Supplementary Data

SNAC1-targeted gene OsSRO1c modulates stomatal closure and oxidative stress tolerance by regulating hydrogen peroxide in rice

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Supplementary Fig. S1. Multiple sequence alignment and sequence comparison. A, Multiple sequence alignment of OsSRO1c and Arabidopsis SRO proteins (RCD1, SRO1-5). B, Multiple sequence alignment of the PARP domain from human PARP1 (HsPARP1), Arabidopsis PARP-1 and -2 (AtPARP1 and AtPARP2), Arabidopsis RCD1, and OsSRO1c. Conserved ADP-ribosyl transferase catalytic triad, composed of three amino acids is indicated by an asterisk (*) below the alignment.
Supplementary Fig. S2. Tissue and organ expression pattern of OsSRO1c. The 16 organs/tissues are as follows: T1, calli; T2, seed; T3, radicle; T4, plumule; T5, nodes; T6, collar; T7, stem; T8, flag leaf; T9, sheath; T10, young panicle at stage 3; T11, young panicle at stage 4; T12, young panicle at stage 5; T13, young panicle at stage 7; T14, stamen; T15, secondary branches; and T16, hull. Error bars indicate SE based on three replicates.
**Supplementary Fig. S3.** Subcellular localization of OsSRO1c. A, Subcellular localization of OsSRO1c in rice protoplast (ZH11 background). 35S:sGFP-OsSRO1c and 35:sCFP-GHD7 were co-transformed into rice etiolated shoot protoplasts. 35S:sGFP was transformed as control. B, Subcellular localization of OsSRO1c in transgenic rice (ZH11 background). OsSRO1c cDNA was fused to GFP and the construct was expressed in transgenic rice under the control of the cauliflower mosaic virus 35S promoter. Confocal image of the root was shown.
Supplementary Fig. S4. Thermal images of detached leaves (A) and drought-stressed seedlings (B) of DJ and ossro1c-1 mutants. Visible image is on the top.
Supplementary Fig. S5. Transcript level of OsSRO1c in amiR-OsSRO1c (A) and OsSRO1c-overexpressing (B) plants. Error bars indicate SE based on three replicates. Red triangle indicates transgenic plants that were selected for further study.
Supplementary Fig. S6. Suppression of OsSRO1c showed increased sensitivity to osmotic stress. A, Increased sensitivity of *ossrol-1* mutants to osmotic stress. B, Plant height of DJ and *ossrole-1* mutant under normal and mannitol treatment (*n* = 10). C, Increased sensitivity of amiR-OsSRO1c plants to osmotic stress. D, Relative plant height of ZH11 and amiR-OsSRO1c plants under mannitol treatment (*n* = 3). Data represent mean ± SE. **P < 0.01, *t* test.
Supplementary Fig. S7. Thermal images of the OsSRO1c-overexpressing plants or ossro1c-2 mutants. Visible image is on the left side.
**Supplementary Fig. S8.** Endogenous ABA content and stomatal responses to ABA in OsSRO1c-overexpressing plants. A, The ABA content of ZH11 and OsSRO1c-overexpressing plants under normal and drought stress \((n = 3)\). Data represent mean ± SE. B, The percentage of three levels of stomatal opening in wild-type (WT), OsSRO1c-overexpressing plants and osso1c-1 mutants under ABA treatment. Thirty-five-day-old plants were treated with 50 μM ABA for 2h. 49 stomata of ZH11, 51 stomata of OsSRO1c-overexpressing plants, 65 stomata of WT and 63 stomata of osso1c-1 were randomly selected for analysis.
Supplementary Fig. S9. Loss of OsSRO1c function enhanced resistance to oxidative stress. A and C, *ossro1c-1* mutant (A) and amiR-OsSRO1c plants (C) enhanced resistance to oxidative stress. B and D, Total chlorophyll contents of wild-type (WT) and *ossro1c-1* mutants (B) or amiR-OsSRO1c plants (D) under normal and MV stress. Data represent mean ± SE (*n* = 3). **P < 0.01, *t* test. FW, fresh weight.
Supplementary Fig. S10. qPCR analysis expression of ROS-scavenging genes in OsSRO1c-overexpressing plants. *Fe-SOD* and *SodCc2* are two superoxide dismutase (SOD) genes. *Glutathione S-transferase* GSTU6 and *peroxidase 16 precursor* were ROS-scavenging genes that up-regulated in *dst* mutant. Error bars indicate SE based on three replicates.
Supplementary Fig. S11. Pairwise interaction test of OsSRO1c with members of rice DREB and NAC family. Interaction was tested by the expression of lacZ reporter gene (blue on X-gal assay). 1 and 2 show two different colonies of each pairwise interaction test.
**Supplementary Fig. S12.** Overexpression of *OsSRO1c* caused increased sensitivity to drought stress. A and C, Increased drought sensitivity of *OsSRO1c*-overexpressing plants (A) and *ossro1c-2* mutants. Four-leaf stage plants were not watered for 10 d, followed by rewatering for 7 d. (C). B and D, Survival rate of wild-type and *OsSRO1c*-overexpressing plants (B) or *ossro1c-2* mutants (D) after drought stress. Data represent mean ± SE (*n* = 3). **P < 0.01, *t* test.
Supplementary Fig. S13. Expression of *DST* in *SNAC1*-overexpressing plants. Error bars indicate SE based on three replicates.

Supplementary Fig. S14. Effect of ABA on seedling growth of *ossro1c-1* mutants (A and B) and *OsSRO1c*-overexpressing plants (C and D). 3-day-germinated seeds transplanted in either MS medium or MS medium supplemented with 3 μM ABA for 7 days. Data represent mean ± SE (*n* = 3).
Supplementary Table S1. Primer sequences used in this study.

| Primer name | Primers sequence | Description |
|-------------|------------------|-------------|
| (a) ChIP-PCR |                 |             |
| PA          | TTCATATGTTTCTGCGGTTTCTTC | TATTTTAGTACGGACGGAAGTTCGT |
| PB          | AAACTCCCGTAACCCTAGGATGATC | CCCAGGCACCACAGCC |
| PC          | ATGCTGAAATTTTGTATTTC | GTGTTCTGAGGGCGGCG |
| (b) T-DNA verification |                 |             |
| M1          | TCGCCGATGTGGGA | GGTAAACTGGTACTTCTTGCT |
| pGARP       | TGGGGTTTCTACAGGACGTAAC | Postech T-DNA-specific |
| M2          | AAGTGGGAGCGGAAGACG | CGGCGATGGCAAAGGT |
| T-DNA-3     | TAATAACGCTGCGGACATCTA | SHIP T-DNA-specific |
| (c) construction for rice transformation |                 |             |
| SNAC1miRI   | AGTATTTACACACGTCCTCAGCATCAGGAGATTCAGTTTGA | P\textsubscript{Ubi}:amiSNAC1 |
| SNAC1miRII  | TGATGCTGAACTGAGTGTAAATACTCTGCTGCTAGCC | |
| SNAC1miRIII | CTATGCTCAGAAGGTGTAAATATCTTCTGCTAGGCTG | |
| SNAC1miRIV  | AATATTACACACTTCCCGAGCATAGGAAATACCTGCTAGGCTG | |
| SROmiRI     | AGTACTGTTCTGATTCTACGGACTCAGGAGATTCAGTTTGA | P\textsubscript{Ubi}:amiOsSRO1c |
| SROmiRII    | TGATGCTAGAATGAGAAGTACCTGCTGCTAGGCTG | |
| SROmiRIII   | CTATGCTCAGAAGGTGTAAATATCTTCTGCTAGGCTG | |
| SROmiRIV    | AATATTACACACTTCCCGAGCATAGGAAATACCTGCTAGGCTG | |
(d) construction for yeast transformation

| Cloning domains of OsSRO1c into GAL4 Y2H system (pDEST32) |
|----------------------------------------------------------|
| **SROHIS** ATAGAATTCAACTCGCCATTATACTATC                  |
| **SROY2H** GGGGACAAGTTTGTACAACAAAAACGAGG                |
| **SROWWW** GGGGACAAGTTTGTACAACAAAAACGAGG                |
| **SROPARP** GGGGACAAGTTTGTACAACAAAAACGAGG                |
| **SRORST** GGGGACAAGTTTGTACAACAAAAACGAGG                |
| **SROWP** GGGGACAAGTTTGTACAACAAAAACGAGG                 |
| **SROP1** GGGGACAAGTTTGTACAACAAAAACGAGG                 |
| **SROP2** GGGGACAAGTTTGTACAACAAAAACGAGG                 |

**pHIS2-** OsSRO1c

**pDEST32-OsSRO1c**
Cloning OsDREB1A, OsDREB1B, OsDREB2A and OsDREB2B into GAL4 Y2H system (pEXE-AD502)

AD502F TATAACGCGTTTGGAATCACT  pEXE-AD502 sequencing primer
AD502R GTAAATTTCTGGCAAGGTAGA  pEXE-AD502 sequencing primer

(e) construction for protoplast transformation

SROSL  CAGTCTAGAGGGAGGGGTGATGGAC  CAGTCTAGACCCTCGGAGTTTCTTGG  P255::OsSRO1c:sGFP (pM999-33)
SROBI  GGTACCTCCGGCGACGTCAAG  GAGCTCAACTAAAGCAGCATCAGAAGA fusion of OsSRO1c to the N-terminal of YFP (pVYNER)
D2BBI  GGATCCGAAGGCAAGGAAGGCA  GGTACCCAAGCCCTCAAAGAACTGAGA fusion of OsDREB2B to the C-terminal of YFP (pVYCE)
UBIBI  TCTAGACCCGCACCCCCG  ACTAGTCTGGTCTTTCTTCTCCCTAGC  fusion of UBA to the C-terminal of YFP (pVYCE)
N8BI  TCTAGAAGTAGTCCACCCCCAACGAC  CTCGAGGGTGATGCTGATCTCTCTCT  fusion of NAC8 to the C-terminal of YFP (pVYCE)
(f) qPCR primers

| LOC             | Primer                     | Primer efficiency |
|-----------------|----------------------------|-------------------|
| LOC_Os03g12820  | TCCCTATGCTTCTGACGGAGAT     | 105.63%           |
| LOC_Os03g12820  | CTCCCACATCGGCGGACA         | 100.83%           |
| LOC_Os03g60080  | CATGGTTCCGTCTGAGCTCA       | 104.23%           |
| LOC_Os03g57240  | ATCCAAAGGAAGGTCAATC        | 101.40%           |
| LOC_Os01g28030  | GTCTCCAGGATCCCTGTCGTC      | 98.96%            |
| LOC_Os10g38470  | CGCCACCAACTGAAGTGACA       | 92.51%            |
| LOC_Os06g48030  | CCATGATGATCTTCGATTCTCAG    | 108.40%           |
| LOC_Os06g05110  | CGACGGGCAAGGAATTTTCTAG     | 108.24%           |
| LOC_Os07g46990  | ATTCCATGTGCACGGCA          | 104.78%           |
| LOC_Os11g06390  | TGGCATCTCTAGCACACATTCC     | 100.60%           |
Supplementary methods S1. Supplementary methods for plasmid construction, rice transformation, stress treatments, physiological measurements, yeast assay, BiFC assay, and RT-PCR.

Stress treatments
To measure the transcript level of the OsSRO1c under stresses, ZH11 plants were grown in the greenhouse with a 14-h-light/10-h-dark cycle. Plants at the four-leaf stage were treated with various stresses and phytohormones treatment. For drought stress, the seedlings were grown for 7 d without water, and sampled at 0 d, 3 d, 5 d and 7 d. For salt stress, the seedlings were irrigated with 200 mM NaCl solution and sampled at 0 h, 3 h, 6 h and 12 h. For cold and heat shock stress, seedlings were transferred, respectively, to a growth chamber at 4°C and sampled at 0 h, 6 h, 12 h and 24 h and 42°C and sampled at 0 min, 10 min, 30 min and 2 h after treatment. For UV stress, seedlings were transferred to a tissue prepared room with UV lights (emission peak 254 nm; 1100 μW/cm² at plant level; TUV30W, Philips, Nederland) and sampled at 0 h, 3 h, 6 h and 12 h. Rice leaves were wounded by cutting into pieces and floated on water at room temperature under continuous light for 1 h, 3 h and 6 h. For oxidative stress, seedlings were irrigated with 1% (v/v) H₂O₂ solution and sampled at 0 h, 2 h, 6 h and 12 h. For ABA treatment, 100 μM ABA were sprayed on leaves and sampled at 0 h, 2 h, 6 h and 12 h.

For stress testing of transgenic lines and mutant, OsSRO1c-overexpressing and amiRNA transgenic plants were selected by germinating seeds on MS medium containing 50 mg L⁻¹ hygromycin. Wild-type and homozygous mutants were grown on MS medium or grown in soil. For drought stress at the seedling stage, mutant/transgenic plants and wild-type plants were growing in the same barrels filled with a mixture of soil and sand (1:1). The water supply was stopped to allow drought stress to develop at four-leaf stage. After all leaves completely rolled and recovery by re-water, surviving seedlings were photographed and analyzed. Drought stress testing at the later tillering stage was performed in a refined paddy field facilitated with a movable rain-off shelter. To evaluate mannitol stress tolerance, gerninated seeds were transplanted in MS medium supplemented with 150 mM mannitol. After 7 d of growth, shoot length was measured. For the oxidative treatment, gerninated seeds were transplanted in MS medium supplemented with 2 μM MV. After 7 d of growth, chlorophyll content was measured.
Physiological measurements

To measure the activity of ROS-scavenging enzymes, soluble proteins were extracted using 50 mM potassium phosphate buffer (pH 7.8). After centrifuged at 12,000 g for 15 min at 4ºC, the supernatant was used as the enzyme extract. Total SOD activity was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) according to the method described previously (Giannopolitis and Ries, 1977). The 3 mL reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 2 µM riboflavin, 10 µM EDTA and 100 µL enzyme extract. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT as monitored at 560 nm. The activity of CAT and POX were measured using kit from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). One unit of CAT activity was defined as the amount of enzyme depleting 1 µmol H2O2 in 1 sec. One unit of POX activity was defined as the amount of enzyme for producing 1 µg substrate in 1 min. Quantitative measurement of H2O2 production was performed using the Amplex Red hydrogen peroxide/peroxidase assay kit (Molecular Probes) following the manufacturer’s instructions. H2O2 was extracted from leaves according to the method described previously (Wu et al., 2012)

Plasmid construction and rice transformation

A full-length cDNA of OsSRO1c isolated from indica rice Guang-lu-ai 4 was obtained from the Rice Indica cDNA Database (http://www.ncgr.ac.cn/ricd/) (Liu et al., 2007). To generate the OsSRO1c overexpression constructs, the full-length cDNA of OsSRO1c was amplified from plasmid by PCR and the sequence-confirmed PCR fragment inserted into vector pCAMBIA1301U (pU1301) under the control of a maize ubiquitin promoter. The OsSRO1c artificial microRNAs (amiR-OsSRO1c) were constructed as described (Warthmann et al., 2008). A 21-mer sequence targeting to the 3’UTR of OsSRO1c was used to replace the endogenous miRNA and miRNA* of osa-MIR528 were designed by Web MicroRNA Designer platform (WMD) (Ossowski et al., 2008). Then the resulting artificial microRNAs were cloned into pU1301. The SNAC1 artificial microRNAs (amiR-SNAC1) were constructed with the same process. To investigate the expression of OsSRO1c, the OsSRO1c promoter region (a fragment of 1.6 kb length upstream of the starting codon of the gene) was
amplified from the genomic DNA of Nipponbare and the sequence-confirmed PCR fragment cloned into pDX2181 to control GFP expression. For subcellular localization, the coding region of *OsSRO1c* cDNA was prepared by PCR, and cloned into the GATEWAY destination vector pH7WGF2.0 binary vector by recombination reaction (Karimi *et al.*, 2007). The primers are listed in Table S1. Both of the constructs were transformed into Zhonghua 11, a japonica rice that can be easily transformed, by the *Agrobacterium*-mediated transformation method (Hiei *et al.*, 1994; Lin and Zhang, 2005). The GFP fluorescence in different tissues of transgenic plants was observed by fluorescence stereomicroscope (MZ FLIII, Leica, Germany) or confocal laser-scanning microscopy (TCS SP2, Leica, Germany).

**In vivo binding assay of SNAC1**

For ChIP assay, wild-type ZH11 were used for chromatin extraction and immunoprecipitation as described previously (Huang *et al.*, 2007). Briefly, 3-leaf-stage rice seedlings were treated with formaldehyde and the nuclei were isolated and sonicated using an Ultrasonic Crasher Noise Isolating Chamber (SCIEN'TZ, Ningbo Science Biotechnology Co.Ltd, China). The soluble chromatin fragments were isolated and reabsorbed with sheared salmon sperm DNA/protein A-agarose (Sigma-Aldrich, USA) to remove nonspecific binding. Immunoprecipitations with anti-SNAC1 rabbit polyclonal antibody (New-East Biosciences) or without any serum were performed as described. The precipitated DNA was analyzed by PCR using specific primer sets (see Table S1). Typically, 26 to 28 cycles of PCR were performed, and the products were analyzed by agarose gel electrophoresis.

**Biochemical assay in yeast**

Yeast one-hybrid assay was performed using the Matchmaker one-hybrid system (Clontech, Palo Alto, CA, USA). *OsSRO1c* promoter fragment was fused upstream to the HIS3 minimal promoter and served as reporter constructs. *SNAC1* was fused to the GAL4 activation domain in the vector pGADT7-Rec2 (Hu *et al.*, 2006) and cotransformed with the reporter vector (pHIS2-P*OsSRO1c*) into yeast cell Y187 for determination of the DNA–protein interactions. Primers used for yeast constructs were listed in Table S1.

The yeast two-hybrid assay was performed using the ProQuest Two-Hybrid
System (Invitrogen, USA). To isolate interact protein of OsSRO1c, the coding region of OsSRO1c was amplified with primers SROY2H. The PCR product was cloned into the entry vector pDONR221 using the BP reaction and then into the vector pDEST32 using the LR reaction to generate bait vector with OsSRO1c fused to the GAL4 DNA binding domain. A prey stress mix (drought, high salt, cold, and ABA treated) cDNA library of rice was constructed by fusing cDNAs with the GAL4 activation domain in the pEXP-AD502 vector according to the manufacturer’s instructions. The yeast strain Mav203 was transformed with the bait plasmid, and the cells containing the bait were transformed with the plasmid DNA of the prey cDNA library according to the method described previously (Gietz et al., 1997). A total of \(2.05 \times 10^5\) transformants were selected on synthetic complete selection medium containing 20 mM 3-AT (3-amino-1, 2, 4-Triazole) and lacking Leu, Trp, and His. Large yeast clones appearing within 7 days were picked out for testing of the \(\text{LacZ}\) reporter gene. Positive clones were isolated and co-transformed with pDEST32 to test their self-activation activities.

To test interaction of OsSRO1c and members of NAC family or DREB family, the full-length cDNA of OsDREB1A, OsDREB1B, OsDREB2A and OsDREB2B proteins were isolated and fusion to the transcriptional activation domain of GAL4. Nine members of stress responsive NAC transcription factors were also fused to the transcriptional activation domain of GAL4 for yeast two-hybrid assay. All these construction were co-transformed with OsSRO1c fused to the GAL4 DNA binding domain.

**Subcellular localization and BiFC assays in rice protoplast**

The rice protoplast isolation and transformation were based on the protocol for maize protoplasts and *Arabidopsis* protoplasts from Sheen’s laboratory (Sheen, 1990; Yoo et al., 2007) with minor modifications. Rice seeds were germinated on half-strength MS medium under light for 3 d, and then grown in the dark at 26 °C for 12 d. Etiolated young seedlings were cut into 0.5 mm pieces using sharp razors. Tissue was immediately incubated in enzyme solution (0.6 M mannitol, 10 mM MES (pH 5.7), 1.5% cellulase RS, 0.75% macerozyme, 0.1% BSA, 1 mM CaCl\(_2\) and 50 μg mL\(^{-1}\) carbenicillin) for 4 h in the dark under gentle shaking (40 rpm). After incubation, protoplasts were passed through a 35 μm nylon mesh filter. One volume of W5 solution (154 mM NaCl, 125 mM CaCl\(_2\), 5 mM KC1, 2 mM MES (pH 5.7)) was
added and the solution was centrifuged for 5 min at 100 g to pellet the protoplasts. After removing the solution, 5 mL cold W5 solution was added to re-suspend the protoplasts and the protoplasts were kept on ice for 30 min. Cells were re-suspended in MMG solution (0.6 M mannitol, 15 mM MgCl$_2$, 4 mM MES (pH 5.7)) for PEG-mediated transformation at $10^6$ cells mL$^{-1}$. Cells were quantified using a hemocytometer. For transformation, 40% PEG (0.6 M mannitol, 100 mM CaCl$_2$, 40% v/v PEG 3350) and 10 µg plasmid were added to the protoplasts for 20 min. After incubation, 5 mL W5 solution was added then the protoplasts were incubated at 28°C in the dark overnight.

To investigate the subcellular localization of the OsSRO1c protein, the full open reading frame of OsSRO1c was cloned into pM999-33 vector, fused with the GFP reporter gene. To confirm the protein interactions by BiFC assays, full-length cDNAs of OsSRO1c, OsDREB2B, OsNAC8, and UBA were inserted into pVYNE(R) (fusion with the N-terminus of YFP) or pVYCE (fusion with the C-terminus of YFP) (Waadt et al., 2008). Plasmids were purified using Plasmid midi kit (QIAGEN, Germany) according to the manufacturer’s protocol. The plasmids were introduced into rice protoplasts according to the method described above. The expression of the fusion construct was monitored after 16 h of incubation in the dark, and images were captured with confocal laser-scanning microscope (TCS SP2, Leica, Germany).

**RNA isolation and RT-PCR**

The TRIZol reagent (Invitrogen, USA) was used according the manufacturer's instructions to extract total RNAs. Before reverse transcription, total RNA was treated with amplification-grade DNase I (Invitrogen, USA) for 15 min to degrade possibly contaminated residual genomic DNA. The cDNA templates were synthesized using Superscript II reverse transcriptase (Invitrogen, USA) according to the manufacturer’s instructions. Real-time quantitative RT-PCR was performed on a 7500 real-time PCR system (Applied Biosystems, USA) using SYBR Premix Ex Taq (TaKaRa, China) according to the manufacturer’s protocol. Rice Actin1 gene (accession no. AK060893) was used as the endogenous control. The relative expression levels were determined as described previously (Livak and Schmittgen, 2001). The gene-specific primers are listed in Table S1.
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