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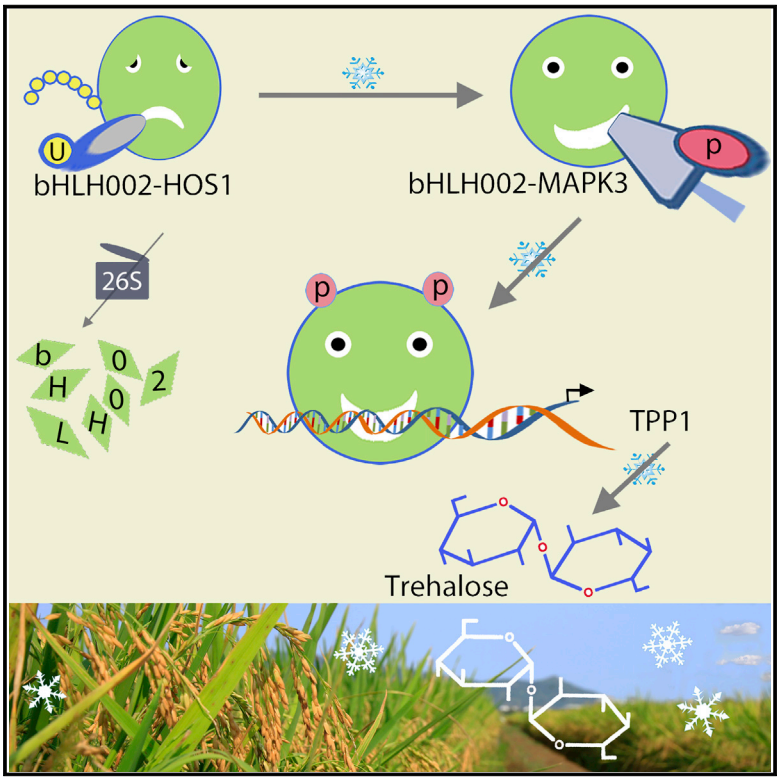
OsMAPK3 Phosphorylates OsbHLH002/OsICE1 and Inhibits Its Ubiquitination to Activate *OsTPP1* and Enhances Rice Chilling Tolerance

Graphical Abstract



Highlights

- OsbHLH002/OsICE1 positively regulates cold signaling via targeting *OsTPP1*
- OsMAPK3 interacts with and phosphorylates the OsbHLH002 protein
- OsMAPK3 interferes with the interaction between OsbHLH002 and HOS1
- OsMAPK3 slows the degradation of OsbHLH002 mediated by OsHOS1 during chilling stress

Authors

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In Brief

The ICE1/bHLH002 transcription factor is a key factor in the ICE1-CBFs-COR signaling pathway, which regulates cold tolerance in plants. Zhang et al. demonstrate that the kinase OsMAPK3 phosphorylates and stabilizes OsbHLH002, which targets *OsTPP1* to increase its transcription, leading to increased trehalose level and enhanced chilling tolerance in rice.

Zhang et al., 2017, Developmental Cell *43*, 731–743
December 18, 2017
https://doi.org/10.1016/j.devcel.2017.11.016

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Developmental Cell
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OsMAPK3 Phosphorylates OsbHLH002/OsICE1 and Inhibits Its Ubiquitination to Activate *OsTPP1* and Enhances Rice Chilling Tolerance

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https://doi.org/10.1016/j.devcel.2017.11.016

SUMMARY

Improvement of chilling tolerance is a major target in rice breeding. The signaling pathways regulating chilling consist of complex networks, including key transcription factors and their targets. However, it remains largely unknown how transcription factors are activated by chilling stress. Here, we report that the transcription factor OsbHLH002/OsICE1 is phosphorylated by OsMAPK3 under chilling stress. The *osbhlh002-1* knockout mutant and antisense transgenic plants showed chilling hypersensitivity, whereas *OsbHLH002*-overexpressing plants exhibited enhanced chilling tolerance. OsbHLH002 can directly target *OsTPP1*, which encodes a key enzyme for trehalose biosynthesis. OsMAPK3 interacts with OsbHLH002 to prevent its ubiquitination by the E3 ligase OsHOS1. Under chilling stress, active OsMAPK3 phosphorylates OsbHLH002, leading to accumulation of phospho-OsbHLH002, which promotes *OsTPP1* expression and increases trehalose content and resistance to chilling damage. Taken together, these results indicate that OsbHLH002 is phosphorylated by OsMAPK3, which enhances OsbHLH002 activation to its target *OsTPP1* during chilling stress.

INTRODUCTION

The ability of plants to tolerate chilling stress is fundamental in determining the growing season and geographical distribution of plants. During rice production, chilling stress affects growth and development, especially at seedling and booting stages (Chinnusamy et al., 2007; Ma et al., 2015; Zhu, 2016). Improvement of chilling tolerance in rice varieties requires clarifying the regulatory mechanisms of chilling signaling pathways. Signal transduction pathways involved in the response to chilling in rice or freezing in *Arabidopsis* have been established. For chilling, membrane protein complexes, such as COLD1-RGA1 (chilling tolerance divergence 1, COLD1; rice G-protein α subunit 1, RGA1), sense cold environments and trigger calcium signaling leading to downstream responses, including activation of transcription factors (the C-repeat binding factors [CBFs]) in rice (Ma et al., 2015). During freezing in *Arabidopsis*, kinase open stomata 1 (OST1)/SNF1-related protein kinase 2.6 (SnRK2.6) phosphorylates ICE1, which activates *CBF* transcription and triggers the defense. For trade-off between defense and development, a cold-responsive protein kinase 1 (CRPK1) phosphorylates 14-3-3λ protein, which interacts with CBFs in the nucleus and negatively regulates freezing tolerance (Guo et al., 2017; Liu et al., 2017). Therefore, the CBFs/DREBs (dehydration-responsive element-binding transcription factors) transcription factor networks for stress defense are shared between freezing and chilling pathways (Dubouzet et al., 2003; Ma et al., 2009; Park et al., 2010; Wang et al., 2008).

In the nucleus, the ICE1-CBF-COR transcriptional network is one of the primary cold acclimation signaling pathways in *Arabidopsis*. In this pathway, CBFs can bind to the CRT/DRE *cis* element in the promoters of *cold-regulated* (*COR*) genes, and thus activate transcription of various *COR* genes (Chinnusamy et al., 2007; Shi et al., 2015; Thomashow, 1999). At the transcription level, *CBFs*, including *CBF1*, *CBF2*, and *CBF3*, are induced rapidly (<1 hr) in response to cold treatment (Chinnusamy et al., 2003; Liu et al., 1998; Stockinger et al., 1997; Thomashow, 1999). *CBFs* are positively regulated by ICE1 and calmodulin-binding transcription activator 3 (CAMTA3) but negatively regulated by R2R3-MYB-like transcription factor 15 (MYB15) and ethylene insensitive 3 (EIN3) (Agarwal et al., 2006; Chinnusamy et al., 2003; Doherty et al., 2009; Shi et al., 2012). ICE1, which is constitutively expressed, can bind to the promoter of *CBF3* and activate its expression (Chinnusamy et al., 2003). The RING finger-type E3 ligase, high expression of osmotically responsive gene 1 (HOS1), mediates the degradation of ICE1 via the 26S-proteasome pathway (Dong et al., 2006). This degradation can be attenuated by sumoylation on ICE1 (Miura et al., 2007). Under cold stress, the phosphorylation of ICE1 by OST1 enhances ICE1 stability and increases its transcriptional activation activity by suppressing HOS1-mediated ICE1 degradation (Ding et al., 2015b).

CBF expression and degradation are regulated by kinases, such as OST1/SnRK2.6 and CRPK1 (Ding et al., 2015b; Liu et al., 2017). Calcium-dependent protein kinase 7 (OsCDPK7), CBL-interacting protein kinase 3 (AtCIPK3), and OsCDPK13 are also involved in cold signaling (Kim et al., 2003; Komatsu et al., 2007; Saijo et al., 2000). The Ca^{2+}-binding

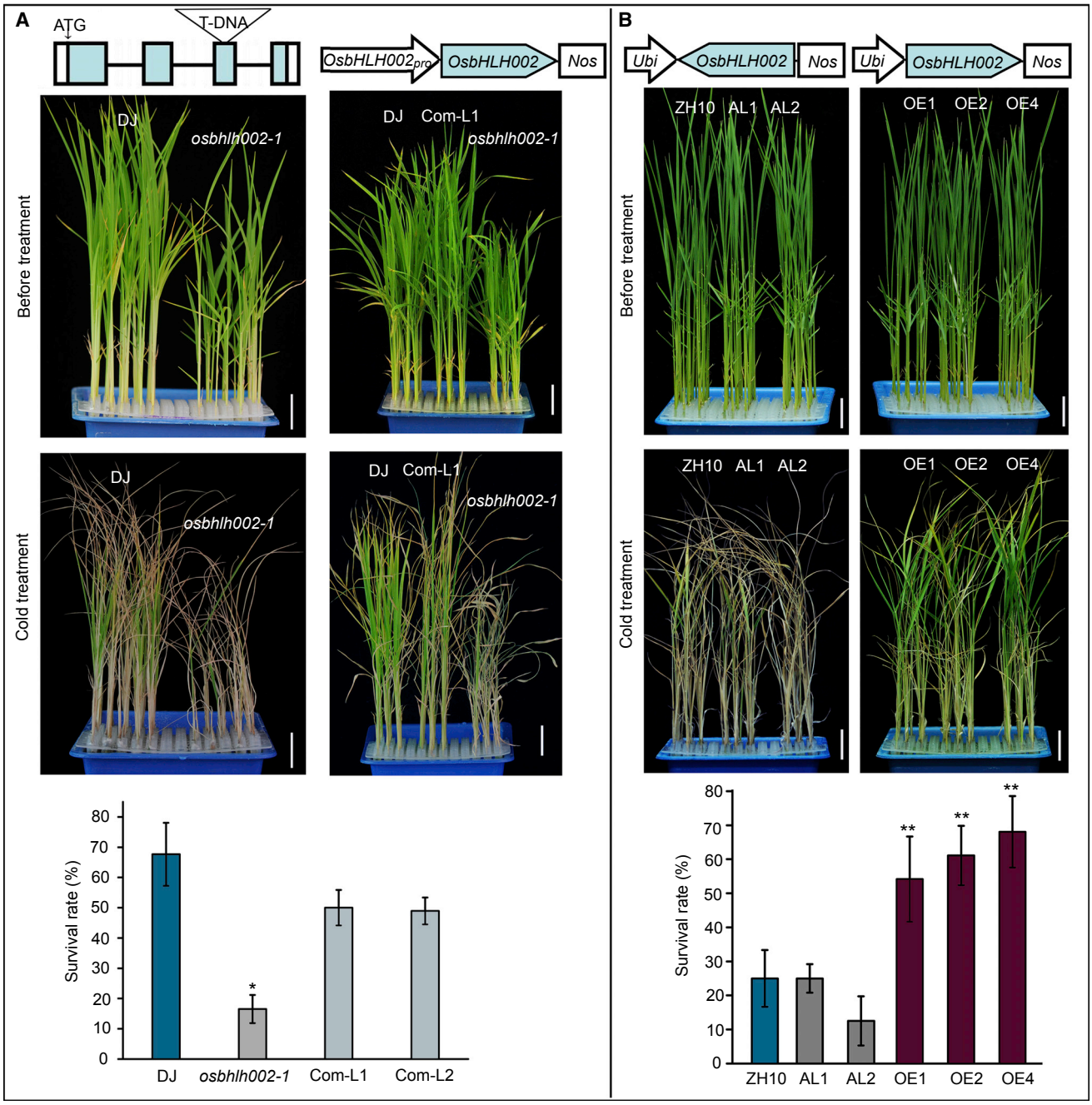

Figure 1. *OsbHLH002* Improves Chilling Tolerance in Rice Seedlings

(A) The *osbhlh002-1* mutant shows chilling sensitivity, and the chilling-sensitive phenotype of *osbhlh002-1* is complemented by expression of *OsbHLH002*. Four-week-old seedlings of wild-type Dongjin (DJ), *osbhlh002-1*, and complementation lines (Com-L1, Com-L2) were treated at 4°C for 84 hr. The survival rate was determined after recovery. Values are means of four replicates ± SD (*$p < 0.05$, t test, n = 30–40 for each replicate). Bar, 2 cm.

(B) The antisense transgenic lines (AL1 and AL2) show chilling sensitivity and the overexpression transgenic lines (OE1, OE2, and OE4) show chilling tolerance. The survival rate was determined after treatment at 4°C for 96 hr and recovery for 7 days. Values are means of four replicates ± SD (**$p < 0.01$, t test, n = 24 for each replicate). ZH10, Zhonghua 10. Bar, 2 cm.

See also Figure S1.

calcium/calmodulin-regulated receptor-like kinase (CRLK1) is a positive regulator of cold tolerance and interacts with AtMEKK2 to regulate the expression of cold-responsive genes such as *CBF1*, *RD29A*, *COR15a*, and *KIN1* (Yang et al., 2010). Multiple members of another kinase family, mitogen-activated protein kinases (MAPKs), including AtMEKK2, OsMAPK3/OsMAPK5/OsMAP1 (encoded by *Os03g17700*), OsMAPK6, OsMEK1/OsMKK6, and MsMKK2, also function in cold signaling (Furuya

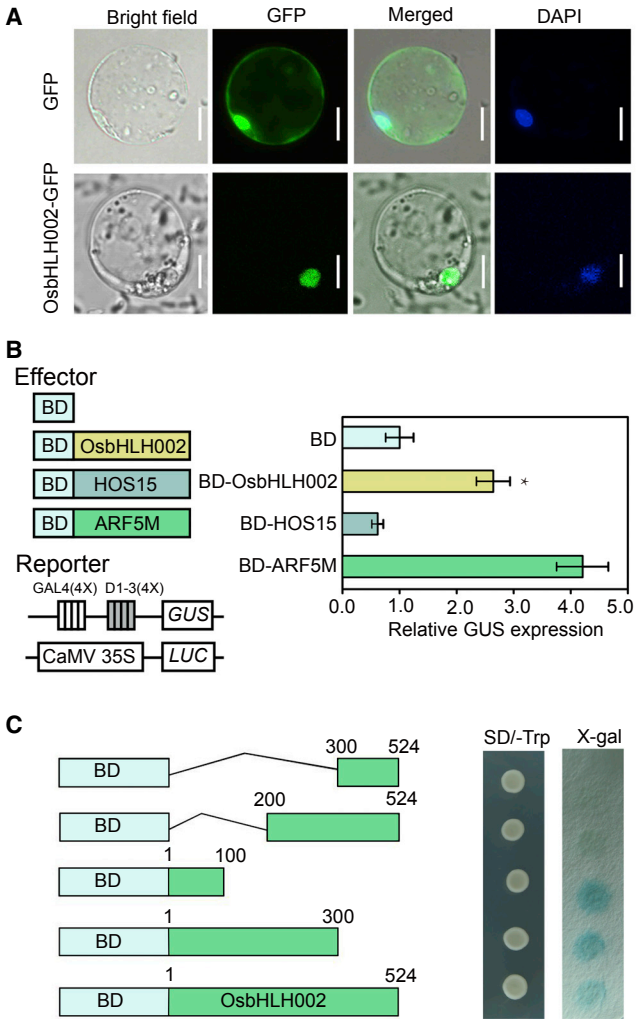

Figure 2. OsbHLH002 Is Localized in the Nucleus and Activates Transcription of the Reporter Gene

(A) Localization of OsbHLH002-GFP in rice protoplasts. Bar, 10 μm.

(B) Transcriptional activity assays in *Arabidopsis* protoplasts. The effector plasmids encode BD-OsbHLH002 fusion proteins, which bind to the promoter in the reporter plasmid. *CaMV35S::LUC* was used as an internal control. ARF5M and HOS15 were used as positive and negative controls, respectively. Data are means of three replicates ± SD (*$p < 0.05$, t test).

(C) Analysis of OsbHLH002 transactivation activity in yeast.

See also Figure S2.

et al., 2014; Gao et al., 2017; Huo et al., 2016; Teige et al., 2004; Xiong and Yang, 2003; Xie et al., 2012). However, the mechanism for the convergence of protein phosphorylation with other cold signaling pathways, such as the extensively studied CBF cascade, remains largely unknown.

The osmotic molecule, trehalose, is produced from glucose by trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) and plays an important role as a stress protectant under abiotic stresses (Wingler, 2002). Overexpression of the trehalose biosynthesis genes *TPS1* and *TPP1* was found to increase trehalose content and enhance rice tolerance to abiotic stresses without altering growth (Garg et al., 2002; Ge et al., 2008; Jang et al., 2003; Li et al., 2011). Exogenous application of trehalose to plants significantly reduces damage caused by abiotic stress in *Arabidopsis* and rice (Mostofa et al., 2015; Nakamura et al., 2011; Yang et al., 2014). In addition, overexpression of *OsTPP1* in maize ears improves yield in well-watered and drought conditions (Nuccio et al., 2015). However, how trehalose metabolism is connected to cold signal pathways remains to be elucidated.

To gain further insight into the molecular mechanisms of chilling signaling, the role of OsbHLH002/OsICE1, a homolog of ICE1 in rice, was investigated in low-temperature signaling. We found that under chilling stress, OsMAPK3 functions to maintain the phosphorylation status of OsbHLH002 and thus prevents its ubiquitination by OsHOS1, leading to increased expression of *OsTPP1* by OsbHLH002 and increased trehalose accumulation.

RESULTS

OsbHLH002 Is a Positive Regulator of Chilling Tolerance

OsbHLH002, also named OsICE1, is a bHLH transcription factor (Li et al., 2006; Nakamura et al., 2011). Phylogenetic analysis showed that OsbHLH002 is a homolog of OsbHLH001 and OsRA1 (Figure S1A). Given what is known about ICE1 and cold tolerance, *osbhlh002-1*, a transfer DNA (T-DNA) insertion line in the *japonica* rice Dongjin (DJ) background was obtained. DNA sequencing revealed that the T-DNA was inserted in the third exon of the *OsbHLH002* gene (Figures S1B and S1C). A qRT-PCR assay showed that *osbhlh002-1* plants had little detectable expression of *OsbHLH002* (Figure S1E). To evaluate the function of *OsbHLH002* in cold stress responses, *osbhlh002-1* and wild-type DJ seedlings were treated at chilling temperature (4°C) and subsequently returned to 30°C for recovery. Survival rates were used as a measure of cold tolerance. After the chilling treatment and recovery, 68% of the wild-type seedlings survived compared with only 18% of the *osbhlh002-1* seedlings (Figure 1A). Transgenic *OsbHLH002* overexpression (OE) and antisense (AL) lines in the *japonica* Zhonghua 10 (ZH10) background were obtained (Figure S1D). The survival rates of *OsbHLH002*-OE lines were more than 50%, however, the survival rates of ZH10 and *OsbHLH002*-AL lines were less than 30% (Figure 1B). There was a significant correlation between chilling tolerance and the expression levels of *OsbHLH002* in the *osbhlh002-1*, OE lines, and ZH10. The staining intensity of trypan blue, a marker of dead cells (Tian et al., 2014), as well as the rate of ion leakage, an indicator of membrane integrity, were dramatically higher in the *osbhlh002-1* mutant than in wild-type seedlings after the chilling treatment (Figures S1F and S1G).

A construct containing *OsbHLH002::OsbHLH002* was introduced into the *osbhlh002-1* for genetic complementation analysis. The expression of *OsbHLH002::OsbHLH002* rescued the chilling-sensitive phenotype of *osbhlh002-1* (Figures 1A and S1H). These results suggest that *OsbHLH002* modulates chilling tolerance in rice.

OsbHLH002 Is Localized in the Nucleus and Has Transactivation Activity

Organ-specific *OsbHLH002* expression data in the Rice eFP Browser (http://bar.utoronto.ca/efprice/cgi-bin/efpWeb.cgi) showed that *OsbHLH002* transcripts were highly abundant in seeds, young panicles, and shoot apical meristems

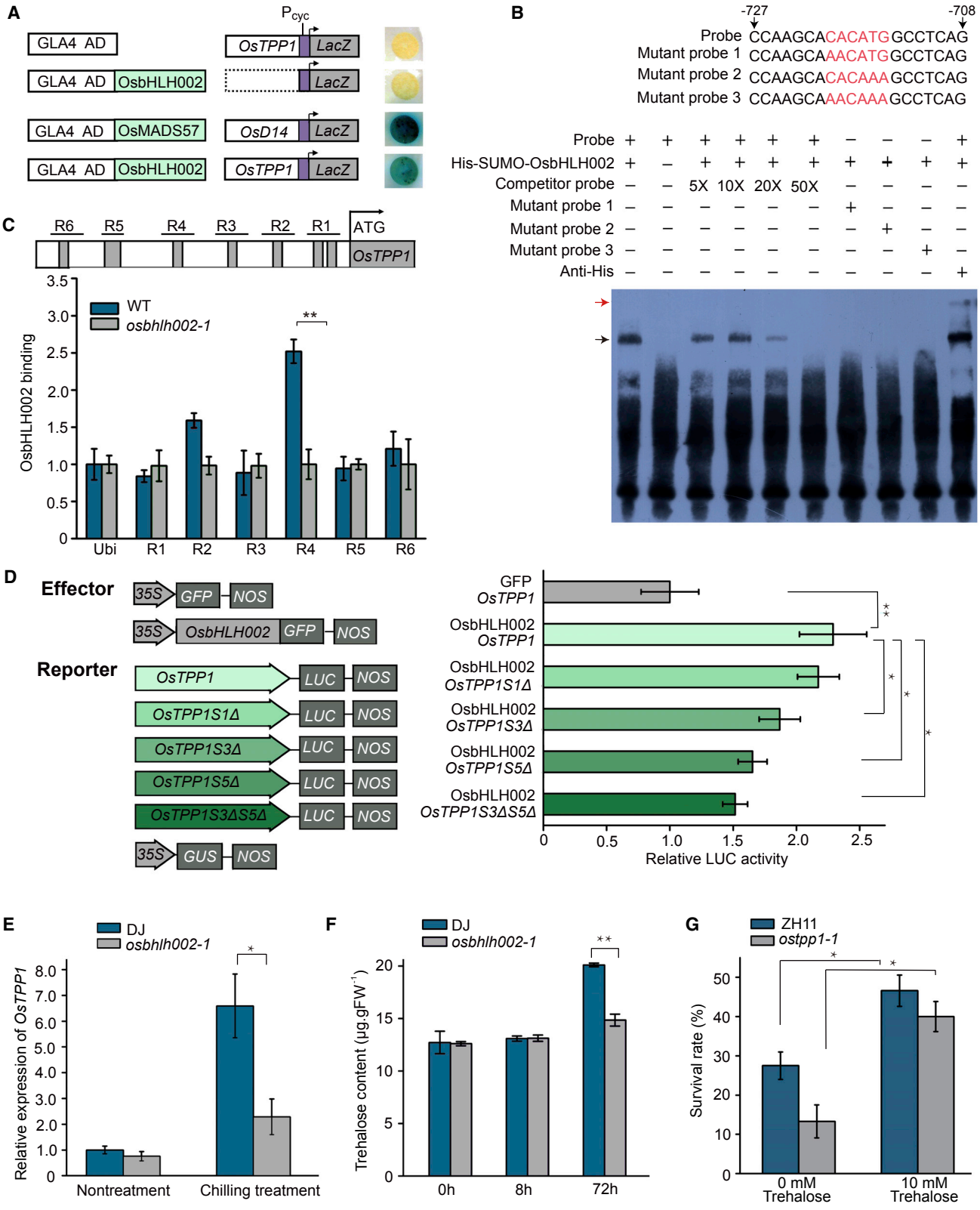

Figure 3. OsbHLH002 Binds to the Promoter of *OsTPP1* and Activates Its Transcription to Increase Trehalose Synthesis

(A) Yeast one-hybrid analysis to test the ability of OsbHLH002 to bind to the *OsTPP1* promoter. OsMADS57 and the *D14* promoter were used as a positive control.

(legend continued on next page)

(Figure S2A), which was confirmed by qRT-PCR (Figure S2B). Subcellular localization analysis using rice protoplasts transiently transformed with a *35S::OsbHLH002-GFP* construct indicated that OsbHLH002-GFP fluorescence completely overlapped with the DAPI-stained nucleus, while control GFP was distributed throughout the cell (Figure 2A). To test its transcriptional activity, the *OsbHLH002* coding sequence was fused in-frame with the GAL4 DNA-binding-domain (BD)-coding sequence in an effector vector (Figure 2B, left panel). GUS staining assays showed that OsbHLH002 activation of the *GUS* reporter was 2.8-fold higher than the BD vector alone (Figure 2B, right panel), indicating that OsbHLH002 has transactivation activity. The transactivation activity was further confirmed by β-galactosidase activity in yeast (Ma et al., 2009), and the result showed that the N-terminal 100 amino acids were critical for transactivation activity (Figure 2C).

OsbHLH002 Directly Targets *OsTPP1*

The bHLH domain of the transcription factor preferentially binds to the motifs such as E-box (CACATG) and G-box (CACGTG) elements (Li et al., 2006). Yeast one-hybrid assays showed that OsbHLH002 can bind to both of these elements (Figure S3A). It was reported that *OsTPP1* encodes a key enzyme for trehalose synthesis and functions to enhance cold tolerance downstream of *OsbHLH002* in rice (Ge et al., 2008; Nakamura et al., 2011). Sequence analysis found that there are eight putative E-box elements in the *OsTPP1* promoter region (Figure S3B). Yeast one-hybrid assays showed that OsbHLH002 could bind to the *OsTPP1* promoter (Figure 3A). Further electrophoretic mobility shift assays (EMSA) showed that the E-box located at −708 bp to −727 bp was preferentially bound by OsbHLH002 (Figure S3B). Therefore, the binding specificity of OsbHLH002 to this E-box sequence (−708 bp to −727 bp) was further evaluated using binding competition assays. In the presence of increasing concentrations of unlabeled competitor probe, the signal intensity of retarded bands corresponding to His-SUMO-OsbHLH002:probe complexes decreased. Addition of anti-His antibody to the reaction resulted in the appearance of one additional retarded band with increased molecular weight (Figure 3B). To investigate the binding of OsbHLH002 to the *OsTPP1* promoter *in vivo*, a chromatin immunoprecipitation (ChIP) assay using an anti-OsbHLH002 antibody was performed. The R4 region containing the sequence from −708 bp to −727 bp was more highly enriched in genomic DNA immunoprecipitated by OsbHLH002 from wild-type than from the *osbhlh002-1* mutant (Figure 3C). These results suggest that OsbHLH002 binds to the E-box located within the −708 bp to −727 bp of *OsTPP1* promoter region.

To detect the effect of OsbHLH002 on the *OsTPP1* transcription, transcriptional regulation activity assay was employed (Guo et al., 2013). The luciferase (LUC) reporter gene was activated when *35S::OsbHLH002* and *OsTPP1::LUC* were co-introduced into *Arabidopsis* protoplasts. In contrast, the strength of *OsTPP1* activation by OsbHLH002 decreased when the E-box elements in the R2 and/or R4 regions were deleted from the *OsTPP1* promoter (Figures 3D and S3B). In addition, qRT-PCR analysis indicated that the expression level of *OsTPP1* in *osbhlh002-1* seedlings was significantly lower than in wild-type DJ after the chilling treatment (Figure 3E). Accordingly, the *osbhlh002-1* seedlings had lower trehalose levels than wild-type following chilling stress (Figure 3F). Exogenous application of trehalose enhanced the chilling tolerance of both wild-type and *ostpp1-1* (Figures 3G and S3C).

To understand the function of *OsTPP1*, the chilling tolerance of T-DNA insertion line *ostpp1-1* was observed. The mutant *ostpp1-1* had a lower survival rate (20%) than wild-type Zhonghua 11 (ZH11) (34%) after chilling treatment (Figures 4A and S4A–S4C). In the progeny from *OsbHLH002*-OE2 X *ostpp1-1*, mutation of *OsTPP1* abolished the chilling-tolerant phenotype of *OsbHLH002* overexpression (Figures 4B and S4D). These results suggest that the function of *OsbHLH002* in rice chilling tolerance is genetically dependent on *OsTPP1*.

OsbHLH002 Interacts with OsMAPK3

OsMAPK3 is a member of the MAPK family and is primarily involved in positively regulating tolerance to abiotic stresses in rice (Singh and Sinha, 2016; Xiong and Yang, 2003). It was reported that OsRAI1, a homolog of OsbHLH002, is one of the substrates of OsMAPK3 (Kim et al., 2012).

Our yeast two-hybrid data showed that OsbHLH002 can interact with OsMAPK3 (Figure 5A). A bimolecular fluorescence complementation (BiFC) assay showed that co-expression of

(B) An EMSA assay to analyze the binding of OsbHLH002 to the E-box element in S5 (−708 to −727) in the *OsTPP1* promoter. The competitor probe was added at 5-, 10-, 20-, and 50-fold molar excess of labeled probes, respectively. The red arrow indicates retarded bands corresponding to complexes containing His antibody, His-SUMO-OsbHLH002, and labeled probes; the black arrow indicates the bands corresponding to complexes containing His-SUMO-OsbHLH002 and labeled probes.

(C) ChIP-qPCR assay of 3-week-old seedlings to show the relative binding strength of OsbHLH002 to different regions in the *OsTPP1* promoter. The relative locations of each region, R1 to R6, are indicated in the upper panel. The *Ubiquitin* (*Ubi*) promoter was used as a control. Data are means of three replicates ± SD (**$p < 0.01$, t test).

(D) Transcriptional activation of *OsTPP1* expression by OsbHLH002 in *Arabidopsis* protoplasts. Schematic diagrams of reporter and effector constructs used in the protoplast transcription system (left panel) and for transcriptional activation activity assays in *Arabidopsis* protoplasts (right panel) are shown. S1Δ, S3Δ, and/or S5Δ indicate deletion of E-box elements in S1, S3, and/or S5 within the *OsTPP1* promoter (see Figure S3B). Luciferase activities were measured in *Arabidopsis* protoplasts co-transfected with the effector plasmid and different combinations of reporters. The activity of protoplasts transfected with the empty effector construct was defined as 1. Values are means of three replicates ± SD (*$p < 0.05$ and **$p < 0.01$, t test).

(E) The relative transcription level of *OsTPP1* in wild-type Dongjin DJ and *osbhlh002-1* mutant seedlings with or without chilling treatment for 12 hr. Data are means of three replicates ± SD (*$p < 0.05$, t test).

(F) Trehalose content in wild-type DJ and *osbhlh002-1* mutant seedlings after chilling treatment for 0 hr, 8 hr, and 72 hr. Data are means of three replicates ± SD (**$p < 0.01$, t test).

(G) Trehalose enhanced rice seedling chilling tolerance. Zhonghua 11 (ZH11) and *ostpp1-1* seedlings with or without 10 mM trehalose treatment were treated at 4°C. The survival rate was determined after recovery. Values are the means of three replicates ± SD (*$p < 0.05$, t test, n = 30–40 for each replicate).

See also Figure S3.

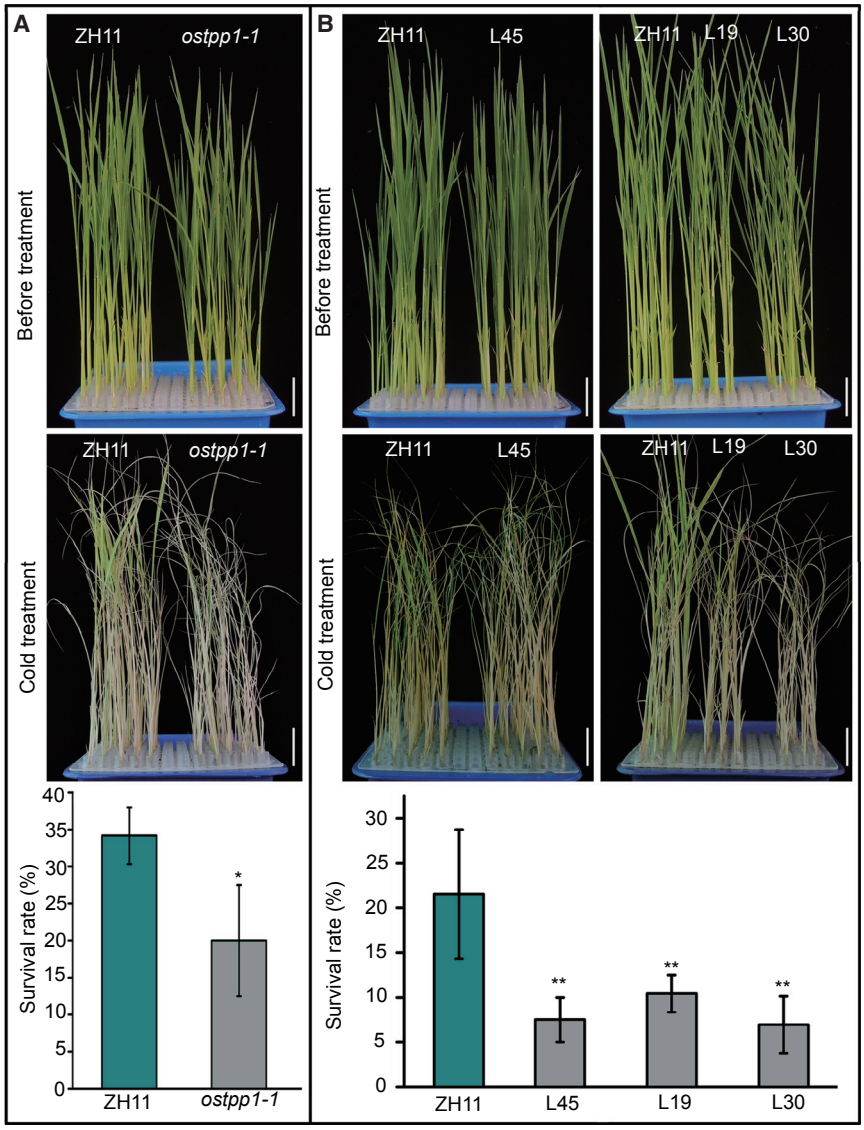

Figure 4. *OsbHLH002* Function in Chilling Tolerance Is Dependent on *OsTPP1*

(A) The *ostpp1-1* mutant shows chilling sensitivity. The survival rate was determined after treatment at 4°C for 84 hr and subsequently recovery at 30°C for 7 days. Data are the means of three replicates ± SD (*$p < 0.05$, t test, n = 30–40 for each replicate). Bar, 2 cm.

(B) Survival rate of the progeny from OE2 × *ostpp1-1*. Three offspring lines (L45, L19, and L30) were obtained from crossing the *OsbHLH002*-overexpression line 2 (OE2) with *ostpp1-1*. Data are the means of three replicates ± SD (**$p < 0.01$, t test, n = 24–40 for each replicate). Bar, 2 cm.

See also Figure S4.

the OsbHLH002-cYFP and OsMAPK3-nYFP in rice protoplasts produced an obvious YFP signal in the nucleus (Figure 5B), and a co-localization assay also showed that the distributions of OsbHLH002 and OsMAPK3 overlapped in the nucleus (Figure S5A). Co-immunoprecipitation (CoIP) assays further showed that a complex containing OsbHLH002-FLAG and OsMAPK3-GFP formed in *Arabidopsis* protoplasts (Figure 5C). The N terminus (amino acids 1–390) of OsbHLH002 and the C terminus (amino acids 301–369) of OsMAPK3 were required for OsbHLH002-OsMAPK3 interaction in yeast (Figures S5B–S5D). Thus, both *in vitro* and *in vivo* data suggest that OsbHLH002 physically interacts with OsMAPK3 in the nucleus.

OsMAPK3 Phosphorylates OsbHLH002 and Promotes Its Transactivation Activity

The interaction between OsbHLH002 and OsMAPK3 raises the question whether OsbHLH002 is a substrate of OsMAPK3. Based on bioinformatics predictions (http://kinasephos.mbc.nctu.edu.tw), OsbHLH002 contains five putative MAPK phosphorylation sites: T404, T406, S407, T412, and S433. An *in vitro* phosphorylation assay was performed to test this possibility. In contrast to the negative controls, in the presence of maltose-binding protein (MBP)-fused OsMAPK3 and His-SUMO-fused OsbHLH002, a single band corresponding to His-SUMO-OsbHLH002 phosphorylated at Ser and/or Thr residues was observed. The signal density of this band was enhanced when the amount of the MBP-OsMAPK3 protein was increased (Figure 5D). The five potential Ser and Thr phosphorylation sites in OsbHLH002 were mutated to Ala (termed OsbHLH002^{5A}), and the phosphorylation status of OsbHLH002^{5A} was assayed *in vitro*. The immunoblotting results showed no phosphorylation signal when OsbHLH002^{5A} was used as the substrate (Figure S5E). Immunoblotting of nuclear proteins with anti-phosphoSer/Thr antibody showed that the level of phosphorylated OsbHLH002 in chilling-treated seedlings (8 hr) was 16.3-fold higher than in untreated ones (Figure 6A). To confirm phosphorylation *in vivo*, OsbHLH002 immunoprecipitated from cold-treated seedlings was dephosphorylated by lambda alkaline phosphatase. After hydrolysis for 60 min, only about 50% of the phosphorylated OsbHLH002 remained (Figure 6B). This suggests that phosphorylation of OsbHLH002 can be induced by chilling.

It is known that the activity of MAPKs is typically activated via phosphorylation. To determine if OsMAPK3 is activated by chilling stress, the phosphorylation level of OsMAPK3 in rice was determined using an antibody against phospho-p42/p44 (Jia et al., 2016). The immunoblotting results showed that high-density bands corresponding to phospho-OsMAPK3 were present in protein extracts of chilling-treated seedlings (Figure 6C), which indicates that, consistent with a previous report (Xiong and Yang, 2003), the kinase activity of OsMAPK3 was activated after chilling treatment.

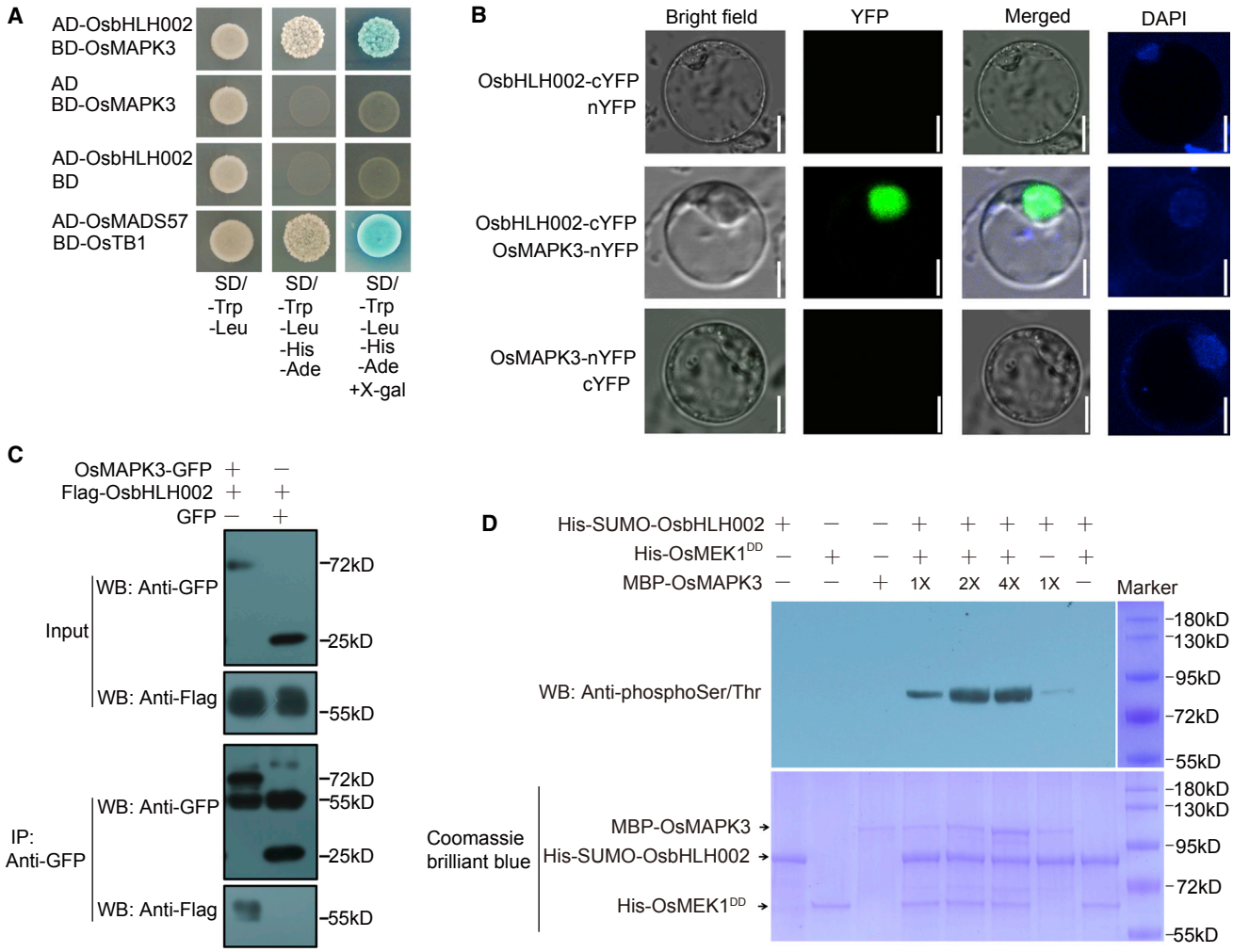

Figure 5. OsbHLH002 Interacts with OsMAPK3

(A) Detection of OsbHLH002-OsMAPK3 interaction with a yeast two-hybrid assay. The AD-OsMADS57 and BD-OsTB1 combination (Guo et al., 2013) was used as a positive control.

(B) Verification of the interaction between OsbHLH002 and OsMAPK3 in rice protoplasts by BiFC assay. Empty nYFP and cYFP were used as negative controls. Scale bar, 10 μm.

(C) Co-immunoprecipitation (CoIP) of OsbHLH002 and OsMAPK3 in *Arabidopsis* protoplasts. Flag-OsbHLH002 (molecular weight [MW], 55 kDa) and OsMAPK3-GFP (MW, 70 kDa) were co-expressed in *Arabidopsis* protoplasts. + or − denote the presence or absence of the protein in each sample.

(D) OsMAPK3 phosphorylates OsbHLH002 *in vitro*. The gel stained with Coomassie brilliant blue was used as a loading control.

See also Figure S5.

To explore the effects of OsMAPK3 on phosphorylation of OsbHLH002, the levels of phospho-OsbHLH002 in the *OsMAPK3*-overexpression line (*MAPK3*-OE) and wild-type DJ were compared by immunoblotting. The relative intensity of phospho-OsbHLH002 bands was higher in *OsMAPK3*-OE lines than that in DJ both before (0 hr) and after 24 hr of cold treatment (Figure 6D). In contrast, the level of phospho-OsbHLH002 in the *OsMAPK3*-antisense line (*OsMAPK3*-AS) (Hu et al., 2015) was lower than in the wild-type Xiushui 11 (XS) after chilling treatment (Figure 6E). These results suggest that OsbHLH002 is a substrate of OsMAPK3. OsMAPK3 activity was enhanced by chilling treatment, which subsequently resulted in an increase in phospho-OsbHLH002 level.

The effect of OsMAPK3 on the transactivation activity of OsbHLH002 was evaluated using GUS reporter assays (Guo et al., 2013). *Arabidopsis* protoplasts co-transformed with OsMAPK3 and BD-OsbHLH002 had higher levels of GUS activity than protoplasts expressing BD-OsbHLH002 alone, indicating that OsMAPK3 increases the transactivation activity of OsbHLH002 (Figure 6F).

To confirm the function of OsMAPK3 in chilling tolerance, the *OsMAPK3*-overexpression lines (OE3 and OE8) were treated at 4°C. The survival rate of *OsMAPK3*-overexpression lines was greater than 40%, while the survival rate of wild-type DJ was about 15% under the same conditions (Figures 6G and S6A). The mutated protein OsbHLH002^{5A} did not rescue the chilling-sensitive phenotype of *osbhlh002-1* (Figures 6H and S6B). These

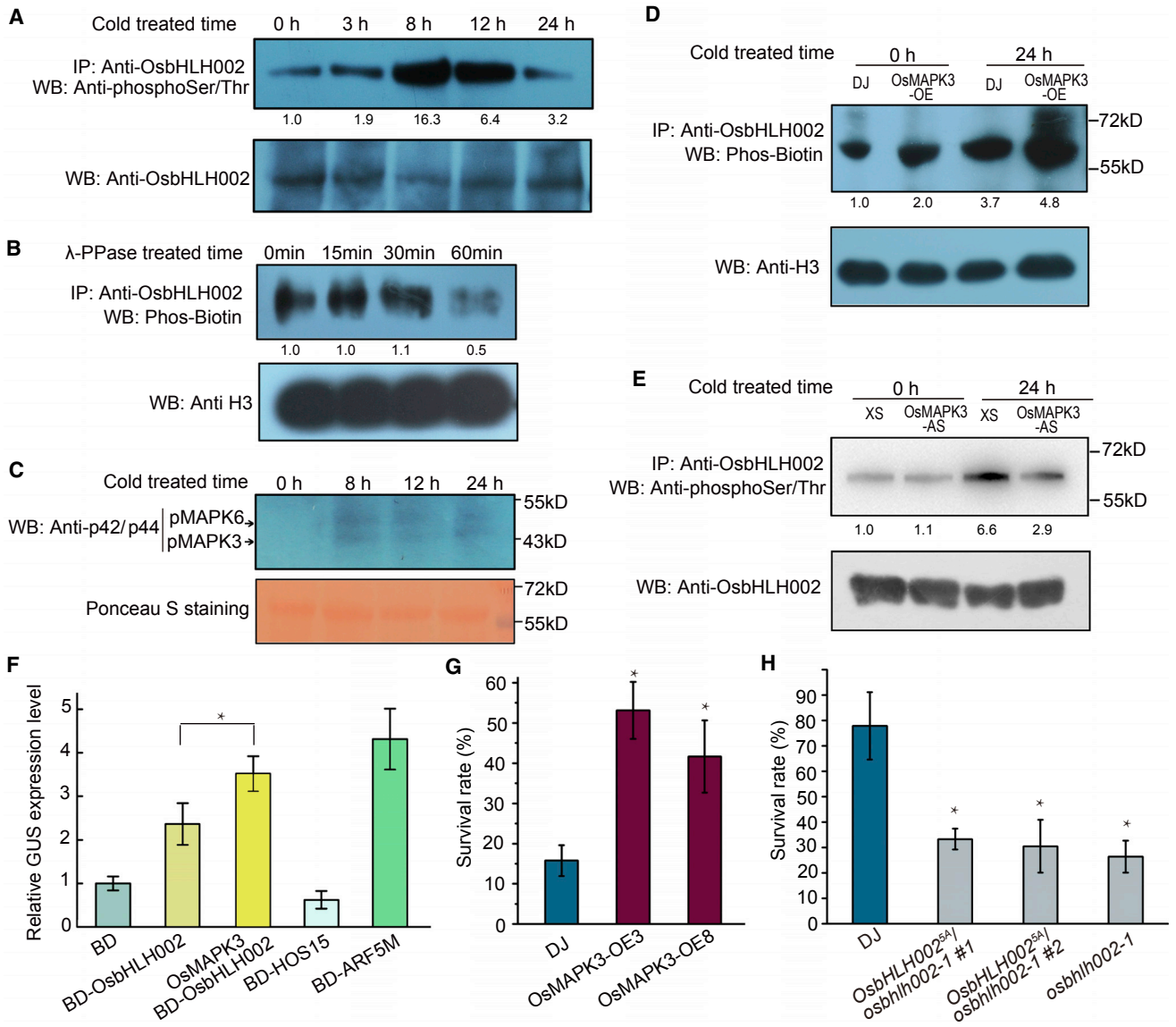

Figure 6. The Chilling-Induced Accumulation of Phosphorylated OsbHLH002 and Increase in Transcriptional Activity Are Mediated by OsMAPK3

(A) Phosphorylation of OsbHLH002 under cold stress *in vivo*. The relative intensity is indicated below each band (normalized to the untreated sample used as 1.0). Nuclear proteins were extracted from 3-week-old rice seedlings treated at 4°C for 0 hr, 3 hr, 8 hr, 12 hr, and 24 hr, respectively. Then the nuclear proteins were immunoprecipitated with anti-OsbHLH002 antibody and separated by SDS-PAGE for immunoblotting analysis using anti-phosphoSer/Thr antibody.

(B) Verification of OsbHLH002 phosphorylation *in vivo* by phosphatase treatment. Three-week-old rice seedlings were treated at 4°C for 24 hr. Nuclear proteins immunoprecipitated with anti-OsbHLH002 antibody were hydrolyzed with lambda protein phosphatase at 30°C for 0, 15, 30, and 60 min, respectively. The proteins were then separated by SDS-PAGE, and immunoblotting analysis was done using biotinylated Phos-tag.

(C) Detection of the phosphorylation level of OsMAPK3 in rice seedlings after chilling treatment. Anti-p-MAPK antibodies (anti-phospho-p42/p44 antibody) were used for immunoblotting.

(D) Detection of the phosphorylation level of OsbHLH002 by immunoblotting in *OsMAPK3* overexpression (*MAPK3*-OE) and wild-type Dongjin (DJ) plants with or without chilling treatment using biotinylated Phos-tag. H3 served as a control.

(E) Detection of the phosphorylation level of OsbHLH002 in *OsMAPK3* antisense (*MAPK3*-AS) and wild-type Xiushui 11 (XS) plants with or without chilling treatment by immunoblotting using anti-phosphoSer/Thr antibody.

(F) OsMAPK3 promotes the transactivation activity of OsbHLH002 in *Arabidopsis* protoplasts. *35S::OsMAPK3-GFP* was co-expressed with *BD-OsbHLH002* for evaluating the effect of OsMAPK3 on the transcriptional activity of OsbHLH002. The effector and reporter plasmids were the same as in Figure 2B. Data are the means of three replicates ± SD (*$p < 0.05$, t test).

(G) Overexpression of *OsMAPK3* in transgenic plants confers chilling tolerance. The survival rate was determined after treatment at 4°C for 108 hr and recovery for 7 days. Values are the means of four replicates ± SD (*$p < 0.05$, t test, n = 20–24 for each replicate).

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results suggest that the phosphorylation of OsbHLH002 by OsMAPK3 is required for chilling tolerance in rice.

OsMAPK3 Inhibits Ubiquitination of OsbHLH002 by OsHOS1

It is known that the E3 ligase OsHOS1 interacts with OsbHLH002 and mediates its degradation (Lourenco et al., 2013). We found that OsbHLH002 interacted with both full-length and the N-terminal 600 amino acids of OsHOS1 in yeast (Figure 7A). A yeast three-hybrid assay showed that the OsHOS1-OsbHLH002 interaction was dramatically reduced in yeast cells expressing OsMAPK3 (Figure 7B). In CoIP assays, Flag-OsbHLH002 was co-expressed with Flag-OsHOS1N-HA in *Arabidopsis* protoplasts and Flag-OsbHLH002 was detected in a complex immunoprecipitated with an anti-HA antibody (Figure 7C). When OsMAPK3-GFP was co-expressed with Flag-OsbHLH002 and Flag-OsHOS1N-HA in *Arabidopsis* protoplasts, the relative amount of immunoprecipitated Flag-OsbHLH002 was lower (0.55) than in the absence of OsMAPK3 (1.0) (Figure 7C). These results suggest that OsMAPK3 disrupts the interaction of OsHOS1 with OsbHLH002.

The ubiquitination of OsbHLH002 by OsHOS1 was verified using purified His-SUMO-OsbHLH002 and GST-OsHOS1. Higher-molecular-mass bands were detected by an anti-His antibody in the reactions containing both His-SUMO-OsbHLH002 and GST-OsHOS1, but not in the reactions lacking GST-OsHOS1 (Figure 7D), suggesting that OsbHLH002 can be ubiquitinated by OsHOS1 *in vitro*. The ubiquitination of OsbHLH002 was also detected in *Nicotiana benthamiana* co-transformed with *OsbHLH002-GFP* and *OsHOS1-HA*, and the level of ubiquitinated OsbHLH002-GFP was significantly decreased after chilling treatment in the presence of OsMAPK3-HA (Figure 7E). In contrast, under a warmer temperature of 22°C, OsMAPK3 had no obvious effect on OsbHLH002 ubiquitination (Figure S7). To further investigate how OsMAPK3 affects the degradation of OsbHLH002 *in vivo*, the rate of OsbHLH002 degradation during chilling treatment in *OsMAPK3*-OE and *OsMAPK3*-AS lines was determined. Immunoblotting showed that the rate of OsbHLH002 degradation was slower in *OsMAPK3*-OE than that in wild-type during chilling treatment. In the *OsMAPK3*-AS line, however, the degradation of OsbHLH002 was faster than in wild-type under the same conditions (Figure 7F). Therefore, these results suggest that OsMAPK3 suppresses OsbHLH002 ubiquitination and thereby slows its degradation.

DISCUSSION

MAPK3 Phosphorylates OsbHLH002, which Leads to Enhanced Cold Tolerance

ICE1 is a key transcription factor to trigger its targets, such as CBFs for freezing tolerance in *Arabidopsis*, which leads the ICE1-CBF-COR signaling pathway (Zhu, 2016). SnRK family kinases OST1 specifically phosphorylate ICE1 under freezing conditions (Ding et al., 2015b). In rice, OsbHLH002 is also one of the major regulators of chilling tolerance, which is supported by our finding that lines with loss of function or overexpression of *OsbHLH002* have decreased and increased chilling tolerance, respectively (Figure 1).

Our data suggest that OsbHLH002 is a substrate of OsMAPK3, which positively regulates chilling tolerance in rice (Xiong and Yang, 2003; Singh and Sinha, 2016). Our *in vitro* and *in vivo* assays both suggest that there is an interaction between OsbHLH002 and OsMAPK3 (Figure 5) that promotes the phosphorylation of OsbHLH002 and enhances its transactivation activity. Overexpression of *OsMAPK3* caused an increase in phospho-OsbHLH002 and enhanced chilling tolerance. The function of OsbHLH002 in chilling tolerance was lost when the five predicted Ser/Thr phosphorylation sites were replaced with Ala (Figures 6H and S6B). Rescue of the dwarf phenotype of *osbhlh002-1* by both *OsbHLH002*^{5A} and *OsbHLH002* may hint that the function of OsbHLH002 in seedling growth is independent of phosphorylation, but chilling tolerance is dependent on the five phosphorylation sites (Figures 1 and S6B). Taken together, our findings support the existence of an alternative phosphorylation pathway where OsMAPK3 phosphorylates OsbHLH002 to mediate chilling tolerance in rice.

OsMAPK3 Represses the Interaction between OsbHLH002 and HOS1 during Chilling

In *Arabidopsis*, the activity of ICE1 is regulated by both SIZ1- and HOS1-mediated sumoylation and polyubiquitination (Chinnusamy et al., 2007). OST1/SnRK2.6 is activated by cold stress and interacts with and phosphorylates ICE1, which in turn stabilizes ICE1 and promotes its activity in the ICE1-CBF transcriptional cascade (Ding et al., 2015b). Moreover, activated OST1 disrupts the ICE1-HOS1 interaction to prevent ICE1 degradation. Therefore, ICE1 is a central component in cold signaling and is regulated by multiple protein modifications, including not only ubiquitination and sumoylation but also phosphorylation in *Arabidopsis* (Dong et al., 2006; Ding et al., 2015b; Miura et al., 2007). In rice, OsHOS1 interacts with OsbHLH002 and is responsible for its degradation (Lourenco et al., 2013). Here we found that OsMAPK3 attenuated the interaction between OsHOS1 and OsbHLH002, which led to reduced ubiquitination and degradation of OsbHLH002 (Figure 7). Our data support the hypothesis that OsMAPK3 inhibits OsHOS1-OsbHLH002 interaction and prevents ubiquitination of OsbHLH002 during chilling stress (Figure 7G). These results indicate that OsMAPK3 regulates both the transactivation activity and stability of OsbHLH002.

The Trehalose Biosynthesis Gene *OsTPP1* Is an Alternative Target of OsbHLH002

Cold-inducible CBF homologs, including OsDREB1B/CBF1, OsDREB1A/CBF3, and OsDREB1F, are key transcription factors that function in rice chilling tolerance. It has been established that *COR* genes are transcriptionally regulated by CBFs (Dubouzet et al., 2003; Ito et al., 2006; Wang et al., 2008). Results of our

(H) The chilling-sensitivity phenotype of *osbhlh002-1* cannot be complemented by OsbHLH002^{5A}. Three-week-old seedlings of wild-type DJ, *osbhlh002-1*, and complementation lines (Com-L1, Com-L2) were treated at 4°C for 84 hr. The survival rate was determined after recovery. Values are the means of three replicates ± SD (*$p < 0.05$, t test, n = 20–24 for each replicate).
See also Figure S6.

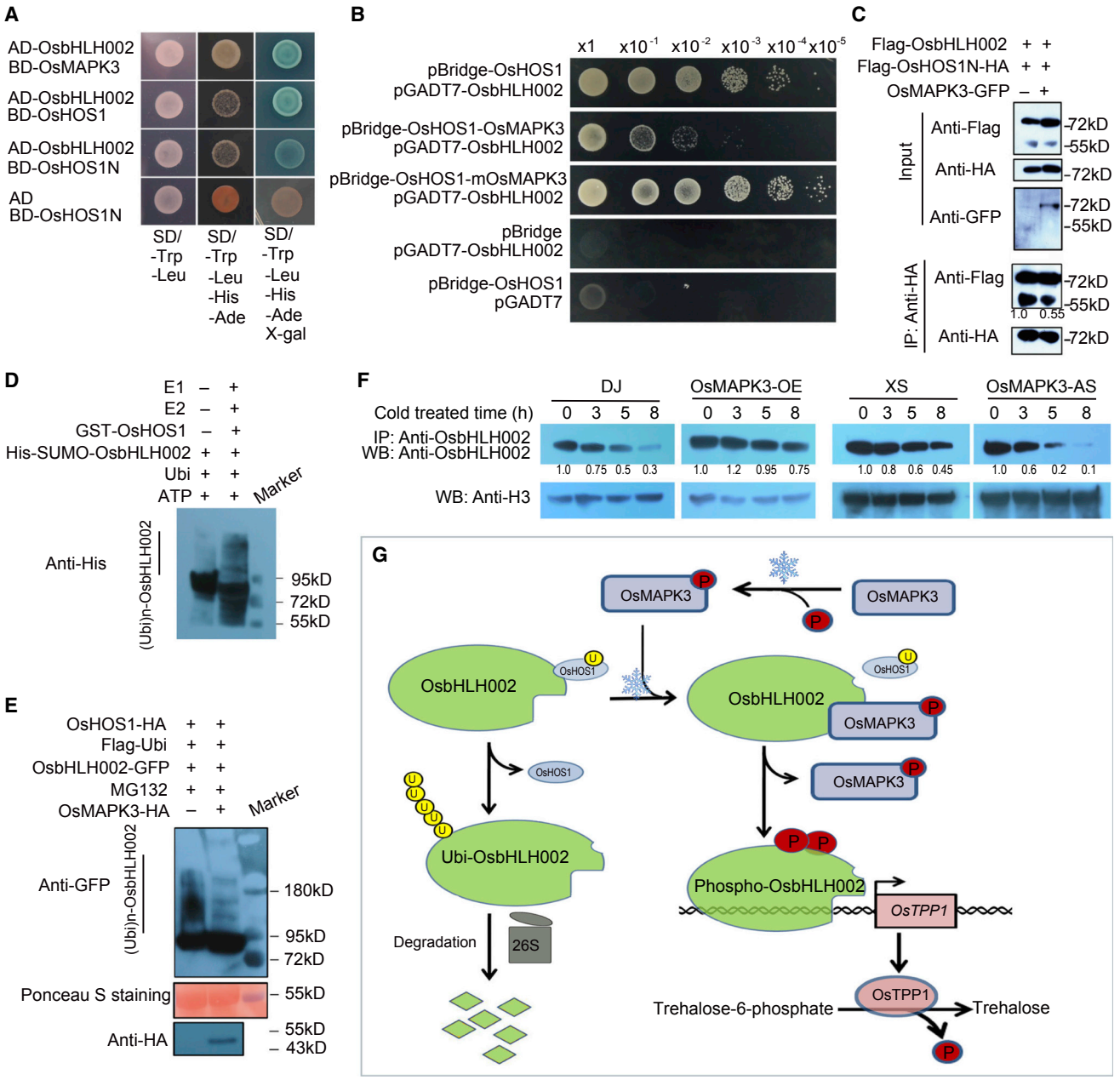

Figure 7. OsMAPK3 Inhibits OsbHLH002-OsHOS1 Interaction and Reduces Ubiquitination of OsbHLH002 under Chilling Stress

(A) OsbHLH002 interacts with both full-length OsHOS1 and the N-terminal 600 amino acids of OsHOS1 (OsHOS1N) in yeast.

(B) Yeast three-hybrid assays showing the effects of OsMAPK3 on the OsHOS1-OsbHLH002 interaction. Yeast cells transformed with different vector combinations as indicated were cultured on SD/-Trp/-Leu/-His/-Met medium. The mOsMAPK3 is a truncated form lacking the C-terminal amino acids 301–369 (Figure S5D).

(C) The effects of OsMAPK3 on the interaction between OsHOS1 and OsbHLH002 in an *Arabidopsis* protoplast transient expression assay. Flag-OsbHLH002 (MW, 55 kDa) and Flag-OsHOS1N-HA (MW, 72 kDa) with or without OsMAPK3-GFP (MW, 70 kDa) were co-expressed in *Arabidopsis* protoplasts. CoIP and immunoblotting analysis were carried out.

(D) The ubiquitinated forms of the His-SUMO-OsbHLH002 fusion protein (MW, 85 kDa) were detected by anti-His antibody *in vitro*.

(E) OsMAPK3 represses the ubiquitination of OsbHLH002 under chilling conditions (4°C for 4 hr). Fusion proteins OsbHLH002-GFP, Flag-Ubi, and OsHOS1-HA were co-expressed in *N. benthamiana* leaves with or without OsMAPK3. After chilling treatment for 4 hr, the total protein was extracted, and ubiquitinated OsbHLH002-GFP was detected with anti-GFP antibody. Ponceau S staining indicates equal loading.

(F) The accumulation of OsbHLH002 under chilling treatment. Three-week-old seedlings of *OsMAPK3*-OE, *OsMAPK3*-AS, Dongjin (DJ), and Xiushui 11 (XS) were treated at 4°C for 0 hr, 3 hr, 5 hr, and 8 hr, respectively. Then nuclear protein was extracted and immunoblots were probed with anti-OsbHLH002 and anti-H3 antibodies. The relative intensity of each band is indicated and that of untreated sample used as 1.0.

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yeast one-hybrid assay and EMSA experiments indicate that OsbHLH002 binds to the promoter of *OsTPP1*, which encodes an enzyme involved in the synthesis of trehalose. Trehalose acts as a highly effective osmoprotectant, and its synthesis is induced as a response to abiotic stresses (Ge et al., 2008). Mutation of *OsbHLH002* led to a decrease in both *OsTPP1* expression and trehalose content under chilling treatment (Figure 3), indicating that OsbHLH002 functions in chilling tolerance at least partially by inducing trehalose accumulation through activation of the *OsTPP1* gene. Accordingly, the chilling tolerance phenotype of *OsbHLH002* overexpression lines was blocked by loss of *OsTPP1* function (Figure 4). Overexpression of *OsTPS1* and *OsTPP1* increases endogenous trehalose content and enhances the tolerance to abiotic stresses in rice (Ge et al., 2008; Li et al., 2011). Exogenous application of trehalose rescued the chilling-sensitive phenotype of *ostpp1-1* (Figure 3G). Therefore, our results suggest that OsbHLH002 can directly target *OsTPP1* and result in trehalose accumulation during cold stress.

Besides *OsTPP1*, other target genes of OsbHLH002 must exist in rice. We found that *osbhlh002-1* seedlings were shorter than wild-type (Figure 1A) and that *ostpp1-1* seedlings were similar to wild-type ZH11 (Figure 4A). One reasonable explanation for the dwarf phenotype of *osbhlh002-1* is that other target genes of OsbHLH002 may function to regulate plant height. In fact, the complementary lines with $OsbHLH002^{WT}$ (Figure 1A) and the mutated gene $OsbHLH002^{5A}$ (Figure S6B) displayed heights similar to wild-type. We hypothesize that $OsbHLH002^{5A}$ still has the ability to control plant height and that $OsbHLH002^{WT}$ functions in both seedling development and chilling tolerance.

In conclusion, our studies revealed that the OsMAPK3-OsbHLH002-OsTPP1 pathway triggers chilling tolerance in rice. When rice is subjected to chilling stress, cold-activated OsMAPK3 phosphorylates OsbHLH002 and retards OsbHLH002 degradation. Phospho-OsbHLH002 directly activates *OsTPP1* transcription, leading to accumulation of the osmoprotectant trehalose and increased chilling tolerance (Figure 7G). This represents an alternative phosphorylation pathway for OsbHLH002/OsICE1 and establishes a linkage of the ICE1-CBF signaling pathway to an osmotic molecule under chilling stress. The identification of this pathway lays the groundwork for greater molecular understanding and improvement of chilling stress tolerance in rice. A number of important questions still remain to be addressed in the future, including what are the upstream regulators that activate OsMAPK3 and whether there is crosstalk between the OsMAPK3-OsbHLH002-OsTPP1 pathway and the OsCBFs-COR pathway in rice.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and one table and can be found with this article online at https://doi.org/10.1016/j.devcel.2017.11.016.

ACKNOWLEDGMENTS

We thank Dr. Yonggen Lou (Zhejiang University) for kindly providing the *OsMAPK3* transgenic seeds and Dr. Qi Xie (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences) and Dr. Feng Qin (Institute of Botany, Chinese Academy of Sciences) for kindly providing us with the *in vitro* ubiquitination plasmids E1 and E2. We would like to thank Dr. J.H. Snyder for examining the English in the manuscript. This work was funded by grants from the Chinese Ministry of Agriculture (2016ZX08009003-002), the Chinese Academy of Sciences (XDA08010205), and the National Natural Science Foundation of China (31400238).

AUTHOR CONTRIBUTIONS

Z.Z. performed the experiments, analyzed data, and wrote the paper. H.L., J.L., F.L., and W.Y. performed some of the experiments and data analysis. K.C. and Y.X. designed the experiments, analyzed data, and wrote the paper.

Received: July 14, 2017
Revised: October 17, 2017
Accepted: November 19, 2017
Published: December 18, 2017

SUPPORTING CITATIONS

The following reference appears in the Supplemental Information: Kong et al. (2012).

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(G) Model of the control of chilling tolerance by the OsMAPK3-OsbHLH002-OsTPP1 pathway. Under chilling conditions, the kinase activity of OsMAPK3 was increased, which leads to the phosphorylation of OsbHLH002. The presence of OsMAPK3 abolishes the interaction between OsbHLH002 and OsHOS1. Phospho-OsbHLH002 directly activates *OsTPP1* transcription to enhance the level of the osmoprotective substance trehalose. See also Figure S7.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-Myc	Beijing Com Win Biotech	Cat#CW0259S
Mouse monoclonal anti-HA	Sigma-Aldrich	Cat#H3663; RRID: AB_262051
Mouse monoclonal anti-His	TIANGEN	Cat#AB102-2
Rabbit polyclonal anti-GFP	Abcam	Cat#ab6556; RRID: AB_305564
Mouse monoclonal anti-Flag	Sigma-Aldrich	Cat#F3165; RRID: AB_259529
Rabbit polyclonal anti-OsbHLH002	Beijing Protein Innovation	Customer order
Rabbit polyclonal anti-p42/p44	Cell Signaling Technology	Cat#9101S; RRID: AB_331646
Rabbit polyclonal anti-H3	Merck Millipore	Cat#07-690; RRID: AB_41739
Rabbit polyclonal anti-phosphoSer/Thr	Abcam	Cat#Ab17464; RRID: AB_443891
Bacterial and Virus Strains		
BL21(DE3)pLysS	TIANGEN	Cat#CB106-02
AH109	Towfly	Cat#6453471VEC
EGY48	NTCC	Cat#NTCC55625
Chemicals, Peptides, and Recombinant Proteins		
Prorein A Agarose, Fast Flow	Merck Millipore	Cat#16-156
Glutathione Sephasose TM 4B	GE Healthcare	Cat#17-0756-01
Ni Sepharose 6 Fast Flow	GE Healthcare	Cat#17-5318-01
Amylose Resin	NEW ENGLAND BioLabs	Cat#E8021V
Phos-tag Biotin BTL-111	Nard Institute, Ltd	Cat#308-97201
MG132	Sigma-Aldrich	Cat#C2211
Cocktail	Roche	Cat#04693132001
Critical Commercial Assays		
Lambda Protein Phosphatase	NEW ENGLAND BioLabs	Cat#P0753S
ATP	Sigma-Aldrich	Cat#A6559
4-Methylumbelliferyl-β-D-glucuronide hydrate	Sigma-Aldrich	Cat#M9130
Luciferase Assay Reagent	Promega	Cat#E1483
Experimental Models: Organisms/Strains		
Rice: *osbhlh002-1* (PFG-3A13049)	this paper	N/A
Rice: *ostpp1-1* (RMD TTL-04Z11LP01)	this paper	N/A
Rice: *MAPK3-AS*	Hu et al., 2015	N/A
Rice: *MAPK3-OE*	this paper	N/A
Rice: *Ubi::OsbHLH002 ostpp1-1*	this paper	N/A
Rice: *OsbHLH002-OE*	this paper	N/A
Rice: *OsbHLH002-AS*	this paper	N/A
Rice: *OsbHLH002::OsbHLH002^{5A}osbhlh002-1*	this paper	N/A
Rice: *OsbHLH002::OsbHLH002 osbhlh002-1*	this paper	N/A
Oligonucleotides		
Yeast one hybrid primers *EcoRI-OsbHLH002-F*: CGGAATTCATGCTGCCGCGGTTTCACGG	Beijing Genomics Institute	Customer order
Yeast one hybrid primers *BamHI-OsbHLH002-R*: CGGGATCCGATCATGGTATGGAACCCGG	Beijing Genomics Institute	Customer order
other primers see Table S1	Beijing Genomics Institute	Customer order

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant DNA		
MBP-MAPK3	this paper	N/A
His-MEK1^{DD}	this paper	N/A
His-SUMO-OsbHLH002	this paper	N/A
His-SUMO-OsbHLH002^{6A}	this paper	N/A
Flag-OsbHLH002	this paper	N/A
GST-HOS1	this paper	N/A
HOS1-HA	this paper	N/A
Flag-OsHOS1N-HA	this paper	N/A
MAPK3-GFP	this paper	N/A
MAPK3-HA	this paper	N/A
MAPK3-Myc	this paper	N/A
OsbHLH002-GFP	this paper	N/A
OsbHLH002-mcherry	this paper	N/A
Flag-Ubi	this paper	N/A
Software and Algorithms		
Image J	National Institutes of Health	1.48u

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Yunyuan Xu (xuyy@ibcas.ac.cn).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

The T-DNA insertion line PFG_3A-13049 (*osbhlh002-1*) in the Dongjin background was obtained from RiceGE (http://signal.salk.edu/cgi-bin/RiceGE/), the Rice Functional Genomics Express Database, in Pohang city, Korea. The T-DNA insertion mutant RMD TTL-04Z11LP01 (*ostpp1-1*) in the ZH11 background was obtained from Rice Mutant Database in Huazhong Agricultural University in Wuhan, China. The *OsMAPK3*-antisense transgenic seeds were kindly provided by Prof. Yonggen Lou (Hu et al., 2015). The primers used for genotyping are listed in Table S1.

Sequence data from this study can be found in the in NCBI GenBank under the following accession numbers: *OsbHLH001* (AK102594), *OsbHLH002* (AK109915), *OsMAPK3* (AJ486975), *OsTPP1* (AK103391), *OsHOS1* (JQ866627), *OsMEK1* (AF216314), wheat E1 (GI: 136632), human E2 (UBCh5b, GI: 1145689), *Ubiquitin* (UBQ14, AT4G02890).

METHOD DETAILS

Growth Conditions and Chilling Treatment

Cold treatments were performed according to methods from our previous reports (Ma et al., 2015) with minor modification. The sterilized rice seeds were soaked in water for 3 days at 30°C. The germinated seeds were then placed into an incubator with Kimura B nutrient solution and grown in a greenhouse under a short-day photoperiod (10-h day/14-h night) with light strength 1.34 ×10,000 lux at 30°C /25°C (day/night) cycles. 3 – 4 weeks later, the seedlings were moved into a water bath maintained at 4 ± 0.5°C, with about 3 cm of the aerial part of the rice seedlings immersed in the water bath for a chilling treatment. The room temperature was controlled at about 22 ± 2°C, the photoperiod conditions were not changed. Depending on the various genetic backgrounds, the duration of the treatment varied from 84 to 108 h. Subsequently, seedlings were returned to greenhouse conditions and allowed to recover. The survival rate (percentage of live seedlings from in all tested plants) was determined. Each experiment was conducted independently at least three times. Significance analysis between samples was determined by Student's t-test.

Trehalose pretreatment was performed according to methods described previously with minor modification (Mostofa et al., 2015). Four-week-old rice seedlings were treated with 10 mM trehalose in the Kimura B nutrient solution for 60 h and then seedlings were transferred into 4°C room for 7 days.

Rice Transformation

For overexpression and antisense analysis, full-length cDNA of *OsbHLH002* in either a sense orientation or an antisense orientation was inserted into pUN1301, a binary vector carries the maize *Ubiquitin* promoter. For complementation analysis, *osbhlh002-1* plants

were transformed with the open reading frame of *OsbHLH002* or *OsbHLH002*^{5A} driven by the *OsbHLH002* promoter (2.0 kb of 5'-untranslated region) in the pCAMBIA2301 vector (Miaolingbio). Full cDNA of *OsMAPK3* was cloned into the pCAMBIA2301 vector and driven by *Ubiquitin* promoter to generate the *OsMAPK3* overexpression construct. *Agrobacterium tumefaciens* (strain EHA105) mediated transformation was used to introduce the constructs into rice (Ma et al., 2009). All primers used in the present study are detailed in Table S1.

Ion Leakage Assay and Trypan Blue Staining

Three-week-old DJ and *osbhlh002-1* seedlings were treated in 4 ± 0.5°C water bath for 3 days. Samples were harvested for the ion leakage assays (Ding et al., 2015b) and for trypan blue staining (Tian et al., 2014). Briefly, after chilling treatment, leave sample from 20 plants with 0.3 g was immersed in 20 mL deionized water and shaken at 200 rpm at room temperature for 2 h, and electrical conductivity (C1) was determined. The samples were then boiled for 20 min, and the total conductivity (C2) was determined. The electrical conductivity of deionized water defined as C0. Relative ion leakage was calculated as (C1-C0)/(C2-C0) X 100%. Three replicates were performed and significant differences were analyzed by Student's *t* test. For trypan blue staining, the leaves were submerged in the second leaves were harvested and submerged in trypan blue solution for 12 h, then boiled in 75% ethanol to decolorize for photography.

Trehalose Extraction and Determination

Shoot samples (1 g) from three-week-old seedlings, either before or after chilling stress treatment for 8 h and 72 h, were ground in liquid nitrogen and extracted in boiling water. After centrifuging, the supernatant was was filtered with a 0.45 μm aperture filter and concentrated for trehalose extraction (Ge et al., 2008). Trehalose content was determined with standard Gas-Liquid-Chromatography-Mass Spectrometery based methods (Albersheim et al., 1967). Three replicates were performed and Student's *t* test was used for statistical analysis.

Transactivation Analysis in Yeast Cells

For transactivation assays in yeast cell, the bait plasmid pGBKT7 containing intact or truncated *OsbHLH002* was used to transform the AH109 yeast strain. After culturing on synthetic medium plates (SD medium) lacking Trp (SD/-Trp) at 30°C for 3 d, the yeast transformants were transferred onto SD agar medium lacking Trp, His, and adenine (SD/-Trp/-His/-Ade) for selecting. The transactivation activity of each protein was evaluated based on the growth status of yeast strain and the activity of β-galactosidase (Ma et al., 2009).

Yeast One-hybrid Assays

Yeast one-hybrid assays were used to examine the binding of OsbHLH002 to the *OsTPP1* promoter, and were performed according to a previously described method (Lin et al., 2007). Briefly, the pGAD424-OsbHLH002 plasmid was co-transformed with the pLacZ-E-box, pLacZ-G-box, and pLacZ-OsTPP1p plasmids, into *Saccharomyces cerevisiae* strain EGY48 using standard transformation techniques respectively. After culturing on SD agar medium lacking Ura and leu (SD/-Ura/-Leu) at 30°C for 2 days. Yeast transformants were transferred onto proper dropout plates containing X-gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) for blue colour development (Guo et al., 2013).

Yeast Two-hybrid Analysis

Yeast two-hybrid analysis was performed according to the Match maker GAL4 Two-Hybrid System 3 manufacturer's manual (Clontech). The prey plasmid, pGADT7-OsbHLH002, was co-transformed with the bait plasmids, pGBKT7-OsMAPK3, pGBKT7-OsHOS1, and pGBKT7-OsHOS1N into *Saccharomyces cerevisiae* strain EGY48, respectively. After culturing on synthetic medium plates (SD medium) lacking Trp and Leu (SD/-Trp/-Leu) at 30°C for 2 d, the transformants were transferred onto SD/-Trp/-Leu containing X-gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) for blue colour development. The primers used are detailed in Table S1.

Yeast Three-hybrid Assays

Yeast three-hybrid analysis was performed according to previously described methods (Lian et al., 2011). The constructs expressing OsHOS1 and bridge protein OsMAPK3 or expressing OsHOS1 and bridge protein mOsMAPK3 (lacking C-terminal 301 to 369 amino acids) were generated. The yeast strain AH109 was transformed with a pair of plasmids, pBridge-HOS1-MAPK3 and pGADT7-OsbHLH002, or with pBridge-HOS1-mMAPK3 and pGADT7-OsbHLH002. pBridge-HOS1 and pGADT7-OsbHLH002 were used as positive control. The pBridge and pGADT7-OsbHLH002, pBridge-HOS1 and pGADT7 were used as negative control. The transformed colonies were screened on synthetic medium (SD/-Met/-Trp) and the transformants were transferred onto plates lacking Trp and Leu (SD/-Trp/-Leu) or plates lacking Trp, Leu, and His (SD/-Trp/-Leu/-His) for growth.

CoIP and Immunoblotting Analysis

To verify the *in vivo* interaction between OsbHLH002 and OsMAPK3, CoIP assays were performed as described previously (Guo et al., 2013). Total proteins were extracted from *Arabidopsis* protoplasts expressing two pairs of plasmids (35S::Flag-OsbHLH002 and 35S::OsMAPK3-GFP or 35S::Flag-OsbHLH002 and 35S::GFP) and incubated with agarose beads and anti-GFP-antibody (Abcam). Proteins bound to the beads were detected with anti-Flag antibody (Sigma).

For the interference assay, proteins were extracted from *Arabidopsis* protoplasts expressing 35S::Flag-OsbHLH002 and 35S::HA-OsHOS1N-Flag with or without 35S::OsMAPK3-GFP, Anti-HA agarose beads were used for purifying the protein complex. After separation and blotting, protein bands were detected with anti-Flag antibody (Sigma), anti-HA antibody (Sigma), and anti-GFP antibody (Abcam), respectively.

Chromatin Immunoprecipitation Assays

To confirm the binding of OsbHLH002 to the *OsTPP1* promoter *in vivo*, chromatin immunoprecipitation (ChIP) assays were performed in two-week-old DJ and *osbhlh002-1* seedlings as described previously (Guo et al., 2013). Briefly, two-week-old rice seedlings of 2 g were treated with 1% formaldehyde for protein–DNA cross-linking. The samples were ground and the chromatin was extracted with anti-OsbHLH002 antibody (1:1,000 dilution, BGI, Beijing). The Protein A-agarose beads were used for purifying DNA-histones-antibody complex. Finally, the enriched DNA fragments were analyzed by qPCR. The primers used are detailed in Table S1.

Subcellular Localization and BiFC Assays

For OsbHLH002 subcellular localization, the *35S::OsbHLH002-GFP* construct was generated on pBI221. For BiFC assays, the coding sequences of *OsMAPK3* and *OsbHLH002* were amplified and fused into the pUC-SPYNE and pUC-SPYCE vectors, respectively (Walter et al., 2004). The plasmids were transformed into rice protoplasts that released from leaf sheaths of 10-day-old etiolated rice seedlings by polyethylene glycol (PEG)-mediated transient expression system (Chen et al., 2006). The transformed protoplasts were observed using a fluorescence microscope (Leica TCS SP5). Images were analyzed with Image LAS-AF software.

EMSA Assays

The amplified CDS of *OsbHLH002* was fused in-frame with the His tags in vector pET32a-SUMO. *In vitro* translation was performed with a SP6 TNT Quick Coupled Transcription/Translation kit (Promega) according to the manufacturer's instructions. Oligonucleotides (Table S1) were synthesized and labeled using a Biotin 3' End DNA Labeling Kit according to the manufacturer's instructions (Pierce). The His-SUMO-OsbHLH002 recombinant protein was purified and incubated with the biotin-11-UTP-labelled DNA fragments. Each fragment contained three repeats of the sequences that cover one E-box in site 1 to site 8 within *OsTPP1* promoter, respectively (Figure S3B). Unlabeled oligonucleotides (5, 10, 20, and 50-fold excesses of labeled probes) were added to the EMSA reactions for the competition assays. The protein-DNA binding and competition reactions, electrophoretic separation, and visualization of bands were conducted as described previously (Liu et al., 2014). The primers used for probes amplified were listed in Table S1.

Transcriptional Activity Assays

The transcription activity assays were performed in transiently-transformed *Arabidopsis* protoplasts according to described previously (Guo et al., 2013). The DNA binding domain (BD) from GAL4 BD (amino acids 1 to 147) was used in this system. The effector region of the plasmid encodes a BD-OsbHLH002 fusion protein was driven by the *35S* promoter. The reporter plasmid *GAL4(4X)-D1-3(4X)::GUS* contains four tandem copies of the GAL4 DNA binding sites and four tandem copies of the constitutive D1-3 elements. Two known proteins, HOS15 and ARF5M, were used as the transcription suppression and activation controls, respectively. The GUS reporter and the luciferase (LUC) reporter were co-transformed with GAL4 BD-OsbHLH002 into *Arabidopsis* protoplasts. The *35S::LUC* reporter was used as an internal control. Relative GUS activity was calculated (GUS/LUC) to determine transactivation activity. In order to observe the effect of OsMAPK3 on the transcriptional activity of OsbHLH002, the *35S::OsMAPK3-GFP* was co-expressed with *BD-OsbHLH002* in *Arabidopsis* protoplast in this assay system.

For detecting transcriptional regulation activity and the binding specificity of OsbHLH002 on the *OsTPP1* promoter, full-length of *OsbHLH002* was fused into the pBI221 vector driven by the *35S* promoter to generate the *35S::OsbHLH002-GFP* vector. The *OsTPP1* promoter was amplified to generate the *OsTPP1::LUC* reporter gene. The LUC/GUS ratio was used to determine the OsbHLH002 transient transactivation to the *OsTPP1* according to the previous report (Guo et al., 2013).

In Vitro Phosphorylation Assays

The amplified CDS of *OsMEK1*^{DD} and *OsMAPK3* were fused in-frame with the His tags in vector pET32a and the MBP in vector pMAL-C2, respectively. Subsequently, the expression of His-OsMEK1^{DD} and MBP-OsMAPK3 in *E. Coli* BL21 cells was induced by isopropyl β-D-1-thiogalactopyranoside. The proteins were purified using His and Amylose Resin beads, respectively (Lourenco et al., 2013).

The *in vitro* phosphorylation assays were performed according to previously-described methods with minor modification (Liu and Zhang, 2004). In brief, recombinant MBP-tagged OsMAPK3 (20 μg) was activated by incubation with recombinant His-OsMEK1^{DD} (0.5 μg) in the presence of 50 μM ATP in 50 μL of reaction buffer (20 mM Hepes, pH 7.5, 10 mM $MgCl_2$, and 1 mM DTT) at 22°C for 1 h. Activated MBP-OsMAPK3 was then used to phosphorylate recombinant OsbHLH002 or OsbHLH002^{5A} proteins (20:1 substrate enzyme ratio) in the same reaction buffer with 25 mM ATP. The reactions were stopped by the addition of SDS-loading buffer after 30 min. After phosphorylation, one half of protein products were separated by 12% SDS-PAGE and the gel was stained with Coomassie Brilliant Blue. The other half of proteins was separated by 12% SDS-PAGE for immunoblotting analysis using anti-phosphoSer/Thr antibody.

In Vivo Phosphorylation Assays

Nuclear protein was extracted from three-week-old seedlings of DJ, *OsMAPK3*-OE, Xiushui 11 (XS), and *OsMAPK3*-AS (Ryu et al., 2010) with or without 4°C chilling treatment. Subsequently, nuclear protein was immunoprecipitated with anti-OsbHLH002 antibody. The immunoprecipitated-proteins were separated by 10% SDS-PAGE for immunoblotting using anti-phosphoSer/Thr antibody or Phos-Biotin (Zhao et al., 2016).

To detect the accumulation of OsbHLH002, nuclear proteins were immunoprecipitated with anti-OsbHLH002 antibody from three-week-old rice seedlings treated at 4°C for 0 h, 3 h, 5 h, and 8 h. Subsequently, samples were separated by 12% SDS-PAGE, and protein was detected using anti-OsbHLH002 antibody.

Ubiquitination Assays

The ubiquitination assays were performed largely as described previously (Ding et al., 2015a; Xie et al., 2002). Approximately 1 μg of the His-SUMO-OsbHLH002 fusion protein was mixed with 100 ng crude wheat (*Triticum aestivum*) E1 (GI: 136632), 250 ng of human E2 (UBCh5b), 2 μg purified *Arabidopsis* ubiquitin (UBQ14,), and 500 ng GST-OsHOS1. The mixture was incubated in reaction buffer (50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 2 mM ATP, and 2 mM DTT) at 30°C for 2 h. Reactions were stopped with 4×SDS sampling buffer by boiling at 100°C for 10 min. Products were separated by 8–16% gradient SDS-PAGE (GeneScript) and immunoblotted with the anti-His antibody.

For *in vivo* ubiquitination assay, OsbHLH002-GFP, Flag-Ubi, OsHOS1-HA were co-expressed in *N. benthamiana* leaves with or without OsMAPK3-HA or OsMAPK3-Myc for three days, then the plants were treated at 4°C or 22°C for 4 h. Total proteins were extracted from *N. benthamiana* leaves at presence of proteasome inhibitor MG132. The proteins were separated by 8–16% gradient SDS-PAGE and detected with anti-GFP antibody (Abcam), anti-HA antibody (Sigma-Aldrich) or anti-Myc antibody (Beijing Com Win Biotech).

QUANTIFICATION AND STATISTICAL ANALYSIS

The chilling survival assay, qRT-PCR, electrolyte leakage assay, transcriptional activity assay, quantitative analysis of trehalose, and statistical analysis were described in the Method Details. Protein quantification in western blotting experiments was achieved by using Image J to measure the intensity of bands. Each experiment was performed independently at least three times.

DATA AND SOFTWARE AVAILABILITY

The raw data used to compose the figures have been deposited as a Mendeley Data set and can be found at the following DOI: https://doi.org/10.17632/bdp82zfz6f.1