Light Harvesting-like Protein 3 Interacts with Phytoene Synthase and Is Necessary for Carotenoid and Chlorophyll Biosynthesis in Rice

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Abstract

**Background:** Carotenoid biosynthesis is essential for the generation of photosynthetic pigments, phytohormone production, and flower color development. The light harvesting like 3 (LIL3) protein, which belongs to the light-harvesting complex protein family in photosystems, interacts with geranylgeranyl reductase (GGR) and protochlorophyllide oxidoreductase (POR) both of which are known to regulate terpenoid and chlorophyll biosynthesis, respectively, in both rice and Arabidopsis.

**Results:** In our study, a CRISPR-Cas9 generated 4-bp deletion mutant osli3 showed aberrant chloroplast development, growth defects, low fertility rates and reduced pigment contents. A comparative transcriptomic analysis of osli3 suggested that differentially expressed genes (DEGs) involved in photosynthesis, cell wall modification, primary and secondary metabolism are differentially regulated in the mutant. Protein-protein interaction assays indicated that LIL3 interacts with phytoene synthase (PSY) and in addition the gene expression of PSY genes are regulated by LIL3. Subcellular localization of LIL3 and PSY suggested that both are thylakoid membrane anchored proteins in the chloroplast. We suggest that LIL3 directly interacts with PSY to regulate carotenoid biosynthesis.

**Conclusion:** This study reveals a new role of LIL3 in regulating pigment biosynthesis through interaction with the rate limiting enzyme PSY in carotenoid biosynthesis in rice presenting it as a putative target for genetic manipulation of pigment biosynthesis pathways in crop plants.

**Keywords:** Carotenoid, Light-harvesting complex, Phytoene synthase, Transcriptome, Cell-wall, Protein-protein interaction

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Introduction

Light harvesting complex (LHC) proteins constitute major light harvesting antenna of photosynthetic eukaryotes. LHC proteins contain a representative LHC motif consisting of 25–30 amino acids (mostly hydrophobic). The LHC motif is also present in a number of transmembrane proteins such as light harvesting like (LIL) proteins (Takahashi et al. 2014). There are multiple LIL proteins in both photosynthetic eukaryotes and cyanobacteria. For example, there are five different types of LIL proteins in the model cyanobacterium *Synechocystis* sp. PCC6803 while there are at least eight different types of LIL proteins in *Arabidopsis* (Jansson 1999; Funk and Vermaas 1999; Liu et al. 2004). Among these LIL proteins, ferrochelatase (FC) and LIL3 have been functionally characterized. FC is responsible for the final step of Fe$^{2+}$ insertion into protoporphyrin during hemoglobin biosynthesis (Funk and Vermaas 1999). LIL3 is involved in the stabilization of geranylgeranyl reductase (GGR), that is a key enzyme for phytyl pyrophosphate (Phytyl PP) formation (Tanaka et al. 2010).

In *Arabidopsis*, there are two LIL3 isoforms, namely AtLIL3.1 and AtLIL3.2, both of which share high sequence similarity (73% identity) with each other at the amino acid level. Both AtLIL3.1 and AtLIL3.2 not only regulate the activity of the photosynthetic pigment biosynthesis but also bind to protochlorophyllides, that are substrates of POR enzymes (Hey et al. 2017; Mark-Jansson and Eichacker 2018). Arabidopsis LIL3 also interacts with POR and GGR, thus modulating the levels of two metabolites, chlorophyllide and phytyl pyrophosphate, that are biosynthesized by POR and GGR, respectively. These two metabolites are essential for chlorophyll and tocopherol synthesis in *Arabidopsis* (Hey et al. 2017). In rice, there is only one LIL3 ortholog and bi-molecular fluorescent complementation (BiFC) assay confirmed the interaction between LIL3 and GGR, similar to the observation in *Arabidopsis* (Zhou et al. 2017). OsLIL3 was also reported to interact with geranylgeranyl diphosphate synthase (OsGGPPS1) but not with OsGRP (OsGGPPS-recruiting protein). OsGRP directs OsGGPPS1 to form a large multiprotein complex in the thylakoid membrane consisting of OsGGR (geranylgeranyl reductase), OsGRP, OsCHLG (chlorophyll synthase) and OsPORB (protochlorophyllide oxidoreductase) (Zhou et al. 2017).

In rice, many leaf color mutants have been extensively studied providing insights into chlorophyll biosynthesis, chloroplast development, tetrapyrole biosynthesis and photosynthesis (Nagata et al. 2005; Adhikari et al. 2011; Deng et al. 2014). One prominent phenotype of leaf color mutants is chlorotic lesion.

Apart from being deficient in photosynthetic pigments due to the disruption of pigment biosynthesis enzyme coding gene, the mutant phenotype can be a result of various stress conditions such nitrate and iron deficiency that affects pigment production (Shao et al. 2020; Shim et al. 2020). Leaf chlorosis is commonly related to chlorophyll biosynthesis and degradation pathways. Thus, deficiency in any step leading to chlorophyll synthesis can possibly result in a reduced chlorophyll content and thereby changes in leaf color. The LIL3 protein associates with the GGR and POR enzymes in both rice and Arabidopsis. These enzymes are involved in the generation of phytylated GGPP, a metabolite essential for both terpenoid and chlorophyll biosynthesis (Zhou et al. 2017; Hey et al. 2017). Thus, LIL3 acts as a common player in both chlorophyll and carotenoid biosynthesis.

In this study, we found that *osil3* mutant has a chlorotic phenotype and aberrant chloroplast development at seedling stages. Next, analysis of *osil3* mutant found that DEGs involved in photosynthesis, cell wall and metabolism were differentially regulated in this mutant and protein-protein interaction assays found that LIL3 interacts with PSY enzymes and this interaction possibly affect PSY gene expression, revealing a novel regulatory mechanism of LIL3 in the carotenoid biosynthesis. Interestingly, LIL3 co-localized with PSY in the thylakoid membrane complex. LIL3 was also found to regulate the transcript levels of other LIL3-interacting protein genes including OR, POR, and GGR as well as the protein abundance of POR and GGR enzymes that were repressed in the *osil3* mutant. This shows the dramatic effect of *LIL3* mutation on plant pigment pathways and its possible use in molecular biotechnology for crop pigment level manipulation.

Results

**OsLIL3 CRISPR-Cas9 Mutant Displays Leaf Chlorosis and Developmental Defects**

CRISPR-Cas9 was used for generation of a rice *osil3* mutant, taking advantage of gene editing protocol (Shan et al. 2014). The single guide RNA, sgRNA 5'-GGCGCCTCGA CAGTCTCCACGGG −3’ was designed for positional targeting of *OsLIL3*. A total of eight independent transgenic lines were obtained and out of these, one CRISPR-Cas9 generated mutant line showed stable homozygous *osil3* mutation and would be hereby referred to as *osil3* (16-#1) or *osil3* (Supplementary data S4). The T2 generation *osil3* (16-#1) homozygous plants displayed chlorotic phenotype at normal growth temperatures (28°C), more prominently at the early seedling stages (Fig. 1a) but this phenotype was less visible at later (adult) stages (Fig. 1b). Therefore, this line was used for all the downstream experiments.
Fig. 1 (See legend on next page.)
Sequencing revealed that *oslil3* mutation in this line was caused by the deletion of four base pairs from the first exon of *OsLIL3* (Fig. 1c), leading to a frameshift in translation. Sequence alignment with WT sequence showed that a deletion of four bases (positions 158–161) in the *OsLIL3* coding sequence (Figure S1-a), resulted in premature translational termination (Figure S1-b). We also examined the top five ranked off-target sites predicted by CRISPR-P (Liu et al. 2017), and off-target mutations were located only to introns or CDS of transposon genes (Supplementary data S1).

Interestingly, the pigment content of *oslil3* was greatly reduced. The mutant showed reduction of 80% of total chlorophylls and 60% of carotenoids, in comparison to the WT plants (Fig. 1e). TEM examination of ultrastructure of third leaf (L3) chloroplasts in *oslil3* and wild-type 3-leaf seedlings grown at 28 °C showed highly disorganized thylakoid lamellar structures in *oslil3* chloroplasts and without grana formation (Fig. 1f).

To account for the pigment reduction in *oslil3*, the effect of OsLIL3 on the regulation of proteins involved in carotenoid biosynthesis (*OsGGR* and *OsPORB*, previously reported interactors of OsLIL3) was investigated. Firstly, the peptide “AMIGFFMATVDLSL” (172–185 amino acids of OsLIL3 protein sequence, used for generating LIL3 antibody) was used for analyzing protein expression in *oslil3* vs WT. The results indicated absence of OsLIL3 in the mutant (Fig. 1d). The immunoblot analysis of OsLIL3-interacting proteins suggested that OsPORB and OsGGR protein levels were also drastically reduced in *oslil3* vs WT at the 3-leaf stage seedlings (Fig. 1d), consistent with the previous findings (Tanaka et al. 2010; Ikey et al. 2017).

To analyze the growth and yield performance of *oslil3* (16-#1), plants grown for 90 days were harvested and different growth parameters were measured. Upon maturity, the *oslil3* plants displayed significant differences in various quantified morphological changes in comparison to WT (Table 1), including reductions in plant height, culm length, ear number, tiller number and fertility rate in the *oslil3* mutant. Overall, *oslil3* was dwarf and yellowish with low fertility, with the chlorotic phenotype being more prominent in the early seedling stages.

| Trait                  | WT             | osilil3 16-#1  |
|------------------------|----------------|---------------|
| Plant height (cm)
| 91.6 ± 6.8          | 64 ± 4.9**    |
| Culm length (cm)
| 53.6 ± 5.5          | 39.3 ± 5.2**  |
| Ear length (cm)
| 18.5 ± 1.3          | 15.8 ± 1.2**  |
| Tiller number
| 8.2 ± 1.9           | 1.2 ± 0.4**   |
| Ear number
| 7.2 ± 2.3           | 1.8 ± 1.1*    |
| Fertility rate (%)    | 86.4 ± 3.5     | 25.3 ± 0.3**  |

*Length from the cotyledonary node to the tip of the top leaf

*Length from the cotyledonary node to the panicle base. *, indicates the *P* < 0.05; **, indicates the *P* < 0.01, Student t-test, *n* = 6 independent plants, values are means ±SD
thylakoid lumen, and anchored component of plasma membrane were the topmost enriched terms.

For specific metabolic pathways, top 20 KEGG pathways involving most number of DEGs are listed (Figure S4). DEGs were mostly enriched in terms such as "plant-pathogen interaction", "phenylpropanoid biosynthesis", "plant hormone signal transduction", "amino sugar and nucleotide sugar metabolism", "biosynthesis of amino acids" among others. Interestingly, the KEGG pathway term "plant-pathogen interaction" contained the greatest number of DEGs (listed in Supplementary data S5). In total, there are 19 genes in this category, including those
encoding calcium-dependent protein kinase (CPK), respiratory burst oxidase (RBOH), calcium-binding protein (CML), nitrite oxide synthase (NAO1), WRKY transcriptional factors (WRKY22 & WRKY33), pathogen related protein 1 (PR1), LRR receptor like kinase FLS2 (FLS2), LRR receptor like kinase EFR (EFR), pto-interacting protein 1 (PII1), disease resistance protein 3 (RPS3), RPS2, RPS5, chitin elicitor-binding protein (CEBiP) and receptor kinase-like protein (XA21).

We mapped the DEGs in our transcriptome analysis to different functional categories in the MapMan tool to visualize the pathways mostly affected by OsLIL3 deficiency. Metabolic pathways in cell wall biosynthesis, light reactions, lipid metabolism, secondary metabolism, amino acid metabolism, tetrapyrroloides biosynthesis, and nucleotide metabolism were mostly changed (Fig. 2b). Specifically, most of the light reaction genes are up-regulated and four genes involved in light reactions are up-regulated and four novel interactors among the enzymes of chlorophyll and carotenoid pathways that are also perturbed in the oslil3 transcriptome. Therefore, Y2H assays were carried out to predict interactions between OsLIL3 and proteins in carotenoid biosynthesis pathways whose genes were identified as being differentially expressed in oslil3. Results suggested that the enzyme phytoene synthase 2 (PSY2, Os12g43130) interacts with OsLIL3 in yeast (Fig. 4a). A co-immunoprecipitation assay in tobacco leaves confirmed an interaction between OsLIL3 and OsPSY2 in vivo (Fig. 4b). An in planta BiFC assay revealed strong yellow fluorescence signals with co-expression of OsLIL3-YFPN and OsPSY2-YFPC constructs (Fig. 4c, top panel). No yellow fluorescent signals were detected in empty YFPN vector + OsPSY-YFPC or in OsLIL3-YFPN + empty YFPC vector controls. There are two other ortholog genes in addition to OsPSY2 (OsPSY1 and OsPSY3), and both of them share 74% identity to OsPSY2 at amino acid levels. To investigate whether OsPSY1 and OsPSY3 can interact with OsLIL3, above methods (Y2H, Co-IP and BiFC) were employed. The data suggests that OsLIL3 can also interact with OsPSY1 and OsPSY3 (Figure S7). In addition, interaction of OsLIL3 with other pigmet metabolism proteins (OsLUT1, OsNCED2, OsCHLI and OsCPOX) was tested but no interactions were detected for these protein pairs in the Y2H assay (Figure S8). Taken together, these results indicated that OsLIL3 interacts physically with all three OsPSYs (inside chloroplasts in planta and also in yeast), suggesting that OsLIL3 may regulate
Fig. 3 (See legend on next page.)
carotenoid biosynthesis via its interaction with OsPSY enzymes.

OsLIL3 and OsPSY2 Co-Localize to Thylakoid Membranes of Chloroplasts

Confocal microscope-based visualization of *N. benthamiana* leaves infiltrated with p35S-OsLIL3-EYFP and p35S-OsPSY2-EYFP revealed the localization of yellow fluorescence signals for both constructs to the chloroplasts of mesophyll cells (Fig. 5a).

Further immunoblot analysis using different subfractions prepared from Percoll-purified chloroplasts extracted from above infiltrated leaves revealed presence of both OsLIL3-EYFP and OsPSY2-EYFP in the thylakoid membranes, but not in the stromal fraction (Fig. 5b).

OsLIL3 Induces Gene Expression of its Interaction Partners Involved in Carotenoid and Chlorophyll Biosynthesis

To further analyze the gene expression of enzymes involved in carotenoid and chlorophyll biosynthesis, 15 candidate genes were selected from DEGs in these pathways. qRT-PCR validation of these genes indicated that gene expression of OsLIL3-interacting genes such as OsPSY (OsPSY1, OsPSY2, and OsPSY3), OsGGR, OsPOR, OsOR, OsCLP, OsCLPC1, OsCLPS1) family genes was greatly suppressed (Fig. 6).

Discussion

Carotenoids refer to a large number of yellow, orange or lipophilic molecules that are synthesized during photosynthesis (Rodríguez-Concepcion et al. 2018). The various functional roles of carotenoids involve: light harvest/capture; protection against abiotic stress conditions (Darijo et al. 2007; Stanley and Yuan 2019); acting as a precursor in hormone biosynthesis (such as for biosynthesis of GA that acts as a stress and developmental signal) (Walter et al. 2010; Sun et al. 2018), quenching of singlet oxygen in photosynthesis II under normal conditions (Tamura and Ishikita 2020), and rendering tolerance to heat and light stress by protecting membranes from reactive oxygen species (ROS) and lipid peroxidation (Johnson et al. 2007; Yokono and Akimoto 2018).

In our study, the CRISPR-Cas9 oslil3 mutant was used for a detailed physiological, transcriptomic, and molecular characterization of rice OsLIL3 gene. The mutant has a 4-bp deletion leading to a frameshift mutation. It displayed a yellowish leaf color phenotype that is more prominent in the seedling stage (Fig. 1a, b and c). oslil3 contained reduced levels of chlorophylls and carotenoids in the mutant vs WT. In addition, OsLIL3 mutation caused aberrant chloroplast development under normal growth conditions. In Arabidopsis, the *lil3.1/lil3.2* double mutant showed reduced chlorophyll contents while the single *lil3.1* or *lil3.2* mutants displayed no significant differences from WT (Hey et al. 2017). Since OsLIL3 mutation leads to a strong reduction in chlorophyll and carotenoid levels it could act as a master regulator of any downstream stress caused by a reduction in pigment levels. Previous investigation of LIL3 homologs in dicot (Arabidopsis AtLIL3.1 and AtLIL3.2) and monocot (rice OsLIL3) revealed a conserved function in chlorophyll and terpenoid biosynthesis through their interaction with GGR and POR enzymes (Tanaka et al. 2010; Hey et al. 2017; Zhou et al. 2017). LIL3 proteins interact directly with different isoforms of POR and GGR enzymes and regulate their protein abundances in rice and Arabidopsis (Hey et al. 2017; Tanaka et al. 2010). The yellow leaf phenotype of *lil3.1/lil3.2* mutant may be caused by the reduced protein levels of POR and GGR enzymes involved in chlorophyll and terpenoid biosynthesis. POR is responsible for reduction of protochlorophyllide to chlorophyllide and GGR is responsible for the generation of intermediate metabolite GGPP (Tanaka et al. 2010; Hey et al. 2017). A similar case may be made for the yellow leaf phenotype of rice *oslil3*. However, the molecular relation between LIL3 levels and these pathway proteins is still disconnected.

A genome-wide transcriptome analysis of *oslil3* identified that of 4288 DEGs in total, 2188 genes were up-regulated and 2100 genes were down-regulated vs WT (Fig. 2a). GO enrichment analysis suggested that OsLIL3 deficiency leads to expression changes in genes localized to thylakoid membrane, lumen and PSI1, in genes involved in aldolate transmembrane transport, chitin metabolism and many amino-sugar catabolic pathways and for genes with chitinase, transporter and sugar modification activity. KEGG pathway analysis suggested similarly
that many metabolic pathways are affected in the osil3 mutant especially plant-pathogen interaction genes (possibly chitin related genes) and in carbon metabolism, photosynthetic light reactions, and isoprenoid pathways and so on (Figure S3 and S4). Interestingly, all of the DEGs mapped in the MapMan reveals that genes in the cell wall biosynthesis were down-regulated and most of the genes in light reactions were up-regulated (Fig. 3a &b). Interestingly, four CESA proteins (CESA1, CESA3, CESA4, CESA6) that are the core components of the cell wall cellulose synthase (Kumar et al. 2016) were down-regulated that will repress the synthetic activities of cellulose in osil3. COBL4 is also another reported gene responsible for cellulose biosynthesis in primary and secondary cell wall and help crystallize cellulose in the walls (Brown et al. 2005). Six FLA genes were also identified as down-regulated in osil3, however, the functional roles of FLA family proteins are largely unknown. The FLA3 were reported to be involved in cellulose biosynthesis in pollen tubes (Andersson-Gunnerås et al. 2006). This could explain the dwarf phenotype observed in osil3. There were distinct differences between

![Fig. 4 Protein-protein interaction between OsLIL3 and OsPSY2. (a), Yeast two hybrid assays. OsLIL3 and LHC domains were fused with Gal DNA-binding domain (BD). OsPSY2 was fused with Gal DNA-activation domain (AD). The two other combinations OsLIL3/empty (pGADT7 vector) and empty (pGBK7 vector)/OsPSY2 were included as negative controls. Yeast cells containing various vector combinations were grown on DDO plates (SD–Leu–Trp) for transformation selection. Yeast cells showing growth and blue color on high selection TDO/X/A plates (SD-His-Leu-Trp + X-alfa-Gal + Aureobasidin A) confirmed positive interactions between the two indicated proteins. (b), OsLIL3-FLAG coimmunoprecipitated with OsPSY2-HA. Total proteins were purified from tobacco leaves expressing OsLIL3-FLAG with or without OsPSY2-HA and immunoprecipitated with an anti-Flag or anti-HA antibody. The presence of OsLIL3-FLAG, and OsPSY2-HA in the complex was determined by immunoblot analysis with Flag and HA antibodies and anti-HA anti-bodies respectively. (c), BIFC-visualized interactions between OsLIL3 and OsPSY2 in N. benthamiana leaves. Abbreviations: Y N, YFP N.Y C, YFP C. Chl, chlorophyll autofluorescence. BF, bright field. All bars = 5 μm.](image-url)
Fig. 5. Subcellular localization of OsLIL3 and OsPSY2 in N. benthamiana leaves. Fluorescent signals of OsLIL3-EYFP and OsPSY2-EYFP indicated by confocal microscopy. In both cases, chlorophyll autofluorescence (Chl) merged with yellow fluorescence signals, suggesting chloroplast localization. All bars = 10 μm. The OsLIL3-EYFP and OsPSY2-EYFP signal were detected using the anti-GFP antibodies, both these two proteins located in the thylakoid membrane fraction but not stroma and envelope fractions compared to marker proteins (Tic110, envelope; RbcL, stroma; Lhca1, thylakoid membrane). Env, envelope membranes; Str, stroma; Tm, thylakoid membrane.
chloroplasts from osil3 and WT based on ultrastructural and physiological characteristics. RNA-seq data comparison of the wp1 (Wang et al. 2016) and osil3 mutant revealed a common pattern of downregulation of secondary metabolic process (phenylpropanoid biosynthesis, flavonoid biosynthesis), lignin biosynthetic process, sterol biosynthetic process, and of up-regulation of chlorophyll organization, amino sugar and nucleotide sugar metabolism and chitin and polysaccharide catabolism. We can conclude that both LIL3 and WP1 are involved in chloroplast organization and pigment biosynthesis that in turn play essential roles in plant growth and in determining the leaf color.

In Arabidopsis, previously it was reported that deficiency of LIL3 levels leads to reduced accumulation level of GGR protein and influences chlorophyll biosynthesis that seemed to support a similar yellowish phenotype. Arabidopsis LIL3 mutation down-regulated genes involved in light reaction pathway and isoprenoid pathways under light stress conditions similar to the findings from our osil3 transcriptomic data (Lohscheider et al. 2015). It also showed reduced abundance of POR enzymes similar to our immunoblot analysis findings (Hey et al. 2017). This suggests a common mechanism of lower abundances of GGR or POR enzymes leading to the yellow leaf phenotype in both studies. Previous studies suggest how rice LIL3 directly regulates isoprenoid enzymes (leading to a yellow leaf phenotype). In rice, LIL3 interacts with POR, GGR, geranylgeranyl diphosphate synthase (GGDPPS)1 and GGPPS recruiting protein (GRP) proteins that are components of a large multiprotein complex in the thylakoid membrane (Zhou et al. 2017). Looking for additional LIL3 novel interacting proteins in the isoprenoid biosynthesis pathway revealed that OsPSY2, phytoene synthase 2, shows positive interaction with OsLIL3 in this study (Fig. 4). The other two ortholog genes namely OsPSY1 and OsPSY3, also have positive interactions with OsLIL3. Phytoene synthase is a major rate limiting enzyme in carotenoid biosynthesis pathway that is controlled by the Clp protease family proteins and ORGANLE (OR) protein in the plastid carotenoid biosynthesis in Arabidopsis (Welsch et al. 2018; Zhou et al. 2017). The OR protein is a post-transcriptional regulator of PSY while the Clp protease degrades and controls the PSY protein turnover in Arabidopsis. LIL3 contains typical LHC domain characterized by about four hydrophobic and α-helical membrane-spanning domains that can bind dozens of proteins related to carotenoid biosynthesis, chlorophyll biosynthesis and validation of genes identified as DEGs in osil3 vs WT this transcriptome analysis. Two reference genes were used as internal controls. The expression levels of genes in WT plants were set to 1. OsAtpB, Os10g21266; OsPSY1, Os06g51290; OsPSY2, Os12g43130; OsPSY3, Os09g38320; OsGGR, Os02g51080; OsPORA, Os04g58200; OsPORB, Os10g35370; OsNECD, Os12g24800; OsLUT1, Os10g39930; OsCPOX, Os04g52130; OsCHLI, Os03g36540; OsOR, Os02g43500; OsCLPC1, Os04g32560; OsCLPS1, Os08g33540; OsLHCA2, Os09g26810.

**Fig. 6** Expression analysis of genes in carotenoid biosynthesis and validation of genes identified in RNA-seq analysis. qRT-PCR analysis of genes related to carotenoid biosynthesis, chlorophyll biosynthesis and validation of genes identified as DEGs in osil3 vs WT this transcriptome analysis. Two reference genes were used as internal controls. The expression levels of genes in WT plants were set to 1. OsAtpB, Os10g21266; OsPSY1, Os06g51290; OsPSY2, Os12g43130; OsPSY3, Os09g38320; OsGGR, Os02g51080; OsPORA, Os04g58200; OsPORB, Os10g35370; OsNECD, Os12g24800; OsLUT1, Os10g39930; OsCPOX, Os04g52130; OsCHLI, Os03g36540; OsOR, Os02g43500; OsCLPC1, Os04g32560; OsCLPS1, Os08g33540; OsLHCA2, Os09g26810.
chlorophyll and three to four different carotenoids (Zapata et al. 2000), thus playing vital roles in photosynthesis and photoprotection. Alteration of PSY expression displayed profound effects on the content of carotenoids. A number of factors were reported to have influences on the expression of PSY gene expression (Cheminant et al. 2011; Toledo-Ortiz et al. 2010). For example, the expression of PSY1 in tomato was regulated by carotenoids and transcriptional factors like RIPENING INHIBITOR (Martel et al. 2011). Gibberellin-regulated DELLA protein regulated the expression of genes involved in carotenoid biosynthesis during de-etiolation process in Arabidopsis (Cheminant et al. 2011). The tomato ERF gene, SIAP2α, regulated the carotenoid accumulation profile by altering carotenoid pathway flux (Chung et al. 2017). Recently, PSY protein levels were shown to be regulated by a negative feedback emerging from carotenoids (Arango et al. 2014). However, the factors that may act as translational or post-transcriptional regulators are still limited. In our study, the mRNA levels of OsPSY1, OsPSY2, and OsPSY3 was significantly down-regulated in oslil3 mutant as compared to WT (Fig. 6) and a positive interaction between LIL3 and PSY proteins which suggests that LIL3 may act as a post-transcriptional regulator of PSYs, representing an important regulatory mechanism underlying carotenoid biosynthesis in plants. There is a possibility that OsPSY genes may be another example of LIL3 anchored protein partner in the chloroplast similar to GGR and POR (Tanaka et al. 2010; Hey et al. 2017). Indeed, the evidence from sub-cellular localization analysis suggests OsPSY2 and OsLIL3 are both co-localized to thylakoid membranes in the chloroplasts (Fig. 5). Further studies need to investigate the protein accumulation level of PORA and PORB was greatly suppressed in osli3 mutant vs WT while the OsPORB expression was about 50% lower (Fig. 6). This differential expression pattern of OsPORB was also detected in our RNA-seq analysis but expression level OsPORB did not significantly change in WT and osli3. As reported, the OsOR protein was reported to be a key post-transcriptional regulator of PSY in carotenoid biosynthesis (Zhou et al. 2015a). This suggests that the regulation of PSY levels may be determined by multiple molecular regulators such as OR and LIL3. Further studies need to investigate the protein accumulation levels of other PSY interacting proteins. Protein-protein interaction assays are required to determine whether the interaction of LIL3-PSY is unique essential in controlling carotenoid biosynthesis. Additionally, the regulation of enzymatic activities of PSYs in osli3 mutant and protein levels of LIL3 in Ospsy mutants are also necessary to be determined to further support our findings.

As we have drafted the abbreviated model of chlorophyll, carotenoid and GA biosynthesis in Fig. 3b, these three pathways are connected by GGPP (Geranylgeranyl diphosphate, an intermediate in these pathway). GGPP acts as an entry point to the biosynthesis of carotenoids, chlorophylls, tocopherols, gibberellins, plastoquinones, polyrenols, diterpenoids, and mitochondria-derived polyterpenes (Nagegowda and Gupta 2020). Overexpression of PORA, PORB or PORC could accumulate more Chls and subsequently caused yellow leaf phenotype in several studies (Kimura et al. 2018; Li et al. 2019; Buhr et al. 2017; Liu et al. 2015). On the other hand, overexpression of PORB produces more chlorophyll metabolites in plant leaves (Zhan et al. 2019) and overexpression of GGR also generates more contents of chlorophylls in tomato leaves (Liu et al. 2015). The chlorophyll contents are reduced in osli3 mutant that is in accordance with the lower accumulation levels of PORB and GGR protein in this mutant.
protect plants against photooxidative damage in Arabidopsis (Pattanayak and Tripathy 2011; Sperling et al. 1997). In crop plants, overexpression of PORB also improves chlorophyll and tocopherol content in both leaves and kernels in maize (Zhan et al. 2019). Overexpression of PSY induced accumulation of phytoene but not of any downstream products in rice (Burkhardt et al. 1997). Thus, any metabolic engineering of the carotenoid biosynthesis could also influence the chlorophylls but would still play positive roles in plant growth and performance under oxidative stress conditions in plants. In summary, our work revealed a novel functional impact of OsLIL3 mutation on carotenoid and chlorophyll biosynthesis. OsLIL3 may regulate carotenoid and chlorophyll biosynthesis not only through its interaction with a thylakoid co-localizing OsPSY2 but also by enhancing the protein levels (OsPORB, OsGGR) and regulating the gene expression (OsPSYs, OsGGR, OsPORA, OsLIL1, OsCPOX, OsCHLI and OsCLP) of enzymes involved in these pathways. Thus, LIL3 may serve as a common hub for modulating levels of enzymes involved in different plant pigment pathways to manipulate its metabolism and growth.

Materials and Methods

Plant Materials and Growth Conditions

The CRISPR-Cas9 mutant osill3 (Oryza sativa ssp. japonica cv. Nipponbare) was generated using CRISPR-Cas9 system at Biogle (http://www.biogle.cn, Hangzhou, China). A single sgRNA targeting OsLIL3 at the location 5'-GCGGCTTCGACACCTCCACGGG-3' was created in the Bsgk03 vector (Bai et al. 2020) containing Cas9, that was thentransformed into Agrobacterium tumefaciens strain EHA105 and the transgenic rice plants were obtained as mentioned previously (Nishimura et al. 2006). Genomic DNA from these transformants was sequenced for verifying precise mutation site in the OsLIL3 sequence using PCR amplification with primer pairs flanking the target site and potential off-targets were also investigated based on the online prediction tool CRISPR-P (http://cbi.hzau.edu.cn/crispr/) (Supplementary data S1). The mutant line 16-1 T2 single transgenic CRISPR-Cas9 plant), is a 4-bp deletion OsLIL3 knock-out mutant as compared to the other lines. As analyzed in the segregation experiment, the 3-bp and 9-bp deletions did not cause any frameshift in translation while only the 4-bp deletion caused a frameshift premature translation termination (Supplementary data S2). This mutant has a chlorotic phenotype and was used for further analysis. Amplified PCR products of size (300–500 bp) were sequenced directly and analyzed using the Degenerate Sequence Decoding method (Ma et al. 2015) to identify the mutated sites.

TEM Analysis of Chloroplast Structures

Third leaf from 1-week-old WT and osill3 mutant seedlings (3-leaf stage) grown at 28 °C were used to prepare samples for transmission electron microscopy (TEM, Philips CM100, Eindhoven, The Netherlands) analysis. Leaf samples were fixed in 2.5% glutaraldehyde cacodylate buffer at 4 °C for 12 h. The fixation was stopped by changing to cacodylate buffer containing 0.1 M sucrose. All subsequent experiments were carried out in the hood. Leaf samples were fixed in 1% osmium tetroxide cacodylate buffer at 28 °C for 2 days, and samples were washed several times with cacodylate buffer, 0.05 M sodium cacodylate in an aqueous solution at pH 7.2–7.4. A dehydration procedure was performed as follows: a) 50% ethanol (10 min), 70% ethanol (10 min), 90% ethanol (10 min), 100% ethanol (10 min) (step repeated 3 times); b) 99% propylene oxide (1 × spin) (step repeated 2 times). The tissues were then infiltrated with 1:2 mixture of epoxy resin/propylene oxide for 1.5 h, 1:1 mixture of epoxy resin/propylene oxide for 1.5 h and 2:1 mixture of epoxy resin/propylene oxide overnight at 28°C. Then, the samples were infiltrated with fresh epoxy resin for 3 h at 37 °C in a vacuum oven. These samples were finally embedded in fresh epoxy resin in plastic capsules and polymerized at 60 °C overnight. Ultra-thin sections were microtomed under the transmission electron microscope (Philips CM100, Eindhoven, The Netherlands).

RNA Extraction and Quality Control

WT and osill3 mutant plant samples were collected at the L3 seedling stage and whole leaf samples were harvested for RNA extraction (3 biological replicates with 6–8 individual plants for each replicate). Leaves from osill3 and WT were rapidly frozen in liquid nitrogen and total RNA was extracted with the EZNA plant RNA kit (Omega Bio-Tek, GA, USA) according to the manufacturer’s instructions. RNA quantity and purity were checked by the kaiaoK5500 Spectrophotometer (Kaiao, Beijing, China). RNA integrity and concentration were analyzed with the RNA Nano 6000 Assay Kit of Bioanalyzer 2100 (Agilent Technologies, CA, USA). RNA concentration for library construction was measured using Qubit RNA Assay Kit in Qubit 3.0 (Life Technologies, Grand Island, NY, USA). Dilutions were prepared at a concentration of 1 μg·μl⁻¹.

Library Preparation and Sequencing

For library preparation, 2 μg total RNA was input into NEB Next Ultra RNA library Prep Kit (New England Biolabs, N.E.B, USA) to generate sequencing libraries based on the manufacturer’s recommendation. In brief, mRNA was purified from the input total RNA using poly-dT oligo-attached magnetic beads. This was followed by RNA fragmentation by addition of divalent...
cations under increased temperature. First strand cDNA synthesis (Takara, Japan) was performed on the fragmented RNA using random hexamer primers and the remaining RNA was degraded using RNase H (Omega Bio-Tek, USA). Subsequently, a second strand cDNA synthesis was performed and the resulting fragments were purified using the QiAQuick PCR kit (QIAGen, Hilden, Germany) followed by terminal repair, A-tailing and adapter addition. Finally, PCR reactions were carried out to complete library preparation.

Library insert size was quantified using StepOnePlus Real-Time PCR system (library valid concentration > 10 nmol·L-1). Sample clustering was performed on a cBot cluster generation system using HiSeq PE Clustering Kit v4-cBot-HS (Illumina, CA, USA). Subsequently, libraries were sequenced on an Illumina HiSeq 4000 platform (Illumina, CA, USA) to obtain 150 bp paired-end reads.

Transcriptome Analysis and Differential Gene Expression

Reads were processed through quality check using FastQC protocol and high-quality reads (“clean reads”) were obtained for mapping to reference genome (ftp://ftp.ensemblgenomes.org/pub/plants/release-24/fasta/orzya_sativa/) in HISAT2 v2.0.5 (Kim et al. 2015). Following this, raw read count for each gene in each sample was obtained with HTSeq v0.6.0, and FPKM (Fragments Per Kilobase Million mapped reads) was calculated from the raw read counts to estimate the gene expression level of all the expressed genes in each sample.

Principal component analysis (PCA) analyses were conducted to assess the transcriptome variance in sample replicates (Tsuyuzaki et al. 2020). Hierarchical clustering organizes samples (experimental units) based on the gene expression within each sample using Poisson model and dissimilarity measure based on likelihood ratio statistics (Si et al. 2014).

Finally, DESeq2 v1.6.3 was used to compare gene expression levels between OsLIL3 and WT. Gene expression level was determined by linear regression calculating the fold changes for sample comparisons. Subsequently, p-value for this fold change was calculated with Wald test. Finally, the p-values were corrected by the BH method to give q-values. Genes passing both cut-offs of q-value ≤0.05 and |log 2 fold change| ≥1 were identified as differentially expressed genes (DEGs). Functional enrichment analyses were performed using MapMan analysis (https://mapman.gabipd.org/), GO functional classification (http://www.blast2go.org/) and KEGG pathway analyses (https://www.genome.jp/kegg/) as performed previously (Song et al. 2020). MapMan analysis maps the DEGs according to the log fold change values on the pathway maps. The rich-ratio in GO analysis is defined as amount of differentially expressed genes enriched in the pathway/amount of all genes in that pathway in the background gene set. The size of the dots represents the number of genes, and the color of the dots represents the false discovery rate (FDR) (Zhang et al. 2020). KEGG analyses plots the number of genes belonging to enriched pathways.

Y2H Assay

Coding regions of OsLIL3 and OsPSY genes were cloned without transit peptide and transmembrane domains and fused in-frame into the bait vector pGBKTK7 or prey vector pGADT7 with corresponding primers (Clontech, Mountain View, USA) and further verified for correct sequence by DNA sequencing. Yeast transformation and selection procedures were performed according to the protocols of the Matchmaker gold yeast two-hybrid (Y2H) system (Clontech, Mountain View, USA). E. coli S. cerevisiae Gold cells (Clontech, Mountain View, USA) that were transformed with different combinations of bait and prey vector constructs were incubated on synthetic defined (SD) plates lacking Leu and Trp for selection of co-transformants. Afterwards, the co-transformed yeast cells were further selected on high selection plates (SD –Leu –Trp –His + X -Agal + Aureobasidin A) to identify blue colonies indicative of positive protein-protein interactions.

BiFC Assay

BiFC is commonly used for detection of protein-protein interaction in living mammalian tissue culture and the fluorescent protein YFP and other GFP derivatives are split into non-overlapping N-terminal or C-terminal part. Full-length coding regions of OsLIL3 were cloned in-frame with the N-terminal fragment of EYFP in the SPYNE vector (amino acids 1–155, pSPYNE). On the other hand, full-length coding regions of different candidate proteins OsPSYs were cloned in-frame with the C-terminal fragment of EYFP in the SPYCE vector (amino acids 156–239, pSPYCE) binary vector. Both construct sequences were confirmed with DNA sequencing. Different combinations of BiFC vectors were transiently expressed in N. benthamiana leaves by Agrobacterium infiltration as described previously (Sparkes et al. 2006). Fluorescent signals in the infiltrated leaves were examined after 48–72 h with a confocal microscope (LSM710, Carl Zeiss Microscopy, Germany).

Co-Immunoprecipitation Assay

Co-immunoprecipitation assay was performed according to the methods described in Jin et al. 2018 with some minor modifications. Briefly, the constructs containing the full length of OsLIL3-FLAG and OsPSYs-HA were generated with PCR amplification and correct gene sequence was confirmed by DNA sequencing. In addition, the combinations of co-infiltration were OsLIL3:FLAG
films.

Chalfont, Buckinghamshire, England) on Kodak X-ray Western Blotting Detection Kit (Amersham, Little Sweden). Signals were detected using an ECL rabbit antibodies (1:25,000; Agrisera, Vännäs, Sweden), POR (1:5000; Agrisera) antibodies against OsLIL3 (1:2000), GGR (1:10000, Agrisera, Vännäs, Sweden), AtpB (1:30000;). The membranes were incubated with horseradish peroxidase or AtpB (1:30000;). The membranes were incubated with anti-HA agarose beads (Thermo Scientific, USA) for 1 h at 4 °C. The supernatant was discarded after centrifugation at 2500 rpm for 2 min at 4 °C. The remaining pellets were rinsed with washing buffer [10 mM Tris/Cl pH 7.5; 150 mM NaCl; 0.25% NP-40] for at least three times and the beads were transferred to a fresh tube, and subsequently resuspended in a 100 μl 2x SDS-sample buffer [100 mM Tris HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol] and boiled. The supernatant was collected for immunoblotting with monoclonal anti-HA anti-body (1:5000, Abcam, Cambridge, UK). Signals were detected using an ECL Western Blotting Detection Kit (Amersham Life Science, Little Chalfont, Buckinghamshire, England) on Kodak X-ray films.

**Immunoblot Analyses of OsLIL3, OsGGR, OsPSY in oslil3 WT and osll3 mutant rice samples were prepared at the L3 seedling stage and used for immunoblot analysis. To isolate total proteins, rice leaves were ground in ice cold isolation buffer [50 mM Tris Cl pH 7.8, 250 mM sucrose, 25 mM KCl, 10 mM MgCl2, 0.5% (v/v) β-mercaptoethanol, 4% SDS, 0.2% bromophenol blue, 20% glycerol] and boiled. The supernatant was collected for immunoblotting with monoclonal anti-HA anti-body (1:10000; Sigma-Aldrich, USA), and monoclonal anti-HA anti-body (1:5000, Abcam, Cambridge, UK). Signals were detected using an ECL Western Blotting Detection Kit (Amersham Life Science, Little Chalfont, Buckinghamshire, England) on Kodak X-ray films.***

**Subcellular Localization of OsLIL3 and OsPSY2**
The OsLIL3 and OsPSY2 coding region were cloned in-frame with the EYFP gene in the pCAMBIA1301-35S-NOS vectors as previously described (Li et al. 2014). *Agrobacterium tumefaciens* (strain GV3101) cultures harboring either 35S::OsLIL3-EYFP and 35S::OsPSY2-EYFP constructs were adjusted to OD600 = 0.4 in MES buffer (10 mM MES, pH 5.5, and 10 mM MgSO4) and infiltrated into leaves of *N. benthamiana* (6-week-old). For agroinfiltration in *N. benthamiana* (strain GV3101) cultures harboring constructs were incubated in 28 °C in 10 ml LB with 100 μg/ml kanamycin for 2 days. Cells were pelleted at 4000 rpm for 15 min, and re-suspended in induction medium at 28 °C overnight. Fluorescent signal in *N. benthamiana* infiltrated leaves was examined after 3 days by confocal microscopy (LSM710 Carl Zeiss Microscopy, Germany).

Chloroplasts were then isolated from infiltrated *N. benthamiana* leaves. Stroma, envelope, and thylakoid membrane fractions were prepared as described previously (Salvi et al. 2008). Protein samples from intact chloroplast as well as the stroma, envelope and thylakoid fractions were separated by 10% SDS-PAGE gel for electrophoretic analysis. The proteins were then transferred onto nitrocellulose membranes (Amersham, Little Chalfont, Buckinghamshire, England). Immunoblot experiments were performed with polyclonal mouse antibodies against GFP (1:3000 dilution; Invitrogen, Life technologies, Carls bad, CA, USA) and rabbit polyclonal antibodies against RbcL, Lhca1, Tic110 (1:5000, 1:2000, 1:1000 dilution, respectively; Agrisera, Vännäs, Sweden), followed by incubation with horseradish peroxidase–conjugated goat anti-mouse IgG antibodies (1:3000 dilution; Invitrogen, Life technologies, Carls bad, CA, USA), or goat anti-rabbit antibodies (1:25,000; Agrisera, Vännäs, Sweden).

**qRT-PCR Validation Assays**
Validation of RNA-seq results was performed for 15 genes selected from DEGs that are involved in photosynthesis and other pathways using quantitative RT-PCR (qRT-PCR). The sequences of the gene primers are provided in Supplementary data S3. Total RNA was extracted as mentioned above for RNA-Seq and first-strand cDNA was synthesized using a Transcript One-Step gDNA Removal and cDNA Synthesis SuperMix kit utilizing oligoT primers (TransGen Biotech, Beijing, China). qRT-PCR was carried out in the ABI Step One Plus Real-Time PCR System (Applied Biosystems, USA) utilizing the SYBR Premix Ex Taq RT-PCR kit (Takara Bio, Japan). qRT-PCR programs were as follows: (i) 95 °C for 30 s, (ii) 40 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 10s and extension at 72 °C for 10s. Rice ATP synthase subunit beta (*OsAtpB,*
Os10g21266) gene was employed as negative controls in this study. The two rice reference gene Osactin1 (Os03g50885) and Osubiquitin (Os03g30920) were used as internal control genes for expression normalization.

**Statistical Analysis**

Statistics was conducted using IBM SPSS Statistics ver. 18 (SPSS Inc., IL, USA) employing Duncan’s Multiple Range test (DMRT) post-hoc test with significance level set to \( P \leq 0.05 \) for sample comparisons. Data is mean ± SE from three independent biological replicates. Student’s t-test was performed to employ the data analysis of the qRT-PCR assay.

**Abbreviations**

LIL3: Light harvesting like protein 3; GGR: Geranylgeranyl reductase; POR: Protochlorophyllide oxidoreductase; PSY: Phytoene synthase; FC: Ferrochelatase; GGPPS1: Geranylgeranyl diphosphate synthase; CHLG: Chlorophyll synthase; RNA-seq: RNA-sequencing; Y2H: Yeast two hybrid assay; BiFC: Bimolecular fluorescence complementation; CoIP: Co-immunoprecipitation assay; TEM: Transmission electron microscopy; PS I: photosystem I; PS II: Photosystem II; LHC: Light harvesting complex I; LIL3: Light harvesting like protein 3; GGR: Geranylgeranyl reductase; POR: Protochlorophyllide oxidoreductase; PSY: Phytoene synthase; LIL3: Light harvesting like protein 3; GGR: Geranylgeranyl reductase; Por: Protochlorophyllide oxidoreductase; PSY: Phytoene synthase; LIL3: Light harvesting like protein 3; GGR: Geranylgeranyl reductase.

**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s12284-021-00474-z.

**Additional file 1** Figure S1. OsLIL3 sequence alignment between oslil3 and WT. (a), Sequence alignment between WT and oslil3 16-#1 mutant at DNA level. The red triangle indicates stop codon in the mutated OsLIL3 sequence that leads to premature translational termination while the blue triangle indicates end of truncated sequence due to intron insertion. (b), amino acid sequence alignment between mutated oslil3 and WT.

**Additional file 2** Supplementary data S1. Off-target prediction in oslil3 16-#1. Supplementary data S2. CRISPR-Cas9 induced mutations. Supplementary data S3. Primers used in this study. Supplementary data S4. Segregation of CRISPR-Cas9 induced mutations in T0 generation. Supplementary data S5. DEGs in Plant-pathogen interaction. KEGG category. Supplementary data S6. Light harvesting genes in WT and WT. Supplementary data S7. Cell wall genes list in oslil3 vs WT. Supplementary data S8. Accession number of genes reported in this work.

**Authors’ Contributions**

FY and JHZ designed experiments. FY performed experiments. FY, DD and FS analyzed data. FY and DD wrote the manuscript. JHZ and TS critically commented and revised it. All authors read and approved the manuscript.

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**Availability of Data and Materials**

All relevant data are provided within the article and its supplementary information files.

**Declarations**

**Ethics Approval and Consent to Participate**

Not applicable.

**Consent for Publication**

Not applicable.

**Competing Interests**

The authors declare that no competing interests exist.

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