Serine hydroxymethyltransferase localised in the endoplasmic reticulum plays a role in scavenging H2O2 to enhance rice chilling tolerance

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Research article

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Abstract

Background: Rice is a temperature-sensitive crop that can sustain dramatic damage from low temperatures. Overexpression of the *Lsi1* gene (*Lsi1*-OX) in rice enhances its chilling tolerance. This study revealed that a serine hydroxymethyltransferase (*Os*SHMT) mainly localised in the endoplasmic reticulum (ER) is involved in increasing tolerance to chilling. **Results:** A higher transcription level of *Os*SHMT was detected in *Lsi1*-OX rice than in the wild type. Histone H1 and nucleic acid binding protein were found to bind to the promoter region of *OsSHMT* and regulate its expression, and the transcription levels of these proteins were also up-regulated in the *Lsi1*-OX rice. Moreover, *Os*SHMT interacts with ATP synthase subunit α, heat shock protein Hsp70, mitochondrial substrate carrier family protein, ascorbate peroxidase 1 and ATP synthase subunit β. *Lsi1*-encoded protein *Os*NIP2;1 also interacts with ATP synthase subunit β, and the coordination of these proteins appears to function in reducing reactive oxygen species, as the H_2O_2 content of transgenic *OsSHMT Arabidopsis thaliana* was lower than that of the non-transgenic line under chilling treatment. **Conclusions:** Our results indicate that ER-localised *Os*SHMT plays a role in scavenging H_2O_2 to enhance the chilling tolerance of *Lsi1*-OX rice and that ATP synthase subunit β is an intermediate junction between *Lsi1* and *Os*SHMT.

Background

Plants require a given temperature range for normal growth and development, and sudden changes in temperature can cause growth inhibition and even death [1]. Cold spells in late spring are examples of sudden drops in temperature over a short time and can adversely affect the growth of rice. Some varieties of rice, mainly those in the indica subspecies, are particularly sensitive to low temperatures [2, 3]. Continuous chilling of crop plants has been shown to result in increased reactive oxygen species (ROS) and cell membrane peroxidation and to affect the expression of chloroplast genes, inhibiting photosynthesis [4]. Such ROS include O^{2-}, H_2O_2 and OH^- [5].

Fang et al. [6] documented that chilling treatment inhibited the expression of chlorophyll synthesis genes and promoted the expression of proteasome genes from temperature-sensitive Dular rice (Oryza sativa ssp. indica), leading to chloroplast damage, chlorophyll degradation, loss of green colour and partial degradation of RNA in leaves. Cui et al. [7] found that the whitening of Dular leaves under chilling stress was associated with the deletion of the gene coding sequence of pentatricopeptide (LOC_Os09g29825.1), which is an RNA-binding protein, and the gene coding sequence was missing 8 bases relative to that from Nipponbare rice, resulting in a frameshift mutation of the coding sequence and deactivation of the protein function. The mutant gene was named DUA1. Inactivation of the DUA1 protein in Dular rice leads to a loss of RNA editing capacity under chilling stress, resulting in rice seedlings that exhibit chloroplast development defects and leaf chlorosis. When the silicon-absorbing gene (Lsi1) was over-expressed in Dular rice, the chilling tolerance of the transgenic line was significantly improved, and the leaves maintained their fresh green colour under chilling treatment. The expression of genes from the photosynthesis pathway of transgenic rice was enhanced under low temperature stress, and the

expression of genes involved in the proteasome was down regulated. In addition, the transcription level of the gene encoding serine hydroxymethyltransferase (SHMT, LOC_Os03g52840) was up-regulated in the transgenic rice, but down-regulated in the wild-type, and the expression of its corresponding miRNA changed in an opposite way, indicating that OsSHMT may be involved in regulating the chilling-tolerance of Dular [6].

SHMT is widely distributed in plants [8]. The enzyme catalyses the reversible exchange between serine and glycine (glycine CH_2-THF H_2O↔serine THF) [9, 10], which are precursors of chlorophyll, glutathione, peptides, tryptophan, lecithin, phospholipids and ethanolamine [11]. Therefore, SHMT plays an important role in the photorespiration processes of plants [12]. Studies have found that a mitochondrial serine hydroxymethyltransferase gene mutation in rice (osshm1) causes blockage of the photorespiration pathway, thereby affecting the Calvin cycle and the efficiency of light energy; excess light in the chloroplast then leads to the accumulation of reactive oxygen species (ROS), resulting in disruption of chloroplast development and fewer and smaller chloroplasts with less grana [13].

In addition, SHMT plays a positive role in regulating plants' resistance to stress. In Arabidopsis, expression of NADH dehydrogenase was shown to be inhibited by chilling, leading to the continuous accumulation of ROS, suppressing the transcription of cold-response genes and hypersensitivity to chilling [14]. SHMT activity contributes to a reduction of ROS accumulation in the chloroplast and therefore reduces oxidative damage [15]; as a second messenger, a reduction in ROS would also prevent the transmission of the chilling signal and reduce the damage caused by ROS. The recessive mutation shmt1-1 in Arabidopsis results in abnormal regulation of cell death, leading to chlorotic and necrotic diseases under various environmental conditions, and mutants that carry the shmt1-1 allele exhibit more H_2O_2 accumulation than wild-type plants under salt stress, resulting in greater chlorophyll loss [16]. To the best of our knowledge, the specific role of OsSHMT in the regulation of rice cold resistance has not been reported.

In this study, an OsSHMT gene (LOC_Os03g52840) from rice was amplified, the subcellular localisation of OsSHMT was investigated, and proteins that bind to the promoter region of OsSHMT were obtained. The proteins that interact with OsSHMT were also investigated to indicate the regulation network of OsSHMT in rice under chilling stress, revealing the possible role of OsSHMT in scavenging H_2O_2 to improve cold resistance in rice.

Results

Chilling tolerance of Dular and Lsi1-OX rice

The Dular and Lsi1-OX transgenic lines presented different tolerances to the chilling treatment of 4 °C for 36 h. Most of the leaves from the Dular rice became whiter, whilst the Lsi1-OX line maintained tolerance to chilling (Fig. 1A). Further studies showed that the Lsi1 overexpression vector was driven by the ubiquitin promoter, and the translate OsNIP2;1 was localised in the cytoplasm (Fig. 1B), which differs

significantly from the original localisation in the cell membrane (Ma et al. 2006). This result suggests that OsNIP2;1 may have more roles than its initial function, which belongs to the aquaporin family and controls silicon accumulation in rice [17]. SEM images of the leaves revealed that silica bodies in the Lsi1-OX rice leaves were bigger than those of the Dular rice (Fig. 1C).

Subcellular localisation of OsSHMT

Overexpression of Lsi1 in Dular rice results in changes in the expression of thousands of genes [6], including the OsSHMT gene. Expression of OsSHMT was up-regulated in the Lsi1-OX rice in comparison with the Dular rice (Fig. 2A). Subcellular localisation of OsSHMT (LOC_Os03g52840) in the rice protoplast then showed that yellow fluorescence was concentrated around the nucleus, and no obvious endonuclear fluorescence was observed, whereas significant yellow fluorescence was seen in the whole nucleus in the rice protoplast transformed with the eYFP vector (Fig. S1). This suggested that OsSHMT was localised on the endoplasmic reticulum around the nucleus. Further co-localisation of the endoplasmic reticulum marker protein with mcherry indicated that OsSHMT was widely distributed in the endoplasmic reticulum, as the mcherry fluorescence from the marker protein and yellow fluorescence from OsSHMT infused with eYFP was completely overlapping (Fig. 2B). OsSHMT is involved in the photorespiratory processes of plants, and existing studies have suggested that the protein is localised to mitochondria, chloroplasts and cytoplasm. The results of this study complement those findings.

Promoter region of OsSHMT and the binding proteins

The promoter region 2597 bp upstream of the CDS of the OsSHMT gene from Dular rice was amplified and labelled with biotin at the 5' flanking region of this DNA fragment (Table S1). According to the DNA pull-down results, compared with the control group at room temperature, chilling treatment of Lsi1-OX induced more proteins to bind to the promoter region of OsSHMT. In contrast, chilling treatment of the Dular rice had no significant effect on the proteins binding to the promoter (Fig. 3A). Identification of the proteins showed that retrotransposon protein (Ty3-gypsy subclass), glyceraldehyde-3-phosphate dehydrogenase, and AT hook motif protein were identified from both the Lsi1-OX and Dular rice under chilling treatment or room temperature conditions; however, some proteins, such as histone H1,nucleic acid binding protein (NABP) and tubulin/FtsZ domain-containing protein (LOC_Os03g51600.1) were only identified from the chilling-treated Lsi1-OX group; another tubulin/FtsZ domain-containing protein (LOC_Os05g34170.2) was identified from the chilling-treated Lsi1-OX and Dular groups and form the Dular group at room temperature. AAA-type ATPase family protein was identified from the Dular group at room temperature but not from the chilling-treated Dular group, and this protein was induced in the chilling-treated Lsi1-OX group in comparison with its control group at room temperature (Table 1). The tubulin/FtsZ domain-containing proteins (LOC_Os03g51600.1, LOC_Os05g34170.2, LOC_Os07g38730.1), histone H1 and NABP were selected to analysis their gene expression level on the two rice. A comparison of the gene transcription levels in Dular and Lsi1-OX rice after chilling treatment for 12, 24 and 36 h in comparison with 0 h, revealed opposite trends in the two rice lines, and the gene expression level was up-regulated in the Lsi1-OX rice in comparison with Dular rice under the same treatment conditions (Fig. 3D).

The results indicate that these genes act in combination to exert a positive role in the regulation of OsSHMT expression.

Table 1

Proteins binding on the OsSHMT gene promoter from Dular and Lsi1-OX

Protein ID	Unique peptide number	Unique spectra number	Coverage	Description
Dular, RT				
LOC_Os03g58470.1	11	121	0.3481	retrotransposon protein, putative, Ty3-gypsy subclass, expressed
LOC_Os04g58730.1	4	6	0.1527	AT hook motif domain containing protein, expressed
LOC_Os03g03720.1	4	4	0.1261	glyceraldehyde-3-phosphate dehydrogenase, putative, expressed
LOC_Os12g37260.1	3	3	0.0434	lipoxygenase 2.1, chloroplast precursor, putative, expressed
LOC_Os11g47970.1	3	3	0.0687	AAA-type ATPase family protein, putative, expressed
LOC_Os04g42320.1	3	3	0.0414	AT hook motif family protein, expressed
LOC_Os07g08710.1	3	3	0.1871	AT hook-containing DNA-binding protein, putative, expressed
Dular, chilling				
LOC_Os03g58470.1	10	130	0.3311	retrotransposon protein, putative, Ty3-gypsy subclass, expressed
LOC_Os04g58730.1	4	8	0.1527	AT hook motif domain containing protein, expressed
LOC_Os07g08710.1	4	9	0.223	AT hook-containing DNA-binding protein, putative, expressed
LOC_Os05g34170.2	4	7	0.1036	tubulin/FtsZ domain containing protein, putative, expressed
LOC_Os03g03720.2	3	4	0.1226	glyceraldehyde-3-phosphate dehydrogenase, putative, expressed
Lsi1-OX, RT				
LOC_Os03g58470.1	10	145	0.3447	retrotransposon protein, putative, Ty3-gypsy subclass, expressed
LOC_Os04g42320.1	6	9	0.0674	AT hook motif family protein, expressed

Protein ID	Unique peptide number	Unique spectra number	Coverage	Description
LOC_Os01g72049.1	5	5	0.1975	retrotransposon, putative, centromere-specific, expressed
LOC_Os05g34170.2	3	4	0.0811	tubulin/FtsZ domain containing protein, putative, expressed
LOC_Os07g38730.1	2	2	0.0444	tubulin/FtsZ domain containing protein, putative, expressed
Lsi1-OX, chilling				
LOC_Os03g58470.1	15	205	0.3823	retrotransposon protein, putative, Ty3-gypsy subclass, expressed
LOC_Os05g51850.1	8	8	0.1413	AT hook-containing DNA-binding protein, putative, expressed
LOC_Os04g58730.1	7	10	0.2506	AT hook motif domain containing protein, expressed
LOC_Os11g47970.1	6	6	0.1309	AAA-type ATPase family protein, putative, expressed
LOC_Os01g72049.1	6	6	0.1975	retrotransposon, putative, centromere-specific, expressed
LOC_Os04g42320.1	6	7	0.0721	AT hook motif family protein, expressed
LOC_Os07g08710.1	5	9	0.2662	AT hook-containing DNA-binding protein, putative, expressed
LOC_Os04g49990.1	4	5	0.2507	AT hook motif domain containing protein, expressed
LOC_Os05g34170.2	4	5	0.1059	tubulin/FtsZ domain containing protein, putative, expressed
LOC_Os08g40150.1	3	3	0.1102	AT hook motif domain containing protein, expressed
LOC_Os04g38600.2	3	3	0.1111	glyceraldehyde-3-phosphate dehydrogenase, putative, expressed
LOC_Os06g04020.1	3	3	0.1458	histone H1, putative, expressed
LOC_Os03g52490.1	2	2	0.098	nucleic acid binding protein, putative, expressed

Proteins interacting with OsSHMT

Based on the GFP-TRAP method to obtain the proteins interacting with OsSHMT, it was found that several proteins co-precipitated with OsSHMT, in comparison with the GFP-vector control (Fig. 4; Table S2). Some of these proteins, including ATP synthase α subunit, ATP synthase β subunit, heat shock protein 70, mitochondrial substrate carrying family protein E, ascorbate peroxidase 1, are defense proteins that interact with OsSHMT protein (Table 2). Further determination of the interaction of OsSHMT with ATP synthase subunit α, ATP synthase subunit β, Hsp70, MSCP and APX from the rice showed that yellow fluorescence was detected in the leaves of tobacco infected with OsSHMT and each of these proteins respectively. No fluorescence was detected in the control group, demonstrating that OsSHMT positively interacts with the above proteins. The results indicate that OsSHMT interacted with defence-related proteins in rice, including APX, MSCP, HSP70 and ATP, to jointly regulate chilling resistance.

Table 2
The target protein from rdr6 interacted with OsSHMT identified by LC-MS

Accession	Description	Sum PEP Score	Coverage	Peptides	PSMs	Unique Peptides
ATCG00480.1	ATP synthase subunit beta	39.443	50.60241	19	35	18
ATCG00120.1	ATP synthase subunit alpha	30.137	26.82446	13	28	11
AT1G07890.2	ascorbate peroxidase 1	9.281	37.6	7	8	7
AT5G02490.1	Heat shock protein 70 (Hsp 70) family protein	15.568	9.035222	5	7	5
AT5G46800.1	Mitochondrial substrate carrier family protein	5.09	10	4	4	4
ATCG00480.1	ATP synthase subunit beta	6.277	8.634538	3	3	3
Supplementary information						
Figure S1 Subcellular localisation of OsSHMT protein in rice protoplast						

H_2O_2 content in the OsSHMT transgenic A. thaliana and wild type

To further indicate the function of OsSHMT in scavenging H_2O_2, wild-type A. thaliana and positive transgenic T3 A. thaliana seedlings were exposed to a temperature of 4 °C for 12, 24 and 36 h. The leaves of the wild type showed more reddish-brown spots after DAB staining. The transgenic line also showed reddish-brown spots, but the spots were small in size and number. Determination of the leaf H_2O_2 content of the transgenic line and wild type of A. thaliana showed that the increase in H_2O_2 in the transgenic line

of A. thaliana after chilling treatment was significantly lower than that of the wild type that underwent the same treatment.

Os NIP2;1 interacts with ATP synthase subunit β from rice

Lsi1 encodes the aquaporins protein OsNIP2;1, which is a nodulin 26-like intrinsic protein (NIP) and is localised in the membrane of the cell. Overexpression of Lsi1 in Dular rice using a ubiquitin promoter resulted in the wide distribution of this protein, including in the nucleus and cytoplasm, enabling OsNIP2;1 to play multiple roles. The interaction between OsNIP2;1 and OsSHMT was also investigated. The results showed no direct interaction between OsNIR2;1 and OsSHMT. However, OsNIP2;1 interacted with ATPsyn-β, a protein that also interacts with OsSHMT, and the results indicate that ATPase-β acts as an intermediate junction between Lsi1 and OsSHMT.

Discussion

Chilling stress is a limiting factor in the survival, geographical distribution and production of many crops. Plants that are subject to low temperatures usually exhibit membrane oxidation, chlorophyll degradation, photosynthesis reduction and growth inhibition. Previous studies have suggested a close linkage between chilling stress and ROS [18, 19], which are mainly generated in the chloroplast, mitochondria and peroxisomes and accumulate under stressful condition [20–22]. ROS are regarded as toxic compounds and secondary messengers in signal transduction. It has been reported that enzymatic components such as ascorbate peroxidase (APX), superoxide dismutase, catalase, guaiacol peroxidase, glutathione reductase, monodehydroascorbate reductase, dehydroascorbate reductase and non-enzymatic antioxidants such as ascorbic acid, reduced glutathione, α-tocopherol, carotenoids, flavonoids and the osmolyte proline play dominant roles in scavenging ROS [23, 24].

SHMT participates in the photorespiration pathway and its inactivation usually results in low rates of photorespiration and accumulation of ROS. In Arabidopsis, the loss of SHMT activity compromised the photorespiratory cycle and led to overproduction of ROS, leading to a susceptibility to salt stress [16]. Our previous studies indicated that overexpression of Lsi1 in chilling-sensitive Dular rice results in enhanced tolerance to low temperature; gene expression of OsSHMT was up-regulated in the Lsi1-OX rice in comparison with Dular rice [6]. Wang et al. [13] found that OsSHMT is localised in the mitochondria, and it has also been reported to localise in the chloroplast, cytoplasm, nucleus, plasma membrane and cytosol [25, 26]; despite these results, our present study indicated that OsSHMT is also localised in the endoplasmic reticulum, which is the main organelle for protein and lipid synthesis, membrane biogenesis, xenobiotic detoxification and cellular calcium storage [27]. Several stress-defence proteins relevant to detoxification were found to interact with OsSHMT, including APX, HSP70, MSCP and ATP synthase. Among these proteins, APX is a ROS-scavenging antioxidative enzyme. The cytosolic ascorbate peroxidase 1 (APX1) in Arabidopsis thaliana is a central component of the H_2O_2-scavenging system. Absence of APX1 in the knock-out line leads to the collapse of the chloroplast H_2O_2 clearance system, thereby increasing levels of H_2O_2 and oxidative proteins [28]. The heat shock protein Hsp70 is a

molecular chaperone protein and plays a critical role in stress tolerance [29]; in rice, chloroplast-localised Hsp70 is essential for chloroplast development under high-temperature conditions [30], whilst overexpression of mitochondrial HSP70 in rice suppresses programmed cell death [31]. It is suggested that the multiple roles of Hsp70 in rice defence contribute to enhancing chilling tolerance.

Moreover, the results of this study indicate that subunit α and subunit β of ATP synthase also interact with OsSHMT. ATP synthase is widely distributed in mitochondria and chloroplasts; it produces ATP from ADP in the presence of a proton gradient across the membrane, which is generated by electron transport complexes of the respiratory chain [32]. The α chain is a regulatory subunit, whilst the catalytic sites are hosted primarily by the β subunits. The bioactivity of ATP synthase in the organelle provides ATP for OsSHMT and its pathways. Mitochondrial substrate carrier family proteins catalyse the passage of hydrophilic compounds such as ADP/ATP across the inner mitochondrial membrane [33].

The results of this study also show that the ATP synthase β subunit interacts with the Nod26-like intrinsic protein OsNIP2;1, which is encoded by the Lsi1 gene. The results indicate that OsNIP2;1 can indirectly cooperate with OsSHMT through the ATP synthase β subunit and that OsSHMT interacts with defence and anti-oxidation related proteins to regulate the chilling resistance of rice and ensure its normal growth and development. The increase in OsSHMT expression and its positive role in scavenging H_2O_2 provides partial assistance to rescue the loss of chilling resistance in Dular rice due to the inactivation of DUA1.

The differential expression level of OsSHMT in Dular and Lsi1-OX rice is considered to be relative to transcription regulators binding to the promoter region. Our results show that NABP and histone H1 participated in regulating OsSHMT expression. NABP binds to DNA and RNA and plays a dual role in regulation of gene expression at the transcriptional and translational levels. Histones in eukaryotes are structural proteins that bind to DNA to construct chromatin nucleosomes. Histone H1 is commonly considered a transcriptional repressor because it prevents transcription factors and chromatin remodelling complexes from entering DNA [34].

In addition, AAA-ATPase family proteins were found to be involved in the regulation of OsSHMT gene expression in rice under temperature changes and in three tubulin chains. Tubulin is the main component of plant microtubules. It is mainly composed of α-tubulin and β-tubulin. Tubulin plays a role in maintaining cell morphology, promoting intracellular transport, participating in cell movement and cell division during development [35]. It plays an important role in the regulation of stress tolerance and thus responds to a variety of intracellular and external stimuli [36, 37]. The transcriptional levels of these genes in the Lsi1-OX rice were higher than those in wild-type Dular, suggesting that these factors have a positive effect in regulating the expression of SHMT genes. Whether these proteins are directly or indirectly linked to the SHMT gene promoter remains to be revealed.

Conclusions

Overexpression of Lsi1 in chilling-sensitive Dular rice resulted in the plasma membrane-localized OsNIP2;1 protein expressed in the cytoplasm, which would enable OsNIP2;1 to perform multiple roles. The expression of OsSHMT was up-regulated in the Lsi1-OX line in comparison with the Dular line. The differential gene expression level of OsSHMT may be transcriptionally regulated by OsNABP, histone H1 and tubulin. In addition, OsSHMT interacts with OsAPX, OsHsp70, OsATP-synα, OsATP-synβ and OsMSCP to scavenge H_2O_2, and OsATP-synβ interacts with OsNIP2;1, although OsSHMT does not directly interact with OsNIP2;1 (Fig. 8), and ATP synthase subunit β is an intermediate junction between Lsi1 and OsSHMT.

Methods

Plant materials and treatments

In this study, Dular rice (Oryza sativa L. subsp. indica) and its transgenic line with Lsi1 overexpression (Lsi1-OX) were used [6]. Arabidopsis thaliana with an RNA-dependent RNA polymerase 6 gene mutation (rdr6) [38] and Nicotiana benthamiana [39] were also used in this study.

The Dular and Lsi1-OX rice seeds were sterilised with 25% sodium hypochlorite solution (W/V) for 30 min and washed with sterile water to remove any residues. The sterilised seeds were soaked overnight at 30 °C in an incubator. The seeds were germinated and sown in black pots filled with a hydroponic nutrient solution suspended in a polyethylene mesh. The pots were placed in an artificial climate chamber for 10 days, during which a temperature of 26 °C was maintained for 14 h and darkness was maintained for 10 h at 22 °C. The relative humidity in the chamber was maintained at about 85%. The nutrient solution was changed weekly, and the pH was maintained between 5.5 and 6.0 throughout the experiment.

When the rice had reached the three-leaves stage, the Lsi1-OX and wild-type Dular that treated at 12 °C/10 °C (day/night) were set as treatment groups, and the control groups were these two rice lines that placed in another chamber with day/night temperatures of 26 °C/22 °C. Both the treatment and the control groups for each rice line had four replicates and they were both repeated three times in the same growth chamber.

Protein Sub-localisation

The coding DNA sequence of OsSHMT (LOC_Os03g52840) was amplified and fused with eYFP in the pCambia2300 to construct a recombinant 35S::OsSHMT-eYFP vector for rice protoplast transformations. Sub-localisation of OsSHMT was detected using confocal laser scanning microscopy. An organelle-specific protein marker (mcherry) was co-localised with OsSHMT to validate the above results. At the same time, a blank vector that contained only the yellow fluorescent protein gene was separately transferred into the rice protoplast to serve as a reference. The rice protoplasts were cultured at 28 °C for 24–48 h. The distribution of yellow fluorescence in the protoplast was observed by laser confocal microscopy to determine the subcellular localisation of OsSHMT protein.

DNA pull-down fishes transcription regulators binding to the promoter of OsSHMT

The second-topmost leaves of the four replicates were respectively sampled at 48 h after chilling treatment. These rice leaves were quickly frozen in liquid nitrogen. The natural leaf proteins of Dular and Lsi1-OX were extracted using Pi-IP buffer (50 mM Tris·Cl, 150 mM NaCl, 1 mM EDTA pH 8.0, 1% Triton X-100, 1 mM PMSF, 1 × EDTA-free Protease Inhibitor Cocktail, Roche, Merck) (Method S1). The promoter region (2549 bp upstream of the CDS of OsSHMT) was cloned from the DNA of Dular, labelled with biotin at the 5' flanking and incubated with the natural proteins. The interacting proteins were then collected using a Dynabeads kilobaseBINDER Kit (Thermo Fisher Scientific). The interacting proteins were separated on 10% SDS-PAGE and identified using Orbitrap Fusion (Thermo Fisher Scientific).

Quantitative Pcr To Determine Gene Expression Level

Total RNA from Dular and Lsi1-OX rice exposed to a temperature of 15 °C for 12, 24 and 36 h was extracted using Trizol and reverse-transcribed into cDNA using TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix. The control groups were grown at 26 °C for 12, 24 and 36 h. Specific primers for tubulin/FtsZ domain containing protein (LOC_Os03g51600, LOC_Os05g34170, LOC_Os07g38730), histone H1 and nucleic acid binding protein (NABP) are listed in Table S3; the β-actin gene was taken as the reference. The qPCR reaction system was prepared using TransStart Tip Green qPCR SuperMix and an Eppendorf realplex^4 instrument. The reaction process was as follows: pre-denaturation at 94 °C for 30 s, denaturation at 94 °C for 5 s, annealing at 55 °C for 15 s, extension at 72 °C for 10 s; 42 cycles. After amplification, a melting curve analysis was performed to verify the specificity of the amplified products, under the following conditions: 95 °C for 15 s, 60 °C for 15 s and an increase to 95 °C over a 10-min period, followed by a 15-s holding period. Threshold cycle values (Ct) were recorded for each candidate mRNA in both the control and test samples. Each candidate mRNA was set with four independent replicates. The relative expression of the gene was calculated by the $2^{-\triangle\triangle Ct}$ method [40].

Arabidopsis thaliana transformation

The CDS of OsSHMT was amplified and inserted into modified pCambia3301 (with 35 s promoter) to construct the recombinant vector for Arabidopsis thaliana transformation using the floral dip protocols described by Clough and Bent [41]. Natural leaf proteins were extracted from a T3 generation homozygote of OsSHMT transgenic A. thaliana and incubated with GFP-Trap agarose (Chromotek) to collect putative interacting proteins.

Bimolecular Fluorescence Complementation (bifc) Validates Rice Protein Interactions

To investigate the possible interactions between OsSHMT and the proteins identified from the Co-IP results, the genes that encode these proteins were cloned from Dular rice and used to construct recombinant vectors fused with the N-terminal domain or C-terminal domain of enhanced yellow fluorescent protein (eYFP). These vectors were then transformed into Agrobacterium (EHA105) for inoculation into the cytoplasm of tobacco (Nicotiana benthamiana) for a transient expression assay.

Laser scanning confocal microscopy was used to investigate the positive interactions of the target proteins.

Dab Staining And Determination Of Ho Content

Detection of H_2O_2 in leaves was conducted using DAB staining; OsSHMT transgenic A. thaliana and its wild type were treated at 4 °C for 12, 24 and 36 h. The treatment was repeated three times in the same growth chamber. Leaves from these two lines were sampled and immersed in 50 mg/L diaminobenzidine (DAB) solution by vacuum-pumping for 1 h and incubated overnight at room temperature. These leaves were then decolourised with 95% ethanol in a water bath at 80 °C, and the reddish brown spots in the transgenic leaves and wild-type leaves were observed using an integrated microscope (Nikon, SMZ18). The H_2O_2 content in the 12 h-treated leaves of the transgenic line and the wild type were determined using an H_2O_2 determination kit (Solarbio Life Sciences).

Primer Sequences

A list of the primers is provided in Table S3.

Abbreviations

APX
ascorbate peroxidase
BiFC
Bimolecular fluorescence complementation
ER
endoplasmic reticulum
OX
overexpression
SHMT
serine hydroxymethyltransferase
NIP
nodulin 26-like intrinsic protein
ROS
reactive oxygen species
NABP
nucleic acid binding protein
HSP70
heat shock protein 70

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All supporting data can be found within the manuscript and its additional supporting files.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

CF and WL designed the experiments. PZ, LL and CF performed most of experiments and analyzed the data. Other authors assisted in experiments and discussed the results. CF and WL wrote the manuscript. All authors read and approved the final manuscript.

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Supplementary Files Legend

Fig. S1 Subcellular localisation of *Os*SHMT protein in rice protoplast

Table S1 Sequence of *OsSHMT* gene promoter from Dular

Table S2 Proteins interacted with *Os*SHMT in the *Os*SHMT transgenic *A. thaliana* using GFP-Trap Co-IP

Table S3 Primers used in this study

Methods S1 Extraction of rice leaf protein

Figures

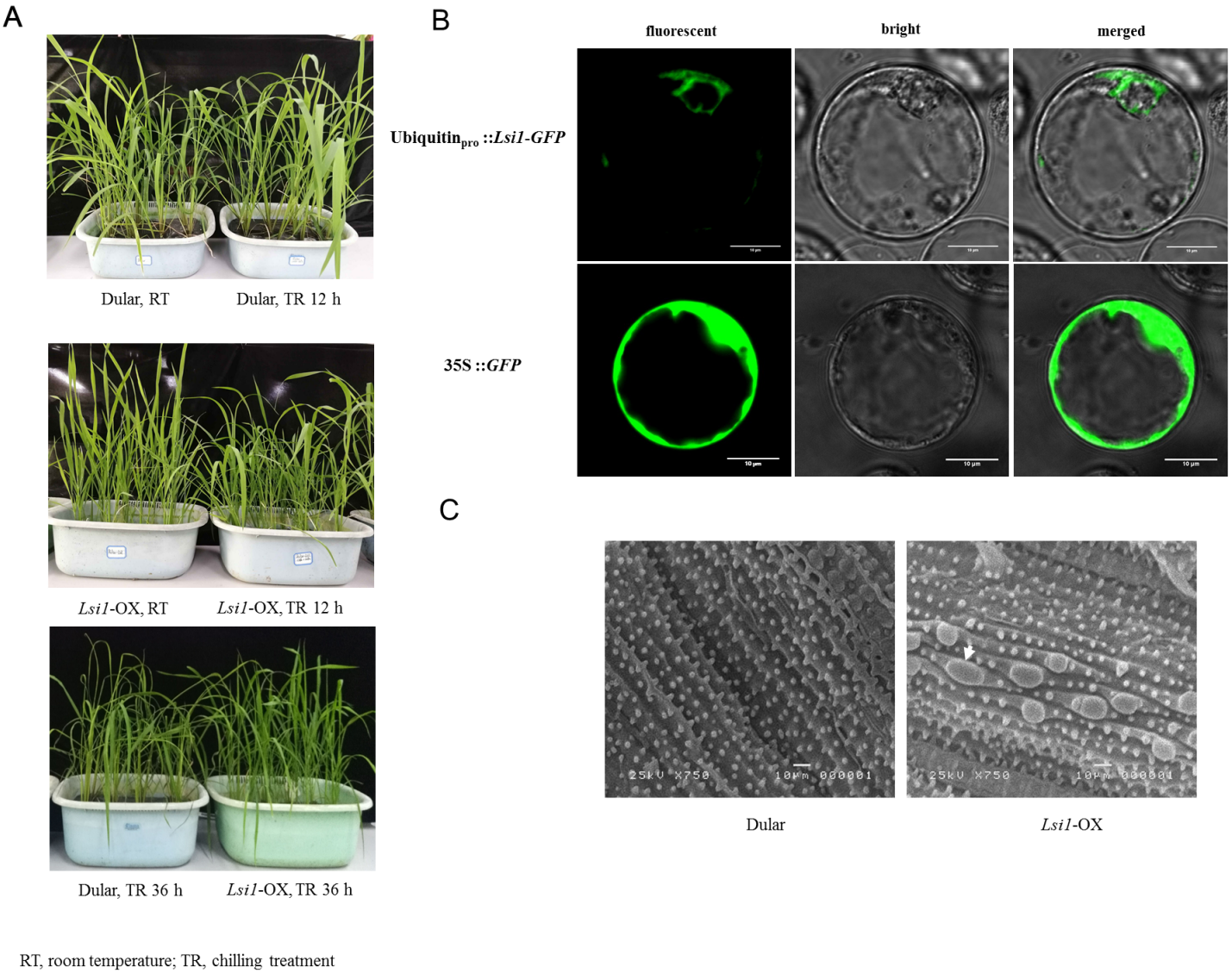

Figure 1

Phenotypic changes in the leaves under chilling treatment (A), sub-cellular localisation of Lsi1 driven by ubiquitin promoter (B) and SEM images of the leaf surface of Dular and Lsi1-OX (C) The Dular rice and

the transformed Lsi1-OX line were treated at 4 °C for 36 h and the phenotypic changes in the leaves of the two rice lines were compared (A). Lsi1 was fused with GFP and inserted into a modified pCambia 1301 vector in which the Lsi1 gene was transcribed by the ubiquitin promoter. The recombinant Lsi1 expression vector was transformed in the rice protoplast and sublocalisation of OsNIP2;1 was detected using laser scanning confocal microscopy (B). SEM images of the surfaces of the two rice leaves were compared to determine the differences in silica deposition (C).

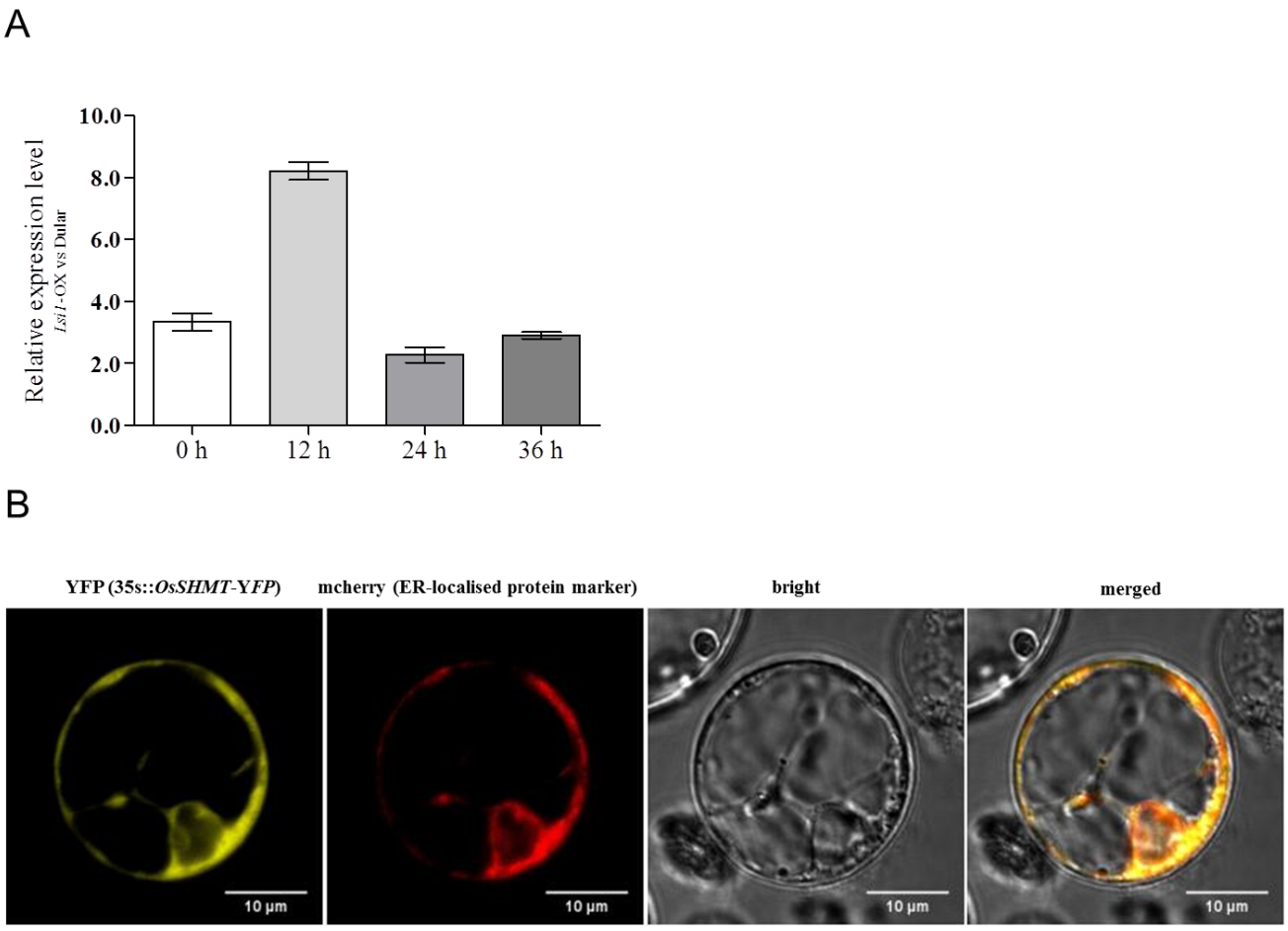

Figure 2

Relative expression of OsSHMT in the Lsi1-OX rice line and Dular rice (A) and subcellular co-localisation of OsSHMT protein with endoplasmic reticulum marker protein (B) Changes in the gene expression level of OsSHMT before chilling treatment and after treatment for 12, 24 and 36 h were compared for Lsi1-OX and Dular rice (A). OsSHMT was fused with YFP and inserted into pCambia 2300 to construct the recombinant vector for OsSHMT expression and detect subcellular protein localisation. The recombinant vector was transformed into rice protoplast for transient expression, together with a mcherry fused ER localised protein as a marker. Yellow fluorescence and mcherry fluorescence were respectively detected and then merged to validate the OsSHMT subcellular localisation (B).

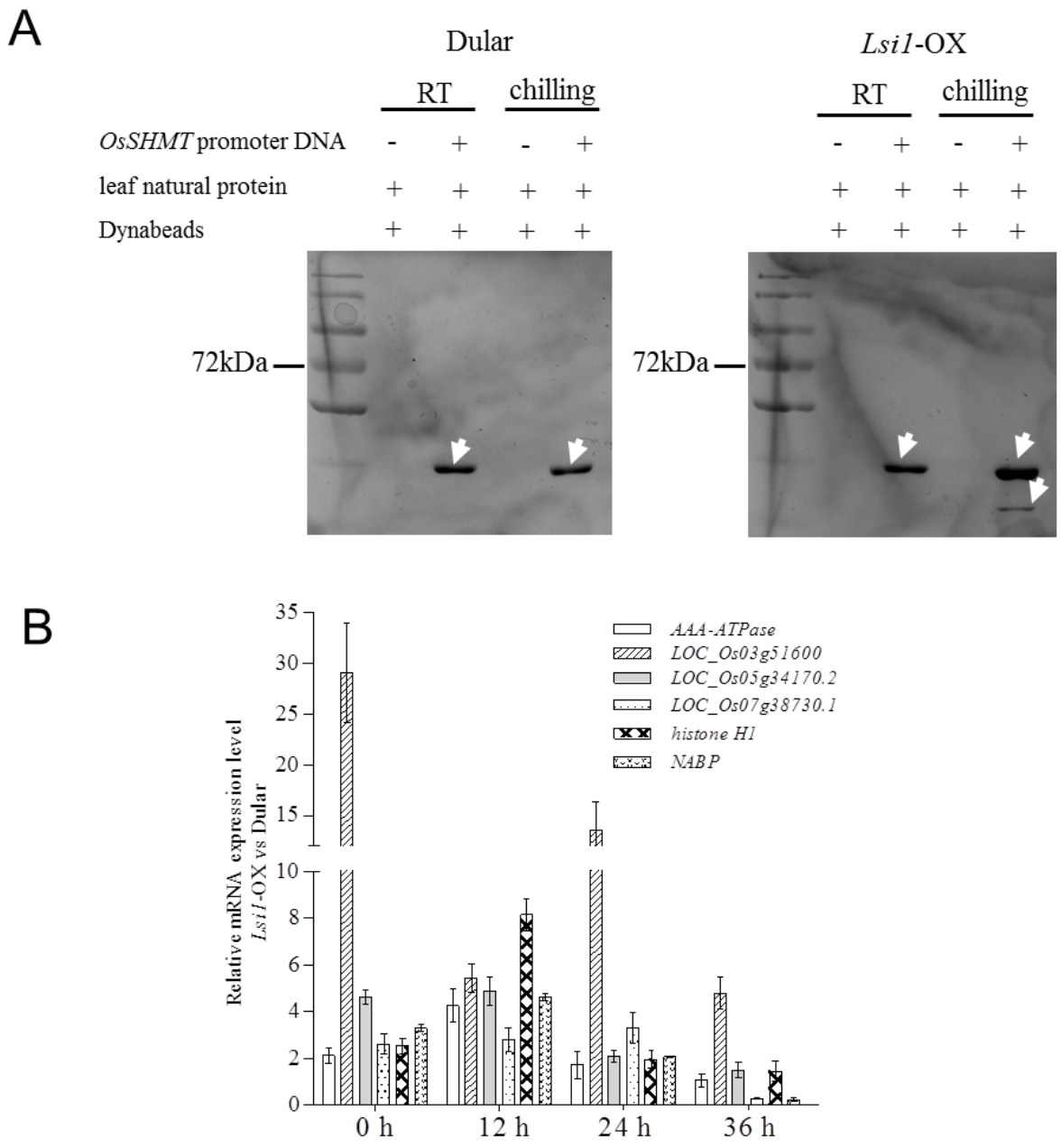

Figure 3

Proteins binding on the OsSHMT-promoter in Dular and Lsi1-OX rice and the differences in transcription level between the two types of rice The promoter region of OsSHMT was amplified using the specific primers with biotin labelled at the 5' end and then fused with Streptavidin-coupled Dynabeads and natural leaf proteins from Dular or Lsi1-OX rice. The protein and DNA complex mixture was extracted and incubated with OsSHMT promoter-containing Dynabeads and fished using a magnetic frame to collect the proteins and then separated by SDS-PAGE (A). qPCR was conducted to determine the change in the transcription levels of AA-ATPase, histone H1, nucleic acid binding protein (NABP) and tubulin/FtsZ

domain containing protein (LOC_Os03g51600, LOC_Os05g34170, LOC_Os07g38730) for Lsi1-OX and Dular rice under chilling treatment of different durations (D).

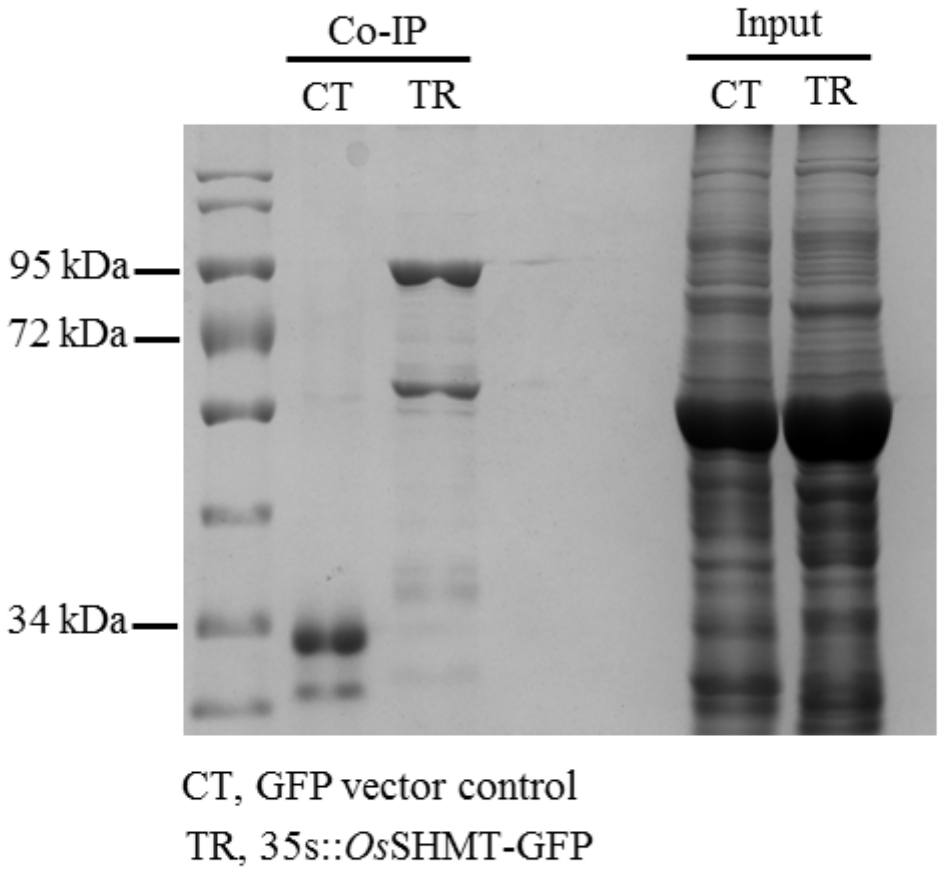

Figure 4

Protein interactions with OsSHMT in Arabidopsis thaliana The recombinant vector of OsSHMT fused with GFP was used to genetically transform Arabidopsis thaliana and T3 homozygous transgenic lines were taken to isolate proteins interacting with OsSHMT. A vector containing only GFP without OsSHMT was used as a control to transform into A. thaliana to obtain the transformed lines. Natural leaf proteins from OsSHMT transgenic A. thaliana and GFP transgenic A. thaliana were respectively extracted and incubated with GFP-Trap agarose beads to reveal the proteins interacting with OsSHMT.

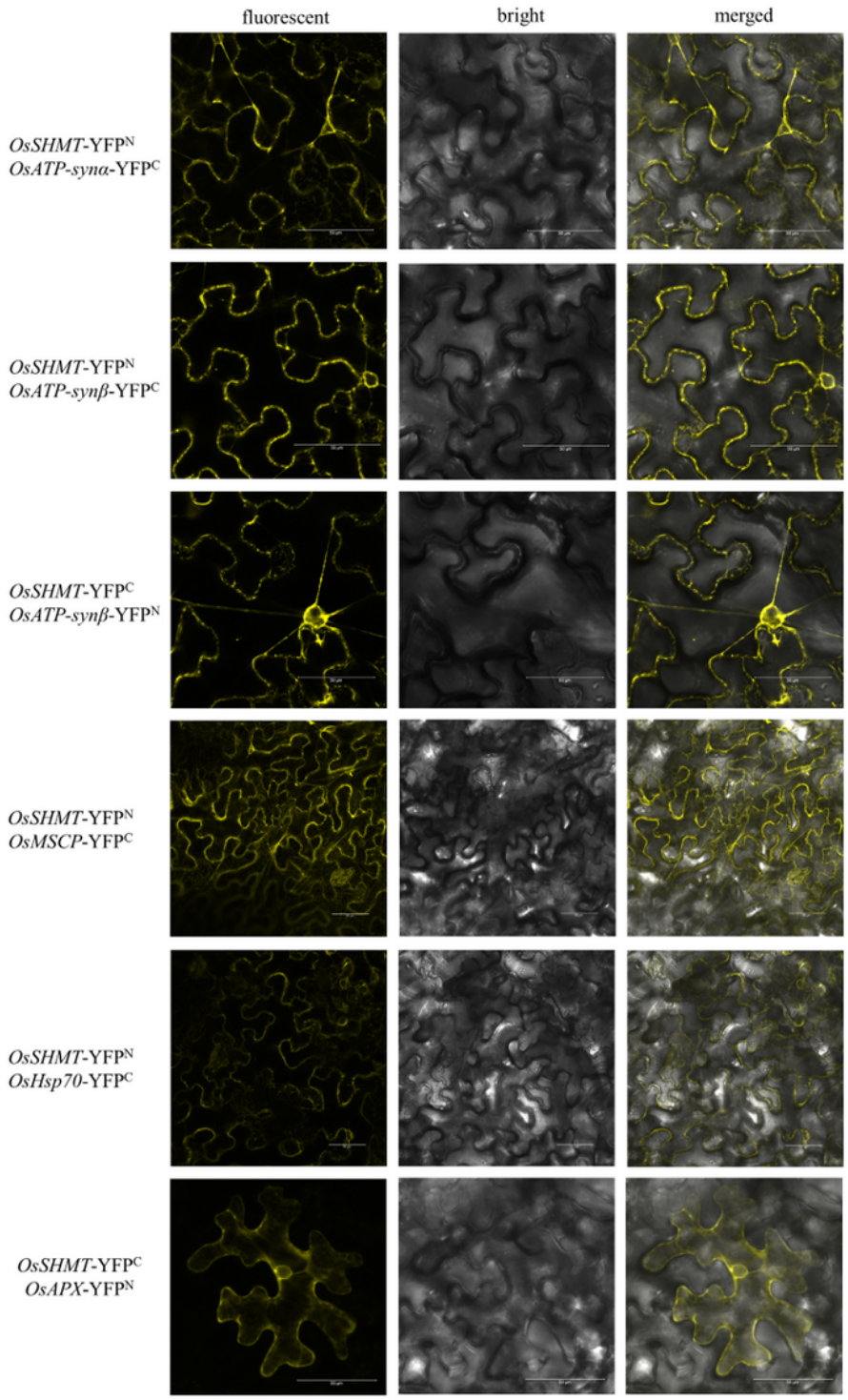

Figure 5

Bimolecular fluorescence complementation validation of the bio-interaction of OsSHMT and ATP synthase subunit α, ATP synthase subunit β, Hsp70, MSCP and APX from rice To validate the positive interaction of OsSHMT with ATP synthase α subunit, ATP synthase β subunit, heat shock protein 70, mitochondrial substrate carrying family (MSCP) protein E and ascorbate peroxidase (APX) in rice, their genes were amplified from Dular rice and respectively infused with the N-terminal or C-terminal of YFP to

construct YFPn- and YFPC-containing recombinant vectors for bimolecular fluorescence complementation (BiFC), according to transient transformation in the leaves of Nicotiana benthamiana. The YFP fluorescence was detected using laser confocal microscopy under 488-nm excitation light.

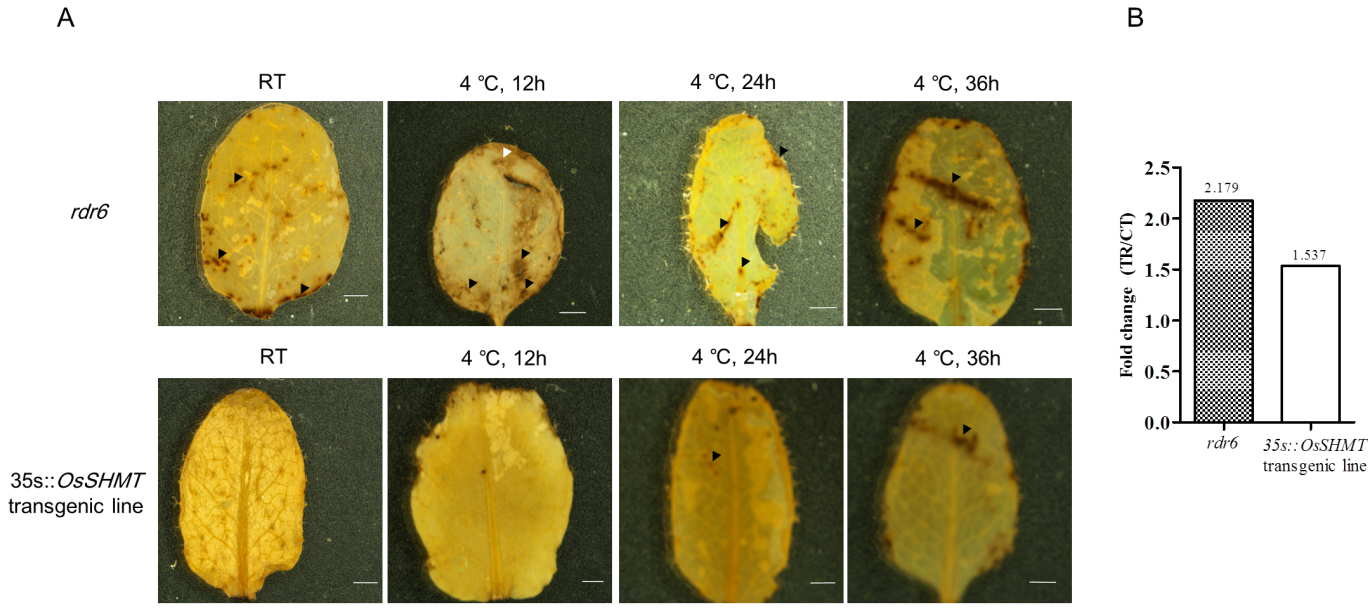

Figure 6

DAB staining of leaves of OsSHMT transgenic and wild type A. thaliana (A) and the H2O2 content in the leaves (B) OsSHMT transgenic Arabidopsis thaliana and its wild-type were exposed to 0 °C for 12, 24 and 36 h, and leaves were sampled and stained with DAB, after which the chlorophyll in the leaves was dissolved using ethanol, leaving dark brown precipitate on the leave. Arrowheads indicate reddish-brown spots. Bar=1mm. The H2O2 content in the leaves was determined using an H2O2 determination kit (Solarbio Life Sciences).

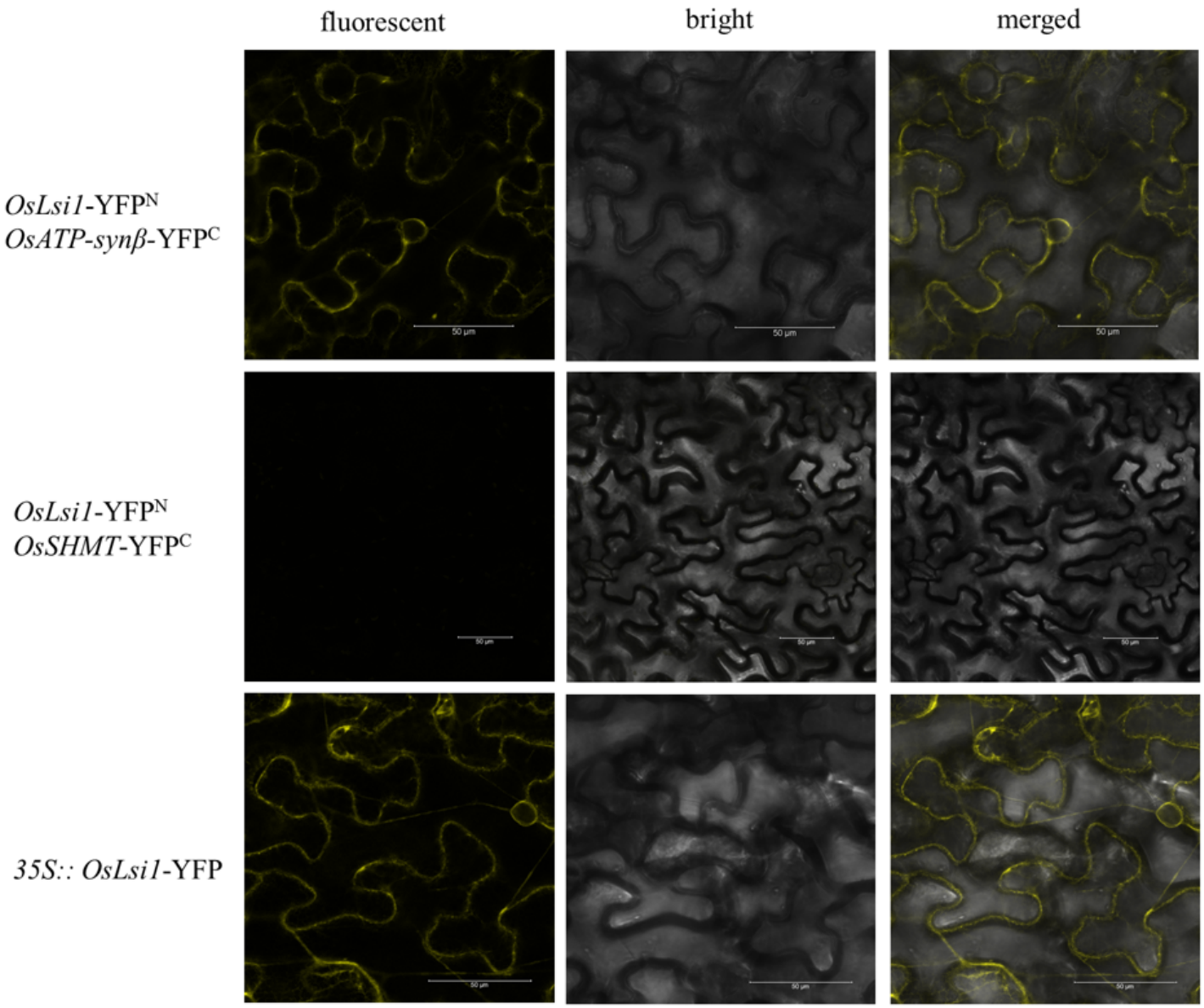

Figure 7

Bimolecular fluorescence complementation validates the bio-interaction of OsNIP2;1 and ATP -synβ Lsi1 was infused with the N-terminal domain of YFP and ATP synthase subunit β and OsSHMT was infused with the C-terminal domain of YFP. Lsi1 with N-terminal domain was then co-expressed with ATP synthase subunit β or OsSHMT with the C-terminal domain of eYFP, respectively, in the leaves of Nicotiana benthamiana for 48 h, and YFP fluorescence was detected using laser confocal microscopy under 488-nm excitation light. The sub-cellular localisation of Lsi1 was also detected in the leaves of Nicotiana benthamiana.

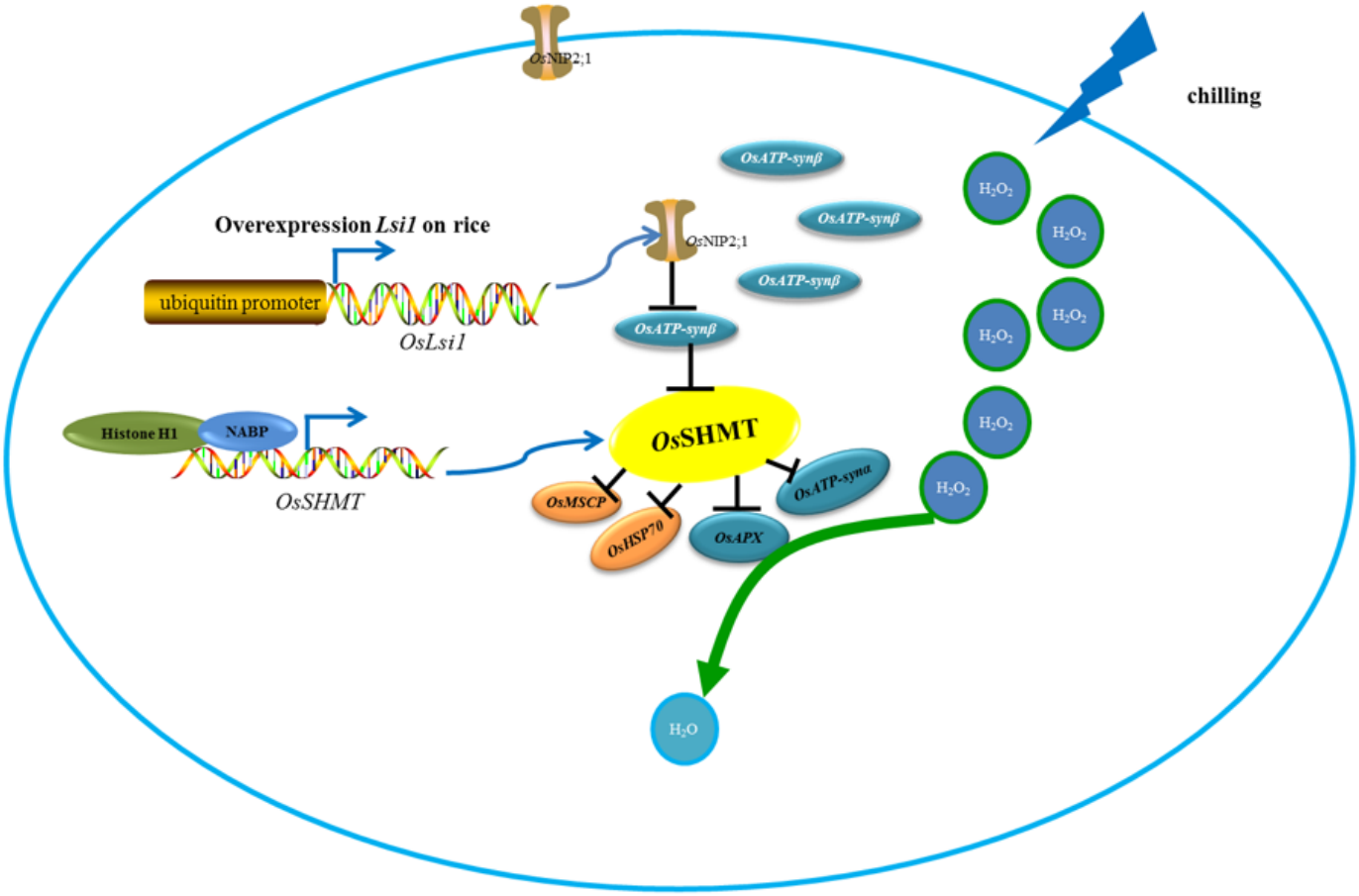

Figure 8

Schematic summary of the role of OsSHMT in the regulation of rice chilling tolerance. OsSHMT interacts with APX, HSP70, MSCP, ATP-synα and ATP-synβ to scavenge H2O2. ATP-synβ also interacts with OsNIP2;1 in the cytoplasm when the ubiquitin promoter is used to overexpress Lsi1 in the rice.

Supplementary Files

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- SupplementaryMethod.docx
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