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Transcription analysis of chlorophyll biosynthesis in wildtype and chlorophyll *b*-lacking rice (*Oryza sativa* L.)

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Abstract

The aim of the present study was to investigate the photosynthetic properties and transcriptomic profiles of wildtype and chlorophyll (Chl) *b*-lacking rice (*Oryza sativa* L.). The plastid ultrastructure of the Chl *b*-lacking rice (*i.e*., loss of starch granules, abundant vesicles, and abundant plastoglobuli) indicated abnormal plastid development, whereas the analysis of transcriptome profiles and differentially expressed genes revealed that gene encoding PsbR (PSII core protein) was downregulated in the mutant, thereby reducing the Chl accumulation of the mutant. Meanwhile, in regards to Chl biosynthesis and degradation pathways, *GluTR* gene was downregulated, whereas *UROD*, *CPOX*, and *MgCH* genes were upregulated. The qPCR results were generally consistent with those of the transcription analysis, except for the finding that *NOL* genes, which regulate Chl *b* degradation, were upregulated. These results suggest that both the reduction in Chl accumulation and increase in conversion rate of Chl *b* to Chl *a* caused Chl *a*/*b* ratio amplification in mutant. The present study also provides evidence for Chl *b* degradation *via* pheophorbide *b*.

Additional key words: grana; next-generation sequencing; photosynthesis; RNA-Seq; transcriptome.

Introduction

Photosynthesis (*i.e*., the process of converting sunlight energy into a chemically stable form), occurs in plants, algae, cyanobacteria, and other photosynthetic bacteria (Jin *et al*. 2003). In higher plants, photosynthesis occurs in chloroplasts, which are specific organelles that synthesize and degrade billion tons of chlorophyll (Chl) annually (Eckhardt *et al*. 2004, Liu *et al*. 2007, Kräutler 2008). Chl is extremely important during the photosynthesis, since it plays an essential role in light absorption and energy transfer (Fromme *et al*. 2003, Tanaka and Tanaka 2006). Therefore, understanding the efficiency of Chl biosynthesis could facilitate increases in photoassimilates accumulation, and ultimately, in crop yield (Mitchell and Sheehy 2006, Huang *et al*. 2013). Recently, the Chl biosynthesis and degradation pathways have been well characterized and found to involve more than 17 identified enzymes and 15 reactions from precursor 5-aminolaevulinic acid (ALA) to the Chl *a* and Chl *b* at Chl cycle until breakdown at a degradation pathway (Masuda and Fujita 2008). Even though Chl *a* along with Chl *b* and carotenoids (Car) are found throughout higher plants, extremely different Chl *a*/*b* ratios have been reported in many plant and algae mutants (Thornber and Highkin 1974, Eskins *et al*. 1983, Rühle *et al*. 1983, Yang *et al*. 1990, Zhang *et al*. 2011). Such Chl-deficient mutants have been extensively used to study the Chl biosynthetic pathway and the biogenesis of the photosynthetic apparatus in barley, maize, pea, sweet clover, wheat, rice, soybean, sugar beet, *Arabidopsis*, and *C. reinhardtii* (Chen *et al*. 2007, Chu *et al*. 2015, Bujaldon *et al*. 2017, Zhu *et al*. 2019).

Rice (*Oryza sativa* L.) is a major crop worldwide. However, the current rice production is insufficient for meeting the world food demand, owning to continuous increases in world population. Consequently, great efforts have been made to enhance the yield of these plants (Gupta 2013). Studies have shown that photosynthesis can affect rice yield (Ambavaram *et al*. 2014). Enhancing the Chl contents was reported to have positive effects on biomass, photosynthetic rate, and grain yield in rice (Wang *et al*. 2008). In fact, such studies have described at least 16 different Chl-deficient rice mutants, which based on Chl *a*/*b* ratios, have been separated into two main types: Type 1, in which Chl *b* is completely absent (Chl *b*-lacking mutants), and Type 2, which possesses Chl *a*/*b* ratios of ~ 10 (Type 2a) or ~ 15 (Type 2b) (Terao *et al*. 1985a,b; 1996). However, the mechanisms that underlie differences

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Abbreviations: Car – carotenoids; *ch1* – Chlorina 1 mutant rice; Chl – chlorophyll; DEGs – different expressed genes; GO – gene ontology; *wt* – Norin No. 8 wildtype rice.
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between the Chl *a*/*b* of Chl-deficient and wildtype rice remain unclear. Currently, the use of next-generation sequencing (NGS) for transcriptome profiling is widely used for the broad assessment of gene expression. In the present study, wildtype (Norin No. 8, *wt*) rice (*Oryza sativa* L.) and a Chl *b*-lacking mutant of *wt* (Chlorina 1, *ch1*), which were provided by Dr. Tomio Terao, were used to investigate the effects of gene expressional patterns on Chl regulation in *ch1* rice. This study compared the characterization, differentially expressed genes (DEGs), and transcription factors (TFs) related to Chl biosynthesis and degradation between *wt* and *ch1* rice. This work is the first to report molecular mechanism underlying Chl *a*/*b* ratio of a Chl *b*-lacking mutants in rice.

Materials and methods

Plant materials: Seeds of wildtype (Norin No. 8, *wt*) and its Chl *b*-lacking mutant (Chlorina 1, *ch1*) rice were graciously provided by Dr. Tomio Terao (Department of Applied Physiology, National Institute of Agrobiological Resources, Tsukuba Science City, Japan). The *ch1* and *wt* rice represent Chl-deficient mutant Type 1 and its wild type, respectively, as described above. The seeds were sown in the greenhouse, and the seedlings were grown for three weeks. Leaves were then collected, immediately frozen in liquid nitrogen, and stored at –80°C for further experiments.

Chl and Car measurement: The leaf material was extracted using 80% (v/v) acetone, according to the method described by Yang *et al*. (1998). The absorbance of the extract was then measured at 480 nm using UV-Vis spectrophotometer (*Hitachi U2800*, Tokyo, Japan) at room temperature to determine total Chl content, Car content, and Chl *a*/*b* ratio.

Ultrastructure microscope: Wildtype and *ch1* leaf tissues were trimmed into small cubes (approx. 0.5 × 0.5 × 0.5 mm). The tissue pieces were immersed in 2.5% glutaraldehyde at 4°C for 24 h followed by 1% OsO_4 for 2 h. The tissues were trimmed to 70 nm using a *Leica EM UC6* ultramicrotome (*Leica Microsystems GmbH*, Wetzlar, Germany), stained using 1% (w/v) uranyl acetate and 1% (w/v) lead citrate (Spurr 1969), and then visualized using a *Phillip Tecnai 12* transmission electron microscope (*JEOL Ltd.*, Japan).

Transcriptome sequencing: For transcription analysis, total RNA was extracted from *wt* and *ch1* leaves using the *RNeasy Plant Mini Kit* (*Qiagen*, Hilden, Germany), following the manufacturer's instructions. For transcriptome sequencing, paired-end cDNA libraries for the *wt* and *ch1* rice were constructed, and the resulting cDNA libraries were sequenced using *BGISEQ-500* platform (*Beijing Genomics Institute*, Shenzhen, China). Adapter sequences, ambiguous nucleotides, and low quality sequences were trimmed from the raw reads using *SOAP* nuke *v. 1.5.2* (https://github.com/BGI-flexlab/SOAPnuke). Both the cDNA library construction and transcriptome sequencing were performed by a commercial service provider (*Tri-I Biotech*, *Inc.*, New Taipei City, Taiwan).

Transcriptome analysis: The clean reads were mapped to the genome of *O. sativa* 'Nipponbare' (http://rapdb.dna.affrc.go.jp/) using hierarchical indexing for the spliced alignment of transcripts in *HISAT2 v. 2.0.4* (http://www.ccb.jhu.edu/software/hisat). Gene expression analysis was performed using *Bowtie2 v. 2.2.5* (http://bowtie-bio.sourceforge.net/Bowtie2/index.shtml), and gene expression levels were calculated using *RSEM v. 1.2.12* (http://deweylab.biostat.wisc.edu/RSEM). *Pearson*'s correlation, hierarchical clustering and illustration of the samples were performed using the cor, hclust, and ggplot2 functions of *R*, respectively. Meanwhile, DEGs were detected using PossionDis, which is based on Poisson distribution with parameters of Fold Change ≥ 2.00 and FDR ≤ 0.001 (Audic and Claverie 1997), and were subject to *Gene Ontology* (GO) and *KEGG* pathway analysis.

Quantitative RT-PCR (qPCR): Total RNA (μg) extracted from *wt* and *ch1* rice was subjected to cDNA synthesis using a *Transcriptor First Strand cDNA Syn-thesis Kit* (*Roche Diagnostic Systems*, Branchburg, NJ, USA) and oligo (dT) primer. Primer sets were designed using *Primer Premiere 6* software (*Premiere Biosoft*, Palo Alto, USA) (Table 1S, *supplement*). qPCR was performed using the *StepOne Plus* Real-Time PCR system (*Applied Biosystems*, *Life Technologies Inc.*, Italy) with *Roche FastStar Universal SYBR Green Master* reagent (*Roche Diagnostic Systems*, Branchburg, NJ, USA). Relative gene expression values were calculated as $2^{-\Delta Ct}$, where ΔCt was calculated as the difference between the target gene Ct and reference gene Ct. The fold change of each gene in leaf tissues was calculated by $2^{-\Delta Ct}{}_{mutant}/2^{-\Delta Ct}{}_{wildtype.}$.

Statistical analysis: The pigment contents and relative gene expression (qPCR results) of *wt* and *ch1* rice were statistically analyzed using the least significant difference (LSD) *t*-test at $p \leq 0.05$, which was performed by *SAS v. 8.0* (*Research Triangle Park*, NC, USA).

Results

Characterization of *ch1* rice: The *wt* leaves were dark green, whereas *ch1* leaves ranged from light green to green (Fig. 1*A*). Overall, the *wt* leaves accumulated about twice as much of Chl (total Chl) as in the *ch1* leaves (~ 91%) and ~ 78% more Car. Furthermore, Chl *b* was absent in the *ch1* leaves, thereby yielding a Chl *a*/*b* ratio of ∞ so only Chl *a* was present (Fig. 1*B*). Chloroplasts from the mesophyll cells of *wt* leaves exhibited normal structure, including distinct thylakoid membranes and stromal lamellae with small starch granules and one or two plastoglobuli (Fig. 2*A*,*B*). By contrast, those of *ch1* leaves were presented as indistinct thylakoid membranes, indistinct or absent stromal lamellae, and abundant vesicles and plastoglobuli (Fig. 2*C*). The stacked thylakoid grana of the *ch1* leaves were also thinner than those of *wt* leaves (Fig. 2*D*).

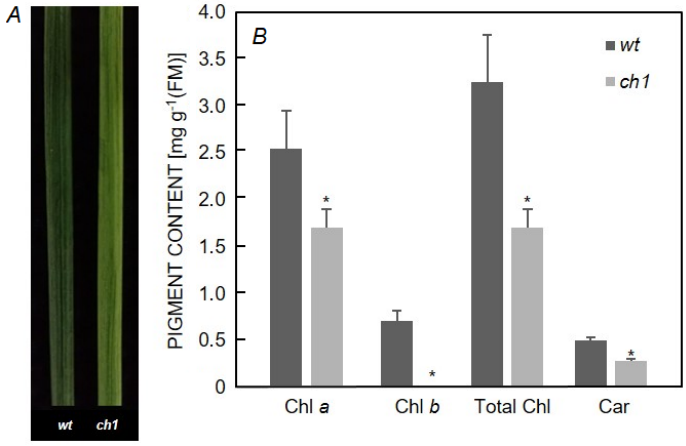

Fig. 1. Leaf coloration and determination of pigments content. (*A*) Leaf coloration of wildtype (*wt*) and Chl *b*-lacking (*ch1*) rice. (*B*) Chlorophyll (Chl) and carotenoid (Car) contents of wildtype (*wt*) and Chl *b*-lacking (*ch1*) rice. *Asterisks* indicate significant differences between the pigment contents of the *wt* and *ch1* rices ($p \leq 0.05$).

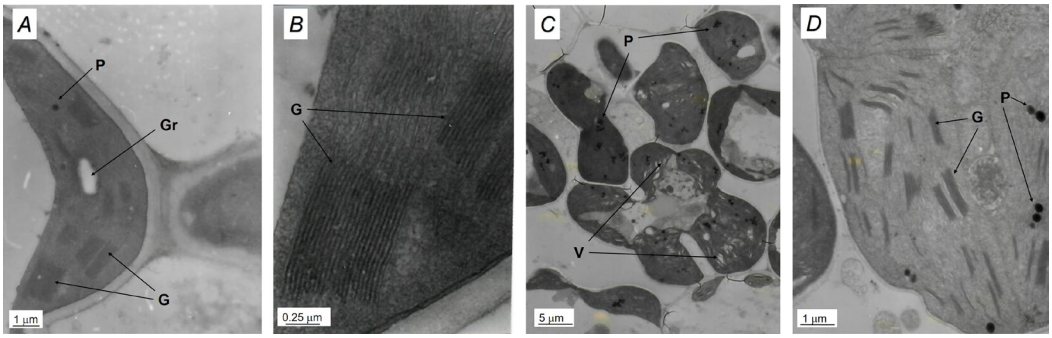

Fig. 2. Chloroplast ultrastructure of wildtype and chlorophyll *b*-lacking rice. (*A*,*B*) Normal chloroplast structure of wildtype (*wt*) rice. (*C*,*D*) Abnormal chloroplast structure chlorophyll *b*-lacking (*ch1*) rice, showing indistinct thylakoid membranes and abundant plastoglobuli. G – grana; P – plastoglobuli; Gr – granulose; V – vacuole.

Genome mapping and gene expression: Approximately 1.27–1.45 GB of clean reads were produced using the *BGISEQ-500* platform, and after filtering, 25.31–28.93 million reads were mapped to the *O. sativa* 'Nipponbare' reference genome (http://rapdb.dna.affrc.go.jp/), with match ratios in the range of 96.84-97.94% (Table 2S, *supplement*). A total of 93.0 and 89.67% of the *wt* and *ch1* libraries, respectively, were mapped to the reference genome, whereas the 80.91 and 80.42% of the libraries were mapped to single location in the reference genome (Table 3S, *supplement*). Approximately 89.6% of genes expressed by *wt* and *ch1* rice were detected in both (Fig. 1S).

Annotation and analysis of different gene expression DEGs: Functional annotation was performed to obtain information protein function annotation, pathway annotation and gene ontology (GO) annotation. Genes with sequence orientation were aligned against *KEGG* database. GO is an international standardized gene functional classification system in which transcripts are attributed to biological processes, cellular components, or molecular function. Fig. 3 shows the distribution of genes among the GO categories. In total, 22,493 genes were mapped, with 7,972 genes assigned to 'biological process', 10,282 genes assigned to 'cellular component', and 4,239 genes assigned to 'molecular function'. Individual genes can be assigned to multiple GO-terms (Fig. 3).

DEGs were identified using the fragments per kb per million reads (FPKM) method, and the Poisson distribution equation, with false discovery rate (FDR) ≤ 0.001 and absolute log2 ratio ≤ 1 (Fig. 4). Total of 1,971; 8,336; and 15,886 genes were identified as upregulated, downregulated, and unaffected in *ch1* leaves, respectively (Fig. 4*A*).

Role of Chl metabolism genes in leaf coloration: DEGs related to chloroplast development and division were identified based on KEGG pathway annotation. Genes related to the FtsZ cell division protein genes and PsbR chloroplast photosystem II protein genes were downregulated in the *ch1* rice. Meanwhile, *PetF* genes related to the FDX electron transfer protein genes were greatly upregulated (Table 1).

A total of 19 DEGs related to Chl biosynthesis and four DEGs related to Chl degradation were identified based on KEGG pathway annotation. The expression levels of the DEGs were determined using hierarchical cluster analysis (Fig. 5). A *UROD* homolog (Osa_4326635), *CPOX* (Osa_4336951), and *MgCH* (Osa_4334537) were significantly downregulated in *ch1* ($\Delta\log 2 = 2.1$, 2.2, and 2.4, respectively) as compared to the *wt* (Fig. 5). These

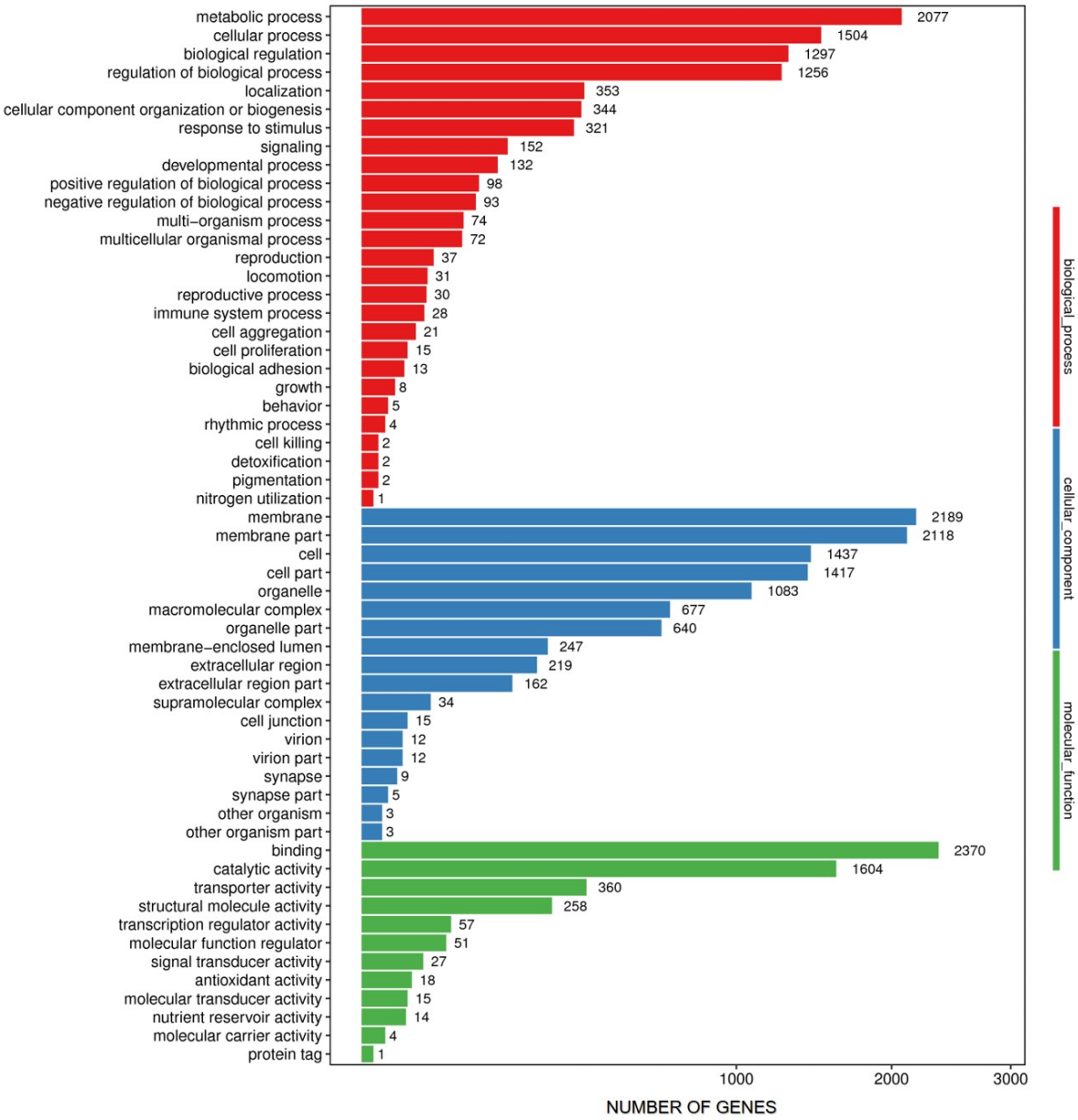

Fig. 3. Gene Ontology classification of differentially expressed genes from wildtype (*wt*) and chlorophyll *b*-lacking (*ch1*) rice.

results suggested that the Chl biosynthesis of *ch1* was similar to that of *wt* rice, even though the *ch1* rice exhibited the significantly lower Chl content (Fig. 1).

Role of transcription factors (TFs) in leaf coloration: TFs are key regulatory proteins that play important roles in the regulation of gene expression. In the present study, 671 DEGs were identified as putative TFs and were associated with a total of 51 TF families. The most abundant TF family was the MYB superfamily (90 DEGs), followed by the MYB-related (62 DEGs), bHLH (48 DEGs), WRKY (46 DEGs), AP2-EREBP (40 DEGs), and NAC (35 DEGs) TF families (Table 2). The MYB, MYB-related, WRKY, and NAC families were represented by 35, 28, 21, and 28 DEGs, respectively, which were downregulated in *ch1* rice. Meanwhile, 19 DEGs were associated with the HLH TFs, most of which were also significantly downregulated in the *ch1* rice, and some of the DEGs which were associated with the AP2-EREBP, C2H2, and TCP families were downregulated in the *ch1* rice, as well (Table 2).

DEGs validation: To validate the identification of DEGs related to Chl biosynthesis and degradation, different expression analysis of the identified DEGs was performed. Eleven of the twelve genes analyzed by qPCR expression patterns were similar to those observed using transcription analysis (Fig. 6). More specifically, *UROD*, *CPOX*, and *MgCH* were significantly upregulated in *ch1*, whereas

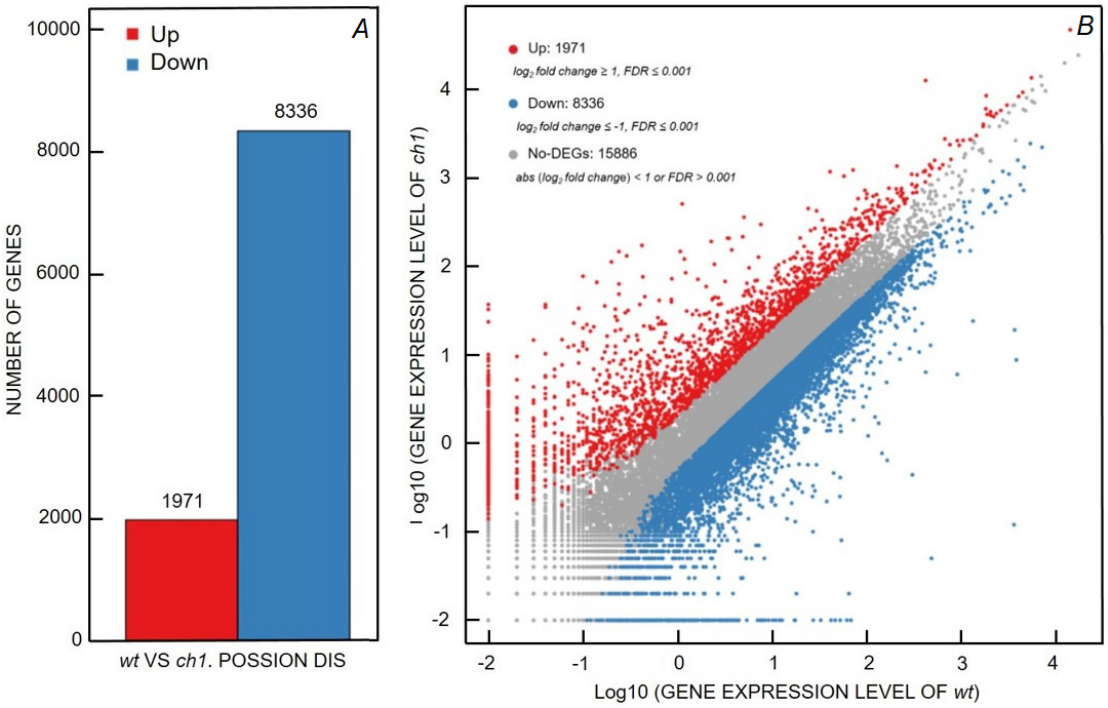

Fig. 4. Differentially expressed genes (DEGs) from wildtype (*wt*) and chlorophyll *b*-lacking (*ch1*) rice. (*A*) Comparison of upregulated and downregulated DEGs. (*B*) Classification of genes, based on differences in expression level.

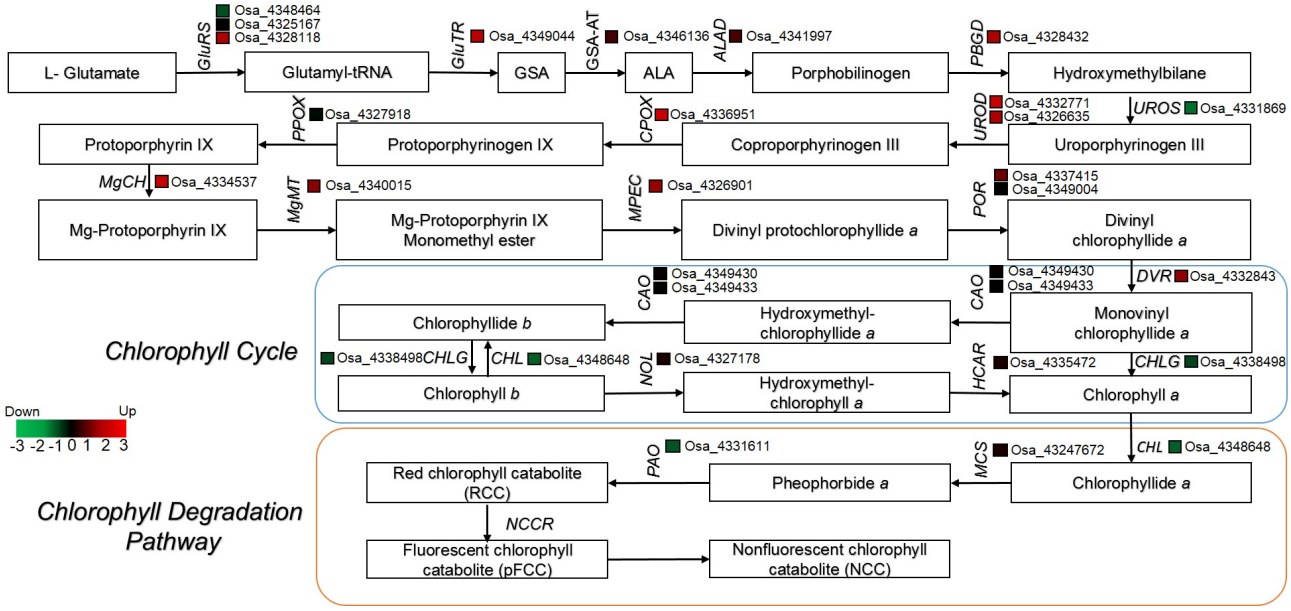

Fig. 5. Expression profiles of differentially expressed genes involved in chlorophyll biosynthesis and degradation. The expression levels of the chlorophyll *b*-lacking (*ch1*) are compared to those of wildtype (*wt*) rice.

GluRS was significantly downregulated. However, in contrast to the results of transcription analysis, the qPCR results indicated that NOL was significantly upregulated in *ch1* (Fig. 6). In general, the qPCR results were consistent with the RNA-Seq data, despite some differences in expression level.

Discussion

Physiological changes and pigment contents analysis: In higher plants, Chl biosynthesis and degradation are involved in leaf coloration. Therefore, Chl-deficient mutants exhibit changes in leaf coloration (Yang *et al*. 2015b).

Table 1. Distribution of unigenes amongst KEGG pathways that contain differentially expressed genes. TF – transcription factor; *ch1* – chlorophyll *b*-lacking mutant (*Chlorina 1*).

Function	Gene ID	TF family	Log_2 fold change	Expression in *ch1*	Annotation
Chlorophyll biosynthesis	Osa_4332771	*UROD*	2.1	Upregulated	uroporphyrinogen decarboxylase
	Osa_4336951	*CPOX*	2.2	Upregulated	coproporphyrinogen III oxidase
	Osa_4334537	*MgCH*	2.4	Upregulated	Mg-chelatase
Photosynthesis	Osa_107276047	*PsbR*	–5.15	Downregulated	photosystem II 10 kDa protein
	Osa_4342395	*PsbR*	–7.2	Downregulated	photosystem II 10 kDa protein
	Osa_4335779	*PetF*	2.8	Upregulated	ferredoxin-1
	Osa_9270637	*PetF*	–5.5	Downregulated	ferredoxin, root R-B1
	Osa_4333746	*PetF*	5.3	Upregulated	ferredoxin-2
Chloroplast division	Osa_4338932	*FtsZ1*	–2.7	Downregulated	Cell division protein FtsZ homolog 1
	Osa_4338932	*FtsZ2*	–2.3	Downregulated	Cell division protein FtsZ homolog 2

Chl-deficient mutants have been reported in many species, including barley, maize, peas, sweet clover, wheat, rice, rockcress, and maidenhair tree (Yang *et al*. 1990, Chen *et al.* 2007, Chu *et al*. 2015, Bujaldon *et al*. 2017, Li *et al*. 2018, Zhu *et al*. 2019). Our results are consistent with those of another study, which reported that Chl *b* was completely absent in a Chl-deficient mutant (Terao *et al*. 1985a). The total Chl and Chl *b* content of the *ch1* leaves remarkably decreased as compared to *wt* leaves. This result suggested that the reductions in total Chl content contributes to leaf color variation at the physiological level.

The analysis of the chloroplast ultrastructure of the *wt* and *ch1* rice revealed differences in thylakoid membrane structure. The *wt* chloroplasts possessed typical thylakoid membranes that were efficient for harvesting and converting light energy, whereas the *ch1* chloroplast possessed abnormal thylakoid membranes. Similarly, chloroplasts of green bamboo leaves contained abundant thylakoid membranes, whereas the thylakoid membranes of Chl *b*-lacking leaves were converted into numerous abnormal vesicles (Yang *et al*. 2015a). In *Anthurium andraeanum*, the mesophyll cells of wildtype plants contained normal chloroplast which contained small starch granules and, thus, large gaps among stroma thylakoids (Yang *et al*. 2015b).

The results obtained in this study also demonstrated differences in the chloroplast structure of *wt* and *ch1* rice. The ultrastructure of *ch1* rice (*i.e*., abundant plastoglobuli, indistinct thylakoid membranes, and reduced starch granules) indicated abnormal development and possibly a reduced accumulation of pigments, which would reduce light energy conversion and account for the coloration difference in the *wt* and *ch1* rice. The *ch1* rice also exhibited thinner stacked grana, caused by a reduction in the thylakoid membranes per granum. Therefore, the Chl *a*/*b* ratio of *ch1* rice may reflect the abnormal plastid development and function of plastid in the *ch1* rice.

Chloroplast-related DEGs and photosynthetic capacity: The normal development of chloroplasts in higher plants requires the coordination of both plastid nuclear genes, and changes in the expression levels of either gene type could affect the biogenesis in Chl metabolism and chloroplast assembling, thus affecting Chl *a*/*b* ratio and leaf coloration (Li *et al*. 2015, Yang *et al*. 2015a). FtsZ proteins, which play important roles in cell and organelle division, also regulate chloroplast division (Schmitz *et al*. 2009). The expression levels of *FtsZ1* and *FtsZ2* were significantly downregulated in the *ch1* rice, which indicated that chloroplast division may be defective. This finding was strongly supported by differences in the chloroplast ultrastructure and leaf coloration of *wt* and *ch1* rice, thereby suggesting that the lower accumulation of Chl contents was responsible for the lower Chl *b* content and higher Chl *a*/*b* ratio of the *ch1* rice. During photosynthesis, light energy is captured by pigments in the LHC proteins, which then transfer absorbed light energy to PSI and PSII reaction center complexes (Goral *et al*. 2012, Zhao *et al*. 2017). In a previous study of a Chl-deficient *A. thaliana* mutant, LHC protein was strongly reduced, or even completely absent, thereby impairing the grana stacking of the plant's chloroplast (Kim *et al*. 2009). The multi-protein and pigment complex known as PSII provides the high redox potential needed to oxidize water, and contains more than 20 subunits, including PsbR (Shi *et al*. 2012). Chloroplast FDX proteins including PetF, play important roles in electron transfer. Enhancing *PetF* genes raises reduced ascorbate contents and lowers H_2O_2 contents under natural conditions in *C. reinhardtii* (Lin *et al*. 2013). Moreover, reducing FDX causes leaf yellowing in transgenic plants grown under high-light conditions (Holtgrefe *et al*. 2003, Hanke and Hase 2008). In the present study, two DEGs (PsbR) were identified as the members of the LHCII gene family, and their expression levels were strongly downregulated in the *ch1* rice, which might indicate that the amounts of light-harvesting Chl proteins were reduced, thereby lowering the photosynthetic capacity. Furthermore, *PetF* genes were strongly upregulated in the *ch1* rice, which might enhance Chl degradation (Table 1).

RNA-Seq analysis and Chl-related DEGs: Both NGS and transcriptome profiling provide information about DEGs (Seo *et al*. 2004, Wang *et al*. 2009). Therefore, the combination of RNA-Seq technology and improved

Table 2. Summary of differentially expressed gene transcription factor genes of chlorophyll *b*-lacking (*ch1*) rice compared to wildtype (*wt*).

Transcription factor family	Total no. genes	No. genes upregulated	No. genes downregulated
zf-HD	5	0	1
WRKY	46	2	21
VOZ	1	0	0
TUB	5	0	3
Trihelix	17	0	1
TIG	2	0	0
Tify	8	0	1
TCP	6	1	2
SRS	3	0	2
Sigma70-like	3	0	0
SBP	5	0	3
S1Fa-like	1	0	0
PLATZ	2	0	1
PBF-2-like	2	0	0
OFP	8	3	0
NAC	35	3	28
MYB-related	62	12	25
MYB	90	17	35
mTERF	23	1	14
MADs	16	0	11
LOB	1	0	0
LIM	4	0	0
HSF	9	0	5
HB	2	1	0
GRF	4	0	0
GRAS	18	0	8
GeBP	3	0	0
G2-like	23	6	15
FHA	9	0	3
FAR1	18	0	4
E2F-DP	3	0	0
DBP	1	0	0
CSD	1	0	0
CPP	6	0	3
CAMTA	3	0	0
C3H	19	3	2
C2H2	20	1	7
C2C2-YABBY	5	1	0
C2C2-GATA	11	2	2
C2C2-Dof	11	0	6
C2C2-CO-like	10	2	1
b2IP	16	2	1
BSD	7	0	1
bHLH	48	4	18
BES1	2	0	1
BBR/BPC	2	0	2
ARR-B	4	0	4
ARF	14	0	4
AP2-EREBP	40	2	8
Alfin-like	12	0	1
ABI3VP1	15	0	5

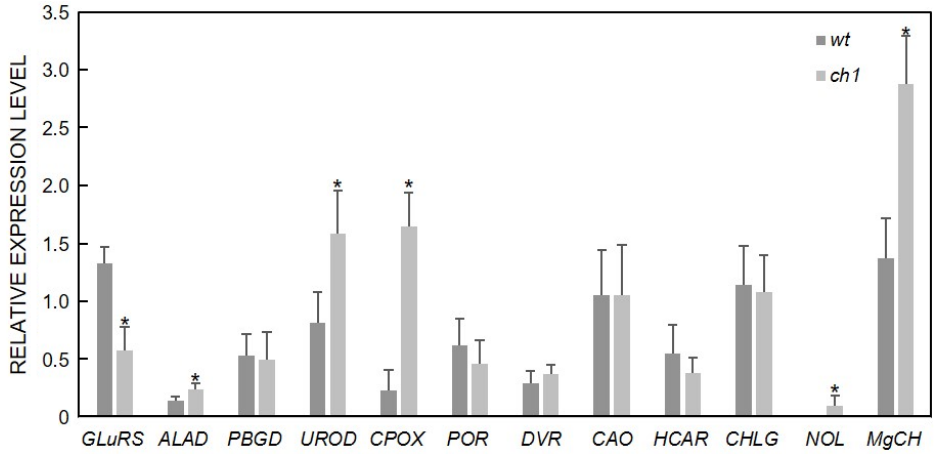

Fig. 6. Expression of levels of 12 differentially expressed genes from wildtype (*wt*) and chlorophyll *b*-lacking (*ch1*) rice. Gene expression was measured using quantitative real-time PCR. *Asterisks* indicate significant differences between the expression levels of the *wt* and *ch1* rices ($p \leq 0.05$).

analysis methods enables the recognition of novel transcript isoforms and the estimation of their roles in altering Chl *a*/*b* ratios. In the present study, transcriptome profile analysis of *wt* and *ch1* rice revealed a total of 26,193 DEGs. It is worth noting that some of DEGs related to Chl biosynthesis and degradation, chloroplast development, photosynthesis, and pigment biosynthesis are likely contributing to the observed differences in Chl *a*/*b* ratios.

Chl is responsible for light harvesting and transferring in antenna systems, and for charge separation and electron transport in reaction center (Ben-Shem *et al*. 2003, Nelson and Yocum 2006). In *A. thaliana*, 27 genes were identified to encode 15 Chl-biosynthesis enzymes (Meier *et al.* 2011), and changes in the expression could generate Chl-metabolism disorders that alter Chl *a*/*b* ratio. In common pathway of Chl biosynthesis metabolism, changes in the expression of *GluRS* could affect Chl contents (Fang *et al*. 2016). Uroporphyrinogen decarboxylase (*UROD*), which is a branch point enzyme in the biosynthesis of tetrapyrroles, catalyzes the decarboxylation of four acetate groups of uroporphyrinogen III, thereby yielding coproporphyrinogen III, which then contributes to heme and Chl biosynthesis (Fan *et al*. 2007). Moreover, Mg-chelatase deficiency is a common factor among many Chl-deficient mutants. For example, the downregulation of *MgCH* genes reduce Mg-chelatase activity in *C. sinensis* (Wang *et al*. 2014).

In the present study, a total of 19 DEGs related to Chl biosynthesis were identified based on KEGG pathway annotation. Among these DEGs, *GluTR* was downregulated in the *ch1* rice. However, *UROD* and *CPOX* were upregulated, an observation that was further confirmed through qPCR, and that suggested that later stages of Chl biosynthesis occurred in the *ch1* rice, whereas early stages were inhibited. Moreover, the expression of MgCH was upregulated due to less early stage products which suggests that the Chl contents of the *ch1* rice needs to be enhanced in order to adapt to light-harvesting needs for conversion to sugar. In addition, the expression of Chl cycle- and degradation-related genes, including *CAO*, *CHLG*, *HCAR*, *CHL*, *MCS*, and *PAO*, were insignificantly different. *Non-yellow coloring1* (*NYC1*) is a stay-green rice mutant in which degradation of Chl during senescence is impaired. Pigment analysis has revealed that degradation of both Chls and carotenoids is repressed in the *NYC1*, in which most LHCII isoforms are selectively retained during senescence. The *NOL* protein is closely related to *NYC1*, and the upregulation of *NOL* has been reported to drastically reduce the Chl *b* content (Kusaba *et al*. 2007, Sato *et al*. 2009). In the present study, qPCR analysis indicated that *NOL* was significantly upregulated in *ch1* rice, although the transcriptome data indicated that it was unaffected. The Chl content of *ch1* rice was significantly reduced as compared to *wt* rice. This strongly suggests that the abnormal chloroplast development of the *ch1* rice affects the accumulation of Chl *b* and that, together with the significant upregulation of *NOL* genes, enhances the degradation of Chl *b* to Chl *a*. This is similar to observation in *G. biloba* leaves, where the upregulation of *NOL*/*NYC* genes was associated with coloration changes from green to light-green (Li *et al*. 2018).

There are also two other possible explanations for the absence of Chl *b* in *ch1* rice. First, because chlorophyllide *a* oxygenase (CAO) is the main enzyme involving to the conversion of Chl *a* to Chl *b* in Chl cycle (Reinbothe *et al*. 2006, Yang *et al*. 2015c). The expression of *CAO* genes plays an important role in the Chl *a*/*b* ratio. In this study, both the transcriptome and qPCR analysis confirmed that there was no significant difference between the *CAO* expression of the *wt* and *ch1* rice, which suggests that *CAO* expression was blocked at the translation level, or that abnormal translation contributed to the formation of nonfunctional CAO proteins. Secondly, many studies reported that the conversion of Chl *b* to Chl *a via* hydroxymethyl Chl *a* is the first step of Chl *b* degradation, and is catalyzed by NOL (Ito *et al*. 1996, Horie *et al*. 2009). In addition, NOL has multi-substrate specificity and is unbound to light-harvesting complexes including Chl *b*, chlorophyllide *b*, pheophorbide *b*, and pheophytin *b* (Shimoda *et al*. 2012). However, pheophytin *b* and pheophorbide *b* were reported not to be produced in chloroplast due to the specificity of Mg-dechelatase (Tanaka and Tanaka 2019). In contrast, another study reported that pheophorbide *b* is accumulated during cell death after incubating a plant whose core antenna complex contained Chl *b* in dark conditions (Shimoda *et al*. 2012).

Therefore, the mechanism underlying pheophorbide *b* presence in plant cells is still unclear. We hypothesized that Chl *b* might be degraded *via* the pheophorbide *b* pathway in *ch1* rice, however, this requires further investigations. The absence of Chl *b* in *ch1* may confirm that the pheophorbide *b* is capable of degrading Chl *b*, which is a pathway that remains poorly understood.

This study also found that grana stacking is normal along with the absence of Chl *b* and reduced levels of Chl *a*. Similar patterns have been reported by previous studies, which suggests that stacking grana affects both LHCII protein and another factor (Nakatani and Baliga 1985, Ouijja *et al*. 1988, Yang and Chen 1996). Accordingly, the results of the present study provide strong evidence to support the conclusion that other than Chl-deficiency-mediated changes in LCH proteins are involved in grana stacking.

Conclusion: This study reported the mechanisms underlying the different Chl *a*/*b* ratios in wildtype rice and Chl *b*-lacking mutant (*ch1*) rice. Lower Chl content and abnormal chloroplasts structure were observed in the *ch1* leaves. Transcription analysis identified 23 DEGs and 671 TFs, which are involved in Chl metabolism, chloroplast development, cell division, and photosynthesis. qPCR analysis was used to validate the DEGs. Changes in the expression of genes related to chloroplast development, Chl biosynthesis, and degradation reduced the accumulation of Chl in the *ch1* rice. This result suggest that the Chl *a*/*b* ratio was amplified both by reduction in Chl accumulation, owing to abnormal chloroplast development, and by the enhanced conversion of Chl *b* to Chl *a*. Moreover, the results of the present study indicated the same Chl-cycle pattern in the wildtype and *ch1* rice, which suggests the involvement of either abnormal *CAO* translation, or another Chl *b* degradation pathway. This study provides insights into the molecular mechanism that underlies different Chl *a*/*b* ratios in rice.

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