Characterization and Complementation of a Chlorophyll-Less Dominant Mutant *GL1* in *Lagerstroemia indica*

Shu'an Wang,^{1,2,*} Peng Wang,^{2,*} Lulu Gao,^{2,*} Rutong Yang,² Linfang Li,²
Enliang Zhang,² Qing Wang,² Ya Li,² and Zengfang Yin¹

Crape myrtle (*Lagerstroemia indica*) is a woody ornamental plant popularly grown because of its long-lasting, midsummer blooms and beautiful colors. The *GL1* dominant mutant is the first chlorophyll-less mutant identified in crape myrtle. It was obtained from a natural yellow leaf bud mutation. We previously revealed that leaf color of the *GL1* mutant is affected by light intensity. However, the mechanism of the *GL1* mutant on light response remained unclear. The acclimation response of mutant and wild-type (WT) plants was assessed in a time series after transferring from low light (LL) to high light (HL) by analyzing chlorophyll synthesis precursor content, photosynthetic performance, and gene expression. In LL conditions, coproporphyrinogen III (Coprogen III) content had the greatest amount of accumulation in the mutant compared with WT, increasing by 100%. This suggested that the yellow leaf phenotype of the *GL1* dominant mutant might be caused by disruption of coproporphyrinogen III oxidase (CPO) biosynthesis. Furthermore, the candidate gene, oxygen-independent CPO (*HEMN*), might only affect expression of upstream genes involved in chlorophyll metabolism in the mutant. Moreover, two genes, photosystem II (PSII) 10 kDa protein (*psbR*) and chlorophyll a/b binding protein gene (*CAB1*), had decreased mRNA levels in the *GL1* mutant within the first 96 h following LL/HL transfer compared with the WT. Hierarchical clustering revealed that these two genes shared a similar expression trend as the oxygen-dependent CPO (*HEMF*). These findings provide evidence that *GL1* is highly coordinated with PSII stability and chloroplast biogenesis.

Keywords: antioxidant enzyme, photosynthesis, chlorophyll b, high light, qRT-PCR

Introduction

THE POPULAR ORNAMENTAL plant Crape myrtle (*Lagerstroemia indica*) is known for its long-lasting midsummer blooms and beautiful colors. The earliest historical records of crape myrtle cultivation are from China, dating back 1700 years (Zhang, 1991). Numerous crape myrtle cultivars are widely used in Chinese landscaping (Wang *et al.*, 2008). Chlorophylls (Chl) are required for harvesting solar energy in photosynthetic antenna systems, and for charge separation and electron transport within reaction centers (Tanaka and Tanaka, 2006).

Chl-less mutant plants are important for exploration of the biochemistry and molecular processes of Chl metabolism and regulation. Chl-less mutants have been identified in almost all higher plants, including rice (*Oryza sativa*) (Zhou *et al.*, 2013) and *Arabidopsis thaliana* (Wang *et al.*, 2016). In *Triticum monococcum*, chlorophyll-deficient mutant (*clm1*) showed

pale yellow leaves, early flowering, reduced number of tillers, trichome length and density, and different shape compared with the wild type (WT) (Ansari *et al.* 2013). In *Cucumis sativus*, chlorophyll-deficient mutant C528 with golden leaf color throughout its development showed relatively weaker and slower growth with a much shorter vine length and smaller foliage in the field (Gao *et al.*, 2016).

GL1 was the first Chl-less mutant described in crape myrtle; it was obtained from a natural yellow leaf bud mutation (Wang *et al.*, 2013). The leaves of *GL1* were orange in May, yellow during June and July, light green in early August, light yellow during the middle of August through September, and yellow in October (Li *et al.*, 2015). The height, cutting root rate, petal length, petal width, and claw length of *GL1* are less than observed in WT plants (Wang *et al.*, 2013, 2016). However, the branch number of *GL1* was significantly more than observed in WT plants (Wang *et al.*, 2013, 2016). Furthermore, the ultrastructure of

¹Southern Modern Forestry Collaborative Innovation Center, College of Biology and the Environment, Nanjing Forestry University, Nanjing, China.

²Institute of Botany, Jiangsu Province and Chinese Academy of Sciences, Nanjing, China.

*These authors are co-first authors.

chloroplasts in the mutant leaves contains ruptured thylakoid membranes, and indistinct or absent stromal lamellae when compared with the WT (Li *et al.*, 2015). Analyses of the mutant leaf transcriptome identified 79 unigenes involved in chlorophyll biosynthesis and degradation, photosynthesis, and chloroplast development (Li *et al.*, 2015).

In higher plants, Chl biosynthesis is a highly coordinated process. It is executed via a series of cooperative reactions catalyzed by numerous enzymes (Beale, 2005). 5-aminolevulinic acid (ALA) synthesis is the first committed step of Chl biosynthesis (Tanaka and Tanaka, 2006). Among three enzymes responsible for ALA synthesis, glutamyl-tRNA reductase (GluTR) is regarded the main target of regulatory mechanisms; its transcription is controlled by light (Ilag *et al.*, 1994; Cornah *et al.*, 2003). Porphobilinogen (PBG) synthase encoded by the 5-aminolevulinic acid dehydratase (*HEMB*) gene catalyzes condensation of two ALA molecules to form the PBG (Beale, 1999, 2005). Uroporphyrinogen III (Urogen III) synthase catalyzes cyclization of 1-hydroxymethylbilane, with a concomitant inversion of the fourth ring of the porphyrin macrocycle, giving rise to Urogen III (Battersby *et al.*, 1979; Beale, 1999). Three enzymes, uroporphyrinogen decarboxylase III (UROD), coproporphyrinogen III oxidase (CPO), and protoporphyrinogen IX oxidase, sequentially convert Urogen III to protoporphyrin IX (Pro IX) (Beale, 1999).

CPO catalyzes oxidative decarboxylation of the two propionate side chains of rings A and B of Coprogen III to vinyl groups (Tanaka *et al.*, 2011). There are two structurally unrelated CPOs: the oxygen-independent CPO, *HEMN*, and the oxygen-dependent CPO, *HEMF* (Troup *et al.*, 1994, 1995). *HEMN* belongs to the radical *S*-adenosylmethionine (*SAM*) family, carries an oxygen-sensitive [4Fe-4S] cluster, and requires *SAM* for catalysis (Layer *et al.*, 2002). *HEMF* belongs to a family of monooxygenases with binuclear irons and requires oxygen for oxidative decarboxylation (Ouchane *et al.*, 2004).

Phenotypic expression of Chl-less mutants is markedly affected by manipulation of environmental factors such as light (Biswal *et al.*, 2012). The Chl content and ratios of chlorophyll/carotenoids of 14 species of Chl-less ornamental plants were shown to be affected by light intensity (Hu *et al.*, 2007). In *Arabidopsis*, the CPO deficiency mutant lesion initiation 2 (*lin2*) has yellow-green leaves when grown under long day conditions and yellow leaves under short day conditions (Ishikawa *et al.*, 2001). The barley (*Hordeum vulgare*), Nanchong Yellow Barley (*NYB*), mutant becomes more yellow and has dwarfing of seedlings in high-light (HL) conditions (Yuan *et al.*, 2010b). Meanwhile, thylakoid structures in chloroplasts are damaged by excessive light in the Chl-less mutant. Plastid development is arrested in HL and can be derepressed by transferring to low-light (LL) conditions in *Ligustrum vicaryi* golden leaves (Yuan *et al.*, 2010a). GOLDEN2-LIKE (*GLK*) transcription factors are required for chloroplast development (Waters *et al.*, 2009). The expression of *GLK1* is markedly lower in pale-green true leaves in the *at131/at16* double *Arabidopsis* mutant than in WT plants grown under medium-light conditions (Maekawa *et al.*, 2015).

Previously, we revealed that leaf color of the *GL1* mutant is affected by light intensity (Zhang *et al.*, 2014). However, the mechanism of the *GL1* mutant on light response re-

mained unclear. In the present study, time series are used to study the dynamic changes of chlorophyll content and its synthesis precursor content in the response of the *GL1* mutant toward HL. Photosynthesis parameters, chlorophyll fluorescence, and expression of genes were measured following transfer from LL to HL (LL/HL) conditions in *GL1* mutant and WT plants. Our findings indicated that the yellow leaf phenotype of the *GL1* dominant mutant might be caused by disruption of Coprogen III oxidase biosynthesis.

Materials and Methods

Plant materials and growth conditions

GL1 dominant mutant was obtained from a natural yellow leaf bud mutant of the *L. indica* cultivar “*Fenjing*” at the Institute of Botany, Jiangsu Province, and the Chinese Academy of Sciences, Nanjing, China (CNBG), in 2003 (Li *et al.* 2015). The mutant and WT “*Fenjing*” cutting seedlings were grown at the CNBG under natural conditions and subsequently irradiated for the times and at the light intensities specified later in the text in 2015.

All plants were initially grown for 8 weeks under LL conditions (40 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$). Subsequently, they were exposed to HL (1000–1500 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$). The third to fifth leaves were harvested from both mutant and WT plants in all experiments. Harvested samples were either immediately analyzed or kept at -80°C until required. Leaf numbers were counted from the top of the shoot.

Chlorophyll and carotenoid determination

Fresh leaves of WT and *GL1* mutant plants were collected at four time points (0, 48, 96, and 168 h after LL/HL transfer). Pigments were extracted from mature leaves according to the methods described by Li (2000). Chl and carotenoids were extracted with 95% ethanol for 3 days, and spectrophotometrically quantified (DU-800; Beckman Coulter, Brea, CA) at 470, 649, and 665 nm.

Assay of antioxidant enzyme activities

Fresh leaves of WT and *GL1* mutant plants were collected at four time points (0, 48, 96, and 168 h after LL/HL transfer). Superoxide dismutase (SOD) activity was measured using the xanthine oxidase method (Sun *et al.*, 1988). Absorbance was determined at 550 nm. Catalase (CAT) activity was measured using the ammonium molybdate spectrophotometric method (Góth, 1991), which is based on the fact that ammonium molybdate can rapidly terminate the H_2O_2 degradation reaction catalyzed by CAT and reacts with residual H_2O_2 to generate a yellow complex; this was monitored by absorbance at 405 nm. Peroxidase (POD) activity was monitored by absorbance at 420 nm. All experiments were carried out using the following kits: SOD assay kit/A001-3, CAT assay kit/A007-2, and peroxidase assay kit/A084-3 (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Content of chlorophyll synthesis precursors

Fresh leaves of WT and *GL1* mutant plants were collected at 09:00 a.m. (0h), 11:00 a.m. (2h), 1:00 p.m. (4h), and 4:00 p.m. (7h). 5-aminolevulinic acid was extracted as described

by Dei (1985). Isolations of PBG and uroporphyrinogen were performed using the method of Bogorad (1962). Coprophen III, Proto IX, Mg-proto, and Pchlide were determined according to the methods of Rebeiz *et al.* (1975). Three replicates were performed for each sample. Contents of Chl precursors were determined by spectrophotometrical methods (DU-800; Beckman Coulter, Brea, CA).

Transmission electron microscopy

Fresh leaves of WT and *GL1* mutant plants were collected at four time points following LL/HL transfer (0, 48, 96, and 168 h). Leaf samples were fixed overnight at 4°C in 3% glutaraldehyde and 0.1 M sodium cacodylate buffer (pH 7.4) and then processed for electron microscopy as described by Sakamoto *et al.* (2009). Ultrathin sections were cut using an ultramicrotome (Ultracut, Reichert-Jung) and observed with a transmission electron microscope (TEM 300; Hitachi Comp., Japan) operating at 75 kV.

Leaf gas exchange measurement

The Li-Cor 6400 portable photosynthesis system (LI-COR Biosciences, Lincoln, NE) with a built-in light source set at 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ was used to determine net photosynthesis (Pn), stomatal conductance (Cond), intercellular CO_2 concentration (Ci), and transpiration rate (Trmmol). All measurements were carried out between 09:00 a.m. and 11:00 a.m., at four time points following LL/HL transfer (0, 48, 96, and 168 h). During measurements, leaf temperature was adjusted to 25°C.

Chlorophyll fluorescence

Chlorophyll fluorescence parameters were measured using an OS1p fluorometer (Opti-Sciences, Hudson, NH). Leaves were dark adapted for 30 min before measurement to ensure all capable PSII reaction centers were fully oxidized. Fluorescence parameters were calculated based on methods described by Genty *et al.* (1989); Hendrickson *et al.* (2004); and Kramer *et al.* (2004) as follows: maximum quantum yield of PSII, $F_v/F_m = (F_m - F_o)/F_m$; potential photochemical efficiency, $F_v/F_o = (F_m - F_o)/F_o$; effective quantum yield of PSII, $Y(II) = (F_m - F_s)/F_m$; Y(NPQ) and Y(NO) represent the quantum yield of regulated and nonregulated energy dissipation in PSII, respectively; $Y(NPQ) = F_s/F_m - F_s/F_m$; $Y(NO) = F_s/F_m$; non-photochemical quenching; $NPQ = (F_m - F_m)/F_m$.

F_o and F_m are the minimum and maximum fluorescence measured after 30 min of dark adaptation. F_m is the maximum fluorescence after light adaptation and is measured on illumination of a saturating pulse; F_s is the steady-state fluorescence after light adaptation. All measurements were carried out at 09:00 a.m. (0 h), 11:00 a.m. (2 h), 1:00 p.m. (4 h), and 4:00 p.m. (7 h).

RNA extraction and quantitative real time-PCR analysis

Fresh leaves of WT and *GL1* mutant plants were collected at four time points after LL/HL transfer (0, 48, 96, and 168 h). Total RNAs were extracted using the TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Total RNA concentration was quantified

using One Drop OD-2000 Spectrophotometer (Nanjing Wuyi Technology Co., Nanjing, China) and quality checked by electrophoresis in a 1% agarose gel.

For each sample, 2 μg of total RNA were reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega, Madison, WI). Reverse transcription was performed at 37°C for 60 min in a 20 μL reaction with a final denaturation step at 95°C for 5 min. Primers were designed using Primer premier 5 (www.premierbiosoft.com/primerdesign; Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/dna) and synthesized by SIPUJIN (Nanjing, China). Reactions were carried out using SYBR Green PCR Master Mix (Roche Applied Science, Mannheim, Germany) on an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA) with three replicates.

The *Actin* gene from *L. indica* was amplified as an internal control for data normalization. The 25 μL reaction volumes contained 12.5 μL SYBR Green PCR Master Mix, 9.5 μL deionized H_2O , 1 μL primers, and 2 μL cDNA. Amplification reactions were initiated with a predenaturation step (95°C for 10 min), followed by 40 cycles of denaturing (95°C for 10 s), annealing (60°C for 35 s), and extension (72°C for 35 s). Data were processed using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). Heat maps for the gene expression profiles were generated using the MultiExperiment Viewer (MeV 4) software package (Saeed *et al.*, 2003).

Sequence analysis of HEMN genes

The *HEMN* protein sequences from *A. thaliana* (At), *O. sativa* (Os), *Populus trichocarpa* (Pt), *Theobroma cacao* (Tc), *Gossypium raimondii* (Gr), *Gossypium arboreum* (Ga), *Vitis vinifera* (Vv), *Brassica napus* (Bn), *Microcoleus* sp. PCC7113, *Zea mays* (Zm), *Malus domestica* (Md), *Eucalyptus grandis* (Eg), *Ziziphus jujube* (Zj), *Fragaria vesca* (Fv), *Capsella rubella* (Cr), *Citrus clementine* (Cc), *Citrus sinensis* (Cs), and *Rivularia* sp. PCC 7116 were downloaded from GenBank (www.ncbi.nlm.nih.gov). The full-length *LiHEMN* cDNA was amplified from the *GL1* mutant and WT by RT-PCR with the gene-specific primers F (5'-TTCTGGAGGAAAATGCTT-3') and R (5'-CTAACATTATGGAGCCACGGT-3'), respectively. The PCR products were checked and sequenced by SIPUJIN (Nanjing, China). Multiple sequence alignment was conducted using DNAMAN version 6.0 (Lynnon Biosoft). A phylogenetic tree was generated in MEGA5 version 5.1 using the neighbor-joining method with 1000 bootstrap replicates.

Statistical analysis

One-way analysis of variance was used to determine the significance between the mean values of the *GL1* mutant and WT. Comparison among means was performed using the Duncan's multiple range test and the two-tailed unequal variance *t*-test in SPSS 17.0 (SPSS, Inc., Chicago).

Results

LL/HL transfer leads to reduction of Chl b content and lesion formation in the *GL1* mutant

Under LL conditions (t_0), the *GL1* dominant mutant leaf color was golden-yellow, while the WT was light yellow-

FIG. 1. Phenotypic characterization of *GL1* mutant after low-light/high-light transfer. (A–D) Phenotype of *GL1* mutant and wild type at four time points (0, 48, 96, and 168 h after LL/HL transfer). (E) Changes in chlorophyll synthesis precursors of *GL1* mutant and wild type at 09:00 a.m. (0 h), 11:00 a.m. (2 h), 1:00 p.m. (4 h), and 4:00 p.m. (7 h). Three replicates were performed for each sample. Ratios of mutant (M) and wild type (WT) were determined using the log₂ transformation method. LL/HL, low light/high light.

green (Fig. 1a). When the mutant and WT were exposed to HL conditions for 1 week (t_{168}), the leaf color of the mutant rapidly changed to a pale-green, whereas it changed to green in the WT (Fig. 1d). Under LL conditions, Chl a content of the mutant was markedly lower than observed in the WT; there was no difference in Chl b content between the mutant and WT (Table 1). Exposure of the LL-grown mutant plants to HL resulted in a drop in Chl a, Chl b, and total Chl content, but raised carotenoid content and the Chl a/b ratio (Table 1). Compared with LL conditions, after 168 h of HL, the Chl a and Chl b contents of the mutant were significantly reduced, by 47.89% and 70.26%, respectively, whereas carotenoid content increased by 62.61% (Table 1). These data suggested that changes of leaf color in the mutant might be caused by a significant reduction of Chl b content.

Lesions appeared on both mutant and WT leaves within the first 48 h of HL (Fig. 1b). Lesions in the mutant gradually became severe, with the leaf margin beginning to die on increased illumination time (Fig. 1c, d). Contrastingly, in the WT, lesion formation after LL/HL transfer peaked at t_{96} ,

and was followed by a slow recovery (Fig. 1c, d). Thus, the yellow leaf was more sensitive to photodamage. To characterize photodamage in more detail, the antioxidant enzyme activities of SOD, CAT, and POD were investigated in *GL1* mutant and WT plants after LL/HL transfer. SOD activity in the mutant gradually decreased following LL/HL transfer (Table 2). CAT activity in the mutant showed the greatest value at t_{48} and was always higher than in WT, except at t_0 (Table 2). Initially, POD activity in the mutant was markedly decreased, but this was followed by a rapid recovery; activity was always higher than in the WT after LL/HL transfer, except at t_{48} (Table 2). After 1 week (168 h) in HL conditions, the activities of SOD, CAT, and POD in the mutant were 0.87-, 1.34-, and 1.94-fold of that observed in the WT, respectively (Table 2). These results further indicated that the *GL1* mutant senses a higher level of photodamage than the WT.

Precursors of the Chl biosynthesis pathway were measured in the mutant and the WT following LL/HL transfer. ALA, PBG, Urogen III, and Coprogen III contents in the

TABLE 1. THE CONTENT OF CHLOROPHYLL AND CAROTENOID AFTER LOW-LIGHT/HIGH-LIGHT TRANSFER

Time (h)	Chla (mg g ⁻¹ FW ⁻¹)		Chlb (mg g ⁻¹ FW ⁻¹)		Car (mg g ⁻¹ FW ⁻¹)		Total Chl (mg g ⁻¹ FW ⁻¹)		Chla/b	
	Mutant	Wild type	Mutant	Wild type	Mutant	Wild type	Mutant	Wild type	Mutant	Wild type
0	7.58 ± 0.19 ^a	8.22 ± 0.09 ^b	2.69 ± 0.14 ^a	2.64 ± 0.15 ^a	1.15 ± 0.04 ^c	1.34 ± 0.05 ^b	10.26 ± 0.32 ^a	10.87 ± 0.24 ^a	2.82 ± 0.10 ^c	3.12 ± 0.14 ^b
48	7.47 ± 0.12 ^a	8.20 ± 0.14 ^b	2.34 ± 0.07 ^a	2.61 ± 0.11 ^a	1.62 ± 0.05 ^b	1.85 ± 0.01 ^a	9.81 ± 0.18 ^a	10.81 ± 0.24 ^a	3.20 ± 0.06 ^c	3.10 ± 0.08 ^b
96	4.05 ± 0.13 ^b	8.44 ± 0.23 ^b	0.97 ± 0.03 ^b	2.72 ± 0.11 ^a	1.82 ± 0.03 ^a	1.80 ± 0.05 ^a	5.02 ± 0.16 ^b	11.17 ± 0.90 ^a	4.19 ± 0.06 ^b	3.36 ± 0.12 ^b
168	3.95 ± 0.30 ^b	9.10 ± 0.16 ^a	0.80 ± 0.17 ^b	2.40 ± 0.12 ^a	1.87 ± 0.06 ^a	1.76 ± 0.05 ^a	4.75 ± 0.45 ^b	11.50 ± 0.16 ^a	5.10 ± 0.78 ^a	4.97 ± 0.84 ^a

The data represent the mean ± SE of $n=3$ replicates.

The letters mean a significant difference at $p < 0.05$ (Duncan's multiple test).

Asterisk means significantly different between mutant and wild type at $p < 0.05$ (two-tailed unequal variance t -test).
SE, standard error.

GLI mutant were significantly higher ($\log_2$ ratios >0) than in the WT, while Proto IX, Mg-protoporphyrin IX (Mg-proto), and protochlorophyllide (Pchlde) contents were lower in LL conditions ($\log_2$ ratios <0) (Fig. 1e; Supplementary Table S2). Among the precursors, Coprogen III had the greatest accumulation, increasing by 100% in the mutant compared with the WT in LL (Fig. 1e; Supplementary Table S2). However, the contents of ProtoIX, Mg-proto, and Pchlde in the mutant were always significantly lower than in the WT ($\log_2$ ratios <0) after LL/HL transfer (Fig. 1e; Supplementary Table S2). These results indicated that in *L. indica*, the *GLI* mutant was a Coprogen III-less mutant. Interestingly, light responses of the precursors were different in the *GLI* mutant, with the PBG content of the mutant being significantly higher than observed in WT ($\log_2$ ratios >0) after the LL/HL transfer (Fig. 1e). However, ALA, Urogen III, and Coprogen III content of the *GLI* mutant was higher compared with WT ($\log_2$ ratios >0) within the first 2 h; this was followed by a rapid decrease ($\log_2$ ratios <0) (Fig. 1e).

The structure of grana lamellae was destroyed in the GL1 mutant after LL/HL transfer

How the *GLI* mutant affected chloroplast development after LL/HL transfer was investigated by comparing the ultrastructure of chloroplasts in the mutant and WT at t_0 , t_{48} , t_{96} , and t_{168} using transmission electron microscopy. At t_0 , the structure of grana lamellae was similar in both *GLI* and WT (Fig. 2). After LL/HL transfer, the structure of grana lamellae was distinctly different in the *GLI* mutant and WT. The length and density of grana lamellae gradually decreased in the mutant on increased illumination times, with absence of grana lamellae structure at t_{168} (Fig. 2). In contrast, the length and density of grana lamellae in the WT gradually increased following the LL/HL transfer (Fig. 2). The data suggested that the structure of grana lamellae was destroyed in the *GLI* mutant after LL/HL transfer.

During LL/HL transfer, damages to photosystem II in the GL1 mutant lead to decreased photochemical capacity

There were no differences in the values of net photosynthesis (Pn), stomatal conductance (Cond), intercellular CO₂ concentration (Ci), and transpiration rate (Trmmol) ($\log_2$ ratios ≈ 0) between the *GLI* mutant and WT in the first 48 h after LL/HL transfer (Fig. 3a; Supplementary Table S3). However, after 96 h in HL, the values of Pn, Cond, and Trmmol ($\log_2$ ratios <0) in the mutant decreased significantly, whereas values of Ci ($\log_2$ ratios >0) increased (Fig. 3a; Supplementary Table S3). In comparison with LL, Pn decreased by 30.29%, Cond by 16.67%, and Trmmol by 29.17%, whereas Ci increased by 58.07% after 1 week (168 h) in HL in the mutant plants (Supplementary Table S3). The above data suggested that the reduction of net photosynthesis in the *GLI* mutant was caused by nonstomatal factors.

Chlorophyll fluorescence parameters were measured to better understand the reasons for decreased net photosynthesis in the mutant following LL/HL transfer. The maximum quantum yield of PSII, as measured by F_v/F_m , did not differ between the *GLI* mutant and WT ($\log_2$ ratios ≈ 0)

TABLE 2. THE ENZYME ACTIVITIES OF SOD, CAT, AND POD IN *GL1* MUTANT AND WILD-TYPE PLANTS AFTER LOW-LIGHT/HIGH-LIGHT TRANSFER (U/MG PROTEIN)

Time (h)	SOD		CAT		POD	
	Mutant	Wild type	Mutant	Wild type	Mutant	Wild type
0	161.84 ± 6.33*a	179.06 ± 4.02a	217.38 ± 32.51*b	327.31 ± 77.78a	31.78 ± 8.17a	30.27 ± 3.89a
48	123.19 ± 2.76*b	96.66 ± 6.50c	263.06 ± 27.14*a	220.30 ± 47.41b	18.74 ± 3.31*c	23.03 ± 1.05b
96	105.55 ± 2.16c	102.53 ± 2.10b	233.52 ± 29.09*b	174.16 ± 18.12c	27.53 ± 2.16*b	17.64 ± 1.08c
168	80.57 ± 3.98*d	93.00 ± 2.22c	159.11 ± 27.04*c	119.05 ± 72.36d	31.37 ± 4.67*a	16.21 ± 1.00c

The data represent the mean ± SE of $n=3$ replicates.

The letters mean a significant difference at $p<0.05$ (Duncan's multiple test).

Asterisk means significantly different between mutant and wild type at $p<0.05$ (two-tailed unequal variance t -test).

SOD, superoxide dismutase; CAT, catalase; POD, peroxidase.

with values being close to 0.8 under LL conditions (Fig. 3b; Supplementary Table S4). However, F_o and F_m values in the mutant were significantly lower than in the WT under LL (Fig. 3b; Supplementary Table S4). The ETR value was also significantly higher in the mutant than in the WT under LL conditions (Fig. 3b; Supplementary Table S4). Following the LL/HL transfer, the F_v/F_m and ETR values in the mutant were decreased, while those of the WT initially decreased rapidly and then recovered (Fig. 3b; Supplementary Table S4). F_o values were increased significantly in the *GL1* mutant, while F_m values were rapidly decreased after the LL/HL transfer (Fig. 3b; Supplementary Table S4). In the mutant plants, the F_v/F_m value decreased by 53.75%, ETR by 34.58%, and F_m by 61.34% after 1 day in HL (7 h), while F_o increased by 16.95% (Supplementary Table S4). The data suggested that the decrease of F_v/F_m was mainly due to a large decrease in maximal chlorophyll fluorescence.

There was no significant difference in NPQ values ($\log_2$ ratios ≈ 0) between the mutant and WT at t_0 following the LL/HL transfer (Fig. 3b). However, NPQ values of the mutant were significantly lower at t_2 ($\log_2$ ratios <0) and markedly higher at t_4 and t_7 ($\log_2$ ratios >0) compared with the WT after LL/HL transfer (Fig. 3b; Supplementary Table S4). Y(II), Y(NO), and Y(NPQ) values of the *GL1* mutant were always lower than in WT plants ($\log_2$ ratios <0) after the LL/HL transfer (Fig. 3b; Supplementary Table S4). The Y(NO) values of the mutant were significantly higher than in WT plants under HL conditions (Fig. 3b; Supplementary

Table S4). In mutant plants grown in HL for 1 day (7 h), Y(II) decreased by 60.53% and Y(NPQ) by 45.16%, whereas Y(NO) increased by 43.90% (Supplementary Table S4). The above data indicated that damages to photosystem II (PSII) in the *GL1* mutant lead to decreased photochemical capacity after LL/HL transfer.

Expression analysis of chlorophyll- and photosynthesis-associated genes in the *GL1* mutant and WT

In our previous study, the leaf transcriptome of the *GL1* mutant was sequenced. This identified 79 unigenes involved in chlorophyll biosynthesis and degradation, photosynthesis, and chloroplast development (Li *et al.*, 2015). In this study, the transcript levels of 23 genes were selected for assay in the mutant and WT after LL/HL transfer. Transcript levels of 11 chlorophyll-associated genes were assayed: glutamyl-tRNA synthetase (*GluRS*), glutamate-1-semialdehyde aminotransferase (*HEMA*), glutamyl-tRNA reductase (*HEMA*), *HEMA*, hydroxymethylbilane synthase (*HEMC*), uroporphyrinogen-III synthase (*HEMD*), *HEMF*, *HEMN*, protoporphyrinogen oxidase (*HEMG*), chlorophyllide a oxygenase (*CAO*), and chlorophyllase (*Chlase*) (Fig. 4). In comparison with the wild type, the expression levels of *HEMA*, *HEMD*, and *HEMN* were decreased in the mutant following the LL/HL transfer (Fig. 4). Interestingly, the expression level of *HEMA* in the mutant was significantly decreased at t_0 and that of *HEMD* greatly

FIG. 2. Ultrastructure of chloroplasts in the *GL1* mutant and wild type after low-light/high-light transfer. gl, grana lamella, scale bar = 200 nm.

FIG. 3. Photosynthetic characters and chlorophyll fluorescence parameters of *GL1* mutant and wild type after low-light/high-light transfer. **(A)** Photosynthetic characters of *GL1* mutant and wild type. **(B)** Chlorophyll fluorescence parameters of *GL1* mutant and wild type. Five replicates were performed for each sample. Ratios of mutant and WT were determined using the log₂ transformation method.

decreased at t_{168} compared with the WT following the LL/HL transfer (Fig. 4). Furthermore, the expression levels of *HEMF* in the mutant increased after the LL/HL transfer (Fig. 4).

To under the structural difference between the *GL1* mutant and WT, the full-length *LiHEMN* cDNA sequences were cloned by RT-PCR. The *LiHEMN* cDNA from mutant and WT was 1431 and 1407bp in length, respectively (Supplementary Fig. S1). Sequence comparison revealed that two inserted fragments were detected from the *GL1* mutant sequence (Supplementary Fig. S1). Based on the phylogenetic tree of *HEMN*, we found that *LiHEMN* and *EgHEMN* showed the closest relationship (Supplementary Fig. S2). Therefore, the data suggested that the *HEMN* gene might be responsible for the yellow leaf phenotype of the *GL1* mutant.

Twelve photosynthesis-associated genes were assessed: cytochrome b6-f complex iron-sulfur subunit (*petC*), ferredoxin—NADP⁺ reductase (*petH*), photosystem I P700 chlorophyll a apoprotein A1 (*psaA*), photosystem I subunit XI (*psaL*), photosystem II CP47 chlorophyll apoprotein (*psbB*), photosystem II CP43 chlorophyll apoprotein (*psbC*), photosystem II 10kDa protein (*psbR*), light-harvesting

complex I chlorophyll a/b binding protein 2 (*LHCA2*), light-harvesting complex II chlorophyll a/b binding protein 1 (*LHCB1*), chlorophyll a/b binding protein gene (*CAB1*), stomatal density and distribution 1 (*SDD1*), and *GLK2*. The transcript levels of *LHCB1*, *psbR*, *CAB1*, *SDD1*, and *GLK2* were significantly changed in the mutant after the LL/HL transfer compared with the WT (Fig. 5). Expression level of *LHCB1* in the mutant was greatly decreased at t_{48} , while the *psbR* gene had significantly decreased mRNA levels at t_{96} (Fig. 5). The *CAB1* gene showed remarkably decreased mRNA levels for the first 96 h after LL/HL transfer, this was followed by significantly enhanced mRNA levels (Fig. 5). In addition, the expression levels of *SDD1* in the mutant were markedly reduced at t_{48} and t_{96} and those of *GLK2* significantly decreased at t_{48} (Fig. 5).

Hierarchical clustering revealed that *HEMN* shared a similar expression trend as *GluRS*, *HEML*, *HEMB*, *HEMC*, *petC*, and *petH* (Supplementary Fig. S3). In addition, *HEMF* showed a similar expression trend to *CAB1* and *psbR* (Supplementary Fig. S3). These findings indicated that the molecular mechanism of the *GL1* dominant mutant is complicated.

Discussion

Chl plays significant role in absorption of sunlight by photosynthetic reaction centers (Liu *et al.*, 2007). Chl-deficient mutants or leaf-color mutants are ideal materials for fundamental research in photosynthesis, photomorphogenesis, hormone physiology, resistance mechanism, and identification of gene functions (Ansari *et al.*, 2013; Guo *et al.*, 2013; Gao *et al.*, 2016). In addition, Chl-deficient mutants could be applicable as a leaf-color marker gene for efficient identification and elimination of false hybrids in commercial hybrid rice and cucumber production (Wan *et al.*, 2015; Gao *et al.*, 2016). Meanwhile, Chl-deficient mutants can be bred as ornamental plant varieties such as *Lindica* “Jinhuang” (Wang *et al.*, 2016). Crape myrtle is well known for its long-lasting midsummer blooms and beautiful colors. *GL1* was the first golden-leaf mutant described in crape myrtle (Wang *et al.*, 2013). We have researched on the mutant for many years. However, the molecular mechanism of *GL1* has not been illuminated.

The yellow leaf phenotype of the *GL1* dominant mutant might be caused by disruption of CPO biosynthesis

Chl metabolism is a highly coordinated process executed via a series of cooperative reactions catalyzed by numerous enzymes (Beale, 2005). CPO catalyzes the oxidative decarboxylation of Coproten III into Proto IX, and is an important regulatory step in the Chl biosynthetic pathway. Coproten III belongs to a group of photosensitive tetrapyrroles, and reducing its levels causes light-dependent yellow leaf and lesion formation in tobacco (*Nicotiana tabacum*), *Arabidopsis*, and rice (Kruse *et al.*, 1995b; Ishikawa *et al.*, 2001; Guo *et al.*, 2013; Wang *et al.*, 2015).

In CPO-deficient *lin2* plants, young leaves are pale green and develop lesions. Lesions begin as a small spot or stripe of collapsed tissue, usually first appearing in the middle part of young leaves (Ishikawa *et al.*, 2001). Similarly, lesions were generated and the leaf color changed from yellow- to pale-green in the *GL1* mutant following LL/HL transfer

FIG. 4. Expression of chlorophyll-associated genes in *GL1* mutant and wild type after LL/HL transfer. The *Actin* gene from *L. indica* was amplified as an internal control for data normalization. Each value represents the mean of three independent replicates $\pm$ SE. The letters mean a significant difference at $p < 0.001$ (Duncan's multiple test). Asterisk means significantly different between mutant and wild type at $p < 0.001$ (two-tailed unequal variance t -test). SE, standard error.

FIG. 5. Expression of photosynthesis-associated genes in *GL1* mutant and wild type after LL/HL transfer. The *Actin* gene from *L. indica* was amplified as an internal control for data normalization. Each value represents the mean of three independent replicates $\pm$ SE. The letters mean a significant difference at $p < 0.001$ (Duncan's multiple test). Asterisk means significantly different between mutant and wild type at $p < 0.001$ (two-tailed unequal variance *t*-test).

(Fig. 1a–d). Thus, the phenotypic characteristics of the *GL1* mutant were similar to those of the *Arabidopsis lin2* mutant. Transgenic tobacco plants with reduced CPO activity are characterized by antioxidative responses and necrotic leaf lesions (Kruse *et al.*, 1995a). In this study, we investigated antioxidant enzyme activities of SOD, CAT, and POD in *GL1* mutant and WT plants following LL/HL transfer. After 1 week (168 h) in HL conditions, SOD, CAT, and POD activities in the mutant were 0.87-, 1.34-, and 1.94-fold of that observed in the WT, respectively (Table 1).

In the rice PORB-deficient (*fgl*) mutant, contents of ALA, Proto IX, and Mg-ProtoIX are greatly decreased under HL conditions (Sakuraba *et al.*, 2013). In the maize (*Zea mays*) UROD-deficient (*Les22*) dominant mutant, there is a two- to threefold increase in uroporphyrin levels, while levels of Coprophen III are unaffected under HL in comparison with WT controls (Hu *et al.*, 1998). Antisense COP RNA in tobacco leads to accumulation of Coprophen III (Kruse *et al.*, 1995a). In the present study, Coprophen III levels in the *GL1* mutant in HL conditions were significantly higher in the first 2 h and this was followed by a rapid decrease, while Proto IX content was unaffected by the shift in light conditions compared with the WT (Fig. 1e; Supplementary Table S2).

In eukaryotes and bacteria, CPO enzymes are encoded by *HEMF* and *HEMN* genes, respectively (Tanaka *et al.*, 2011). The transcript levels of *HEMF* and *HEMN* were assayed in WT and *GL1* mutant using qRT-PCR in the present study. Compared with the WT, the expression levels of *HEMN* were remarkably decreased, whereas *HEMF* showed enhanced mRNA levels after the LL/HL transfer (Fig. 4). Sequence comparison revealed that two inserted fragments were detected from the *GL1* mutant sequence (Supplementary Fig. S1). Therefore, the *GL1* mutant is a CPO-deficient mutant in *L. indica*. The *HEMN* gene may be responsible for the yellow leaf phenotype of the mutant.

Analysis of the complete *Arabidopsis* genome revealed 15 enzymes, encoded by 27 genes, for biosynthesis of Chl from glutamyl-tRNA to Chl b (Nagata *et al.*, 2005). In higher plants, *HEMF* genes have been cloned from maize (Williams *et al.*, 2006) and *Arabidopsis* (Ishikawa *et al.*, 2001). However, the role of *HEMN* in higher plants remained unknown. In the present study, the leaf color of *GL1* mutant might be caused by a deficiency of *HEMN*. To study the relationship of *HEMN* and Chl-related genes, the transcripts levels of 11 Chl-associated genes were assayed using qRT-PCR. In comparison with the WT, the expression levels of *HEMA* and *HEMD* in the *GL1* mutant were decreased at all times except for t_{168} following LL/HL transfer (Fig. 4).

Previously, we found that *HEMA* and *HEMD* had significantly reduced mRNA levels in the mutant in different months (Li *et al.*, 2015). *HEMA1* transcripts are reduced at different levels in the rice chlorophyll synthase-deficient mutant (*yg11* mutant) at the seedling stage (Wu *et al.*, 2007). Furthermore, *HEMA1* expression is markedly downregulated under medium-light conditions in the *Arabidopsis atl31/atl6* mutant (Maekawa *et al.*, 2015). Hierarchical clustering revealed the *HEMN* gene shared a similar expression trend as *GluRS*, *HEML*, *HEMB*, and *HEMC* (Supplementary Fig. S1). This suggested that expression of *HEMN* affected expression of upstream genes.

GL1 mutant and chloroplast development

In the Chl-less mutant, chloroplast development is always hindered following LL/HL transfer. In *L. vicaryi* golden leaves, plastid development is arrested in HL conditions and can be derepressed by transferring to LL (Yuan *et al.*, 2010a). Under LL conditions, the structures of *Arabidopsis atl31/atl6* plastids are similar to those of WT, whereas in medium-light conditions, the *Arabidopsis atl31/atl6* leaf plastids in the apex section show an almost total loss of stromal thylakoids and a reduction in grana stacks (Maekawa *et al.*, 2015). In this study, the length and density of grana lamellae in the *GL1* mutant gradually decreased with increased illumination time (Fig. 2). Following the LL/HL transfer, the grana lamellae structure was absent in the *GL1* mutant at t_{168} (Fig. 2). This provides evidence that both *GL1* and chloroplast development are highly coordinated.

The *CAB* gene family is a marker for chloroplast development and is tightly controlled by both light and plastid signals (Dijkwel *et al.*, 1997). In the rice *yg11* mutant, *CAB1* transcript levels are decreased at the young seedling stage (Wu *et al.*, 2007). Similarly, *CAB1* transcripts are significantly reduced in the rice *vyl* mutant (Dong *et al.*, 2013). In contrast, *CAB* gene transcripts are not significantly reduced in the *Arabidopsis atl31/atl6* double mutant (Maekawa *et al.*, 2015). In this study, greatly decreased *CAB1* mRNA levels were observed in the *GL1* mutant compared with WT within the first 96 h after LL/HL transfer, and there was then a significant enhancement of mRNA levels (Fig. 5). According to the hierarchical clustering, *HEMF* shows a similar expression trend with *CAB1* (Supplementary Fig. S1).

The *GLK* gene family encodes a pair of partially redundant nuclear transcription factors required for expression of nuclear photosynthetic genes and for chloroplast development (Waters *et al.*, 2008). In an *Anthurium andraeanum* etiolated mutant, the expression level of *GLK* is lower than observed in WT (Yang *et al.*, 2015). *GLK1* is specifically downregulated in the *Arabidopsis atl31/atl6* double mutant under medium-light conditions (Maekawa *et al.*, 2015). In the present study, *GLK2* showed decreased mRNA levels at t_{48} in the mutant compared with WT following LL/HL transfer (Fig. 5). This suggests *GL1* may regulate chloroplast biogenesis through *CAB1* and *GLK2*.

GL1 mutant and PSII stability

Chl-less mutants make valuable tools for photosynthetic studies (Havaux and Tardy, 1999). PSII functions as a light-driven water plastoquinone oxidoreductase (Liu *et al.*, 2009). The *Fv/Fm* ratio is a useful relative measure of maximal quantum efficiency of PSII photochemistry, providing a simple and rapid way of monitoring environmental stress (Baker, 2007). Other fluorescence parameters, such as ETR, NPQ, and Y(II), are also important for evaluating PSII stability. In the rice *vyl* mutant, the values of ETR and NPQ are significantly lower than in WT, while the *Fv/Fm* value is similar; this is because of smaller amounts of Chl in the *vyl* mutant than in WT at the various growth stages (Dong *et al.*, 2013). In a maize yellow-green mutant, values of ETR, NPQ, and *Fv/Fm* were markedly reduced at different growth stages compared with WT, consistent with differences in the Chl content (Zhong *et al.*, 2015). In the present study, the *Fv/Fm* value in the mutant plants was

lower by 53.75%, ETR by 34.58%, and Y(II) by 60.53% after 1 day in HL (7 h) (Fig. 3b; Supplementary Table S4). Meanwhile, Chl a and Chl b contents of the mutant were reduced by 47.89% and 70.26%, respectively, after 168 h in HL conditions (Table 2).

PSII is a supramolecular pigment-protein complex embedded in the thylakoid membrane. It catalyzes light-induced electron transfer from water to plastoquinone (Suorsa *et al.*, 2006). *PsbR*, a fourth nuclear-encoded lumenal protein, was first identified in PSII complexes of spinach (Ljungberg *et al.*, 1984). Absence of *psbR* decreases the oxygen evolution capacity of thylakoid membranes, indicating *psbR* is essential for photosynthetic water splitting and electron transfer in PSII (Suorsa *et al.*, 2006; Allahverdiyeva *et al.*, 2007). In the Chl-less tea (*C. sinensis*) variety “*Baijiguan*,” HL intensity inhibits expression of *psbR*, thus affecting PSII stability, chloroplast development, and chlorophyll biosynthesis (Wu *et al.*, 2016). In our previous study, the mRNA levels of *psbR* were greatly suppressed in the mutant in different months (Li *et al.*, 2015).

In the present study, the *psbR* gene showed decreased mRNA levels in the mutant at t_{96} following LL/HL transfer compared with the WT (Fig. 5). In addition, hierarchical clustering revealed that *HEMF* and *psbR* shared a similar expression trend (Supplementary Fig. S1). This provides evidence that *GLI* and PSII stability is highly coordinated.

Acknowledgments

This work was supported by the Agricultural science and technology independent innovation fund of Jiangsu province (grant no. CX(14)2032), Sanxin forestry engineering of Jiangsu province (grant no. LYSX[2015]13), the doctorate fellowship foundation of Nanjing Forestry University, and the Natural Science Foundation of Jiangsu Province (grant no. BK20161382).

Disclosure Statement

The authors declare that there are no conflicts of interest regarding the publication of this article.

References

- Allahverdiyeva, Y., Mamedov, F., Suorsa, M., Styring, S., Vass, I., and Aro, E.M. (2007). Insights into the function of *PsbR* protein in *Arabidopsis thaliana*. *Biochim Biophys Acta* **1767**, 677–685.
- Ansari, M.J., Al-Ghamdi, A., Kumar, R., Usmani, S., Al-Attal, Y., Nuru, A., Mohamed, A.A., Singh, K., and Dhaliwal, H.S. (2013). Characterization and gene mapping of a chlorophyll-deficient mutant *clm1* of *Triticum monococcum* L. *Biol Plantarum* **57**, 442–448.
- Baker, N.R. (2007). Chlorophyll fluorescence: a probe of photosynthesis in vivo. *Annu Rev Plant Biol* **59**, 89–113.
- Battersby, A.R., Fookes, C.J., Gustafson-Potter, K.E., Matcham, G.W., and McDonald, E. (1979). Proof by synthesis that unrearranged hydroxymethylbilane is the product from deaminase and the substrate for cosynthetase in the biosynthesis of uro'gen-III. *J Chem Soc Chem Commun* **24**, 1155–1158.
- Beale, S.I. (1999). Enzymes of chlorophyll biosynthesis. *Photosynth Res* **60**, 43–73.
- Beale, S.I. (2005). Green genes gleaned. *Trends Plant Sci* **10**, 309–312.
- Biswal, A.K., Pattanayak, G.K., Pandey, S.S., Leelavathi, S., Reddy, V.S., and Tripathy, B.C. (2012). Light intensity-dependent modulation of chlorophyll b biosynthesis and photosynthesis by overexpression of chlorophyllide a oxygenase in tobacco. *Plant Physiol* **159**, 433–449.
- Bogorad, L. (1962). Porphyrin synthesis. *Methods Enzymol* **5**, 885–895.
- Cornah, J.E., Terry, M.J., and Smith, A.G. (2003). Green or red: what stops the traffic in the tetrapyrrole pathway? *Trends Plant Sci* **8**, 224–230.
- Dei, M. (1985). Benzyladenine—induced stimulation of 5—aminolevulinic acid accumulation under various light intensities in levulinic acid—treated cotyledons of etiolated cucumber. *Physiol Plant* **64**, 153–160.
- Dijkwel, P.P., Huijser, C., Weisbeek, P.J., Chua, N.H., and Smeekeens, S.C. (1997). Sucrose control of phytochrome A signaling in *Arabidopsis*. *Plant Cell* **9**, 583–595.
- Dong, H., Fei, G.L., Wu, C.Y., Wu, F.Q., Sun, Y.Y., Chen, M.J., Ren, Y.L., Zhou, K.N., Cheng, Z.J., Wang, J.L., Jiang, L., Zhang, X., Guo, X.P., Lei, C.L., Su, N., Wang, H., and Wan, J.M. (2013). A rice virescent-yellow leaf mutant reveals new insights into the role and assembly of plastid caseinolytic protease in higher plants. *Plant Physiol* **162**, 1867–1880.
- Gao, M.L., Hu, L.L., Li, Y.H., and Weng, Y.Q. (2016). The chlorophyll-deficient golden leaf mutation in cucumber is due to a single nucleotide substitution in *CsChlI* for magnesium chelatase I subunit. *Theor Appl Genet* **129**, 1961–1973.
- Genty, B., Briantais, J.M., and Baker, N.R. (1989). The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *BBA Gene Subjects* **990**, 87–92.
- Góth, L. (1991). A simple method for determination of serum catalase activity and revision of reference range. *Clin Chim Acta* **196**, 567–573.
- Guo, C.Y., Wu, G.H., Xing, J., Li, W.Q., Tang, D.Z., and Cui, B.M. (2013). A mutation in a coproporphyrinogen III oxidase gene confers growth inhibition, enhanced powdery mildew resistance and powdery mildew-induced cell death in *Arabidopsis*. *Plant Cell Rep* **32**, 687–702.
- Havaux, M., and Tardy, F. (1999). Loss of chlorophyll with limited reduction of photosynthesis as an adaptive response of Syrian barley landraces to high-light and heat stress. *Funct Plant Biol* **26**, 569–578.
- Hendrickson, L., Furbank, R.T., and Chow, W.S. (2004). A simple alternative approach to assessing the fate of absorbed light energy using chlorophyll fluorescence. *Photosynth Res* **82**, 73–81.
- Hu, G., Yalpani, N., Briggs, S.P., and Johal, G.S. (1998). A porphyrin pathway impairment is responsible for the phenotype of a dominant disease lesion mimic mutant of maize. *Plant Cell* **10**, 1095–1105.
- Hu, H.Z., Zhang, R., Shang, A.Q., Zhao, L.J., and Lu, Z.M. (2007). Response of pigment content of golden-leaf plants to light intensity. *Acta Horti Sin* **34**, 717.
- Ilag, L.L., Kumar, A.M., and Söll, D. (1994). Light regulation of chlorophyll biosynthesis at the level of 5-aminolevulinic acid formation in *Arabidopsis*. *Plant Cell* **6**, 265–275.
- Ishikawa, A., Okamoto, H., Iwasaki, Y., and Asahi, T. (2001). A deficiency of coproporphyrinogen III oxidase causes lesion formation in *Arabidopsis*. *Plant J* **27**, 89–99.
- Kramer, D.M., Johnson, G., Kiirats, O., and Edwards, G.E. (2004). New fluorescence parameters for the determination of

- QA redox state and excitation energy fluxes. *Photosynth Res* **79**, 209–218.
- Kruse, E., Mock, H.P., and Grimm, B. (1995a). Coproporphyrinogen III oxidase from barley and tobacco—sequence analysis and initial expression studies. *Planta* **196**, 796–803.
- Kruse, E., Mock, H.P., and Grimm, B. (1995b). Reduction of coproporphyrinogen oxidase level by antisense RNA synthesis leads to deregulated gene expression of plastid proteins and affects the oxidative defense system. *EMBO J* **14**, 3712–3720.
- Layer, G., Verfürth, K., Mahlitz, E., and Jahn, D. (2002). Oxygen-independent coproporphyrinogen-III oxidase *HemN* from *Escherichia coli*. *J Biol Chem* **277**, 34136–34142.
- Li, H. (2000). *Principle and Technology of Plant Physiological and Biochemical Experiments*. Higher Education Press, Beijing, pp. 134–138.
- Li, Y., Zhang, Z.Y., Wang, P., Wang, S.A., Ma, L.L., Li, L.F., Yang, R.T., Ma, Y.Z., and Wang, Q. (2015). Comprehensive transcriptome analysis discovers novel candidate genes related to leaf color in a *Lagerstroemia indica* yellow leaf mutant. *Genes Genom* **37**, 851–863.
- Liu, H., Frankel, L.K., and Bricker, T.M. (2009). Characterization and complementation of a *psbR* mutant in *Arabidopsis thaliana*. *Arch Biochem Biophys* **489**, 34–40.
- Liu, W.Z., Fu, Y.P., Hu, G.C., Si, H.M., Zhu, L., Wu, C., and Sun, Z.X. (2007). Identification and fine mapping of a thermosensitive chlorophyll deficient mutant in rice (*Oryza sativa* L.). *Planta* **226**, 785–795.
- Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**, 402–408.
- Ljungberg, U., Åkerlund, H.E., Andersson, B. (1984). The release of a 10-kDa polypeptide from everted photosystem II thylakoid membranes by alkaline tris. *FEBS Lett* **175**, 255–258.
- Maekawa, S., Takabayashi, A., Reyes, T.H., Yamamoto, H., Tanaka, A., Sato, T., and Yamaguchi, J. (2015). Pale-green phenotype of *at131 at16* double mutant leaves is caused by disruption of 5-aminolevulinic acid biosynthesis in *Arabidopsis thaliana*. *PLoS One* **10**, e0117662.
- Nagata, N., Tanaka, R., Satoh, S., and Tanaka, A. (2005). Identification of a vinyl reductase gene for chlorophyll synthesis in *Arabidopsis thaliana* and implications for the evolution of Prochlorococcus species. *Plant Cell* **17**, 233–240.
- Ouchane, S., Steunou, A.S., Picaud, M., and Astier, C. (2004). Aerobic and anaerobic Mg-protoporphyrin monomethyl ester cyclases in purple bacteria. *J Biol Chem* **279**, 6385–6394.
- Rebeiz, C.A., Mattheis, J.R., Smith, B.B., Rebeiz, C.C., and Dayton, D.F. (1975). Chloroplast biogenesis: biosynthesis and accumulation of protochlorophyll by isolated etioplasts and developing chloroplasts. *Arch Biochem Biophys* **171**, 549–567.
- Saeed, A., Sharov, V., White, J., Li, J., Liang, W., Bhagabati, N., Braisted, J., Klapa, M., Currier, T., and Thiagarajan, M. (2003). TM4: a free, open-source system for microarray data management and analysis. *Biotechniques* **34**, 374.
- Sakamoto, W., Uno, Y., Zhang, Q., Miura, E., and Kato, Y. (2009). Arrested differentiation of proplastids into chloroplasts in variegated leaves characterized by plastid ultrastructure and nucleoid morphology. *Plant Cell Physiol* **50**, 2069–2083.
- Sakuraba, Y., Rahman, M.L., Cho, S.H., Kim, Y.S., Koh, H.J., Yoo, S.C., and Paek, N.C. (2013). The rice faded green leaf locus encodes protochlorophyllide oxidoreductase B and is essential for chlorophyll synthesis under high light conditions. *Plant J* **74**, 122–133.
- Sun, Y., Oberley, L.W., and Li, Y. (1988). A simple method for clinical assay of superoxide dismutase. *Clin Chem* **34**, 497–500.
- Suorsa, M., Sirpiö, S., Allahverdiyeva, Y., Paakkari, V., Mamedov, F., Styring, S., and Aro, E.M. (2006). *PsbR*, a missing link in the assembly of the oxygen-evolving complex of plant photosystem II. *J Biol Chem* **281**, 145–150.
- Tanaka, A., and Tanaka, R. (2006). Chlorophyll metabolism. *Curr Opin Plant Biol* **9**, 248–255.
- Tanaka, R., Kobayashi, K., and Masuda, T. (2011). Tetrapyrrole Metabolism in *Arabidopsis thaliana*. *Arabidopsis Book* **9**, e0145.
- Troup, B., Hungerer, C., and Jahn, D. (1995). Cloning and characterization of the *Escherichia coli hemN* gene encoding the oxygen-independent coproporphyrinogen III oxidase. *J Bacteriol* **177**, 3326–3331.
- Troup, B., Jahn, M., Hungerer, C., and Jahn, D. (1994). Isolation of the *hemF* operon containing the gene for the *Escherichia coli* aerobic coproporphyrinogen III oxidase by in vivo complementation of a yeast *HEM13* mutant. *J Bacteriol* **176**, 673–680.
- Wan, C.M., Li, C.M., Ma, X.Z., Wang, Y., Sun, C.H., Huang, R., Zhong, P., Gao, Z.Y., Chen, D., Xu, Z.J., Zhu, J.Q., Gao, X.L., Wang, P.R., and Deng, X.J. (2015). *GRY79* encoding a putative metallo- β -lactamase-trihelix chimera is involved in chloroplast development at early seedling stage of rice. *Plant Cell Rep* **34**, 1353–1363.
- Wang, J., Ye, B., Yin, J., Yuan, C., Zhou, X., Li, W., Min, H., Wang, J., Chen, W., and Peng, Q. (2015). Characterization and fine mapping of a light-dependent leaf lesion mimic mutant 1 in rice. *Plant Physiol Bioch* **97**, 44–51.
- Wang, M., Song, P., Ren, X., and Zhang, Q. (2008). Recent advances in *Lagerstroemia indica* resources and breeding. *J Shandong For Sci Technol* **(2)**, 66–68.
- Wang, S.A., Wang, P., Zhang, Z.Y., Yang, R.T., Ma, L.L., and Li, Y. (2013). Study on propagation by cutting of *Lagerstroemia indica* L. ‘Jinwei’. *North Hortic* **11**, 72–75.
- Wang, S.A., Yang, R., Wang, P., Wang, Q., Li, L.F., Li, Y., and Yin, Z.F. (2016). ‘Jinhuang’ yellow-leaf crape myrtle. *HortScience* **51**, 595–596.
- Wang, Y.W., Chen, S.M., Wang, W.J., Huang, X.Q., Zhou, C.F., Zhuang, Z., and Lu, S. (2016). The DnaJ-like zinc finger domain protein *PSA2* affects light acclimation and chloroplast development in *Arabidopsis thaliana*. *Front Plant Sci* **7**, 360.
- Waters, M.T., Moylan, E.C., and Langdale, J.A. (2008). *GLK* transcription factors regulate chloroplast development in a cell-autonomous manner. *Plant J* **56**, 432–444.
- Waters, M.T., Wang, P., Korkaric, M., Capper, R.G., Saunders, N.J., and Langdale, J.A. (2009). *GLK* transcription factors coordinate expression of the photosynthetic apparatus in *Arabidopsis*. *Plant Cell* **21**, 1109–1128.
- Williams, P., Hardeman, K., Fowler, J., and Rivin, C. (2006). Divergence of duplicated genes in maize: evolution of contrasting targeting information for enzymes in the porphyrin pathway. *Plant J* **45**, 727–739.
- Wu, Q., Chen, Z., Sun, W., Deng, T., and Chen, M. (2016). De novo sequencing of the leaf transcriptome reveals complex light-responsive regulatory networks in *Camellia sinensis* cv. *Baijiguan*. *Front Plant Sci* **7**, 332.
- Wu, Z., Zhang, X., He, B., Diao, L., Sheng, S., Wang, J., Guo, X., Su, N., Wang, L., Jiang, L., Wang, C., Zhai, H., and Wan,

- J. (2007). A chlorophyll-deficient rice mutant with impaired chlorophyllide esterification in chlorophyll biosynthesis. *Plant Physiol* **145**, 29–40.
- Yang, Y., Chen, X., Xu, B., Li, Y., Ma, Y., and Wang, G. (2015). Phenotype and transcriptome analysis reveals chloroplast development and pigment biosynthesis together influenced the leaf color formation in mutants of *Anthurium andraeanum* “Sonate”. *Front Plant Sci* **6**, 139.
- Yuan, M., Xu, M.Y., Yuan, S., Chen, Y.E., Du, J.B., Xu, F., Zhang, Z.W., Guo, Z.C., Zhao, Z.Y., and Lin, H.H. (2010a). Light regulation to chlorophyll synthesis and plastid development of the chlorophyll—less golden—leaf privet. *J Integr Plant Biol* **52**, 809–816.
- Yuan, M., Yuan, S., Zhang, Z.W., Xu, F., Chen, Y.E., Du, J.B., and Lin, H.H. (2010b). Putative mutation mechanism and light responses of a protochlorophyllide oxidoreductase-less barley mutant NYB. *Plant Cell Physiol* **51**, 1361–1371.
- Zhang, Q. (1991). Studies on cultivars of crape myrtle (*Lagerstroemia indica*) and their uses in urban greening. *J Beijing For Univ* **(13)**, 57–66.
- Zhang, Z.Y., Wang, P., Wang, S.A., Li, L.F., Ma, Y.Z., and Li, Y. (2014). Study on structure and physiological basis of leaf colouring in *Lagerstroemia indica* L. ‘Jinwei’. *North Hortic.* **4**, 68–72.
- Zhong, X.M., Sun, S.F., Li, F.H., Wang, J., and Shi, Z.S. (2015). Photosynthesis of a yellow-green mutant line in maize. *Photosynthetica* **53**, 499–505.
- Zhou, Y., Gong, Z.Y., Yang, Z.F., Yuan, Y.A., Zhu, J.Y., Wang, M., Yuan, F.H., Wu, S.J., Wang, Z.Q., and Yi, C.D. (2013). Mutation of the light-induced yellow leaf 1 gene, which encodes a geranylgeranyl reductase, affects chlorophyll biosynthesis and light sensitivity in rice. *PLoS One* **8**, e75299.

Address correspondence to:

Ya Li, PhD

Institute of Botany

Chinese Academy of Sciences

No. 1 Qianhu Houcun

Zhongshanmen Wai, Nanjing, 210014

Jiangsu Province

China

E-mail: yalicnbg@163.com

Zengfang Yin, PhD

Southern Modern Forestry

Collaborative Innovation Center

College of Biology and the Environment

Nanjing Forestry University

No. 159 Longpan Road

Nanjing, 210037

Jiangsu Province

China

E-mail: zfyin@njfu.edu.cn

Received for publication November 1, 2016; received in revised form January 17, 2017; accepted February 2, 2017.