The rice TRIANGULAR HULL1 protein acts as a transcriptional repressor in regulating lateral development of spikelet

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As a basic unit of rice inflorescence, spikelet has profound influence on grain size, weight and yield. The molecular mechanism underlying spikelet development has not been fully elucidated. Here, we identified four allelic rice mutants, s2-89, xd151, xd281 and xd425, which exhibited reduced width of spikelet, especially in the apical region. Map-based cloning revealed that all these mutants had missense mutation in the TRIANGULAR HULL1 (TH1) gene, encoding an ALOG family protein. TH1 has been shown to regulate the lateral development of spikelet, but its mode of action remains unclear. Microscopic analysis revealed that the reduction in spikelet width was caused by decreased cell size rather than cell division. The TH1 protein was shown to localize in the nucleus and possess transcriptional repression activity. TH1 could form a homodimer and point mutation in the s2-89, xd281 and xd425 mutant inhibited homodimerization. The transcriptional repression activity of TH1 was partially relieved by the His129Tyr substitution in the s2-89 mutant. Fusion of an exogenous EAR transcription suppression domain to the mutant protein TH1 s2-89 could largely complemented the narrow spikelet phenotype. These results indicate that TH1 functions as a transcription repressor and regulates cell expansion during the lateral development of spikelet.

Rice (Oryza sativa L.), a model monocot with the smallest genome of major cereals, provides the staple food for over half of the world’s population. Increasing grain yield is a major target in the current rice breeding programs. Grain yield potential mainly depends on four major components: grain weight, panicle (or tiller) number per plant, grain number per panicle, and ratio of filled grains1. Grain weight is considerably determined by grain shape and size, which is specified by grain length, width, thickness and the length/width ratio.

Rice grain weight is nearly completely governed by genetic factors. With the advancement of the rice genome sequencing project and development of advanced mapping population, many quantitative trait loci (QTLs) for grain shape and size have been cloned using map-based cloning approach. Most of the cloned QTL genes affect grain size by influencing cell division in the spikelet hull. GRAIN SIZE 3 (GS3), a major QTL for grain length and weight in rice, encodes a putative transmembrane protein, which functions as a negative regulator of grain and organ size24. GRAIN LENGTH 3 (GL3), a novel serine/threonine phosphatase, regulates grain length by mediating cell division through affecting the phosphorylation status of cell cycle proteins4. GRAIN WIDTH 2 (GW2), a QTL for rice grain width and weight, encodes a RING-type E3 ubiquitin ligase which negatively regulates cell division by targeting its substrates for degradation5. GRAIN WIDTH 5 (GW5), a major QTL underlying rice width and weight, encodes a novel nuclear protein which likely acts in the ubiquitin-proteasome pathway to regulate cell division during seed development6. GRAIN WIDTH 8 (GW8) is synonymous with OsSPL16, which encodes a protein that is a positive regulator of cell proliferation. Higher expression of this gene promotes cell division and grain filling7. GRAIN SIZE 5 (GS5) encodes a putative serine carboxypeptidase and functions as a positive regulator of grain width, such that higher expression of GS5 is correlated with larger grain size8. In addition to cell division, cell expansion also plays important role in grain size. POSITIVE REGULATOR OF GRAIN LENGTH 1 (PGL1) and POSITIVE REGULATOR OF GRAIN LENGTH 2 (PGL2) positively regulate the rice grain length.
through increasing cell length in lemma and palea\textsuperscript{9,10}. The studies above suggest that grain size is rigidly controlled by the size of spikelet hull.

Spikelet is a basic unit of the grass inflorescence architecture, whose development and morphogenesis has profound influence on grain size and weight. Grass species, such as wheat, rice, maize and barley, have a highly specialized inflorescence architecture which is apparently distinct from eudicots\textsuperscript{11,12}. In rice, a spikelet is composed of two rudimentary glumes, two empty glumes (or sterile lemmas) and a floret which consists of one lemma, one palea, two lodicules, six stamens and one carpel\textsuperscript{13-16}. Among these floral organs, lemma and palea play a critical role in determining the grain shape, size and yield, because the space enclosed by the lemma and palea determines the size of spikelet hull. Although the grass reproductive organs (stamens and carpels) appear to be largely conserved with eudicots, the non-reproductive floral organs (lemma, palea and lodicules) are considerably different from the sepal and petals found in flowers of most eudicots and many other monocots\textsuperscript{17}. Currently, the widely-accepted ABCDE model describes the development of floral organs, which is based on early mutant studies in two eudicot species, Antirrhinum majus\textsuperscript{18} and Arabidopsis thaliana\textsuperscript{19}. Some studies provide evidence in support of the hypothesis that grass lemma and palea are evolutionarily equivalent to eudicot sepal, whereas other studies suggest that lemma and palea should be regarded as bract and prophyll, respectively\textsuperscript{20}. Hence, it is necessary to isolate the corresponding genes related to the origin and mechanism of lemma and palea development.

Genetic and molecular studies have allowed the identification of several mutants and genes that play key roles in regulating spikelet development through forward and reverse genetic approaches\textsuperscript{21,22,23,24}. Spikelet development is controlled by the action of numerous transcription factors, the majority of which belong to the MADS-box family\textsuperscript{23,24}. The AP1/FUL-like subfamily of MADS-box genes RAP1B/OsMADS14 and RAP1A/OsMADS15 have class A-like floral homeotic gene functions in specifying palea/lemma and lodicule identities\textsuperscript{25}. The SEPALLATA(SEP)-like MADS gene LEAFY HULL STERILE1 (LHS1)/OsMADS1 specifies the identity of both the lemma and palea and regulates the determinacy of the spikelet meristem\textsuperscript{26}. Another SEPELLATA (SEP)-like MADS gene, PANICLE PHYTOMER2 (PAP2)/OsMADS34 may act together with ELE/G1 in controlling sterile lemma development\textsuperscript{27,28}. The AGL6-like MADS-box gene MOSAIC FLORAL ORGANS1 (MFO1)/OsMADS6 gene, a major determinant of palea architecture, has a central role in spikelet development and is involved in floral meristem determination\textsuperscript{28}. In addition, several grass-specific genes also play key roles in regulating spikelet development. The DEPRESSED PALEA1 (DP1) gene encodes a nuclear-localized AT-hook DNA binding protein and plays a grass-specific role of chromatin architecture modification in flower development\textsuperscript{29}. The RETARDED PALEA1 (REPI) gene has a specific role in the regulation of palea development. The reduced cell size in the palea of the rep1 mutant implies a role of REPI similar to other TCP proteins in regulating cell expansion and differentiation\textsuperscript{30}. Further study found that REPI is downstream of and regulated by DP1\textsuperscript{29}. In addition, the rice EXTRA GLUME1 (EGI) gene, a putative lipase gene, specifies empty glume fate and floral meristem determination\textsuperscript{31}. The LONG STERILE LEUMLA1 (G1) protein contains an ALOG domain and belongs to a recently described class of transcription factor. The gl1 mutant shows the striking phenotype of sterile lemmas transformed into lemmas\textsuperscript{32}. Thus, mutations in the genes mentioned above caused dramatic abnormality in the spikelet. By contrast, in the triangular hull1 (th1) mutant, the floral organ identity and patterning are normal, but the lemma and palea become narrower especially in the apical region, forming a beak-like spikelet\textsuperscript{33,34}. Several allelic mutants of th1 named beak like spikelet1 (bs1), beak-shaped grain1 (bg1) and abnormal flower and dwarf1 (afd1) were also reported\textsuperscript{35-37}. The TH1 gene\textsuperscript{32} encodes a nuclear protein with a conserved ALOG domain of unknown function\textsuperscript{32}. It is still unclear how TH1 specifically regulates the lateral development of the lemma and palea.

In the present study, we isolated four allelic mutants of TH1, namely, s2-89, xd151, xd281 and xd425 from the EMS-mutagenized japonica cv. Nipponbare and Xu Dao3, respectively. Detailed morphological analysis indicates that the reduction in spikelet width was caused by decreased cell size rather than cell division in the lemma and palea. Cellular and biochemical studies show that the TH1 protein is localized in the nucleus, functions as a homodimer, and has transcription repression activity. Point mutations in the ALOG domain disrupted dimerization and compromised its transcription repression activity. Phenotypic analysis of transgenic plants harboring the 4 × EAR-TH1\textsuperscript{2-89} or 4 × VP16-TH1\textsuperscript{12-89} fusion constructs further supported that TH1 functions as a transcriptional repressor. This work provides a new perspective of TH1 function in regulating spikelet development and biochemical function of the ALOG family proteins.

Results
We identified four allelic mutants of TH1, i.e., s2-89, xd151, xd281 and xd428. Because they show similar phenotypes, we mainly describe s2-89 below.

Morphological phenotype of the s2-89 mutant. The s2-89 mutant was isolated from the M1 population of japonica cv. Nipponbare mutagenized by the ethyl methane sulphonate (EMS). Compared with the wild type, the s2-89 mutant showed no apparent difference in the vegetative phase. At maturity, phenotype of the s2-89 mutant was also not conspicuously different from that of the wild type in many respects such as plant type, panicle structure and other agronomic traits (Fig. 1a,b, see Supplementary Table S1). The most striking phenotype of the s2-89 mutant was the abnormal grain morphology. The unhulled grain of the s2-89 mutant exhibited a pointed beak-like shape and the hulled grain displayed a triangle-like shape (Fig. 1c). After measuring the grain traits, we found that the s2-89 mutant is reduced in grain width and thickness relative to the wild type, whereas there is no significant change in grain length, which leads to the increased grain length-width ratio. In addition, the 1000-grain weight of the s2-89 mutant is only 53.72% of that of wild type (Fig. 1d–h, see Supplementary Table S1). These results collectively indicated that grain weight is affected predominantly by the abnormal grain morphology.

In addition to the s2-89 mutant, we obtained other three similar beak-shaped grain mutants from EMS-mutagenized japonica cultivar Xu Dao3, referred as xd151, xd281 and xd425 (see Supplementary
Figure S2a). The grain width of these mutants was reduced by 4.36%, 8.72% and 9.66% of that of wild type, respectively. The grain thickness of these mutants was reduced to varying degrees by 4.44%, 6.22% and 11.56%, respectively. However, the grain length of these mutants was increased by 11.75%, 11.05% and 0.84% of that of wild type, respectively. Consistent with the grain size, the 1000-grain weight of these mutants was also reduced (see Supplementary Figure S2b–f, Table S2).

Comparison of cell size and cell number in WT and s2-89 spikelet hull. Given that the slender grain phenotype of the s2-89 mutant was more obvious in the apical than the middle region, we compared the cross-sections of the s2-89 and wild type spikelet hull at the apical region (Fig. 2a–c). The perimeter length of both lemma and palea was significantly reduced at the apical region (Fig. 2d and g). Both palea and lemma comprise outer parenchyma cell layer (opc) and inner parenchyma cell (ipc) layer (Fig. 2b,c). Because the inner parenchyma cells are regular in shape and large in size, we counted the number of inner parenchyma cells and measured their size. Although the grain width is reduced, the number of inner parenchyma cells along the grain width direction was slightly increased in both the lemma and palea of the s2-89 mutant (Fig. 2e and h). However, size of the inner parenchyma cell was reduced by about 50% (Fig. 2f and i). These observations demonstrated that the reduced width of the s2-89 spikelet hull was mainly due to the decrease in cell size.

To further reveal the characteristics of the spikelet in the s2-89 mutant, the lemma and palea of the wild type and s2-89 mutant spikelets were observed through scanning electron microscopy (SEM). These two organs have a rough outer epidermal surface covered with convex structures named tubercles. The average number of tubercles per unit area of outer epidermal surface of lemma and palea in the s2-89 mutant was increased substantially than the wild type (Fig. 3a–e). The average length and width of tubercle in the s2-89 mutant decreased significantly, compared with that of wild type (Fig. 3f,g). These results indicate that the decrease in cell size was mainly responsible for the pointed beak-shaped grain of this mutant.

Both cell proliferation and cell expansion is essential for the control of organ size. Cyclin B 1;1 (CYCB1; 1) is strongly expressed in cells with high mitotic activity, and has been used as a marker of G2/M of the cell cycle. To examine whether cell division activity is reduced in the spikelet hull of the s2-89 mutant, we compared the
CYCB1;1-GUS staining pattern in the wild type and s2-89 mutant background, respectively. There was no obvious difference in the GUS staining pattern between the mutant s2-89 and wild type (see Supplementary Figure S3). This result suggests that cell division may not be responsible for the narrower spikelet hull in the s2-89 mutant.

The s2-89 mutant is a novel allelic mutant of TH1. To investigate the inheritance pattern, the s2-89 mutant was crossed with the wild type Nipponbare. In the F2 population, the ratio of plants with normal grain to those with beak-like shape grain was 208:65, which was fit for the ratio of 3:1 (χ² = 0.148 < χ² 0.05 = 3.84), indicating that the mutant phenotype was controlled by a single recessive gene. To identify the gene, we used a map-based cloning approach. Primary mapping using 32 F2 mutant individuals derived from a cross between the s2-89 mutant and the indica variety Dular revealed that the mutation locus localizes between two insertion-deletion (InDel) markers R2-14 and R2-16 on chromosome 2. For fine mapping, we developed six new InDel markers and the gene was narrowed to a 48 kb interval between molecular markers C2-3 and C2-4 (Fig. 4a).

Based on the available MSU Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/), seven predicted candidate genes were located in this region. Among them, the LOC_Os02g56610 locus has been proved to be the TH1/BSG1/BL51/AFD1 gene in several previous studies33,35–37. The th1/bsg1/bl51/afd1 mutants produce narrow spikelet, similar to the s2-89 mutant. To test whether the s2-89 mutant had a mutation in the TH1 gene, the candidate gene was amplified and sequenced from both the s2-89 mutant and wild type genomic DNA. A transition from C to T in the coding sequence of the TH1 gene was detected, resulting in a change of the 129th amino acid from His to Tyr (Fig. 4b). We further sequenced the TH1 gene from the other three mutants xd151, xd281, xd425 and the corresponding wild type Xu Dao3. In the xd151 mutant, a transition from G to A occurred.
Figure 3. SEM observation of the outer surface of the wild type and s2-89 spikelet. (a,b) SEM analysis of the outer surface of the WT (a) and s2-89 (b) lemmas. (c,d) SEM analysis of the outer surface of the WT (c) and s2-89 (d) paleas. (e) The average number of tubercles per mm² on the outer surface of palea (Pa) and lemma (Le). Double asterisks indicate significant differences between WT and s2-89 at P < 0.01 by Student’s t test. (f,g) The average length and width of tubercles. Data are given as mean ± SE. (n = 10). Double asterisks and asterisk indicate significant differences between WT and s2-89 at P < 0.01 and P < 0.05 by Student’s t test. Each scale bar is indicated.
Figure 4. Identification and characterization of the TH1 gene. (a) The TH1 locus was mapped to a region between markers R2-15 and R2-16 on chromosome 2. The gene was further delimited to a 48-kb genomic region between markers C2-3 and C2-4. There are seven predicted ORFs within this region. (b) The TH1 gene structure. White and black box represent untranslated region and coding sequence, respectively. Lines represent intron. The start codon (ATG) and the stop codon (TGA) are indicated. The xd151, xd281, xd425 and s2-89 mutation sites in the TH1 gene are shown. (c) Comparison of the size of TH1 cDNA between WT and the xd151 mutant. The first intron was retained within the mRNA transcript. (d) Sequence alignment of the TH1 protein with three functionally characterized member of the ALOG family, i.e., Arabidopsis LSH1, rice TAW1 and G1. The conserved ALOG domain and nuclear localization sequence (NLS) are underlined. Mutations in × 281, xd425 and s2-89 are indicated by red arrowhead. (e) Complementation of grain morphology. Two transgenic lines B195-2 and B195-5 are shown in which the wild type TH1 genomic DNA was introduced into the s2-89 mutant.
at the 5′ splicing site of the first intron. RT-PCR amplification of cDNA revealed that the TH1 transcript became larger in the xd151 mutant than wild type (Fig. 4c). Sequence analysis confirmed that the first intron was retained within the 5′-UTR of the TH1 gene (Fig. 4b, lower panel). In the xd281 mutant, a transition from C to T was detected, causing the 80th amino acid Arg to be substituted by Trp. In the xd425 mutant, a transition from G to A was found, resulting in a change of the 124th amino acid from Gly to Gln (Fig. 4b). Furthermore, in a functional complementation experiment, transformation of the wild-type TH1 genomic fragment into the s2-89 mutant was able to rescue the beak-shaped grain phenotype (Fig. 4e). Based on these results, we concluded that mutations in TH1 are responsible for the beak-shaped grain phenotype.

The TH1 gene encodes a protein with a conserved ALOG domain of unknown function. Sequence alignment of TH1 with three previously identified members of the ALOG family, i.e., Arabidopsis LIGHT-DEPENDENT SHORT HYPOCHOTYL1 (LSH1), rice LONG STERILE LEMMA (G1) and TAWAWA1 (TAW1)32,40,41, showed that the central ALOG domain is highly conserved whereas the N- and C-terminus are highly diverse (Fig. 4d). The three missense mutations in the s2-89, xd281 and xd425 mutant are present in the ALOG domain (Fig. 4d). In the quantification analysis of grain related traits, grains of these three allelic mutants were found to be more severely distorted than that of the xd151 mutant (see Supplementary Figure S2), which has an intact ALOG domain (Fig. 4b). This result suggests that these amino acids and the ALOG domain are critical for the proper function of the TH1 protein.

Expression patterns of TH1. We used real-time PCR analysis to examine the spatial expression pattern of TH1. The result indicated TH1 was highly expressed in young panicles, especially in the lemma and palea of spikelets. Although TH1 was also expressed in other tissues such as root, culm, leaf blade, leaf sheath and tiller bud, the expression level was relatively lower (see Supplementary Figure S3). This result indicated that TH1 is predominantly expressed in a spikelet-specific manner, which is consistent with the fact that the s2-89 mutant exhibited abnormal phenotype mainly in the spikelet. This result is also consistent with a previous TH1 promoter driven GUS reporter assay where strong GUS signals were detected in the lemmas and paleas of young spikelets41. In another assay using in situ hybridization, TH1 was shown to be highly expressed in the primordia of the lemma and palea and also detected in the primordia of the rudimentary glume, sterile lemma, lodicule and stamen29.

Nuclear localization and dimerization of TH1. Previous bioinformatic study has predicted that the ALOG domain proteins may act as transcription factors or recruiters of repressive chromatin42. If TH1 acts as a transcription factor, nuclear localization is a prerequisite to execute its function. A nuclear localization signal (KKKKKK) was predicted immediately after the ALOG domain (Fig. 4d), using the web-based tools such as NucPred43 and WoLFPSORT44. To test this possibility, the wild type and mutant alleles of the TH1 gene were fused in-frame with the green fluorescent protein (GFP) under the control of the CaMV 35S promoter in the transient expression vector pAN580. The fusion vectors were transformed into rice leaf protoplasts. The fluorescent signal of TH1-GFP was targeted mainly to the nucleus. Interestingly, green fluorescence of the three mutant TH1 proteins, i.e., TH1s2-89-GFP, TH1 xd281-GFP and TH1 xd425-GFP was also mainly detected in the nucleus (Fig. 5). This result indicates that these three substitutions have no effect on the TH1 subcellular localization.

Considering the fact that transcription factors usually function as a homodimer45, we examined whether TH1 can form a homodimer using the yeast two-hybrid assay. As shown in Fig. 6, interaction between BD-TH1 and AD-TH1 was detected, suggesting that TH1 can interact with itself to form a homodimer. However, the three TH1 mutant proteins TH1s2-89 (His129Tyr), TH1 xd281 (Arg80Trp) and TH1 xd425 (Gly124Gln) lost the ability to interact with itself. Therefore, Arg80, Gly124 and His129 are critical for the homodimerization of TH1.

TH1 acts as a transcriptional repressor in regulating spikelet development. To test whether TH1 possesses the capacity to activate transcription, a fusion of TH1 coding region with the yeast GAL4 DNA binding domain was expressed in the yeast strain Y187 containing the β-galactosidase reporter gene driven by the GAL4 UAS (GAL4 binding site). If TH1 is a transcription activator, the β-Galactosidase reporter gene will be expressed and the filter will be stained blue in the Colony-lift Filter Assay. However, we could not detect any β-Galactosidase activity using either the wild type or mutant TH1 protein, although it was easily detected in the positive control (Fig. 7a). These results suggest that TH1 could not act as a transcriptional activator, at least in yeast.

To determine whether TH1 acts as a transcriptional repressor, we employed the luciferase transient expression assays in rice protoplasts. For the effector plasmids, coding sequence of the wild type TH1 and mutant TH1s2-89 was inserted into the p35S-GAL4DB plasmid46 to generate fusion proteins with the GAL4 DNA binding domain (GAL4 DB). The reporter plasmid contains a CaMV 35S promoter, 5 × GAL4 UAS (GAL4 binding site), a minimal TATA region and the firefly luciferase gene (Fig. 7b). Each effector plasmid was co-transfected with the reporter plasmid into rice protoplasts and luciferase activity was measured. As a control, fusion of the activation domain of the herpes simplex virus VP16 protein47 or the EAR transcription repression domain43 with the reporter plasmid into rice protoplasts and luciferase activity was measured. As a control, fusion of the activation domain of the herpes simplex virus VP16 protein47 or the EAR transcription repression domain43 with the reporter plasmid into rice protoplasts and luciferase activity was measured. As a control, fusion of the activation domain of the herpes simplex virus VP16 protein47 or the EAR transcription repression domain43 with the reporter plasmid into rice protoplasts and luciferase activity was measured. As a control, fusion of the activation domain of the herpes simplex virus VP16 protein47 or the EAR transcription repression domain43 with the reporter plasmid into rice protoplasts and luciferase activity was measured. As a control, fusion of the activation domain of the herpes simplex virus VP16 protein47 or the EAR transcription repression domain43 with the reporter plasmid into rice protoplasts and luciferase activity was measured. As a control, fusion of the activation domain of the herpes simplex virus VP16 protein47 or the EAR transcription repression domain43 with the reporter plasmid into rice protoplasts and luciferase activity was measured. As a control, fusion of the activation domain of the herpes simplex virus VP16 protein47 or the EAR transcription repression domain43 with the reporter plasmid into rice protoplasts and luciferase activity was measured.

Interestingly, fusion of TH1 with GAL4 DB or GAL4DB-VP16 resulted in about two-fold or three-fold reduction in luciferase activity, indicating that TH1 has transcription repression activity (Fig. 7c). However, the substitution of His129Tyr in TH1s2-89 relieved the repression activity. Fusion of TH1s2-89 with GAL4 DB increased rather than decreased luciferase activity. Fusion of TH1s2-89 with GAL4DB-VP16 reduced luciferase activity to a less extent (Fig. 7c). The loss of transcription repressive activity of the TH1s2-89 mutant protein is probably due to the lack of homodimerization of TH1s2-89 (Fig. 6). However, we can not rule out the possibility that the s2-89 mutation may alter domain folding thus affect protein function.

To confirm the requirement of the TH1 transcription repression activity in spikelet development, we performed a complementation experiment in the s2-89 mutant. The exogenous EAR transcription suppression domain48 and VP16 transcription activation domain49 was fused to the N-terminus of the mutant protein TH1s2-89. 7
(Fig. 8a), respectively. These constructs were transformed into the s2-89 mutant. The *EAR-TH1*\_s2-89* transgenic plants largely complemented the spikelet and grain phenotype of the s2-89 mutant (Fig. 8b–f). Consistent with the transgenic phenotype, the TH1\_s2-89*-EAR fusion protein exhibited transcription repressive activity in luciferase transient expression assays in rice protoplasts (Fig. 7c). Therefore, fusion of an exogenous transcription suppression domain to the mutant protein TH1\_s2-89* restores the complementation ability. In contrast, transgenic plants expressing *VP16-TH1*\_s2-89* exhibited more slender spikelet phenotype, compared with the wild type and mutant control (Fig. 8b–f). These results indicate transcription repression activity of TH1 acts to promote the lateral development of rice spikelet.

**Discussion**

Rice grain size is an important agronomic trait and nearly completely governed by genetic factors. Several genes involved in rice grain development have been isolated, such as GS3, GL3, GW2, GW5 and GW8\_2–8. These studies suggest that spikelet architecture has profound influence on grain size and weight. In this study, we isolated four grain shape related mutants, s2-89, xd151, xd281 and xd425, which showed an unusually slender spikelet. Map-based cloning approach revealed that these mutants have single base mutation in the TH1 gene, which is previously reported to function in the control of rice spikelet shape and grain size33–37. In this study, we made two novel insights about the function of TH1. Firstly, TH1 determines grain shape and size by regulating cell expansion in the lemma and palea of rice spikelet. Secondly, TH1 functions as a transcriptional repressor in spikelet development.

Each rice spikelet consists of a floret with one carpel, six stamens and two lodicules subtended by the lemma and palea, and two depressed empty glumes at a position opposite to each other above the rudimentary glumes. It was previously reported that TH1 has a specific role in regulating lemma and palea development33–37. In our study, the s2-89, xd151, xd281 and xd425 mutants bore beak-shaped grains with reduced width, thickness and weight (Fig. 1, Supplementary Figure S2), as for other mutant alleles of TH1\_s2-89*. Young florets in the th1 mutant and corresponding wild type at different developmental stages had been observed by SEM. TH1 seems not to
influence the initiation of floral-organ primordia, but affects the enlargement of the lemma and palea at late development stage of spikelet35,36. Meanwhile, in the th1 mutants, the marginal region of palea (mrp) looks normal and the alterations are due to shape changes of the lemma and the rest of palea (body of palea, bop) 36. In addition, the lodicules of th1 florets were elongated and green34,36,37. Further observations showed that the surface of elongated lodicules was rough, crumpled and possessed some protrusions that were similar with those in the wild type hulls36. In this study, we determined whether the reduced spikelet size is caused by regulations of cell size or cell number in th1 spikelet. Our histological observation showed that the s2-89 mutant exhibited smaller inner parenchyma cell in both the lemma and palea, compared with the wild-type (Fig. 2). The number of inner parenchyma cell was slightly increased rather than decreased in the s2-89 mutant (Fig. 2). SEM observation also revealed that the average length and width of tubercle on the outer surface of lemmas and paleas was decreased significantly in the mutant s2-89, compared with that of wild-type (Fig. 3). Moreover, the similar staining pattern of the CYCB1;1-GUS mitotic marker in the wild type and s2-89 mutant background indicated cell division may not be responsible for the narrower spikelet in the s2-89 mutant (see Supplementary Figure S3). To sum up, the reduced width of the th1 spikelet hull resulted mainly from the decrease in cell size. TH1 most likely functions as an upstream modulator of cell expansion during lateral development of lemma and palea in rice.

Previous studies have reported numerous genes involved in the regulation of non-floral spikelet organ development in rice, such as LSH1/OsMADS1, MFO1/OsMADS6, RAP1B/OsMADS14, RAP1A/OsMADS15, PAP2/OsMADS34, DP1, REP1, EG1 and G1. Mutation in these genes causes severe morphological alterations in various spikelet organs. For example, in the mfo1 mutant, the identity of palea and lodicule is disturbed, and mosaic organs were observed. The number of vascular bundles increased to five to six in the mfo1-1 palea and the interlocked lemma/palea structure was destroyed28,49,50. In the lsh1 mutant, palea and lemma are elongated and leafy that exhibits a feature of open hull22. Detailed histological analyses demonstrate clearly that LSH1/OsMADS1 regulates lemma development by controlling epidermal cell fate and proliferation and by influencing internal cell differentiation. In the palea, LSH1/OsMADS1 largely regulates internal cell layer differentiation51. In the pap2 mutant, elongation of sterile lemmas and rudimentary glumes was observed in all spikelets examined52. These

Figure 6. Homodimer formation of TH1 protein detected via yeast two-hybrid assay. The WT and three mutant alleles of the TH1 gene were cloned into both the bait vector pGBK7 and the prey vector pGADT7T and co-transformed into the yeast strain Y187. A decimal serial dilution of the yeast colony was spotted onto the selective medium DDO (SD/-Trp-Leu) or QDO (SD/-Trp-Leu-Ade-His). The colony will survive on the QDO medium only if the bait and prey interact with each other. pGADT7-RecT and pGBK7-53 was used as positive control, while pGADT7-RecT and pGBK7-Lam as negative control.
genes are associated with meristem function and affect spikelet formation at the early stages by controlling cell number. In contrast, in the rep1 mutant, the development of palea is significantly retarded and its palea exhibits vascular pattern similar to that of lemma and the mutant floret presents bilateral symmetry along the Le/Pa axis. Detailed histological analyses demonstrate clearly that loss of function of REP1 may dramatically affect the palea cell growth and expansion, especially in the epicuticula, the innermost cell layer, and vascular tissues30. In our study, the th1 mutation only resulted in a slight change in the shape of the lemma and palea. SEM observation of early stages of floret development in several allelic mutants of TH1, such as bls1-1 and afd1, showed that TH1 does not function at stages of floral-organ initiation and patterning but is required for lateral development of the lemma and palea in the final stages34–36. Our observation that TH1 controls cell size during the lateral development of lemma and palea is consistent with previous finding that TH1 is not associated with meristem function.

TH1 belongs to the ALOG family protein, which has 10 and 11 members in the rice and Arabidopsis genome, respectively40. One previous bioinformatic study has predicted that the ALOG family protein may act as transcription factors or recruiters of repressive chromatin42. Three ALOG family members have been functionally characterized, i.e., the Arabidopsis LSH141, the rice G1 and TAW1 protein32,40. All these three proteins are shown

Figure 7. TH1 acts as a transcriptional repressor in the transient expression assay. (a) Transcriptional activation assay in yeast cell. Yeast co-transformants were incubated on the selective medium (SD/-Ade/-His/-Leu/-Trp plus X-α-Gal) at 30°C for 3 d. If TH1 is a transcription activator, the β-Galactosidase reporter gene will be expressed and the filter will be stained blue in the Colony-lift Filter Assay. pGADT7-RecT & pGBK7-53 (positive control); pGADT7-RecT & pGBK7-Lam (negative control). (b) Constructs used in transient expression assay in rice leaf protoplasts. Six different effector plasmids were made by fusing TH1, TH1S2-89 with activator domain VP16, repressor domain EAR or the GAL4 DNA binding domain (GAL4-DB). The reporter plasmid Pro35S-GAL4:LUC contains a CaMV 35S promoter, 5 × GAL4 binding site, a TATA box in front of the firefly luciferase (LUC) gene and a nopaline synthase terminator. The p35S:REN plasmid expressing the Renilla luciferase was used as an internal control. (c) Relative luciferase activities in rice leaf protoplasts using TH1, TH1S2-89, VP16, TH1-VP16, TH1S2-89-VP16, EAR, TH1-EAR and TH1S2-89-EAR as effector compared with GAL4-DB control. Error bars indicate SE (n = 3). Double asterisks indicate significant difference at P < 0.01 by Student’s t test.
be localized into the nucleus. The rice G1 and TAW1 are shown further to have slight but significant activity as a transcriptional activator \(^{32,40}\). In this study, we showed that TH1 is also nuclear-localized (Fig. 5) and has the ability to form the homodimer (Fig. 6), which is consistent with the attributes of most transcription factors. Homodimerization of TH1 seems to be mediated by the ALOG domain, because all three point mutations in the ALOG domain abolished its ability to form the homodimer (Fig. 6). Interestingly, we found that TH1 repressed rather than enhanced transcriptional activity in rice protoplasts using a trans-activation assay based on the yeast GAL4 system (Fig. 7). The transcriptional repression activity of TH1 was relieved by the His129Tyr substitution in the s2-89 mutant (Fig. 7). Fusion of exogenous EAR transcription suppression domain to the mutant protein TH1 s2-89 could largely complemented the narrow spikelet phenotype, whereas fusion with the exogenous VP16 transcription activation domain enhanced the narrow spikelet phenotype (Fig. 8). These results suggest that TH1 is involved in transcriptional regulation as repressor. However, noticeable transcription repression domains such the EAR motif \(^{48}\) could not be identified within the TH1 protein (Fig. 4). Previously, the ALOG domain was postulated to function as a DNA-binding domain derived from a novel class of DIRS-1-like retrotransposons \(^{42}\). If this is the case, binding of the ALOG proteins to target DNA might create a repressive chromatin state thus suppress transcription of the target gene. Further studies will be required to identify the target genes regulated by TH1. TH1 likely functions as an upstream modulator of cell expansion via the regulation of downstream target genes during rice lemma and palea development.

Some previously reported allelic mutants such as th1-2\(^{33}\), bls1-1, bls1-2\(^{35}\) have large-fragment deletion in which the TH1 gene is completely deleted. Other allelic mutant such as th1-1\(^{33}\), th1-6569, bgI-1\(^{37}\) and afd1\(^{36}\)

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**Figure 8.** Requirement of the TH1 transcriptional repression activity in spikelet development. (a) Schematic representation of the vectors transformed into the s2-89 mutant. Four copies of transcription activation domain VP16 or repression domain EAR were fused with the TH1 s2-89 mutant protein. (b) Spikelet morphology of transgenic lines expressing the VP16-TH1 s2-89 fusion protein (B192-2) and the EAR-TH1 s2-89 fusion protein (B193-2) in s2-89 mutant. (c) Comparisons of grain length, grain width, length-width ratio and grain thickness in WT, s2-89 and the transgenic lines. Data are means ± SE (n = 20). Double asterisks indicate significant differences at P < 0.01 by Student's t test. Each scale bar is indicated.
have small-fragment deletion which cause frame-shift mutation in the TH1 gene. These deletion mutants provide little information about the functional domains and critical amino acids required for the proper function of the TH1 protein. In this study, the s2-89, xd281 and xd425 mutant all contain missense point mutations in the ALOG domain. The three TH1 mutant proteins TH1 s2-89 (His129Tyr), TH1 xd281 (Arg80Trp) and TH1 xd425 (Gly124Gln) lost the ability to interact with itself (Fig. 6), indicating that Arg80, Gly124 and His129 are critical for the homodimerization of TH1. Moreover, we found that the His129Tyr substitution relieved the transcriptional repression activity of TH1 (Fig. 7c). Therefore, our study identified several critical amino acids in the ALOG domain essential for the TH1 function.

Methods

Plant materials and growth conditions. Four rice mutants, s2-89, xd151, xd281 and xd425, with beak-like spikelet were identified from an M2 population mutagenized with ethyl methane sulfonate (EMS). The s2-89 mutant was derived from japonica cv. Nipponbare and xd151, xd281 and xd425 from japonica cv. Xu Dao3. Corresponding cultivars were used as the wild type strains for phenotype comparison. The s2-89 mutant was crossed with the indica variety Dular for genetic analysis and gene mapping. All of the parents, F1 hybrids and corresponding F2 individuals were grown in paddy fields at Beijing and Hainan under natural conditions.

Analysis of the mutant phenotype. Plant phenotypes were photographed with a Canon digital camera. Harvested rice grains were air-dried and stored at room temperature before testing. At least 30 mature grains were measured for grain length, width and thickness using calipers. Grain weight was calculated on the basis of 200 grains and converted to 1,000-grain weight.

Scanning electron microscopy (SEM) observation. The rice spikelet samples of the wild type and mutant were harvested at heading stage in normal growth conditions. The rice spikelet samples were fixed in 3.5% glutaraldehyde solution and then dehydrated through an ethanol series. After dehydration process, the samples were dried by critical-point drying method and sputter-coated with platinum, and then observed using a variable pressure scanning electron microscope (Hitachi, Tokyo).

Histological analysis and GUS staining. The rice young spikelet hulls of wild type and the mutant at booting stage were fixed in FAA solution at 4 °C overnight, dehydrated in a graded ethanol series, and substituted with xylene. Finally, the samples were embedded in paraplast (Sigma, St. Louis, MO). Thin sections (8-10 μm) were prepared using a rotary microtome, dewaxed in xylene, hydrated through a graded ethanol series, stained with xylene. Finally, the samples were embedded in paraplast (Sigma, St. Louis, MO). Thin sections (8-10 μm) were prepared using a rotary microtome, dewaxed in xylene, hydrated through a graded ethanol series, stained with 1% fast green and observed using a light microscope and photographed. The cell length and width of lemma and palea were measured using the Image J software.

Map-based cloning. The s2-89 mutant was crossed with the indica variety Dular to develop the F2 population. Mutant individuals showing the beak-like spikelet in the F2 population were used for map-based cloning. Primary mapping was conducted with 180 pairs of InDel markers using 32 F2 mutant individuals. Fine mapping was performed using new markers developed based on genomic polymorphism between Dular and Nipponbare. The candidate gene was amplified and sequenced from both the s2-89, xd151, xd281 and xd425 mutants and wild type genomic DNA. The primer sequences for the INDEL markers and sequencing the candidate gene are listed in the Supplementary Table S3.

RNA extraction and quantitative real-time PCR analysis. Total RNA was extracted from various rice tissues and organs of the wild type and mutant plants using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and was reverse transcribed using the M-MLV Reverse Transcriptase kit (Invitrogen), following the manufacturer’s instructions. Quantitative real-time PCR analysis was performed with a SYBR Premix Ex Taq2 (TaKaRa) kit and run on an Applied Biosystems 7500 Real-Time PCR System. The rice Ubiquitin gene (LOC_Os03g13170) was amplified as an internal control for loading normalization. The amplification program was as follows: 95 °C for 3 min, followed by 40 cycles at 95 °C for 30 s, and 60 °C for 1 min. All primers used are listed (see Supplementary Table S3). Relative transcript levels were calculated using the 2−ΔΔCT quantification method44.

Plant expression vector construction and rice transformation. To make the genomic DNA complementation vector, the 3,872 bp genomic DNA fragment including 2,354 bp upstream of start codon and 771 bp downstream of stop codon was amplified from the wild type Nipponbare using primers TH1-CF/R (see Supplementary Table S3) and cloned into the EcoRI and PmlI sites of the plant expression vector pCAM-BIA305.1. To make plant expression vectors harboring EAR-TH1−∆∆ or VP16-TH1−∆∆, the full-length coding sequence of TH1 was amplified from the s2-89 mutant using primers attB-TH1F/R (see Supplementary Table S3). PCR product was cloned into the plant expression vector LP041nEAR-hyg-asRED or LP042nVP64-hyg-asRED using the Gateway system55, in which four copies of the transcriptional repression motif EAR or activation motif VP16 were fused at the N-terminus of TH1. The genomic DNA complementation, EAR-TH1−∆∆ and VP16-TH1−∆∆ constructs were transformed into the s2-89 mutant using an Agrobacterium Tumefaciens-mediated transformation method. The CYCB1;1-GUS fusion construct was constructed as described46,47. A translational fusion of the 1990 bp fragment upstream of the OsCYCB1;1 (LOC_Os01g59120) start codon and a 912 bp fragment of the ORF, starting at the ATG start site, which encodes an N-terminal 124 amino acids containing a mitotic degradation box, was amplified using primers CYCB1;1 F/R (see Supplementary Table S3). The 2902 bp PCR product
was digested with HindIII and NcoI, and inserted into the binary vector pCAMBIA1305.1 as an in-frame fusion with the Gus gene. The CYCB1;1–GUS fusion construct was transformed into the s2-89 heterozygous mutant. Gus staining was conducted in the T1 plants showing the wild type and s2-89 mutant phenotype, respectively.

**Transcription activation activity assay in a yeast two-hybrid system.** The full-length TH1 coding region from the wild type and the mutants was amplified by PCR using primer BD-TH1F/R (see Supplementary Table S3) and cloned into the pGBK7 vector (Clontech). The resultant constructs were co-transformed with the empty pGADT7 vector (Clontech) into yeast strain AH109 containing the GAL4-UAS-β-galactosidase reporter gene. The transformants were grown on the solid medium lacking leucine and tryptophan. The β-Galactosidase activity was detected using the Colony-lift Filter Assay according to the manufacturer’s user manual. For different formation assays, the full-length TH1 coding region from the wild type and the mutants was amplified by PCR using primer AD-TH1F/R (see Supplementary Table S3) and cloned into the pGADT7 vector. The resultant constructs were co-transformed with the pGBK7 vector containing different TH1 alleles into yeast strain AH109. The transformants were grown on selective solid medium SD-Leu-Trp (DDO) or SD-Leu-Trp-His-Ade (QDO), Yeast colony co-transformed with pGBK7–s3 and pGADT7–T (Clontech) was used as positive control, while yeast colony co-transformed with pGBK7–Lam and pGADT7–T (Clontech) was used as negative control.

**Transient expression assays.** Protoplast isolation and transient expression assays were performed as described previously. For subcellular localization experiments, the TH1 coding region from the wild type and mutants was amplified using primers Actin-Prom:TH1CDS:GFPF/R (see Supplementary Table S3) and ligated into the SpeI and NcoI sites of the transient expression vector pAN580, which contains the open reading frame of enhanced green fluorescent protein (EGFP) driven by the CaMV 35S promoter. The recombinant plasmids were introduced into protoplasts isolated from wild type rice seedlings and the transformed protoplasts were incubated at 28°C for 16 h. Green fluorescence of the GFP fusion protein was observed under a Zeiss LSM 510 META confocal microscope.

The transcriptional activity of TH1 was examined using the luciferase transient expression assays in rice protoplasts. The reporter plasmid Pro35S–GAL4:LUC contains a CaMV 35S promoter, 5 × GAL4 binding site, a TATA box in front of the firefly luciferase (LUC) gene and a nopaline synthase terminator. The empty effector plasmid p35S–GAL4BD:VP16 and p35S–GAL4BD:EAL (see Supplementary Figure S1) was adapted from the pSAT–GAL4DB plasmid. The coding sequence of TH1 from the wild type and s2-89 mutant was inserted into the BglII and BamHI sites to generate the p35S–GAL4DB–TH1 construct, or the BglII and KpnI sites to generate the p35S–GAL4DB–TH1–VP16 and p35S–GAL4DB–TH1–EAL constructs. Each transformation used 5 μg of reporter plasmid, 4 μg of effector plasmid and 1 μg of p35S–REN plasmid expressing the Renilla luciferase as an internal control. The activities of firefly and Renilla luciferases were measured sequentially from a single sample using the Dual-Luciferase Reporter assay kit (Promega, Cat. no. E1910). The relative activity of experimental reporter was expressed as the LUC/REN ratio.

**Data availability.** All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

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Author Contributions
X.L. and P.P. designed the project. P.P., L.L., J.F., J.Z., S.Y. performed the experiments and analyzed the data. P.P. and X.L. wrote the manuscript. All authors reviewed the manuscript.

Additional Information
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