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Identification of genes involved in spontaneous leaf color variation in *Pseudosasa japonica*

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ABSTRACT. Spontaneous leaf color variation in bamboo provides the opportunity to study the mechanisms of leaf color formation and the breeding of ornamental bamboos. Despite the fact that many genes are known to be involved in leaf color variation in model plants, molecular mechanisms governing natural leaf color variation in bamboo have remained obscure. This study aimed to identify the genes responsible for the occurrence of such phenomena in bamboo using the suppression subtractive hybridization (SSH) method between green and albino leaves in *Pseudosasa japonica* f. A total of 1062 and 1004 differentially expressed transcripts were obtained from the forward and reverse SSH libraries, respectively. Subsequently, 59 differentially expressed unigenes with potential roles in leaf color formation, predicted via computational analysis of their functional relevance, were selected for further analysis using qPCR. Ten genes, involved in photosynthesis, plastid development, and cation signal transduction, showed 2-fold changes in expression levels between green and albino leaves. Further expression pattern analyses of these genes at three developmental stages revealed much lower expression

abundance of *Lhca1*-encoded chlorophyll a/b binding protein in the albino leaves than in the green leaves. Our results suggest that, together with the concatenated negative pressure for subsequent photosynthetic processes, the albino phenotype is at least partly attributable to chloroplast inner membrane damage or to the impairment of photosynthetic pigment accumulation, which results from low *Lhca1* expression.

Key words: Leaf color; *Pseudosasa japonica*; Photosynthesis; Quantitative real-time polymerase chain reaction; Suppression subtractive hybridization

INTRODUCTION

Bamboo, a woody perennial grass, has more than 1250 species in 75 genera that are unevenly distributed worldwide in various parts of the humid tropical, sub-tropical and temperate regions (Rai et al., 2011). Bamboo is extensively cultivated not only for its economic productivity, but also for its important ecological value, including its ornamental properties for gardening and environmental conservation, its high protection potential, and its role in water and soil conservation. Given the ornamental property of bamboo, spontaneous color alterations of leaves and culms in various bamboo genera have been widely utilized to increase its ornamental value (e.g., the white, yellow, green, and striped leaves as well as yellow, purple, red, green, and striped culms). *Pseudosasa japonica* Markino, a member of the subfamily Bambusoideae, is an extraordinary ornamental bamboo that originated in Japan, and it is now widely planted in Japan and China. In *P. japonica*, a spontaneous leaf color alteration that changes its native green leaves to the green-white longitudinal striped leaves and albino leaves (hereafter referred as to *P. japonica* f.) is observed, resulting in an increase in its ornamental value for gardens and landscapes.

Three main physiological processes are involved in the pigmentation of the specific tissues of plants (Morosinotto et al., 2005). 1) Metabolism and accumulation of pigments (chlorophylls, carotenoids, flavonoids, and heme): Chlorophyll, responsible for green color in most plants, is a principal pigment in chloroplasts due to its participation in photosynthesis (Sundberg et al., 1997; Wu et al., 2007). The metabolism of all pigments is complex, so functional mutations occurring in any part will cause pigment synthesis failure and further changes to the color phenotype of plant tissue. For instance, leaf color was altered when *ChlH* and *IMMUNE*, which play roles in chlorophyll and carotenoid biosynthesis, respectively, were functionally disrupted (Hudson et al., 1993; Jung et al., 2003; Sakamoto, 2003). 2) Development and degradation of chloroplast: Chloroplasts contain about 80 proteins encoded by chloroplast genes and over 3000 proteins encoded by nuclear genes (Waters and Langdale, 2009). The chloroplast development requires coordinated expression of both nuclear- and chloroplast-encoded genes (Rosso et al., 2009).The mutated genes in chloroplast development, including mutants of *FtsH2* (filament temperature sensitive; Rosso et al., 2009), *CHM* (chloroplast mutator; Jose et al., 1992), chlorophyll a/b binding protein *Lhcb1* (Streatfield et al., 1999), *PAC* (pale cress; Meurer et al., 1998), and *ATD2* (atase2 deficient; Vander Graaff et al., 2004), were all found to be associated with leaf color alternation. 3) Signal transduction between the chloroplast and the nucleus (including plastid-to-nucleus retrograde signaling and the nuclear signaling) (La Rocca et al., 2004, 2007): Mg-protoporphyrin IX (a chlorophyll synthesis precursor) acts as the signaling molecule in pathways, and it accumulates in the chloroplasts and cytosol of the cell after treatment with the

herbicide norflurazon. A prominent case for nuclear-chloroplast signal transduction in leaf color mutants included evidence that Mg-protoporphyrin IX accumulated in chloroplasts because of stress conditions, which resulted in the impairment of chlorophyll synthesis. Moreover, the accumulation of Mg-protoporphyrins IX led to the downregulation of nuclear genes related to photosynthesis (Strand et al., 2003; La Rocca et al., 2001, 2004, 2007). With the exception of the above mechanisms, some leaf cell structural changes also lead to leaf color variation. In some Begonia cultivars, intercellular space can be enlarged below the adaxial epidermis or below the adaxial water storage tissue, creating light areas on a leaf. In addition, chlorenchyma cell shape and chloroplast distribution within chlorenchyma cells differ between light and green areas. Chloroplasts from both areas showed dense stacking of grana and stroma thylakoid membranes (Sheue et al., 2012).

Although the genetic mechanisms of leaf color variation in *Arabidopsis thaliana* and rice (*Oryza sativa*) was well-illustrated (Sakamoto, 2003), only a few orthologous genes related to leaf color variation were identified in bamboo (Lin et al., 2006). Lin et al. (2006) identified 10 non-photosynthesis-related (with the exception of the Rubisco small subunit) bamboo genes that are differentially expressed in a bamboo (*Bambusa edulis* Murno) albino mutant. To do so, they employed a combination of suppressive subtractive hybridization (SSH) and microarray hybridization analyses, which mainly involved the stress response and photaesthesia. Subsequent research further showed that three chloroplast genes, NAD(P)H-quinone oxidoreductase chain 4, chloroplast 50S ribosomal protein L14, and ATP synthase beta chain, were supposed candidate genes for the albino mutants (Liu et al., 2007). However, current bamboo leaf color variation mechanisms were mainly derived from research on bamboo albino mutants from tissue culture lines. In this study, three types of leaf color in the bamboo *P. japonica* f. were observed, including full green, striped, and albino. These variations were spontaneously induced and unstable because the area, number, and location of the longitudinal white stripes in leaves were unstable. Furthermore, some albino leaves of several bamboo plants could recover green color during long-term tissue culture and manual cultivation. In order to illustrate the mechanisms of spontaneous bamboo leaf color variation, we used cDNA derived from complete green leaves and albino *P. japonica* f. leaves to develop an SSH library enriched for differentially expressed genes involved in leaf color variation.

MATERIAL AND METHODS

Plant materials

P. japonica f. from which the leaf samples were collected grew under natural conditions in the field at 'Jiyong' bamboo station, Zhejiang A&F University, Lin'an, Zhejiang, China. All materials used in this study were collected from the complete green and the albino plant at the same rhizome. For the construction of SSH libraries, juvenile leaves from shoots (5-6 cm in length) with green and albino leaves were sampled [Figure 1 (GJL and AJL) and Table 1]. For verification of the identified differentially expressed genes obtained by SSH, the same materials were used in quantitative real-time polymerase chain reaction (qPCR) analysis (Figure 1). To dissect the expression patterns of candidate genes during the leaf growth and development, two leaf types (green and albino) at three developmental stages [juvenile leaves (I), intermediate leaves between juvenile and adult leaves (II), and adult leaves that originated from the same axillary bud (III)] were used for qPCR analyses (Table 1). For electron microscopy, both the green and the albino tissues from adult leaves from the same developmental period (GAL, AAL) were also sampled (Table 1). In detail, adult leaves

were collected 1 week after growing out from new auxiliary buds and full expanding, and the intermediate leaves were about 8 cm in length at that point.

Electron microscopy

The micro-ultrastructure of chloroplasts in green and albino adult leaf tissues was observed under transmission electron microscope (TEM) (H-7650, HITACHI, Japan). Leaf tissue was cut into 1.0 x 1.0-mm^2 blocks. These tissues were immediate fixed in 2.5% glutaraldehyde in 100 mM phosphate buffer, pH 7.0, in the dark for 24 h. After post-fixation dehydration and resin-embedding procedures (Rascio et al., 1991), those sections were stained with lead citrate and uranyl acetate, and they were then observed by TEM.

Total RNA extraction and mRNA isolation

Total RNA was extracted from leaves using an RNeasy Plant Mini Kit (Qiagen, USA) according to the manufacture instructions. The integrity of total RNA was determined by electrophoresis on 1.0% agarose gels and quantified by a NanoDrop spectrophotometer (ND-7000, USA). The mRNA was purified using a FastTrack® MAG mRNA Isolation Kit for SSH library construction. In qRT-PCR analysis, total RNA was extracted and detected with the above methods, and first-strand cDNA was synthesized using the Reverse Transcription System (RT Master Mix, TaKaRa, Japan).

Construction of SSH library

SSH libraries were constructed using a PCR-Select™ cDNA Subtraction kit (Clontech, USA) based on the manufacturer instructions. The cDNAs from the green and albino leaves were mutually used as testers and drivers, and they were forward and reverse subtracted. Subsequently, these subtracted cDNAs were cut by *Rsa*I, mixed for 1.5 h at 37°C, and then ligated to adaptor 1 (5'-CTA ATA CGA CTC ACT ATA GGG CTC GAG CGG CCG CCC GGG GCA GGT-3') and adaptor 2 (5'-ACC TCG GCC GCG ACC ACG CTG CCC TAT AGT GAG TCG TAT TAG-3'). After two rounds of subtractive hybridization, two rounds of suppression PCR amplification were successively carried out. The efficiency of suppression subtractive hybridization was determined by gel electrophoresis. The purified PCR products were transformed into *Escherichia coli* DH5 α (Invitrogen, USA), and the recombinant clones were plated onto LB medium containing ampicillin, X-Gal, and IPTG, and were incubated overnight at 37°C. All of the white clones were picked for further analyses and sequencing.

Sequencing and expressed sequence tag (EST) analysis

Raw sequences were trimmed from the vector and assembled by CAP3 (Huang and Madan, 1999). The passed singles and contigs were renamed PjESTF-# or PjESTR-# (# represented serial number). The BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) and the Blast2GO software (http://www.blast2go.com/b2ghome) were used to search for homologous genes and gene annotations and for gene ontology (GO) data mining. Functional gene categories were defined via the Web Gene Ontology Annotation Plotting (WEGO, http://wegoGenomics.org.cn/cgi-bin/wego/index.pl). According to functional relevance, we screened ESTs for their association with pigment metabolism and transport, chloroplast development and degradation, and signal transduction of nuclear-chloroplast interactions (Waters and Langdale, 2009; He et al., 2013).

Gene expression by qPCR and data analysis

An iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) was used in qPCR analyses to detect expression levels (three replicates). The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) was used to calculate gene expression levels calibrated by the housekeeping gene *actin3* that was used as the endogenous control. The expression abundance of each gene was a relative expression level shown along the y-axis, which was calculated using the formula "E_x/E_{GJL}", where E_x represents the expression level of each sample, and the expression level of sample "GJL" (juvenile leaves of green type) was used as a control. The primers used in qPCR analyses are listed in **Table S1**.

RESULTS

Leaf color variation pattern in *P. japonica* f.

P. japonica f. served as a natural form of *P. japonica* due to its spontaneous leaf color variations across a long-term cultivation process. Three types of leaf colors (green, striped, and albino) were observed in *P. japonica* f. plants regenerated from the same rhizome. Over years of observation, the *P. japonica* f. striped leaves exhibited irregular performances, including varied stripe locations, area, and number, and some albino leaves could recover green coloration during long-term tissue culture and manual cultivation. Interestingly, each type of leaf color (green, striped, or albino) was unique to all of the leaves growing out from the same auxiliary bud. Therefore, the type of leaf color could be determined by the color on the first leaf that grew from each branch. This characteristic can facilitate sample collection for a leaf color type in the early stages of development. In this study, full green and albino leaves at three development stages were selected for the analysis of genes related to expression patterns of color variation in leaf growth and development (Figure 1).

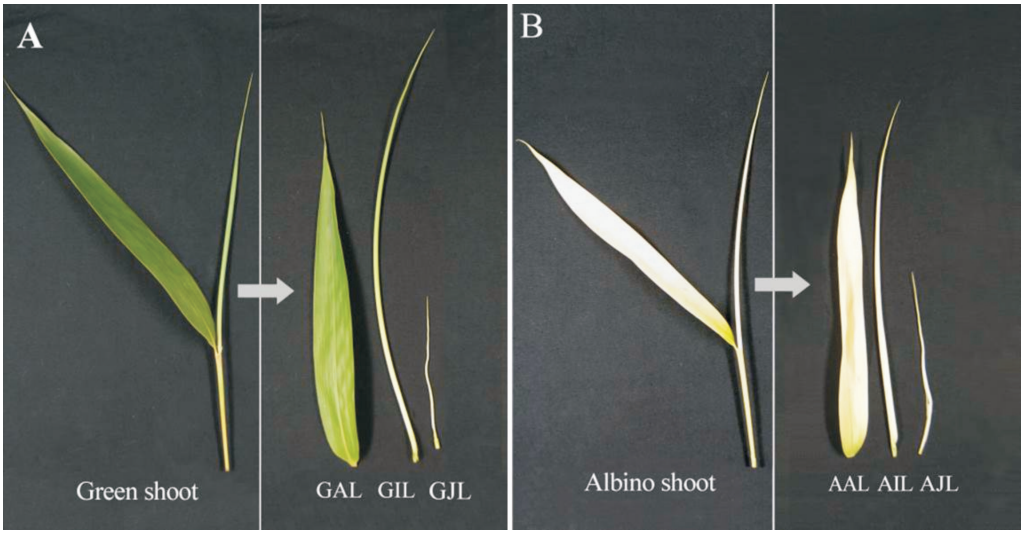

Figure 1. Two types of leaf color variation in *Pseudosasa japonica* f. **A.** and **B.** Represent the green and albino leaves, respectively. The leaves next to the shoot in each type represent three development stages: the adult leaf, the intermediate leaf, and the juvenile leaf from left to right (the abbreviations are shown in Table 1).

Chloroplast ultrastructure

The chloroplast ultrastructure from green and albino tissues of the bamboo leaves showed observable differences. All chloroplasts in green leaves had abundant thylakoid membranes, with dense stacking of grana that entirely filled the plastids (Figure 2A). In contrast, the chloroplasts in albino leaves had an intact outer membrane, but thylakoid membranes were converted into many abnormal vesicles, and no lamella and grana were observed (Figure 2B), which suggests that the inner membranes were damaged.

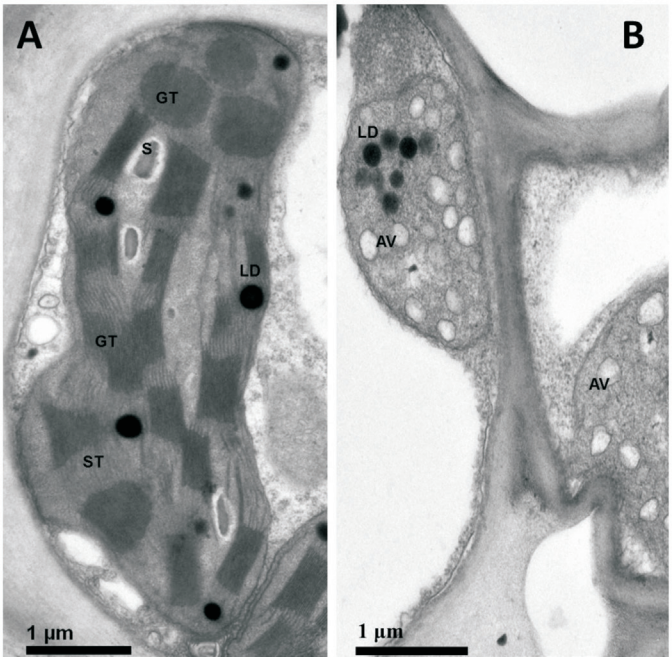

Figure 2. Ultrastructure of chloroplasts in mesophyll cells of bamboo *Pseudosasa japonica* f. **A.** and **B.** Represent the green and albino adult leaves, respectively. S = starch grain; AV = abnormal vacuole; LD = liquid droplet; ST = stroma thylakoid; GT = grana thylakoid.

SSH library construction and sequence data analysis

For investigation of the mechanism of leaf color variation in *P. japonica* f., green and albino leaf shoots from the plants were used for the construction of SSH libraries. Approximately 1521 colonies from the forward SSH library and 1394 colonies from the reverse library were obtained and sequenced. A total of 1062 and 1004 high-quality ESTs were obtained from the forward (named PjESTF-#) and the reverse libraries (named PjESTR-#), respectively (**Table S2**). Of these high quality ESTs, 281 (13.4%) were successfully assembled into contigs, while the remainder (86.6%) were singletons.

Based on additional sequence homology analyses by BLASTx in NCBI's GenBank(http://blast.ncbi.nlm.nih.gov/Blast.cgi), 457 (PjEST-F) and 604 (PjEST-R) ESTs showed high homology to the coding genes in NCBI (E-value $<10^{-5}$), and four ESTs in each library blasted to the same

coding gene. Based on the known protein function, 107 (PjEST-F) and 237 (PjEST-R) EST homologous proteins were annotated, which corresponded to 759 and 1701 GO terms identified by the Blast2Go software, respectively. The functional classifications of the obtained ESTs were clustered into 40 slims (http://wego.genomics.org.cn/) within the three ontology categories, including molecular function, biological processes, and cellular components (Figure 3). Among all of these slims, cell, cell part, and organelle were three main components in the forward and reverse libraries. Moreover, the proportion of the three main components in all slims were similar between both libraries, indicating the important role of "cell-related" unigenes in bamboo leaf growth and development.

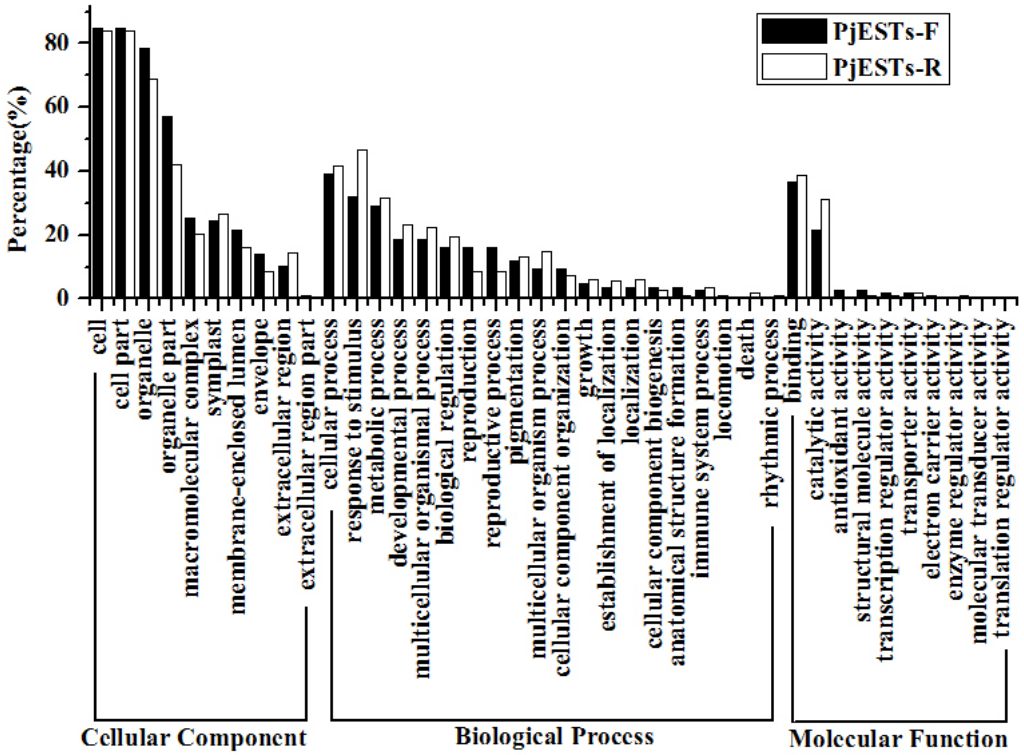

Figure 3. Classification of the 344 ESTs from the forward SSH library and the reverse library according to Gene Ontology Consortium (http://wego.genomics.org.cn/cgi-bin/wego/index.pl).

Verification of differentially expressed genes by qPCR

There are three major physiological processes for leaf color variation, including pigment metabolism and transport, chloroplast development and degradation, and signal transduction on nuclear-chloroplast interaction (Waters and Langdale, 2009). Therefore, some genes were not associated with the above major physiological processes, and they were filtered out manually, including genes involved in basal metabolic process, stimulus response, and immune system processes. The genes involved in leaf growth and development, with emphasis on leaf colorizations,

were selected. Finally, 59 unigenes were chosen for further expression level verification between the green and albino leaves using qPCR (**Table S3**). Results showed that 10 (16.4%) of 59 unigenes exhibited a 2-fold change in their expression between the green and albino leaves (**Table S3**). Specifically, eight unigenes were associated with the photosynthetic processes [e.g., *Light harvesting complex 1* (*Lhca1*, PjESTF-622), *plastocyanin* (*PC*, PjESTF-497), *cofactor assembly of complex C* (*CCB1*, PjESTF-161), *ferritin 2* (*FER2*, PjESTF-733), *clp-protease* (*ClpP*, PjESTR-793), *uridylate kinase* (*UMPK*, PjESTF-477), *ribulose bisphosphate carboxylase small chain 2B* (*Rbcs*, PjESTF-298), and *glyceraldehyde 3-phosphate dehydrogenase* a subunit (*GAPA*, PjESTF-566)]. The remaining two unigenes, plastid-localized *phosphoenolpyruvate enolase* (*ENO1*, PjESTF-51) and *cation exchanger* (*CAX1*, PjESTR-501), were individually involved in plastid development and signal pathways, respectively.

Expression patterns of 10 candidate genes during leaf color variation

To further dissect the contribution of these 10 genes to spontaneous leaf color variation in bamboo, the green and albino leaves were selected for expression pattern analysis by qPCR. The two different types of leaves were collected at three development stages, including the juvenile leaf (stage I), the intermediate leaf (stage II), and the adult leaf (stage III) stages (Table 1). As shown in Figure 4, the 10 candidate genes were categorized into four groups based on the similarity of their expression patterns.

Table 1. Materials used in expression pattern analyses for 10 candidate genes.

Leaf color	Developmental stage	Shape and size	Abbreviation	Stage No.
Green	Juvenile leaf	Small and round	GJL	I
Green	Intermediate leaf*	Long and round	GIL	II
Green	Adult leaf	Slender	GAL	III
Albino	Juvenile leaf	Small and round	AJL	I
Albino	Intermediate leaf*	Long and round	AIL	III
Albino	Adult leaf	Slender	AAL	III

*The leaf developmental stage was between juvenile and adult.

The *Lhca1*, *PC*, *Rbcs*, and *GAPA* genes were included in group A. During growth and development, continuously increased expression levels of these four genes in the two leaf types were observed. These four genes significantly reduced their expression levels in the albino relative to the green leaves, especially at stage III (Figure 4A). *CCB1*, *FER2*, *ClpP*, and *UMPK* were classified into the group B. The expression levels of these four genes in the albino leaves were discernibly higher than those in green leaves at stage II, but they were at the same levels in both leaf types at stage III (Figure 4B).

ENO1 and *CAX1* belonged to group C and group D, respectively. A similar expression pattern of *ENO1* was detected at the three development stages of both leaf types, but the expression level of *ENO1* in the albino was approximately 2-fold higher than that in the green leaves, according to the developmental stage (Figure 4C). Similarly, *CAX1* expression continuously increased during leaf growth and development in the two leaf types, but its expression level in albino leaves was much higher than that in green leaves at stages II and III (Figure 4D).

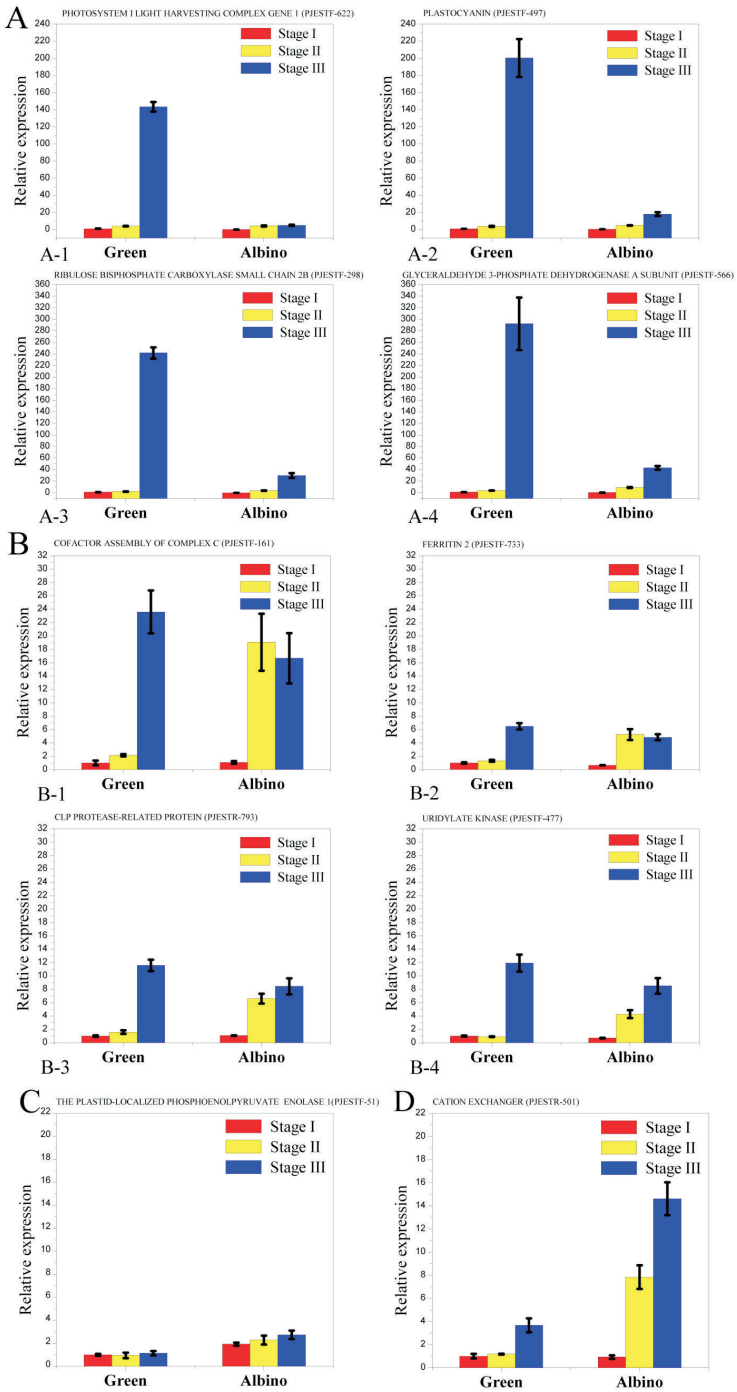

Figure 4. Expression profile analysis of 10 candidate genes at different developmental stages in the green and albino leaves based on qPCR analyses. The data points represent means ± SD of relative gene expression.

DISCUSSION

The chloroplast ultrastructure observations show that the albino leaves contain non-functional chloroplasts that are possibly due to damaged inner membranes. The SSH technology identified 10 differentially expressed genes when compared between plants with green and albino leaves, including eight nucleus-encoded genes related directly to photosynthesis and two chloroplast-encoded genes related to plastid development and cation exchange. These 10 differentially expressed genes were further divided into four groups based on their roles in a set of biological processes. The gene expression patterns of green and albino leaves from every group were similar.

Genes associated with the assembly of light-harvesting complex, plastocyanin biosynthesis, and the Calvin cycle

Lhca1, *PC*, *Rbcs*, and *GAPA* (Group A) exhibited similar expression modes in which their expression levels continuously increased along with leaf growth and development for both leaf types. However, their expression levels in albino leaves were much lower than those in the complete green leaves at stage III (Figure 4A). All four genes were essential cofactors involved in plant photosynthesis. LHCA, the outer antenna system of photosystem I, was thought to bind chlorophyll (chl) a, chl b, and xanthophylls to harvest light and transfer the excitation energy to the reaction center in PSI (Ben-Shem et al., 2003). In bamboo plants, *Phyllostachys pubescens*, the Lhca protein contained four subunits, Lhca1-4. The expression level of *Lhca1* was highest in new leaves, but that of *Lhca2*, *3*, and *4* was only detectable in old leaves, bamboo shoots, and culms (Tang, 2008). In this study, there were no genes homologous to *Lhca2-4* detected, but the much lower *Lhca1* expression levels in albino leaves, compared to that in complete green leaves at stage III, indicated that the light-harvesting complex could not be adequately formatted in the albino leaf. As a result, chlorophylls and xanthophylls could not bind to the light-harvesting complex, so that the unbound photosynthetic pigments would be degraded (**Table S4**), showing albinism (Cornah et al., 2003). The loss of xanthophylls would lead to the loss of chloroplast photo-protection in albino leaf cells (Mayfield, 1986), which was associated with chloroplast inner membrane damage in adult albino leaf cells (Figure 2B). Similar results were detected in the barley mutant, where the reduction of *Lhc* mRNA levels led to photo-oxidative damage in plastids (La Rocca et al., 2001).

In addition, the low expression level of *Lhca1* also revealed that PSI could not be sufficiently assembled in albino leaves, which would influence the biosynthesis of PC, NADPH, and ATP produced by the light reaction. Moreover, this would subsequently affect the carboxylation and reduction reaction regulated by *Rbcs* and *GAPA* genes in the Calvin cycle (Morosinotto et al., 2005). Therefore, the expression of the nuclear genes *Rbcs* and *GAPA* was regulated by light (Sparla et al., 2004), and *Rbcs* expression was also linked to chloroplast membrane photo-damage (La Rocca et al., 2001, 2004). The reduction of expression levels of the three genes (*PC, Rbcs*, and *GAPA*) at stage III might indicate that the functional chloroplast could not be biosynthesized and assembled in the albino leaf (Figure 2B). Lin et al. (2006) also detected low *Rbcs expression levels* in albino tissue culture lines (*Bambusa edulis*), which was associated with aberrant chloroplasts (Figure 5).

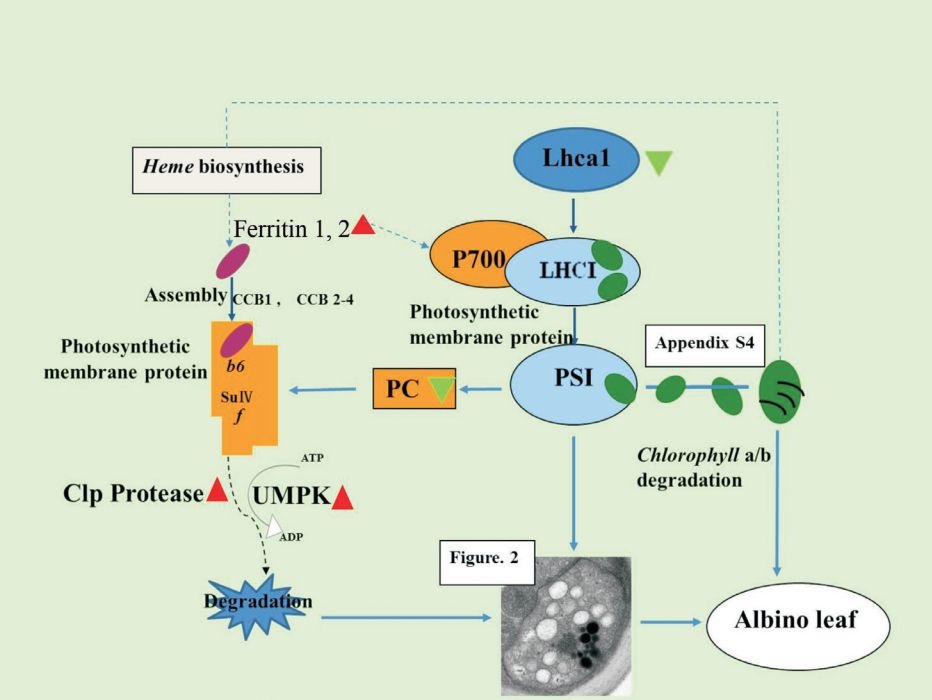

Figure 5. Hypothetical gene regulatory network for the mechanisms associated with albino leaf observations in bamboo. The differentially expressed genes are marked with triangles. The red up-triangles represent relatively high expression levels of the genes in the albino leaf, while the green down-triangles represent relatively low expression levels in the albino leaf.

Genes associated with assembly and degradation of the chloroplast cytb_6f complex

The expression levels of the group B genes in the albino leaves were much higher than that in the green leaves at stage II (Figure 4B). CCB proteins are known to be associated with heme binding, stabilization of the chloroplast cytb_6f complex, and a plastoquinol-plastocyanin oxidoreductase that links PSI and PSII in the electron transport chain (Kuras et al., 1997). *FER*-encoded ferritin is the major, and perhaps only, iron storage protein used as the iron source for *de novo* synthesis of cytochromes and FeS proteins in the photosynthetic apparatus during chloroplast development (Merchant and Dreyfuss, 1998). *ClpP* played a critical role in the removal of damaged proteins, and it controlled the degradation rate of the cytb_6f complex in chloroplasts (Majeran et al., 2000). *UMPK* was tightly linked with the ATP metabolic process, and it determined the essential function of *clpP* for cell growth and division (Sjögren and Clarke, 2011). For the degradation of cytb_6f complex, C*lpP*-mediated degradation was an ATP-dependent proteolysis (Majeran et al., 2000), suggesting a positive correlation between C*lpP* and *UMPK*. Taken together, all four genes are involved in the assembly or degradation of the cytb_6f complex. The high expression levels of *CCB* and *FER* genes in the albino leaves showed high biosynthesis levels of *ci-heme*. This is expected considering that the abundance of *CCB* was positively correlated with the biosynthesis of *ci-heme*, and that the biosynthesis of *ci-heme*

is required for Fe^{2+}, which is offered by *FER*-encoded ferritin (Tarantino et al., 2010). However, the high expression abundance of *CCB* and *FER* did not indicate the increased content of the chloroplast cytb_6f complex in the albino leaf cells, because *ClpP* and *UMPK* expressed at a high levels in those leaves were positively correlated with the degradation of the cytb_6f complex (Majeran et al., 2000; Sjögren and Clarke, 2011) (Figure 5).

Genes associated with plastid development and cation exchange

The *ENO1*-encoded protein was the enolase located in plastids, which is a key enzyme for the production of phosphoenolpyruvate (PEP) from phosphoglycerate (2-PGA) (Canback et al., 2002). The expressed *ENO1* was detected in the immature plastids of some non-photosynthetic cells, including the developing endosperm of *Ricinus communis* (Miernyk and Dennis, 1984), the trichome of *Arabidopsis thaliana* (Prabhakar et al., 2009), and the leaf sheath at the tiller base of rice, but it was not detected in mature chloroplasts (Fukayama et al., 2014). In this study, the relative expression abundance of *ENO1* was significantly high in albino leaves, which indicated the high content of immature plastids in the tissue (Figure 4C).

The expression of *CAX1* continuously increased during leaf growth and development in both leaf types, and its expression level in albino leaves was much higher than that of green leaves at stages II and III (Figure 4D). *CAX1* encoded a cation exchanger-cation (Ca^{2+})/H^+ antiporter, which exports cations from the cytosol to vacuoles to maintain ion homeostasis (Hirschi, 1999). In tobacco, high-level expression of *CAX1* caused severe symptoms of Ca^{2+} deficiency and increased the observation of albino leaves (Pittman and Hirschi, 2003). This suggested that when *CAX1* is expressed at high levels, it might play a role in the formation of albino leaves in bamboo, but the detailed regulation mechanism is not clear.

In conclusion, only 59 genes were correlated with leaf color formation via screening for potential functional relevance, but more than 1000 differentially expressed genes were identified between green and albino leaves by SSH. Ten genes with roles in photosynthesis, chloroplast formation, and cation signal transduction showed more than a 2-fold expression level change between the green and albino leaves, providing information for understanding the molecular mechanisms of spontaneous leaf color variation in bamboo. Our results suggest that the albino phenotype is at least partly attributable to chloroplast inner membrane damage or impairment of photosynthetic pigment accumulation, resulting from low *Lhca1* expression together with concatenated negative pressure for subsequent photosynthesis processes (Figure 5).

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

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