

Temperature-mediated shifts in chlorophyll biosynthesis in leaves of chlorophyll *b*-lacking rice (*Oryza sativa* L.)

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Abstract

Extreme temperatures have become a threat to crop yields. To maintain plant growth and yield, chlorophyll (Chl) biosynthesis plays a crucial role in adaptation to temperature stress. This study investigated the influence of temperature on the biosynthesis and characteristics of pigments (Chl *a*, Chl *b*, and carotenoids) in the leaves of Chl *b*-lacking mutant rice (Chlorina 1, *chl1*) and wild-type rice (Norin No.8, *wt*). The *chl1* showed thinner stacked grana caused by a decrease in thylakoid membranes per granum at 15 °C, whereas the destacked grana were observed at 35 °C after 12 h incubation. However, the grana are stacked normally, along with the absence of Chl *b*, and a significantly decreased amount of Chl *a* in both *wt* and *chl1* were observed after heat stress exposure, demonstrating that light-harvesting complex II proteins are involved in grana stacking. *Chl1* was sensitive to 15 °C during the first 4 h of incubation but it subsequently adapted to the cold environment. In addition, there were no significant differences in the photosynthesis between *wt* and *chl1* after 12 h incubation at 35 °C. Differentially expressed gene (DEGs) analysis revealed that *GlurS* expression decreased, which resulted in a decline in Chl biosynthesis in *wt* and *chl1* at 35 °C. At 8 h and 12 h, there were no significant differences in the expression of DEGs involved in Chl biosynthesis and degradation between *wt* and *chl1* at 15 °C. ALAD expression in *wt* and *chl1* at 15 °C decreased until it was undetectable. These findings suggested that *chl1* may adapt to temperatures ranging from 15 °C to 35 °C.

Keywords: chlorophyll *b*-lacking mutant; grana; photosynthesis; temperature sensitivity

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Introduction

Extreme temperatures caused by climate change, including cold stress and heat stress, affect the development, growth, and seasonal behaviors of plants, and can consequently impact crop yield (Raza *et al.*, 2019; Ding *et al.*, 2020). Under unsuitable growth temperature, thermomorphogenesis occurs and produces rapid changes in morphology. Vernalization and cold stratification processes can be observed as increases in flowering or seed germination under cold, moist temperatures, and may trigger cold acclimatization. In contrast, global warming frequently accelerates heat stress, which affects a part of the plant life cycle, particularly photosynthesis and carbon assimilation. High-temperatures have been found to decrease net photosynthetic rate, which is attributed to either stomatal or nonstomatal limitation (Hassan, 1999; Shangguan *et al.*, 1999; Yordanov *et al.*, 1999; Kadam *et al.*, 2014). Rubisco activase, a heat-stable enzyme in higher plants involved in carbon assimilation and metabolism in the chloroplast stroma, was found to be inactivated at higher temperatures (Salvucci and Crafts-Brandner, 2004; Sharkey, 2005). Moreover, the key physiological indicator of heat stress was found to be loss and detachment of thylakoid membrane integrity (Gounaris *et al.*, 1984; Semenova, 2004; Yamamoto *et al.*, 2008; Ding *et al.*, 2020). In addition, photosystem II (PS II) is the most heat-sensitive component within the chloroplast thylakoid membrane (Hu *et al.*, 2020). The function of the electron transport, cleavage of the D1 reaction center-binding protein in PSII, and oxygen-evolving complex were found to be altered by heat stress (Havaux and Tardy, 1997; Klimov *et al.*, 1998; Yamane *et al.*, 1998; Wahid *et al.*, 2007; Allakhverdiev *et al.*, 2008; Lin *et al.*, 2013). Thus, the chloroplast was suggested as a key sensor to detect elevated temperatures because it is highly sensitive to damage from heat stress (Yu *et al.*, 2012).

Chlorophylls (Chl) and carotenoid (Car) are the predominant plastid pigments with approximately 10^9 tons of Chl being biosynthesized and degraded annually (Rüdiger, 1997; Eckhardt *et al.*, 2004; Liu *et al.*, 2007). Chl biosynthesis pathways have been well-documented (Nguyen *et al.*, 2020) and are summarized in Figure 1. Chl is extremely important during photosynthesis because they play a central role in light absorption and energy transfer (Fromme *et al.*, 2003). Chl *a* is the most abundant pigment and presents in all higher plants. The second-most abundant pigment is Chl *b*, which accounts for approximately one-quarter of the total chlorophyll in higher plants, and differs from Chl *a* only in the presence of a formyl group at the C-7 position instead of a methyl group. Chl *a* plays an important role in both light absorption and primary photochemical reactions, whereas Chl *b* is involved only in light absorption (Michel *et al.*, 1983; Voitsekhovskaja and Tyutereva, 2015; Landi *et al.*, 2020). Furthermore, Chl *b* also plays a major role in sustaining the accumulation of polypeptides associated with pigments and can serve as an intermediate to improve the efficiency of energy transfer between Chl *a* and carotenoid (Car) (Bellemare *et al.*, 1982; Terao *et al.*, 1985; Allen *et al.*, 1988). In addition, the binding of Chl *b* to the antenna proteins is needed for stable anchoring of the antenna complexes in thylakoid membranes (Voitsekhovskaja and Tyutereva, 2015).

Rice (*Oryza sativa* L.) is one of the most important food crops in the world. However, its yield is affected by the extreme temperatures caused by climate change and global warming (Horie, 2019). Under extreme temperatures, the photosynthetic machinery of rice undergoes modifications to adapt to the stress. Therefore, studies have focused on investigating the influence of temperature stress on the photosynthetic machinery of rice (Soda *et al.*, 2018). Understanding the cold and heat tolerance in rice is one of the solutions for improving the rice yield (Cruz *et al.*, 2013; Fahad *et al.*, 2018). The breeding of mutated plants is achieved by effective treatments such as transfer-DNA insertion mutagenesis, physical mutagenesis (ionizing radiation), and chemical mutagenesis (ethyl methane sulphonate and EMS) (Liu *et al.*, 2016). These mutations play an important role in evaluating plant life cycles, crop yield, plant characteristics, gene identification, and gene mapping genetics (Huang *et al.*, 2014). Studies have reported that Chl-deficient mutants are light- (Hopkins *et al.*, 1980; Markwell *et al.*, 1986; Allen *et al.*, 1988; Greene *et al.*, 1988) and temperature-sensitive (Yang *et al.*, 1990; Markwell and Osterman, 1992). Moreover, temperature sensitivity is a general characteristic of Chl-deficient *Melilotus alba* (Yang *et al.*, 1990) and *Arabidopsis thaliana* (Markwell and Osterman, 1992). Previous

studies have introduced and elucidated the transcriptomic profiles and photosynthetic properties of Chl *b*-lacking mutant rice (Chlorina 1, *chl1*) and wild-type rice (Norin No.8, *wt*) (Nguyen *et al.*, 2020). However, the sensitivities under temperature stress on the Chl biosynthesis mechanism of Chl *b*-lacking rice remains unclear. To further investigate these characteristics, the present study aimed to examine the influence of growth temperature on physiological characteristics and differentially expressed genes (DEGs) encoding 12 enzymes that play important role in Chl biosynthesis at 15 °C and 35 °C in both *wt* and *chl1* rice. The interactions between *wt* and *chl1* and temperature were also discussed in this work.

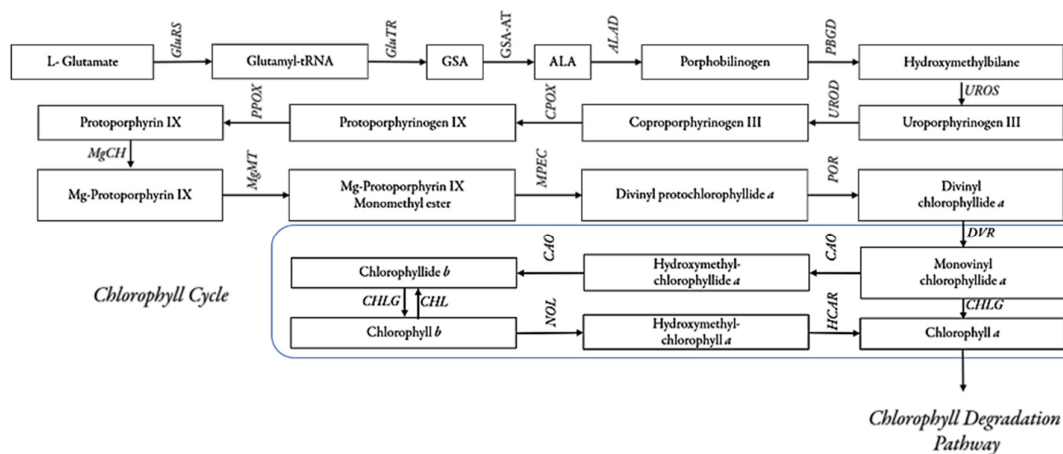


Figure 1. Chl biosynthesis pathway

Chl biosynthesis is composed of 3 separated sections: (1) Common pathway: The synthesis of protoporphyrin IX from the first committed precursor, 5-aminolevulinic acid (ALA). ALA is synthesized from L-glutamate via multiple reactions catalyzed by glutamyl-tRNA reductase (GluRS), glutamyl-tRNA reductase (GluTR), and glutamate 1-semialdehyde aminotransferase (GSA-AT). Subsequently, intermediates, such as porphobilinogen, hydroxymethylbilane, uroporphyrinogen III, coproporphyrinogen III, protoporphyrinogen IX, and protoporphyrin IX, are produced through the reactions catalyzed by porphobilinogen synthase (ALAD), hydroxymethylbilane synthase (PBGD), uroporphyrinogen decarboxylase (UROD), coproporphyrinogen III oxidase (CPOX), and protoporphyrinogen oxidase (PPOX), respectively. (2) The Mg branch: Mg-chelatase (MgCH) catalyzed to insert Mg²⁺ into protoporphyrin IX to form Mg-protoporphyrin IX. Subsequently, chlorophyllide *a* is produced through multiple reactions catalyzed by different enzymes such as Mg-protoporphyrin IX methyltransferase (MgMT), Mg-protoporphyrin IX monomethyl ester cyclase (MPEC), protochlorophyllide reductase (POR), and 3,4-divinyl protochlorophyllide *a*-9-vinyl reductase (DVR). (3) Chl cycle: This section includes the interconversion of Chl *a* and Chl *b*, which is catalyzed by chlorophyllide *a* oxygenase (CAO), chlorophyll synthase (CHLG), chlorophyll *b* reductase (NOL), and 7-hydroxymethyl chlorophyll *a* reductase (HCAR) (Masuda and Fujita, 2008; Lai *et al.*, 2016; Nguyen *et al.*, 2020).

Materials and Methods

Plant materials and growth condition

Seeds of *wt* and *chl1* were graciously provided by Dr. Tomio Terao (Department of Applied Physiology, National Institute of Agrobiological Resources, Tsukuba Science City, Japan). The seeds were sown and observed in a growth chamber (*Firstek*, Taiwan), and the seedlings (3 plants) were grown for 42 d at 25 °C at a relative humidity (RH) of ≥ 80% and a 12/12 photoperiod with the light intensity of 2000 μmol s⁻¹ m⁻². Then, sets of *wt* and *chl1* plantlets were incubated at different temperatures (15 °C or 35 °C) for 12 h under the same RH, light intensity, and photoperiod. Leaves (3 leaves per plant) were then collected every 4 h, frozen in liquid nitrogen, and kept at -80 °C for next uses.

Pigments content assessment

Leaf samples (fresh leaves) were extracted using 80% (v/v) acetone and homogenized using bullet blender tissue homogenizer (Next Advance Inc., New York, USA) at 4 min °C for 5 min. The absorbances of the extract were then measured at 663.6 nm, 646.6 nm, and 440.5 nm using a UV-Visible spectrophotometer (Hitachi U2800, Tokyo, Japan) at room temperature to determine total Chl content, carotenoid (Car) content, and the ratio of Chl *a* to Chl *b* (Chl *a/b*), respectively. The pigment contents were calculated, as follows (Yang *et al.*, 1998):

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

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

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

$$\text{Car} = 4.69 \times A_{440.5} - 0.267 \times \text{Total Chl} \quad (4)$$

where $A_{663.6}$, $A_{646.6}$, and $A_{440.5}$ are the absorbances at 663.6 nm, 646.6 nm, and 440.5 nm, respectively.

Transmission electron microscopy

The ultrastructure of *wt* and *chl1* leaf samples was observed using transmission electron microscopy. Each set of leaves were trimmed into small fragments (0.5 × 0.5 × 0.5 mm) and sample fragments were fixed in 2.5% glutaraldehyde at 4 °C for 24 h followed by in 1% OsO₄ for 2 h. All fragment sets were then fixed up to 70 nm using a Leica EM UC6 ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany), stained using 1% (w/v) lead citrate and 1% (w/v) uranyl acetate (Spurr, 1969), and photographed using a Phillip Tecnai 12 transmission electron microscope (JEOL Ltd., Japan).

Quantitative RT-PCR (qPCR)

Total RNA was extracted from *wt* and *chl1* leaves using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), following the manufacturer’s instructions. Total RNA (µg) extracted from *wt* and *chl1* was subjected to cDNA synthesis using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostic Systems, Branchburg, NJ, USA) and oligo (dT) primer. Primer sets were designed using Primer Premiere software version 6.0 (Pre-miereBiosoft, Palo Alto, USA) (Table 1). qPCR was performed using the StepOne Plus Real-Time PCR system (Applied Biosystems, Life Technologies Inc, Italy) with iTaq Universal SYBR Green Supermix reagent (Bio-Rad, CA, USA) to analyze 12 genes involved in Chl biosynthesis and degradation (Nguyen *et al.*, 2020). Relative gene expression values were calculated as 2^{-ΔCt}, where ΔCt was calculated as the difference between the cycle threshold (Ct) of a target gene and the reference gene. The fold change in each gene in leaf tissues was calculated as 2^{-ΔCt_{mutant}}/2^{-ΔCt_{wild-type}}.

Table 1. Primer sequences used in qPCR in this study

Marker	Forward sequence (5'-3')	Reverse sequence (5'-3')
<i>GLuRS</i>	GCTCTCCGCTCAAGTGAATACC	GATCAGTCCAGTCCTCCACCTT
<i>ALAD</i>	GGCATGGGCTTCTTGATGAGG	CCAGAGCAACATCCGTGTAGAC
<i>PBGD</i>	GAAGGATTGACATTGCCGTCCA	GTCTACGCAGAGAAGCACTTCC
<i>UROD</i>	GACGGCGTCATCCTGTTCTC	TGCGTAGAATCTTGAGCGACTC
<i>CPOX</i>	AAGCACCGTAATGAGCGTCCG	TCAGTTGTTGCCATGCCTTGT
<i>POR</i>	TCATCCTCGGCTCCATCACC	CTCCTGCATCGTCAGCATGTT
<i>DVR</i>	CAGGTTTCATCAAGGTGCCGAT	CGTCATCTCGTCGCTGTAATC
<i>CAO</i>	GCATGGACTTCTGCTGGACAA	AGATGCCAGTGGTTGACAAGAC
<i>HCBR</i>	GCAGGAAGCAAGACATGGATGA	GCATTGGAGCAAGCCTGTCA
<i>CHLG</i>	GGGCACTGTTGTTAGCAGGG	GCCAATGTAGCTCGCACCAA
<i>NOL</i>	CACTGCTTCTCCTGGAATGGTC	GCAAGATCCTTGGTGGTGTCAA
<i>MgCH</i>	AAGATGGTTGCCGAAGTGGATG	ATGTCTGGAGCTGCTTCTCA
UBQ10*	CTCATCTCCTCTCCTCGCATCA	CCACCAATCGGATCTAGCAACA

* Reference gene

Statistical analysis

The pigment contents and relative gene expressions (qPCR results) of *wt* and *chl* were statistically analyzed using least significant difference (LSD) *t*-tests at $p \leq 0.05$. All tests were performed by SAS version 8.0 (Research Triangle Park, NC, USA).

Results

Effects of temperature on pigment contents

The 35~40 cm and 25~30 cm height *wt* and *chl* plantlets were obtained after 6 weeks incubation at 25 °C, respectively. Each set of *wt* and *chl* plantlets was transferred to a growth chamber with a controlled temperature of 15 °C (*wt-15*, *chl-15*) or 35 °C (*wt-35*, *chl-35*) for 12 h. The leaves were taken for analysis every 4 h. The results showed that leaf coloration differed between *wt* and *chl* at both 15 °C and 35 °C. From 0 – 12 h, the *wt* leaves remained dark green to light green, whereas the *chl* leaves ranged from light green to yellow-green (Figure 2) at both 15 °C and 35 °C.

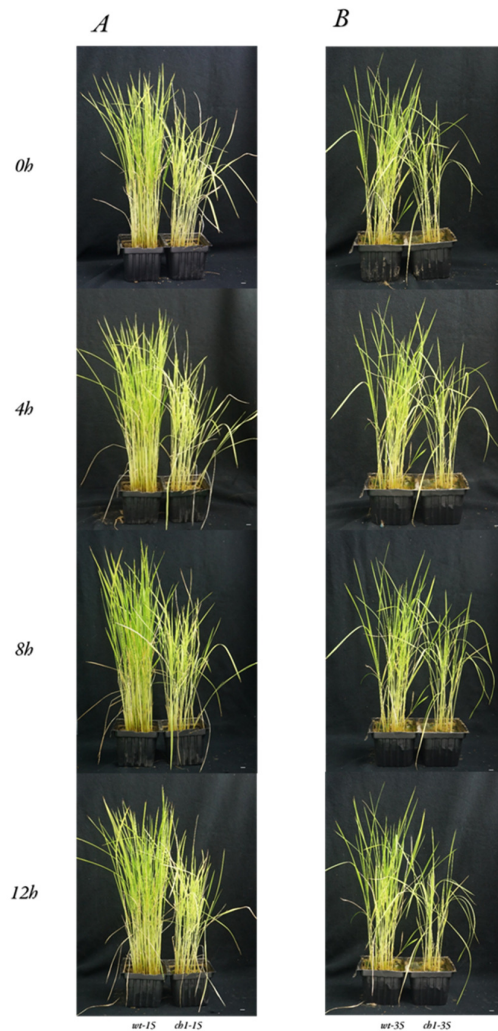


Figure 2. Effects of growth temperature on plantlet morphology
Changes in wild-type rice (*wt-15*, *wt-35*) and chl *b*-lacking rice (*chl-15*, *chl-35*) plantlets grown at (A) 15 °C and (B) 35 °C at 0 h, 4 h, 8 h, and 12 h incubation. Bar = 1 cm.

The tip of *chl1* became dry compared to *wr* leaves after 12 h at 35 °C, whereas both *chl1* and *wr* remained healthy at 15 °C. Overall, the *wr* leaves accumulated roughly twice the amount of total Chl as *chl1* leaves at the start of incubation (0 h). Over 12 h at 15 °C, the total Chl content of *wr-15* and *chl1-15* decreased remarkably from 4.5 mg g⁻¹ and 2.63 mg g⁻¹ to 0.9 mg g⁻¹ and 0.32 mg g⁻¹, respectively. Total Chl also significantly decreased in *wr-35* and *chl1-35* at 35 °C, from 4.31 mg g⁻¹ to 0.34 mg g⁻¹ and from 2.66 mg g⁻¹ to 0.17 mg g⁻¹, respectively (Figure 3A). Meanwhile, Chl *a* content also decreased in all samples (Figure 3B). Chl *b* was not detected in *chl1* in any treatment over 12 h, whereas Chl *b* significantly decreased in *wr* before it became absent at 8 h (for 35 °C treatment) or 12 h (for 15 °C treatment) (Figure 3C). Hence, Chl *a/b* of ∞ over 12 h in both treatments, whereas the Chl *a/b* in *wr* increased to ∞ at 12 h (for 15 °C treatment) and 8 h (for 35 °C treatment) (Table 2). However, the Car content accumulated in *wr* leaves was roughly twice to four times greater than that in *chl1* at 0 h. However, the Car content of *chl1-15* slightly increased from 0.42 to 0.45 mg g⁻¹, while Car content in *chl1-35* significantly dropped from 0.42 to 0.28 mg g⁻¹. Besides, the Car content of the *wr-15* and *wr-35* increased rapidly at 15 °C (0.71 mg g⁻¹) and 35 °C (0.67 mg g⁻¹), respectively (Figure 3D).

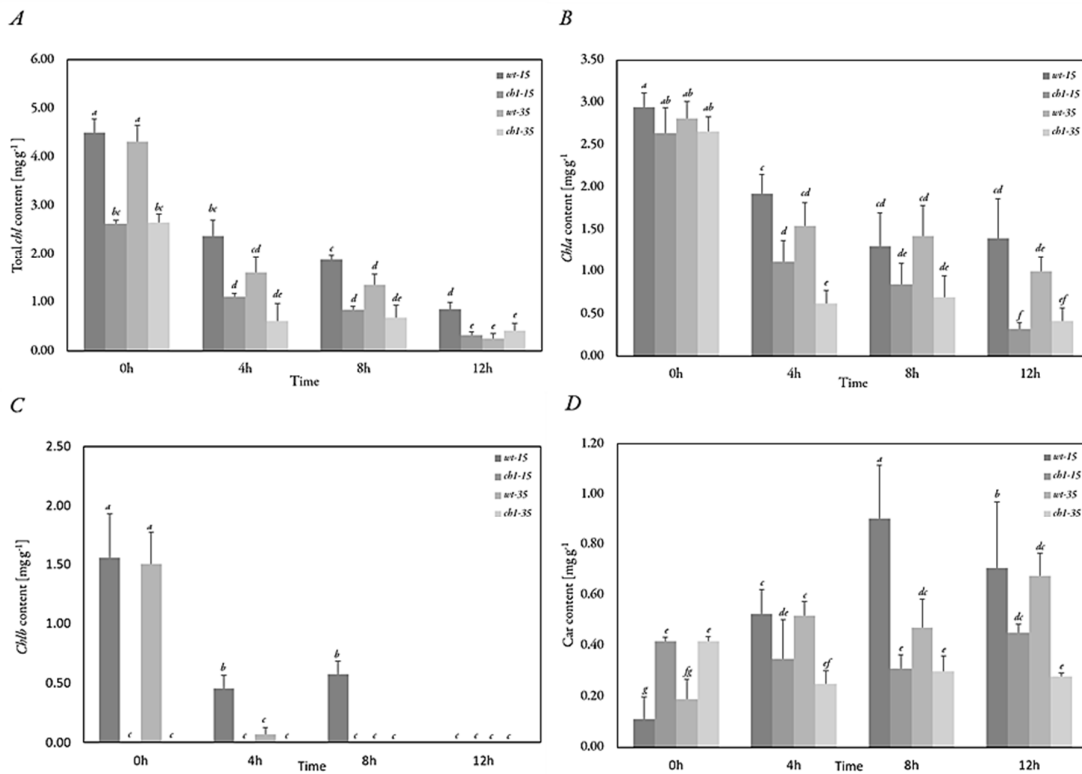


Figure 3. Effects of temperature on (A) total Chl, (B) Chl *a*, (C) Chl *b*, and (D) Car contents of wild-type rice (*wr-15*, *wr-35*) and Chl *b*-lacking rice (*chl1-15*, *chl1-35*) grown at 15 °C or 35 °C. Vertical bars show the standard deviations. Variables with the same letter mean no significant difference at $p \leq 0.05$ by LSD test.

Table 2. Chl *a/b* of wild-type and Chl *b*-lacking rice grown at 15 °C or 35 °C

Strain	Chl <i>a/b</i>							
	15 °C				35 °C			
	0 h	4 h	8 h	12 h	0 h	4 h	8 h	12 h
<i>wr</i>	1.89	4.20	2.27	∞	1.87	21.29	∞	∞
<i>chl1</i>	∞	∞	∞	∞	∞	∞	∞	∞

wr, wild-type rice (Norin No.8); *chl1*, chlorophyll *b*-lacking mutant (Chlorina 1).

Temperature effect on chloroplast development

To examine the influences of temperature on chloroplast development, the chloroplast ultrastructures of *wt* and *chl* grown at different temperatures were analyzed. At the start of both temperature treatments, chloroplasts from the mesophyll cells of *wt* leaves exhibited a normal structure, including distinct thylakoid membranes and stromal lamellae with small starch granules and one or two plastoglobuli (Figure 4A). However, the chloroplasts of *chl* leaves presented indistinct or absent stromal lamellae, indistinct thylakoid membranes, and abundant vesicles and plastoglobuli (Figure 4A). The stacked thylakoid grana of the *chl* leaves were also thinner than those of the *wt* leaves (Figure 4A). After 12 h incubation, destacked thylakoid grana were observed in *chl-35* (Figure 4C), while thinner and stacked thylakoid grana remained in *chl-15* (Figure 4B).

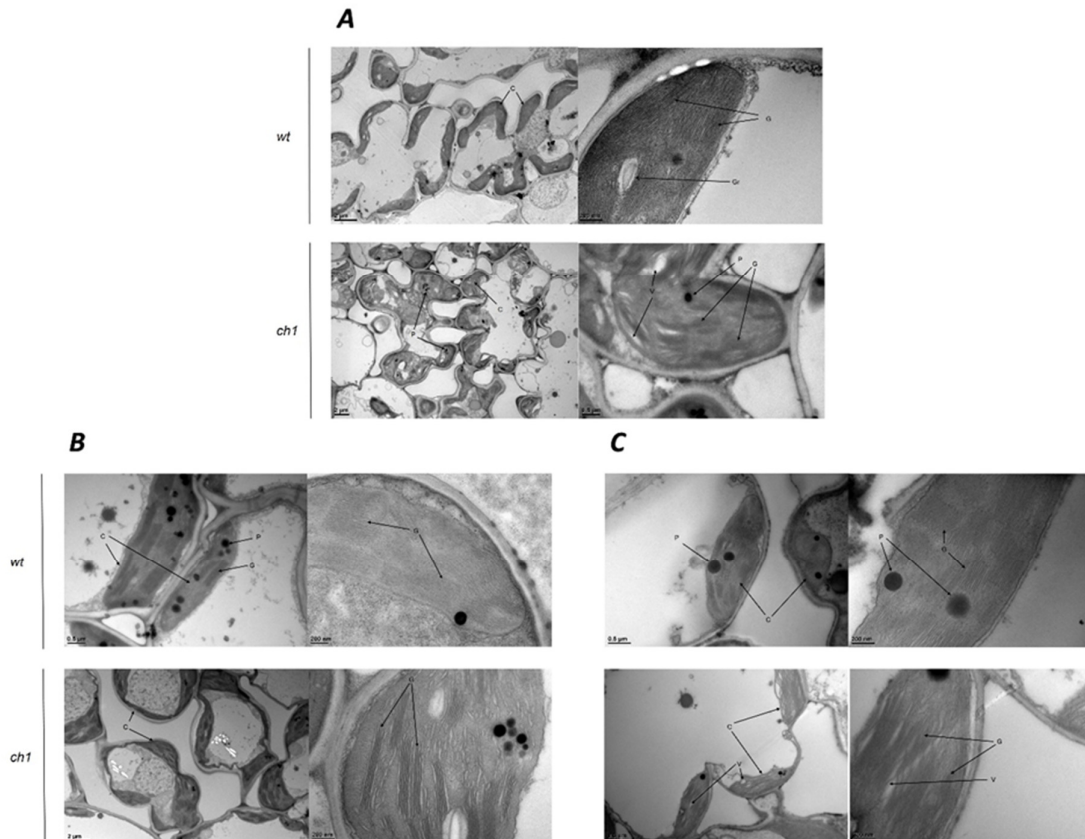


Figure 4. Effects of temperature on the chloroplast ultra-structures of wild-type and chlorophyll *b*-lacking rice

A. Normal chloroplast structure of *wt* and abnormal chloroplast structure of *chl*, showing thinner thylakoid membranes and abundant plastoglobuli at 0 h. Normal chloroplast structure of *wt* and abnormal chloroplast structure of *chl* grown at (B) 15 °C or (C) 35 °C for 12 h, showing indistinct thylakoid membranes and abundant plastoglobuli. C. Chloroplast; G, grana; P, plastoglobuli; Gr, granulose, V, vacuole

Differentially expressed gene (DEGs) determined by qPCR

The expression of 11 of 12 genes related to Chl biosynthesis and degradation were analyzed by qPCR (Figure 5). Among the genes, *UROD*, *CPOX*, and *NOL* were significantly up-regulated in *chl* at 0 h. Overall, the relative gene expression levels in the rice (both *wt* and *chl*) grown at 35 °C were higher than that of rice grown at 15 °C. *ALAD*, *CPOX*, *DVR*, and *NOL* were not detected after 4 h incubation at 15 °C in *chl-15*. In contrast, the expression levels of *ALAD*, *CPOX*, *DVR*, and *NOL* did not differ significantly over 12 h at 35 °C in *chl-35*.

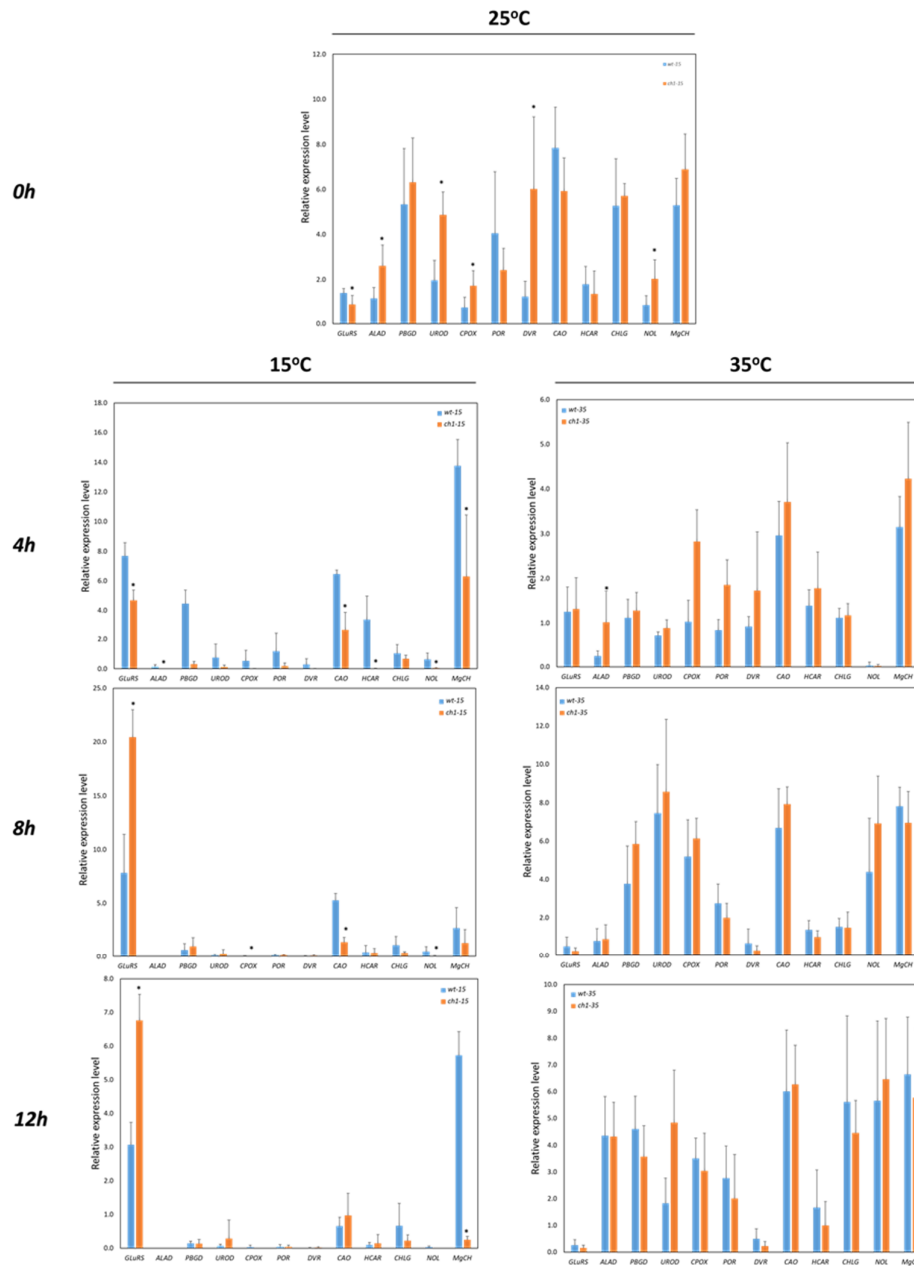


Figure 5. Expression of levels of 12 differentially expressed genes in wild-type and chlorophyll b-lacking rice. Gene expression was measured using quantitative real-time PCR. Asterisks (*) indicate significant differences in expression levels between the *wt* and *chl* ($p \leq 0.05$)

Discussion

Temperature stress results in changes in plant growth and physiology, and consequently, limits agricultural productivity (Kadam *et al.*, 2014; Xin *et al.*, 2015; Petrov *et al.*, 2016). Both heat and cold stresses cause multi-step damages to the photosynthetic apparatus of plants. Cold stress has been shown to inhibit photosynthesis in the albino rice mutant (Allen and Ort, 2001). Nevertheless, the molecular mechanisms involving in the influences of temperature stress on plant development are not completely understood in Chl

b-lacking mutant. In this study, *wt* and *chl1* were grown at low and high temperatures to examine the responses of Chl *b*-lacking mutants to temperature stress. Compare to *wt*, *chl1* plantlets were remarkably shorter and less healthy, with light green to yellow-green leaf coloration under both temperature treatments (15 °C and 35 °C). Moreover, after 12 h incubation, the tips of *chl1-35* leaves were dry compared to *chl1-15* and *wt-35*, suggesting that high temperatures damaged plant development. This result is similar to that observed for heat-stress sensitive albino rice (Qiu *et al.*, 2018).

When plants are exposed to extreme temperature, a high amount of reactive oxygen species (ROS) is produced, damaging the cellular structures and consequently causing plant senescence and death (Cheng *et al.*, 2016). The peroxidation of membrane lipids caused by O₂ and H₂O₂ resulted in destruction of chloroplast and degradation of plastid pigments (Zhao *et al.*, 2011). In addition, chloroplast microstructure, photosynthetic metabolism, and energy production are damaged by cold and chilling conditions (Bilska and Sowiński, 2010), which inhibit photosynthesis and cause a serious threat to crop development. Under chilling stress, the efficiency of photosynthetic electron transfer in plants is drastically reduced, resulting in a burst of ROS that directly induces cellular oxidative damages and increases membrane rigidity (Nakashima *et al.*, 2007; Chaves *et al.*, 2009; Lawlor and Tezara, 2009; Xie *et al.*, 2009). To respond to those damages, Car plays a vital role in the pathways that protect the photosynthetic apparatus from various extreme environmental factors. Under extreme heat conditions, Car mitigates the effects of intense heat by scavenging ROS produced during photooxidative stress (Strzalka *et al.*, 2003). In addition, chloroplast plays a crucial role in photosynthetic machinery, where numerous metabolic reactions occur. However, it is effortlessly affected by environmental stressors, such as heat (Zhu, 2016). Heat stress causes partial changes in membrane fluidity, which is attributed to electron transport activity, and leads to photosynthesis inhibition (Raison *et al.*, 1982; Havaux and Tardy, 1996; Murakami *et al.*, 2000). In this study, total Chl decreased dramatically over 12 h in both temperature treatments, suggesting that temperature stress affects both *wt* and *chl1*. In higher plants, photosynthesis temperature is usually based on natural atmosphere encountering. Consequently, the optimum temperature range typically matches the average daytime temperature (Berry and Bjorkman, 1980; Poorter, 2004). Additionally, the photosystems, the photosynthetic machinery, principally PSII with its oxygen-evolving complex, the ATP producing process, and the carbon metabolism process, are the three major stress-sensitive site (Aro *et al.*, 1993; Bukhov and Carpentier, 2000; Nishiyama *et al.*, 2005; Mohanty *et al.*, 2007; Murata *et al.*, 2007; Allakhverdiev *et al.*, 2008). The analyses of the chloroplast ultrastructure revealed differences in thylakoid membrane structure between *wt* and *chl1* grown at both 15 °C and 35 °C for 12 h. The *wt* chloroplasts possessed typical thylakoid membranes that were efficient for harvesting and converting light energy, whereas the *chl1* chloroplasts possessed abnormal thylakoid membranes. Similarly, chloroplasts of green bamboo leaves contain abundant thylakoid membranes, whereas the thylakoid membranes of Chl *b*-lacking bamboo leaves are converted into numerous abnormal vesicles (Yang *et al.*, 2015a). In *Anthurium andraeanum*, the mesophyll cells of wild-type plants possess normal chloroplasts containing small starch granules and, thus, large gaps among stroma thylakoids (Yang *et al.*, 2015b). The results obtained in the present study also demonstrated differences in the chloroplast structures of *wt* and *chl1* rice. The ultrastructure of *chl1* (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 efficiency and account for the coloration differences observed between *wt* and *chl1*. The number of thylakoid membranes in *chl1* significantly reduced, leading to thinner stacked grana. Therefore, the enhancement in Chl *a/b* of *chl1* might reflect abnormal chloroplast development and function. In addition, heat stress typically leads to a loss of thylakoid membrane integrity, particularly the unstacking of thylakoid membranes (Gounaris *et al.*, 1983; Semenova, 2004; Allakhverdiev *et al.*, 2008).

In this work, the unstacked grana were observed in *chl1-35* compared to *chl1-15*, suggesting that chloroplast development in chlorophyll *b*-lacking mutants is sensitive to high temperatures. The current study also found that grana stacking, along with an absence of Chl *b*, is normal in both *wt* and *chl1* at 15 °C after 12 h, whereas unstacked grana were observed in *chl1* at 35 °C. This result suggested that high temperatures affect

chloroplast development in *chl*. Studies have reported that grana stacking is affected both LHC II proteins and other factors (Nakatani and Baliga, 1985; Ouijja *et al.*, 1988; Wood *et al.*, 2019). The present study indicated that high temperatures might be one of the factors, along with LCHII, that affected grana stacking in rice.

This study examined the expression level of 12 important genes that code enzymes involved in Chl biosynthesis in rice (Nguyen *et al.*, 2020). Those genes include GluRS, ALAD, PBGD, UROD, CPOX, POR, DVR, CAO, HCAR, CHLG, NOL, and MgCH. Over 12 h, this study found no significant differences in the expression level of DEGs relative to Chl biosynthesis and degradation between *wt-35* and *chl-35*. This result suggests that *chl* is not sensitive to environmental temperatures ranging from 15 °C to 35 °C. GluRS expression decreased in *wt-35* and *chl-35*, and consequently, lead to a decrease in Chl biosynthesis in both *wt-35* and *chl-35*. This is because at the early stage of Chl biosynthesis, GluRS encodes glutamyl-tRNA reductase, which catalyzes the conversion of glutamyl-tRNA into glutamate-semialdehyde (GSA), an important intermediate in the Chl biosynthesis cycle (Verkamp *et al.*, 1992; Zhao *et al.*, 2014). More specifically, *chl* was observed to be sensitive to 15 °C at 4 h incubation but adapted to the cold environment at 8 h and 12 h. There was no significant difference in the expression of DEGs related to Chl biosynthesis and degradation between *wt-15* and *chl-15* at 8 h and 12 h. Moreover, even though *GluRS* expression rapidly increased, *ALAD* expression decreased until it could not be detected in *wt-15* and *chl-15*, suggesting that its production was blocked in the early stages of the experiment. Thus, total Chl of *wt-15* and *chl-15* rapidly decreased over 12 h at 15 °C. This could be explained that *ALAD* encodes an aminolevulinic acid dehydratase, which catalyzes the reaction to condense two ALA to form porphobilinogen (Sassa, 1982). The intermediate porphobilinogen subsequently undergoes multiple enzymatic reactions to form Chl *a* and Chl *b* (Willows and Hansson, 2003; Masuda and Fujita, 2008). This study suggests that low- and high-temperatures may affect DEGs associated with chloroplast development rather than Chl biosynthesis. Although many studies reported that Chl-deficient mutants of plants such as *Morus alba* and *Arabidopsis thaliana* are sensitive to temperatures (Yang *et al.*, 1990; Markwell and Osterman, 1992), this study showed that Chl *b*-lacking and wild-type rice adapted to environmental temperatures in ranging from 15 °C–35 °C.

Conclusions

This study reported the biosynthesis and characteristics of pigments in the leaves of *wt* and *chl* under temperature stress. Ultrastructure analysis in *chl* revealed the thinner stacked grana at 15 °C, whereas destacked grana were found at 30 °C after 12 h incubation. Under temperature stress, Chl *b* is undetectable, whereas a significant deficiency in Chl *a* was found in both *wt* and *chl*. Photosynthesis capacity was insignificantly different in *chl* as compared to *wt* after 12 h incubation at 35 °C. However, *chl* was sensitive to 15 °C during the first 4 h of incubation. This study suggested that *chl* may adapt to temperatures ranging from 15 °C to 35 °C.

Authors' Contributions

Conceptualization: CMY, MKN, and THS; Methodology: CMY, MKN, and THS; Validation: MKN, SHL, JWJ, HCN, and ZWY; Formal analysis: MKN, THS, and HCN; Investigation: MHK, SHL, JWJ, and ZWY; Writing: MKN and HCN; Supervision: CMY; Project administration: MKN, 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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