The OsABCI7 transporter interacts with OsHCF222 to stabilize the thylakoid membrane in rice

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Short title
OsABCI7 is indispensable for thylakoid stability

One-sentence summary
The OsABCI7 transporter interacts with OsHCF222 at thylakoid membranes to regulate reactive oxygen species homeostasis, thereby maintaining thylakoid membrane stability.

Author contributions
YH and JW designed the experiments; YH, YS, XZ, XX, HW, LL, ZZ, HS and ZW performed the experiments. YH analyzed the data. YH and JW wrote the manuscript.
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ABSTRACT

The thylakoid membrane is a highly complex membrane system in plants and plays crucial roles in the biogenesis of the photosynthetic apparatus and plant development. However, the genetic factors involved in chloroplast development and its relationship with intracellular metabolites are largely unknown. Here, a rice (*Oryza sativa*) chlorotic and necrotic leaf 1 (*cnl1*) mutant and map-based cloning revealed that a single base substitution followed by a 6-bp deletion in the *ATP-binding cassette transporter 1 family member 7* (*OsABCI7*) resulted in chlorotic and necrotic leaves with thylakoid membrane degradation, chlorophyll breakdown, photosynthesis impairment and cell death in *cnl1*. Furthermore, the expression of *OsABCI7* was inducible under lower temperatures, which severely affected *cnl1* chloroplast development, and etiolated *cnl1* seedlings were unable to recover to a normal green state under light conditions. Functional complementation and overexpression showed that *OsABCI7* could rescue the *cnl1* chlorotic and necrotic phenotype. *OsABCI7* interacted with HIGH CHLOROPHYLL FLUORESCENCE 222 (*OsHCF222*) to regulate cellular reactive oxygen species (ROS) homeostasis for thylakoid membrane stability. *OsABCI7* localized to thylakoid membranes while *OsHCF222* targeted to endoplasmic reticulum and chloroplasts. Exogenous application of ascorbic acid eased the yellowish leaf phenotype by increasing chlorophyll content and alleviating ROS stress in *cnl1*. Unlike *cnl1*, the CRISPR/Cas9-mediated *OsHCF222* knockout lines showed chlorotic leaves but were seedling lethal. Our results provide insight into the functions of ABC transporters in rice, especially within the relationship between ROS homeostasis and stability of thylakoid membranes.
INTEGRATION

In plant leaves, mature chloroplasts develop from proplastids, a type of colorless and small precursor, with surrounded inner and outer envelopes and rudimentary inner membranes. These rudimentary membranes elaborate into an inerratic thylakoid membrane system during leaf development. As the photosynthetic machinery, the thylakoid membrane complex is composed of four major protein complexes: photosystem II (PSII), cytochrome b6f (Cytb6f), photosystem I (PSI) and ATP synthase. Thylakoid membrane biogenesis is involved in a variety of processes, such as gene transcriptional regulation, post-translational regulation and modification, and protein complex assembly (Lyska et al., 2013). In chloroplasts, the thylakoid membrane is regularly stacked to increase the membrane area for a higher photosynthetic efficiency, and a well-developed thylakoid membrane system is also closely associated with chloroplast development (Kobayashi et al., 2007). To date, many genes exhibiting crucial regulatory roles in chloroplast development under low temperatures have been identified (Wang et al., 2016; Sun et al., 2017; Liu et al., 2018; Cui et al., 2019), while genetic factors affecting the relationship between chloroplast development and intracellular metabolites are largely unknown.

Endogenous reactive oxygen species (ROS) are mainly produced at the PSI and PSII reaction centers in thylakoids. Usually, an oxygen molecule obtains an electron and is reduced to superoxide anion (O$_2^-$), and its disproportionation by a superoxide dismutase (SOD) produces hydrogen peroxide (H$_2$O$_2$) (Asada et al., 1974). To protect chloroplasts from the direct damage by H$_2$O$_2$, the newly produced thylakoidal H$_2$O$_2$ is promptly scavenged, hence, H$_2$O$_2$ is not accumulated in intact chloroplasts (Asada, 2006). In this process, H$_2$O$_2$ is reduced into water by ascorbate acid (AsA) catalyzed by the plant- and algae-specific H$_2$O$_2$-scavenging enzyme, ascorbate peroxidase (APX) (Asada, 1992). Additionally, catalase (CAT) can directly reduce H$_2$O$_2$ into water. Once the balance of ROS production and scavenging is broken, oxidative damage induced by ROS preferentially occurs in chloroplast thylakoids, leading to chlorophyll degradation as well as DNA damage (Ahmad et al., 2010; Gill & Tuteja, 2010; Soares
et al., 2016). Besides biotic and/or abiotic stresses, mutations of relevant genes are important causes for inducing ROS bursts, resulting in various morphological variations, such as premature senescence, yellowing and spotted leaves (Huang et al., 2016; Xu et al., 2018; Sathe et al., 2019; Wang et al., 2019).

The ATP-binding cassette (ABC) transporter is an ancient and large protein superfamily consisting of eight major subgroups (ABCA–ABCH). In plants, the members of ABCA–ABCG subgroups are widely distributed while the ABCH members are absent (Garia et al., 2004). ABC proteins, with six conserved typical motifs including ABC signature, Walker-A, Walker-B, D-loop, H-loop and Q-loop sequence, are well known for their functions in intracellular/extracellular substrate transporting (Huang et al., 2009). Not only limited to the transport of ions, lipids, monosaccharides, hormone secondary metabolites and xenobiotics, recent studies have demonstrated that ABC proteins are also involved in plant defense response, pathogen resistance and male reproduction (Matsuda et al., 2012; Zhao et al., 2015; Garroum et al., 2016). Plants share the specific bacterial-type ABC transporters with prokaryotes, designed as ABCI subgroup. In Arabidopsis (Arabidopsis thaliana), both chloroplast-localized AtABCI10 and AtABCI11 (AtNAP14) proteins participate in the regulation of chloroplast metal homeostasis, and their corresponding T-DNA mutants are totally devoid of chlorophylls with severely deformed leaf structures, aberrant chloroplasts and defective photosynthetic capacity (Shimoni-Shor et al., 2010; Voith von Voithenberg et al., 2019). In rice (Oryza sativa), OsABCI8 is indispensable for chloroplast development by engaging iron transportation and homeostasis (Zeng et al., 2017). OsABCI12 (STAR2) interacts with STAR1 to form a complex responsible for the transport of UDP-glucose and detoxification of aluminum (Huang et al., 2009).

The rice high-chlorophyll-fluorescence (hcf) mutants are unable to utilize absorbed light efficiently (Meurer et al., 1996). Due to lack of photosynthetic autotrophy, most hcf mutants are seedling-lethal yet able to survive on sucrose-supplemented medium (Schult et al., 2007; Link et al., 2012; Hartings et al., 2017). Here, we isolated an ABC transporter I family member 7 (OsABCI7) which interacts with HIGH CHLOROPHYLL FLUORESCENCE 222 (OsHCF222) to
maintain thylakoid membrane stability via regulating ROS homeostasis in rice.

RESULTS

cnl1 is defective in plant growth and development

The chlorotic and necrotic leaf 1 (cnl1) mutant was isolated from an EMS-induced indica rice Zhongjian 100 (wild-type, WT) mutant bank. Under natural field conditions, cnl1 exhibited chlorotic and necrotic phenotypes on older leaves with the vestigial thylakoid membrane ultrastructure and lower levels of chlorophyll contents approximately 40 days after sowing (Fig. 1A–D, S1A–C). Due to abnormal chlorophyll degradation, cnl1 showed significantly compromised net photosynthesis rate, higher intercellular CO2 concentration and transpiration rate compared with WT (Fig. S1D–G).

In addition, cnl1 also exhibited a poorer performance in major agronomic traits compared with WT (Fig. 1E–I). The II and III internode lengths were remarkably decreased in cnl1 compared to WT at the heading stage (Fig. 1J, 1K), and microscope observation directly revealed that the shorter stature of cnl1 resulted from the reduced length of cells in the stems (Fig. 1L–N). These results indicated that the cnl1 mutation resulted in multiple deficiencies in plant growth and development.

CNL1 encodes rice ABC transporter I family member 7 (OsABCI7)

To determine the genetic control of the cnl1 phenotype, we crossed cnl1 with WT, two japonica cultivars (ReYan 1 and ORO, respectively). As shown in Table 1, the WT F1 plants and expected mendelian 3:1 (WT:mutant) ratios in F2 populations indicated monogenic and recessive genetic control of the mutant trait.

We selected 755 F2 mutant-type individuals derived from the cross cnl1×ReYan 1 to map the mutation. The OsABCI7 locus preliminarily mapped to the long arm of chromosome 11 between the markers RM287 and RM229, and we narrowed the location to a 116-kb region containing 12 open reading frames (ORFs) (Fig. 2A; Table S1). Sequencing of these 12 ORFs revealed a single nucleotide substitution (C
A) followed by a 6 base-pair deletion at the junction of the 8th exon and 8th intron of *LOC_Os11g29850*, resulting in a 17-bp alternative splicing deletion at the end of the 8th exon, which finally led to a frame shift and premature stop codon (Fig. 2B, S2, S3A). According to the Rice Annotation Project Database (https://rapdb.dna.affrc.go.jp/), *LOC_Os11g29850* belongs to the ABC transport superfamily ABCI subgroup member 7 (referred to *OsABCI7*). The structural changes of OsABCI7 were intuitively visible by modeling the three-dimensional structures of WT (OsABCI7) and mutant (ΔOsABCI7) proteins (Fig. S3B). Additionally, the derived cleaved amplified polymorphic sequences (dCAPS) analysis was performed to confirm the mutation in the genomic DNA (gDNA), and the agarose electrophoresis verified the presence of the 17-bp deletion in the complementary DNA (cDNA) of ΔOsABCI7 (Fig. 2C, 2D). OsABCI7 encodes an ATP-binding cassette transport protein and contains all typical conserved motifs of ABC transporters, namely, the Walker-A, Q-loop, ABC signature, Walker-B, D-loop, and H-loop motifs (Fig. S2).

To confirm whether the mutation was responsible for the *cnl1* phenotype, we performed a complementation test by introducing 5.9-kb genomic DNA containing the promoter and the full genomic coding region of OsABCI7 into *cnl1*-derived calli. All 15 independent positive T0 complementary lines showed the normal green phenotype, mimicking the WT (Fig. 2E). We also overexpressed the CDS of OsABCI7 in *cnl1* using the vector pCAMBIA1300-UBI (Zhang et al., 2018), which confers maize (*Zea mays*) Ubiquitin 1 promoter and the coding region for an N-terminal hemagglutinin (HA) epitope tag to the CDS (Fig. S4A, S4B), and the mutant phenotype was rescued in all 24 T0 positive overexpression lines (Fig. 2E). These results demonstrated that ΔOsABCI7 was responsible for the *cnl1* phenotype and the CDS of OsABCI7 encoded a functional protein. Compared with WT, the expression of OsABCI7 was significantly downregulated in *cnl1* but recovered to WT levels in complementary plants, and the expression level of OsABCI7 in overexpression plants was upregulated by about 6-fold in WT (Fig. 2F). To investigate whether the low expression of OsABCI7 would lead to the *cnl1* phenotype,
we also performed RNAi analysis of OsABCI7 and obtained 21 T₀ positive lines which exhibited significantly down-regulated OsABCI7 expression with normal WT phenotype (Fig. S4D–H). We therefore concluded that the low OsABCI7 expression was unable to cause cnl1 phenotype, and the rescues of cnl1 by complementation and overexpression were due to the recovered OsABCI7 function and not the recovered OsABCI7 expression. Furthermore, similar expression levels of OsABCI7 were detected from the tip to the base of the yellowing flag leaves, indicating low relevance between OsABCI7 expression and natural leaf yellowing (Fig. S4C).

Leaf yellowing and senescence are largely accompanied with ROS accumulation (Buchanan-Wollaston et al., 2003). Thus, we first measured the levels of ROS, including H₂O₂ and O₂⁻, and found that ROS were higher in cnl1 compared with WT (Fig. 2G, 2H). We then detected the activities of ROS scavenging enzymes and the contents of malondialdehyde (MDA) and soluble proteins. The activities of APX and CAT, two key H₂O₂ scavenging enzymes, as well as soluble protein contents, were significantly lower in cnl1 than those of WT (Fig. 2I, 2J, S5A). In contrast, the level of MDA, an indicator of ROS-induced lipid peroxidation, was significantly higher in cnl1 than that of WT (Fig. 2L). Peroxidase (POD) activity was higher in cnl1 compared with WT while SOD activity was similar between the two genotypes (Fig. 2K, S5B). These results suggested that ΔOsABCI7 disrupted the balance between ROS production and scavenging. Moreover, ROS accumulation in cnl1 was also confirmed by nitrotetrazolium blue chloride (NBT) and 3,3’-diaminobenzidine (DAB) staining (Fig. S6C). As expected, the ROS contents and ROS scavenging enzyme activities in the complementary and overexpression line recovered to WT levels (Fig. 2G–L). In addition, chlorophyll fluorescence and chlorophyll levels in the complementary and overexpression lines also recovered to the WT levels (Fig. S6A, S6B; Table S2). Taken together, our results demonstrated that both the normal expression and overexpression of OsABCI7 in the cnl1 background could fully rescue the physiological and biochemical defects of cnl1. Notably, in contrast to WT, the overexpression plants exhibited prominently decreased H₂O₂ and O₂⁻ contents, and increased APX activities (Fig. 2G–I). We speculated that ΔOsABCI7 was involved in
chloroplast-specific ROS scavenging since the H$_2$O$_2$ scavenging largely relies on APX in chloroplasts (Asada, 2006).

**Table 1** Genetic control of the *cnl1* phenotype

| Cross          | F$_1$   | No. F$_2$ individuals | $\chi^2$ (3:1) |
|----------------|---------|-----------------------|---------------|
|                |         | Wild-type  | Mutant-type  |               |
| *cnl1*×WT     | Wild-type | 127         | 34          | 1.29          |
| *cnl1*×ReYan 1| Wild-type | 923         | 283         | 1.51          |
| *cnl1*×ORO    | Wild-type | 399         | 112         | 2.59          |

**DNA damage and DNA damage response are induced in *cnl1***

The programmed cell death (PCD) phenotype was detected in *cnl1* by trypan blue staining (Fig. S6C). Since PCD associates with DNA breakdown, we conducted the terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay to determine the nuclear DNA fragmentation in the flag leaves of WT and *cnl1*. Expectedly, few TUNEL positive signals were detected in WT, yet numerous nuclei exhibited TUNEL positive signals in *cnl1* (Fig. S7). Furthermore, we performed comet assay (single cell gel electrophoresis assay), a simple and effective method for evaluating DNA damage in cells (Speit & Hartmann, 2006), to test DNA damage in WT and *cnl1*. Consistent with the TUNEL assay, significantly increased DNA damage was found in *cnl1* compared with WT (Fig. 3A–C).

DNA repair and DNA replication occur in response to DNA damage. Indeed, 8 upregulated-marker genes associated with DNA repair and replication were found in *cnl1* (Fig. 3D). Poly (ADP-ribose) polymerase (PARP), ataxia telangiectasia mutated (ATM) and ATM-rad3-related (ATR) are classified to the ATM/ATR-mediated signaling response (Zhou & Elledge, 2000; Culligan et al., 2006). DNA repair protein *Rad51* (RAD51A2 and RAD51C) belong to the RecA/Rad1 protein family, which is required for the homologous recombination (HR) repair pathway (Durrant et al., 2007). *RNRL1* and *RNRS1* encode the large and small subunits of ribonucleotide
reductase (RNR), respectively, regulating the deoxyribonucleotide production rate for DNA synthesis and repair (Yoo et al., 2009). *RNA-dependent RNA polymerase 6* (*RDR6*) acts in the biogenesis of multiple small RNAs (Song et al., 2012). In conclusion, Δ*OsABCI7* induced PCD, severe DNA damage and up-regulated expression of marker genes associated with DNA repair and replication.

*OsABCI7* is constitutively expressed and *OsABCI7* localizes to the thylakoid membrane

To validate if *OsABCI7* localized to chloroplasts as predicted by TargetP 1.1 Server (http://www.cbs.dtu.dk/services/TargetP/), we fused the full CDS of *OsABCI7* to the N-terminus of GFP driven by the CaMV35S promoter and transiently expressed the construct in rice protoplasts. The *OsABCI7*-GFP fusion protein indeed localized to chloroplasts (Fig. 4A). Notably, the Δ*OsABCI7*-GFP fusion protein also localized to chloroplasts, indicating the mutation of *OsABCI7* did not change its chloroplast localization (Fig. 4A). Additionally, we truncated constructs to analyze the chloroplast transit peptide (cTP), and nearly all fusion constructs with varying lengths of *OsABCI7*, such as *OsABCI7*1–30aa, apparently localized to the chloroplast except for *OsABCI7*30–276aa and *OsABCI7*50–276aa (Fig. 4A). These demonstrated that the *OsABCI7*1–30aa may contain a cTP indispensable for *OsABCI7* chloroplast targeting.

To detect the precise localization of *OsABCI7*, we performed immunoblot analysis using total proteins, chloroplasts, thylakoid fractions and stromal fractions extracted from *OsABCI7* overexpression plants. A 32 kDa protein was absent in stromal fractions while present in total proteins, chloroplasts and thylakoid membrane fractions, confirming the thylakoid membrane localization of *OsABCI7* (Fig. 4B).

We then detected *OsABCI7* expression levels in different organs using RT-qPCR. *OsABCI7* was expressed in all organs at different growth stages (Fig. 4O). Consistent with the RT-qPCR results, GUS expression was detected in all organs of *OsABCI7*pro::GUS transgenic plants (Fig. 4C–N), further supporting the constitutive expression of *OsABCI7*. 
OsABCI7 is required for chloroplast development under cold stress and light-dependent chlorophyll synthesis

Chloroplast development and chlorophyll synthesis are affected by light and temperature in rice (Wang et al., 2016; Sun et al., 2017; Wang et al., 2017). WT and cnl1 were planted in a growth chamber under different temperature conditions. At 30 °C, cnl1 plants displayed a WT-like phenotype though the chlorophyll content of cnl1 was significantly lower than that of WT (Fig. 5A, S8A). In contrast, cnl1 showed pale green leaves at 25 °C, and the pale green phenotype aggravated at 20 °C with rapid decrease of chlorophyll contents compared with WT (Fig. 5B, 5C, S8A). We then explored the kinetic expression of OsABCI7 in WT and cnl1 under low temperature treatment at 10 °C, and OsABCI7 expression increased rapidly by 2-fold after 2 h, by 2.5-fold after 4 h treatment, and recovered to the control level after 6 h of treatment (Fig. 5D), while OsABCI7 expression increased 2-3 fold from 4 to 10 h treatment in cnl1 (Fig. S8B). The results indicated that OsABCI7 transcription was induced by low temperature and likely acted as a signal factor responding to cold stress.

We also investigated the ultrastructure of chloroplasts in mesophyll cells of WT and cnl1 under different temperatures using Transmission electron microscope (TEM). At 30 °C, intact chloroplast structure and well-formed grana thylakoids were observed in WT and cnl1 (Fig. 5E). In contrast, distinctly degraded chloroplasts and reduced grana thylakoids were found in cnl1 at 25 °C and 20 °C compared with WT (Fig. 5F, 5G). These suggested that the developmental defects associated with the pale green phenotype in cnl1 resulted from chloroplast breakdown induced by low temperatures. Furthermore, OsABCI7 was essential for light-dependent chlorophyll synthesis during the greening of etiolated seedlings (Fig. 5H–K), while OsABCI7 transcription in WT and cnl1 was not relevant to the greening process (Fig. 5L). Collectively, the results suggested that OsABCI7 was involved in light-dependent chlorophyll synthesis and required for chloroplast development under low temperature.

Thylakoid membrane complexes are impaired in cnl1
Thylakoid membrane biogenesis is regulated and assisted by many plastome- and nucleus-encoded genes, and thylakoid membrane protein complexes are composed of PSII, PSI, Cyb6f complex, and ATP synthase (Hartings et al., 2017). Upon the low photosynthetic capacity in *cnl1* (Fig. S1D–G; Table S2), the accumulation of major components of thylakoid membrane protein complexes in WT and *cnl1* were determined by immunoblot to test whether thylakoid membrane complexes were impaired in *cnl1*. We tested the core subunits of PSII and PSI complexes (plastid encoded: PsaA/B and PsbA/D), nucleus-encoded light-harvesting antenna of PSI (LHCI) chlorophyll a/b-binding proteins Lhca1–4, and nucleus-encoded LHCII type II chlorophyll a/b-binding protein Lhcb2. A pronounced reduction was observed for the levels of all these subunits except for PsaB, Lhca2 and PsbA (Fig. 6A). Additionally, the plastome-encoded NAD(P)H dehydrogenase subunit 5 (NdhF) and NAD(P)H-quinone oxidoreductase subunit H (NdhH), as well as nucleus-encoded plastocyanin (PC), β-subunit (AtpB) and epsilon-subunit (AtpE) of ATP synthase, showed notably decreased levels in *cnl1* compared with WT (Fig. 6A). The observed reduction of almost all examined photosynthetic proteins in *cnl1* might be caused by a generally impaired rate of chloroplast protein synthesis in the mutant. Besides the thylakoid membrane proteins, soluble chloroplast proteins also should be affected by this defect. Hence, we further performed polyacrylamide sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to analyze the total proteins extracted from WT and *cnl1* flag leaves. As a soluble representative of total proteins, the level of large subunit of Rubisco (RbcL) was much lower in *cnl1* than that of WT (Fig. 6B). Decreased accumulation of the abundant RbcL protein indicated that the reduction of different thylakoid membrane proteins in *cnl1* is the consequence of a generally defective synthesis rate of chloroplast proteins. Taken together, the results demonstrated that the thylakoid membrane protein complexes were impaired in *cnl1*.

We also detected the plastid- and nucleus-encoded transcripts of thylakoid membrane proteins by RT-qPCR, and 15 out of 25 thylakoid membrane protein-encoding genes were significantly upregulated, while *PsaF*, *Rieske*, *AtpH* and *AtpI* were significantly downregulated in *cnl1* compared to WT (Fig. 6C). Intriguingly,
the immunoblot analysis showed that nearly all detected thylakoid membrane proteins were reduced in cnl1, which was not consistent with the RT-qPCR results as most genes were upregulated. We speculated that the impaired thylakoid membrane protein accumulation was likely caused by a posttranslational defect in cnl1. The abnormal mRNA levels of photosynthesis-related genes might also imply a feedback mechanism upon the posttranslational downregulation of thylakoid membrane proteins (Fig. 6D).

OsABCI7 interacts with OsHCF222 in chloroplasts

To determine the functional pathway of OsABCI7, we performed a yeast two-hybrid (Y2H) assay to screen for proteins that interact with OsABCI7. A total of 132 colonies were found positive by expressing the β-galactosidase (lacZ) reporter gene. Among 67 positive colonies sequenced, 9 corresponded to rice LOC_Os03g30092. In the rice database (http://rice.plantbiology.msu.edu/), we found an orthologous gene of LOC_Os03g30092 in Arabidopsis named HIGH CHLOROPHYLL FLUORESCENCE 222 (AtHCF222), hence, we referred to rice LOC_Os03g30092 as OsHCF222. To verify the localization of OsHCF222, GFP fused to the C-terminus of OsHCF222 was transformed into rice protoplasts. Confocal microscopy showed GFP signals of OsHCF222-GFP fusion proteins co-localized both with the red auto-fluorescent signals of chloroplasts and mCherry-HDEL signals (Fig. S9), revealing that OsHCF222 dually targeted to the chloroplasts and endoplasmic reticulum (ER), consistent with the previous localization of AtHCF222 (Hartings et al., 2017).

The conserved H-loop motif was deleted in ΔOsABCI7 (Fig. 7A, S2). In yeast (Saccharomyces cerevisiae) cells and a bimolecular fluorescence complementation (BiFC) assay, we observed the interaction between OsABCI7 and OsHCF222, but no interaction between ΔOsABCI7 and OsHCF222 (Fig. 7B, 7D, S10). We therefore speculated that the H-loop motif of OsABCI7 was required for the interaction. Using Y2H and BIFC assay, we also examined the interaction between OsABCI7 with/without H-loop motif and OsHCF222. The results exhibited that both OsABCI7
(+ H-loop) and OsABCI7 (− H-loop) could not interact with OsHCF222 (Fig. 7B, 7D, S10). These suggested that the C-terminus of OsABCI7 was essential for the interaction between OsABCI7 and OsHCF222. Additionally, this interaction was furtherly confirmed by co-immunoprecipitation (Co-IP) and BiFC assay in vivo, indicating that OsABCI7 interacts with OsHCF222 in rice chloroplasts and Nicotiana benthamiana cells (Fig. 7C, 7D, S10). All these demonstrated the interactions of OsABCI7 and OsHCF222 in vivo and vitro.

OsHCF222 is required for biogenesis of the thylakoid membrane

To confirm whether both OsABCI7 and OsHCF222 are essential for chloroplast development, we used CRISPR/Cas9 editing to generate Cr-OsABCI7 and Cr-OsHCF222 mutants in the cultivar Kitaake background and identified 13 Cr-OsABCI7 and 18 Cr-OsHCF222 knockout plants (Fig. 8A, 8B). Expectedly, all Cr-OsABCI7 knockout plants showed similar phenotypes to cnl1 with degraded chlorophyll contents (Fig. 8C, 8E, S11), while all Cr-OsHCF222 knockout plants exhibited severe growth retardation and much paler leaf color compared with Kitaake, and, strikingly, homozygous Cr-OsHCF222 plants were lethal approximately 12 days after germination (DAG) under conditions of soil cultivation (Fig. 8D, 8F). Thus, all progenies of Cr-OsHCF222 homozygous plants were obtained from Cr-OsHCF222 heterozygous plants. Among 16 and 12 T1 plants randomly-chosen from two independent heterozygous Cr-OsHCF222 lines, four and three homozygous plants were found, respectively, showing a mendelian 3:1 (wild-type:mutant) ratio that indicated a single gene recessive nature of OsHCF222. On sucrose-supplemented half Murashige and Skoog (MS) culture medium, the growth of Cr-OsHCF222 plants was slightly retarded and the leaves were paler than those of Kitaake, whereas on sucrose-free half MS medium, the Cr-OsHCF222 plants showed more severe phenotypes than in sucrose-supplemented half MS medium (Fig. S12A). These indicated that the CRISPR/Cas9-mediated mutations of OsHCF222 resulted in non-autotrophic seedling-lethal mutants.

The levels of thylakoid proteins in the representative Cr-OsABCI7-2 and
Cr-OsHCF222-1 lines were compared by immunoblot analysis. In both CRISPR/Cas9 mutants, the levels of Lhcb2, Lhca3 and Lhca4 were similar to those of Kitaake, while pronounced protein reductions were found for Lhca1, Lhca2, Ndhl and AtpB in Cr-OsHCF222-1 compared with Kitaake and Cr-OsABCI7-2 (Fig. 8G). In contrast, PsbD and PsaB were highly accumulated in Cr-OsABCI7-2 and Cr-OsHCF222-1, respectively (Fig. 8G). Compared to Kitaake, the expression of photosynthesis-related genes varied in Cr-OsABCI7-2 but were all downregulated in Cr-OsHCF222-1 (Fig. S12b). Moreover, we also analyzed the expression levels of 25 thylakoid membrane genes in both mutants, and 22 genes except PsaF, Lhcb2 and Rieske were up-regulated in Cr-OsABCI7-2 compared with Kitaake, and a similar upregulated expression pattern was detected in Cr-OsHCF222-1 compared with Kitaake (Fig. 8H). This suggested that the higher transcriptional levels of thylakoid membrane genes in Cr-OsABCI7-2 and Cr-OsHCF222-1 did not contribute to the synthesis of thylakoid proteins. Considering OsABCI7/OsHCF222 interaction occurs in chloroplasts, the main ROS-generating organelle, we measured the H₂O₂ contents and found both Cr-OsABCI7-2 and Cr-OsHCF222-1 had significantly increased H₂O₂ levels compared with Kitaake (Fig. 8I, 8J).

**Exogenous antioxidants alleviate the cnll phenotype**

Considering the significant reduction of ROS levels in OsABCI7 overexpression plants and significant accumulation in Cr-OsHCF222 knockout plants (Fig. 2G, 2H, 8J), we hypothesized that OsABCI7 may interact with OsHCF222 to regulate the balance of ROS production and scavenging in chloroplasts. To demonstrate this, cnll seedlings were treated with the exogenous antioxidants dimethyliourea (DMTU) and ascorbic acid (AsA) (Asada, 2006; Wang et al., 2018). Under normal hydroponic conditions, cnll exhibited chlorophyll degradation and H₂O₂ accumulation compared to WT (Fig. S13), while the cnll seedlings treated with 1mM exogenous AsA displayed an eased yellowish phenotype with significantly alleviated chlorophyll degradation (Fig. 9A, 9B). We then measured the contents of H₂O₂ and O₂⁻ in cnll under different antioxidant treatments. Higher levels of H₂O₂ and O₂⁻ were both...
detected in untreated cnl1 seedlings compared with cnl1 seedlings treated with 1 mM
AsA (Fig. 9C, 9D). As for DMTU treatment, only O$_2^-$ but not H$_2$O$_2$ quenching was
observed in treated cnl1 seedlings (Fig. 9A–D). Upon these results, we inferred that
excessive ROS was responsible for the cnl1 phenotype, and the exogenous AsA
alleviated the yellowish phenotype of cnl1 by relieving the ROS stress.

DISCUSSION

OsABCI7 functions in chloroplast development but its function may differ
between monocots and dicots

Many leaf pigmentation mutants are usually associated with chloroplast
development in rice (Wang et al., 2016; Lv et al., 2017; Sun et al., 2017; Liu et al.,
2018; Cui et al., 2019). In this study, we isolated the constitutively expressed
OsABCI7, which encodes a chloroplast-localized ABC transporter protein (Fig. 4).
Due to the mutation of OsABCI7, cnl1 plants mainly showed chlorotic and necrotic
leaves with chloroplast structure disintegration, chlorophyll degradation and
photosynthesis impairment, finally leading to the deteriorated yield performance (Fig.
1A–I, S1). The chloroplast development of temperature-sensitive mutants, like tsv,
wsl5 and dua1, is easily hindered by low temperatures in rice (Sun et al., 2017; Liu et
al., 2018; Cui et al., 2019). For cnl1, we also observed yellowish leaves with
significantly decreased chlorophyll level and degradation of chloroplast ultrastructure
at 20 °C and altered mRNA levels of OsABCI7 in response to the low temperature
stress (Fig. 5A–G, S8). Unlike most temperature-sensitive rice mutants, ΔOsABCI7
leads to disabled light-dependent chlorophyll synthesis in cnl1 during the greening
process of etiolated seedlings (Fig. 5I–K). Taken together, we conclude that OsABCI7
plays a crucial role in chloroplast biogenesis, especially under low temperature stress
and in the greening process.

The homologue of rice OsABCI7, Arabidopsis AtABCI11 (AtNAP14),
participates in the regulation of chloroplast metal homeostasis and attaches to the
inner envelop membrane of the chloroplast; compared to the wild-type (Col-0), metal
elemental contents including iron (Fe), Zinc (Zn), molybdenum (Mo), copper (Cu) are higher, while manganese (Mn) is lower in Atabci11 mutant shoot tissues, whereas Fe and Zn contents in Atabci11 were lower than those of Col-0 in root tissues (Shimoni-Shor et al., 2010; Voith von Voithenberg et al., 2019). Hence, we determined the contents of metal elements, including magnesium (Mg), potassium (K), Fe, Cu and Zn in the shoot and root tissues of WT and cnl1. Inconsistent with the metal distribution of Atabci11 mutants, only significant differences in Mn (1.2-fold in shoot and 2.2-fold in root) and Cu (0.8-fold in shoot and root) were detected (Fig. S14). Mn is integral to photosynthetic electron transport, redox processes and ROS scavenging in plants, while excessive Mn is toxic to the photosynthetic apparatus and inhibits chloroplast protein and chlorophyll synthesis (Millaleo et al., 2013; Eisenhut et al., 2018). Cu is an important minor element found in chloroplasts as a cofactor with Cu/Zn-superoxide dismutase (Cu/Zn-SOD), and Cu/Zn-SOD contributes to the reduction of oxidative stress (Bowler et al., 1994; Cohu et al., 2009). The observed Mn overload and lower Cu concentration in cnl1 may explain the chlorotic and necrotic leaf phenotypes, which are likely due to the increased ROS stress generated by the over-accumulation of Mn and decreased levels of Cu/Zn-SOD. As a transporter, OsABCI7 plays an important role in regulating chloroplast metal homeostasis (Fig. S14). Based on this, we proposed that OsABCI7 could be a component of the chloroplast transporter complex. In addition, unlike the chloroplast inner envelop membrane-localized AtABCI11, OsABCI7 targets to the thylakoid membrane in chloroplasts (Fig. 4B). Considering the pea (Pisum sativum) samples used for the in vivo localization detection of AtABCI11 (Voith von Voithenberg et al., 2019), further studies need to be conducted in Arabidopsis to confirm the true in vivo localization of AtABCI11. In conclusion, these deviations of OsABCI7 and AtABCI11 might suggest different regulatory manners between monocots and dicots.

OsABCI7 is required for ROS-mediated thylakoid membrane stability

During the photosynthesis process at the thylakoid membrane, O₂ molecules are reduced to O₂⁻ by trapping electrons derived from water in PSI, and O₂⁻ is
disproportionated to H$_2$O$_2$, catalyzed by chloroplastic SOD (Asada, 2006). Both over-accumulated ROS and a disrupted ROS scavenging system were detected in cnl1 indicating the impairment of ROS homeostasis (Fig. 2G–K). As the consequence of over-accumulation of ROS, DNA fragmentation and DNA damage repair response activation were observed in cnl1, which exhibited an enhanced PCD manifested by trypan blue staining, TUNEL assay and comet assay (Figs 3, S6C, S7). Accumulating evidence also demonstrates that ROS and cell death usually occur together (Roy et al., 2012; Luo et al., 2013; He et al., 2018a; Qiu et al., 2019; Zafar et al., 2020).

ROS are extremely active and able to interact with many kinds of target molecules and metabolites, like DNA, proteins, lipids, pigments and other cellular molecules, resulting in a number of destructive cell processes (Ashraf, 2009). An accurate control of steady-state H$_2$O$_2$ concentration is critical to cell homeostasis, and H$_2$O$_2$ is directly scavenged by the antioxidant AsA with the catalysis of APX in healthy chloroplasts (Zhen et al., 2011; Ni et al., 2018). Notably, endogenous ROS contents of OsABCI7 overexpression plants were significantly lower than those of WT, which was well explained by the higher level of APX activity in overexpression plants compared to WT (Fig. 2G–I). We therefore speculated that OsABCI7 is closely associated with the APX-related ROS scavenging pathway. As a consequence of OsABCI7 disruption, newly produced ROS could not be eliminated effectively in APX-pathway, leading to over-accumulation of ROS in chloroplasts. Conversely, over-accumulated ROS directly gave rise to thylakoid membrane damage, chlorophyll breakdown and photosynthetic protein degradation, which may be the reason for the initiation of the cnl1 phenotype (Fig. 1A–D, 6A, 6B). The above hypothesis was further confirmed by the easing of the cnl1 phenotype under the treatment of exogenous antioxidant AsA (Fig. 9). In conclusion, these findings suggested that OsABCI7 is essential for ROS-mediated thylakoid membrane stability.

OsABCI7 interacts with OsHCF222 at the thylakoid membrane to regulate ROS homeostasis

HCF proteins are required for the formation of thylakoid membrane complexes
in plants (Schult et al., 2007; Link et al., 2012; Hartings et al., 2017). The Arabidopsis
AtHCF222 encodes a small protein homologous to the zinc-finger domain of
Escherichia coli chaperone DnaJ-like protein (Hartings et al., 2017). OsHCF222
shares 59% protein identity to AtHCF222 and is also characterized by eight conserved
cysteine-rich (CR) domains arranged in four CxxC motif clusters (Fig. S15). Between
the Cys residues of four CxxC motifs, two Zn(II) ions are coordinated to form a
topology with two C4-type zinc finger structures (Shi et al., 2005). As members of the
heat shock protein (HSP) family, DnaJ proteins serve as co-chaperones and involve in
different substrate bindings, playing important roles in multiple biotic and abiotic
stress responses (Liu & Whitham, 2013; Pulido & Leister, 2018).

Our results revealed physical interaction between OsABCI7 and OsHCF222 in
vitro and vivo (Fig. 7B–D, S10). AtHCF222 is a dually ER- and chloroplast-localized
protein and has been detected in leaf whole-cell membrane fractions (Hartings et al.,
2017). In the present study, OsHCF222 dually localized to the ER and chloroplasts,
and OsABCI7 was detected in thylakoid membrane fractions by immunoblot (Fig.
4B). Upon these findings, we speculated that OsABCI7 interacts with OsHCF222 at
thylakoid membranes to regulate ROS homeostasis for maintaining thylakoid integrity.

To test this assumption, CRISPR/Cas9 editing was performed to obtain the
corresponding knockout mutants of OsABCI7 and OsHCF222. Unlike the
Cr-OsABCI7 plants, Cr-OsHCF222 lines displayed chlorotic leaves and
seedling-lethality (Fig. 8C, 8D). In spite of the major differences in seedling-lethality
and photosynthetic protein accumulation between the two types of knockout lines,
ROS over-accumulation occurred in both Cr-OsABCI7 and Cr-OsHCF222 mutants
(Fig. 8G, 8I, 8J). Rice DnaJ proteins are involved in chloroplast development and
H₂O₂-induced DNA damage (Yamamoto et al., 2005; Zhu et al., 2015). Thus, we
conclude that both OsABCI7 and OsHCF222 are essential for the thylakoid
membrane development, and the OsABCI7/OsHCF222 complex plays a vital role in
ROS homeostasis regulation at thylakoids. This explained that thylakoid degradation
of cnl1 was closely associated with the disruption of OsABCI7/OsHCF222 complex
formation in cnl1.
In summary, our data support the idea that OsABCI7 interacts with OsHCF222 to form a complex functioning at the thylakoid membranes in chloroplasts. We propose that the interaction is crucial to maintain thylakoid stability by regulating ROS homeostasis in chloroplasts. In cnl1, the disrupted interaction causes a rapid ROS burst resulting in thylakoid instability, chloroplast breakdown and cell death which might in turn reinforce ROS accumulation but in a controllable manner as cnl1 itself and the knockout lines are viable. On the other hand, OsHCF222 knockout lines are unviable, probably indicating that OsHCF222 is not only required for thylakoid stability but also for thylakoid membrane complex formation as demonstrated in Arabidopsis (Schult et al., 2007; Link et al., 2012; Hartings et al., 2017).

MATERIALS AND METHODS

Plant materials and growth conditions

The cnl1 mutant was obtained from an ethane methyl sulfonate (EMS)-induced indica rice (Oryza sativa) Zhongjian100 (wild-type, WT) mutant bank. cnl1 mutants were crossed to the japonica rice ReYan 1, ORO and WT, respectively. All the parents, F₁ plants and F₂ individuals were grown in the paddy field at Zhejiang province for genetic analysis and/or gene mapping. For the exogenous antioxidant treatments, plants were hydroponically cultured with Yoshida rice nutrient salt mixture (Coolaber, NSP1040) after germination in a growth chamber at 30 °C, 14 h light/26 °C, 10 h dark cycle. The cnl1 seedlings without antioxidant treatment were used as the control (CK). The nutrient solution containing either 1mM dimethythiourea (DMTU) or 1mM ascorbic acid (AsA) was used for antioxidant treatments.

Chlorophyll measurement, transmission electron microscopy and paraffin sectioning

Chlorophyll content was determined by measuring the absorbance at 652 nm using a SpectraMax i3x Multi-Mode Microplate Reader (MOLECULAR DEVICES, Sunnyvate, CA, USA) as described previously (Kim et al., 2006). Leaves were
collected to perform transmission electron microscopy (TEM) according to a previous report (He et al., 2018a). The third internodes of the main stem from WT and cnl1 at the mature stage were used for paraffin sections as described previously (He et al., 2017).

Malondialdehyde, ROS content and antioxidant activity measurement

The contents of malondialdehyde (MDA), as well as the activities of ROS scavenging enzymes, including peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT), were determined using the kits (A003-1-2, A084-1-1, A001-1-1, and A007-1-1) following the manufacturer’s instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). H2O2 content was determined using the H2O2 assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), and the level of O2− was quantified using a kit (R30343) following the manufacturer’s instructions (Shanghai yuanye Bio-Technology Co., Ltd).

Map-based cloning

The mutant F2 individuals from the cnl1/ReYan 1 cross were selected for mapping the OsABCI7 locus. Bulked segregant analysis was adopted to rapidly locate the mutation as follows: Equal amounts of leaf blades from each of ten WT plants and ten cnl1 plants were sampled for DNA extraction to form a WT DNA pool and a mutant DNA pool, respectively. The parents and the two DNA pools were subjected to preliminary linkage analysis of the mutation by genotyping 362 polymorphic simple sequence repeat (SSR) markers covering 12 chromosomes. Subsequently, 755 cnl1 individual plants from the F2 population were genotyped to determine the physical location of OsABCI7. Primers used for mapping are listed in Table S3.

RNA extraction and reverse transcription quantitative PCR (RT-qPCR)

Total RNA was extracted using a NucleoZOL Reagent Kit (MACHEREY-NAGEL, Düren, Germany) according to the manufacturer’s instructions. One μg total RNA was reverse-transcribed using the ReverTra Ace
RT-qPCR RT Master Mix with genomic DNA (gDNA) Remover Kit (Toyobo, Osaka, Japan). Rice Ubiquitin (LOC_Os03g13170) was used as an internal control. Primers used for RT-qPCR are listed in Table S3.

**Generation of transgenic plants**

For the complementation of *cnl1*, the WT allele containing the 1788-bp promoter region, entire genomic DNA region (3384-bp) and 711-bp downstream of *OsABCI7* was cloned into the vector pCAMBIA1300. To generate the overexpression lines, the 831-bp coding sequence (CDS) of *OsABCI7* was cloned into the vector pCAMBIA1300-UBI under the control of the maize (*Zea mays*) Ubiquitin 1 promoter. For the promoter activity assay of *OsABCI7*, the *OsABCI7* promoter was inserted into the vector pCAMBIA1381Z containing the β-glucuronidase gene (*GUS*). The CRISPR/Cas9 constructs for *OsABCI7* and *OsHCF222* were generated according to a previous report (Ma et al., 2015). The genetic transformations were conducted using rice embryogenic calli through *Agrobacterium tumefaciens*-mediated transformation (Hiei & Komari, 2008). Primers used for vector constructions are listed in Table S3.

**Subcellular localization and chloroplast-localized signal peptide analysis**

To determine the subcellular localization of *OsABCI7* and *OsHCF222*, the CDS of *OsABCI7* and *OsHCF222* amplified from WT were fused to the N-terminus of *green fluorescent protein* (*GFP*) in the pAN580 (GFP) vector, respectively. To determine the chloroplast-localized signal peptide region, different truncated CDS of *OsABCI7* were also fused to the N-terminus of *GFP* in the pAN580 vector. All these constructs were separately introduced into rice protoplasts for transient expression, and the GFP fluorescence signals were detected using a Zeiss lsm710 confocal laser scanning microscope (Carl Zeiss, Inc., Jena, Germany).

**OsABCI7**<sub>Pro</sub>:*GUS* staining assay

Different tissues from *OsABCI7*-promoter-*GUS* transgenic plants were collected at different growth stages and stained using a GUS staining Kit (Coolaber, SL7160),
following the manufacturer’s instructions. Images were photographed using a stereomicroscope (LEICA MC120 HD, Wetzlar, Germany).

**Comet assay**

The alkaline comet assay was performed according to Wang & Liu (2006) using the Comet assay kit from Trevigen (Gaithersburg, MD, USA). The SYBR green I nucleic acid gel stain (S9430) purchased from Sigma-Aldrich company (Saint Louis, MO, USA) was used for staining. Thirty to seventy comets were counted in each slide, and DNA damage was classified into four degrees as previously reported (Yuan et al., 2010).

**Yeast two-hybrid assay**

The cDNA library constructed from Nipponbare embryogenic calli and the screening kit (Clontech, Mountain View, CA, USA) were used for yeast two-hybrid assays. All protocols were carried out strictly according to the manufacturer’s user manual. The full CDS of OsABCI7 and OsHCF222 amplified from WT cDNA and the mutant full CDS amplified from cnl1 were cloned into the pGBKT7 or pGADT7 vector, respectively. The truncated CDS of OsABCI7 with/without specific domains from the WT were also cloned into the pGBKT7 vector. The yeast strain Y2HGold (Saccharomyces cerevisiae) was used in the yeast two-hybrid assay. Primers used are listed in Table S3.

**Bimolecular fluorescence complementation (BiFC) assay**

For BiFC assays, the entire CDS of OsABCI7, 90 bp of the N-terminal OsABCI7 CDS, the CDS of ΔOsABCI7 and OsABCI7 with the H-loop motif were cloned into the 1300-YN vector. The entire CDS of OsHCF222 was cloned into the 2300-YC vector. The new constructs were electroporated into A. tumefaciens strain GV3101 for transient expression in Nicotiana benthamiana leaves and were also introduced into rice protoplasts for transient expression. The YFP fluorescent signals in the N. benthamiana leaves were detected approximately 48 h after transfection, and the YFP
signals in rice protoplasts were observed about 36 h after expression.

**Protein extraction and immunoblot analysis**

Total proteins were extracted as previously described (He et al., 2018b) and quantified into the same concentration using the BCA Protein Assay Kit (Coolaber, SK1070). For intact chloroplast isolation, 1 g fresh leaf tissue from *OsABC17* overexpression seedlings was collected for chloroplast isolation using the chloroplast isolation kit (BestBio, BB-3622) following the manufacturer's instructions. To directly isolate the thylakoid membranes, 1 g fresh leaf tissue was homogenized on ice with 5 mL extraction buffer (0.4 M sucrose, 10 mM NaCl, 2 mM MgCl₂, 50 mM HEPES), then filtered through two layers of 40 μM nylon membrane and centrifuged for 10 min at 4 °C, 5000 g. After discarding the supernatant, the sediment was rinsed with 1 mL extraction buffer, then centrifuged for 10 min at 4 °C, 5000 g, repeating this step twice. Finally, the sediment was re-suspended with the extraction buffer and stored at -80 °C. Antibodies used for immunoblot analysis of thylakoid membrane proteins were purchased from Agrisera (Vännäs, Sweden), and the products and catalog numbers are as follows: anti-PsaA (AS06172), anti-PsaB (AS10695), anti-Lhca1 (AS01005), anti-Lhca2 (AS01006), anti-Lhca3 (AS01007), anti-Lhca4 (AS01008), anti-PsbA (AS132669), anti-PsbD (AS06146), anti-Lhcb1 (AS01004), anti-Lhcb2 (AS01003), anti-NdhH (AS132712), anti-NdhF (AS132711), anti-PC (AS06141), anti-AtpB (AS05085), anti-AtpE (AS101586). The protein levels were detected using a Super ECL Western Blotting Substrate (Coolaber, SL1350).

**Co-immunoprecipitation (Co-IP) assay**

For the Co-IP assay, the full CDS of *OsABC17* was cloned into pYBA1132-GFP and the CDS of *OsHCF222* was cloned into pYBA1132-HA, respectively. The constructs were transiently expressed in *N. benthamiana* leaves via *A. tumefaciens*-mediated transfection. About 60 h after transfection, fresh *N. benthamiana* leaves were harvested for total protein extraction using the buffer (0.4 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 25 mM β- mercaptoethanol, 0.5% Triton X-100).
with proteinase inhibitor cocktail (Roche, 04693159001). The supernatant of total proteins was collected after centrifugation at 12,000 g for 20 min at 4°C. The Pierce HA-tag Magnetic IP/Co-IP Kit (88838X) was used for the Co-IP assay following the manufacturer’s manual. Anti-GFP (Abmart, M200045) and anti-HA (CWBIO, CW0092M) antibodies were used for detection in immunoblot analysis.
ACCESSION NUMBERS

Sequence data from this article for the cDNA and genomic DNA of OsABCI7 and OsHCF222 can be found in the GenBank/EMBL/Gramene data libraries under accession number LOC_Os11g29850 and LOC_Os03g30092, respectively.

SUPPLEMENTAL DATA

Supplemental Figure S1. Phenotypic and photosynthetic analysis of WT and cnl1.
Supplemental Figure S2. Coding sequence of OsABCI7 and amino acid sequence of OsABCI7.
Supplemental Figure S3. Mutation analysis of OsABCI7.
Supplemental Figure S4. Overexpression of OsABCI7 and analysis of OsABCI7-RNAi lines.
Supplemental Figure S5. Soluble protein contents and SOD activities in WT, cnl1, complementary and overexpression lines.
Supplemental Figure S6. Chlorophyll contents and histochemical analysis.
Supplemental Figure S7. TUNEL assay of WT and cnl1.
Supplemental Figure S8. Chlorophyll contents and OsABCI7 expression analysis.
Supplemental Figure S9. Subcellular localization of OsHCF222.
Supplemental Figure S10. Bimolecular fluorescence complementation for the interaction of OsABCI7 and OsHCF222 in N. benthamiana.
Supplemental Figure S11. Knockout analysis of OsABCI7 by CRISPR/Cas9 technology.
Supplemental Figure S12. Phenotypes of OsHCF222 knockout line and expression of photosynthesis-related genes in OsABCI7 and OsHCF222 knockout lines.
Supplemental Figure S13. Chlorophyll and H2O2 contents in WT and cnl1 seedlings.
Supplemental Figure S14. Levels of metal elements in shoots and roots of WT and cnl1.
Supplemental Figure S15 Alignment of HCF222 orthologs from different plant species.
**Supplemental Table S1.** List of open reading frames in the 116 kb target region.

**Supplemental Table S2.** Chlorophyll fluorescence parameters measured in flag leaves of WT, cnl1, complementary and overexpression lines.

**Supplemental Table S3.** Primers used in this study.

**FIGURE LEGENDS**

**Figure 1.** Phenotypic characterization of cnl1. (A) WT and cnl1 at the tillering stage (45 d after field transplanting). Insets show magnified views of the four leaves (L1–L4) from top to bottom of WT and cnl1. Bar, 20 cm. (B, C) Chloroplast ultrastructure in mesophyll cells of L4 in WT (B) and cnl1 (C) in (A). (D) Chlorophyll contents in different leaves of WT and cnl1. L1–L4 represent the four leaves shown in (A) (from left to right), respectively. (E–I) Major agronomic traits of WT and cnl1. (J) Comparison of main stems from WT and cnl1. Arrows indicate the node positions. Bar, 10 cm. (K) Internode lengths of main stems in WT and cnl1 at the mature stage. (L) Longitudinal sections of the corresponding parts of internode III from WT and cnl1 as marked by the white boxes in (J). Bars, 100 μm. (M, N) Comparison of cell length (M) and cell width (N) of internode III in WT and cnl1. Data are means ± SD (n = 5), **P < 0.01 and *P < 0.05 by Student’s t test.

**Figure 2.** Map-based cloning of OsABCI7 and ROS detection in cnl1. (A) Mapping of the OsABCI7 locus. The molecular markers and recombinant numbers are displayed. The candidate gene OsABCI7/LOC_Os11g29850 is depicted in red. (B) OsABCI7 allele structure in WT and cnl1. The white boxes, filled boxes and black lines represent untranslated regions, exons and introns, respectively. The red stars indicate the nucleotide substitution and red arrowheads indicate the splicing site of the 8th intron in WT and cnl1. pF and pR indicate the forward and reverse primer sites in (D). (C) dCAPs confirmation of the mutation. PCR products were digested by SalI from WT but not from cnl1 and detected by 6% polyacrylamide gel. (D) PCR analysis...
showing the 17-bp splicing deletion of the 8th exon. The smaller band of cnl1 cDNA resulted from the 17-bp alternative upstream splicing deletion as indicated in (B). gDNA, genomic DNA; cDNA, complementary DNA. Agarose gel concentration was 3.5%. (E) Phenotypes of complementary (CP) and overexpression (OE) lines at 40 d after sowing. Insets show magnified views of the bottom second leaves. Bar, 10 cm. (F) Relative expression of OsABC17 in WT, cnl1 and transgenic plants. Rice UBIQUITIN was used as an internal control. Data are means ± SD (n = 3). (G–K) ROS levels, ROS scavenging enzyme activities and MDA contents in flag leaves of WT, cnl1 and transgenic plants at the heading stage. H2O2 content (G), O2− content (H), APX activity (I), CAT activity (J), POD activity (K), MDA content (L). Data are means ± SD (n = 3). Different letters indicate significant differences by one-way ANOVA and Duncan’s test (P < 0.05).

Figure 3. DNA damage detection and transcriptional level analysis of DNA damage repair- and replication-associated genes. (A, B) Typical comets for DNA damage in nuclei of flag leaves from WT (A) and cnl1 (B) at the heading stage. (C) Four types of DNA damage extents in each nucleus are indicated by the units 1%, 10%, 30%, or 50%. An increased unit associated with a larger comet tail. Frequency distribution of four types of DNA damage extents in WT and cnl1. Data are means ± SD (n = 3), **P < 0.01 by Student’s t-test. (D) Transcriptional level analysis of DNA repair- and replication-associated genes by RT-qPCR. Rice Ubiquitin was used as an internal control. Data are means ± SD (n = 3).

Figure 4. Subcellular localization and expression pattern of OsABC17. (A) GFP signals of different constructs in rice protoplasts. The full OsABC17 protein contains 276 amino acids. ΔOsABC17 represents the mutant OsABC17 protein. Bars, 5 μm. (B) Immunoblot analysis of OsABC17. Protein samples were from OsABC17 (fused HA-tag) overexpression seedlings. Antiserum against HA-tag (anti-HA), D2 (thylakoid marker) and β-actin were used in blotting. TP, total protein; Chl, chloroplast; Thyl, thylakoid membrane; Fraction II, stroma fraction. (C–N) GUS
staining of OsABCI7\textsubscript{P\textsubscript{ro}::GUS} transgenic plants. Root (C), leaf blade (D), node V (E), culm (F), leaf sheath (G), leaf blade transverse slice (H), stamen (I), seeds at different stages of development (J–N). Bars, 1 mm (B–F, H–M), 100 μm (G). (O) Relative expression of OsABCI7 in various organs at different growth stages. Data are means ± SD (n = 3).

**Figure 5. Phenotypic characterization of WT and cnll at different temperatures and light-dependent chlorophyll synthesis of WT and cnll.** (A–C) Phenotypic comparison of WT and cnll seedlings at different temperatures. The WT and cnll were grown in growth chambers after germination for 10 d at 30 °C (A) and 25 °C (B) and 16 d at 20 °C (C) with constant temperatures and 14 h light/10 h dark cycles (400 μmol m\(^{-2}\) s\(^{-1}\)). Bars, 2 cm. (D) Kinetic analysis of OsABCI7 expression in WT at 10 °C. Data are means ± SD (n = 3), **P < 0.01 by Student’s t test. (E–G) Ultrastructure of chloroplasts in mesophyll cells of WT and cnll at different temperatures. All the cells were from the fully emerged third leaves of WT and cnll seedlings at the three-leaf stage. Bars, 2 μm in cell view and 1 μm in enlarged view. (H–L) Chlorophyll contents (H), greening speed (I–L) and OsABCI7 expression (L) of etiolated WT and cnll seedlings during the greening process. Seven-d etiolated seedlings were exposed to light (250 μmol m\(^{-2}\) s\(^{-1}\)) for 12 h. Bars, 2 cm. Data are means ± SD (n = 3). Different letters indicate significant differences by one-way ANOVA and Duncan’s test (P < 0.05).

**Figure 6. Levels of thylakoid membrane proteins and expression of photosynthetic genes.** (A) Levels of thylakoid membrane proteins detected in total proteins from flag leaves of WT and cnll at the heading stage. The WT dilution series contains 30 μg of proteins (1x) or the indicated dilutions, and the cnll contains 30 μg proteins. Antibodies used for detection are indicated on the left; the asterisk indicates an unspecific signal detected by the NdhF antibody. (B) Total proteins of flag leaves from WT and cnll mutant separated by SDS-PAGE and indicated by Coomassie Brilliant Blue. (C) Levels of plastid- and nucleus-encoded mRNAs...
related to thylakoid membrane proteins. (D) Relative expression analysis of photosynthetic genes in WT and cnl1. Data are means ± SD (n = 3).

Figure 7. Interaction of OsABC17 and OsHCF222. (A) Diagrams of the wild-type OsABC17, mutant OsABC17 (ΔOsABC17), OsABC17 with H-loop motif (OsABC17 (+ H-loop)), and OsABC17 without H-loop motif (OsABC17 (– H-loop)). The number of amino acids is indicated at the top. (B) Y2H assay for the interaction of OsABC17 and OsHCF222. The pGBK7T-53 and pGADT7-T pair is the positive control, the pGBK7-Lam and pGADT7-T pair is the negative control, and the pGBK7-OsABC17 and pGADT7 pair is the self-activation test. SD/-TL, synthetic dropout medium without Trp and Leu; SD/-TLHA, synthetic dropout medium without Trp, Leu, His and Adenosine; X-α-gal, 5-Bromo-4-chloro-3-indoxyl-α-D-galactopyranoside; AbA, Aureobasidin A. (C) Co-IP assay confirmation of OsABC17 and OsHCF222 interaction. (D) BiFC assay for OsABC17 and OsHCF222 interaction in rice protoplasts. YN-OsHAL3/YC-OsHAL3 is the positive control, YN-OsHAL3/YC-OsHCF222 and YN-OsABC17/YC-OsHAL3 are the negative controls, YN-OsABC171-30aa/YC-OsHCF222 is a negative control to ensure that the YFP signals of YN-OsABC17/YC-OsHCF222 are caused by the interaction between OsABC17 and OsHCF222 instead of the chloroplast co-localization of OsABC17 and OsHCF222. Bars, 5 μm.

Figure 8. Characterization of CRISPR/Cas9-edited OsABC17 and OsHCF222 mutations in the Kitaake background. (A, B) CRISPR/Cas9-mediated mutations at the target sites of OsABC17 and OsHCF222 in representative knockout lines. The sgRNA target sequence is underlined in green and the PAM motif is indicated in orange, respectively. (C) Phenotypes of flag leaves in Kitaake and Cr-OsABC17 knockout lines at 60 d after transplanting. Bar, 2 cm. (D) Seedlings of T1 Cr-OsHCF222 knockout lines at 12 d after germination. Bar, 2 cm. (E, F) Chlorophyll contents of Cr-OsABC17 and Cr-OsHCF222 knockout lines in (C) and (D). Different
letters indicate significant differences by one-way ANOVA and Duncan’s test ($P < 0.05$). Data are means ± SD ($n = 3$). (G) Levels of thylakoid membrane proteins tested in total proteins extracted from Kitaake, Cr-OsABC17 and Cr-OsHCF222 knockout lines at 8 DAG. (H) Detection of mRNA levels of plastid- and nucleus-encoded genes related to thylakoid membrane proteins in Cr-OsABC17-2 and Cr-OsHCF222-I lines at 8 DAG. (I, J) Measurements of $H_2O_2$ contents in Cr-OsABC17-2 and Cr-OsHCF222-I at 8 DAG. Data are means ± SD ($n = 3$), **$P < 0.01$ by Student’s $t$ test.

**Figure 9. Responses of cnll to exogenous antioxidant treatment.** (A) Phenotypes of fully expanded top-second leaves of cnll under 1 mM DMTU and 1 mM AsA treatments. cnll was hydroponically cultured for 4 d after germination and then treated with 1 mM DMTU or 1 mM AsA for 8 d. CK, control. Bar, 1 cm. (B–D) Chlorophyll (B), $H_2O_2$ (C) and $O_2^-$ contents (D) of cnll in (A). Data are means ± SD ($n = 3$). NS, no significance; *$P < 0.05$ and **$P < 0.01$ by Student’s $t$ test.

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Figure 1. Phenotypic characterization of *cnll*. (A) WT and *cnll* at the tillering stage (45 d after field transplanting). Insets show magnified views of the four leaves (L1–L4) from top to bottom of WT and *cnll*. Bar, 20 cm. (B, C) Chloroplast ultrastructure in mesophyll cells of L4 in WT (B) and *cnll* (C) in (A). (D) Chlorophyll contents in different leaves of WT and *cnll*. L1–L4 represent the four leaves shown in (A) (from left to right), respectively. (E–I) Major agronomic traits of WT and *cnll*. (J) Comparison of main stems from WT and *cnll*. Arrows indicate the node positions. Bar, 10 cm. (K) Internode lengths of main stems in WT and *cnll* at the mature stage. (L) Longitudinal sections of the corresponding parts of internode III from WT and *cnll* as marked by the white boxes in (J). Bars, 100 μm. (M, N) Comparison of cell length (M) and cell width (N) of internode III in WT and *cnll*. Data are means ± SD (*n* = 5), **P < 0.01 and *P* < 0.05 by Student's t-test.
Figure 2. Map-based cloning of OsABCI7 and ROS detection in cnl1. (A) Mapping of the OsABCI7 locus. The molecular markers and recombinant numbers are displayed. The candidate gene OsABCI7/LOC_Os11g29850 is depicted in red. (B) OsABCI7 allele structure in WT and cnl1. The white boxes, filled boxes and black lines represent untranslated region, exons and introns, respectively. The red stars indicate the nucleotide substitution and red arrows indicate the splicing site of the 8th intron in WT and cnl1. pF and pR indicate the forward and reverse primer sites in (D). (C) dCAPs confirmation of the mutation. PCR products are digested by SalI from WT but not from cnl1 and detected by 6% polyacrylamide gel. (D) PCR analysis showed the 17-bp splicing deletion of 8th exon. The smaller band of cnl1 cDNA was resulted from the 17-bp alternative upstream splicing deletion as indicated in (B). gDNA, genomic DNA; cDNA, complementary DNA. Agarose gel concentration is 3.5%. (E) Phenotypes of complementary (CP) and overexpression (OE) lines at 40 d after sowing. Insets show magnified views of the bottom second leaves. Bar, 10 cm. (F) Relative expression of OsABCI7 in WT, cnl1 and transgenic plants. Rice UBIQUITIN
was used as an internal control. Data are means ± SD (n = 3). (G–K) ROS levels, ROS scavenging enzyme activities and MDA contents in flag leaves of WT, cnl1 and transgenic plants at the heading stage. H2O2 content (G), O2− content (H), APX activity (I), CAT activity (J), POD activity (K), MDA content (L). Data are means ± SD (n = 3). Different letters indicate significant differences by one-way ANOVA and Duncan’s test (P < 0.05).
Figure 3. DNA damage detection and transcriptional level analysis of DNA damage repair- and replication-associated genes. (A, B) Typical comets for DNA damage in nuclei of flag leaves from WT (A) and cnl1 (B) at the heading stage. (C) Four types of DNA damage extents in each nucleus are indicated by the units 1%, 10%, 30%, or 50%. An increased unit correlated with a larger comet tail. Frequency distribution of four types of DNA damage extents in WT and cnl1. Data are means ± SD (n = 3), **P < 0.01 by Student’s t test. (D) Transcriptional level analysis of DNA repair- and replication-associated genes by qPCR. Rice Ubiquitin was used as an internal control. Data are means ± SD (n = 3).
Figure 4. Subcellular localization and expression pattern of OsABCI7. (A) GFP signals of different constructs in rice protoplasts. The full OsABCI7 protein contains 276 amino acids. ΔOsABCI7 represents the mutant OsABCI7 protein. Bars, 5μm. (B) Western blot analysis of OsABCI7. Protein samples were from OsABCI7 (fused HA-tag) overexpression seedlings. Antiserum against HA-tag (anti-HA), D2 (thylakoid marker) and β-actin were used in blotting. TP, total protein; Chl, chloroplast; Thy, thylakoid membrane fraction; Str, stroma fraction. (C–N) GUS staining of OsABCI7::GUS transgenic plants. Root (C), leaf blade (D), node V (E), culm (F), leaf sheath (G), leaf blade transverse slice (H), stamen (I), seeds at different stages of development (J–N). Bars: 5 mm (H–M), 100 μm (G). (O) Relative
expression of OsABCI7 in various organs at different growth stages. Data are means ± SD (n = 3).
The images depict various analyses of plant growth under different conditions and treatments. Images A, B, and C show the growth of wild-type (WT) and cnl1 plants at 30 °C, 25 °C, and 20 °C, respectively, with a clear difference in growth patterns between the two genotypes.

Graph D illustrates the relative expression of OsABC1/7 under low temperature and control conditions, showing a significant increase in expression at 4 hours.

Images E, F, and G depict cellular structures of WT and cnl1 plants at 30 °C, 25 °C, and 20 °C, respectively, with enlarged views highlighting cell morphology and chloroplast content.

Graph H presents chlorophyll content measurements (mg g⁻¹ FW) for WT and cnl1 plants at 0 h, 6 h, and 12 h, showing a marked increase in chlorophyll content in cnl1 plants compared to WT at 12 h.

Images I, J, and K show the growth of WT and cnl1 plants at 0 h, 6 h, and 12 h, respectively, with a vivid coloration difference.

Graph L illustrates the relative expression of OsABC1/7 at 0 h, 6 h, and 12 h, with a clear distinction between WT and cnl1 plants, indicating a significant difference in expression levels.
Figure 6. Levels of thylakoid membrane proteins and expressions of photosynthetic genes. (A) Levels of thylakoid membrane proteins detected in total proteins from flag leaves of WT and cnl1 at the heading stage. The WT dilution series contains 30 μg of proteins (1x) or the indicated dilutions, and the cnl1 contains 30 μg proteins. Antibodies used for detection are indicated on the left; the asterisk indicates an unspecific signal detected by the NdhF antibody. (B) Total proteins of flag leaves from WT and cnl1 mutant were separated by SDS-PAGE and indicated by coomassie brilliant blue (CBB) staining. (C) Levels of plastid- and nucleus-encoded mRNAs related to thylakoid membrane proteins. (D) Relative expression of photosynthetic genes in WT and cnl1. Data are means ± SD (n = 3).
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