Integrated analyses of rice dark response and leaf color control reveal links with porphyrin and chlorophyll metabolism

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Abstract

Background: Light is a key regulatory signal for rice growth and development. Under dark stress, rice shows leaf yellowing. Whole genome transcriptomic analysis will identify differentially expressed genes (DEG) in dark-treated rice seedlings and DEG-enriched metabolic pathways. Rice leaf color is an essential agronomic trait. Traditional genetic experiments have reported over a hundred of leaf color control (LCC) genes and some of them were also regulated by light signal. Thus, an integrated analysis for the two set of data will be helpful for illustration of the mechanism for both dark-response and leaf color control.

Results: Transcriptome changes in response to dark treatment were surveyed by RNA-Seq analysis. About 13,115 differentially expressed genes (DEGs) were identified. One hundred and fifty rice LCC genes were collected. It was found that 102 LCC genes (68.0%) were also dark-response DEGs, which suggests an overlap between dark response and LCC networks. Fifty DEG overlapped LCC genes was associated with chloroplast development. KEGG analysis revealed enrichment of LCC genes in porphyrin and chlorophyll metabolism (PCM) (18/44, 40.9%). Of the 18 LCC genes in the PCM pathway, 15 were dark-response DEGs (83.3%). More interestingly, all of them are involved in a central PCM sub-pathway, chlorophyll biosynthesis.

Conclusions: Integrated analysis for dark stress-response and leaf color control identified the correlation between the two processes and mutually supported evidences were obtained. It was found that PCM pathway, particularly chlorophyll biosynthesis process, plays important roles in rice LCC and dark stress-response. This study provides important clues for understanding the mechanisms of dark response and leaf color control and identifying additional LCC genes.

Background

Light is the essential environmental factor for normal plant growth and development, both as an energy source processed via photosynthesis and as a signal for many biological processes. When cultivated in regular day-night cycles (diurnal cycle), rice grows with green leaves. In contrast, rice seedlings grown under dark treatment elongate more quickly and have yellow or pale colored leaves. Dark-stress is a severe stress, producing serious physiological damage that affects plant growth and development.

Through the course of evolution, plants have established efficient mechanisms for responding to light signals (Demarsy et al., 2017). Photoreceptors play essential roles, sensing light signals and initiating signal transduction. Three types of photoreceptors have been identified in Arabidopsis: five subtypes of red / far red light receptor phytochromes (PhyA–PhyE), blue light receptor cryptochromes (Cry1 and Cry2) and phototropins (Phot1 and Phot2) and UV-B specific photoreceptors (UVR8) (Demarsy et al., 2017; Yang et al., 2018). In light signal transduction pathways, dozens of elements that are downstream of photoreceptors, such as chromatin remodeling factors, histone modifying enzymes, transcription factors (such as PIFS, HY5 and FHY3), splice factors, protein degradation factors (such as COP1, SPAs and LR8), factors interacting with exogenous environmental signals and endogenous hormones have been

identified. These elements are coordinated to transmit light signals accurately and quantitatively, thereby regulating seed germination, photomorphogenesis, chloroplast development, shade response, stomatal opening and closing, flowering, biological rhythm and aging to ensure normal plant growth and development (Demarsy et al., 2017). On the basis of sequence similarity, homolog photoreceptor genes for Phy (Kay et al., 1989), Cry (Zhang et al., 2006b), Phot (Kanegae et al., 2000) and UVR8 (Fernández et al., 2016) have been identified in rice. However, limited information has been reported about regulatory elements downstream of these photoreceptors.

Rice is the world's most important food crop and a model monocotyledon plant for basic research. In rice, leaf color mutations occur with high frequency and can be recognized easily. Further, they are a useful resource for genetic studies and breeding applications. So far, over one hundred leaf color mutants have been isolated in rice. They are classified by phenotypes: albino, yellow, temperature-sensitive, bright green, stay green, stripe, purple and turning green (Wu et al., 2015). Rice leaf color control genes (LCC) are mainly involved in chlorophyll metabolism and chloroplast development. The chlorophyll (Chl) synthesis pathway involves dozens of genes, and genes encoding Chl synthesis enzymes have been identified in higher plants (Nagata et al., 2005). Every Chl metabolic pathway gene mutation that has led to the inactivation or deletion of its encoded enzyme has changed the proportion of chlorophyll contents and produced leaf color variation. In rice, dozens of genes identified as Chl biosynthesis enzymes have been cloned, including OsYGL18 (Wang et al., 2017c), OsChlH, OsChlD and OsChlI (Zhang et al., 2006a; Inagaki et al., 2015), OsPORA and OsPORB (Sakuraba et al., 2013), OsYGL1 (Wu et al., 2007), OsIsP (Huang et al., 2017), OsDVR (Wang et al., 2010), OsCAO1 and OsCAO2 (Lee et al., 2005; Yang et al., 2016b), OsCRD1 (Wang et al., 2017b), OsGluRS (Liu et al., 2007), OsIsP (Chen et al., 2018), OsV5B (Liu et al., 2016b) and OsMTS1 (Hong et al., 2018). Several genes associated with chlorophyll degradation, like OsNOL (Kusaba et al., 2007), OsNYC3 (Morita et al., 2009) and OsSGRL (Rong et al., 2013), have also been cloned. Chloroplast organelles are unique to higher plants and responsible for photosynthesis. Abnormal chloroplasts develop when genes associated with chloroplast development are mutated, influencing the amount and proportion of chlorophyll and other photosynthetic pigments. At present, cloned chloroplast development genes include OsLC7 (Chen et al., 2016b), OsZN (Li et al., 2010), OsTLP27 (Kang et al., 2015), OsCpn60 α (Jiang et al., 2014b), OsGRY79 (Wan et al., 2015), OsYSS1 (Zhou et al., 2017), OsPGL12 (Chen et al., 2019), OsYS83 (Chen et al., 2016a), OsNTRC (Perez-Ruiz et al., 2006), OsVYL (Dong et al., 2013), OsSRP43 (Lv et al., 2015), OsSRP54 (Zhang et al., 2013a), OsETI1 and OsETI2 (Mao et al., 2011), OsClpP5 (Tsugane et al., 2006), OsV5A (Zhou et al., 2013a), OsS6K (Sun et al., 2016), OsHAD1 (Liu et al., 2018), OsHAP3A (Miyoshi et al., 2003), OsYGL8 (Zhu et al., 2016), OsDG2 (Jiang et al., 2014a), OsYLD1 (Deng et al., 2017), OsNOA1 (Yang et al., 2011), OsPAPST1 (Xu et al., 2013) and OsCSP41b (Mei et al., 2017). Mutation of key enzymes in the heme synthesis pathway resulted in significantly reduced chlorophyll biosynthesis in rice. Genes encoding heme oxygenases OsHO1 and OsHO2 have been cloned (Chen et al., 2013; Li et al., 2014). Carotenoid content is a determinant of leaf color. A number of essential carotenoid synthesis genes have been cloned, including OsPDS (encoding phytoene desaturase), OsZDS (encoding zeta-carotene desaturase), OsCRTISO (encoding carotenoid isomerase) and beta-OsLCY (encoding lycopene beta-cyclase) (Fang et al., 2008). Improved understanding of functions and cross-talk

of genes involved in chlorophyll synthesis and degradation and chloroplast development would help clarify mechanisms controlling leaf color and facilitate improvements of photosynthesis efficiency.

Whole-genome transcriptome analysis, involving deep sequencing or DNA microarrays, is a method used to reveal differential gene transcription at varying locations and times or under various stresses. It is useful for identifying clues that enable subsequent investigations of molecular mechanisms. Using oligonucleotide arrays, transcriptional profiling was performed under four light treatments (blue, green, red and white), as well as dark treatment, in rice. The results showed that the expression of transcription factors, such as bHLH, MYB, C2H2, ERF, NAC and WRKY, changed significantly, and carbohydrate degradation decreased under dark treatment (Lakshmanan et al., 2015). It was also revealed that both Phy A and Phy C cooperatively regulate transient gene expression in red-light treated rice seedlings (Kiyota et al., 2012). It has been observed that middle mesocotyl elongation was almost completely inhibited when germinating seeds were exposed to low-intensity light. RNA-Seq analysis revealed that most of the differential expressed genes (DEGs) were associated with hormone changes that occur in response to light exposure (Feng et al., 2017). Comparative transcriptome profiling for low-light tolerant and sensitive rice varieties was carried out via RNA-Seq analysis, and DEGs induced by low-intensity light at the tillering stage were identified (Sekhar et al., 2019). Rice leaf color mutant accessions were analyzed by whole-genome resequencing and transcriptomic approaches, and the identified DEGs were classified into different categories, including genes related to macronutrient (e.g. magnesium and sulfur) transport and genes related to flavonoid biosynthesis (Kim et al., 2015).

In this study, transcriptomic analysis using an RNA-Seq approach was carried out for yellow leaves of dark-treated rice seedlings. DEGs were identified and DEG-enriched metabolic pathway analysis was performed with KEGG. Furthermore, data for previously reported LCC genes were collected for KEGG analysis, and metabolic pathways that include both LCCs and dark-response DEGs were identified. It was found that the transcript abundance of most LCC genes changed under dark treatment, suggesting an overlap between leaf color control and dark response. Therefore, integrated analyses were carried out, producing supporting, supplementary evidence for this hypothesis. This study provides important clues for improving mechanistic understanding of dark response and leaf color control and identifying additional LCC genes.

Materials And Methods

Plant materials, cultivation and dark treatment

Rice variety TP309 was used in this study. After being soaked in water for 3 days, seeds were surface sterilized and then germinated in soil (soil: vermiculite = 1:1). Rice seedlings were grown for 5 days in a 30°C growth chamber under a 12-h light/12-h dark cycle ($60 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Then, seedlings were transferred to 24-h dark (constant dark, CD) conditions until sample collection.

Measurement of plant height and chlorophyll content

Phenotypic photos were taken at different time points during constant dark treatment. The plant height was measured for more than 10 randomly selected seedlings. The mean and standard deviation were calculated. Rice leaves were harvested, sliced and soaked in 80% acetone for 48 h in the dark. The optical density was recorded at 470 nm, 646 nm and 663 nm with an ultraviolet spectrophotometer. The chlorophyll a, chlorophyll b and carotenoids contents were calculated in accordance with Lichtener and Wellburn's method (Lichtenthaler and Wellburn, 1983). Three replicates were measured.

Total RNA extraction from rice seedlings and RNA-Seq analysis

Rice seedlings under CD treatment for 0, 3 and 6 days were collected for total RNA extraction and library construction. Transcriptomic analysis was performed using Illumina Novaseq 6000 by Novegene Co., Ltd (Beijing, China).

Identification of differentially expressed genes (DEGs) and KEGG-enrichment analysis

Genes with log2 fold change (absolute value) >1 and P-value <0.05 were defined as differentially expressed genes. The Kyoto Encyclopedia of Genes and Genomes (KEGG, <https://www.genome.jp/kegg/>) pathway network was used to identify DEG-enriched metabolic pathways under dark treatment. The DEG-enriched KEGG analysis was performed by ClusterProfilerR software. Rich factor (RF), the number of DEGs normalized to the total number of genes in a specific metabolic pathway (#GMP), was calculated for all metabolic pathways. Bubble charts were drawn for the 20 pathways with the highest P-value.

Identification of transcription factors among DEGs

Transcription factors among DEGs were identified with PlantTFDB v5.0 (planttfdb.cbi.pku.edu.cn), and their relative expression levels were obtained from the RNA-Seq data.

Collection of leaf color control (LCC) genes

Rice LCC genes were collected by literature mining. The gene name, locus number, annotation and references for cloned LCC genes are summarized in Table 2. KEGG analysis of LCC genes was carried on the basis of DEG enrichment analysis. The number of LCC genes measured as a proportion of the #GMP is referred to as the mutant rich factor (mRF).

Heat Map

LCC genes were functionally classified by their protein's annotated function, and different colors were assigned to indicate expression on the basis of log2 (fold change) values. Red and green colors depict up- and down-regulated genes, respectively. The LCC heat map was made with EXCEL software (Microsoft Corporation, USA).

Metabolic pathway chart

LCC gene locus numbers were input to obtain corresponding EC (Enzyme Commission) numbers from the KEGG database (<https://www.genome.jp/kegg/pathway.html>). KEGG Mapper software was used to assign a color to each gene on the basis of the transcript abundance, as determined by RNA-Seq analysis. On the basis of log₂ (fold change) values, red lines represent up-regulated DEGs, green lines represent down-regulated DEGs, and yellow lines represent DEGs with discrepant up- and down-regulation at 3 d and 6 d under constant dark.

Results

Phenotypic characterization and transcriptomic analysis of dark-treated rice seedlings

Rice seedlings grown in normal light condition (12-h light/12-h dark) for 5 days were switched to dark treatment, and samples were collected at 0 (CK), 3 (CD_3d) and 6 days (CD_6d). The seedling height and chlorophyll a, chlorophyll b and carotenoid content were measured. Under CD stress, rice seedlings showed more leaf yellowing and taller plant height than CK (Fig. 1A). The plant height increased 19.2% and 11.5% at CD_3d and CD_6d, respectively, compared with control (Fig. 1B). The chlorophyll a, chlorophyll b and carotenoid contents under 6d of CD significantly decreased by 82.0%, 100.0% and 70.4%, respectively (Fig. 1C, 1D and 1E). Taken together, yellowed leaves, increased plant height and decreased chlorophyll content are typical characteristics of plant skotomorphogenesis, suggesting that CD stress resulted in deficient rice growth and development.

RNA-Seq transcriptome analysis was carried out for CK, CD_3d and CD_6d rice seedlings. The results revealed 33,755 non-redundant transcripts, accounting for 59.2% of all predicted rice genes (about 57,023). A total of 13,115 DEGs were identified, which accounts for 38.9% of all non-redundant transcripts (Additional file: Table S1). Approximately 8,242 DEGs were detected between CK and CD_3d, of which 4,467 were up-regulated and 3,775 were down-regulated. Additionally, there were 8,266 DEGs between CK and CD_6d, of which 3,834 were up-regulated and 4,432 were down-regulated. There were more up-regulated DEGs than down-regulated DEGs for CD_3d, while there were fewer up-regulated DEGs than down-regulated DEGs for CD_6d (Fig. 2A). There were 4,849 CD_3d only and 4,873 CD_6d only DEGs. A total of 3,393 genes were DEGs in both CD_3d and CD_6d (Fig. 2B). It can be inferred that the early stages of dark treatment induced the transcription of many genes, while longer periods of dark treatment led to increased numbers of down-regulated genes. This switch from up-regulation to down-regulation is an indication that as the CD treatment is extended, rice seedling growth becomes more inhibited and the expression of additional genes is down regulated.

DEG-enriched KEGG metabolic pathway analysis

KEGG metabolic pathway analysis of dark-response DEGs was performed by ClusterProfiler R. DEG-enriched pathways and corresponding rich factors (RF) for CD_3d and CD_6d are listed in Additional file: Table S2. The 20 most significantly enriched pathways are shown in Fig. 3. In CD_3d, the term with the highest RF was cutin, suberine and wax biosynthesis. There were 11 up-regulated DEGs and 6 down-regulated DEGs. The second highest RF term was glyoxylate and dicarboxylate metabolism with 13 up-

regulated DEGs and 22 down-regulated DEGs. The third highest RF term was amino sugar and nucleotide sugar metabolism with 49 up-regulated DEGs and 13 down-regulated DEGs. In CD_6d, the pathway with highest RF was photosynthesis-antenna proteins with 13 down-regulated DEGs, the second highest RF term was monobactam biosynthesis with 7 down-regulated DEGs, and the third highest RF term was photosynthesis with 1 up-regulated DEG and 28 down-regulated DEGs. Comparison the terms from 3d and 6d, 7 out of 20 were overlapped, which supported the unique dark-stress response at different time points.

Next, the CD_3d and CD_6d DEGs were combined, and RF was recalculated for each pathway using non-redundant DEGs. The 20 pathways with the highest RF are shown in Fig. 4, and the highest RF pathways include photosynthesis-antenna proteins; carotenoid biosynthesis; cutin, suberine and wax biosynthesis; monobactam biosynthesis; porphyrin and chlorophyll metabolism (PCM); other glycan degradation; one carbon pool by folate; glyoxylate and dicarboxylate metabolism; and photosynthesis and biotin metabolism. Data for additional pathways are listed in Additional file: Table S3.

Identification of transcription factors among dark-response DEGs

Because transcription factors (TFs) play important roles in many biological processes, transcription factors among the dark-response DEGs were identified from matches with the *Oryza sativa subsp. japonica* transcription factor database in PlantTFDB (planttfdb.cbi.pku.edu.cn). Of the 8,242 DEGs in CD_3d, 449 (5.4%) were annotated as TFs belonging to 48 TF families, of which 296 were up-regulated and 153 were down-regulated. Of the 8,266 DEGs from CD_6d, 394 (4.8%) were annotated as TFs belonging to 47 families, of which 262 were up-regulated and 132 were down-regulated. Combined, 661 non-redundant TFs, representing 52 TF families, were identified as DEGs at the two dark treatment time points. Note, as shown in Table 1, the TF families that were most affected by CD were the bHLH, MYB, C2H2 and WRKY TF families. In particular, there were 47 bHLH among 449 TFs (10.5%) in CD_3d and 36 bHLH among 394 TFs (9.1%) in CD_6d. TF transcript levels were further analyzed. The most significant differentially expressed TFs under 3 d of dark treatment include AP2, B3, bHLH, G2-like, GeBP, HSF, LBD, M-type_MADS, MYB_related and SBP family members. Among them, the expression of a SBP (LOC_Os06g44860) increased by 754-fold, and the expression of a HSF (LOC_Os01g53220) decreased by 478-fold. The most significant differentially expressed TFs for 6 d dark treatments include ARR-B, B3, GRAS, LBD and MYB_related family members. Among them, the transcript abundance of an ARR-B (LOC_Os04g28160) increased by 625-fold, and the expression of a MYB_related (LOC_Os01g47370) decreased by 350-fold. Future functional investigations of TFs with the largest expression changes should be of interest to dark-response research.

Collection of rice leaf color control (LCC) genes

Rice leaves showed green color under normal growth condition. Numerous leaf color mutants have been identified via traditional forward genetics and breeding programs in rice. Information on 150 LCC genes was collected from literature, and loc#, gene name and references are summarized in Table 2 and Additional file: Table S6. As shown in Table 2, the phenotypes of known LCC mutants are albino (26),

yellow (52), temperature-sensitive (14), stay green (16), stripe (15), spot (9), purple (1) and turning green (17). On the basis of their annotation, LCC genes mainly participate in chlorophyll synthesis, chloroplast development and photosynthesis.

Transcription analysis of LCC genes in dark-treated rice

RNA-Seq measurements of CD stress transcript levels for 150 LCC genes were surveyed. This analysis revealed that 102 of 150 LCC genes (68.0%) were also dark-response DEGs, of which 18 were up-regulated at both 3d and 6d, 77 were down-regulated at both 3d and 6d, and 7 had opposing up- and down-regulation at the two time points (Additional file: Table S6). Thus, the transcript abundance of most LCC genes changed in response to dark stress.

The 102 LCC DEGs were functionally classified on the basis of annotations. A heat map was drawn showing log₂ (fold change) values under 3 d and 6 d dark treatments (Fig. 5). In Fig. 5, it can be seen that there are 50 chloroplast development related genes, including three that were up-regulated and 46 that were down-regulated at both time points. There were 15 chlorophyll synthesis-related LCC genes, including three that were up-regulated and eight that were down-regulated at both time points. There were 10 photosynthesis-related LCC genes and five chlorophyll degradation-related LCC genes.

These data support that chloroplast development, chlorophyll synthesis and photosynthesis LCC genes play important roles in leaf yellowing under CD treatment and, in particular, down-regulated genes in these pathways. Moreover, aging, active oxygen scavenging, light signal transduction and carotenoid synthesis are also involved in the yellowing of leaves. Significant down-regulated expression was observed for most LCC genes under constant dark treatment, which implies that these LCC genes play a positive role in light signal responses.

KEGG analysis of LCC genes

Although hundreds of LCC genes have been cloned through traditional genetic approaches, larger-scale understanding of molecular mechanisms controlling leaf color remains poor. Thus, LCC genes were functionally classified and assigned to KEGG *Oryza sativa japonica* (*Japanese rice*) pathways. Similar to the calculation of rich factor in KEGG enrichment analysis, the number of LCC genes as a proportion of the total number of genes in a specific metabolic pathway (#GMP) was calculated as mutant rich factor (mRF). Fig. 6 shows pathway names and mRF values. The highest mRF pathway was PCM, accounting for 40.9% (18/44), followed by betalain biosynthesis (16.7%; 1/6) and photosynthesis-antenna proteins (6.7%; 1/15). The mRF for other metabolic pathways was less than 6% (Additional file: Table S7). Based on this result, it can be postulated that the PCM pathway plays important roles in regulating rice leaf color. KEGG analysis of the collection of LCC genes brought focus to specific metabolic pathways, providing a broader understanding of leaf color mechanisms and clues for identifying novel LCC genes.

Integrated analysis of dark-response DEGs and LCC genes

Among the 150 LCC genes, the transcript abundance of 102 genes changed under CD treatment, indicating an overlap between dark-response and leaf color control. Thus, it is reasonable and necessary to carry out integrated analysis of the two biological networks. Fig. 7 highlights pathways that include both dark-enriched DEGs and LCC genes, and the name or locus number of each gene is shown. A correlation between the two sets of data can be seen. As shown in Fig. 7, there was an overlap between dark-response DEGs and LCCs for a number of metabolic pathways. In the PCM pathway (Dosa00860), there are 27 dark response DEGs and 18 are LCC genes. Fifteen of these are both LCC genes and dark-response DEGs: *OsNYC1*, *OsGGR2*, *OsCRD1*, *OsHY2*, *OsLYL1*, *OsDVR*, *OsChlI*, *OsChlD*, *OsPORA*, *OsSGRL*, *OsYGL1*, *OsYGL18*, *OsSGR*, *OsRCCR1* and *OsPGL*. In other words, the number of LCC genes overlapped with dark-response DEGs normalized to the total number of dark-response DEGs is 55.6% (15/27) and it is 83.3% when normalized to total number of LCC genes (15/18), which provides strong evidence supporting a connection between PCM and both dark response and LCC. Taken together, integrated analysis provided a tool for specific examination of genome-wide transcriptome data within the context of metabolic pathways established using traditional forward genetics data. The complementary sets of data mutually support one another.

The chlorophyll biosynthesis process in the PCM pathway plays important roles in rice leaf color control

To further examine the function of LCC genes and DEGs in PCM, a flow chart was drawn, on the basis of the KEGG data, to highlight the position and distribution of the 15 LCC dark-response DEGs (Fig. 8). Fig. 8 and Additional file: Table S8 shows that the LCC overlapped dark-response DEGs were neither evenly nor randomly distributed throughout the metabolic pathway. Instead, almost all of them were concentrated to chlorophyll biosynthesis, which is conserved in higher plants. Thus, it can be postulated that this process is the key part of the PCM pathway responsible for both LCC and dark response, and additional genes that participate in this process are potential LCC genes.

Discussion

To understand the effect of light on rice growth, RNA-Seq analysis was carried out for rice leaves treated with constant dark, and DEG-enriched KEGG pathways with high rich factors were identified. Meanwhile, a collection of rice LCC genes identified and cloned by traditional genetics were analyzed for mutant-enriched pathways with KEGG. It was found that 102 of 150 LCCs (68.0%) were DEGs under CD treatment, suggesting an overlap between dark response and leaf color control networks. An integrated analysis of the two sets of data found that 83.3% of the LCCs in PCM pathways were also dark-response DEGs. More importantly, most of the LCC genes participate in chlorophyll synthesis, which suggests that chlorophyll synthesis, as a central part of the PCM pathway, plays an important role in both leaf color control and dark response.

The mechanisms of dark response in rice

Darkness is a severe stress. Rice leaves become yellow and unhealthy. Results obtained in this study and reported literature have shown that chlorophyll a, chlorophyll b and carotenoid contents are significantly

reduced under CD treatment.

RNA-Seq analysis of dark-treated leaves revealed down-regulation of several genes involved in chlorophyll and carotenoid synthesis, including *OsChlI*, *OsChlH* and *OsYGL1*. Their transcript abundances decreased 13.3-, 5.5- and 5.30-fold, respectively. It has been reported that *OsChlI* and *OsChlH* encode the CHLI and CHLH subunits of Mg^{2+} -protoporphyrin IX chelatase (Mg^{2+} -chelatase) (Zhang et al., 2006a; Inagaki et al., 2015), and *OsYGL1* encodes chlorophyll synthase (Wu et al., 2007). These are key enzymes for chlorophyll synthesis and leaf color control. In addition, the expression of β -*OsLCY*, encoding lycopene β -cyclase, decreased by 2.4-fold. The expression of *OsPDS*, encoding phytoene desaturase, decreased by 3.5-fold, and the expression of *OsZDS*, encoding ζ -carotene desaturase, decreased by 2.9-fold. These three genes have been identified as key enzymes in carotenoid synthesis, with β -*OsLCY* also being a known LCC gene (Fang et al., 2008).

CD-enriched DEGs in photosynthesis-related pathways were detected by RNA-Seq analysis in this study. Four of the 10 most highly DEG-enriched metabolic pathways in dark response are associated with photosynthesis. For the photosynthesis-antenna proteins pathway, all 13 DEGs were down-regulated, including *OsLhca4*, which was down-regulated approximately 217-fold. It has been reported that this gene encodes a light-harvesting complex I (LHCI) subunit, and it is also an LCC gene (Yamatani et al., 2018). In PCM, 21 out of the 27 DEGs were down-regulated under dark treatment, among which, LOC_Os10g28370 was down-regulated 69-fold. In glyoxylate and dicarboxylate metabolism, *OsRBCS4*, which encodes a small subunit of rubisco, was down-regulated 3001-fold and is also an LCC gene (OGAWA et al., 2012). In the photosynthesis pathway, 30 out of 33 DEGs were down-regulated, among which, LOC_Os12g10570 was down-regulated 90-fold. RNA-Seq analysis has previously been carried out for different light treatments (white, red, blue and green). It was found that photosynthesis-related genes were significantly down-regulated and carbohydrate degradation was pronounced in darkness (Lakshmanan et al., 2015), consistent with the results obtained in this study.

The mechanisms of leaf color control

Leaf color is an important agronomic trait that is directly related to rice growth and grain yield. In this study, data on 150 LCC genes were consolidated from the literature. According to LCC gene transcription data, most LCC genes were down-regulated under constant dark treatment, which demonstrates that light signals can play positive roles in regulating LCC gene expression. Light is the upstream initiator of signal transduction in plants. PIFs (phytochrome interacting factors) are a class of bHLH transcription factors that can interact with phytochrome (Phy). Light signals regulate PIFs protein stability; that is, the protein is stable in the dark and degrades in the light (Demarsy et al., 2017). In rice, six PIF genes have been identified and designated as OsPIL11 to OsPIL16 (Nakamura et al., 2007; Piao et al., 2015). It was reported that OsPIL13 is an LCC gene, with expression controlled by circadian rhythms (Nakamura et al., 2007). *OsPIL13* binds to the promoters of two Chl biosynthetic genes, *OsPORB* and *OsCAO1*, and induces the transcription of downstream genes (Sakuraba et al., 2017). *OsPIL15* is responsible for regulating rice tiller angle in response to light and gravity, and *OsPIL15* expression is negatively regulated in etiolated

seedlings exposed to light (Xie et al., 2019). In this study, up-regulated expression for *OsPIL 11*, *OsPIL 13* and *OsPIL 16* was detected, and bHLH TFs were found to be CD-response DEGs at a higher percentage than any other TF family, suggesting that bHLH TFs play important roles to propagate light signal transduction in rice.

Darkness can lead to extensive stress responses, including loss of green leaf color. Direct correlations between light and leaf color mutation have been reported. For example, *OsLYL 1*, encoding a geranylgeranyl reductase, was induced by light and suppressed by dark (Zhou et al., 2013b). *OsYGL 18* encodes a putative magnesium protoporphyrin IX methyltransferase (ChlM). When an *OsYGL 18* deletion mutant (*ygl18*) was transferred from dark to light, chlorophyll content increased and its expression was up-regulated (Wang et al., 2017c). *OsZN* encodes a thylakoid-bound protein of unknown function, and its mRNA level in constant light is higher than that in CD, indicating that *OsZN* transcription is controlled by light (Li et al., 2010). In addition, an *OsOTP51* mutant, encoding a pentatricopeptide repeat protein, showed dramatic changes in PSI structure and function, which led to severe photoinhibition (Ye et al., 2012). In this study, it was found that expression of these genes was down-regulated after CD treatment, which suggests that light signals positively regulate LCC genes.

Although more than 100 LCC genes have been cloned by traditional genetic methods, it has been difficult to assess the role of specific LCC genes in the context of metabolic pathways. In this study, LCC genes were surveyed with KEGG metabolic pathway analysis. LCC gene-enriched pathways were identified by calculating the mutant rich factor (mRF). It was found that the mRF was highest for PCM, which suggested that PCM plays an important role in leaf color control, and additional genes from the PCM pathway may be LCC genes.

The feasibility and importance of integrated analysis of transcriptome and genetic data

Genome-wide transcriptome analysis can detect stress-related DEGs, and KEGG enrichment analysis can further identify DEG-enriched metabolic pathways. However, clues obtained by transcriptomic analysis require verification from genetic analyses and functional studies. Traditional forward genetics approaches can efficiently identify genes related to specific mutant phenotypes, but it is difficult to integrate a group of mutant-related genes into specific metabolic pathways. In this circumstance, it was possible to obtain mutual supporting, complementary evidence through integration of two sets of data. The integrated analysis can get focused view from transcriptomic data and a magnified view from genetic data.

In this study, RNA-Seq analysis of CD-treated rice seedlings was carried out and DEGs were identified. Data on 150 LCC genes previously cloned by traditional genetic means were collected, and 102 LCC genes were found to be dark-response DEGs, which suggests an overlap between dark response and leaf color control networks. Furthermore, KEGG analysis of LCC genes showed that the mRF of LCC genes was the highest for PCM, and most PCM LCC genes (83.3%) were dark-response DEGs. Meanwhile, the rich factor for PCM was among the highest revealed by RNA-Seq analysis of dark stress. Thus, it can be concluded that PCM plays an important role not only in dark response but also in leaf color control. RNA-

Seq data provided understanding of leaf color mutations, and traditional genetic research provided complementary data towards clarifying the mechanism of dark-stress response.

Genome-wide analysis is aimed at investigating all genes. When facing different biological questions in the same organism, data from different sources can be integrated for coordinated analysis. With the rapid accumulation of transcriptome data, which can be combined with genetic data that has been collected over many years, integrated analysis can now be carried out to a greater extent, which will contribute to improved understanding of rice biology.

Dissection of the PCM pathway

DEGs can be allocated to specific metabolic pathways via KEGG analysis; however, a metabolic pathway may contain dozens or even hundreds of genes that are connected with multiple interrelated processes. Analysis of the location and distribution of DEGs is helpful for making functional associations with specific processes. In other words, more accurate and precise results can be obtained if metabolic pathways are dissected. In this study, DEGs derived from whole-genome transcriptomic analysis and LCC genes collected from traditional genetics literature were associated with the PCM pathway. Further, the DEGs and LCC genes were neither randomly nor evenly distributed throughout the pathway. Instead, they were concentrated in the chlorophyll biosynthesis part of the pathway (Nagata et al., 2005). On this basis, it can be postulated that chlorophyll biosynthesis is the key process for leaf color regulation. Analyzing dissected metabolic pathways is important because it helps narrow the range of target genes and leads to comprehensive understanding of gene functions.

Conclusions

In this study, transcriptomic analysis using an RNA-Seq approach was carried out for yellow leaves of dark-treated rice seedlings. DEGs and DEG-enriched metabolic pathways were analyzed. Data for reported LCC genes were collected. Metabolic pathways that included both LCCs and dark-response DEGs were identified. It was found that the transcript abundance of most LCC genes changed under dark treatment, suggesting an overlap between leaf color control and dark response. KEGG analysis revealed enrichment of LCC genes in porphyrin and chlorophyll metabolism (PCM). Interestingly, all of the overlapped LCC genes and DEGs were concentrated at chlorophyll biosynthesis in the central of PCM, indicating that PCM pathway, particularly chlorophyll biosynthesis process, plays important roles in rice LCC and dark stress-response. This study provides important clues for understanding mechanisms of dark response and leaf color control and identifying additional LCC genes.

Supplementary Information

The datasets supporting the conclusions of this article are included within the article and its additional file.

Additional file: Table S1. Transcriptional data for rice under constant dark stress. **Table S2.** DEG-enriched metabolic pathway analysis by KEGG. **Table S3.** List of metabolic pathways containing dark-response DEGs. **Table S4.** List of TFs among dark-response DEGs. **Table S5.** Transcript abundance of TFs that are dark-response DEGs. **Table S6.** List of rice leaf color control genes. **Table S7.** KEGG analysis of rice leaf color control genes. **Table S8.** Integrated analysis of dark response and leaf color control networks.

Abbreviations

CD: constant dark; Chl: chlorophyll; DEG: differentially expressed gene; GMP: genes in a specific metabolic pathway; KEGG: Kyoto encyclopedia of genes and genomes; LCC: leaf color control; mRF: mutant rich factor; PCM: porphyrin and chlorophyll metabolism; Phy: phytochrome; PIF: phytochrome interacting factor; PIL: rice phytochrome-interacting factor-like; RF: rich factor.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

No applicable.

Availability of data and material

All data generated or analyzed during this study are included in this published article or its supplementary information files or are available from the corresponding authors on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

LG, LL, DS and YM designed the experiments; WT, CY, ZZ, LY, YG, XS and XH performed the experiments; WT, CY and ZZ analyzed the data; LG, LL, CY and WT wrote and edited the manuscript.

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Tables

Table 1. Transcription factors identified among dark-response DEGs.

TF_Family	Up-regulated		Down-regulated	
	3d	6d	3d	6d
bHLH	39	26	8	10
MYB	21	16	13	9
C2H2	19	18	8	5
bZIP	18	18	9	9
ERF	14	23	12	9
NAC	14	20	13	11
WRKY	12	19	19	10
HD-ZIP	10	5	2	6
GRAS	9	5	3	4
MYB_related	9	6	9	6
Dof	8	3	5	3
G2-like	8	5	7	4
HSF	8	9	3	1
TCP	8	1		
B3	7	4	2	3
ZF-HD	7	3	1	2
LBD	6	9	1	2
SBP	6	2	1	2
TALE	6	2	5	5
CO-like	5	4	7	3

Transcription factors were identified with PlantTFDB v5.0 (planttfdb.cbi.pku.edu.cn). The list shows the number of TF genes identified as DEGs after 3 d and 6 d of dark treatment for 20 TF families. Data for other TF families are listed in additional file: Table S4 and S5.

Table 2. List of rice leaf color control genes.

Gene Name	Reference	Gene Name	Reference	Gene Name	Reference
<i>OsABC1-2</i>	(Gao et al., 2012)	<i>OsHY2</i>	(Yoshitake et al., 2015)	<i>OsSHMT1</i>	(Wu et al., 2015)
<i>OsAK1</i>	(Wei et al., 2017)	<i>OsHYR</i>	(Ambavaram et al., 2011)	<i>OsSIG2A</i>	(Yu et al., 2019)
<i>OsAL1</i>	(Zhang et al., 2016b)	<i>OsIspE</i>	(Chen et al., 2018)	<i>OsSLL1</i>	(Zhang et al., 2009)
<i>OsAL2</i>	(Liu et al., 2016a)	<i>OsIspF</i>	(Huang et al., 2017)	<i>OsSPP</i>	(Yue et al., 2010)
<i>OsALD-Y</i>	(Zhang et al., 2016a)	<i>OsKS2</i>	(Ji et al., 2013)	<i>OsSRP43</i>	(Lv et al., 2015)
<i>OsALS3</i>	(Lin et al., 2015a)	<i>OsLAS</i>	(Zhang et al., 2017a)	<i>OsSRP54</i>	(Zhang et al., 2013a)
<i>OsAM1</i>	(Sheng et al., 2014)	<i>OsLC7</i>	(Chen et al., 2016b)	<i>OsSWL1</i>	(Tsugane et al., 2014)
<i>OsAPX2</i>	(Zhang et al., 2013b)	<i>OsLCY</i>	(Fang et al., 2008)	<i>OsTCD10</i>	(Wu et al., 2016b)
<i>OsARVL4</i>	(Wang et al., 2016c)	<i>OsLhca4</i>	(Yamatani et al., 2018)	<i>OsTCD11</i>	(Wang et al., 2017a)
<i>OsASL1</i>	(Gong et al., 2013)	<i>OsLMM24</i>	(Zhang et al., 2019)	<i>OsTCD5</i>	(Wang et al., 2016f)
<i>OsASL2</i>	(Lin et al., 2015b)	<i>OsLS1</i>	(Qiu et al., 2019)	<i>OsTCM1</i>	(Lin et al., 2018b)
<i>OsBE1</i>	(Wang et al., 2014)	<i>OsLYL1</i>	(Zhou et al., 2013b)	<i>OsTCM5</i>	(Zheng et al., 2016)
<i>OsBGL11(t)</i>	(Wang et al., 2013)	<i>OsMADS26</i>	(Lee et al., 2008)	<i>OsTDC3</i>	(Kanjaphachao et al., 2012)
<i>OsBT1-3</i>	(Hu et al., 2017)	<i>OsMPR25</i>	(Toda et al., 2012)	<i>OsTLP27</i>	(Kang et al., 2015)
<i>OsCAO1</i>	(Lee et al., 2005)	<i>OsMTS1</i>	(Hong et al., 2018)	<i>OsTRXM</i>	(Chi et al., 2008)
<i>OsCDC48</i>	(Huang et al., 2016)	<i>OsMYC2</i>	(Giri et al., 2017)	<i>OsTSC1</i>	(Shi et al., 2018)
<i>OsCGA1</i>	(Hudson et al., 2013)	<i>OsNAP</i>	(Liang et al., 2014)	<i>OsTSCD1</i>	(Liu et al., 2015)
<i>OsChlD</i>	(Zhang et al., 2006a)	<i>OsNaPRT1</i>	(Wu et al., 2016a)	<i>OsTSV3</i>	(Lin et al., 2018a)
<i>OsChlH</i>	(Inagaki et al., 2015)	<i>OsNOA1</i>	(Yang et al., 2011)	<i>OsV1</i>	(Kusumi et al., 2011)
<i>OsChlI</i>	(Zhang et al., 2006a)	<i>OsNOL</i>	(Kusaba et al., 2007)	<i>OsV2</i>	(Kusumi and Iba, 2014)
<i>OsClpP5</i>	(Tsugane et al., 2006)	<i>OsNTRC</i>	(Perez-Ruiz et al., 2006)	<i>OsV3</i>	(Yoo et al., 2009)
<i>OsCpn60α</i>	(Jiang et al., 2014b)	<i>OsNYC1</i>	(Kusaba et al., 2007)	<i>OsV4</i>	(Gong et al., 2014)
<i>OsCpn60α1</i>	(Kim et al., 2014)	<i>OsNYC3</i>	(Morita et al., 2009)	<i>OsV5A</i>	(Zhou et al., 2013a)
<i>OsCRD1</i>	(Wang et al., 2017b)	<i>OsONAC106</i>	(Sakuraba et al., 2015)	<i>OsV5B</i>	(Liu et al., 2016b)
<i>OsCRL6</i>	(Wang et al., 2016e)	<i>OSOTP51</i>	(Ye et al., 2012)	<i>OsVAL1</i>	(Zhang et al., 2018b)
<i>OsCYO1</i>	(Tominaga et al., 2016)	<i>OsPAPST1</i>	(Xu et al., 2013)	<i>OsVYL</i>	(Dong et al., 2013)
<i>OsDG2</i>	(Jiang et al., 2014a)	<i>OsPDF1B</i>	(Moon et al., 2008)	<i>OsW01S</i>	(Li et al., 2018b)
<i>OsDOS</i>	(Kong et al., 2006)	<i>OsPGL12</i>	(Chen et al., 2019)	<i>OsWGL2</i>	(Qiu et al., 2018a)
<i>OsDVR</i>	(Wang et al., 2010)	<i>OsPHR2</i>	(Guo et al., 2015)	<i>OsWLP1</i>	(Song et al., 2014)
<i>OsEF8</i>	(Feng et al., 2014)	<i>OsPhyB</i>	(Piao et al., 2015)	<i>OsWLP2</i>	(Lv et al., 2017)
<i>Osetl1</i>	(Mao et al., 2011)	<i>OsPIL1</i>	(Sakuraba et al., 2017)	<i>OsWLS5</i>	(Zhao et al., 2019)
<i>Osetl2</i>	(Mao et al., 2011)	<i>OsPL</i>	(Akhter et al., 2019)	<i>OsWP1</i>	(Wang et al., 2016d)
<i>OsFBP1</i>	(Koumoto et al., 2013)	<i>OsPLS2</i>	(Wang et al., 2018)	<i>OsWP3</i>	(Li et al., 2018a)
<i>OsFDC2</i>	(Li et al., 2015)	<i>OsPORA</i>	(Sakuraba et al., 2013)	<i>OsWRKY53</i>	(Hu et al., 2015)
<i>OsFLA</i>	(Ma et al., 2019)	<i>OsPORB</i>	(Sakuraba et al., 2013)	<i>OsWSL</i>	(Tan et al., 2014)
<i>OsFLN2</i>	(Qiu et al., 2018b)	<i>OsPPR1</i>	(Gothandam et al., 2005)	<i>OsWSL12</i>	(Ye et al., 2016)
<i>OsFRDL1</i>	(Yokosho et al., 2009)	<i>OsPPR6</i>	(Tang et al., 2017)	<i>OsWSL3</i>	(Wang et al., 2016b)
<i>OsGATA12</i>	(Lu et al., 2017)	<i>OsPS1-F</i>	(Ramamoorthy et al., 2018)	<i>OsWSP1</i>	(Zhang et al., 2017b)
<i>OsGF14e</i>	(Manosalva et al., 2011)	<i>OsPAC2</i>	(Wang et al., 2016a)	<i>OsYGL1</i>	(Wu et al., 2007)
<i>OsGGR2</i>	(Kimura et al., 2018)	<i>OsRAI</i>	(Zheng et al., 2019)	<i>OsYGL18</i>	(Wang et al., 2017c)
<i>OsGIC</i>	(Kamau et al., 2015)	<i>OsRCCR1</i>	(Tang et al., 2011)	<i>OsYGL8</i>	(Zhu et al., 2016)
<i>OsGLK1</i>	(Nakamura et al., 2009)	<i>OsREL2</i>	(Yang et al., 2016a)	<i>OsYLD1</i>	(Deng et al., 2017)
<i>OsGluRS</i>	(Liu et al., 2007)	<i>OsRLIN1</i>	(Sun et al., 2011)	<i>OsYS83</i>	(Chen et al., 2016a)
<i>OsGRA(t)</i>	(Chen et al., 2007)	<i>OsRLS1</i>	(Jiao et al., 2012)	<i>OsYSA</i>	(Su et al., 2012)
<i>OsGRA78</i>	(Wang et al., 2019)	<i>OsRNRS1</i>	(Chen et al., 2015)	<i>OsYSL13</i>	(Zhang et al., 2018a)
<i>OsGRY79</i>	(Wan et al., 2015)	<i>OsRopGEF10</i>	(Yoo et al., 2011)	<i>OsYSL16</i>	(Zheng et al., 2012)
<i>OsHAD1</i>	(Liu et al., 2018)	<i>OsS6K</i>	(Sun et al., 2016)	<i>OsYSS1</i>	(Zhou et al., 2017)
<i>OsHAP3A</i>	(Miyoshi et al., 2003)	<i>OsSGL1</i>	(Hitoshi et al., 2012)	<i>OsZ2</i>	(Han et al., 2012)
<i>OsHO1</i>	(Chen et al., 2013)	<i>OsSGR</i>	(Jiang et al., 2007)	<i>OsZ3</i>	(Kim et al., 2018)
<i>OsHO2</i>	(Li et al., 2014)	<i>OsSGRL</i>	(Rong et al., 2013)	<i>OsZN</i>	(Li et al., 2010)

Detailed information is listed in additional file: Table S6.

Figures

Figure 1

Phenotype of rice seedlings under constant darkness treatment. Rice seedlings grown for 5 days under normal light condition were treated with constant dark. Photographs and measurements of plant height, as well as chlorophyll and carotenoid content, were collected at 0, 3 and 6 d. (A) Photographs of rice seedlings cultivated at constant dark for 0, 3d and 6d. (B) Plant height of rice seedling under dark treatment. (C) Chlorophyll a content in rice leaves under dark treatment. (D) Chlorophyll b content in rice

leaves under dark treatment. (E) Carotenoids content in rice leaves under dark treatment. CK: Control; CD: Constant dark. 3d: Constant dark treatment for 3 days. 6d: Constant dark treatment for 6 days. Results were deemed significant at $P < 0.01$. Column heights and bars represent the mean and standard deviation of triplicates.

Figure 2

Comparative analysis of differentially expressed genes (DEGs) in rice seedlings in response to dark treatment. (A) Number of up-regulated and down-regulated DEGs under constant dark for 3 and 6 days. (B) Venn diagram for DEGs under constant dark for 3 and 6 days.

Figure 3

DEG-enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of dark treatment. (A) CD_3d vs CK. (B) CD_6d vs CK. CD_3d and CD_6d refer to 3 and 6 days after constant dark treatment.

Figure 4

Rich factors for DEG-enriched metabolic pathways identified by KEGG analysis of dark-treated rice. Twenty pathways with highest rich factor value were listed. More pathways were listed in additional file: Table S3.

Figure 5

The expression profile of LCC genes that are dark-response DEGs. 3d and 6d refer to 3 and 6 days under constant dark treatment, respectively. Red and green colors depict up- and down-regulation, respectively. The scale shows log₂ (fold change) values.

Figure 6

Mutant rich factors (mRF) for metabolic pathways associated with rice leaf color control genes. #LCC: number of leaf color control genes in a specific metabolic pathway; #GMP: Total number of genes in a specific metabolic pathway. mRF: #LCC/#GMP. The 20 metabolic pathways with the highest mRF values are listed here. Additional pathways are listed in additional file: Table S7.

Figure 7

Integrated analysis of dark response and leaf color control. Gene names in red (italic) are LCC genes that were also dark-response DEGs. Dosa# indicated the number of pathways in KEGG database. The percentages at the bottom represent the number of LCC genes overlapped with dark-response DEGs normalized to the total number of dark-response DEGs. Ten pathways are listed here, and detailed information for additional pathways is listed in additional file: Table S8.

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- [SupplementaryTableL2.xlsx](#)