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Regular Paper

Rice FLUORESCENT1 Is Involved in the Regulation of Chlorophyll

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(Received March 19, 2019; Accepted June 20, 2019)

Chlorophyll biosynthesis plays essential roles in photosynthesis and plant growth in response to environmental conditions. The accumulation of excess chlorophyll biosynthesis intermediates under light results in the production of reactive oxygen species and oxidative stress. In this study, we identified a rice (*Oryza sativa*) mutant, *oxidation under photoperiod* (*oxp*), that displayed photobleached lesions on its leaves, reduced growth and decreased chlorophyll content during light/dark cycles or following a dark-to-light transition. The *oxp* mutant accumulated more chlorophyll precursors (5-aminolevulinic acid and protochlorophyllide) than the wild type in the dark, and more singlet oxygen following light exposure. Several singlet-oxygen-responsive genes were greatly upregulated in *oxp*, whereas the expression patterns of *OsPORA* and *OsPORB*, two genes encoding the chlorophyll biosynthesis enzyme NADPH:protochlorophyllide oxidoreductase, were altered in de-etiolated *oxp* seedlings. Molecular and complementation studies revealed that *oxp* is a loss-of-function mutant in LOC_Os01g32730, a homolog of *FLUORESCENT* (*FLU*) in *Arabidopsis thaliana*. Rice PHYTOCHROME-INTERACTING FACTOR-LIKE14 (OsPIL14) transcription factor directly bound to the *OsFLU1* promoter and activated its expression. Dark-grown transgenic rice seedlings overexpressing *OsPIL14* accumulated more chlorophyll and turned green faster than the wild type upon light illumination. Thus, OsFLU1 is an important regulator of chlorophyll biosynthesis in rice.

Keywords: Chlorophyll biosynthesis • FLU • Light signaling • Photobleaching • Photoperiod sensitive • Rice.

Introduction

Chlorophylls are the major pigments that harvest light energy and drive electron transfer in photosynthesis. The early steps of chlorophyll biosynthesis, from glutamate to the protoporphyrin IX, are shared with heme biosynthesis in the tetrapyrrole biosynthesis pathway (Tanaka and Tanaka 2007). After these initial steps, magnesium chelatase inserts an Mg^{2+} ion into protoporphyrin IX to form Mg-protoporphyrin IX, thus directing the pathway into the chlorophyll branch. In angiosperms, chlorophyll biosynthesis is paused in darkness at the protochlorophyllide (Pchlide) intermediate step. Light activates NADPH:protochlorophyllide oxidoreductase (POR) activity, converting Pchlide to chlorophyllide, which in turn is catalyzed by several enzymes into chlorophyll *a* and *b* (Tanaka and Tanaka 2007). Overaccumulation of free Pchlide in plants during darkness or as a result of compromised POR function may lead to the production of singlet oxygen ($^1O_2$) upon light irradiation, which causes photobleaching and cell death (Op den Camp et al. 2003, Buhr et al. 2008, Tang et al. 2012). Although the chlorophyll biosynthesis pathway has been elucidated, its regulation remains unclear.

Transcriptional and posttranslational regulation are thought to play important roles in modulating tetrapyrrole biosynthesis (Tanaka et al. 2011, Brzezowski et al. 2015). A number of light signaling transcription factors are directly or indirectly involved in regulating the expression of the chlorophyll biosynthesis genes and the seedling greening response. PHYTOCHROME-INTERACTING FACTOR1 (PIF1) and PIF3 are negative regulators of chlorophyll biosynthesis, whereas FAR-RED ELONGATED HYPOCOTYL3 (FHY3), FAR-RED IMPAIRED RESPONSE1 (FAR1) and ELONGATED HYPOCOTYL5 (HY5) are positive regulators of this process (Stephenson et al. 2009, Tang et al. 2012, Chen et al. 2013). The *pif1* and *pif3* mutants accumulate high levels of Pchlide in darkness and generate singlet oxygen after light irradiation, causing photobleaching of their cotyledons (Huq et al. 2004, Stephenson et al. 2009, Chen et al. 2013). By contrast, the *fhy3* and *fhy3 far1* mutants contain low concentrations of Pchlide in darkness and turn green rapidly, even when exposed to high light (Tang et al. 2012). PIF1 directly activates *PORC* expression, whereas FHY3 and FAR1 directly regulate *HEMB1* transcription (Moon et al. 2008, Tang et al. 2012). PIF1, PIF3 and HY5 are also able to directly bind to the specific promoter sequence of many

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Plant Cell Physiol. 60(10): 2307–2318 (2019) doi:10.1093/pcp/pcz129, Advance Access publication on 10 July 2019,
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reactive oxygen species (ROS)-responsive genes to control their expression after light treatment (Chen et al. 2013).

FLUORESCENT (FLU) was identified as a negative regulator of chlorophyll biosynthesis in an *Arabidopsis thaliana* mutant screen. Etiolated *flu* seedlings accumulate excessive amounts of Pchlide and are bleached when transferred to light. The *flu* plants grow only in continuous light and cannot survive in light/dark cycle conditions (Meskauskiene et al. 2001). A genetic screen identified *executer 1* (*ex1*) mutant, which can suppress the *flu* phenotype (Wagner et al. 2004). FLU interacts with the C-terminus of glutamyl-tRNA reductase (GluTR) to repress the biosynthesis of 5-aminolevulinic acid (ALA) (Goslings et al. 2004, Zhang et al. 2015), the universal precursor for tetrapyrrole biosynthesis in all organisms, which represents a key point for its posttranslational regulation of chlorophyll biosynthesis. FLU belongs to the tetratricopeptide repeat (TPR) family of proteins and contains three TPR motifs in its C-terminal region (Bohne et al. 2016). Singlet oxygen is a type of ROS that has a short half-life and is highly toxic to plant cells (Triantaphylidès et al. 2008). Studies of the conditional *Arabidopsis flu* mutant and its suppressors have suggested that its role in chlorophyll biosynthesis involves singlet oxygen-mediated signaling (Kim and Apel 2013). An ortholog of *FLU* in barley (*Hordeum vulgare*), *TIGRINA d*, has also been reported (Lee et al. 2007). However, the role of FLU in rice is unknown.

Here, we identified a mutant, *oxidation under photoperiod* (*oxp*), in rice (*Oryza sativa*), which exhibited an oxidative stress response and had reduced chlorophyll levels when grown under photoperiodic conditions or following a dark-to-light transition. The *oxp* phenotype was caused by a deletion in *OsFLU1*. We found that the transcript levels of *OsPORA* and *OsPORB* and several singlet-oxygen-responsive genes are altered in the *oxp* mutant. Furthermore, the PHYTOCHROME-INTERACTING FACTOR-LIKE14 (OsPIL14) transcription factor directly controls *OsFLU1* expression and regulates chlorophyll biosynthesis. This study demonstrates that OsFLU1 is involved in modulating chlorophyll biosynthesis and is regulated by a PIL transcription factor in rice.

Results

The *oxp* mutant displays a photobleaching phenotype under photoperiodic conditions

We obtained a rice T-DNA insertion mutant from Pohang University of Science and Technology (An et al. 2003). When grown under continuous light conditions at 25°C, the mutant seedlings were indistinguishable from the Dongjin (DJ) wild-type plants (Fig. 1A); however, when grown under a 12 h light/12 h dark photoperiod, the young leaves and leaf sheaths of the mutants exhibited lesion-like photobleaching shortly after germination (Fig. 1B). We compared the effect of different light/dark cycles on the mutant. As shown in Fig. 1C, D, 20 min of darkness per day did not cause the photobleaching phenotype in the mutant; however, when grown under conditions of an hour of darkness or more each day, the mutant seedlings displayed the photobleaching phenotype, which was most severe under 12 h light/12 h dark conditions. The chlorophyll content and plant height of the mutant decreased when grown in photoperiods with a longer dark period (Fig. 1E, F). We, therefore, designated the mutant as *oxp*, for *oxidation under photoperiod*.

Next, the *oxp* mutant was backcrossed with the wild type. The selfed F2 progeny segregated with an approximately 3:1 ratio (385:131 plants) of wild type:mutant phenotypes, suggesting that *oxp* contains a single recessive mutation.

The *oxp* phenotype is regulated by light intensity

To test whether light intensity could affect the *oxp* phenotype, DJ and *oxp* were grown under a 12 h light/12 h dark photoperiod with different light intensities for 10 d. As shown in Fig. 2A, B, under low-light conditions (30 μmol/m^2/s), the *oxp* leaves were slightly yellower, with minimal lesions. When the light intensity was increased to 140 μmol/m^2/s, the *oxp* phenotype became stronger, and the mutants showed almost photobleached under 240 μmol/m^2/s light conditions. The *oxp* plants were much shorter than the wild type under 240 μmol/m^2/s light conditions (Fig. 2C).

Etiolated *oxp* seedlings are hypersensitive to light

We also examined the greening process of etiolated seedlings. No differences were observed between the 7-day-old *oxp* mutant and DJ seedlings grown in darkness (Fig. 3A); however, after 1 day of light exposure, the wild-type seedlings accumulated chlorophylls and became yellow-green, whereas the *oxp* mutants remained pale and did not accumulate chlorophyll (Fig. 3A, B). After 6 d of light illumination, all of the wild-type seedlings had turned green, whereas only a few *oxp* plants survived (Fig. 3A). These observations indicate that the *oxp* mutation impairs the heterotrophic-to-autotrophic transition of rice seedlings.

ALA is the first committed precursor for tetrapyrrole biosynthesis in all organisms (Tanaka and Tanaka 2007). We found that the *oxp* mutant had significantly higher levels of ALA than the DJ wild type (Fig. 3C). Pchlide is accumulated in the dark and the conversion of Pchlide to chlorophyllide by POR enzymes is a key step in chlorophyll biosynthesis during the dark-to-light transition (Runge et al. 1996, Tanaka et al. 2011). When grown in darkness for 7 d, the *oxp* seedlings accumulated much higher levels of Pchlide than DJ (Fig. 3D). After light exposure for 10 min, the *oxp* mutant contained more Pchlide and more chlorophyllide (and chlorophyll) than the wild-type plants (Fig. 3E). *OsPORA* (Os04g58200) expression was repressed by light in both the dark-grown wild-type and mutant seedlings, whereas *OsPORB* (Os10g35370) transcription was activated by light; however, *OsPORA* expression was downregulated to a greater degree in the *oxp* mutant, whereas *OsPORB* expression was upregulated to a lesser degree (Fig. 3F). These data suggest that the *oxp* mutation compromises the biosynthesis of chlorophyll intermediates.

The *oxp* mutant accumulates ROS

We investigated ROS production by detecting the fluorescence of the ROS indicator 2′,7′-dichlorodihydrofluorescein diacetate

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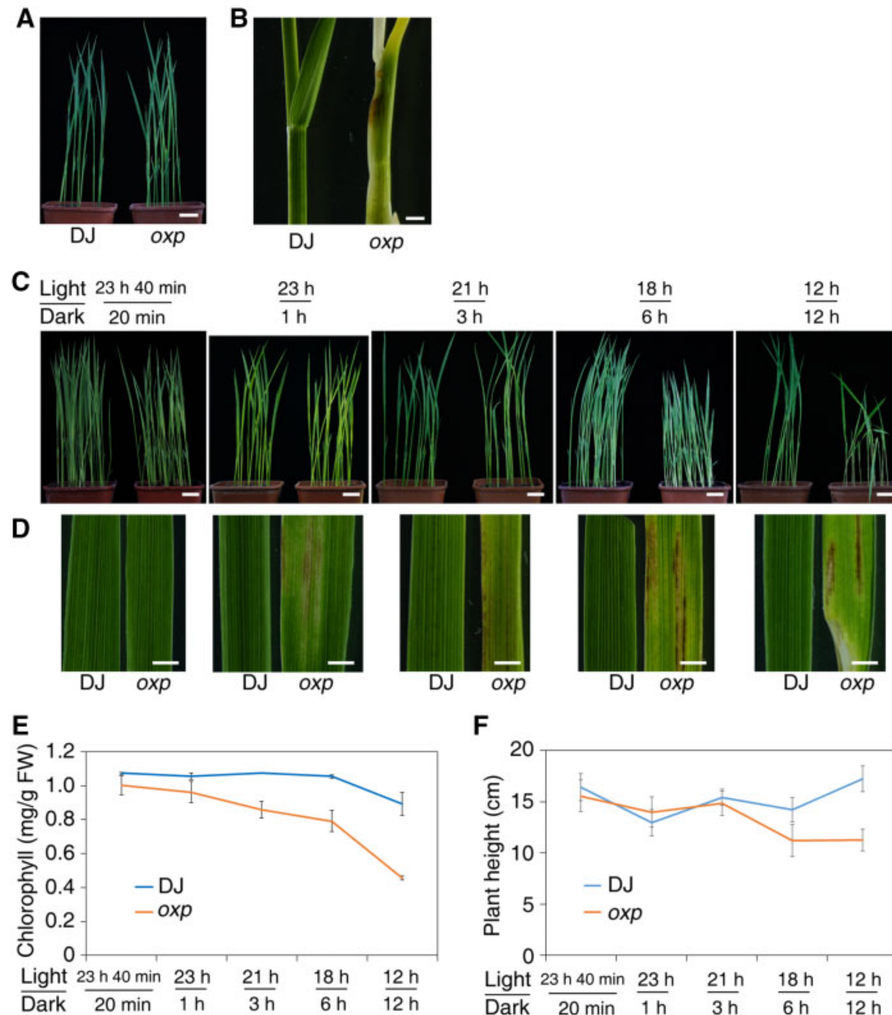

Fig. 1 Rice *oxp* mutants are sensitive to light under photoperiod conditions. (A) Seedling morphology of DJ (wild type) and *oxp* mutant plants grown under continuous light for 10 d. Bar, 2 cm. (B) Photobleaching phenotype of the leaves of *oxp* plants grown under long-day conditions (16 h light/8 h dark) for 10 d. Bar, 2 cm. (C) Seedling morphology of DJ (left) and *oxp* (right) plants grown under different photoperiod conditions for 7 d. L, light; D, dark. Bars, 2 cm. (D) Photobleaching phenotype of the leaves of the seedlings shown in (C). Bars, 2 mm. (E) Chlorophyll contents of the seedlings shown in (C). FW, fresh weight. (F) Plant height of the seedlings shown in (C). For (E, F), data are mean ± SD of three replicates.

(H_2DCFDA) in the *oxp* and wild-type leaves. Dark-grown seedlings showed low H_2DCFDA fluorescence levels with no difference between DJ and *oxp*; however, after a 2-h light exposure, much more H_2DCFDA fluorescence was detected in *oxp* than in the wild type (**Fig. 4A**). Moreover, after illumination, the etiolated *oxp* mutants produced more singlet oxygen, as visualized using the fluorescent probe Singlet Oxygen Sensor Green (SOSG) (**Fig. 4B**). To examine the molecular effect of the ROS production, we investigated the expression of three ROS-responsive marker genes, *Os01g61080*, *Os05g39720* and *Os02g08440*. These genes belong to the WRKY transcription factor family, and the expression of their putative homologs in *Arabidopsis* is greatly induced by ROS (Su et al. 2018). Following exposure to light, these genes were dramatically upregulated in *oxp* in comparison with their expression in the wild type (**Fig. 4C**). Taken together, these data indicate that OXP inhibits ROS production and ROS-responsive gene expression.

OXP encodes FLUORESCENT1

The *oxp* mutant was originally generated by T-DNA insertion; however, one T-DNA inserted into Os02g42314 was not associated with the *oxp* phenotype, indicating that the photobleaching phenotype was caused by a mutation/insertion/deletion in another gene. We reasoned that the light-sensitive phenotypes of *oxp* were similar to the phenotypes displayed in the *flu* mutant in *Arabidopsis* (Meskauskiene et al. 2001). In rice, we identified two loci with homologous sequences to *Arabidopsis FLU*, Os01g32730 (designated as *OsFLU1*) and Os02g37470 (designated as *OsFLU2*). We, therefore, carried out an Reverse Transcription quantitative (RT-qPCR) analysis to determine the levels of *OsFLU1* and *OsFLU2* in the *oxp* mutant. The *OsFLU1* transcript levels in *oxp*

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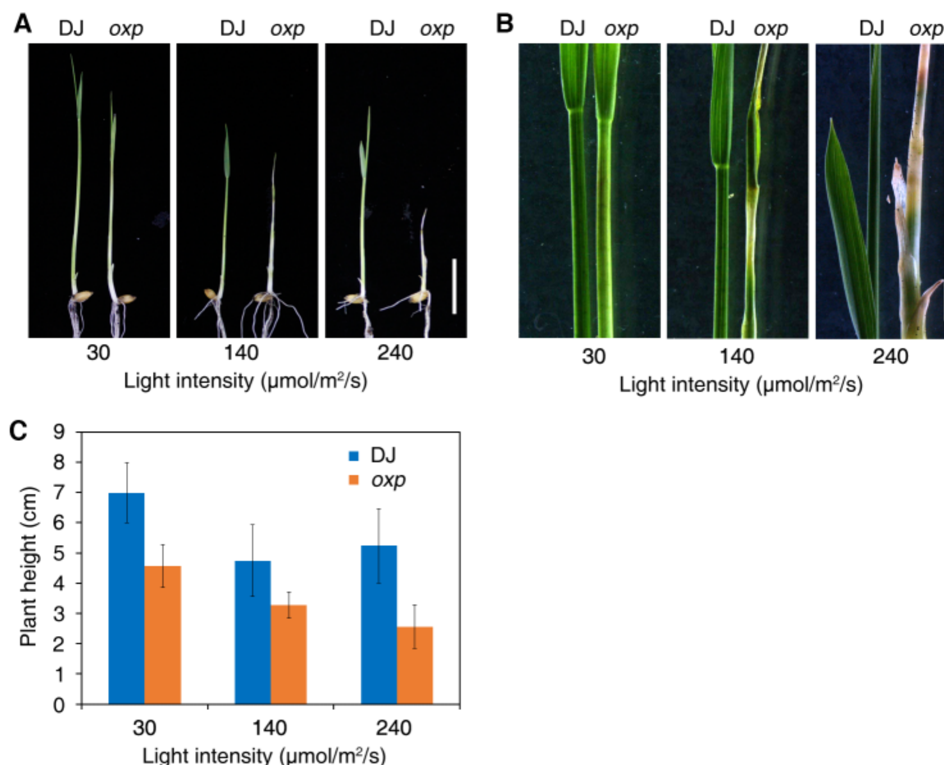

Fig. 2 The *oxp* mutants are sensitive to high light. (A) Seedling morphology of DJ (wild type) and *oxp* plants grown under 12 h light/12 h darkness with different light intensities for 7 d. Bar, 2 cm. (B) Magnification of the seedling phenotypes shown in (A). Bar, 0.5 cm. (C) Plant height of the seedlings shown in (A). Data are mean ± SD of three replicates.

were greatly reduced to about 10% of that of the wild type, whereas the levels of *OsFLU2* were not significantly affected (Fig. 5A).

The genomic sequence of *OsFLU1* comprises five exons and four introns, and encodes a putative protein with 324 amino acids. To determine whether the genomic sequence of *OsFLU1* was altered in *oxp*, we performed a sequencing analysis of this locus, revealing that the mutant had a 53-bp deletion in this gene, including 35 bp of the first exon and 18 bp of the first intron (Fig. 5B). Taken together with the expression analysis, this finding suggests that *OsFLU1* is mutated in *oxp*.

To determine whether the photobleaching phenotype of *oxp* was caused by the loss function of *OsFLU1*, we expressed *OsFLU1* driven by its own promoter in multiple transgenic *oxp* plants. *OsFLU1* expression was partially restored in the *oxp/OsFLU1p:OsFLU1* transgenic lines, which rescued the photo-oxidation and reduced height phenotypes of *oxp* (Fig. 5C, D; two representative lines shown). Furthermore, the expression of two ROS-responsive genes (*Os01g61080* and *Os05g39720*) and two putative pathogen resistance genes (*Os11g37970* and *Os11g37960*) were largely complemented to almost wild-type levels (Fig. 5E–H). This genetic and molecular evidence demonstrates that *OXP* indeed encodes OsFLU1.

Expression and protein localization of *OsFLU1*

We then investigated whether the expression patterns of *OsFLU1* and *OsFLU2* are regulated by light. Wild-type rice plants were grown in continuous darkness or light for 7 d. We found that the transcription of *OsFLU1* was lower in plants in the dark than in those grown in the light, whereas *OsFLU2* expression was not affected by these light conditions (Fig. 6A). When 7-day-old etiolated seedlings were exposed to light for 5 h, the *OsFLU1* expression increased, whereas *OsFLU2* expression decreased (Fig. 6A). These data suggest that *OsFLU1* transcription is activated by light. To determine its protein subcellular localization, OsFLU1 was fused with GFP (green fluorescent protein) and introduced into rice mesophyll cell protoplasts. As shown in Fig. 6B, the OsFLU1-GFP fluorescence colocalized with the chlorophyll auto-fluorescence, suggesting that OsFLU1 is located in the chloroplasts.

Phylogenetic analysis of FLU homologs

To study the origin and evolutionary history of *FLU* homologs, we searched *FLU* for homologs from 58 published genomes of green plants (Supplementary Fig. S1; Table S2). In total, from these 58 plant genomes, we identified 85 FLU homologs. The number of *FLU* homologs in diverse species varied from 0 (*Selaginella moellendorffii*) to 6 (*Citrus sinensis* and *Triticum aestivum*) (Supplementary Fig. S1). There is one *FLU* homolog in the unicellular green alga *Chlamydomonas reinhardtii*, indicating that the *FLU* gene family originated more than 725 million years ago in the ancestor of green plants (Guo 2013). There is one *FLU* homolog in *Arabidopsis*, but this gene is duplicated in grasses; rice thus has two homologs, *OsFLU1* and *OsFLU2* (Fig. 6C). FLU has 53.4% and 48.3% identity with OsFLU1 and

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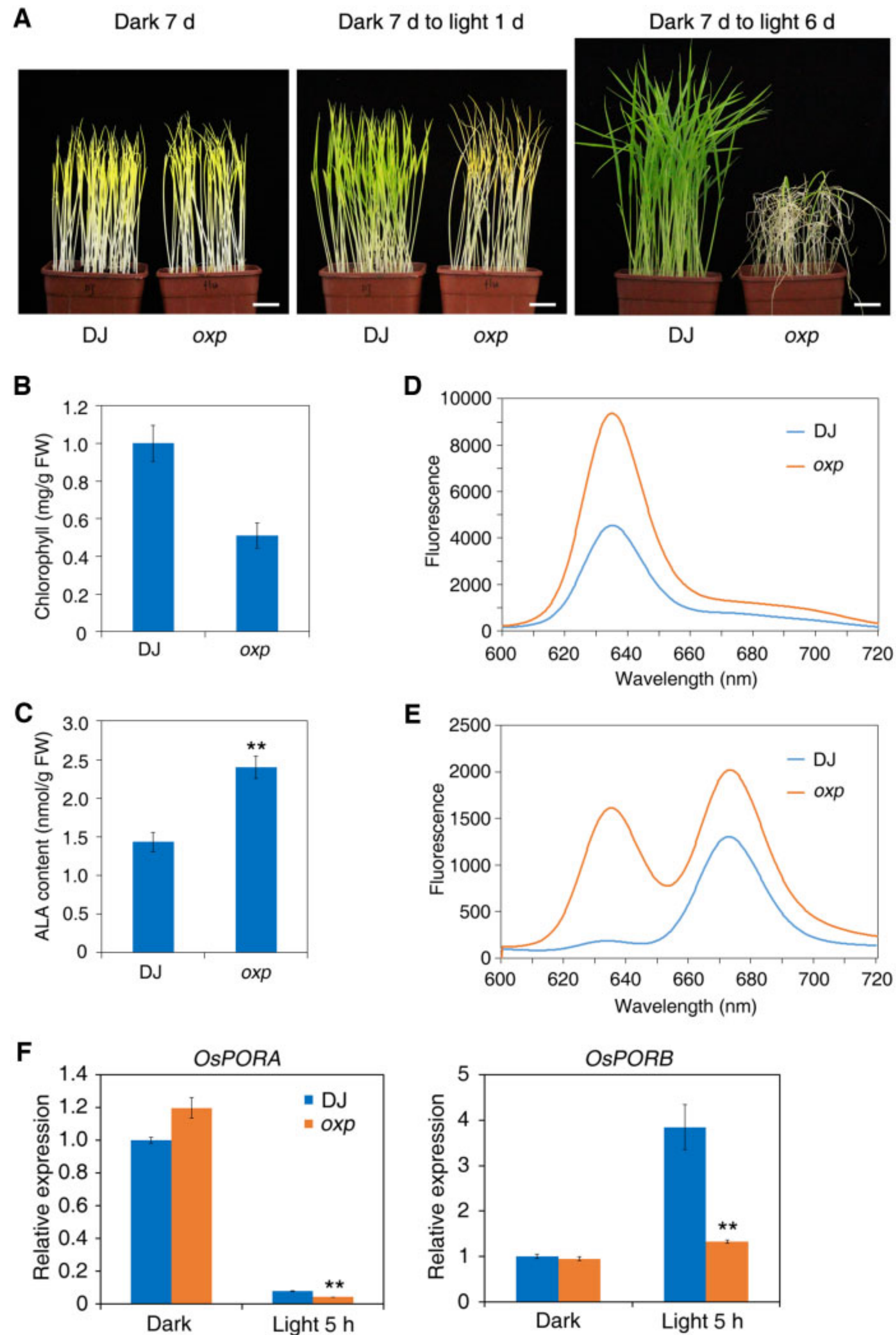

Fig. 3 Etiolated *oxp* seedlings are hypersensitive to light. (A) Seven-day-old dark-grown DJ (wild type) and *oxp* seedlings were exposed to light (100 μmol/m^2/s) for 1 or 6 d. Bars, 2 cm. (B) Chlorophyll levels of 7-day-old etiolated seedling after 2 d of light exposure. FW, fresh weight. (C) ALA contents of 7-day-old etiolated seedlings. For (B, C), data are mean ± SD of three replicates. FW, fresh weight. Pchlide or/and chlide (and chlorophyll) fluorescence of seedlings grown in darkness for 7 d (D) or further illuminated with light (300 μmol/m^2/s) for 10 min (E). Experiments were repeated at least three times and one representative experiment is shown. (F) RT-qPCR analysis of *OsPORA* and *OsPORB* genes. Seedlings were grown in darkness for 7 d and then exposed to light for 5 h. For (A, D–H), data are mean ± SD of three biological replicates. Asterisks indicate significant differences from DJ, revealed using Student's *t*-test ($P < 0.01$).

OsFLU2, respectively; whereas OsFLU1 and OsFLU2 have 47.3% identity (Supplementary Fig. S2).

The constitutive expression of *OsFLU1* and *OsFLU2* complement the *flu* mutant in *Arabidopsis*

To investigate whether the OsFLUs have the same function as *Arabidopsis* FLU, we constructed *OsFLU1* and *OsFLU2* binary vectors and introduced them into the *Arabidopsis flu* mutant background. Multiple transgenic lines for each transgene were obtained. As previously reported (Meskauskiene et al. 2001), the etiolated *flu* seedlings did not turn green after light illumination; however, the light-exposed etiolated *flu/OsFLU1-OE* and *flu/OsFLU2-OE* transgenic plants produced green cotyledons similar to the wild type (Col; Supplementary Fig. 3A).

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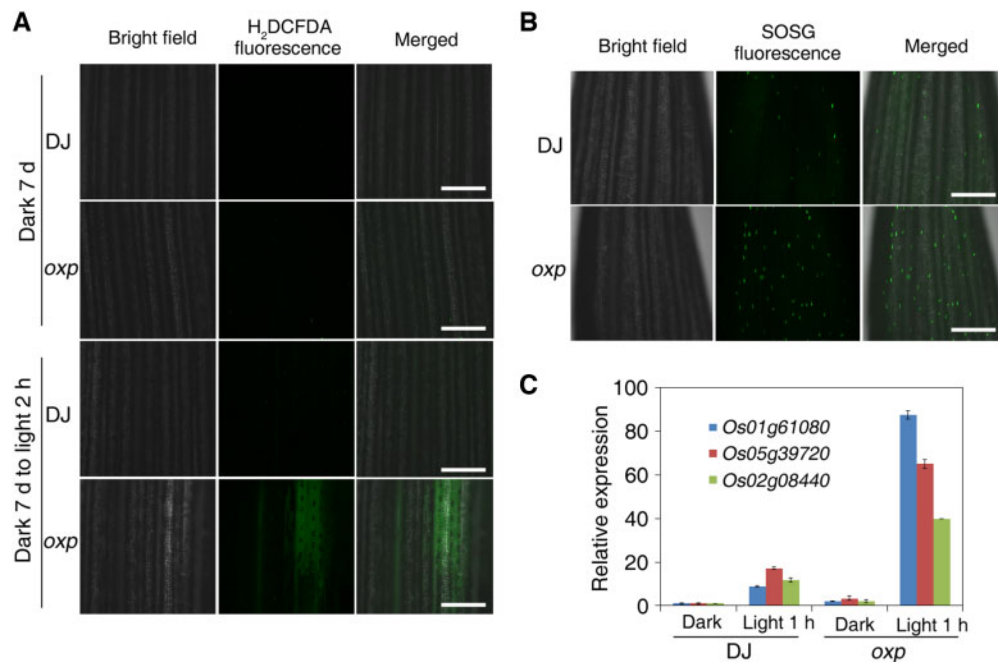

Fig. 4 The *oxp* mutant accumulates ROS. (A) H_2DCFDA (ROS-indicating) fluorescence of 7-day-old dark-grown DJ (wild type) or *oxp* seedlings or those subsequently exposed to light (300 μmol/m^2/s) for 2 h. Bar, 1 mm. (B) SOSG fluorescence of 7-day-old dark-grown seedlings or those subsequently exposed to light (300 μmol/m^2/s) for 2 h. Bar, 1 mm. (C) RT-qPCR analysis of ROS-responsive genes. Seedlings were grown in darkness for 7 d and then exposed to light (200 μmol/m^2/s) for 1 h. Data are mean ± SD of three biological replicates.

When grown under photoperiodic conditions (16 h light/8 h darkness), the *flu* plants were bleached; however, the phenotypes of the *flu/OsFLU1-OE* and *flu/OsFLU2-OE* transgenic plants resembled those of the wild type (Supplementary Fig. 3B, C). Taken together, these observations confirm that OsFLU1 and OsFLU2 function similarly to FLU in *Arabidopsis*.

OsPIL14 directly activates *OsFLU1* expression

A promoter analysis revealed that two putative G-box (CACGTG) motifs are located in the promoter region of *OsFLU1* (**Fig. 7A**). G-box is a *cis*-element to which basic helix-loop-helix (bHLH) transcription factors can bind (Li et al. 2006). PIF proteins are a subfamily of bHLH factors that play critical roles in regulating plant development and responses, including chlorophyll biosynthesis, chloroplast development, photomorphogenesis and ROS response (Huq et al. 2004, Shin et al. 2009, Stephenson et al. 2009, Chen et al. 2013). Six *OsPIL* genes, designated as *OsPIL11* to *OsPIL16*, were identified in the rice genome (Nakamura et al. 2007). We generated transgenic lines constitutively expressing these genes (*UBQp:OsPILs-GFP*; except *OsPIL13*) in the Zhonghua 11 (ZH11) wild-type background. To examine whether these genes were involved in regulating chlorophyll biosynthesis, transgenic lines and wild-type plants were grown in darkness for 4 d and then exposed to light (1,200 μmol/m^2/s) for 1 d. Although the etiolated ZH11 seedlings remained pale, the *OsPIL14OE* plants (Supplementary Fig. S4) turned green and accumulated significantly more chlorophyll than the wild type, whereas the other *OsPILsOE* lines only had slight effects (**Fig. 7B, C;** data not shown), suggesting that OsPIL14 promotes chlorophyll biosynthesis during seedling de-etiolation.

Using a chromatin immunoprecipitation (ChIP) assay, we investigated whether OsPIL14 could bind to *OsFLU1*. As shown in **Fig. 7D**, after pull down with the GFP antibody, an *OsFLU1* promoter fragment containing the two G-boxes (P1), but not another promoter region (P2) or the *OsUBQ5* control, was greatly enriched in samples from the *OsPIL14OE* in comparison with ZH11. Next, we purified the GST-OsPIL14 (fused with glutathione S-transferase) recombinant protein and performed an electrophoresis mobility shift assay (EMSA). GST-OsPIL14, but not GST alone, could bind with biotin-labeled oligonucleotides containing G-box2 to slow the migration of the probes; however, no mobility shift signal was found when the G-box was mutated to ACATAC (**Fig. 7E**). Furthermore, an RT-qPCR analysis revealed that the *OsFLU1* transcript levels were increased in *OsPIL14OE* in comparison with the wild type (**Fig. 7F**). These data suggest that OsPIL14 directly binds to the specific promoter region of *OsFLU1* to activate its expression.

Discussion

Our genetic study identified a mutant, *oxp*, which was sensitive to light and displayed an oxidative stress response. We revealed that *OXP* encodes one of the two FLU homologs in rice, and determined that the function of OsFLU1 was disrupted in the *oxp* mutant. A phylogenetic analysis revealed that *FLU* genes are

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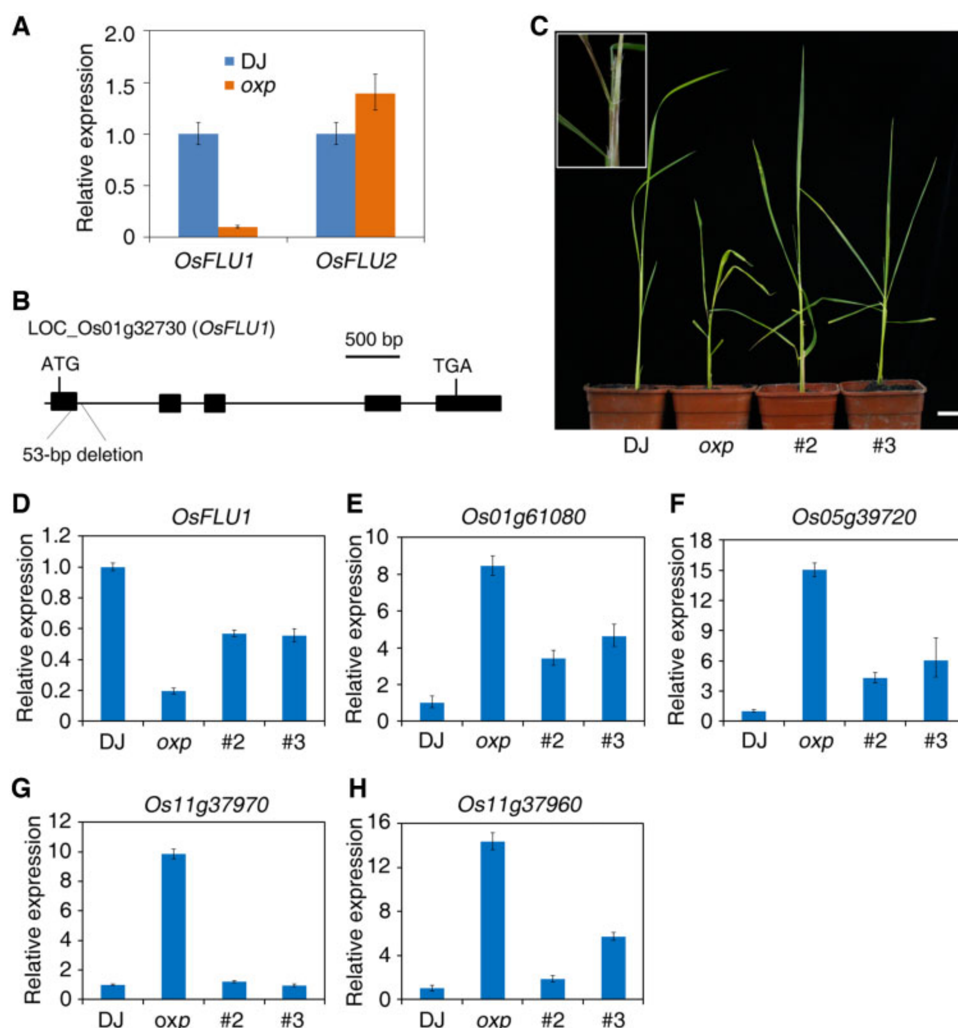

Fig. 5 The *oxp* phenotype is caused by a deletion in *OsFLU1*. (A) Relative expression of *OsFLU1* and *OsFLU2* in DJ (wild type) and *oxp* mutant plants. (B) *OsFLU1* gene structure. Bars denote exons. The *oxp* phenotype was caused by a 53-bp deletion of part of the first exon and intron. (C) Seedling phenotype of *oxp/OsFLU1p:OsFLU1* complementation lines (representative lines #2 and #3 are shown). Insert image shows the lesion-like phenotype of *oxp*. Bar, 2 cm. (D) RT-qPCR analysis of *OsFLU1* in the complementation lines. (E–H) RT-qPCR analysis of ROS-responsive genes in the complementation lines. For (C–H), plants were grown under 12 h light/12 h darkness for 10 d. For (A, D–H), data are mean ± SD of three biological replicates.

distributed in green plants (**Fig. 6**). Both *OsFLU1* and *OsFLU2* were able to complement the *flu* mutant phenotype in *Arabidopsis* (Supplementary Fig. S2), suggesting that OsFLU1 and OsFLU2 have similar functions to FLU. Nonetheless, *OsFLU1* and *OsFLU2* were differentially regulated by light and were distributed in distinct phylogenetic clades (**Fig. 6**), indicating that these two genes may have divergent functions. Further research is required to reveal the role of *OsFLU2* in rice.

The *oxp* mutant accumulated high levels of ALA and Pchlide in the dark but low levels of chlorophyll after exposure to light (**Fig. 3**). Furthermore, the expression patterns of *OsPORA* and *OsPORB* were disrupted by the *oxp* mutation (**Fig. 3**). These results demonstrate that OsFLU1 is essential for regulating chlorophyll biosynthesis in rice. Consistently, the OsFLU1 protein is localized in the chloroplast. FLU acts as a negative feedback regulator of the Mg^{2+}-branch of tetrapyrrole biosynthesis in *Arabidopsis* (Meskauskiene et al. 2001). It suppresses ALA biosynthesis by directly posttranslationally regulating the activity and stability of GluTR, the rate-limiting enzyme of this pathway, in both light and darkness (Hou et al. 2018). The molecular and biochemical roles of OsFLU1 and FLU require further investigation.

ROS are produced in chloroplasts, mitochondria, peroxisomes and the apoplasts of plants and can damage lipids, proteins and DNA when present in excessive levels (Apel and Hirt, 2004). In chloroplasts, ROS production is tightly associated with light-dependent photosynthetic reactions. If the function of the chlorophyll biosynthetic enzymes is disrupted, oxidative stress responses can occur; for example, transgenic *Arabidopsis* plants with reduced levels of *HEMB1*, encoding a 5-aminolevulinic acid dehydratase, exhibited increased ROS accumulation, cell death and high levels of stress-responsive gene expression (Wang et al. 2016). Antisense transgenic tobacco (*Nicotiana tabacum*) plants with a reduced activity of either uroporphyrinogen decarboxylase (UROD) or coproporphyrinogen oxidase display necrotic leaf lesions, constitutive *PR* expression, and an

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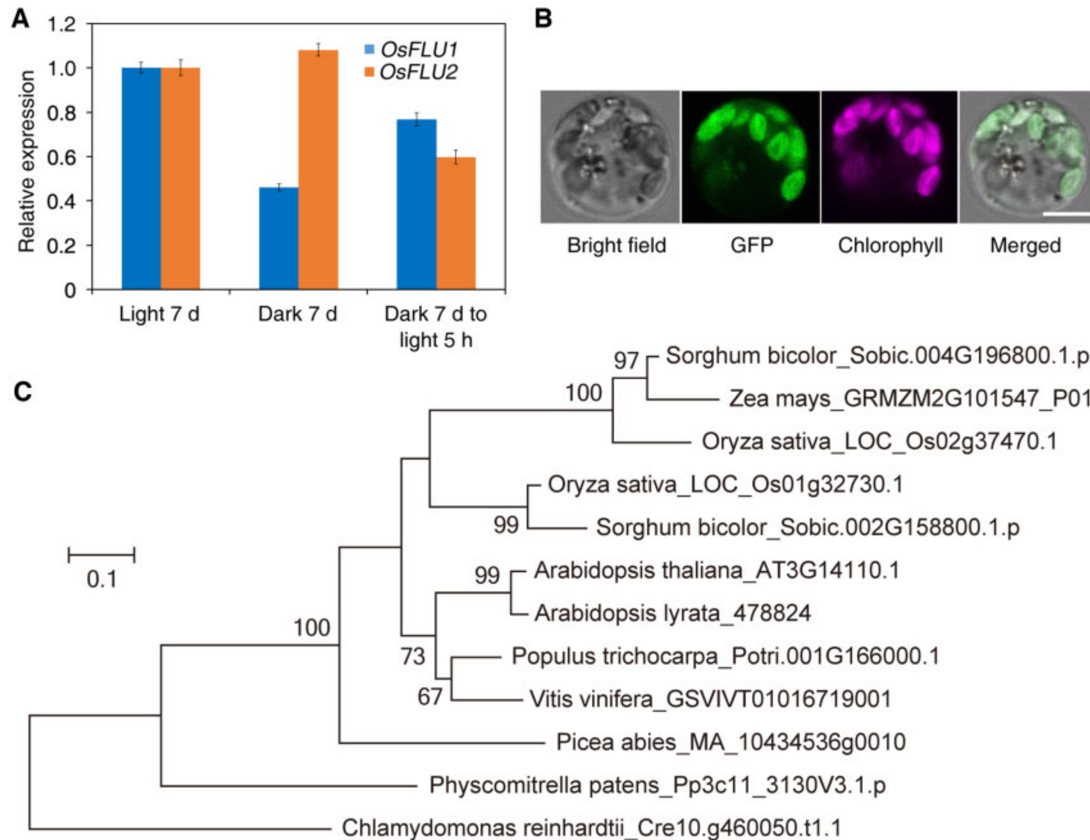

Fig. 6 *OsFLU1* expression, OsFLU1 protein localization and *FLU* gene phylogenetic analysis. (A) RT-qPCR analysis of *OsFLU1* and *OsFLU2* expression in DJ (wild type) plants grown in different light conditions. Data are mean ± SD of three biological replicates. (B) OsFLU1-GFP protein localization in rice protoplasts. GFP and chlorophyll auto-fluorescence are shown in green and purple, respectively. Bar, 5 µm. (C) Phylogenetic tree of FLU homologs in 10 representative green plant species. Bootstrap values are indicated at branches.

increased resistance to pathogens (Mock et al. 1999). The maize (*Zea mays*) *les22* mutant, which has a partial deficiency in UROD displays a necrotic phenotype (Hu et al. 1998) that is mainly due to the production of ROS triggered by the accumulation of chlorophyll intermediates when these plants are subjected to light conditions. Singlet oxygen, a nonradical and highly reactive ROS unique to plant chloroplasts, was found to be specifically and conditionally generated by the *Arabidopsis* loss-of-function *flu* mutants (Meskauskiene et al. 2001). Consistent with this finding, we also detected the accumulation of singlet oxygen in the *oxp* mutant leaves (**Fig. 4**).

ROS also act as redox signaling molecules involved in the metabolism and physiology of both plants and animals (Schieber and Chandel 2014, Waszczak et al. 2018). Chloroplasts are the major sites of plant ROS production, a process which can activate chloroplast-to-nucleus retrograde signaling pathways to initiate either programmed cell death or a response to environmental conditions. Different types of ROS signals trigger both common and distinct transcriptomic changes and responses (Gadjev et al. 2006, Galvez-Valdivieso and Mullineaux 2010). We found that the expression of three genes encoding WRKY transcription factors and two putative pathogen resistance genes were greatly induced in the *oxp* mutant (**Figs. 4, 5**). The production of singlet oxygen in the *flu* mutant was previously reported to activate a signaling pathway involving EX1 (Wagner et al. 2004). The *oxp* mutation in *OsFLU1* provides a new resource that can be used to study the effects of singlet oxygen and its signaling pathways in crop plants.

Although FLU was previously reported to play essential roles in modulating chlorophyll biosynthesis and the oxidative stress response, the regulation of FLU itself was elusive. In this study, we revealed that the transcription of *OsFLU1* is directly controlled by the transcription factor OsPIL14, which binds to the specific G-box motif in the promoter region of *OsFLU1* to activate its expression (**Fig. 7**). Consistently, OsPIL14 was also found to regulate the abundance of chlorophyll and the seedling greening process (**Fig. 7**). OsPIL14 is a bHLH transcription factor that shares sequence similarity with the PIF proteins in *Arabidopsis* (Li et al. 2006). *Arabidopsis* PIF1 and PIF3 control chlorophyll biosynthesis and ROS signaling at least partially by directly regulating the expression of the chlorophyll biosynthesis genes and ROS-responsive genes (Huq et al. 2004, Stephenson et al. 2009, Chen et al. 2013). The red/far-red light photoreceptors, known as phytochromes, interact with the PIF factors and trigger their degradation in light (Leivar and Quail 2011). In rice, PIL proteins likely interact with the phytochromes as well, indicating that the transcription of *OsFLU1* might be controlled by the phytochromes and the light signaling pathway. In concordance with this notion, the expression level of *OsFLU1*, but not *OsFLU2*, was upregulated by

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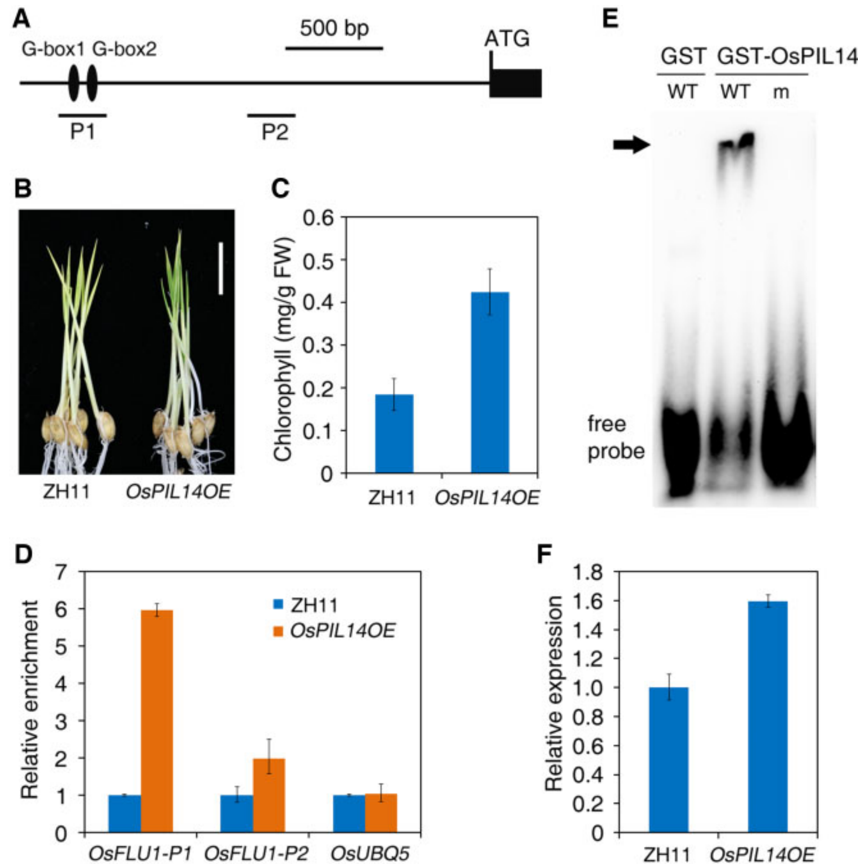

Fig. 7 *OsFLU1* is bound and regulated by the transcription factor OsPIL14. (A) Promoter diagram of *OsFLU1*. Two putative G-box motifs are located in the promoter. P1 and P2 show the regions used for the ChIP qPCR assay. (B) Greening phenotype of ZH11 and *OsPIL14OE* plants. Bar, 2 cm. (C) Chlorophyll contents of plants in (B). FW, fresh weight. For (E, F), seedlings were grown in darkness for 4 d and then illuminated with high light (1,200 µmol/m^2/s) for 1 d. (D) ChIP assay of Zhonghua 11 (ZH11, wild type) and *OsPIL14OE* transgenic plants grown in darkness for 2 weeks. A GFP antibody was used for the immunoprecipitation. *OsUBQ5* serves as a negative control. (E) EMSA of GST or GST-OsPIL14 recombinant proteins incubated with biotin-labeled fragments containing the wild-type G-box2 (CACGTG) motif or a mutant version (ACATAC). Arrow indicates the shifted band. (F) RT-qPCR analysis of *OsFLU1* in ZH11 and the *OsPIL14OE* plants grown in darkness for 10 d. For (C, D, F), data are mean ± SD of three biological replicates.

light (Fig. 6), demonstrating that these two genes are differently regulated by light. Other transcription factors or regulators are also likely involved in controlling the expression of these genes.

As the intermediate products of chlorophyll biosynthesis are sensitive to light and can cause photo-oxidation in plant cells, the biosynthesis pathway and oxidative response must be precisely regulated in response to light and other environmental conditions. The elucidation of the function and regulatory mechanism of OsFLU1 provides insight into these processes, and may facilitate the future improvement of the environmental adaptation and productivity of rice and other crops.

Materials and Methods

Plant materials

The rice *oxp* mutant is in the Dongjin background (*O. sativa* L. var. japonica cv. Dongjin). The *OsPIL14OE* transgenic lines are in japonica cv. Zhonghua 11 background. Rice plants were grown under short-day (10 h light/14 h dark, 150 µmol/m^2/s) conditions at 28°C in a growth chamber or as otherwise described in the text. *Arabidopsis flu* mutant is of Columbia-0 (Col) ecotype. *Arabidopsis* plants were grown under long-day (16 h light/ 8 h dark, 80 µmol/m^2/s) conditions at 22°C in a growth chamber.

Chlorophyll content measurement

Rice samples were ground to powder in liquid nitrogen and chlorophylls were extracted by incubating in 1 ml of methanol for 30 min in darkness. The mixture was centrifuged at 12,000 rpm for 10 min, and the supernatant was transferred to a 1.5-ml tube. All the procedures were carried out under green light. Total chlorophyll contents (chla + chlb) were measured in a spectrophotometer, and the amount was calculated using the equation Chlorophyll = 22.12 OD_{652} + 2.71 OD_{665} (Porra et al. 1989).

Pchlide determination

Rice seedlings were grown in darkness for 7 d or exposed to light for an additional 10 min. Ten seedlings were collected and homogenized in 500 µl of ice-cold 80% acetone and incubated in darkness for 4 h. After spin at 5,000 × *g* for 5 min, 150 µl of the supernatant was mixed with 350 µl of glycol. Pchlide and chlorophyllide fluorescence was excited at 440 nm and scanned from 600 to 700 nm by a fluorescence spectrophotometer (Hitachi) at room temperature.

ALA level determination

Plants were grown in the dark for 7 d. The content of ALA was determined as previously described (Goslings et al. 2004). In brief, plant samples were homogenized and resuspended in 500 µl of 20 mM potassium phosphate buffer (pH 6.8). After spin at 16,000× *g* at 4°C for 10 min, 400 µl supernatant was transferred to a new tube and mixed with 100 µl of ethyl acetoacetate followed by

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boiling for 10 min. The solution was mixed with 500 μl Ehrlich's reagent (0.2 g p-dimethylaminobenzaldehyde, 8.4 ml acetic acid, 1.6 ml 70% perchloric acid) and centrifuged for 5 min. The supernatant was transferred to a cuvette and measured at 553 nm in a spectrophotometer. ALA content was calculated by a coefficient = 7.45×10^4/mol/cm.

H_2DCFDA fluorescence

H_2DCFDA (2′,7′-dichlorodihydrofluorescein diacetate) fluorescence was used to monitor the production of total ROS as previously described (Tang et al. 2012). Etiolated rice seedlings were illuminated with light for 2 h and leaves were incubated with 10 μM H_2DCFDA in 10 mM Tris-HCl, pH 7.2 solution, for 10 min at room temperature. Samples were observed on a fluorescence microscope and H_2DCFDA fluorescence was captured by a charge-coupled device camera (Leica).

SOSG fluorescence

Rice seedlings were grown in darkness for 7 d and then exposed to light for an additional 30 min. The leaves were immersed in a solution of 10 μM SOSG (Invitrogen) in 50 mM phosphate buffer, pH7.5, for 2 h in darkness and then transferred to light for 3 h. Imaging of singlet oxygen production was captured using a laser scanning confocal microscope (Leica) as described (Flors et al. 2006).

RT-qPCR

Rice total RNA was extracted by RNA extraction kit (Tiangen, Beijing China) and the first-strand cDNA was synthesized by reverse transcriptase (Invitrogen). Real-time PCR was performed using the cDNA samples and the SYBR Premix ExTaq kit (Takara) on a LightCycler 480 (Roche) following the manufacturer's instructions. Three biological replicates were performed for each sample and the expression levels were normalized to those of *OsUBQ5* control. PCR primer sequences are shown in Supplementary Table S1.

Plasmid construction

The open reading frames of *OsFLU1*, *OsFLU2* and *OsPIL14* were amplified from rice cDNA and the promoter sequence of *OsFLU1* was obtained from rice genomic DNA (ZH11 wild type). The primers with additional restriction sites were listed in Supplementary Table S1. The fragments were cloned into the pEASY-Blunt vector (TransGen, Beijing China), resulting in pEASY-OsFLU1/OsFLU2/OsPIL14/OsFLU1p, respectively. The coding sequences of *OsFLU1*, *OsFLU2* and *OsPIL14* were released from the corresponding pEASY vectors and cloned into pCAMBIA1302 (www.cambia.org/daisy/cambia/585), generating UBQp:OsFLU1-GFP (OsFLU1OE), UBQp:OsFLU2-GFP (OsFLU2OE), and UBQp:OsPIL14-GFP (OsPIL14OE), respectively. The UBQ promoter in OsFLU1OE was replaced by OsFLU1 promoter to generate OsFLU1p:OsFLU1. The coding sequence of *OsFLU1* was inserted into pSAT6-AFP-N1 (Tzfira et al. 2005) to produce pSAT6-OsFLU1.

Plant transformation

The binary constructs were electroporated into *Agrobacterium tumefaciens* strain EHA105 (for rice) and GV3101 (for *Arabidopsis*). *OsFLU1p:OsFLU1* was introduced into the *oxp* mutant and *OsPIL14OE* was transferred to Zhonghua 11 (ZH11) background. *OsFLU1OE* and *OsFLU2OE* were introduced into the *Arabidopsis flu* mutant via the floral dip method. Transgenic plants were selected on MS plates in the presence of 50 mg/l kanamycin or hygromycin. Homozygous lines were used in all experiments.

Protein localization in protoplasts

Rice protoplasts were isolated from 10-day-old seedlings as previously described (Bart et al. 2006). Transformation and transient expression assay were performed according to a previous method (Xu et al. 2016). The pSAT6-OsFLU1 plasmid was transformed into rice protoplasts and incubated in the dark at 28°C for 16 h before examination. GFP and chlorophyll auto-fluorescence imaging were visualized using a laser scanning confocal microscope (Leica).

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed according to the method from Xu et al. (2016). In brief, recombinant GST or GST-OsPIL14 protein were expressed and purified from *Escherichia coli*. The synthetic DNA oligonucleotide probes were labeled with biotin and incubated with GST-OsPIL14 or GST proteins in the presence or absence of excess unlabeled competitors for 20 min at room temperature. The DNA–protein complexes were separated on 6.5% nondenaturing polyacrylamide gels and transferred to a positive nylon membrane. Finally, the protein–DNA signals were visualized by chemiluminescence using the LightShift Chemiluminescent EMSA Kit (ThermoFisher).

Chromatin immunoprecipitation

ChIP assays were carried out according to modified method from Saleh et al. (2008). Two grams of 7-day-old etiolated *OsPIL14OE* and ZH11 seedlings were incubated in 37 ml cross-link buffer (0.4 M sucrose, 10 mM Tris–HCl pH 8, 1 mM PMSF, 1 mM EDTA and 1% formaldehyde) followed by vacuum for 30 min. Cross-link was stopped by the addition of 2.5 ml of 2 M glycine with vacuum for 5 min. Chromatins were isolated in 25 ml nuclei isolation buffer (0.25 M sucrose, 15 mM PIPES, pH 6.8, 5 mM $MgCl_2$, 60 mM KCl, 15 mM NaCl, 1 mM $CaCl_2$, 0.9% Triton X-100, 1 mM PMSF, one-tablet of protease inhibitor cocktail). The pellets were resuspended with 500 μl of nuclei lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% SDS, 0.1% sodium deoxycholate, 1% Triton X-100, one-tablet of protease inhibitor cocktail) and sonicated with a bioruptor for 30 min on ice (30 s on/30 s off cycles). Take about 100 μl chromatin as input control. The chromatin samples were precipitated with GFP antibody at 4°C overnight with slight rotation and the protein G beads used to collect the immunoprecipitated complexes. Samples were incubated with lysis buffer at 65°C for 4 h and the proteins were digested with 14 mg/ml proteinase K. The DNA fragments were recovered and quantified by quantitative PCR using primers as described (Supplementary Table S1). Relative enrichment was normalized to the value of input.

Phylogenetic analysis

Three *FLU* amino acid sequences (AT3G14110.1 in *A. thaliana*, LOC_Os01g32730.1 and LOC_Os02g37470.1 in *O. sativa*) were used as query to search against the other 57 plant species implemented in Phytozome (https://phytozome.jgi.doe.gov/) and *Picea abies* (Nystedt et al. 2013), with the method previously reported (Guo 2013). Protein sequences were aligned and the maximum likelihood tree was constructed based on the Jones–Taylor–Thornton (JTT) model using Mega 6 with 1000 replicates of bootstrap (Tamura et al. 2013).

Supplementary Data

Supplementary data are available at PCP online.

Acknowledgments

We are grateful to Dr. Chanhong Kim for providing the Arabidopsis *flu* mutant.

Funding

The Ministry of Agriculture of China [2016ZX08009-003]; the National Key Research and Development Program of China [2016YFD0100405 to R.L.]; the Strategic Priority Research Program of the Chinese Academy of Sciences [XDA08020103 to Y.G.].

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Disclosures

The authors have no conflicts of interest to declare.

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