Two rice receptor-like kinases maintain male fertility under changing temperatures

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Plants employ dynamic molecular networks to control development in response to environmental changes, yet the underlying mechanisms are largely unknown. Here we report the identification of two rice leucine-rich repeat receptor-like kinases, Thermosensitive Sensitive Genic Male Sterile 10 (TMS10) and its close homolog TMS10L, which redundantly function in the maintenance of the tapetal cell layer and microspore/pollen viability under normal temperature conditions with TMS10 playing an essential role in higher temperatures (namely, 28 °C). tms10 displays male sterility under high temperatures but male fertility under low temperatures, and the tms10 tms10L double mutant shows complete male sterility under both high and low temperatures. Biochemical and genetic assays indicate that the kinase activity conferred by the intracellular domain of TMS10 is essential for tapetal degeneration and male fertility under high temperatures. Furthermore, indica or japonica rice varieties that contain mutations in TMS10, created by genetic crosses or genome editing, also exhibit thermo-sensitive genic male sterility (TGMS) traits that have been used in crop hybrid seed production systems in crops.

Significance

By affecting male fertility in crops, climate temperature change has a major impact on global food security. Here we show the role of two rice leucine-rich repeat-receptor-like kinases, TMS10 and TMS10L, which redundantly control male fertility under fluctuating temperatures. This finding provides insights into how plants overcome adversary temperature changes to achieve normal male fertility and a new genetic resource for crop hybrid seed production.

Author contributions: W.L. and D.Z. designed research; J.Y., J. Han, and Z.Y. performed data; and J.Y., Y.-J.K., J. Hu, and D.Z. wrote the paper.

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in determining the identity and number of tapetal cells and meioocytes (17, 20, 21).

The tapetum is a somatic helper-cell layer next to the microsporocytes that secretes enzymes to help with the release of microspores from tetrads and provides nutrients for pollen development (22). After meiosis, tapetal cells initiate programmed cell death (PCD)-driven degeneration, which is required for pollen grain maturation, as abnormal tapetal enlargement and premature or delayed degradation of tapetal cells frequently result in male sterility (23). In this study, we report the discovery of two LRR–RLKs, TMS10 and TMS10L, which can redundantly operate in tapetal development, with TMS10 specifically preserving this role in both tapetal degeneration and pollen wall formation. TMS10 plays male sterility under high temperatures (23), whereas TMS10L is sensitive to temperature changes but does not have a specific role in male fertility (24).

**Results**

**tms10 Is a Temperature-Sensitive Male Sterile Mutant.** To identify regulators of rice male fertility during changing temperatures, we isolated a 60Co γ-ray radiation-mutagenized TGMS mutant in ssp. japonica cv. 9522 for its complete male sterility at restrictive temperatures (24). Under high temperatures (25–32 °C) in the paddy field during summer in Shanghai, tms10 and wild type (WT) exhibited normal vegetative growth and flower development (SI Appendix, Fig. S1 A–E). However, tms10 had white and smaller anthers that do not produce viable pollen grains, as revealed by I2–KI staining (Fig. 1).

**TMS10 Is Required for Tapetum Development and Microspore Formation.** Transverse section analysis demonstrated that, at an average temperature of 28 °C, tms10 had enlarged tapetal cells and displayed aborted pollen development at stage 9 of anther development, after the microspores were released from the tetrads (Fig. 1 G–I). This suggested that the deficiency caused by the tms10 mutation occurs at the postmeiotic stage, a finding that differs from previously reported TGMS mutants such as tm5 and PA64S that showed defects during the formation of microspore mother cells (MMC) (9, 11). In support of this, 4',6-diamidino-2-phenylindole staining for the observation of chromosome behaviors confirmed that tms10 had a normal meiotic process under 28 °C, as evidenced by the normal formation of tetrads that each contained four microspores (SI Appendix, Fig. S1J).

Transmission electron microscopy (TEM) showed that, at stages 8b and 9, WT anthers formed tetrads, released the free microspores, and had a condensed and degenerated tapetal layer with abundant lipidic granules (Ubisch bodies) attached to their inner surface (Fig. 1 J and SI Appendix, Fig. S2 A, C, E, G, I, K, M, and O). In contrast, tapetal cells in tms10 exhibited no obvious degeneration and became highly vacuolated, containing no visible Ubisch bodies and showing a multinuclei cell cluster pattern at stages 8b and 9 under high temperature (Fig. 1 K and L and SI Appendix, Fig. S2 D, H, L, and P).

At these stages, microspores of tms10 seemed to develop normally, but lacked primexine, a structure of the template for pollen wall formation (SI Appendix, Fig. S2 B, F, J, and N). Further analysis using scanning electron microscope revealed that, under the average temperature of 28 °C, tms10 had less wax and cutin deposition on anther epidermis, fewer Ubisch bodies on the inner surface of tapetal cells, and less accumulation of sporopollenin on the surface of microspores compared with the WT (SI Appendix, Fig. S2Q).

**TMS10 Encodes an LRR–RLK Highly Expressed in the Anther.** To clone the TMS10 gene, we collected 281 male sterile individuals from the F3 mapping population by crossing the mutant with *O. sativa* ssp. indica cv. 9311. Map-based cloning revealed that the tms10 locus was located within three bacterial artificial chromosome clones on chromosome 2 (Fig. 2A). Sequence analysis revealed a 7-bp deletion in the fourth exon (Fig. 2B) of a putative LRR–RLK
conducted to analyze the spatiotemporal expression pattern of TMS10 and showed that TMS10 is expressed mainly in the early stages of anther and leaf development (SI Appendix, Fig. S3A). In situ hybridization analysis revealed TMS10 expression in almost all layers of the anther wall and MMs at stage 5. At stages 7 and 9, the transcripts of TMS10 were detectable in anther wall layers and MMs/microspores and conjunction tissues, with particularly strong signals in tapetal cells (SI Appendix, Fig. S4F). Consistently, in tms10 lines complemented by pTMS10::TMS10gDNA-GFP also revealed TMS10 expression in both anther sac and conjunction tissues, but not in meiotic cells or microspores (Fig. 2D and SI Appendix, Fig. S4G). Inconsistent results in TMS10 transcripts were detected in MMs/microspores by in situ assay, but the TMS10-GFP fused protein was undetectable in MMs/microspores, which indicated that there might be some posttranscriptional regulation to inhibit TMS10 protein formation in MMCs/microspores.

Reverse transcription–quantitative PCR (RT-qPCR) was conducted to analyze the spatiotemporal expression pattern of TMS10 and showed that TMS10 is expressed mainly in the early stages of anther and leaf development (SI Appendix, Fig. S3A). In situ hybridization analysis revealed TMS10 expression in almost all layers of the anther wall and MMs at stage 5. At stages 7 and 9, the transcripts of TMS10 were detectable in anther wall layers and MMs/microspores and conjunction tissues, with particularly strong signals in tapetal cells (SI Appendix, Fig. S4F). Consistently, in tms10 lines complemented by pTMS10::TMS10gDNA-GFP also revealed TMS10 expression in both anther sac and conjunction tissues, but not in meiotic cells or microspores (Fig. 2D and SI Appendix, Fig. S4G). Inconsistent results in TMS10 transcripts were detected in MMs/microspores by in situ assay, but the TMS10-GFP fused protein was undetectable in MMs/microspores, which indicated that there might be some posttranscriptional regulation to inhibit TMS10 protein formation in MMCs/microspores.

To understand the evolution of TMS10 in plants, we performed a phylogenetic analysis using the full-length TMS10 protein sequence and 43 homologous sequences retrieved from 10 plant species from public databases. TMS10 was found in a clade that contained both dicot and monocot proteins with no reported functions (26) (Fig. 2A). The phylogeny (Fig. 2B) included its 3,031-bp native promoter, 5,804-bp 5′-untranslated region (UTR), and lines between boxes are introns. Red arrow indicates the mutation site in tms10.

Asterisk, stop codon. (LOC_Os02g18320, annotated in www.granme.org/), which caused a frame shift that resulted in the pretermination of protein translation (Fig. 2C). To confirm the identity of this gene, we transformed the full-length genomic DNA of the putative TMS10 gene—which included its 3,031-bp native promoter, 5,804-bp coding region, and 400-bp 3′-untranslated region (UTR) (pTMS10::TMS10gDNA)—into the tms10 mutant for complementation. All 50 transgenic plants that expressed the putative TMS10 gene showed rescue of the mutant phenotype at 28 °C (Fig. 3A), confirming that the mutation in tms10 is responsible for the male sterile phenotype in tms10.
Fig. 3. TMS10 has in vitro kinase activity required for anther fertility. (A) TMS10 protein structure. Green, blue, and purple boxes represent LRR, transmembrane (TM), and kinase (K) domains, respectively. KC, kinase domain plus C terminus (amino acids 284–607). JKC, intracellular domain (amino acids 241–607) including the juxtamembrane (J), kinase (K), and C-terminal domains (C). mJKC, kinase-dead intracellular domain (amino acids 241–607 with K312E). (B and C) In vitro kinase assays of TMS10. Derivatives (Left) and Coomasie Brilliant Blue staining (Right) in B; derivatives (Top) and Coomasie Brilliant Blue staining (Bottom) in C. MBP, myelin basic protein. BR1-phosphorylated MBP used as the positive control. (D–F) Complementary of tms10 at 28 °C. Compared with rescued anther fertility by TMS10 (pTMS10::TMS10gDNA or TMS10-9×myc (pTMS10::TMS10gDNA-9×myc) in tms10 (D, E, G, and H), mTMS10-9×myc (pTMS10::TMS10gDNAK312E-9×myc) in tms10 failed to rescue male fertility (F and I). (Scale bars, 1 mm in D–F; 200 μm in G–I.) (J) Immunoblot analysis to qualitatively show the expression level of TMS10-9×myc and mTMS10-9×myc in tms10 in plants containing the pTMS10::TMS10gDNA-9×myc and pTMS10::TMS10gDNAK312E-9×myc transgenes growing under 28 °C.

TMS10 Is Localized to the Plasma Membrane and Has Kinase Activity. TMS10 encodes a 607-aa LRR–RLK (34), which has four LRRs in its extracellular region, a single transmembrane domain, and a cytoplasmic region that contains the juxtamembrane, kinase, and C-terminal domains (Fig. 3A). TMS10 is localized mainly to the plasma membrane, as demonstrated by confocal microscopic analysis of stably or transiently expressed TMS10-GFP fusion protein in rice anther, rice protoplasts, tobacco leaves, and onion epidermis cells (Fig. 2D and SI Appendix, Fig. S3 B and C). Furthermore, we compared the localization of transiently expressed TMS10-GFP in tobacco leaves under 22 °C and 28 °C and confirmed that the protein’s plasma membrane localization was not affected by temperature changes (SI Appendix, Fig. S4B).

To determine whether TMS10 has kinase activity, we used recombinant proteins that contained the TMS10 kinase domain (K), kinase with C-terminal domain (KC), and cytoplasmic region (JKC) to conduct in vitro kinase assays. JKC can phosphorylate a universal substrate, myelin basic protein (MBP), and this kinase activity is dependent on TMS10’s juxtamembrane domain (Fig. 3A–C). Sequence alignment showed that the kinase domain is highly conserved in TMS10 and its homologs in rice (TMS10L) and Arabidopsis (SI Appendix, Fig. S3A). Mutation of K312 (JKCK312E, mJKC), a conserved residue at the ATP-binding site, abolished the phosphorylation activity of TMS10 in vitro (Fig. 3A and B), demonstrating the critical role of this conserved residue in TMS10’s kinase activity. To test the biological significance of K312 in TMS10, mTMS10-9×myc (pTMS10::TMS10gDNAK312E-9×myc) was introduced into tms10. TMS10-9×myc (pTMS10::TMS10gDNA-9×myc) can rescue anther fertility in tms10 under high temperature (Fig. 3 D and F). However, mTMS10-9×myc failed to rescue tms10’s male sterility under high temperature in all 35 transgenic lines (Fig. 3D and F), suggesting that

the kinase activity of TMS10 is required for controlling tapetal function and pollen formation under high temperatures. Mass spectrometry (MS) analysis of bacterially expressed recombinant JKC and mJKC revealed that, of the three putative phosphorylation sites, T392, T576, Y458, and S646 are auto-phosphorylated in recombinant JKC but not in mJKC (SI Appendix, Table S2), supporting the auto-phosphorylation of TMS10. These results suggest that K312 and the juxtamembrane domain of TMS10 are essential for its kinase activity and that T392, T576, Y458, and S646 are the potential phosphorylation sites for TMS10.

TMS10L and TMS10 Play Redundant Roles in Anther Development Under Low Temperature. The rice genome has a close homolog of TMS10 within the TMS10 clade (TMS10L, LOC_Os03g49620, Fig. 2E), which shares a similar domain structure and 88% amino acid sequence identity with TMS10. TMS10L has a predicted extracellular domain with four LRRs, one transmembrane domain, and an intracellular kinase domain that also contains the conserved ATP activation loop and especially the conserved K310 function of TMS10L, we used the CRISPR-Cas9 system to create two tms10L alleles that contain mutations within the second exon: tms10L-1 has a 17-bp deletion that causes a pretranslational termination in the LRR domain, and tms10L-2 has an 18-bp deletion that causes a 6-aa deletion in the LRR domain (Fig. 4A). The double mutants tms10 tms10L-1 and tms10 tms10L-2 were later generated by genetic crosses.

Both tms10L-1 and tms10L-2 showed normal fertility under high and low temperatures (Fig. 4 B and C). However, tms10 tms10L-1 and tms10 tms10L-2 double mutants were male sterile under both high and low temperatures (Fig. 4 B and C). Semithin section
discuss the TGMS trait in indica and japonica subspecies.

Discussion

Rice is cultivated in many countries. However, temperature has been a limiting factor in achieving high yield. Global climate warming generally has negative effects on the length of both vegetative and reproductive growth periods and on rice yield. Pollen formation is highly sensitive to temperature stress during flowering, which therefore poses a serious threat to current and long-term crop yields (3, 39, 40). Our work identified two homologous LRR-RLKs, TMS10 and TMS10L, which appear to redundantly buffer the adverse effect of fluctuating temperatures on rice male development. TMS10 (but not TMS10L) is essential to the maintenance of normal male fertility under higher temperatures (i.e., 28 °C), while TMS10L together with TMS10 may play an important role in male fertility control under lower temperatures. These findings provide an understanding of how plants develop male organs via genotype-environment temperature interactions.

The use of hybrid plants has significantly transformed agricultural practices and seed production in the past decades (12, 38). In the efforts to increase crop yield, male sterility has played a central role in cytoplasmic male sterility (CMS) and photoperiod-sensitive/thermosensitive genic male sterile (P/TGMS) in hybrid breeding programs (12, 38). Compared with traditional CMS-based three-line hybrid breeding, two-line hybrid breeding is based on nuclear encoded genes, thus widening the choice of restorer lines in producing F1 hybrids. Discovering new resources and mechanisms will provide additional tools to implement P/TGMS traits in hybrid seed production. To date, nine types of TGMS lines have been isolated, several of which have been widely used in two-line hybrid breeding (24). For example, PA64S was shown to be caused by a nucleotide mutation in a 21-nt small RNA osa-smR5864w (9). The TGMS line tms5 is controlled by a mutation in RNase Z3 that is involved in UbA40 mRNA degradation (11). In this study, we identified tms10 mutation(s) as TGMS alleles and discovered that TMS10 encodes a LRR-RLK with its kinase activity essential for anther development and fertility under high environmental temperatures. Anther phenotypes start to show in tms10 after meiosis, and therefore at a stage later than that in tm5 (11), PA64S (9), or UGPase-suppressed lines (41). TMS10 regulates tapetal degeneration and pollen development, which is different from previously reported LRR-RLKs that function in male reproduction, all of which are involved in tapetal cell identity specification during anther development (25, 31, 32).

Our results suggested that, through its kinase activity, TMS10 plays a major role in tapetal cell degeneration and pollen...
formation under high temperatures. Due to functional redundancy, LRR–RLKs coordinately participate in many developmental processes. For example, AtSERK1 and AtSERK2 are functionally redundant in regulating early tapetal-layer formation in Arabidopsis (32, 33), and their extracellular LRR domain has specific functions that cannot be replaced by that of AtSERK3 although the kinase domain of these three SERKs are interchangeable (42). The observation that under low temperatures tms10 tms10 functions redundantly with TMS10 under low temperature during anther development and pollen formation. LRR–RLKs normally dimerize to transphosphorylate (18, 35). However, TMS10 and TMS10L do not interact directly in our assays, so whether and how they function together to regulate anther development under low temperature remains to be elucidated. In the BR signal transduction cascade, BR signal is perceived by a heterodimer of two LRR–RLKs, BR-INSSENSITIVE1 (BRI1) that has 25 LRRs and its coreceptor BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1) that contains 5 LRRs (36, 43). It will be exciting to reveal the partner(s) of TMS10 and/or TMS10L, which could be unknown LRR–RLKs with longer LRRs and capable of forming a heterodimer with TMS10 and/or TMS10L to transphosphorylate and magnify the signals in promoting tapetal PCD and normal pollen development. Identifying components of the TMS10- and TMS10L-mediated signaling pathway in the thermosensitive control of rice male fertility will uncover how LRR–RLKs buffer the adverse effect of changing temperatures on male developmental programs in plants. Finally, the finding that tms10 confers the TGMS trait in both japonica and indica cultivars can help to widen the genetic resources for manipulating male fertility, a key factor in hybrid seed production.

Materials and Methods

Details of materials and methods are provided by SI Appendix, SI Materials and Methods, including plant materials and growth conditions, plasmid construction and transformation, phenotypic analysis of anther and GUS staining, phylogenetic analysis, RT-qPCR analysis, in situ hybridization, yeast two-hybrid (Y2H) assays, subcellular localization, protein expression, purification and immunoblot analysis, in vitro kinase assays, and MS analysis.

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13. Li SQ, Yang DC, Zhu YG (2007) Characterization and use of male sterility in hybrid rice production cascade, BR signal is perceived by the heterodimer of two LRR–RLKs, BRI1 that has 25 LRRs and its coreceptor BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1) that contains 5 LRRs (36, 43). It will be exciting to reveal the partner(s) of TMS10 and/or TMS10L, which could be unknown LRR–RLKs with longer LRRs and capable of forming a heterodimer with TMS10 and/or TMS10L to transphosphorylate and magnify the signals in promoting tapetal PCD and normal pollen development. Identifying components of the TMS10- and TMS10L-mediated signaling pathway in the thermosensitive control of rice male fertility will uncover how LRR–RLKs buffer the adverse effect of changing temperatures on male developmental programs in plants. Finally, the finding that tms10 confers the TGMS trait in both japonica and indica cultivars can help to widen the genetic resources for manipulating male fertility, a key factor in hybrid seed production.
1 Supporting Information

2 Materials and Methods

3 **Plant Materials and Growth Conditions.** Rice cultivars used in this study are cv. 9522 (*japonica*), 9311 (*indica*), KY131 (*japonica*), HHZ (*indica*) and restorer line JP69 (*indica*). Rice plants were grown in the paddy field in Shanghai (30°N, 121°E) in summer and Sanya (18°N, 109°E) in winter. Rice plants were grown in the paddy field until their inflorescence length reached ~ 0.5 cm in which the anther primordium was formed at stage 3 of anther development, and then transferred to growth chamber until anthesis (stage 14) for treatment. Anther development stages were valued by flower and anther length or anther section (1). Temperature and photoperiod treatments during reproductive stage were carried out in the growth chamber of China National Rice Research Institute and phytotron (Conviron, GR48, Canada), at an average temperature of 23 °C, 24 °C and 28 °C, photoperiod of 11.5 h, 12.5 h, 13.5 h and 14.5 h, and 75% relative humidity (detailed information in *SI Appendix*, Table S1). For material collection, plants were germinated on plates and cultured in growth chamber for 2 weeks before they were moved to phytotron (Conviron, GR48, Canada) for nutrient solution culture at 14 h light photoperiod, 28 °C and 75% relative humidity for vegetative growth for 50 days to keep tillers growing. Then growth conditions were set as 12 h light, 28 °C and 75% relative humidity to induce reproductive growth for ~ 2 weeks, before plants were subjected to do treatment under 75% relative humidity with 12 h light/22 °C or 12 h light/28 °C.

4 **Map-based Cloning of tms10.** 281 mutants from the F2 population created by crossing the mutant tms10 (*O. sativa* ssp. *japonica* cv.9522) with *O. sativa* ssp. *indica* cv. 9311, were used for map-based cloning with our marker system (2). The mutated site were located on chromosome 2 between primer Y1 and Y2, then narrowed down to Y7 and Y8, within the region covered by three BACs (AP005694, AP005533 and AP004001) (Fig 2A, Primers referred as *SI Appendix*, Table S3). Further sequence analysis of the
genes between mapping primer Y7 and Y8 by PCR using primer set S4F/S4R (SI Appendix, Table S3) revealed a mutation in LOC_Os02g18320 (called TMS10).

Plasmid Construction and Transformation. A genomic fragment that contained 3,031-bp promoter, 5,804-bp full length genomic DNA and 401-bp 3' UTR of TMS10 was amplified from Nipponbare BAC clone OSJNBa0018M09 and inserted into pCAMBIA1301 to generate pTMS10::TMS10gDNA. To analyze TMS10 protein localization and accumulation, we generated three constructs that contained the 3,031-bp TMS10 promoter and full length gDNA translationally fused with GUS (pTMS10::TMS10gDNA-GUS), GFP (pTMS10::TMS10gDNA-GFP) and 9×myc (pTMS10::TMS10gDNA-9×myc), respectively. Each of the GUS, GFP, and 9×myc tag contained the BstBI site and was cloned into BstBI-linearized backbone between the C-terminus (without stop codon) and the 401-bp 3’ UTR region of TMS10 by In-Fusion (Clontech) cloning technology, using primers listed in SI Appendix, Table S3. Site-directed mutagenesis of pTMS10::TMS10gDNAK312E-9×myc was created by mutation of 4517A>G in pTMS10::TMS10gDNA-9×myc, resulting in TMS10K312E-9×myc that was transformed into tms10. CRISPR-Cas9 constructs targeting 43th-61th nt (5’-TGGCAATCAGCTTTCGGAT-′3) of the second exon of TMS10 and 107th-126th nt (5’-CTGGTAACCAGCTTTCTGAC-′3) of the second exon of TMS10L were made as previously described (3). All vectors were transformed into Agrobacterium tumefaciens strain EHA105 and introduced individually into rice callus by A. tumefaciens-mediated transformation (4). The calli derived from spikelet primordia of the male sterile lines of tms10 or the wild type embryo were infected with A. tumefaciens (5), co-cultured for three days in the dark and selected twice on screening media containing 40 mg/L hygromycin for 12 days. Green shoots were then induced on differentiation media for 30 days, and regenerated shoots were shifted to rhizogenic media to get whole plant (4). Primers used in construction and identification of transgenic lines are listed in SI Appendix, Table S3.

Phenotypic Analysis of Anther and GUS Staining. Anther samples for semi-thin sectioning, TEM and SEM were collected in the growth chamber at 28 °C and 22 °C,
and sample preparation and observation were performed as previously described (5). Inflorescences of *pTMS10::TMS10gDNA-GUS* complemented *tms10* plants were stained by GUS solution at 37 °C overnight in the dark, then de-stained by 75% ethanol for 2-hour each time for 3 times. Images were captured by a Leica MZ8 dissecting microscope.

**Phylogenetic Analysis.** The full length protein sequence of TMS10 was used to search for homologous sequences in public databases (NCBI-BLASTP). 44 sequences from 11 species were obtained and used for sequence alignment and Neighbor-Jointing (N-J) phylogenetic tree construction, using MEGA4 and 1000 replicates. The entire intracellular domain sequences of TMS10 and TMS10L from rice and *Arabidopsis* were used for alignment to show the conserved phosphorylation residue K\textsuperscript{312} and K\textsuperscript{310} within the kinase domain of TMS10 and TMS10L, which corresponds to K\textsuperscript{317} in *AtSERK1* (6).

**RT-qPCR Analysis.** Total RNA was isolated from WT and *tms10* grown under 28 °C and 22 °C, using Trizol reagent (Generay) and protocol provided by the supplier. 1 µg of RNA per sample were used to synthesize cDNA using the PrimeScript RT reagent kit with genomic DNA eraser (Takara). RT-qPCR was performed (7) using gene-specific primers (DL5F/ DL5R or TMS10LRTF/ TMS10LRTR) for quantifying TMS10 or TMS10L expression, respectively. *OsACTIN* was used as a normalizer (for normalization corresponding to the total RNA level) in the RT-qPCR assays.

**In Situ Hybridization.** Full length cDNA sequence of GFP was used as the probe to detect TMS10 expression in anthers of the complemented line, *pTMS10::TMS10gDNA-GFP* grown under 22 and 28 °C. RNA hybridization and immunological detection of the hybridized probes were performed as previously described using the specific antibody against GFP (5). Images were obtained using the Olympus Nikon E600 microscope.

**Yeast Two-Hybrid (Y2H) Assays.** Full-length cDNA of TMS10 and TMS10L were cloned into the DUALmembrane system vector *pBT-STE* and *pPR3-C*, respectively,
following procedures provided by the DUALmembrane starter kits User Manual. Primers used in construction are listed in SI Appendix, Table S3.

**Subcellular Localization.** For localization in onion epidermis, 1,824-bp full-length cDNA of TMS10 was cloned into the CaMV 35S promoter-containing PA7-GFP vector at XhoI/SpeI to generate 35S::TMS10cDNA-GFP. Using the Bio-Rad biolistic system (Hercules, CA, USA), the plasmid was mixed with gold particles and bombarded into onion epidermal cells, which were later kept overnight in the dark at 28 °C. Rice protoplasts were isolated from the sheath of two-week-old wild-type seedlings and transformation was performed as previously described (8). GFP fluorescence signal was observed after plasmolysis with 0.8 M mannitol. For protein localization in tobacco leaves, TMS10cDNA-GFP was cloned into pHB vector that contained the double CaMV 35S promoter to generate 2×35S::TMS10cDNA-GFP. TMS10L cDNA was ligated into the PHB-YFP vector through infusion system to get 2×35S::TMS10LcDNA-YFP. Plasmids were respectively transformed into A. tumefaciens strain GV3101, and then transiently infiltrated onto three-week-old Nicotiana benthamiana leaves, before the plants were placed at 22 °C or 28 °C for 48 h. GFP or YFP fluorescence signal was observed using a confocal laser scanning microscope (Zeiss LSM510), with excitation wavelength at 488 nm or 514 nm and emission wavelength at 505-530 nm or 535-546 nm.

**Protein Expression, Purification and Immunoblot Analysis.** The cDNA fragments encoding various truncated intracellular domain of TMS10, namely, K (850-1,674 bp), KC (850-1,824 bp), JKC (721-1,824 bp), mJKC (721-1,824 bp, with G934A), were cloned into pET-28a at EcoRI/SalI. The intracellular domain of OsBRI1 (2,218-3,363 bp) was cloned into pMAL-c2x. These constructs were individually transformed into Escherichia coli BL21 (DE3), and the expression of recombinant proteins were induced with 0.5 mM IPTG for 6 h at 37 °C. The supernatant was used for protein purification using Ni⁺-NTA (Aogma) or MBP resin (Biolabs) and the recombinant protein was eluted with 120 mM imidazole and 10 mM maltose, which was then removed by ultrafiltration. The purified proteins were re-suspended in enzyme storage buffer (50
mM Tris–HCl, pH 7.5, 20 mM MgCl$_2$, 30% glycerol). Rice anther materials were collected from plants grown under 22 °C and 28 °C and stored at -80 °C. Samples were ground completely in equal volume of 2×loading buffer, boiled and then loaded on 10% SDS gels. Target bands were detected by myc (ABclonal), GFP (Aogma) and tubulin (Beyotime) antibodies with 1: 2000 to 1:5000 dilutions.

**In Vitro Kinase Assays and MS Analysis.** *In vitro* kinase assays were performed as previously described (8) with modifications. Kinase and substrate were mixed in kinase buffer (20 mM Tris–HCl, pH 7.5, 10 mM MgCl$_2$, 10 mM MnCl$_2$, 20 µM ATP, and 1 mM DTT, 1 µCi [γ-32P] ATP) at 37 °C for 1 h. Reaction was terminated by adding equal volume 2×SDS loading buffer and proteins were separated by 12% SDS-PAGE. Phosphorylated proteins were detected through autoradiography and total protein was detected by Coomassie Blue staining (9). After *in vitro* kinase assays, JKC and mJKC proteins were separated by SDS-PAGE and analyzed by MS to identify the phosphorylation site (10).


**Fig. S1.** *tms10* is male sterile at high temperature (28 °C). (A) Whole plants at the heading stage. Scale bars = 15 cm. (B, C) Spikelet at the flowering stage. Scale bars = 1 cm. (D, E) Mature florets before anthesis. Scale bars = 2 mm. (F, G) Single anther of WT (F) and *tms10* (G) at stage 13. Scale bars = 1 mm. (H, I) I2-KI staining of pollen grains in WT and F1 at stage 13. Scale bars = 200 μm. (J) Chromosome behaviors of male meiocytes of WT and *tms10* under 28 °C. Scale bar = 5 μm. (K, L) I2-KI staining
of pollen grains in *tms10* at stage 13 from *tms10* (*K*) and WT (*L*) plants treated with 12 different combinations of temperatures and photoperiods. Each treatment contained ten plants (*n* = 10). In *tms10*, mature pollen grains were not formed at 28 °C irrespective of the photoperiod, whereas visible pollen grains were observed at 23 °C and 24 °C. Scale bars = 200 µm.
Fig. S2. TEM and SEM analysis of the anther and pollen at 28 °C. (A-P) Transverse sections of WT anther and tms10 anther at stage 8b (A-H) and stage 9 (I-P) are shown. (E-H) and (M-P) are higher magnification of the boxed regions in (A-D) and (I-L) respectively. Scale bars = 5 µm in A-D and I-L; 1 µm in E-H, M-P. E, epidermis; En, endothecium; Ml, middle layer; T, tapetal cell; V, vacuole. Red stars indicate the nuclei, and red and blue arrows point to pollen exine in WT and tms10, respectively. (Q) SEM analysis of anther and pollen grains at stage 13. Scale bars for anther epidermis, 10 µm; for anther inner surface and pollen surface, 3 µm; for pollen grains, 20 µm.
**Fig. S3.** Expression pattern of the *TMS10* gene and subcellular location of the TMS10 protein. (A) RT-qPCR analysis in anthers and other tissues of WT plants grown at 28 °C. Values are shown as mean ± s.d. (*n* = 3). S, anther development stage. S5-S8b divided according to flower sizes and sections, S9-S13 divided according to anther sizes and sections (1, 7). (B, C) TMS10 protein subcellular localization in rice protoplasts (B) and onion epidermis cell treated with 0.8 M mannitol for plasmolysis (C). Scale bars = 5 µm in B.
Fig. S4. Effects of temperature on expression pattern, protein accumulation and protein localization of TMS10. (A) RT-qPCR analysis of TMS10 expression. Values are shown as mean ± s.d. (n = 3). (B) Transient expression of the TMS10-GFP fusion protein in tobacco leaves grown under 28 °C and 22 °C. Scale bars = 50 µm. (C) GUS staining in pTMS10::TMS10gDNA-GUS/tms10 flowers grown under 28 °C and 22 °C, tms10 flowers as the negative control (NC). Scale bar = 1 mm. S3-S9 represent the anther development stage 3-9 judged on the flower and anther size (1, 7). (D-E) Immunoblot analysis of the recombinant TMS10 protein in pTMS10::TMS10gDNA-9×myc/tms10
flowers (D) and pTMS10::TMS10gDNA-GFP/tm10 flowers (E), using the antibodies against myc and GFP, respectively. Tubulin was used as control. (F) **In-situ** hybridization of TMS10 in anther under 28 °C and 22 °C. Scale bar = 50 µm. (G) TMS10-GFP signals in anther under 28 °C and 22 °C. Scale bar = 25 µm. Mc, Meiotic cell; Tds, Tetrads; Msp, Microspore parietal cell.
Fig. S5. Sequence alignment of TMS10, TMS10L and its close homologs, yeast two-hybrid analysis, TMS10L expression and TMS10L protein location. (A) Alignment of amino acid sequences of the intracellular domain from reported LRRII-RLKs in *Arabidopsis* and TMS10 new clade in *Arabidopsis* and rice. Purple box indicate the kinase domain, red box amino acids (AVKRI) indicate the conserved ATP activation loop. (B) TMS10 and TMS10L do not form homo- or hetero- dimers in the DUAL membrane yeast two-hybrid system. TMS10-Cub and TMS10L-Cub were transformed with NubG and NubI as negative and positive control, respectively. No activation activity of the report gene *HIS3* and *ADE2* was seen by co-transformation of TMS10-Cub and TMS10L-NubG, TMS10-Cub and TMS10-NubG, TMS10L-Cub and TMS10L-NubG, or TMS10L-Cub and TMS10-NubG. (C) TMS10L relative expression in WT and *tms10* under 28 °C and 22 °C. Values are shown as mean ± s.d. (*n* = 3). (D) Transient expression of TMS10L-YFP in tobacco leaves under 28 °C and 22 °C. Scale bar = 25 μm.
Fig. S6. Anther development in *tms10 tms10l* exhibit irregularities under low temperature at stage 10. Under 22 °C, anther development of *tms10 tms10l-1* and *tms10 tms10l-2* were normal at stage 8b and 9 and started to collapse at stage 10 when the microspores of WT and *tms10* became vacuolated. Scale bars = 20 µm.
Fig. S7. Generation of new tms10 alleles and use of TMS10 in hybrid rice breeding. (A) Genomic sequences of the four CRISPR-Cas9-generated tms10 alleles in cv. KY131. Red dotted lines indicate the nucleotides deletion and blue nucleotides are inserted sequences. (B) Amino acid sequence at the mutated sites of the four new tms10 alleles in cv. KY131. Asterisks indicate translational termination. (C) Spikelet and anther phenotypes of tms10-2 in japonica cv. KY131 at 28 °C (HT) and 22 °C (LT). (D) Scheme of the generation of the tms10 substitution line in HHZ. tms10 mutation was genotyped by PCR and sequencing of the mutated site of tms10 using primer S4F/S4R, and tms10 in indica background was selected by indica traits. ♀, female parent line; ♂, male parent line; ×, cross; ⊘, self-pollination. (E) Genomic sequences around the mutation sites in tms10 in HHZ background (BC2F4). (F) Spikelet and anther phenotypes of tms10 in indica cv. HHZ at 28 °C (HT) and 22 °C (LT). (G, H) Whole plant (G) and mature panicles (H) of parental lines and F1 hybrid at 28 °C, with tms10 (cv. 9522) as the female and JP69 as the male parental line. Scale bars, 20 cm in G; 5 cm in H.
cm in H. (I-K) Tiller number (I), number of grains per plant (J), and 1,000-grain weight (K) in F₁ and male parental line JP69. Values are shown as mean ± s.d. (n = 14 in I, J; n = 3 in K). (L, M) Whole plant (L) and mature panicles (M) of parental lines and F₁ hybrid at 28 °C, with tms10 (HHZ) as the female and JP69 as the male parental line. Scale bars, 20 cm in L; 5 cm in M. (N-P) Number of tillers (N), grains per strain (O) and 1,000-grain weight (P) in F₁ and male parent line JP69. Values are shown as mean ± s.d. (n = 14 in N, O; n = 3 in P). P values were calculated by Student’s t-test. Scale bars, 2 mm in flowers; 200 µm for I₂-KI-stained anthers in C, F.
Table S1. Treatment of *tms10* and WT in the growth chamber with a relative humidity 75%.

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Table S2. Identification of phosphorylation sites within the TMS10 intracellular domain identified by mass spectrometry.

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Residues in red are the phosphorylation sites identified by MS analysis.
Table S3. Primers used in this study.

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PK9F  AGGAATTCTGTTAAAGGGAGGAGGAAAAG  pET-28a-JKC /mJKC construction
PK6R  AAACGCGTCGACTCATCTACCTCCAGACAACTCT  pET-28a-JKC /mJKC construction
POS8F  AACTCGAGATGAGGGAGCTGCGCGTCGC  35S::TMS10-GFP construction
POS8R  AAACTAGTACTCTACCTCCAGACAACTCTATTGC  35S::TMS10-GFP construction
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TMS10CH1R  AAACATCCGAAAGCTGATTGCCAC  TMS10 CRISPR-cas9 construction
DL5F  CCGGCTGGCGTACAATAATC  TMS10 RT-qPCR
DL5R  GAGCAGCTACAATGAGAAGT  TMS10 RT-qPCR
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S4R  ACCAGGAGACATTAGCAAGAGA  tms10 mutant genotyping
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S5R  CATCGATTTCACACAACAAAAC  mTMS10-9×myc (K312E) genotyping
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