Cyclophilin OsCYP20-2 with a novel variant integrates defense and cell elongation for chilling response in rice

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Summary

- Coordinating stress defense and plant growth is a survival strategy for adaptation to different environments that contains a series of processes, such as, cell growth, division and differentiation. However, little is known about the coordination mechanism for protein conformation change.
- A cyclophilin OsCYP20-2 with a variant interacts with SLENDER RICE1 (SLR1) and OsFSD2 in the nucleus and chloroplasts, respectively, to integrate chilling tolerance and cell elongation in rice (Oryza sativa) (FSD2, Fe-superoxide dismutase 2).
- Mass spectrum assay showed that OsNuCYP20-2 localized at the nucleus (nuclear located OsCYP20-2) was a new variant of OsCYP20-2 that truncated 71 amino-acid residues in N-terminal. The loss-of function OsCYP20-2 mutant showed sensitivity to chilling stress with accumulation of extra reactive oxygen species (ROS). In chloroplasts, the full-length OsCYP20-2 promotes OsFSD2 forming homodimers which enhance its activity, eliminating the accumulation of ROS under chilling stress. However, the mutant had shorter epidermal cells in comparison with wild-type Hwayoung (HY). In the nucleus, OsCYP20-2 caused conformation change of SLR1 to promote its degradation for cell elongation.
- Our data reveal a cyclophilin with a variant with dual-localization in chloroplasts and the nucleus, which mediate chilling tolerance and cell elongation.

Introduction

Temperature is a key factor influencing plant growth and geographical distribution. Chilling tolerance influences crop growth and production, and is regulated by a complex signaling transduction pathway, including abscisic acid (ABA) signaling, the Ca2+-influx signal-dehydration responsive element binding (DREB) pathway and reactive oxygen species-mitogen-activated protein kinase (ROS-MAPK) cascade (Ma et al., 2015; Zhu, 2016; Zhang et al., 2017; Ding et al., 2018; Guo et al., 2018; Zhang et al., 2019). Trade-off between defense and development is an adaption strategy during evolution. OsMADS57 functions in regulation of chilling tolerance and lateral meristem differentiation in rice (Oryza sativa) (Chen et al., 2018). OsMADS57 and TEOSINITE BRANCHED1 (TB1) binds directly to the promoter of OsWRKY94 (a defense gene) and DWARF14 (D14, a organogenesis gene) to mediate chilling stress and development in rice (Arite et al., 2009; Lin et al., 2012; Guo et al., 2013; Chen et al., 2018). As an example of trade-off for biotic stress and development, IPA1 (Ideal plant architecture1) regulates yield and immunity by its phosphorylation/dephosphorylation protein status (Wang et al., 2018). In the signaling downstream, C-repeat binding factors (CBFs) play important roles in both plant growth and freeze stress (Park et al., 2015; Zhao et al., 2016). Cyclophilin (CYP) has peptidyl-prolyl cis-trans isomerase (PPIase) activity, which regulates the cis and trans peptide bond conformations of the proline residues of target proteins, to affect their ability and stability involved in hormone signaling pathways and stress response, including heat, salt, wounding, gibberellic acid (GA), indole-3-acetic acid (IAA) and brassinosteroid (BR) signaling (Matouschek et al., 1995; Wang et al., 2010; Kim et al., 2012; Park et al., 2013; Zhang et al., 2013; Kumari et al., 2015; Lee et al., 2015b; Chaowanont et al., 2016; Cui et al., 2017). However, the function of CYP in cross-talk between hormone signaling and chilling tolerance is little known.

Chilling stress has an adverse effect on cell elongation and differentiation, which induced ROS production, changed
membrane lipid composition and fluidity, damaged proteins and nucleic acids, and changed osmolytes (Thomashow, 1999; Browse & Xin, 2001). ROS, mainly including superoxide (O$_2^-$), O$_2$ and hydrogen peroxide (H$_2$O$_2$), at lower concentrations have been found to act as molecular signals regulating plant growth under normal conditions (Mittler, 2017; Qi et al., 2018; Tian et al., 2018; Yang & Guo, 2018). H$_2$O$_2$ is important for maintaining stem cell and promote stem cell differentiation at lower level (Wang et al., 2015; Xia et al., 2015; Zeng et al., 2017). At higher concentrations, however, ROS degrade polyunsaturated lipids, oxidize protein and damage cells with accumulation under biotic and abiotic stress conditions (Pamplona, 2011). Chloroplasts are sensitive to environmental stresses, producing redundant ROS damaging cells (Mignolet-Spruyt et al., 2016). Superoxide dismutase (SOD) could convert O$_2^-$ to O$_2$ and H$_2$O$_2$, and relieve O$_2$ causing damage to plants (Fridovich, 1978, 1983). Overexpression of SOD increased plant tolerance to low temperature, freezing, water and salt (NaCl) stress (Mckersie et al., 1993, 1999, 2000; Guan et al., 2017).

Cell elongation is regulated by light and temperature and multiple hormones, including GA and BR (Lau & Deng, 2010; Bai et al., 2012; Zhong et al., 2012; Takatsuka & Umeda, 2014). In O. sativa, dozens of dwarf genotypes mediating cell elongation have been identified, including mutants of genes involved in GA and BR biosynthesis and signaling pathways (Sasaki et al., 2002; Ueguchi-Tanaka et al., 2005). In the GA signaling pathway, GID2 (GIBBERELLIN INSENSITIVE DWARF2), an F-box subunit of the SCF E3 complex specifically interacts with phosphorylated SLENDER RICE1 (SLR1), a DELLA protein, to repress its degradation (Gomi et al., 2004; Hirano et al., 2010). DELLA proteins in general, as negative regulators of GA signaling, depend on its transcriptional repression to regulate plant growth (Sun, 2011; Floss et al., 2013; Zhang et al., 2014; Gomez et al., 2016). However, less is known about the connection between GA signaling and stress response.

In this study, we reported the cyclophilin OsCYP20-2 with a novel variant with dual-localization at the nucleus and chloroplasts, which ensured plant growth and enhanced chilling tolerance by recruiting different targets in different organelles. In chloroplasts, OsCYP20-2 targeted OsFSD2, a superoxide dismutase to promote OsFSD2 forming homodimers enhancing its activity in scavenging excessive ROS under chilling stress (FSD2, Fe-superoxide dismutase 2). In the nucleus, OsCYP20-2 changed the conformation of SLR1 and promoted its degradation to dis-inhibit its repression to growth. Therefore, OsCYP20-2 coordinates plant growth and chilling tolerance by its different localizations.

Materials and Methods

Plant materials and growth conditions

The T-DNA insertion mutant line PFG_1A-23625.R (oscyp20-2) in an Oryza sativa cv Hwayoung (HY) background was obtained from RiceGE, the Rice Functional Genomics Express Database, in Korea (An et al., 2003). Rice plants were grown in a glasshouse with a 30°C day : 25°C night cycle. For the analysis of gibberellic acid (GA) and brassinosteroid (BR) response experiments, rice seeds were sterilized with 10% NaClO and grown on ¼ Murashige & Skoog (MS) medium with the indicated concentrations of GA$_3$ at 30°C. Seedlings were measured 1 wk after germination. The primers used for genotyping are listed in Supporting Information Table S1.

Chill stress treatment

Rice seedlings were cultured on Kimura B solution and grown in the glasshouse with a 28–30°C day : 25°C night cycle. After 14 d, seedlings was placed in a 4°C water bath for 80 h and returned to growth conditions for one week recovery. Then, the survival rates were calculated as described in a previous study (Zhang et al., 2017).

Genetic complementation test

The promoter region and the coding region of OsCYP20-2 gene (CYP, cyclophilin) were cloned from wild-type (WT) genomic DNA and constructed into the vector pCAMBIA23A. The construct and the empty vector pCAMBIA23A were transformed into the oscyp20-2 mutant by Agrobacterium-mediated transformation (Ge et al., 2004). The primers used for complementation test are presented in Table S1.

Semi-quantitative real-time PCR (semi-qRT-PCR) and qRT-PCR

Total RNA was extracted from rice seedlings using TRNzol A+ reagent (Tiangen, Beijing) according to the manufacturer’s instructions. cDNA synthesis was performed using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Promega) according to the manufacturer’s protocol. For real-time PCR, 2 µg total RNA was used for reverse transcript with M-MLV RT (Promega). The cDNA samples were diluted to 2–8 ng µl$^{-1}$. Triplicate quantitative assays were performed on 5 µl of each cDNA dilution with the SYBR Green Real-time PCR Master mix (Toyobo, Osaka, Japan) and an MX3000P Real-Time PCR System according to the manufacturer’s protocol (Stratagene, San Diego, CA, USA). The relative quantification method (Delta-Delta CT) was used to evaluate quantitative variation between the replicates examined. All primers used in this study are presented in Table S1.

2-Dimensional electrophoresis and immunoblotting

Isoelectric focusing for the first dimension was carried out using 7-cm-long immobilized pH gradient (IPG) strips (GE Healthcare, Boston, MA, USA) with a linear pH gradient from 3 to 10. Chloroplast proteins and nucleus proteins were separated by 12% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane. The OsCYP20-2 protein was detected by anti-OsCYP20-2 polyclonal antibody; anti-SLR1 and anti-OsBZR1 polyclonal antibodies were ordered from the Beijing Protein Institute BPI.
Mass spectrometric analysis of OsCYP20-2 sequence

The total protein of HY was extracted by buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl2, 10% glycerol, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride) and cultured with protein A/G beads at 4°C for 2 h. Discarding the beads, the supernatant was cultured with antibody of OsCYP20-2 (1: 2000) and subsequently mixed with protein A/G beads at 4°C overnight. Then, the supernatant was discarded and the beads washed three times with the buffer. The protein A/G beads were mixed with loading buffer and the protein separated by SDS-PAGE.

The gel containing OsCYP20-2 was cut at a suitable position and embedded in a clean Eppendorf tube. The gel was cleaned with high-performance liquid chromatography (HPLC)-grade water three times and then dehydrated with acetonitrile (ACN). One milliliter 10 mM DTT diluted with 25 mM NH4NO3 was added and the gel was incubated in a water bath at 56°C for 1 h. The supernatant was discarded and 55 mm iodine acetyl amine (IAM) added before placing the gel in darkness for 45 min. The gel was dehydrated with 25 mM NH4NO3, 50% ACN and ACN sequentially, before the position of OsNutCYP20-2-0.1 mg ml−1 trypsin was added and it was cultured in a 37°C water bath overnight. The gel was analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) and the peptide sequence was aligned against the NCBI rice protein database.

Superoxide dismutase (SOD) activity assay

In order to measure SOD activity, Maltose binding protein (MBP)-OsFSD2 was mixed with glutathione-s-transferase (GST) or GST-OsCYP20-2 in reaction buffer (50 mM Hepes, 13 mM methionine, 0.025% Triton X-100, 0.1 mM EDTA, 2 μM riboflavin and 75 μM nitroblue tetrazolium (NBT, pH 7.6)). It was then placed in a light box for 20 min and measured at 560 nm as described previously (Chu et al., 2005).

Yeast-two-hybrid and -three-hybrid assays

A yeast-two-hybrid (Y2H) cDNA library of 10-d old rice seedlings was constructed according to the manufacturer’s protocols (Clontech, Mountain View, CA, USA). The open reading frame (ORF) of OsCYP20-2 was amplified and constructed in-frame into the pGBK7 vector. The rice cDNA library in the vector pGADT7 was screened, and isolation of the positive clones involved used the Matchmaker system (Clontech). The full-length cDNA of DELLA protein SLENDER RICE1 (SLR1) was amplified and inserted into the pGADT7 vector. Yeast strain AH109 (Clontech) was transformed with pGADT7-SLR1 and pGBK7-OsCYP20-2 plasmids by the lithium acetate (LiAc)-mediated method. Transformations were plated on SD/Ade-His-Leu-Trp selection medium. Colonies showing a positive signal subsequently were examined by activating the lacZ reporter gene.

The yeast-three-hybrid (Y3H) method was performed as described previously (Ding et al., 2015). Vectors containing OsFSD2 with or without bridge protein Os-Nu-CYP20-2 were constructed. A pair of plasmids containing pBridge-OsFSD2 and pGADT7, or with pBridge-OsFSD2-Nu-CYP20-2 and pGADT7, were transformed to yeast strain AH109. Then the colonies were transformed onto plates contain synthetic medium (SD/-Met/-Trp) lacking Trp and Leu, or Trp, Leu and His for growth. The primers used are listed in Table S1.

Fusion protein preparation

cDNA of the OsCYP20-2 gene was amplified and cloned into the pGEX4T-1 vector to generate pGEX4T-1-OsCYP20-2 containing the GST-OsCYP20-2 fusion construct driven by the lacI promoter. The GST-OsCYP20-2 fusion protein was induced by 1 mM isopropyl β-d-thiogalactopyranoside (IPTG) for 5 h and purified by glutathione affinity chromatography as described in the Bulk and RediPack GST purification kit from Pharmacia (New York, NY, USA). The cDNA of SLR1 was inserted into pET-28a, which was used to express SLR1-His purifying by Ni sepharose (GE, USA). all primers used are presented in Table S1.

Bimolecular fluorescence complementation (BiFC)

A BiFC assay was carried out according to the described protocol (Waadt et al., 2008). The truncated chloroplast positioning signal peptide of OsCYP20-2 and the whole coding sequence of OsCYP20-2 were cloned into the PSPYNE vector, and the whole coding sequence of SLR1 and OsFSD2 were cloned into the PSPYCE vector. The corresponding plasmids were electroporated into Agrobacterium (strains GV3101) which were then co-infiltrated into tobacco leaves (Liu et al., 2010). The co-infiltrated tobacco leaves could observe green fluorescent protein (GFP) fluorescence. The GFP fluorescence was excited at 488 nm by use of a confocal microscope (ZEISS LSM 510 META, Zeiss) and detected with 510–550 and 635–680 nm filters. All primers used in this study are listed in Table S1.

PPIase activity assay

A peptidyl-prolyl cis-trans isomerase (PPIase) activity assay was carried out as described previously (Fischer et al., 1989). The typical assay mixture consisted of 35 Mm Hepes buffer, pH 8.0, 100 μM N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide dissolved in 60% (v/v) aqueous dimethyl sulfoxide, 10 μM chymotrypsin, and GST-OsCYP20-2 or GST at 10 nM. All components of the assay mixture except for the chymotrypsin were combined and pre-incubated at room temperature. Chymotrypsin was added and quickly mixed to initiate the reaction, and an absorbance reading at 390 nm was collected every 20 s over 3 min by nucleic acid and protein analyzer (Beckman Coulter DU640).

Fourier transform infrared spectroscopy (FTIR)

The FTIR spectroscopy was performed as described previously (Li et al., 2009). The fusion proteins MBP-SLR1, GST-
OsCYP20-2 and GST were purified and dissolved in phosphate buffered saline (PBS) as described previously (Zhang et al., 2013). The PBS was replaced with D_{2}O buffer containing 50 mM K_{2}HPO_{4}/KD_{2}PO_{4} (pH 7.0) 10 times by the Microcon YM-3 Centrifugal Filter Unit (Millipore) and Amicon Ultra-0.5 ml, 10 kDa Centrifugal Filter Unit (Millipore). FTIR spectra were collected by use of a spectrometer (ABB-BOMEM, Bureau, QC, Canada) equipped with a broadband Mercury-Cadmium-Telluride detector cooled by liquid N_{2}.

**In vitro turnover assay**

The degradation analysis of SLR1-His in *vitro* was performed as described previously (Jing et al., 2015). Briefly, 14-d-old seedlings were prepared to extract total protein, which was ground in liquid N_{2} cultured in extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.01% TritonX-100 and 1 mM phenylmethanesulfonyl fluoride). Then, it was centrifuged at 12 000 g for 30 min at 4°C. Purified 1 μg SLR1-His protein was cultured with total protein extracts with or without MG132 at 4°C with gentle rotation. The mixture was collected at different time and detected by antibody of His.

**3,3’-Diaminobenzidine (DAB), NBT and Trypan blue staining**

We performed DAB and NBT assay with a minor modification from a previously published method (Kaut et al., 2016). Briefly, we cut the second leaf of seed from the base and dipped it into 6 mM NBT solution dissolved in 10 mM sodium citrate (pH 6.0). Besides, the leaf was dipped into DAB solution (1 mg ml^{-1}, pH 3.8) preparing in distilled water. For Trypan blue staining, the leaves were dipped into Trypan blue solution (10 mg ml^{-1}) prepared in distilled water. The stained samples were cultured at room temperature for 12 h and boiled in 75% ethanol for removing chlorophyll (Zhang et al., 2017). At least 10 seedlings were used for staining.

**Results**

**OsCYP20-2 mutant causes cell elongation and chilling sensitivity change**

In order to dissect new function CYP20-2 in rice, we selected 14 related genes in 10 species for comparison and construction of a phylogenetic tree. The amino acid alignment showed that rice OsCYP20-2 possesses all seven of the typical amino acid residues involved in the peptidyl-prolyl cis-trans isomerase (PPIase) activity of cyclophilins (Fig. S1a). However, the phylogenetic tree placed OsCYP20-2 on a different branch from its orthologs in wheat and AtCYP20-2 in *Arabidopsis* (Fig. S1b), which may hint that OsCYP20-2 has a potential divergent function.

The T-DNA insertion mutant of *OsCYP20-2* (*oscp20-2 T-DNA line 1, oscp20-2t1*) was obtained from the Rice Functional Genomics Express Database of Korea (Jeong et al., 2002). Genomic DNA sequencing assay indicated that the T-DNA was inserted in the first intron of *OsCYP20-2*, 50 bp from the transcriptional initiation site (Fig. S1c). The semi-qRT-PCR and qRT-PCR showed that *OsCYP20-2* was rarely detected in the *oscp20-2t1* mutant (Fig. S1,c,d). Phenotypically, *oscp20-2t1* displayed a semi-dwarf phenotype relative to the WT HY cultivar, including shorter plant height throughout the entire growth cycle, which was rescued by *OsCYP20-2* in genetic complementation assay (Figs 1a, S1d). A genetic segregation test showed that the segregation ratio (352 normal: 130 dwarf; χ² = 0.94 < χ²,0.05 = 3.84, P > 0.05) was consistent with a 3 : 1 ratio, indicating that the dwarf phenotype of *oscp20-2t1* was caused by a recessive mutation in *OsCYP20-2*. Plant height differences generally are caused by differences in cell division and elongation. A microscopy assay showed that *oscp20-2t1* had much shorter epidermal cells in the second leaf sheath (63.9 μm) than the WT (90.3 μm) and the complemented lines (79.9 μm; Fig. 1b). These findings suggest that *OsCYP20-2* may be involved in cell elongation in plant growth and development.

In a previous study, OsCYP20-2 was shown to confer tolerance to salt, drought and high light in ectopic expression in tobacco and *Arabidopsis* (Kim et al., 2012). *AtCYP20-2* was induced at transcription level under cold treatment (Romano et al., 2004; Goulas et al., 2006). As an ortholog of AtCYP20-2, OsCYP20-2 may participate in cold response. To study the role of *OsCYP20-2* in chilling stress response, the seedlings were treated at chilling temperature (4°C) for 80 h. Then, they were turned back to 30°C for recovery and statistical survival rate after 7 d (Zhang et al., 2017). The results showed only 7% seedling survival in *oscp20-2t1*; by contrast, there was 50% survival in WT HY of the genetic background *japonica* (Fig. 1c). Transgenic overexpression (OE) lines of *OsCYP20-2* under the genetic background *japonica* Zhonghua 10 (ZH10) were obtained (Fig. S1,d). The survival rate of WT ZH10 was merely 5% after the chilling treatment, whereas that of the OsCYP20-2 OE lines showed >18% survival under the same treatment (Figs 1c, S1,d). The staining colors of Trypan blue (showing dead cells) and DAB (an indicator of ROS), were more deeper in the *oscp20-2t1* mutant than HY after the treatment (Fig. S1,e,f). In sum, OsCYP20-2 positively regulates chilling tolerance in rice and functions either in chilling stress or in plant growth.

The variant of OsCYP20-2 showed a nucleus sublocalization

Western blot assay in WT HY showed that there were two bands of different size recognized by our specific antibody of OsCYP20-2 at 17 and 23 kD (Figs 2a, S2a). Then, we extracted chloroplast and nucleus protein from HY seedlings separately to detect OsCYP20-2 via western-blot and performed 2D electrophoresis assay. The data from this assay revealed that OsCYP20-2 could be detected in chloroplasts and the nucleus with two different molecular masses: 23 kDa and pI 7.8, and the variant with 17 kDa and pI 9.3 (Figs 2b, S2,b,c). The OsCYP20-2 isoforms on the immunoblot in-gel were digested using trypsin for assay with quadrupole time-of-flight mass spectrometry (Q-TOF-MS). The MS data showed that the variant lacked the first
71 amino acids (AAs) from the N-terminus of the full-length protein which was named as OsNuCYP20-2 (nuclear localized OsCYP20-2). The 71 AAs from the N-terminal of OsCYP20-2 matched the peptide predicted as the chloroplast signal peptide (CSP; Figs 2c, S2d).

In order to further explore the subcellular localization of the OsCYP20-2 variants, GFP fusion protein was transiently expressed in tobacco (Nicotiana tabacum) leaf cells with nuclear-localized gene H2B fused with mCherry (Howe et al., 2012). OsCYP20-2-GFP was observed in chloroplasts and the nucleus. OsNuCYP20-2-GFP was visible in the nucleus, whereas CSP-GFP was localized in the chloroplast (Fig. 2d,e). The GFP alone as a negative control was distributed throughout the cells (Fig. 2d,e). To explore the different localizations of OsCYP20-2, we explored the expression of different forms of OsCYP20-2 under cold stress with or without CHX (cycloheximide) treatment. The data showed that CYP20-2 was induced under cold stress without CHX treatment, whereas CYP20-2 showed degradation with CHX treatment under cold stress (Fig. 2f,g). This may hint that the cold-induced pattern was contributed by the biosynthesis. To determine the activity of CYP, the recombinant fusion proteins GST-OsCYP20-2 and GST-OsNuCYP20-2 were used in an assay for PPIase activity (Fig. S2e). The assay showed that PPIase activity of GST-OsNuCYP20-2 was close to that of GST-OsCYP20-2, which was much higher than that of GST alone (Fig. S2f). Therefore, OsCYP20-2 was localized in the chloroplasts and the variant in the nucleus, whereas both possessed PPIase activity and were induced under cold stress.

OsCYP20-2 isofoms interact with SLR1 and OsFSD2

In order to screen for potential targets of OsCYP20-2, we performed a Y2H assay. The data showed there were 30 potential interaction proteins including DELLA proteins SLR1, and the iron superoxide dismutase (SOD-Fe). According to its functional domain, SLR1 can be divided into DELLA (39–63 AAs from the N-terminal of SLR1 protein sequences), THVYNP (85–118 AAs), polyS/T/N (177–235 AAs) and GRAS (236–625 AAs).
Fig. 2 OsCYP20-2 locates in chloroplasts and the nucleus of rice (Oryza sativa) (CYP, cyclophilin). (a) Immunoblotting analysis of the expression of OsCYP20-2 in wild-type (WT) Hyawoung (HY), a complementation line, and oscyp20-2t1, using Histone3 as an internal control. The separated chloroplast and nuclear protein of HY was immunized by antibody of OsCYP20-2, PsbA (a marker gene locating in chloroplast, LOC_08g35420) and Histone3. (b) Chloroplast proteins and nuclear proteins of HY were separated by two-dimensional electrophoresis and detected with polyclonal antibodies to OsCYP20-2. (c) Alignment of full-length amino acid sequences between OsCYP20-2 and nuclear-localized OsCYP20-2 (OsNuCYP20). Representative mass spectrometer spectra of the first tryptic peptides from OsNuCYP20. The detection of a series of y and b ions identified their sequence as PKEVDLQSK.CSP, chloroplast signal peptide. (d) Subcellular localization of green fluorescent protein (GFP) fused with different portions of the OsCYP20-2 protein when transiently expressed in tobacco epidermis leaves. Plasmids carrying CsVMV:CYP20-2-GFP, CsVMV: NuCYP20-2-GFP, CsVMV: CSP-GFP or CsVMV: GFP were transformed into tobacco epidermis leaves along with the nuclear marker gene H2B-mCherry. CYP20-2-GFP fluorescence signals were merged with H2B-mCherry (a histone located in nucleus) or chloroplast auto-fluorescence. It showed that the fluorescence intensity was merged with H2B-mCherry or chloroplast auto-fluorescence, which were co-localized with the nucleus and chloroplasts, respectively. IMAGEJ was used to calculate fluorescence intensity. Y-axes represent pixel intensity. Bars, 20 μm. (e) The CYP20-2-GFP, CSP-GFP, NuCYP20-2-GFP and GFP transient transformed tobacco leaves were isolated for immunoblot assay from (d). H2B-mCherry was used as loading control. CSP, chloroplast signal peptide. (f) The expression level of OsCYP20-2 under 4°C treatment with or without 5 μg ml⁻¹ CHX (cycloheximide). Rubisco was used as loading control. To better show the result, the expression level of CYP20-2 is a long exposure image, whereas NuCYP20-2 is a short exposure image used to measure relative expression level by IMAGEJ. (g) The relative protein expression level shown in (f).
domains. The truncated SLR1 proteins were used to perform another Y2H assay which showed that the GRAS domain of SLR1 is necessary for the interaction in the yeast cells (Fig. 3a). However, a bimolecular fluorescence complementation (BiFC) assay showed that OsNuCYP20-2 interacted with SLR1 in the nucleus in vivo (Figs 3b, S3d). Moreover, a pull-down assay showed the interaction between SLR1 and OsCYP20-2 (Fig. 3c). To examine the genetic relationship between OsCYP20-2 and SLR1, we crossed an SLR1 knock-out rice line (slr1, background Zhonghua 11), as male parent, with oscyp20-2t1 as the female parent. Homozygous slr1 oscyp20-2t1 double mutants had a similar plant height to the slr1 mutant, and taller than oscyp20-2t1 (Figs 3d, S3a). Taken together, these results indicated that SLR1 interacts with OsNuCYP20-2 in the nucleus.

In the rice genome, there were two paralogs of SOD-Fe genes Os06g025000 and Os06g051100 encoding FSD1 and FSD2 (Li et al., 2019). Both FSD1 and FSD2 were constructed into prey vector AD. The Y2H assay showed that OsFSD2 rather than OsFSD1 interacted with OsCYP20-2 (Figs 3a, S3c). BiFC assay showed that OsCYP20-2 interacted with OsFSD2 in chloroplasts in N. benthamiana epidermal cell (Figs 3c, S3a). In vivo, OsCYP20-2 interacted with OsFSD2 in pull-down assay (Fig. 3b). The data suggested that OsCYP20-2 interacts with OsFSD2 in chloroplasts. Taken together, OsCYP20-2 interacts with SLR1 and OsFSD2 in the nucleus and chloroplasts, respectively.

OsCYP20-2 enhances OsFSD2 activity to improve chilling tolerance

Superoxide dismutase forms homodimers or tetramers to protect plants from abiotic and biotic stress by scavenging excessive O$_2^-$ (Alscher et al., 2002; Asensio et al., 2012; Li et al., 2017; Xikeranuu et al., 2019). In rice, expression patterns showed that OsFSD2 was expressed in roots, young stems, young leaves, mature leaves and flowers (Fig. S4a). OsFSD2 was localized in chloroplasts and the gene expression was induced under 4°C mature leaves and flowers (Fig. S4a). OsFSD2 was localized in roots, young stems, young leaves, mature leaves and flowers (Fig. S4a). OsFSD2 was localized in chloroplasts and the gene expression was induced under 4°C mature leaves and flowers (Fig. S4a).

OsFSD2-OE (OE7, OE8 and OE10) and OsFSD2-RNAi (RNAi1 and RNAi2) lines, as well as Dongjin (DJ) in the japonica background were prepared, and DJ were exposed to 4°C for 80 h and returned to normal condition for recovery at least one week. The results showed that DJ accumulated redundant ROS that damaged cells after chilling stress, visualized by DAB (diaminobenzidine), NBT (nitroblue tetrazolium) and Trypan blue staining assays, which showed sensitivity to chilling tolerance comparing with OsFSD2-OE lines (Fig. 4c–h). Moreover, OsFSD2-RNAi lines showed opposite phenotype to OsFSD2-OE lines. This suggested that OsFSD2 positively influences chilling tolerance in rice. When the SOD activity of HY and oscyp20-2t1 were measured, results showed the former was greater than the latter (Fig. S4i). Above all, OsCYP20-2 enhances the activity of OsFSD2 to scavenge redundant ROS, which protect the seedlings from chilling stress.

OsCYP20-2 changes SLR1 conformation and promotes its degradation

Given that OsCYP20-2 possessed PPlase activity and interacted with SLR1, FTIR spectroscopy was used to investigate whether CYP20-2 could catalyze the peptidyl-prolyl isomerization of SLR1 proteins. In the FTIR assay, monitoring of the changes in protein secondary structure showed that SLR1 had several characteristic absorption peaks on the secondary derivative spectrum (Fig. 5a), which were identified as described previously (Li et al., 2009): 1625 cm$^{-1}$ (β-sheet (β-sheet 1)), 1637 cm$^{-1}$ (β-sheet (β-sheet 2)), 1646 cm$^{-1}$ (random coil/loop (random coil/loop)) and 1654 cm$^{-1}$ (α-helices). When SLR1 was interacting with OsCYP20-2, the secondary derivative peaks were 1625 cm$^{-1}$ (β-sheet 1), 1635 cm$^{-1}$ (β-sheet 2), 1644 cm$^{-1}$ (random coil/loop) and 1657 cm$^{-1}$ (α-helices), indicating that the absorption peak of β-sheet 2 was red-shifted by SLR1 binding with OsCYP20-2, whereas the α-helix peak at 1654 cm$^{-1}$ was blue-shifted by 3 cm$^{-1}$, suggesting that the secondary structure change in the interacting pair mainly involved SLR1. The red shift in the SLR1 absorption spectrum indicated that the secondary structure of SLR1 is open and accessible to the polar solvent D$_2$O; however, the blue shift (from 1654 to 1657 cm$^{-1}$) after the addition of OsCYP20-2 showed that CYP20-2 binds SLR1 at its corresponding secondary structure, the α-helices (Fig. 5a). The blue shift is due to hydrophobic interaction between the substrate and the enzyme OsCYP20-2, suggesting that OsCYP20-2 is responsible for changing the secondary structure conformation of SLR1.

In order to investigate the protein stability of SLR1, we assessed SLR1 protein concentrations in an immunoblot assay. The concentrations of SLR1 protein were significantly higher in oscyp20-2t1 than in HY (Fig. 5b), which was consistent with the reduced response of oscyp20-2t1 to GA$_3$ confirmed by assays of amylase in seeds, root-length inhibition and elongation of the second leaf sheath inhibition (Figs 5f–h, S5). Besides, qRT-PCR analysis revealed nearly equal expression of SLR1 in HY, oscyp20-2t1 and complementary lines (Fig. 5c), which suggested that the differences in OsCYP20-2 level might connect with SLR1 degradation. To test this hypothesis, we performed an in vitro degradation assay to detect the stability of SLR1. The purified

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recombinant SLR1-His were incubated in protein extract from WT HY and oscyp20-2t1 to test the degradation sensitivity to the proteasome inhibitor MG132. In the presence of OsCYP20-2, SLR1-His showed accelerated degradation and weakened response to MG132 compared with that of the absence of OsCYP20-2 (Fig. 5d). This result may hint that the changed conformation of SLR1 led to decreased sensitivity of the degradation to MG132.

SLR1, as a negative regulator of GA signaling, inhibited cell elongation to impact on plant growth. Under low temperature conditions, DELLA proteins could inhibit plant growth and improve freezing tolerance in Arabidopsis (Achard et al., 2008). In immunoblotting assay, the result showed that the SLR1 protein was induced under 4°C treatment, whereas oscyp20-2t1 accumulated more SLR1 protein than WT HY (Fig. 5e). OsCYP20-2 and its variant were induced under the chilling stress (Fig. 2f). Taken together, these results indicate that OsCYP20-2 can change the conformation of SLR1 and promote its degradation, which partly relieves SLR1 inhibition to growth under chilling stress.

**Discussion**

During stress response, plants endure multiple physiological and biochemical changes, including reduced and arresting growth, as well as accumulation of redundant reactive oxygen species (ROS). It is a great challenge for plants to coordinate growth and stress defense. The data herein show that the cell growth under chilling stress was integrated by the variant of OsCYP20-2 (CYP, cyclophilin) in the nucleus and its full-length protein in chloroplasts in rice (Oryza sativa). The mutant oscyp20-2t1 showed sensitivity to chilling stress with accumulation of extra ROS blocking cell growth with accumulation of more SLENDER RICE 1 (SLR1) protein compared with wild-type (WT) Hwayoung (HY) (Fig. 1). In chloroplasts, OsCYP20-2 is induced by the chilling treatment to enhance OsFSD2 forming homodimers for elevating its activity, which scavenges excessive ROS to enhance cold tolerance of rice (FSD2, Fe-superoxide dismutase 2). During chilling stress, SLR1, as a negative regulator plant growth, accumulates to block plant growth; in addition, OsCYP20-2 was induced under cold treatment. In the nucleus,
OsCYP20-2 changes the conformation of SLR1 and promotes its degradation. During chilling stress to adaption, WT HY accumulates little SLR1 compared with oscyp20-2t1 (Fig. 5e). In sum, OsCYP20-2 scavenges excessive ROS in chloroplasts and eliminates SLR1 accumulation in the nucleus during chilling stress to coordinate chilling tolerance and growth.

During biotic and abiotic stress, the expression of CYP was induced by heat, drought, cold, salt, wounding and fungal infection (He et al., 2004; Dominguez-Solis et al., 2008; Kim et al., 2013; Zhou et al., 2016). It is likely that OsCYP21-4 regulates peroxidase activity function in oxidative stress tolerance (Lee et al., 2015a). OsCYP2 accommodates activities of antioxidant enzymes to adjust ROS concentration, which enhances salt tolerance in rice (Ruan et al., 2011). OsCYP2-O-1 scavenges spare ROS and maintains ion homeostasis during stress (Kumari et al., 2015). It seems that CYP influences ROS concentration during stress. The mutant oscyp20-2t1 shows sensitivity to chilling stress with accumulation of excessive ROS (Figs 1c, 5e).
2 was induced under chilling stress and positively regulated chilling tolerance (Fig. 2f). This hints that OsCYP20-2 enhances chilling tolerance of rice by scavenging redundant ROS during chilling stress. During the stress, O$_2$ is reduced to superoxidase (O$_2^-$/C0$_2$) which is catalyzed to form hydrogen peroxide (H$_2$O$_2$) by superoxide dismutase (SOD) (Scandalios, 1993). Then, catalase (CAT) catalyzes H$_2$O$_2$ to H$_2$O relieving the O$_2^-$/C0$_2$ damage on the plant. Overexpression of SOD enhances multiple stresses in plant (McKersie et al., 1993, 2000; Guan et al., 2017). In rice, there were eight annotated SOD genes with two iron SODs, SOD-Fe1 and SOD-Fe2 (Li et al., 2019). Our data showed that OsCYP20-2 interacted with OsFSD2 in the chloroplast, which promoted

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**Fig. 5** Rice (Oryza sativa) OsCYP20-2 changes SLENDER RICE 1 (SLR1) conformation and promotes its degradation (CYP, cyclophilin). (a) Second-derivative infrared spectra of SLR1 with or without GST-OsCYP20-2, with MBP-SLR1 + GST-OsCYP20-2 minus the background absorption of MBP + GST; spectra were obtained in a D$_2$O medium (GST, glutathione-S-transferase). (b) Protein concentrations of SLR1 in Hyawoung (HY), oscyp20-2t1 and a complementary line. H3 was used as loading control. (c) The mRNA expression levels of SLR1 in HY, oscyp20-2t1 and the complementary line. Values are expressed as mean ± SD, n = 3. (d) OsCYP20-2 promotes SLR1 degradation *in vitro*. Total protein extracted from wild-type (WT) (HY) and oscyp20-2t1 was incubated with 1 µg SLR1-His recombinant protein, with or without 50 µM MG132, at 4°C for different lengths of time. Rubisco was used as loading control. (e) The expression level of SLR1 in HY and oscyp20-2t1 exposed to 4°C at different times. Histone3 was used as loading control. (f) oscyp20-2t1 was insensitive to GA$_3$ response in α-amylase activity. GA$_3$ induction of α-amylase activity was measured in WT HY and oscyp20-2t1 mutant rice seedling. Error bars indicate ±SD for ≥10 seeds. (g) Root length in HY and oscyp20-2t1 in response to different concentrations of GA$_3$. Error bars indicate ±SD for at least 10 seedlings. (h) Elongation of the second leaf sheath in HY and oscyp20-2t1 in response to different concentrations of GA$_3$. Error bars indicate ±SD for ≥13 seedlings.
OsFSD2 forming homodimers and enhanced its activity (Figs 3a, e, 4a,b). OsFSD2 was shown to positively regulate chilling tolerance by controlling ROS concentration (Fig. 4c,e). Above all, OsCYP20-2 interacts with OsFSD2 in chloroplasts to scavenge redundant ROS under chilling stress, which positively influences chilling tolerance in rice.

In wheat (*Triticum aestivum*), TaCYP20-2 adjusts the accumulation of Rht protein affecting plant height (Li *et al.*, 2010). The OsCYP20-2 mutant showed shorter cell length compared with HY (Fig. 1a,b). Cell elongation and differentiation are the major regulators influencing growth and development, which are complex traits controlled by multiple factors including transcription factors and phytohormones (Kim *et al.*, 2009; Li *et al.*, 2018; Liu *et al.*, 2018). Subsequently, we found that OsCYP20-2 interacted with SLR1 in the nucleus by cellular and genetic assays (Fig. 3a–d). Cyclophilin changes the conformation of target protein to influence its activity and stability (He *et al.*, 2004). In vitro, OsCYP20-2 showed peptidyl-prolyl cis-trans isomerase (PPIase) activity and changed the conformation of SLR1 in Fourier-transform infrared (FTIR) spectroscopy assay (Figs 5a, S2f). The SLR1 protein accumulated in the oscyp20-2t1 mutant, which is consistent with its insensitivity to giberellic acid (GA) response (Figs 5b, S5). Taken together, OsCYP20-2 interacts with SLR1

**Fig. 6** The model of rice (*Oryza sativa*) cyclophilin OsCYP20-2 integrates chilling tolerance and plant growth (CYP, cyclophilin). OsCYP20-2 has dual-localization in chloroplasts and the nucleus. At normal temperature, OsCYP20-2 enhances OsFSD2 activity to scavenge reactive oxygen species (ROS) and promotes SLENDER RICE 1 (SLR1) degradation to ensure plant growth (FSD, Fe-superoxide dismutase). At low temperature, the induced OsCYP20-2 enhances the activity of OsFSD2 to eliminate extra ROS and promotes SLR1 degradation to inhibit its redundant accumulation, thus balancing plant growth and chilling stress.
in the nucleus and changes its conformation to influence plant growth in rice.

In Arabidopsis, SIGMA FACTOR BINDING PROTEIN1 (SIB1) has dual localization in chloroplasts and the nucleus to regulate PHANs (photosynthesis-associated nuclear genes) and PHNPs (photosynthesis-associated plastid genes) expression influencing cell death (Lv et al., 2019). In rice, we revealed by biochemical and cellular assay that the OsCYP20-2 variant is localized in the nucleus (Fig. 2). Under chilling stress, OsCYP20-2 was induced to positively influence chilling tolerance (Figs 1c, 2f). In chloroplasts, OsCYP20-2 enhances the ability of OsFSD2 to scavenge redundant ROS during chilling stress (Figs 4b, S1e). Meanwhile, OsCYP20-2 promotes SLR1 degradation in nucleus, which attributes to accumulation of less SLR1 protein in HY compared with oscyp20-2t1 under 4°C treatment (Fig. 5b,e). During adaption to chilling stress, OsCYP20-2 influences chilling tolerance and plant growth via its two variants in different localizations.

Due to the absent N-terminal sequence of OsNuCYP20-2 compared with OsCYP20-2 as chloroplast signal peptide (CSP), we speculated that full-length OsCYP20-2 is processed proteolytically upon chloroplast localization (Fig. 2c). We hypothesized at least two possible pathways for the production of OsCYP20-2. First, owing to the absence of CSP in OsNuCYP20-2 protein sequences, OsNuCYP20-2 may be retrieved from the plastids by retrograde movement as a secretory pathway or unknown translocon (Nevarez et al., 2017). Second, OsNuCYP20-2 could be transported from plastids to the nucleus by stromules connecting these two compartments (Kwok & Hanson, 2004; Isemer et al., 2012). As more and more genes to be described both locating in nucleus and plastids, it is urgent to clarify these proteins as anterograde or retrograde signaling (Krause & Krupinska, 2009; Krause et al., 2012). From our data, we consider that two forms of OsCYP20-2 may be from the proteolytic process.

In conclusion, OsCYP20-2 together with its variant function on coordination between cell elongation and chilling defense. The OsCYP20-2-OsFSD2-SLR1 module integrates chilling stress and plant growth in rice. OsCYP20-2 has dual localizations in chloroplasts and the nucleus. In chloroplasts, OsCYP20-2 enhances OsFSD2 activity to relieve O2•− damage during chilling stress. In the nucleus, OsCYP20-2 promotes SLR1 degradation to partly release its inhibition to plant growth under cold treatment in rice. During chilling stress, OsCYP20-2 targeted SLR1 and OsFSD2 in the nucleus and chloroplasts, respectively, to coordinate plant growth and chilling tolerance. OsCYP20-2, as a new regulator of cell elongation and SOD activity, balanced the growth and chilling tolerance during cold treatment. It is meaningful and efficient for plants to balance growth and abiotic stress by the dual CYP localization, and this further enhances our understanding of the diversity functions and communication of proteins with dual or multiple subcellular localizations. Our work highlights a new model for plants to coordinate chilling stress and plant growth by its variant and the localizations (Fig. 6).

Some important questions for future studies include how OsCYP20-2 produces two different protein variants and how H2O2 communicates with nuclear-localized proteins to enact defense against chilling stress.

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**Author contributions**

KC, YX, MB, YZhang and ZW designed the project; QG, YZhang and YZhao performed the physiological, biochemical, and genetic experiments; YN performed the genetic transformations; QQ, WL and BW generated the rice genetic hybrids; SL and YW analyzed the protein conformations; KC, QG and YZhang analyzed the data; and QG, KC, YX and YZhang wrote the paper. QG and YZhang contributed equally to this work.

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Research 2465

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**Supporting Information**

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**Fig. S1** Amino acid and phylogenetic analysis of OsCYP20-2 and clarification of transgenic material of OsCYP20-2.

**Fig. S2** Detecting the location of OsCYP20-2 and its enzyme activity.

**Fig. S3** OsCYP20-2 interacted with SLR1 and OsFSD2.

**Fig. S4** OsFSD2 forms homodimers and locates in the chloroplast.

**Fig. S5** oscyp20-2r1 shows insensitivity to GA3 response.

**Table S1** List of primer sequences (5' to 3') used in this study.

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