

Supplementary Materials for

Reconfiguring the wild-rice *RBA1* locus breaks the trade-off between chilling tolerance and blast resistance in rice

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Materials and Methods

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Other supplementary materials for this manuscript:

Supplementary Tables S1-S5 (Excel)

Materials and Methods

Plant Materials, Growth Conditions, and Evaluation of Yield-Related Traits

The rice line SL11 was developed by crossing the recurrent parent PL (*Oryza sativa ssp. indica*) with the donor parent GX2 (*Oryza rufipogon*, accession number YD2-0636), and selecting for homozygous GX2 alleles at the *RBA1* locus using marker-assisted selection. The near-isogenic line (NIL) PA64s^{GX2} was generated by introducing homozygous GX2 alleles into the PA64s background of *RBA1* through marker-assisted selection. LYP9^{GX2} represents the F₁ hybrid generation resulting from the cross between PA64s^{GX2} and 93-11.

For disease resistance assessment, 140 *indica* cultivars were cultivated in natural nursery fields in Hangzhou (120°E, 30°N). All agronomic trait evaluations were conducted in paddy fields in Shenzhen (114°E, 22°N) with a spacing of 20 × 20 cm between plants. Key agronomic traits, including spikelet number per plant, panicle number per plant, yield per plant, and thousand-grain weight, were measured for each line. Phenotypic measurements were taken from three independent lines, and statistical significance was assessed using a two-sided paired Student's *t*-test.

Evaluation of Chilling Tolerance

To evaluate chilling tolerance in the mapping population, each F₂ line was self-pollinated to generate F₃ seeds. To break dormancy, the F₃ seeds were treated at 43°C for one week. Chilling tolerance evaluations for LYP9 and LYP9^{GX2} were conducted using hybrid seeds generated from crosses between PA64s and 93-11, and between PA64s^{GX2} and 93-11, respectively.

For germination, seeds were soaked in water at 30°C for 3 days, then the germinated seeds were transferred to a greenhouse and grown in a 1/2 Kimura B nutrient solution at a day/night temperature of 28°C/25°C. Seedlings at the two-leaf stage for *indica* varieties and the three-leaf stage for *japonica* varieties were subjected to chilling treatment at 4°C ± 0.5°C for various durations. Specifically, GX2, mapping plants, and *indica* cultivars were exposed to chilling for 60 h, while the *japonica* variety ZH11 and its transgenic lines were treated for 96 h.

Following chilling treatment, seedlings were returned to the greenhouse conditions described above and allowed to recover for 5–10 d. Survival rates were calculated as the percentage of surviving seedlings relative to the total number of plants tested. All experiments were independently conducted in triplicate.

QTL Analysis and Map-Based Cloning

For the identification of quantitative trait loci (QTLs) associated with chilling tolerance, an F₂ population was generated by crossing PL with SL11, resulting in 94 individuals genotyped with 13 DNA markers on chromosome 11. QTL analysis was performed using the Composite Interval Mapping (CIM) method implemented in QTL IciMapping Version 4.2 with default parameters¹, which mapped *RBA1* to a 3.9-Mb interval between markers CT11-12 and CT11-9. Then, we examined the association between *RBA1* genotype and the phenotypic segregation ratio using markers CT11-12 and CT11-14.

Individuals homozygous for the GX2 allele at the *RBA1* locus were chilling tolerant (survival rate 60-90%), whereas heterozygous and PL homozygotes remained sensitive (< 30%) (Fig. S1h), indicating that chilling tolerance conferred by *RBA1* is recessively inherited. To refine the *RBA1* locus, we first performed preliminary mapping within a population of 1,928 F₂ plants, narrowing the candidate region to a 250-kb interval between markers CT11-12 and CT11-13. By further expanding the F₂ population to 14,256 individuals, we successfully narrowed the candidate region to a 21.2-kb segment between markers CT11-18 and CT11-20.

Ion Leakage Assay

Seedlings were subjected to chilling treatment at 4°C for 24 h, after which ion leakage was assessed with slight modifications to previously established protocols. Five fully expanded leaves from each seedling were cut into equal-sized segments, immersed in 8 mL of distilled water in a 10 mL tube, and the initial electrical conductivity (S₀) was measured immediately.

The tubes were then shaken at 100 rpm at room temperature for 1 h, and the electrical conductivity (S₁) was recorded. Following S₁ measurement, the tubes were placed in boiling water for 20 minutes, then allowed to cool naturally to room temperature. The tubes were again shaken at room temperature for 1 h, and the final conductivity (S₂) was measured. Relative ion leakage was calculated using the formula $(S_1 - S_0) / (S_2 - S_0)$.

Vector Construction and Transformation

Gene sequence was acquired from the Rice Genome Annotation Project (<https://rice.uga.edu/>). Transposable element and ORFs on the 25.7-kb fragment were analyzed using CENSOR tool (<https://www.girinst.org/censor/index.php>) and FGENESH 2.6 software, respectively. To knock out genes *ORF1*, *ORF2*, and *ORF3*, a 20-nucleotide guide sequence was selected as the target using the Target Design tool (<http://skl.scau.edu.cn/targetdesign/>). Oligonucleotides containing the target sequences were synthesized and ligated into a CRISPR-Cas9 vector. These constructs were then transformed into PL calli.

For complementation analysis, five overlapping genomic fragments (C1–C5) were amplified from PL genomic DNA using KOD FX Polymerase (Toyobo) and cloned into the pCAMBIA1300 vector (KpnI/BamHI sites) using the pEASY®-Basic Seamless Cloning and Assembly Kit (TransGen), following the manufacturer's instructions. To generate overexpressing transgenic lines, full-length complementary DNA (cDNA) of *ORF1*, *ORF2*, and *ORF3* was amplified from PL and ligated into the p1300-GFP vector (BamHI/PmlI sites) under the control of the Ubiquitin promoter and NOS terminator. Regarding the construction of the *cORF1* vector, the approximately 2.5-kb promoter region and the *ORF1* coding sequence were PCR-amplified from the PL and subsequently cloned into the p1300 vector. All primers used in this study are listed in Supplementary Table 5. Rice transformation was carried out via *Agrobacterium tumefaciens* (strain EHA105)-mediated transformation.

Histochemical Detection of H₂O₂ and O₂⁻

Seedlings were exposed to chilling stress or pathogen infection for 24 h and then

harvested for superoxide determination. Hydrogen peroxide (H₂O₂) and superoxide (O₂⁻) were visualized by staining with 3,3'-diaminobenzidine (DAB, Coolaber) and nitroblue tetrazolium (NBT, Coolaber), respectively. For DAB staining, leaves were vacuum-infiltrated in a solution containing 1 mg/mL DAB in 10 mM sodium acetate buffer (pH 5.5) and incubated in the dark at room temperature for 12 h. For NBT staining, leaves were immersed in 0.5 mg/mL NBT in 10 mM potassium phosphate buffer (pH 7.8) for 12 h in the dark at room temperature. The reactions were stopped by transferring the leaves to boiling 95% ethanol for 10 minutes, followed by soaking in 70% ethanol until chlorophyll was completely removed. The cleared leaves were then photographed.

Determination of Peroxidase (POD) Activity

Seedlings subjected to chilling stress or pathogen infestation for 24 h were harvested for POD activity measurement. Control plant enzyme activities were also measured from seedlings grown under normal conditions for 24 h. POD activity was assessed according to the Peroxidase Activity Assay Kit protocol (Solarbio). Absorbance values were recorded at 470 nm using a spectrophotometer (Eppendorf).

Pathogen Infection Assays

For field-based disease assessments of 140 *indica* cultivars, disease severity was scored using the 0–9 scale according to Bonman et al.². Plants with scores of 0–3 were classified as resistant, 4–5 as moderately susceptible, and 6–9 as susceptible.

For greenhouse disease assays, four-week-old plants were spray-inoculated with *Magnaporthe oryzae* (*M. oryzae*) spore suspensions (2×10^5 spores/mL with 0.02% Tween-20) in a dark chamber at 25°C with 90% humidity for 24 h. After inoculation, the plants were maintained at 28°C under a 12-h light/12-h dark cycle with 90% humidity for seven days.

For punch inoculation, leaf surfaces were wounded using a mouse ear punch, and 5 µL of *M. oryzae* spore solution (5×10^5 spores/mL) was applied to the wounded area with a transfer pipette. Leaves were placed in a culture dish containing 0.1% 6-Benzylaminopurine (6-BA) sterile water to maintain moisture for seven days. Lesion area percentages were quantified using ImageJ software (<http://rsbweb.nih.gov/ij/>). Relative fungal DNA levels were calculated through DNA-based quantitative PCR by comparing the threshold cycle (CT) of the *M. oryzae* *Pot2* transposon sequence against the CT of the rice ubiquitin gene (*LOC_Os03g13170*)³. Each inoculation experiment was conducted three times independently.

RNA Extraction and Quantitative RT-PCR

Total RNA was extracted using TRIzol reagent following the manufacturer's instructions (Invitrogen). For reverse transcription, 1 µg of total RNA was used with a ReverTra Ace kit (Toyobo). Quantitative real-time PCR (qRT-PCR) was performed using the SYBR Green Master Mix kit (Vazyme) on a CFX96 Optical Reaction Module (Bio-Rad). *OsACTIN1* was used as the internal reference gene. Gene-specific primers are listed in Supplementary Table 5. Each experiment was repeated in triplicate, with

similar results obtained.

Western Blot Analysis

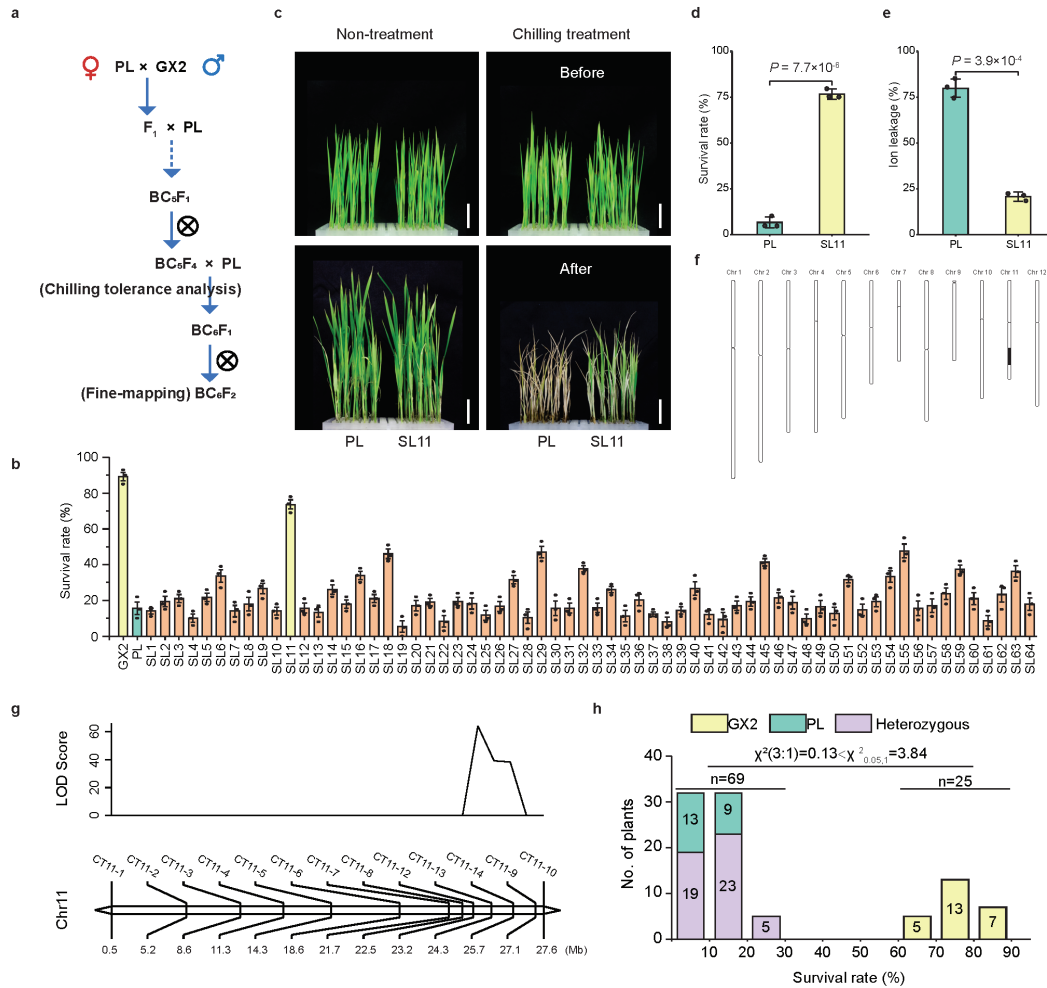
Rice leaves were ground into a fine powder in liquid nitrogen, and total proteins were extracted using a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 20% glycerin, 0.1% NP-40, 1 mM DTT, 1 mM PMSF, and a 1× protease inhibitor cocktail. Proteins were separated on 10% SDS-PAGE gels and transferred onto PVDF membranes (Millipore). Membranes were blocked in 5% nonfat dry milk in TBS with 0.1% Tween-20 for 2 h at room temperature. After blocking, membranes were incubated with an anti-GFP antibody (1:3000, Roche) for 1 h, followed by incubation with anti-mouse IgG-HRP (1:20000, Yeasen, 33201ES60).

Evolution and Haplotype Analysis of the *RBA1* Locus

High-quality rice genome sequences were obtained from the Rice Resource Center (<https://ricerc.sicau.edu.cn/>) and the Rice Super PIRdb (<http://www.ricesuperpir.com/>). Variations in the *RBA1* locus were analyzed using MUMmer4⁴. Haplotype of the *RBA1* locus in modern cultivated rice without genome data were identified based on PCR method. Details of all rice materials are provided in Supplementary Tables 1-3.

Statistical Analysis and Reproducibility

All data are presented as means ± s.d. The number of samples or replicates (n) is specified in figure legends. For pairwise comparisons, statistical significance was determined using a two-tailed Student's *t*-test in Excel 2021, with exact *P* values provided. For multiple-group comparisons, significance was assessed using One-way ANOVA and Tukey's HSD test in Origin 2024, with significant differences indicated by different letters.



Supplementary Fig. S1 Chilling-tolerant phenotype of parents and construction of NILs.

a, Strategy for constructing chromosome fragment substitution lines (CSSLs) using GX2 as donor and PL as recurrent parent. **b**, Chilling tolerance of GX2, PL and 64 individuals of CSSLs after 60 h at 4°C and 7 d recovery; values are means $\pm$ SD ($n = 3$ biological replicates). **c**, Chilling tolerance of SL11 and PL after chilling treatment. Scale bars, 3 cm. **d**, Survival rates of SL11 and PL as in **c**. **e**, Ion leakage of SL11 and PL after chilling treatment. **f**, Graphical genotype of SL11 (Black, GX2; white, PL). **g**, QTL mapping of chilling tolerance in the F₂ population. **h**, Genotypes and chilling phenotypes of 94 random F₂ individuals from SL11 $\times$ PL. The survival rate of F₂ individual was predicted by using F₃ seeds. Colors represent homozygous GX2/SL11 (yellow), homozygous PL (green), and heterozygous (purple). In **d** and **e**, values are means $\pm$ SD ($n = 3$ biological replicates); P values were calculated by two-sided Student's t -test.



Supplementary Fig. S2 Genomic structure and protein sequence of *ORF1*.

a, Genomic sequence of *ORF1*. Exon sequences are highlighted in yellow. Start and stop codons are red. **b**, Protein sequence of *ORF1*. Protein domains predicted using the CDD tool (<https://www.ncbi.nlm.nih.gov/cdd/>) are indicated with color letters. Blue, CC domain; Orange, NB-ARC domain; Green, LRR domain.

a *ORF2*
 ATGGAGACCGCCTGGGTCTGGTGGACATTGCCGTGAAGCTCTTCAACCTTCTCGATGAGAGCTACGAGCAGGGGAAGGGGCTCGCCGCCGGC
 ATCGACTTCATCAGGAGGGAGCTCCGGTCCATCAAGGAAGCCATCAGCATGCGCTCCGGGACGGCGGATCAGGAGGGGATCCGGAGCTGGAT
 GGCTGACCTGAGGGATATATGCATGAAGATCGAGAAGCTGCGTCGACCTCTACAACCTGAGGGTCGTCGTCGCGCAAGCACAGGAAGAGCC
 AGGGGTCGTCCGCGACGCCATCCACCATCTAACCTCAGGACAACGCTTGCCCGCGGCAAGCTCTCCGAGGACATCGAGGCGATCAGGAAGC
 TCGTCGAGCAAGCTAATCCAGGGTCACCGCGTATCATCCCAAGCTCAGCAAGCCACCATCCACAAGAACAGCAGCACTGTCAACAATGGAG
 CCACCCGCCATGCCAGTGAGGCTCAGGCTGACACCCCAAGCGGAGCTGTCAGGCTGCTGTCTCAGGGCGACGGCAAGGCG
 AAGCAGCTCAGGGTATCGCCATTGATGGGCTGGTGGATCCGGGAAGACTGTTCTTGCTAAGGTGTACAGGCAAGTCAACCGAGAGTTC
 GATTGCTGGGCTGGGTGTACGCTCGACGACGTCGATGAACCTCTAGCCGACATACTTCGGAATTACGCGAGCAGATACCGCAGTCATCGA
 AAGATCTGGGCGGCGTTTGAGAGATTTCTGGAGAATAAGAGGTGA

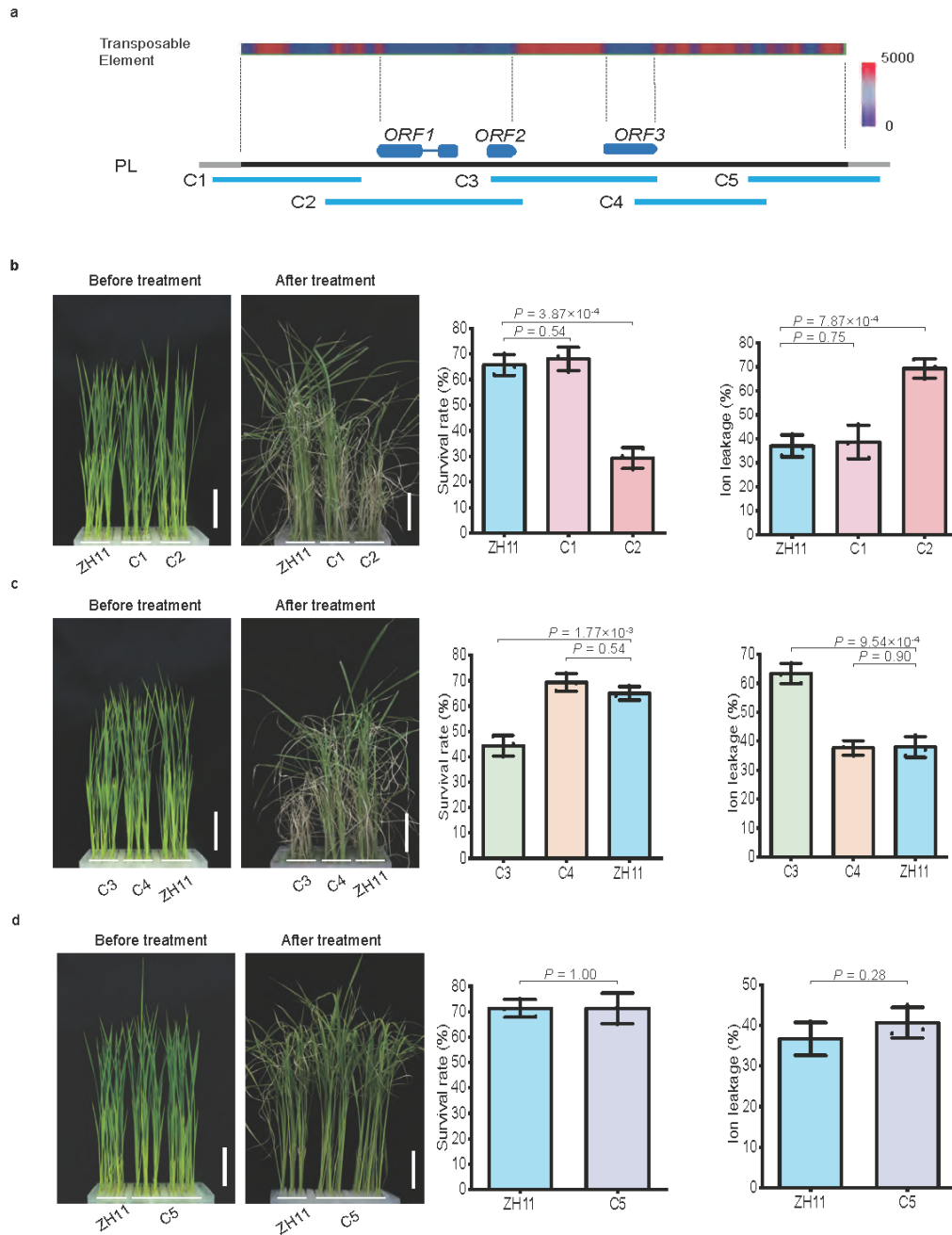
b METALGLVDIAVKLFNLLDESIEQKGLAAGIDFIRRELRSIKEAISMRSGTADQEGIRSWMADLRDICMKIENCVDLYNLRVVVGKHKRKPVGVRDAIH
 HLTLRITTLARGKLSIEDIEAIRKLVEQANSRVTAHYHPQAQQTIIHKNSSTVNNGATRHASEAHPDGLDTPKAELVRLLSQGDGKAKQLRVIAIDGPGGSGK
 TVLAKVSYEQVNRFECDWAWVSASDDVDLADILREFSEQIPQSSKDLGRRLRDFLENKR

c *ORF3*
 ATGAGTAGCAGGATCATTTGTGACCACAACAATAGCAAATAGCAAGGAAGTCCACCTGAATTGCCGGGTTGAGAAGATGCAGCCACTCGAC
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 ATGGCATGCTTGTGCTCCCTAAGTATGTATCACAAGGACAATGTTTTAGGAGGAAAAGCCTGATCCGGAGATGGTTGGCTGAAAGGGTAATCA
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 CAATGGACAGGTGAAGAGGTTTCGAGTTCACCGAATAATGCTCGAGTTCATCATAAACAAGGCGCACTCTGAGAATTATGTCAATTGGATCAGT
 ATCCATGAAGATGGCATTATGGGAGACTCGGGACATGGGAGAAATATCAATCGGCTCATTCTCCATAATAACAGCTCAAGACCTCCCAAGATAG
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 CGGAATATACCTCTTCTGTTCTGTGTCGATCGAATTCGGGTCTTCAGGTAACCTGGATTTCCTGGATAAGCTGGAAGTCCCATTCCTCATGTTCTCTC
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 AGGGTTTGTCTGCGGAGTGAGGGGTTGCTGAACCTGAAGCGCTGTGCTTGTGCTGCTGCGGAGCTGCCAAGATTGTATCCAGCACAAGGC
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 GCCTCCAGCTTCTGTTGAGTGCAGGAGGATTGAAGCGGCAAGTCAAGGCGCATCCCGTGAAGCAAAATTCGTGGAGAAACCAACCC
 GTCGCCACCGGCATCTAGCGGGAGCTGACGAGAAGCATAAAGATTCAAGCACCGACCAAGCTGATGA

d MSSRIIVTTTNEQIARNCHLNCRVQKMQPLDQLSARALLTRKAFGEDGCPIDLEESLDMILNTCECLPLAIVNMADHIKGRKLRHSQCQVHACENLRFLL
 DAKNEAFLGMHQVLDRLSYNSLNNHMACLLSLTMYHKDINVFRKSLIRRWLAERVIVTDGQSEEDVANQCFTDLVDHNIILPTDVSINGQVQRFRVH
 RIMLEFIINKAHSENYYNWSIHEDGIMGDSGHGENINRLIHNSSRPPKIGERFDLSCVRSITCFGLASKSLMYFKDYTLRLSLDLEGCSNLHDEHLETLC
 KSSMLRYSIRGNVGVSRIPKMFKLHLETLDTRGTSVRTLPMEIQLPELTNLGLFQITSSSSMRFLSQKICRLQTLAGFFVDESPNFVKALPRMPKLN
 KVKISCRQTAAPSNEVITDLLISLKECFKDLSPRNIPLRSLIDFGSSGNLDFLDKLEVPFPCSLQSLKLRGQLTRLPKFIESANLKLCLSGSNLWEALSRLQ
 CFKSLLYLKLTEQEPAAFLGAEGFVWRSGGFVNLKRLCLVVAELPKVIMQHKAMPGLSLQLCFRLGSISIDHLLRLEEVVASSFRGVAEDLRQVEA
 HPVPRKPFVEKPHSPASRRELRSRIQSTDHERD

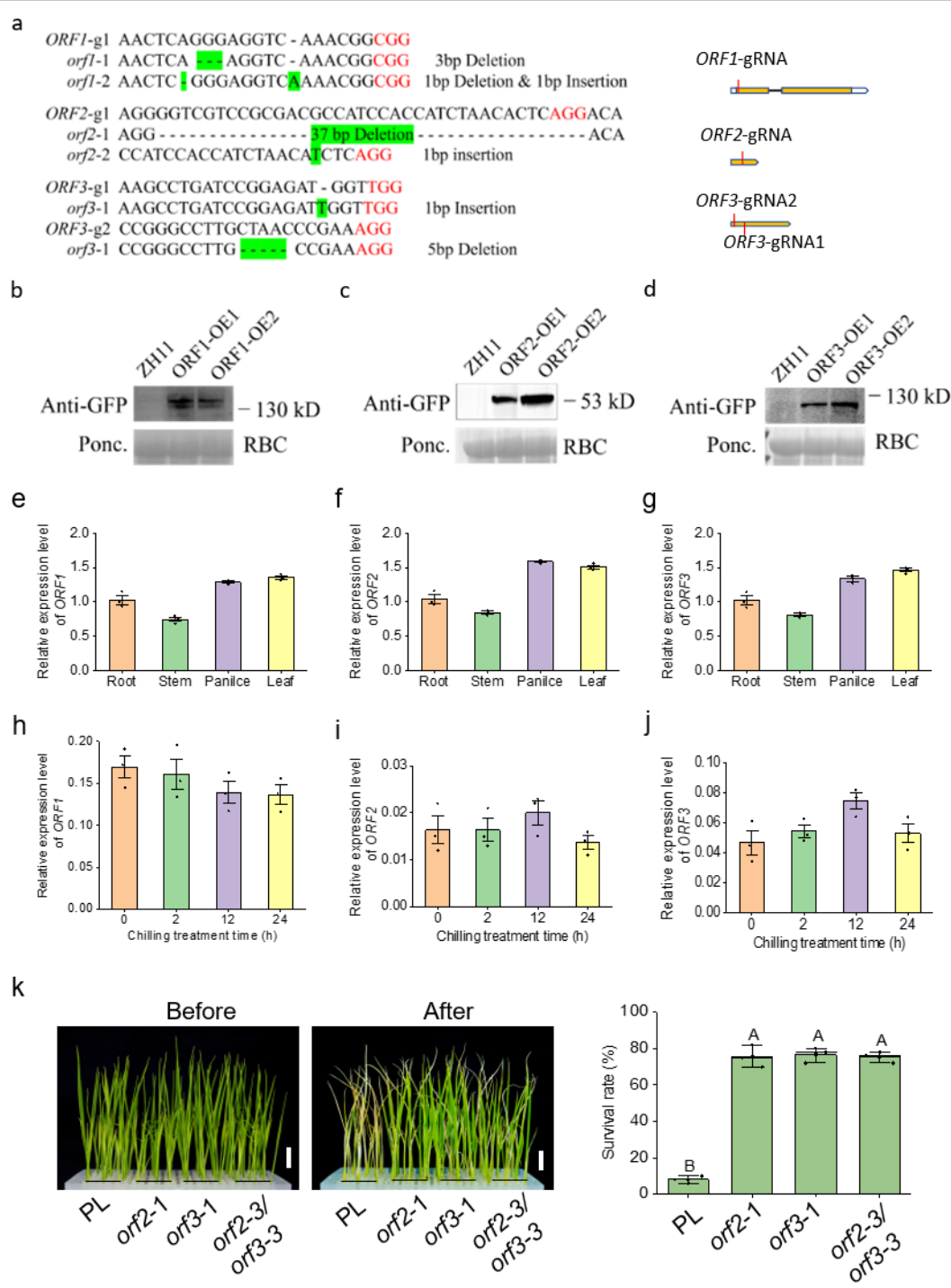
Supplementary Fig. S3 Coding sequences and protein sequences of *ORF2* and *ORF3*.

a, Coding sequence of *ORF2*. Start and stop codons are red. **b**, Protein sequence of *ORF2*. **c**, Coding sequence of *ORF3*. Start and stop codons are red. **d**, Protein sequence of *ORF3*. Protein domains predicted using the CDD tool are indicated with color letters. Blue, CC domain; Orange, NB-ARC domain; Green, LRR domain.



Supplementary Fig. S4 Chilling tolerance of the complementation lines in ZH11 background.

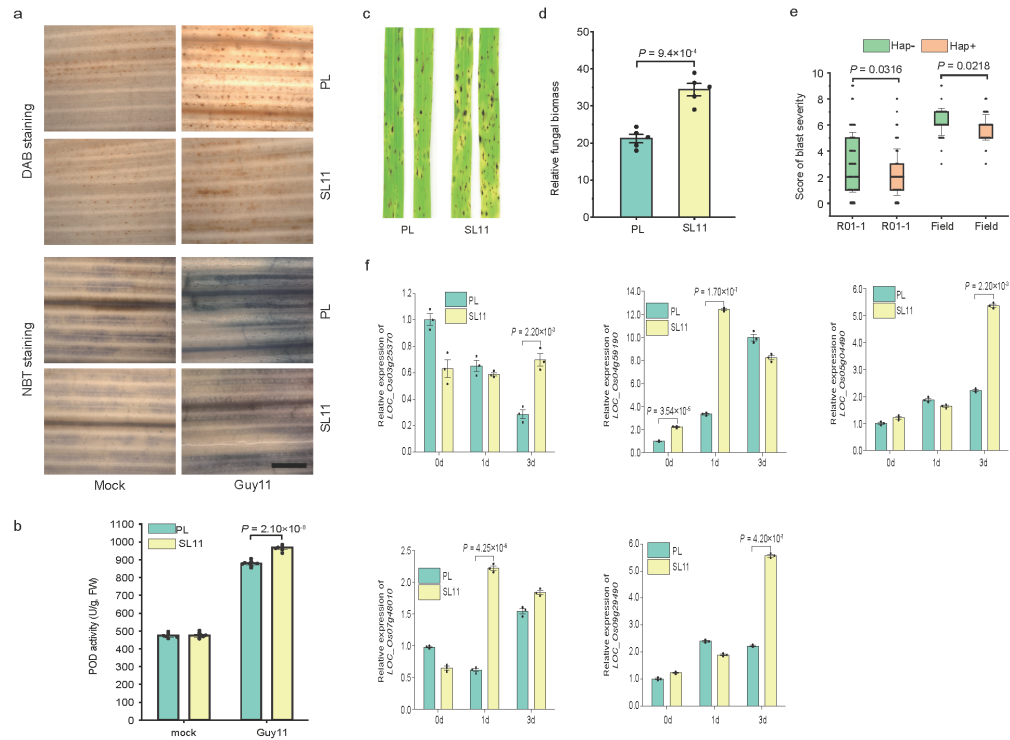
a, Transposable element analysis and ORF prediction of the 25.7-kb insertion in PL, showing ORFs (*ORF1*–*3*) and five fragments (C1–C5) used for transformation. The black line presents the 25.7-kb insertion in PL compared to GX2. **b–d**, Chilling tolerance of complementation lines C1–C5 and ZH11 after 96 h at 4°C and 10 d recovery (left), survival rates (middle), and ion leakage after 24 h at 4°C (right). Scale bars, 3 cm. Values are means $\pm$ SD ($n = 3$ biological replicates); P values were calculated by two-sided Student's t -test.



Supplementary Fig. S5 Identification and expression analysis of *ORF* transgenic lines.

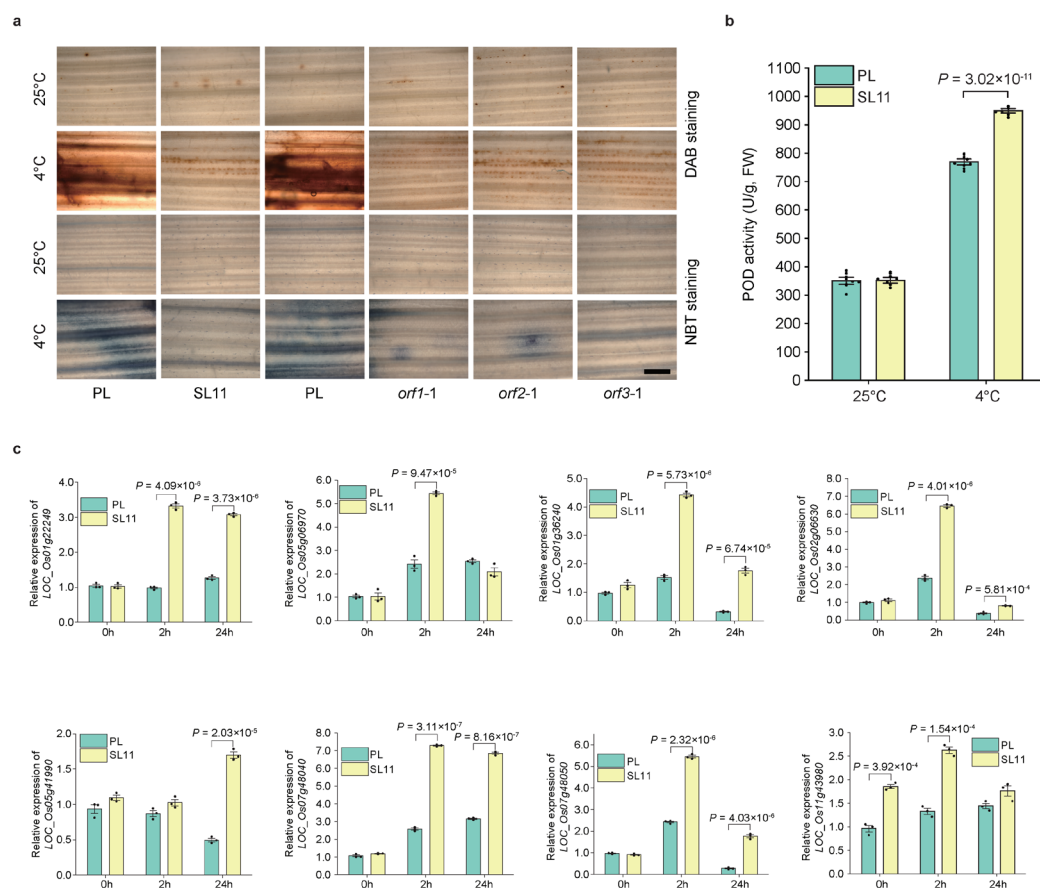
a, Genotypes of *orf1*, *orf2* and *orf3* mutants in PL background. **b–d**, Immunoblot confirmation of *ORF1*-, *ORF2*- and *ORF3*-overexpression lines in ZH11 using anti-GFP antibodies; RuBisCo (RBC) stained with Ponceau S (Ponc.) as loading control. **e–g**, Expression patterns of *ORF1*, *ORF2* and *ORF3* in different tissues of PL. **h–j**, Expression levels of *ORF1*, *ORF2* and *ORF3* in PL under 4°C treatment at different

time points. **k**, Chilling tolerances of PL and *orf2-3/orf3-3* seedlings after 60 h at 4°C followed by 7 d of recovery. One-way ANOVA followed by Tukey's HSD test. Different letters mean $P < 0.01$. Scale bars, 2 cm. In **e–k**, values are means $\pm$ SD ($n = 3$ biological replicates).

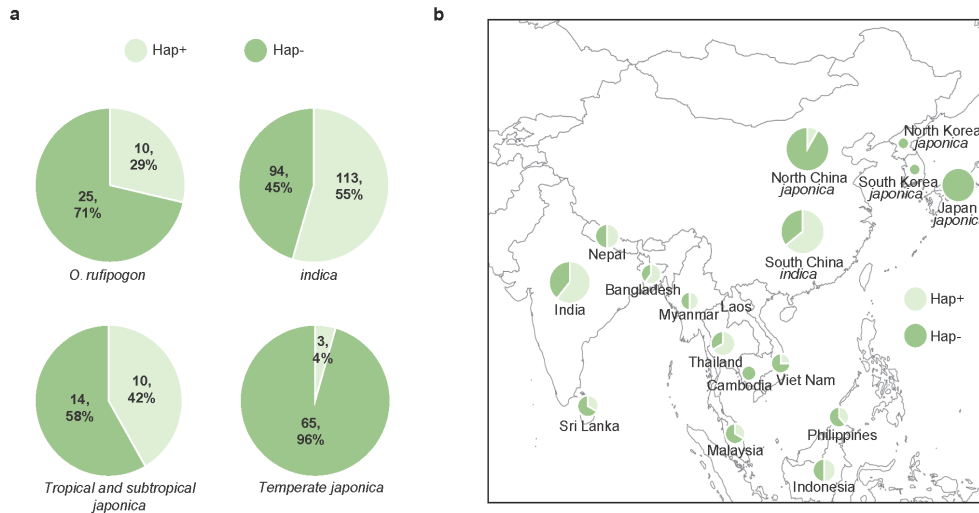


Supplementary Fig. S6 *RBA1* enhances blast resistance through ROS accumulation.

a, NBT and DAB staining of PL and SL11 leaves after spray inoculation with or without the *M. oryzae* isolate Guy11. Scale bar, 0.5 mm. **b**, Peroxidase (POD) activity of PL and SL11 leaves 1 d post-inoculation. **c**, Blast resistance of PL and SL11 plants 7 d post-inoculation. **d**, Relative fungal biomass of samples as in **c**, $n = 5$ biological replicates. **e**, Blast severity levels of 140 *indica* accessions in greenhouse and natural nursery conditions. Hap⁻ indicates absence and Hap⁺ presence of the 25.7-kb insertion. **f**, Relative expression of five *POD* genes in PL and SL11 leaves at different time points post spray inoculation with the *M. oryzae* isolate Guy11. In **b**, **d** and **f**, values are means $\pm$ SD ($n = 9$ (b), 5 (d) or 3 (f) biological replicates); P values were calculated by two-sided Student's *t*-test.

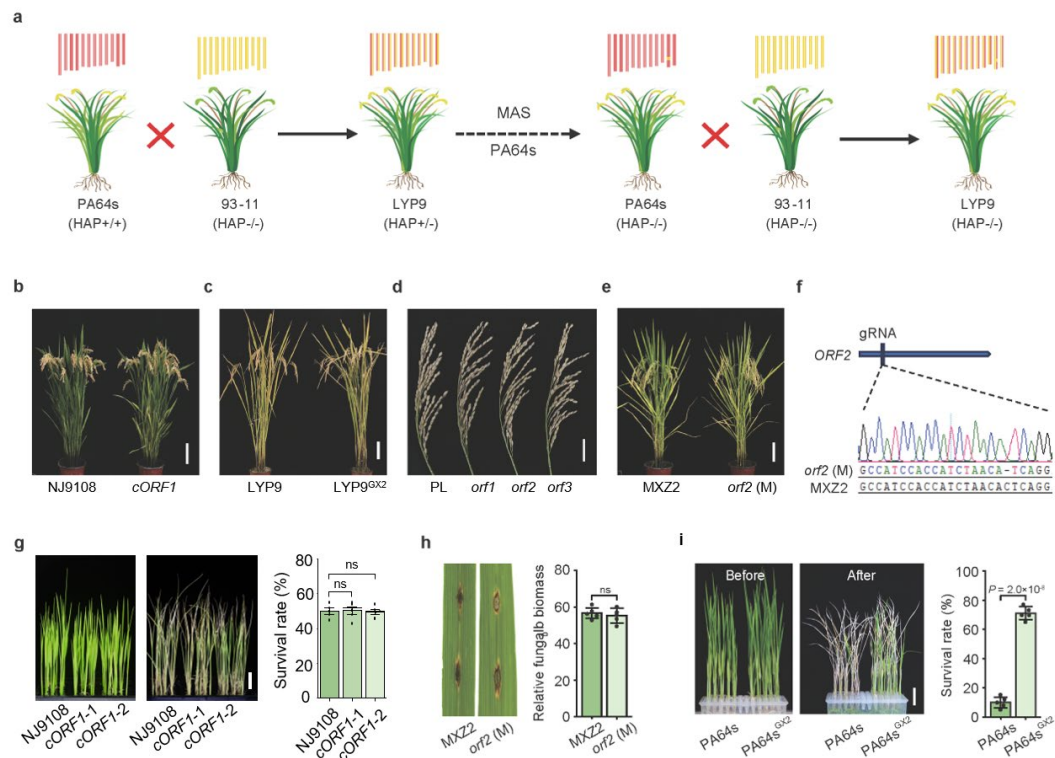


Supplementary Fig. S7 *RBA1^{PL}* promotes ROS accumulation under chilling stress.
a, NBT and DAB staining of seedling leaves grown at 25°C and 24 h at 4°C. Scale bar, 0.5 mm. **b**, POD activity of PL and SL11 at 25°C and after 24 h at 4°C. **c**, Expression of eight *POD* genes in PL and SL11 under 4°C at different time points. Values are means $\pm$ SD ($n = 9$ (b) or 3 (c) biological replicates); P values were calculated by two-sided Student's t -test.



Supplementary Fig. S8 Genetic variation and geographic distribution of *RBA1*.

a, Frequency of the PAVs of *RBA1* in *O. rufipogon*, tropical japonica, temperate japonica, and indica. Details of related materials are provided in Supplementary Tables 1 and 2. **b**, Geographic distribution of *RBA1* haplotypes in Asian rice cultivars. Credits for panel b: background tiles from Stamen Design under a Creative Commons CC BY 3.0 license, map plotted using ggmap and data from an Open Data Commons Open Database License (ODbL). PAVs of *RBA1* are denoted as Hap- representing the absence of the 25.7-kb insertion and Hap+ representing the presence of the 25.7-kb insertion. Details of related materials are provided in Supplementary Table 3.



Supplementary Fig. S9 Phenotypes of plants with different alleles of *RBA1*.

a, Breeding scheme for improving the *RBA1* locus in the PA64s sterile line and LYP9 hybrid rice. **b**, Phenotypes of mature NJ9108 and *cORF1* plants. Scale bars, 15 cm. **c**, Phenotypes of mature LYP9 and LYP9^{GX2} plants. Scale bars, 15 cm. **d**, Mature panicles of *orf1*, *orf2*, *orf3* and PL plants. Scale bars, 3 cm. **e**, Phenotypes of mature MXZ2 and *orf2*(M) plants. Scale bars, 15 cm. **f**, Genotypes of *orf2*(M) mutant in MXZ2 background. **g**, Chilling tolerance of NJ9108, *cORF1*-1 and *cORF1*-2 seedlings after 84 h at 4°C and 7 d recovery. Scale bars, 3 cm. **h**, Blast resistance of MXZ2 and *orf2*(M) plants 7 d post punch inoculation with Guy11. **i**, Chilling tolerance of PA64s and PA64s^{GX2} seedlings after 60 h at 4°C and 5 d recovery. Scale bars, 3 cm. In **g**–**i**, values are means $\pm$ SD ($n = 5$ biological replicates); P values were calculated by two-sided Student's t -test; ns, no significant difference.

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4. Marçais, G. et al. *PLOS Computational Biology* **14**, e1005944 (2018).