OsDXR interacts with OsMORF1 to regulate chloroplast development and RNA editing of chloroplast genes in rice

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Abstract Plant chlorophyll biosynthesis and chloroplast development are two complex processes regulated by exogenous and endogenous factors. In this study, we identified *OsDXR*, a gene expressing a reductoisomerase that positively regulates chlorophyll biosynthesis and chloroplast development in rice. *OsDXR* knock-out lines displayed the albino phenotype and could not complete the whole life cycle process. *OsDXR* was highly expressed in rice leaves, and subcellular localization indicated that OsDXR was a chloroplast protein. Many genes involved in chlorophyll biosynthesis and chloroplast development were differentially expressed in the *OsDXR* knock-out lines compared to the wild type. Moreover, we found that the RNA editing efficiency of *ndhA*-1019 and *rpl2*-1 were significantly reduced in the *OsDXR* knock-out lines. Furthermore, OsDXR interacted with the RNA editing factor OsMORF1 in a yeast two-hybrid screen and bimolecular fluorescence complementation assay. We demonstrate that disruption of the plastidial 2-C-methyl-derythritol-4-phosphate pathway results in defects in chloroplast development and RNA editing of chloroplast genes.

Keywords: rice, OsDXR, Chloroplast development, RNA editing, OsMORF1

1. Introduction

Isoprenoids are essential for plant growth and development, and tens of thousands of these compounds have been isolated from archaea, bacteria and eukaryotes (Rodríguez-Concepción 2014; Tarkowská and Strnad 2018). Isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are two basic five-carbon molecules required for isoprenoid biosynthesis. In higher plants, there are two isoprenoid synthetic pathways located in plastids and the cytoplasm, namely the cytoplasmic methyl valerate (MVA) pathway and the 2-C-methyl-derythritol-4-phosphate (MEP) pathway (Rohdich *et al*. 2001). Chlorophylls, gibberellins, and abscisic acids are synthesized via the MEP pathway (Okada *et al*. 2002). Previous studies have shown that DOXP reductoisomerase (DXR) can catalyze the conversion of 1-deoxy-D-xylulose-5-phosphate (DOXP) into 2-C-methyl-D-erythritol 4-phosphate (MEP) (Carretero-Paulet *et al*. 2002). In *Peppermint*, overexpression of *DXR* increases the biosynthesis of essential oils (Mahmoud *et al*. 2001), while in *Arabidopsis*, the T-DNA insertion mutant of *DXR* was albino and dwarf, could not complete seedling establishment (Xing *et al*. 2010), the

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number of trichomes were reduced, and stomata closure was affected (Xing *et al*. 2010). However, the function of DXR has not been reported in crops, especially in rice.

Posttranscriptional processing, including RNA editing, RNA splicing, RNA cleavage, and RNA stability, plays an important role in regulating plant chloroplast development and chlorophyll biosynthesis (Barkan and Goldschmidt-Clermont 2000). RNA editing converts cytidine nucleotides (C) to uridine (U) in the transcripts of plastidial and mitochondrial genes, and is affected by temperature, fungal infection, and stresses (Karcher and Bock 2002; García-Andrade *et al*. 2013; Rodrigues *et al*. 2017). Many studies have shown that pentatricopeptide repeat (PPR) proteins, multiple organellar RNA editing factors (MORFs), and thioredoxin z play an important role in plant RNA editing (Takenaka *et al*. 2012; Huang *et al*. 2020; Wang *et al*. 2021). *OsPGL1* encodes a dual-localized PPR protein and affects chloroplast and mitochondrial RNA editing (Xiao *et al*. 2018). *WP2* encodes a thioredoxin z protein that affects the efficiency of RNA editing in many plastidial-encoded genes (Wang *et al*. 2021). Recently, the MEP pathway gene *OsHMBPP*/*OsHDR* has been shown to influence plastidic RNA editing and interact with OsMORF8 (Liu *et al*. 2020), suggesting that the MEP pathway might affect plastidic RNA editing.

There is only one DOXP reductoisomerase (DXR) in rice, OsDXR. In this study, OsDXR was identified to be involved in regulating rice chlorophyll biosynthesis and chloroplast development. To elucidate the mechanism, we demonstrated that OsDXR interacts with the RNA editing factor, OsMORF1, resulting in decreased RNA editing of two chloroplast genes.

2. Materials and methods

2.1. Plant materials

Nipponbare, a *japonica* variety of rice (*O*. *sativa*), was used as a wild-type (WT) plant. We developed two *OsDXR* knock-out lines through the CRISPR/Cas9 system (Lu *et al*. 2017). The mutation target (5'-TTCCTCGACTCCAACAG-3') was constructed in a 1305-CRISPR plasmid vector using the AraI enzyme, and introduced into Nipponbare by *A*. *tumefaciens*-mediated transformation. Fragments containing the target were PCR amplified (5'-GAGTCTCAGATTCCCATCTCGTC-3' and 5'-CTGCGGATTATCTTGAAACAGG-3') and sequence verified. All plant seedlings were grown in a growth chamber with 14 h light and 10 h dark at 30/25 ˚C, respectively.

2.2. Chlorophyll content analysis

Chlorophylls were extracted from 10-day-old WT and *OsDXR* knock-out leaves as previously described (Porra *et al*. 1989) with some modifications. Briefly, ~0.2 g of leaves harvested from WT and *OsDXR* knock-out leaves were placed in a 15 mL centrifugal tube in 5 mL extraction buffer (95% ethanol), and stored in the dark for 48 h. All pigment solutions were combined and centrifuged for 2 min. The light absorbance of three biological replicates was measured at 663 and 647 nm.

2.3. Transmission electron microscopy (TEM)

Leaf samples of 10-day-old WT and the *OsDXR* knock-out seedlings were collected and fixed in 4% glutaraldehyde, and then vacuumed for 1 h. The samples were dehydrated through a series of alcohol solutions, and imaged by TEM (Hitachi, Tokyo, Japan) as previsouly described (Liu *et al*. 2020).

2.4. Sequence analysis

Protein sequences homologous to OsDXR were searched using the BLAST search program (www.ncbi.nlm.nih.gov/BLAST/), and aligned using the DNAMAN software.

2.5. Subcellular localization

To examine the subcellular localization of OsDXR, the full-length ORF of *OsDXR* was amplified with the primers 5' -CGGAGCTAGCTCTAGAATGGCGCTCAAGGTCGTCTC-3' and 5'-TGCTCACCATGGATCCACAGGTAC AGGGCTGA-3' and introduced into pAN580-GFP at the XbaI and BamHI sites. Transformation was performed as previsouly described (Liu *et al*. 2020). The GFP fluorescence of rice protoplasts was observed by confocal laser scanning microscopy (LSM700; Zeiss). Chlorophyll autofluorescence was used as a control.

2.6. RT-PCR and qPCR analysis

Total RNA was extracted from roots, stems, leaves, and panicles of WT plants with an RNA Prep Pure Plant kit (Tiangen, Beijing, China). First-strand cDNA was reverse transcribed with a RT primer mix. Real-time PCR was performed using a SYBR Premix *Ex Taq*™ kit (TaKaRa) on a CFX96 Touch Real-time PCR Detection System with three biological replicates. The primers used to analyze the expression level of *OsDXR* were 5'-AAACGAGGGACAGAAGAGCA-3' and 5'-GAACCGGTTGAGCCAACAAT-3'. The primers for chlorophyll biosynthesis (Zeng *et al*. 2020), chloroplast development-related genes (Wang *et al*. 2017), and MEP pathway genes (Liu *et al*. 2020) were obtained as reported previously. The rice *UBQ* gene was used as an internal control. The primers used for *UBQ* were 5'-GCTCCGTGGCGGTATCAT-3' and 5'-CGGCAGTTGACAGCCCTAG-3'.

2.7. RNA editing assay

To analyze RNA editing, total RNA from 10-day-old WT and *OsDXR* knock-out plants were isolated and treated with DNase I (CWBIO, Jiangsu, China). The RNAs were reverse transcribed with random primers. cDNA fragments containing RNA editing sites were amplified by RT-PCR. Primers were used to detect RNA editing sites of chloroplast genes as previously described (Wang *et al*. 2017). The RT-PCR products were sequenced directly and C to T changes were compared using the BioXM 2.6 Software.

2.8. Yeast two-hybrid screen and bimolecular fluorescence complementation (BIFC) assay

The full-length cDNA of *OsDXR* (5'-CATGGAGGCCGAATTCATGGCGCTCAAGGTCGTCTC-3' and 5'-GCAGGTCGACGGATCCCTAGACAGGTACAGGGCTGA-3') and seven MORF genes were amplified and cloned into the EcoR I and BamH I sites of pGBKT7 and pGADT7 vectors with a ClonExpress II One Step Cloning Kit (Nanjing Vazyme Biotech Co., Ltd.), respectively. Different combinations of plasmids were introduced into the yeast strain, AH109, following the manufacturer's protocol (Clontech, PT1172-1). The primers for the pGADT7 vector were obtained as reported previously (Liu *et al*. 2021). For the BiFC assay, *OsDXR* (5'-CATTTACGAACGATAGTTAATTAAATGGCGCTCAAGGTCGTCTC-3' and 5'-CACTGCCACCTCCTCCACTAGTGACAGGTACAGGGCTGA-3') and *LOC_Os11g11020* were cloned into pVYNE, and pVYCE, respectively. VYL interacts with OsClpP4 in rice chloroplast (Dong *et al*. 2013). YN-OsDXR/YC-VYL and YN-OsClpP4/YC-OsMORF1 were used as the negative controls. Recombinant green fluorescence signals from *N. benthamiana* were examined as previously described (Waadt *et al*. 2008).

2.9. Statistical analysis

In this study, all experiments were performed with three biological repeats. The results are presented as mean ± SD in the figures. * and ** indicate signifificant differences at $P<0.05$ and $P<0.01$, respectively.

3. Results

3.1. Characterization of the OsDXR protein in rice

The *OsDXR* gene consists of a 5989 bp open-reading frame, comprising 12 exons and 11 introns. The OsDXR

protein has 473 amino acids with a calculated molecular weight of 51 kD. The first 49 amino acids were predicted to be a chloroplast transit peptide by ChloroP (http://www.cbs.dtu.dk/services/ChloroP/). Multiple amino acid sequence alignments by DNAMAN software indicated that OsDXR has similarities among many species (Fig. 1), including *Arabidopsis* At5g62790, with 80.1% sequence homology (Xing *et al*. 2010). The results indicate that the OsDXR protein is highly conserved in plants.

3.2. Characterization of *OsDXR* knock-out mutants

To investigate the function of *OsDXR* in rice development, we developed two *OsDXR* knock-out lines, *dxr-1* and *dxr-2*, by CRISPR/Cas9-targeted mutagenesis. Two and four bases in *dxr-1* and *dxr-2* were deleted, respectively, leading to a frameshift mutation and premature termination (Fig. 2-A; Appendix A). Subsequent analysis was performed using *dxr-2* due to it being the shortest mutant protein. In addition, we detected the potential off-target sites and did not find any mutations in any of the potential off-target sites (Appendix B). Both *dxr-1* and *dxr-2* displayed an albino phenotype and ultimately died (Fig. 2-B). *OsDXR* expression was remarkably reduced in *dxr-1* and *dxr-2* (Fig. 2-C). Consistent with the albino leaves, the contents of chlorophyll *a* and *b* in the mutant were significantly reduced compared with the WT (Appendix C).

In order to determine whether the structure of chloroplasts was affected in the *dxr-2* mutant, we used TEM to observe the structure of chloroplasts in WT and *dxr-2* seedlings. Chloroplast morphology of the WT seedlings was normal, and the thylakoid lamellae were orderly arranged (Fig. 3-A and B). However, the chloroplasts in the *dxr-2* mutant were abnormal, and were lacking complete thylakoid lamellae (Fig. 3-C and D). These results suggest that *OsDXR* is essential for chloroplast development in rice.

3.3. Expression pattern and subcellular localization of OsDXR

To examine the expression pattern of *OsDXR*, we first analyzed the expression of *OsDXR* in the rice expression database (http://bar.utoronto.ca/efprice/cgi-bin/efpWeb.cgi). As shown in Appendix D, *OsDXR* is expressed in various organs, including leaves, panicles, and seeds. To verify this result, we extracted RNA from the panicles, stems, leaves, and roots and used quantitative RT-PCR to analyze the expression of *OsDXR*. *OsDXR* was highly expressed in leaves and stems (Fig. 4-A). To determine the subcellular localization of the OsDXR protein, we generated a transient expression system in rice protoplasts. Strong green fluorescence signals from OsDXR were co-localized with the chloroplast (Fig. 4-B), indicating that *OsDXR* is constitutively expressed in various tissues and that the OsDXR protein localizes to chloroplasts, providing additional evidence for its role in chloroplast development.

3.4. Expression of chlorophyll biosynthesis and plastid development-related genes were altered in the *dxr* mutant

Genes expressed in plastids by the nucleus and plastid coordinate together to produce normal chloroplasts and synthesize chlorophyll. To examine whether the loss of function of *OsDXR* affects the expression of chlorophyll biosynthesis and chloroplast development-related genes, we performed quantitative RT-PCR to analyze the expression level of these genes in WT and *dxr* plants. Quantitative RT-PCR analysis indicated that the expression level of genes related to chlorophyll biosynthesis and chloroplast development were significantly altered in the *dxr* mutant. For instance, the expression of chlorophyll biosynthetic genes, including *HEMA*, *DVR*, *CHLM* and *PORA*, were significantly reduced in *dxr* compared to WT (Fig. 5). Additionally, the expression level of plastid development-related genes, including *rbcL*, *NDHB*, and *V1*, were significantly reduced in *dxr* compared to WT. However, the expression of chlorophyll biosynthesis genes, such as *CRD* and *CHLG*, and the plastid development related gene, *psaA*, increased relative to the WT (Fig. 5)

To investigate whether the expression levels of the MEP pathway genes were affected by the *OsDXR*

mutation, we performed qRT-PCR to measure the expression level of the other genes of the MEP pathway. Compared with the wild type, the expression levels of *CMK*, *DXS*, *HDS*, *CMS*, and *MCS* were significantly down-regulated (Appendix E).

3.5. RNA Editing of *rpl2*-1 and *ndhA*-1019 were impaired in the *dxr* mutants

Previous studies have shown that RNA editing and other posttranscriptional modifications are involved in the regulation of plant chlorophyll synthesis and chloroplast development (Tang *et al*. 2017; Cui *et al*. 2019). To determine if RNA editing is altered in the *OsDXR* mutants, we examined the editing efficiency of 18 editing sites in the rice chloroplast genome in WT, *dxr-1*, and *dxr-2* seedlings. The editing efficiency of *rpl2*-1 and *ndhA*-1019 were greatly reduced (Fig. 6). Chloroplast *rpl2* and *ndhA* encode ribosomal protein subunit L2 and NADPH dehydrogenase, respectively. *rpl2* participates in the peptidyl-transferase reaction in the *Escherichia coli* ribosome, and *ndhA* influences NDH activity (Nierhaus 1982; Lin *et al*. 2017). The other 16 editing sites displayed normal editing in the WT and *dxr* mutants (Appendix F).

3.6. OsDXR interacted with the multiple organellar RNA editing factor OsMORF1

To explore the function of OsDXR, we performed a yeast two-hybrid (Y2H) screen to identify OsDXR-interacting proteins. We constructed a yeast cDNA library from Nipponbare seedlings and screened the yeast using OsDXR as a target. We obtained 58 colonies that grew well on media lacking Leu/Trp/His/Ade, and found that 5 colonies corresponded to rice LOC_Os11g11020. Additionally, We found an orthologous gene of *LOC_Os11g11020* in *Arabidopsis* named multiple organellar RNA editing factor 1 (MORF1). Hence, we referred to rice LOC_Os11g11020 as OsMORF1. Since there are seven MORF genes in the rice genome, we used the Y2H assay to assess the interaction between OsDXR and the other six rice MORF proteins and only observed an interaction between OsDXR and OsMORF1 in the Y2H assay (Fig. 7-A). Moreover, using the BiFC assay with *N. benthamiana*, a green fluorescence signal was only observed in the protein group of OsDXR/OsMORF1, compared with the YN-OsDXR/YC-VYL and YN-OsClpP4/YC-OsMORF1 combinations (Fig. 7-B). Therefore, Y2H and BiFC assays revealed that OsDXR specifically interacts with OsMORF1.

4. Discussion

OsDXR genes have been isolated from many plants, including *Arabidopsis*, peppermint, and mint through T-DNA insertion mutants, homologous cloning, and transgene overexpression (Lange and Croteau 1999; Mahmoud and Croteau 2001; Xing *et al*. 2010). So far, only three genes, *IspE*, *IspF* and *OsHMBPP*, involved in the MEP pathway have been cloned, but no *OsDXR* gene has yet been identified in rice (Chen *et al*. 2018; Huang *et al*. 2018; Liu *et al*. 2020). In rice, numerous genes responsible for an albino phenotype have been isolated, such as *OsCAF1*, *YSA*, *OsPPR16*, and *RA1* (Su *et al*. 2012; Zhang *et al*. 2019; Zheng *et al*. 2019; Huang *et al*. 2020). In this study, we constructed two *OsDXR* knock-out mutants, which exhibited the albino phenotype and had abnormal chloroplasts. In the two mutants, two and four bases deletions in the *OsDXR* gene resulted in a frameshift mutation and premature termination.

In *Arabidopsis*, the T-DNA insertion *DXR* mutant (Xing *et al*. 2010) displays an albino phenotype, grows purple cotyledons, and exhibits impaired chloroplast development. Mutations in genes of the MEP pathway, including *DXS*, *IspD*, *IspE*, *IspF*, *IspG* and *IspH*, also show the albino phenotype (Hsieh and Goodman 2005, 2006; Hsieh *et al*. 2008; García-Alcázar *et al*. 2017). These studies indicated that cytoplasmic isoprenoids from the MVA pathway could not effectively compensate for the lack of plastid isoprenoids in *Arabidopsis*. In rice, the *IspE* mutant, *gry340*, showed a green-revertible phenotype, and the *IspF* mutant, *505ys*, exhibited a yellow-green phenotype during the whole growth period (Chen *et al*. 2018; Huang *et al*. 2018). Rice *IspE* and *IspF* mutants could set seeds at maturity, whereas the *Arabidopsis IspE* and *IspF* mutants died at seedling stage. The rice *IspH*

mutant, *las1*, had an albino phenotype, which was consistent with the phenotype of the *Arabidopsis IspH* mutant (Hsieh and Goodman 2005; Liu *et al*. 2020). In rice, the *DXR* mutants died at seedling stage and could not complete the whole life cycle, which were different from *Arabidopsis DXR* mutants. These results suggest a divergence in the function of the MEP pathway genes in dicotyledon *Arabidopsis thaliana* and monocot rice. In addition, we found that there are 112 SNPs and 9 InDel variants in the genome of *OsDXR* with the software RiceVarMap V2.0. Based on the SNP variants of *OsDXR*, 15 subhaplotypes of *OsDXR* were found (Appendix G), suggesting that the genetic diversity of *OsDXR* is rich.

The *IspH/LAS1* mutation affects plastdic RNA editing and IspH/LAS1interacts with the MORF family protein Os09g33480 (Liu *et al*. 2020). In our study, we found that the RNA editing efficiency of *rpl2*-1 and *ndhA*-1019 in the *dxr* mutants was significantly reduced compared with the WT. In addition, OsDXR interacted with OsMORF1 *in vivo*, suggesting that OsDXR may be a component of an RNA editing complex. These results indicate that proteins in the MEP pathway might regulate rice chlorophyll biosynthesis and chloroplast development *via* their interactions with the MORF family of proteins, which will be the focus of our future research.

5. Conclusion

We characterized two rice *OsDXR* mutants and confirmed that *OsDXR* positively regulates chloroplast development in rice. Our results suggest that *OsDXR* may be involved in regulating the expression of chlorophyll biosynthesis and plastid development-related genes, and interacts with the RNA editing factor OsMORF1.

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Declaration of competing interest

The authors declare that they have no competing interests.

References

Barkan A, Goldschmidt-Clermont M. 2000. Participation of nuclear genes in chloroplast gene expression. *Biochimie*, **82**, 559-572.

Carretero-Paulet L, Ahumada I, Cunillera N, Rodríguez-Concepción M, Ferrer A, Boronat A, Campos N. 2002. Expression and molecular analysis of the *Arabidopsis DXR* gene encoding 1-deoxy-D-xylulose 5-phosphate reductoisomerase, the first committed enzyme of the 2-C-methyl-D-erythritol 4-phosphate pathway. *Plant Physiology*, **129**, 1581-1591.

Chen N, Wang P, Li C, Wang Q, Pan J, Xiao F, Wang Y, Zhang K, Li C, Yang B, Sun C, Deng X. 2018. A single nucleotide mutation of the *IspE* gene participating in the MEP pathway for isoprenoid biosynthesis causes a green-revertible yellow leaf phenotype in rice. *Plant Cell Physiology*, **59**, 1905-1917.

Cui X, Wang Y, Wu J, Han X, Gu X, Lu T, Zhang Z. 2019. The RNA editing factor DUA1 is crucial to chloroplast development at low temperature in rice. *New Phytologist*, **221**, 834-849.

Dong H, Fei G L, Wu C Y, Wu F Q, Sun Y Y, Chen M J, Ren Y L, Zhou K N, Cheng Z J, Wang J L, Jiang L, Zhang X, Guo X P, Lei C L, Su N, Wang H, Wan J M. 2013. A rice *virescent-yellow leaf* mutant reveals new

insights into the role and assembly of plastid caseinolytic protease in higher plants. *Plant Physiology*, **162**, 1867-1880.

García-Alcázar M, Giménez E, Pineda B, Capel C, García-Sogo B, Sánchez S, Yuste-Lisbona F J, Angosto T, Capel J, Moreno V, Lozano R. 2017. Albino T-DNA tomato mutant reveals a key function of 1-deoxy-D-xylulose-5-phosphate synthase (DXS1) in plant development and survival. *Scientific Reports*, **7**, 45333.

García-Andrade J, Ramírez V, López A, Vera P. 2013. Mediated plastid RNA editing in plant immunity. *Plos Pathogens*, **9**, e1003713.

Hsieh M H, Chang C Y, Hsu S J, Chen J J. 2008. Chloroplast localization of methylerythritol 4-phosphate pathway enzymes and regulation of mitochondrial genes in *ispD* and *ispE* albino mutants in *Arabidopsis*. *Plant Molecular Biology*, **66**, 663-673.

Hsieh M H, Goodman H M. 2005. The *Arabidopsis IspH* homolog is involved in the plastid nonmevalonate pathway of isoprenoid biosynthesis. *Plant Physiology*, **138**, 641-653.

Hsieh M H, Goodman H M. 2006. Functional evidence for the involvement of *Arabidopsis IspF* homolog in the nonmevalonate pathway of plastid isoprenoid biosynthesis. *Planta*, **223**, 779-784.

Huang R, Wang Y, Wang P, Li C, Xiao F, Chen N, Li N, Li C, Sun C, Li L, Chen R, Xu Z, Zhu J, Deng X. 2018. A single nucleotide mutation of *IspF* gene involved in the MEP pathway for isoprenoid biosynthesis causes yellow-green leaf phenotype in rice. *Plant Molecular Biology*, **96**, 5-16.

Huang W, Zhang Y, Shen L, Fang Q, Liu Q, Gong C, Zhang C, Zhou Y, Mao C, Zhu Y, Zhang J, Chen H, Zhang Y, Lin Y, Bock R, Zhou F. 2020. Accumulation of the RNA polymerase subunit *RpoB* depends on RNA editing by OsPPR16 and affects chloroplast development during early leaf development in rice. *New Phytologist*, **228**, 1401-1416.

Karcher D, Bock R. 2002. Temperature sensitivity of RNA editing and intron splicing reactions in the plastid *ndhB* transcript. *Current Genetics*, **41**, 48-52.

Lange B M, Croteau R. 1999. Isoprenoid biosynthesis via a mevalonate-independent pathway in plants: Cloning and heterologous expression of 1-deoxy-D-xylulose-5-phosphate reductoisomerase from peppermint. *Archives of Biochemistry and Biophysics*, **365**, 170-174.

Lin C S, Chen J J W, Chiu C C, Hsiao H C W, Yang C J, Jin X H, Leebens-Mack J, de Pamphilis C W, Huang Y T, Yang L H, Chang W J, Kui L, Wong G K, Hu J M, Wang W, Shih M C. 2017. Concomitant loss of NDH complex-related genes within chloroplast and nuclear genomes in some orchids. *Plant Journal*, **90**, 994-1006.

Liu X, Cao P H, Huang Q Q, Yang Y R, Tao D D. 2020. Disruption of a rice chloroplast-targeted gene *OsHMBPP* causes a seedling-lethal albino phenotype. *Rice*, **13**, 51.

Liu X, Yi X, Yang Y R, Huang Q Q. 2021. A rice geranylgeranyl reductase is essential for chloroplast development. *Journal of Integrative Agriculture*, **20**, 2592-2600.

Lu Y, Ye X, Guo R, Huang J, Wang W, Tang J Y, Tan L T, Zhu J K, Chu C C, Qian Y W. 2017. Genome-wide targeted mutagenesis in rice using the CRISPR/Cas9 system. *Molecular Plant*, **10**, 1242-1245.

Mahmoud S S, Croteau R B. 2001. Metabolic engineering of essential oil yield and composition in mint by altering expression of deoxyxylulose phosphate reductoisomerase and menthofuran synthase. *Proceedings of the National Academy of Sciences of the United States of America*, **98**, 8915-8920.

Nierhaus K H. 1982. Structure, assembly, and function of ribosomes. *Current Topics in Microbiology and Immunology*, **97**, 81-155.

Okada K, Kawaide H, Kuzuyama T, Seto H, Curtis I.S, Kamiya Y. 2002. Antisense and chemical suppression of the nonmevalonate pathway affects ent-kaurene biosynthesis in *Arabidopsis*. *Planta*, **215**, 339-344.

Porra R J, Thompson W A, Kriedemann P E. 1989. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: Verification

of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, **975**, 384-394.

Rodrigues N F, Fonseca G C, Kulcheski F R, Margis R. 2017. Salt stress affects mRNA editing in soybean chloroplasts. *Genetics and Molecular Biology*, **40**, 200-208.

Rodríguez-Concepción M. 2014. Plant isoprenoids: A general overview. *Methods in Molecular Biology*, **1153**, 1-5.

Rohdich F, Kis K, Bacher A, Eisenreich W. 2001. The non-mevalonate pathway of isoprenoids: Genes, enzymes and intermediates. *Current Opinion Chemical Biology*, **5**, 535-540.

Su N, Hu M L, Wu D X, Wu F Q, Fei G L, Lan Y, Chen X L, Shu X L, Zhang X, Guo X P, Cheng Z J, Lei C L, Qi C K, Jiang L, Wang H, Wan J M. 2012. Disruption of a rice pentatricopeptide repeat protein causes a seedling-specific albino phenotype and its utilization to enhance seed purity in hybrid rice production. *Plant Physiology*, **159**, 227-238.

Takenaka M, Zehrmann A, Verbitskiy D, Kugelmann M, Härtel B, Brennicke A. 2012. Multiple organellar RNA editing factor (MORF) family proteins are required for RNA editing in mitochondria and plastids of plants. *Proceedings of the National Academy of Sciences of the United States of America*, **109**, 5104-5109.

Tang J, Zhang W, Wen K, Chen G, Sun J, Tian Y, Tang W, Yu J, An H, Wu T, Kong F, Terzaghi W, Wang C, Wan J M. 2017. OsPPR6, a pentatricopeptide repeat protein involved in editing and splicing chloroplast RNA, is required for chloroplast biogenesis in rice. *Plant Molecular Biology*, **95**, 345-357.

Tarkowská D, Strnad M. 2018. Isoprenoid-derived plant signaling molecules:biosynthesis and biological importance. *Planta*, **247**, 1051-1066.

Waadt R, Schmidt L K, Lohse M, Hashimoto K, Bock R, Kudla J. 2008. Multicolor bimolecular fluorescence complementation reveals simultaneous formation of alternative CBL/CIPK complexes in planta. *The Plant Journal*, **56**, 505-516.

Wang Y, Ren Y, Zhou K, Liu L, Wang J, Xu Y, Zhang H, Zhang L, Feng Z, Wang L, Ma W, Wang Y, Guo X, Zhang, X, Lei C, Cheng Z, Wan J M. 2017. *WHITE STRIPE LEAF4* encodes a novel P-Type PPR protein required for chloroplast biogenesis during early leaf development. *Frontiers in Plant Science*, **8**, 1116.

Wang Y, Wang Y, Ren Y, Duan E, Zhu X, Hao Y, Zhu J, Chen R, Lei J, Teng X, Zhang Y, Wang D, Zhang X, Guo X, Jiang L, Liu S, Tian Y, Liu X, Chen L, Wang H, Wan J M. 2021. *White panicle2* encoding thioredoxin z, regulates plastid RNA editing by interacting with multiple organellar RNA editing factors in rice. *New Phytologist*, **229**, 2693-2706.

Xiao H, Xu Y, Ni C, Zhang Q, Zhong F, Huang J, Liu W, Peng L, Zhu Y, Hu J. 2018. A rice dual-localized pentatricopeptide repeat protein is involved in organellar RNA editing together with OsMORFs. *Journal of Experimental Botany*, **69**, 2923-2936.

Xing S, Miao J, Li S, Qin G, Tang S, Li H, Gu H, Qu L J. 2010. Disruption of the 1-deoxy-D-xylulose-5-phosphate reductoisomerase (*DXR*) gene results in albino, dwarf and defects in trichome initiation and stomata closure in *Arabidopsis*. *Cell Research*, **20**, 688-700.

Zhang Q, Shen L, Wang Z, Hu G, Ren D, Hu J, Zhu L, Gao Z, Zhang G, Guo L, Zeng D, Qian Q. 2019. OsCAF1, a CRM domain containing protein, influences chloroplast development. *International Journal of Molecular Science*, **20**, 4386.

Zheng H, Wang Z, Tian Y, Liu L, Lv F, Kong W, Bai W, Wang P, Wang C, Yu X, Liu X, Jiang L, Zhao Z, Wan J M. 2019. *Rice albino 1*, encoding a glycyl-tRNA synthetase, is involved in chloroplast development and establishment of the plastidic ribosome system in rice. *Plant Physiology and Biochemistry*, **139**, 495-503.

Zeng Z Q, Lin T Z, Zhao J Y, Zheng T H, Xu L F, Wang Y H, Liu L L, Jiang L, Chen S H, Wan J M. 2020. *OsHemA* gene,encoding glutamyl-trna reductase (GluTR) is essential for chlorophyll biosynthesis in rice (*Oryza sativa*). *Journal of Integrative Agriculture*, **19**, 612-623.

Fig. 1 Comparison of amino acid sequences of four DXR homologs. The following sequences were compared: *Oryza sativa LOC_Os01g01710* (OsDXR); *Setaria italica* XP_004967950.1; *Zea mays* XP_008655547.1; *Arabidopsis thaliana* NP_201085.1; *Sorghum bicolor* XP_021311303.1. The amino acids in dark blue are conserved.

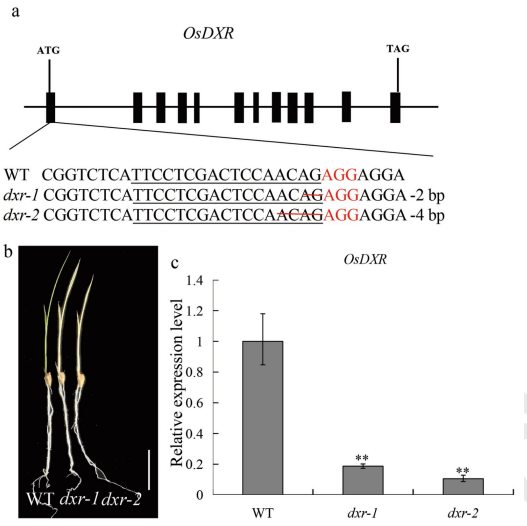

Fig. 2 Disruption of the *OsDXR* gene results in chlorophyll biosynthesis defects in rice. A, Sketch map of the target gene, *OsDXR*, and the mutations of the knockout lines. The 19-bp gene-specific target site and PAM are underlined and shaded red. Red lines represent the missing base. B, the phenotype of *dxr-1* and *dxr-2* at the seedling stage (bar=5 cm). C, the expression level of *OsDXR* in the *dxr* mutants. ** indicates signifificant differences at $P<0.01$.

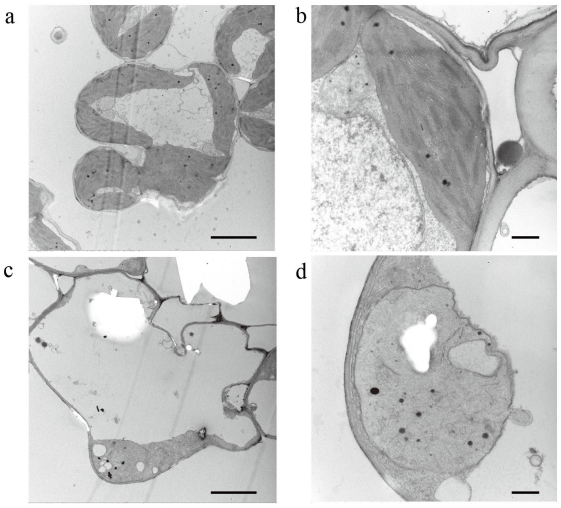

Fig. 3 Chloroplast ultrastructure of WT (A and B) and *dxr* (C and D) leaves. Bars, A and C, 2 μm; B and D, 500 nm.

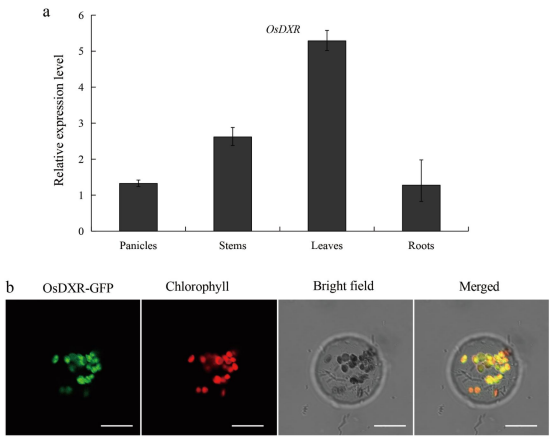

Fig. 4 Expression analysis and subcellular localization of OsDXR. A, expression level of *OsDXR* in various rice organs. B, localization of the OsDXR-GFP protein in rice protoplasts. Bar=10 μm.

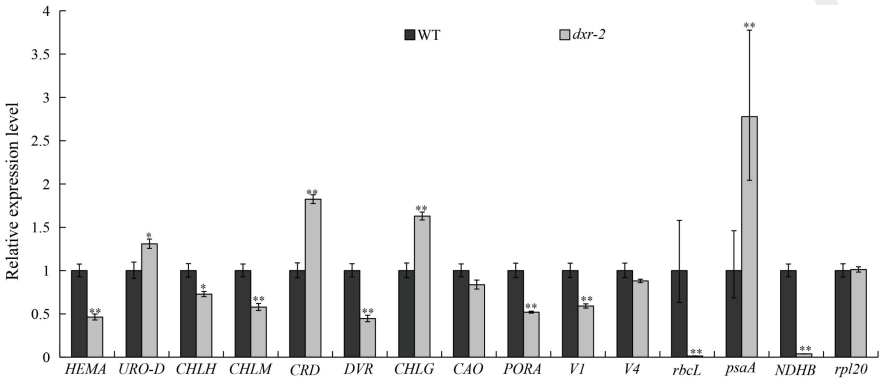

Fig. 5 Expression levels of chlorophyll biosynthesis and plastid development-related genes in WT and *dxr-2* leaves. Total RNA was extracted from the leaves of WT and *dxr-2* mutant plants. Values are presented as the mean±SD of three biological replicates. * and ** indicate signifificant differences at $P<0.05$ and $P<0.01$, respectively.

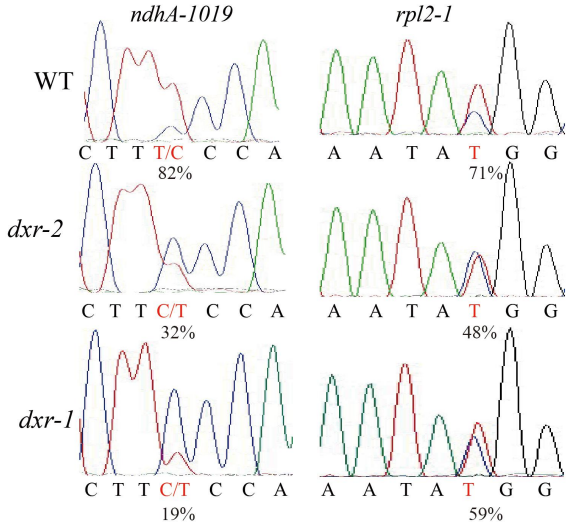

Fig. 6 RNA editing analyses of *rpl2*-1 and *ndhA*-1019 in WT, *dxr-1*, and *dxr-2*. Red labeled nucleotides indicate the editing sites.

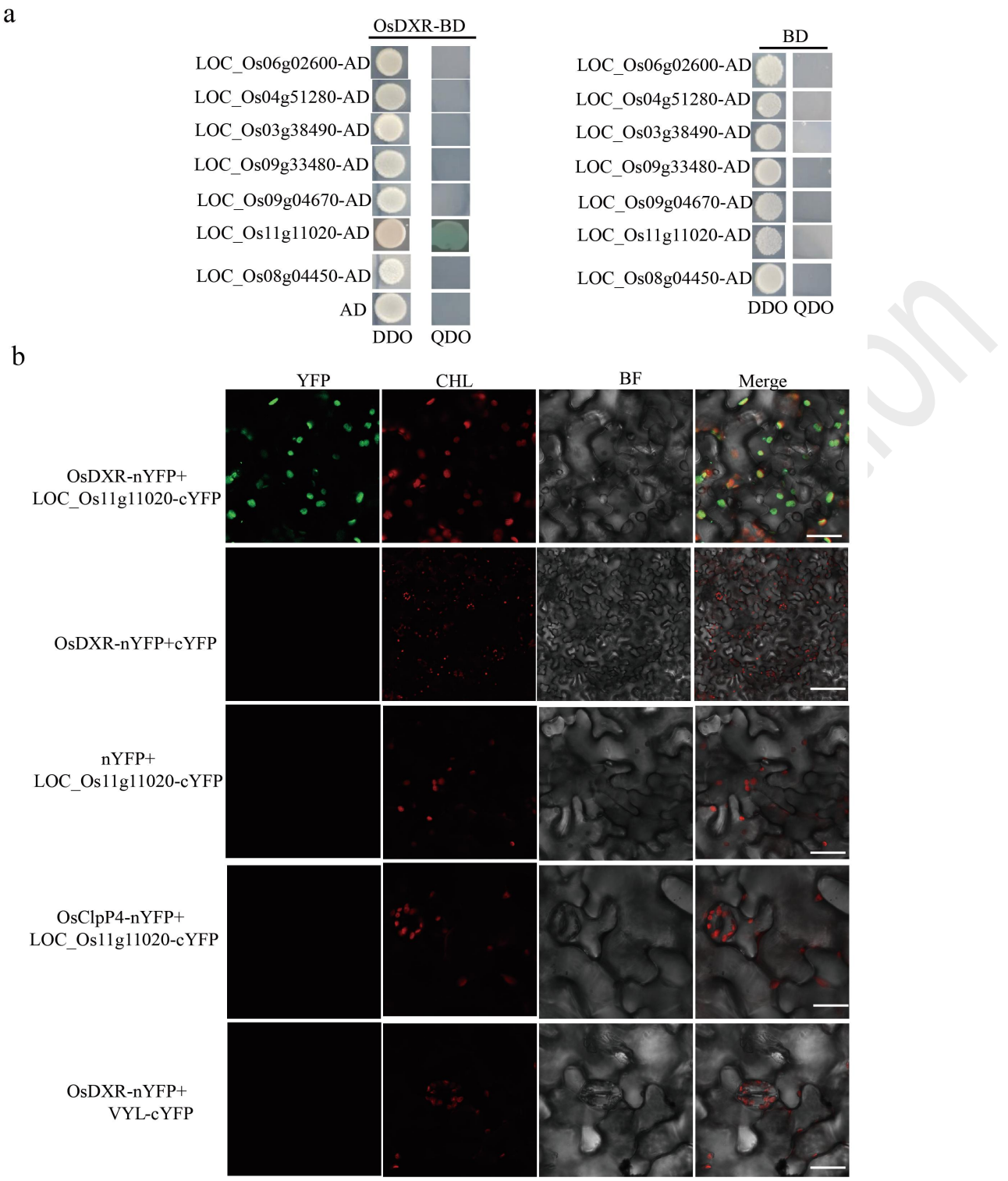

Fig. 7 Interaction identification of OsDXR with OsMORF1. A, Yeast two-hybrid assay of the interaction between OsDXR and OsMORF1. DDO and QDO indicate SD–Leu/–Trp dropout plates and SD–Leu/–Trp/–His/–Ade dropout plates, respectively. QDO contains 40 μg mL^{-1} X-α-Gal. B, BiFC assays of the interaction between OsDXR and OsMORF1 in *N. benthamiana* leaves. Bar=10 μm.