxymethylbilane synthase
	Osa_4327918	<i>PPOX</i>	-1.08	Down-regulated	Protoporphyrinogen oxidase
	Osa_4340015	<i>MgMT</i>	-1.08	Down-regulated	Mg-protoporphyrin IX methyltransferase
	Osa_4337415	<i>POR</i>	-1.51	Down-regulated	Protochlorophyllide reductase <i>a</i>
	Osa_4349004	<i>POR</i>	-1.25	Down-regulated	Protochlorophyllide reductase <i>a</i>
Photosynthesis	Osa_4342395	<i>PsbR</i>	-3.81	Down-regulated	Photosystem II 10 kDa protein
Antenna protein	Osa_4324705	<i>Lhcb1</i>	1.54	Up-regulated	Photosynthesis-antenna protein

Role of TFs

TFs play an important role in gene expression regulation. In this work, 289 DEGs were determined as putative TFs, which are associated with 39 TF families. The MYB superfamily (45 DEGs) was found to be the most well-represented TF family, followed by the MYB-related (33 DEGs), NAC (34 DEGs), bHLH (31 DEGs), AP2-EREBP (13 DEGs), and WRKY (11 DEGs) families (Table 2). In ch14, 38, 28, 29, 27, and 12 DEGs in the MYB, MYB-related, bHLH, NAC, WRKY, and AP2-EREBP families were found to be down-regulated, respectively. Similarly, this study detected 13, 12, 9, 8, 8, and 6 DEGs in the AP2-EREBP, MADs, ARF, G2-like, GRAS, and C2H2 TF families, respectively; most of which were also significantly down-regulated in ch14. In addition, some DEGs associated with the FAR1 and mTERF TF families were down-regulated in ch14 (Table 2).

Table 2. Summary of DEGs associated with TF family in ch14 compared with wt rice

TF family	Total number of genes	Number of up-regulated genes	Number of down-regulated genes
ABI3VP1	9	6	3
Afin-like	1	1	0
AP2-EREBP	13	8	5
ARF	4	0	4
ARR-B	4	0	4
BBR/BPC	2	0	2
bHLH	31	20	11
bZIP	4	0	4
C2C2-Dof	6	0	6
C2C2-GATA	1	0	1
C2C2-YABBY	4	0	4
C2H2	6	0	6
C3H	1	0	1
CSD	1	0	1
E2F-DP	2	0	2
FAR1	14	12	2
G2-like	8	0	8
GRAS	8	1	7

GRF	4	1	3
HB	1	0	1
HSF	2	0	2
MADS	12	4	8
mTERF	13	0	13
MYB	45	30	15
MYB-related	33	24	9
NAC	34	7	27
OFP	4	4	0
PBF-2-like	1	0	1
PLATZ	1	0	1
SBP	16	16	0
SRS	3	0	3
TCP	1	1	0
Tify	4	4	0
Trihelix	5	5	0
WRKY	11	8	3

RT-qPCR validation of DEGs

The identification of DEGs relative to Chl degradation and biosynthesis was validated by performing RT-qPCR expression analyses of the selected DEGs. Examination of the expression patterns of 12 of the 19 genes revealed that these genes corresponded to those observed in the RNA-Seq data (Figure 6). This result corresponded with the transcription analysis data. *GSA-AT* (Osa_4346136), *PBGD* (Osa_4328432), *PPOX* (Osa_4327918), *MgMT* (Osa_4340015), and *POR* (Osa_4337415, Osa_4349004) were observed to be down-regulated in ch14, whereas most of the genes showed non-significant changes (Figure 6).

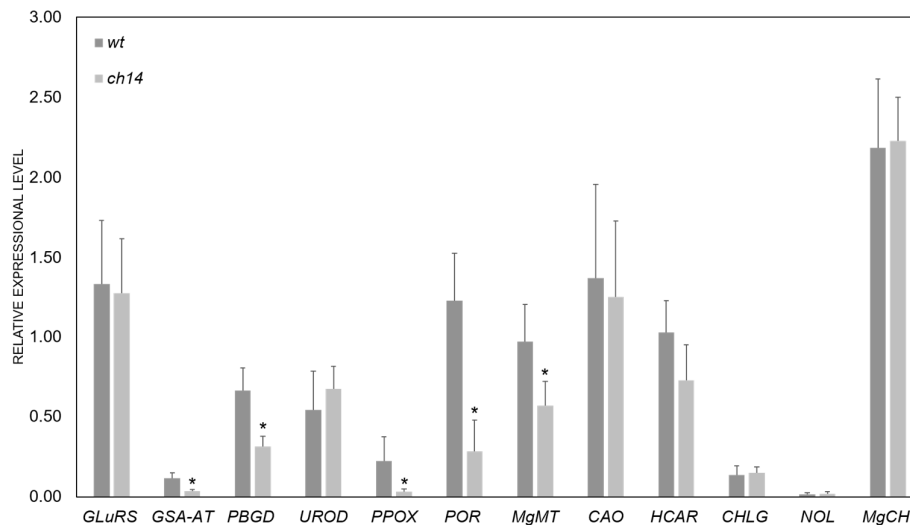


Figure 6. Expression levels of 12 DEGs in wt and ch14 rice. Asterisks (*) show significant differences between the expression levels of the wt and ch14 ($p \leq 0.05$)

Discussion

Pigment contents and chloroplast development

Chl-deficient mutants have been widely employed to investigate Chl biosynthesis and photosynthetic machinery properties of plants (Von Wettstein *et al.*, 1995; Chu *et al.*, 2015; Zhu *et al.*, 2019). Pigment content, Chl *a/b*, and chloroplast development are all linked to the color of leaves in higher plants (Li *et al.*, 2018). Accordingly, a variety of leaf colorations have been observed in Chl-deficient mutants and the corresponding wt plants such as *Arabidopsis thaliana*, *Ginkgo biloba*, and *O. sativa*. In this regard, previous studies have separated Chl-deficient rice mutants into two types, which are based on the Chl *a/b* (Terao *et al.*, 1985; Yamazaki, 2010). In the present work, ch14, a Chl *b*-deficient mutant type 2b, was used for studying Chl biosynthesis and degradation. Variations in leaf coloration were also found in ch14 and wt rice. Ch14 had distinctly paler green leaves, while the wt rice had leaves of dark green. In particular, ch14 displayed a dwarf phenotype, as well as significant reductions in total Chl, Chl *b*, and Car contents in leaves, with a Chl *a/b* of up to approximately 15.7, as compared to wt. Thus, the observed variance in leaf coloration might be because of variations in Chl content and Chl *a/b* between the ch14 and wt rice. Moreover, the presence of Chl *b* in ch14 at a low level could account for the darker coloration compared with the light-green leaves of Chl *b*-lacking mutant (Nguyen *et al.*, 2020). In higher plants, the processes of photosynthesis and Chl biosynthesis occur in the specialized chloroplasts organelle (Rüdiger *et al.*, 2002; Dubreuil *et al.*, 2018). Abnormal development in these structures is likely to cause a drawback of photosynthetic ability and chlorophyll synthesis in the leaves (Liu *et al.*, 2018b; Nguyen *et al.*, 2020). In this regard, numerous studies have reported that irregular chloroplast development (i.e., indistinct thylakoid membrane, non-existent granules, abundant vesicle, and numerous plastoglobuli) in Chl-deficient mutants, including *Bambusa vulgaris*, *G. biloba*, and *O. sativa*, affects Chl *a/b*, Chl contents, and leaf coloration (Li *et al.*, 2018; Nguyen *et al.*, 2020). Furthermore, the efficiency of light absorption and energy conversion in the photosynthetic apparatus is determined by the integrity of the stacked grana of chloroplasts (Wu *et al.*, 2014). In this work, ch14 leaves had distinctly abnormal chloroplast development with thinner stacked grana, thus causing a lower Chl content and deficiencies in the light-harvesting and conversion in ch14 compared with wt rice. A similar pattern has been observed in Chl *b*-lacking rice, where abnormal chloroplast production and thinner grana were linked to reduced pigmentation, raised Chl *a/b*, and lowered photosynthetic ability (Nguyen *et al.*, 2020). As a result, at the physiological level, the dwarf phenotype and leaf color change in ch14 rice are supposed to be closely correlated with a lower Chl level, a higher Chl *a/b*, and inappropriate chloroplast growth, which are thought to lead to a lower photosynthetic capability and thus can have an adverse influence on rice growth and yield.

Chloroplast-related DEGs and photosynthetic capacity

Transcriptome profiles show which genes are differentially expressed between different genotypes of a person of interest (Wang *et al.*, 2009). Transcriptome profiles of wt and ch14 rice showed a total of 2105 and 1489 DEGs, respectively. Among them, several DEGs related to photosynthetic machinery, chloroplast production, and pigment biosynthesis metabolism may be linked to the observed difference in the Chl *a/b*. The coordination of plastid and nuclear genes in plants plays a vital role in normal chloroplast development and differences in the levels of chloroplast-related genes expression, which affects the biogenesis of chloroplast assembly and pigment contents, thus resulting in changes in Chl accumulation, Chl *a/b*, photosynthetic capacity, and leaf coloration (Yang *et al.*, 2015; Li *et al.*, 2015). In higher plants, the light-harvesting complex (or antenna complex, LHC) proteins play a crucial role in the photosynthetic apparatus, which absorbs and regulates the flow of light energy to photosystems I (PSI) and II (PSII) (Zhao *et al.*, 1968; Goral *et al.*, 2012). PSII is a multi-protein-pigment complex that includes the PSII core reaction center dimer, LHCII, as well as minor light-harvesting complexes with over 20 subunits, including *PsbR* (Shi *et al.*, 2012; Amarnath *et al.*, 2016). Moreover, LCHII has been reported to be an instrumental factor that regulates the stacking of grana thylakoids in chloroplasts (Bennett, 1991; Allen, 1992; Standfuss *et al.*, 2005). Previous studies have found that a decrease in or absence of LHC proteins in Chl-deficient mutants of *A. thaliana*, *H. vulgare*, and *O. sativa* causes impairment in stacked grana (Yang and Chen, 1996; Jenny *et al.*, 2003; Kim *et al.*, 2009). Consequently, Chl-deficient mutant chloroplasts are supposed to have moderately formed or non-existent stacked grana

(Barber, 1982; Yang and Chen, 1996). In this study, the expression of *PsbR* (Osa_4342395) genes, which could imply a reduction in LHCII level of proteins and consequently caused a decrease in photosynthetic capability, decreased by 3.81 in ch14. A similar trend was found in the Chl *b*-lacking rice, in which *PsbR* genes were down-regulated, lowering photosynthesis (Nguyen *et al.*, 2020). The stacked grana in ch14 were thinner than those in the wt, which is consistent with a loss of LCHII. Thus, the high Chl *a/b* in ch14 may signify the irregular chloroplast formation and function.

RNA-Seq analysis and chlorophyll-related differentially expressed genes

Chl *a* and Chl *b* are important for light absorption and energy transfer in higher plants (Nelson and Yocum, 2006). The former is the primary pigment involved in light-harvesting and primary photochemical reactions, while the latter is only involved in light-harvesting (Voitsekhovskaja and Tyutereva, 2015; Landi *et al.*, 2020). It has been previously established that at least 28 genes encoding 22 enzymes are involved in Chl biosynthesis and degradation in rice. Therefore, changes in certain DEGs were associated with disorders in Chl metabolism and altered Chl *a/b* ratios (Nguyen *et al.* 2020). Particularly, during the early stages of Chl biosynthesis, glutamyl-tRNA synthetase (*GLuRS*) catalyzed to form Glutamyl-tRNA (*Glu-tRNA*) from L-glutamate. In this pathway, *Glu-tRNA* is transformed to δ -aminolevulinic acid (*ALA*) by the catalyzing of Glu-tRNA reductase (*GluTR*) and glutamate 1-semialdehyde aminotransferase (*GSA-AT*) (Levicán *et al.*, 2007) (Figure 5). Consequently, changes in the expression of *GLuRS* affected the synthesis of early products in the Chl biosynthetic pathway and may contribute to the shorter stature and yellow leaves in rice (Liu *et al.*, 2007; Li *et al.*, 2016). In this study, no significant changes in the expression of *GluRS* and *UROD* were observed, indicating that the synthesis of early products of Chl biosynthesis is unchanged in ch14 compared to wt. This finding contrasts with a previous study on Chl *b*-lacking rice, in which *GluRS* was down-regulated, resulting in inhibition of product synthesis in the early stage of Chl biosynthesis (Nguyen *et al.*, 2020). Two ALA molecules are condensed to form porphobilinogen (*PBG*) by activities of δ -aminolevulinic acid (*ALAD*) (Warren *et al.*, 1998). Porphobilinogen deaminase (*PBGD*) plays a role in the polymerization of four *PBG* molecules from head-to-tail, which subsequently forms hydroxymethylbilane. Hydroxymethylbilane is unstable and then converted to the first cyclic tetrapyrrole, uroporphyrinogen III (*Urop III*) by uroporphyrinogen III synthetase (*UROS*) (Masuda and Fujita, 2008; Blankenship, 2014). At the final stage of the common pathway, protoporphyrinogen oxidase (*PPOX*) catalyzed the oxidation of protoporphyrinogen IX to produce protoporphyrin IX (*Proto IX*) in the soluble stroma of chloroplasts (Masuda and Fujita, 2008). *GSA-AT*, *PBGD*, and *PPOX* were found to be down-regulated in the common pathway, lowering the product of early stages in the Mg branch of Chl biosynthesis. Consequently, it may cause low Chl *a* and Chl *b* contents in ch14 as compared to wt. In the Mg branch, Mg-protoporphyrin IX methyltransferase (*MgMT*) catalyzed the conversion of Mg-protoporphyrin IX to Mg-protoporphyrin IX monomethyl ester (*Mg-protoME*). Moreover, divinyl protochlorophyllide a, a key intermediate in the Chl biosynthesis pathway (Nomata *et al.*, 2005; Masuda and Fujita, 2008), is catalyzed by protochlorophyllide reductase (*POR*), a light-dependent protein present in angiosperms, to yield monovinyl chlorophyllide a (*Chlide*) under light condition (Fujita, 1996; Apel, 2001). In ch14, *MgMT* (Osa_4340015) and *POR* (Osa_4337415, Osa_4349004) were found to be dramatically down-regulated, which might have an influence on disrupting *Chlide* synthesis in the Chl cycle. Consequently, the synthesis of Chl *a* and Chl *b* were blocked or dramatically decreased in ch14 compared to the wt. Contrastingly, in the Chl cycle, no genes was significantly differentially expressed in both ch14 and wt rice. Furthermore, the detection of Chl *b* in ch14 leaves indicated that Chl *b* was normally synthesized from Chl *a*. Therefore, the enzyme *CAO*, *CHL*, *NOL*, *HCAR*, and *CHLG* involved in the Chl cycle were normally translated and functioned. A similar trend was observed in Chl *b*-lacking rice, with the exception of the *NOL* gene, which was up-regulated substantially and thus may have aided in the conversion of Chl *b* to Chl *a* (Nguyen *et al.*, 2020). The observation of Chl *b* in the leaves of ch14 may signify that the expression of *NOL* in this mutant differed insignificantly from that in wt. As a result, the substantial decrease in Chl content in ch14 rice was most likely due to abnormal chloroplast production and a decrease in early intermediates caused

by the down-regulating *GSA-AT*, *PBGD*, *PPOX*, *MgMT*, and *POR* expression. However, there is non-significantly changed in DEGs involved in the Chl cycle including *CAO*, *NOL*, *CHLG*, and *CHL*. In addition, the presence of Chl *b* in ch14 indicated the normal expression of *CAO*. Therefore, the high Chl *a/b* could be explained by the alternative Chl *b* degradation pathway. The accumulation of pheophorbide *b* occurs during cell death after growing a plant in dark conditions (Shimoda *et al.*, 2012). The rising of Chl *a/b* in ch14 together with the normal function of enzymes (*CAO*, *NOL*, *CHLG*, and *CHL*) related in Chl cycle may suggest that the pheophorbide *b* is capable of degrading Chl *b*. Therefore, the degradation of Chl *b* might proceed through the pheophorbide *b* pathway in ch14 rice.

Conclusions

This work examined the transcriptomic profiles and photosynthetic characteristics of Chl *b*-deficient mutant type 2b rice. The ch14 was characterized by a dwarf phenotype along with green leaves, a dropped pigment accumulation, a higher Chl *a/b*, and abnormal chloroplast structure as compared to the wt. 3,594 genes were differentially expressed in ch14, among which there were 309 TFs relative to Chl degradation and biosynthesis, chloroplast formations, and the photosynthesis capacity. This study indicates that the low level of Chl in ch14 may be attributed to abnormal chloroplast development, thus reducing photosynthetic capacity. The high level of Chl *a/b* in ch14 might involve the alternative degradation pathway via pheophorbide *b*.

Authors' Contributions

Conceptualization: CMY, MKN, HCN, and THS; Funding acquisition: CMY; Investigation: MKN, SHL, JWJ, and GTP; Methodology: CMY, MKN, HCN, and THS; Project administration: MKN and CMY; Supervision: CMY; Validation: MKN, SHL, JWJ, GTP, and HCN; Writing - original draft: KMN; Writing - review and editing: KMN, HCN, and CMY. All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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Supplementary Files

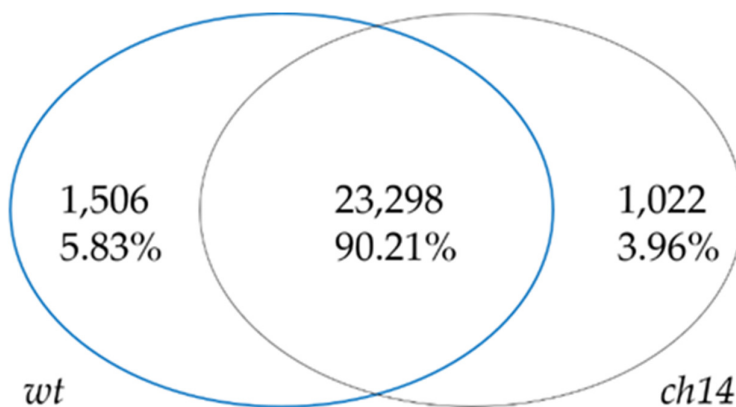


Figure S1. Comparison of gene expression in wildtype rice (wt) and chlorophyll *b*-deficient type 2b rice (ch14)

Table S1. Primer sequences used for RT-qPCR

Marker	Forward sequence (5'-3')	Reverse sequence (5'-3')
<i>GLuRS</i>	GCTCTCCGCTCAAGTGAATACC	GATCAGTCCAGTCCTCCACCTT
<i>GSA-AT</i>	GAGAATGTGTTGGCTGAGA	GGCTGTGGAGGAATGAAG
<i>PBGD</i>	GAAGGATTGACATTGCCGTCCA	GTCTACGCAGAGAAGCACTTCC
<i>UROD</i>	GACGGCGTCATCCTGTTCTC	TGCGTAGAATCTTGAGCGACTC
<i>PPOX</i>	GAATGCTTAATTGACGGAGAG	GAACTGTGGTATGGCTTGT
<i>POR</i>	TCATCCTCGGCTCCATCACC	CTCCTGCATCGTCAGCATGTT
<i>MgMT</i>	CTCTACTTCGACTTCCTCAA	GAACTGCGTGGAGATGAA
<i>CAO</i>	GCATGGACTTCTGCTGGACAA	AGATGCCAGTGGTTGACAAGAC
<i>HCBR</i>	GCAGGAAGCAAGACATGGATGA	GCATTGGAGCAAGCCTGTCA
<i>CHLG</i>	GGGCACTGTTGTTAGCAGGG	GCCAATGTAGCTCGCACCAA
<i>NOL</i>	CACTGCTTCTCCTGGAATGGTC	GCAAGATCCTTGGTGGTGTCAA
<i>MgCH</i>	AAGATGGTTGCCGAAGTGGATG	ATGTCCTGGAGCTGCTTCTCA
<i>UBQ10*</i>	CTCATCTCCTCTCCTCGCATCA	CCACCAATCGGATCTAGCAACA

*Reference gene

Table S2. Summary of genome mapping

Sample	Total clean reads	Total mapping ratio [%]	Uniquely mapping ratio [%]*
ch14	29,397,372	96.49	87.64
wt	25,305,788	96.85	89.19

* Unique mapping ratio: Percentage of reads that map to only one location in the reference genome

wt: wildtype *Oryza sativa* Norin No. 8; ch14: chlorophyll *b*-deficient mutant type 2b

Table S3. Summary of gene mapping ratio

Sample	Total clean reads	Total mapping ratio [%]	Uniquely mapping ratio [%]*
ch14	25,397,372	88.31	78.89
wt	25,305,788	88.94	79.56

* Unique mapping ratio: Percentage of reads that map to only one location in the reference genome

wt: wildtype *Oryza sativa* Norin No. 8; ch14: chlorophyll *b*-deficient mutant type 2b