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A role for *TRIANGULAR HULL1* in fine-tuning spikelet morphogenesis in rice

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The lemma and palea, which enclose the pistil, stamens, and lodicules, are the most conspicuous organs in the rice spikelet. We isolated a mutant line (ng6569) in which the lemma and palea were narrower than those of the wild type, and found that the mutant had a defect in *TRIANGULAR HULL1* (*TH1*), which encodes a nuclear protein with an ALOG domain. Detailed morphological analysis indicated that the *th1* mutation caused a reduction in the size of tubercles, which are convex structures on the surface of the lemma and palea. This reduction was more pronounced in the apical region of the lemma than in the basal region, resulting in the formation of a beak-like spikelet. By contrast, the number of tubercle rows and their spatial distribution on the lemma were not affected in the *th1* mutant. Thus, the *TH1* gene seems to be involved in fine-tuning the morphogenesis of the lemma and palea. In situ hybridization analysis revealed that *TH1* was highly expressed in the primordia of the lemma and palea, but only weakly expressed in the primordia of the sterile lemma and rudimentary glume. We then examined the effect of *th1* mutation on the lemma-like structure formed in the *long sterile lemma/glume1* (*g1*) and *extra glume1* (*eg1*) mutants. The result showed that the *th1* mutation strongly affected the morphology of the extra lemma of *eg1*, but had no significant effect on the transformed lemma of *g1*.

Key words: flower development, lemma, morphology, rice, spikelet

INTRODUCTION

Flower morphology and inflorescence architecture are highly diverse among angiosperms. Rice (*Oryza sativa*) produces a unique inflorescence consisting of spikelets and branches (Hirano et al., 2014; Tanaka et al., 2013, 2014). The spikelet is an inflorescence unit composed of a floret, two sterile lemmas, and two rudimentary glumes. The floral organs, namely the carpel, stamen and lodicule (a petal homolog), are enclosed by the lemma and palea within each floret. Although a pair of glumes surround several spikelets in other grass species, the so-called rudimentary glumes of rice are highly degenerate and tiny. Therefore, the lemma and palea are the most conspicuous organs in rice spikelets. These two organs have a rough epidermal surface covered with convex structures called tubercles (Tanaka et al., 2014; Terrell et al., 2001; Yoshida et al., 2009). The tubercles are aligned in vertical rows that alternate with longitudinal furrows (Terrell et al., 2001), and rigid, prickle-like trichomes are formed in the furrows.

The molecular genetic mechanism of flower development is well characterized in eudicots such as *Arabidopsis thaliana* and *Antirrhinum majus* (Lohmann and Weigel, 2002; Prunet and Jack, 2014). The ABC model, which describes the mechanism of floral organ specification, is a central paradigm in flower development (Coen and Meyerowitz, 1991) and is likely to be largely applicable to the specification of floral organs in rice, which are formed in the specialized inflorescences described above (Hirano et al., 2014; Tanaka et al., 2014). Class B genes in rice specify the lodicule and stamen, whereas *Arabidopsis* class B genes specify the petal and stamen (Nagasawa et al., 2003; Yao et al., 2008). As for class C genes, *OsMADS3* and *OsMADS58* are involved in stamen specification and flower meristem determinacy, respectively, in rice (Yamaguchi et al., 2006). A major difference in flower development between *Arabidopsis* and rice is the gene required for carpel specification: whereas the class

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C MADS-box gene *AGAMOUS* is responsible for carpel specification in *Arabidopsis*, the *YABBY* gene *DROOPING LEAF* fulfills this function in rice (Yamaguchi et al., 2004).

Rapid progress has been made recently in pinpointing the genes that regulate the identity and morphology of non-floral spikelet organs, including the lemma, palea, and sterile lemma (Hirano et al., 2014; Tanaka et al., 2014). For instance, *LEAFY HULL STERILE1* (*LHS1*)/*OsMADS1* specifies the identity of both the lemma and palea, in addition to regulating the determinacy of the spikelet meristem (Jeon et al., 2000). *DEPRESSED PALEA1* (*DP1*) predominantly regulates the development of the palea (Jin et al., 2011), whereas extra lemma-like organs are formed in the *extra glume1* (*eg1*) mutant (Li et al., 2009). The sterile lemma is homeotically transformed into a lemma in the *long sterile lemma*/*glume1* (*g1*) mutant, suggesting that *G1* specifies sterile lemma identity (Yoshida et al., 2009). Thus, mutations in these genes cause dramatic alterations in the spikelet. By contrast, the *triangular hull1* (*th1*) mutation causes a slight change in the shape of the lemma and palea (Li et al., 2012).

In this study, we found that *TRIANGULAR HULL1* (*TH1*) regulates the size of tubercles and trichome density in the lemma. The reduction in lemma width in *th1* seems to result from the reduction in tubercle size. This observation suggests that *TH1* fine-tunes the morphogenesis of the lemma and palea. Analyses of *th1 g1* and *th1 eg1* double mutants revealed that the *th1* mutation affects the morphology of ectopic lemmas, i.e., the homeotically transformed lemma in *g1* and the extra lemma in *eg1*. However, the effect of the *th1* mutation differed between the two ectopic lemmas.

MATERIALS AND METHODS

Plant materials The mutant line ng6569 was identified among mutants which had been roughly selected from the *TOS17* mutant panel (Miyao et al., 2007). Seeds of the *g1-1* and *eg1* mutants were kindly provided by Dr. Atsushi Yoshimura (Kyushu Univ.) (Yoshida et al., 2009). Nippongare was used as wild-type strain for comparing phenotypes and in situ expression analysis.

Morphological analyses To analyze spikelet morphology, photographs of spikelets were taken using an SZX10 stereomicroscope (Olympus, Tokyo, Japan). The images were analyzed using ImageJ software (NIH, Bethesda, MD), and each part of the spikelet was measured according to the ImageJ instruction.

Observation of epidermal surface of the lemmas and ectopic lemmas was performed using scanning electron microscopy (SEM), as described previously (Suzaki et al., 2004; Toriba et al., 2010). Spikelets were fixed in the

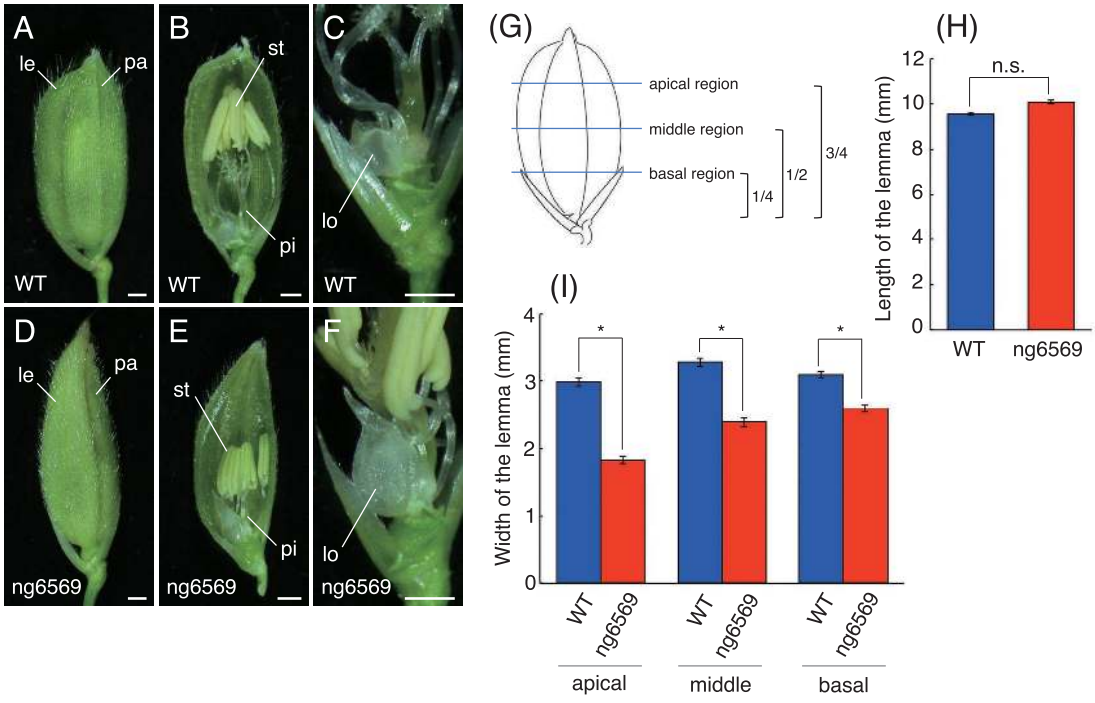

Fig. 1. Spikelet phenotypes of *th1-6569* (ng6569) and wild type. (A, B, D, E) Spikelet phenotypes of wild type (A, B) and *th1-6569* (D, E). (C, F) Close-up view of the lodicule of wild type (C) and *th1-6569* (F). (G) The three regions measured in this study. (H) Longitudinal length of the lemma. (n.s., not significant by Student's t-test at $P < 0.01$, $n = 20$; bar, standard error (SE).) (I) Width of the lemma at the three positions shown in (G). (Asterisk, significant by Student's t-test at $P < 0.0001$, n = 20; Bar, SE.) le, lemma; lo, lodicule; pa, palea; pi, pistil; sl, sterile lemma; st, stamen. Scale bars: 1 mm.

PFA solution containing 4% (w/v) paraformaldehyde, 0.25% glutaraldehyde and 0.1 M sodium phosphate buffer (pH 7.2) at 4°C overnight. After replacement with 3-methylbutyl acetate, the samples were dried at their critical point, sputter-coated with platinum, and observed with a scanning electron microscope (model JSM-6510LV; JEOL, Tokyo, Japan) at an accelerating voltage of 10 kV.

In situ hybridization To make the *TH1* probe, a 453-bp fragment corresponding to the 3' untranslated region and part of the C-terminal region was amplified with the primers 5'-CTCGTCCGCCGGCGCCGGAGC-3' and 5'-GCCCAAATTAAAGCATTTGGCATATA-3', and cloned into the pCRII vector (Invitrogen, Carlsbad, CA). To generate an antisense probe, RNA was transcribed with T7 RNA polymerase and labeled with digoxigenin (Roche, Mannheim, Germany). Immature inflorescences of rice were fixed with the PFA solution for about 16 h at 4°C. After dehydration and substitution with xylene, the samples were embedded in Paraplast Plus (McCormick Scientific, St. Louis, MO) and sectioned at 10-µm thickness by a rotary microtome. Paraffin removal, protease treatment, hybridization, washing, and immunological detection of the transcripts were performed according to the procedure described by Kouchi and Hata (1993).

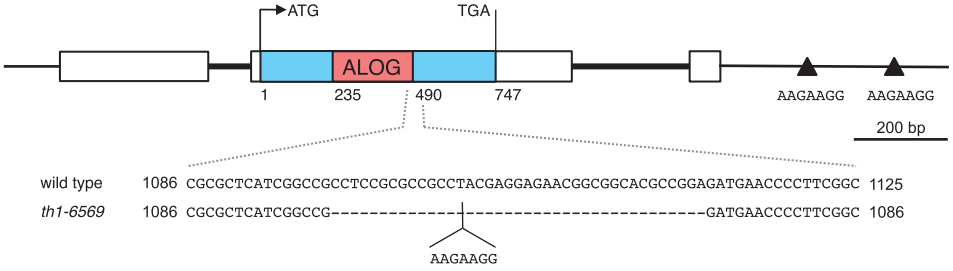

Fig. 2. Identification of the gene responsible for the mutation in *th1-6569*. Schematic representation of *TH1*. Boxes indicate exons, and thick and thin lines indicate introns and untranscribed regions, respectively. Colored boxes indicate the translated region, and the red box shows the ALOG domain. Triangles indicate the positions of sequences that are identical to the 7-bp insertion within the 39-bp deletion (denoted as gaps in the mutant sequence). The numbers below the translated region indicate amino acid positions.

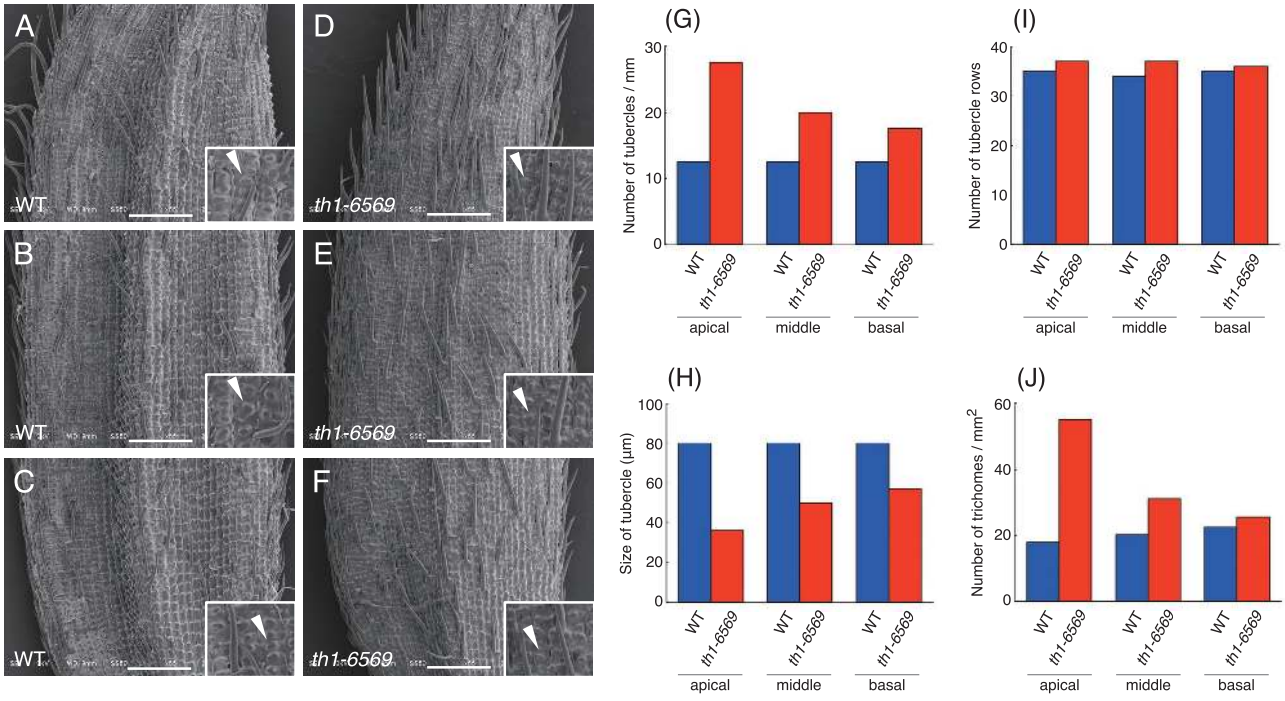

Fig. 3. Characteristics of the epidermal surface of the lemma. (A–F) SEM images of the epidermal surface of the lemma of wild type (A–C) and *th1-6569* (D–F). (A, D), (B, E), and (C, F) indicate apical, middle, and basal regions of the lemma, respectively. Insets show enlarged views, with arrowheads indicating tubercles. (G) Number of tubercles per unit width. (H) Size of the tubercles. (I) Number of tubercle rows per lemma width. (J) Trichome density. Scale bars: 500 µm.

RESULTS

A recessive morphological mutant showing slender spikelets We isolated a recessive mutant line (ng6569) that showed abnormal spikelet morphology. Both the lemma and the palea were more slender than those of the wild type, and the spikelet exhibited a pointed beak-like shape (Fig. 1, A and D). Whereas the pistil and stamens were normal (Fig. 1, B and E), the lodicules of the mutant were pointed and larger than those of the wild type (Fig. 1, C and F).

We then compared several dimensions of the lemma in the ng6569 line and wild type (Fig. 1G). The longitudinal length of the lemma was unaffected by this mutation (Fig. 1H), but its width was significantly reduced (Fig. 1I). In particular, the apical region of the lemma was strongly affected. Similarly, the width of the palea was reduced predominantly at the apical region (Fig. 1, D and E). Therefore, the pointed beak-like spikelet of this mutant appears to result from a reduction in the width of the apical region of the lemma and palea.

ng6569 has a mutation in *TH1*, which encodes a protein containing the ALOG domain While we were analyzing the ng6569 line, a mutant named *triangular hull1* (*th1*) was reported (Li et al., 2012). *th1* produces narrow spikelets, similar to those in the ng6569 line. To test whether the ng6569 line had a mutation in *TH1*, we amplified and sequenced DNA segments from the genomic region of *TH1* in the ng6569 line. We found that the *TH1* gene of the ng6569 line had a 39-bp deletion and a 7-bp insertion within the deleted region (Fig. 2). Two DNA regions with the same nucleotide sequence as that of the 7-bp insertion were found downstream of *TH1*. The simultaneous deletion of a DNA segment and insertion of an extra, unrelated DNA segment is often caused by the integration and subsequent excision of transposons (Kikuchi et al., 2003; Wessler et al., 1990). The inserted "filler DNA" is derived from the sequence near the integration site of the transposon. It is therefore possible that the mutation detected in *TH1* results from an integration/excision event of an unknown transposon.

TH1 encodes a nuclear protein with an ALOG domain, and identical to *OsG1L6* (Os02g0811000) in the rice ALOG gene family, which is previously reported (Yoshida et al., 2009). The mutation in ng6569 was found in the region corresponding to the C-terminal portion of the ALOG domain and its flanking region, and gave rise to a frameshift mutation in the *TH1* gene. Thus, the slender spikelet in ng6569 appears to be caused by the mutation in *TH1*, and we refer henceforth to the ng6569 line as the *th1-6569* mutant.

Epidermal morphology of the *th1-6569* mutant To analyze its morphological characteristics, we examined the epidermal surface of the lemma in *th1-6569* by SEM. The wild-type lemma produced a number of tubercles and trichomes on its abaxial surface (Fig. 3, A–C) (Terrell et al., 2001; Yoshida et al., 2009). Whereas these structures were also observed in *th1-6569*, they were altered in size and number (Fig. 3, D–F).

First, we counted the tubercles along the horizontal axes. The number of tubercles per unit width was increased in the lemma of *th1-6569* in all regions of the lemma examined, compared to the wild-type lemma (Fig. 3G). Consistent with this observation, the tubercle size along the horizontal axis was reduced in *th1-6569* (Fig. 3H). By contrast, the number of rows of tubercles per unit lemma width in each region was unaffected in *th1-6569* (Fig. 3I). Therefore, tubercle patterning was normal in *th1-6569* and it is unlikely that *TH1* regulates the

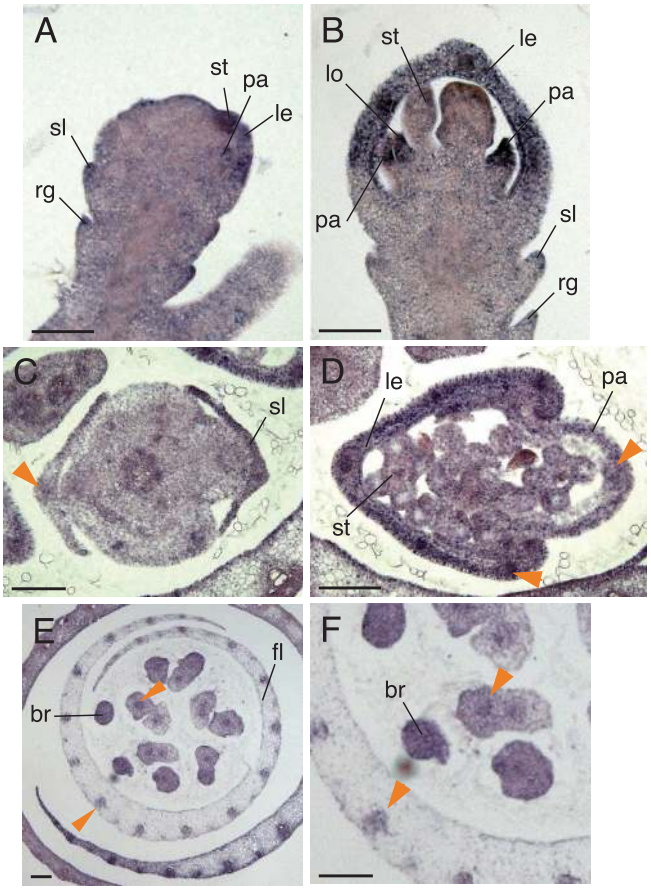

Fig. 4. Spatial expression patterns of *TH1*. (A, B) Longitudinal sections of developing spikelets in the early (A) and middle (B) developmental stages. (C, D) Cross-section of a developing spikelet through the basal (C) and middle (D) regions. (E) Section showing the leaves and inflorescence branches. (F) Close-up view of a part of (E). Arrowheads indicate vasculature. br, inflorescence branch; fl, flag leaf; le, lemma; lo, lodicule; st, stamen; pa, palea; rg, rudimentary glume; sl, sterile lemma. Scale bars: 100 μm.

alignment of the tubercles.

SEM observations revealed that trichome density was increased in the lemma of *th1-6569* (Fig. 3J). The trichome density was considerably higher in the apical region of the lemma than in the middle and basal regions.

Spatial expression pattern of *TH1* To examine the spatial expression patterns of *TH1* during spikelet development, we performed in situ hybridization analysis. In early development, *TH1* transcripts were detected in the primordia of the rudimentary glume and sterile lemma (Fig. 4A). Signals were also detected in the primordia of the lemma, palea, and stamen (Fig. 4A). In the subsequent stage, strong *TH1* expression was observed in the primordia of the lodicule, palea, and lemma (Fig. 4B). The *TH1* transcripts were largely distributed in the distal part of the lemma and were not detected in the basal part. In cross sections, *TH1* signals were detected in the sterile lemma (Fig. 4C), lemma and palea (Fig. 4D). The signal was particularly strong in the lemma.

TH1 expression was also observed in the leaves surrounding a developing inflorescence (Fig. 4E). The *TH1* signal was localized to the vascular bundle in leaves (Fig. 4, E and F). Whereas *TH1* transcripts were detected

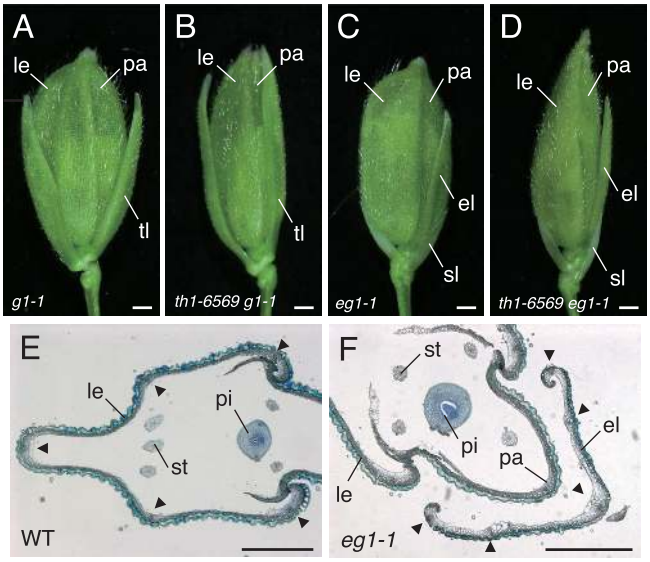

Fig. 5. Aberrant spikelet phenotypes of *g1-1*, *eg1-1*, *th1-6569 g1-1* and *th1-6569 eg1-1*. (A–D) Spikelets of the *g1-1* (A), *th1-6569 g1-1* (B), *eg1-1* (C) and *th1-6569 eg1-1* mutants (D). (E, F) Cross-section of the spikelets of wild type (E) and *eg1* mutant (F). el, extra lemma; le, lemma; st, stamen; pa, palea; pi, pistil; sl, sterile lemma; tl, homeotically transformed lemma. Scale bars: 1 mm (A–D), 500 μm (E, F).

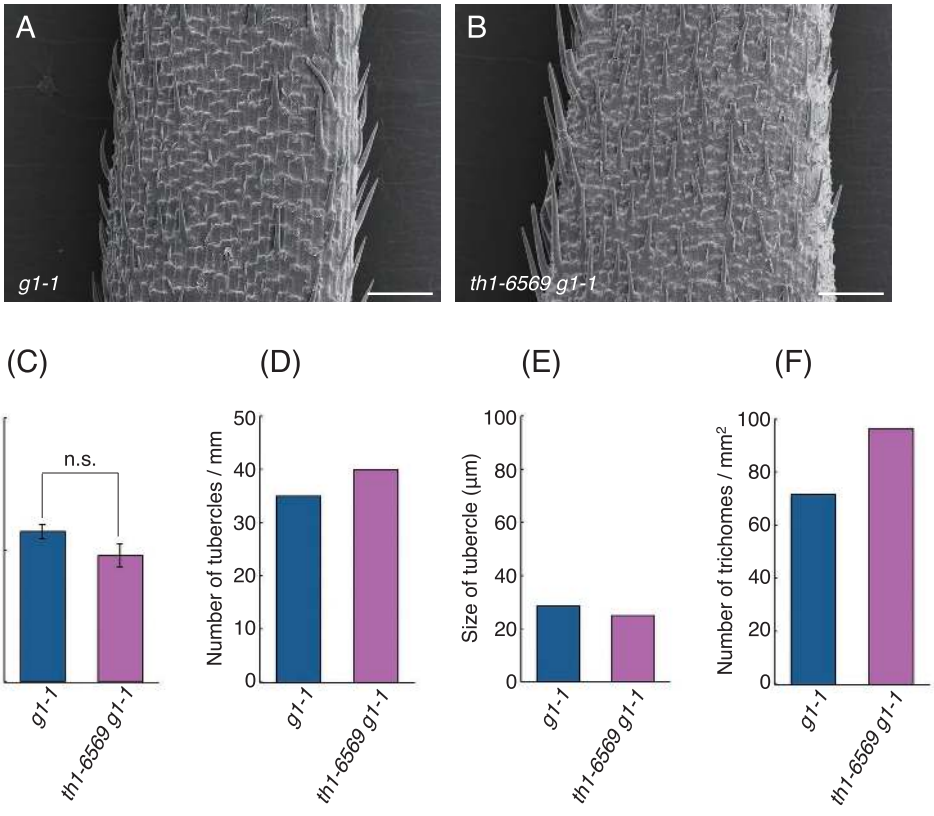

Fig. 6. Effect of the *th1* mutation on the transformed lemma generated by the *g1* mutation. (A, B) SEM images of the epidermal surface of the homeotically transformed lemma in *g1-1* (A) and the *th1-6569 g1-1* double mutant (B). (C) Width of the transformed lemma (n.s., not significant by Student's t-test at $P < 0.01$, n = 20; Bar, SE). (D) Number of tubercles per unit width of transformed lemma. (E, F) Tubercle size (E) and trichome density (F) in the transformed lemma. Scale bars: 200 μm.

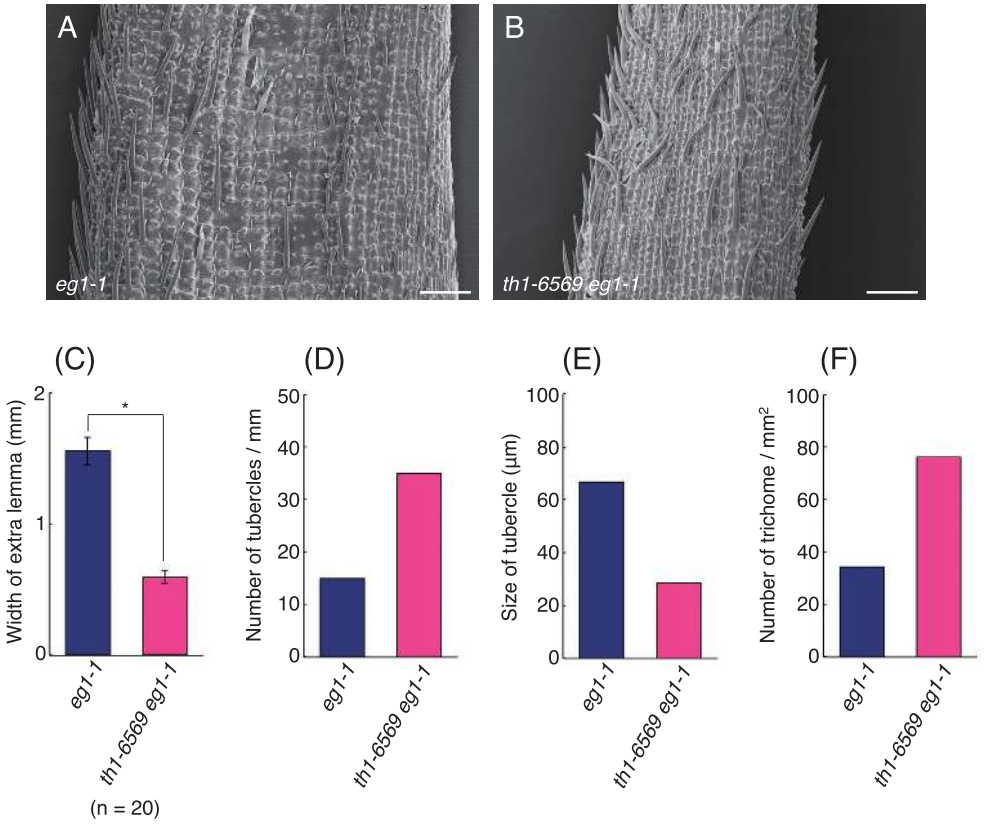

Fig. 7. Effect of the *th1* mutation on the extra lemma generated by the *eg1* mutation. (A, B) SEM images of the epidermal surface of the extra lemma in the *eg1-1* mutant (A) and the *th1-6569 eg1-1* double mutant (B). (C) Width of the extra lemma (asterisk, significant by Student's t-test at $P < 0.0001$, n = 20; Bar, SE). (D) Number of tubercles per unit width of the extra lemma. (E, F) Tubercle size (E) and trichome density (F) in the extra lemma. Scale bars: 200 μm.

throughout the inflorescence branches, strong signals were localized to the central region, at the putative provascular bundle. *TH1* expression was also elevated in the vasculature of the lemma, palea and sterile lemma (Fig. 4, C and D).

Effect of the *th1* mutation on mutants producing ectopic lemmas In the *g1* mutant, the sterile lemma is homeotically transformed into the lemma (Fig. 5A) (Yoshida et al., 2009). We asked here whether the *th1* mutation affects the morphology of this homeotically transformed lemma. We generated a *th1 g1* double mutant, in which the regular lemma and palea appeared to be more slender than those of the *g1* single mutant (Fig. 5, A and B; Fig. 6). However, no statistically significant difference ($P < 0.01$) was observed in the width of the homeotically transformed lemma between *g1* and *th1 g1* (Fig. 6C). The tubercle number per unit width and tubercle size were unchanged, whereas the density of trichomes appears to be slightly increased in the *th1 g1* double mutant (Fig. 6, D–F). Thus, the *th1* mutation does not substantially affect the morphology of the transformed lemma in *g1*.

The *eg1* mutant forms an extra organ in the region between the sterile lemma and the lemma/palea or in the lateral region of the spikelet (Fig. 5C) (Li et al., 2009). Morphological analyses showed that the extra organ in *eg1* has characteristics of the wild-type lemma: the marginal region of the extra organ curled inwards, and the organ contained five vascular bundles (Fig. 5F), similar to the wild-type lemma (Fig. 5E). The *th1* mutation also affected the lemma and palea in the *eg1* mutant background, resulting in a spikelet that was more slender than that in *eg1* (Fig. 5, C and D; Fig. 7). The extra lemma was narrower in the *th1 eg1* double mutant than in the *eg1* single mutant (Fig. 7C). The tubercles were much smaller in the extra lemma of the double mutant, resulting in a marked increase in the number of tubercles per unit width (Fig. 7, D and E). In addition, trichome density was higher in the extra lemma of the *th1 eg1* double mutant (Fig. 7F). Thus, mutation of *th1* strongly affects the morphology of the extra lemma in the *th1 eg1* mutant.

DISCUSSION

In this study, we isolated a morphological mutant line, ng6569, which showed an unusually slender lemma and

palea. Sequence analyses revealed that the ng6569 line had a mutation in *TH1*, the loss-of-function of which was reported to result in a similar spikelet phenotype to that observed in ng6569 (Li et al., 2012). A similar mutant, which has a mutation in *TH1* and is named *BEAK LIKE SPIKELET1* (*BLS1*), has been reported (Ma et al., 2013). To unify the gene nomenclature, we referred to our mutant as an allele of *th1* (*th1-6569*), considering the priority of *TH1* in the literature.

The *th1-6569* mutant had a defect in a gene encoding a protein with an ALOG domain. The ALOG domain was defined based on the conserved amino acid sequence in the *Arabidopsis LIGHT-DEPENDENT SHORT HYPOCOTYLS1* (*LSH1*) and rice *G1* genes (Yoshida et al., 2009; Zhao et al., 2004). The ALOG domain is distributed throughout land plants and is highly conserved (Yoshida et al., 2009). Although little is known about the ALOG family in *Arabidopsis*, recent reports have demonstrated that ALOG family genes play important roles in rice (Li et al., 2012; Ma et al., 2013; Yoshida et al., 2009, 2013). As described above, a founding member of the ALOG family, *G1*, specifies sterile lemma identity by repressing lemma characteristics. Another member of this family, *TAWAWA1* (*OsG1L5*), regulates the transition from branch meristem to spikelet meristem (Yoshida et al., 2013). *TH1* is associated with structural features of the epidermal surface and lateral growth of the lemma and palea, as described here (Figs. 1 and 2) and in previous works (Li et al., 2012; Ma et al., 2013). Thus, the function of *ALOG* genes may be closely associated with the developmental programs of inflorescences and spikelets in rice.

The spatial expression patterns of *TH1* were previously studied using promoter-GUS analysis (Li et al., 2012; Ma et al., 2013). Because these analyses showed the localization of GUS reporter activity only after the completion of spikelet development, *TH1* expression during spikelet development and its detailed spatial distribution in organ primordia remained to be elucidated. In addition, promoter-GUS analyses do not necessarily report the true expression pattern of genes, because, in many cases, rice genes require long upstream sequences and/or introns for proper expression (Ohmori et al., 2011). To address this, we analyzed the spatial and temporal patterns of *TH1* transcripts by in situ hybridization. The in situ analysis revealed that *TH1* was expressed in all spikelet organs, from the early stages of spikelet development. Strong expression was detected in the lodicule and lemma primordia in the middle to later stages (Fig. 4, B and D). This strong *TH1* expression may be required for the normal morphological development of the lemma and lodicule. The narrow lemma in the *th1* mutant seems to be associated with a reduction in tubercle size. Tubercles are convex structures that accumulate a high amount of silicon. How tubercle size is regulated is unknown, and it would be interesting to determine whether *TH1* controls the size or the number of cells underlying tubercles. *TH1* expression was detected in the vasculature in the lemmas, paleas, inflorescence branches, and leaves. No defects were observed in the vascular patterning of lemmas/paleas or leaves in the *th1* mutant. Because the rice genome possesses 10 members of the ALOG family, it is possible that other ALOG paralogs largely compensate for the loss-of-function of *TH1*.

The width of the lemma was reduced in *th1-6569*, as for other alleles (Li et al., 2012; Ma et al., 2013). Our results suggest that the spatial distribution of tubercles is not affected by the *th1* mutation (Fig. 4). By contrast, lemma width and tubercle size changed along the longitudinal axis, with the apical region being more severely affected by this mutation than the middle and basal regions (Figs. 1 and 2). A comparison of the three regions of the lemma revealed that the decrease in lemma width towards the apex corresponded with a proportional decrease in tubercle size in the *th1* mutant (Fig. 8). This result strongly supports the idea that the reduction in lemma width is correlated to the reduction in tubercle size (along horizontal axis) in *th1*. By contrast, along the longitudinal axis of the lemma, there was no difference in tubercle number or tubercle size between wild type and *th1-6569* (Fig. 3. A–F). Thus, *TH1* appears to specifically regulate the width of the lemma by controlling tubercle size (breadth). In *th1*, the trichome density was increased in the apical region of the lemma. Thus, *TH1* function seems to mainly affect the morphology of the apical region of the lemma.

The *g1* and *eg1* mutants produce aberrant lemma-like organs (Li et al., 2009; Yoshida et al., 2009). We found that the *th1* mutation had different effects on the homeotically transformed lemma of the *g1* mutant and the extra lemma of the *eg1* mutant. In the *th1-6569 eg1* double mutant, the width of the additional lemma was reduced

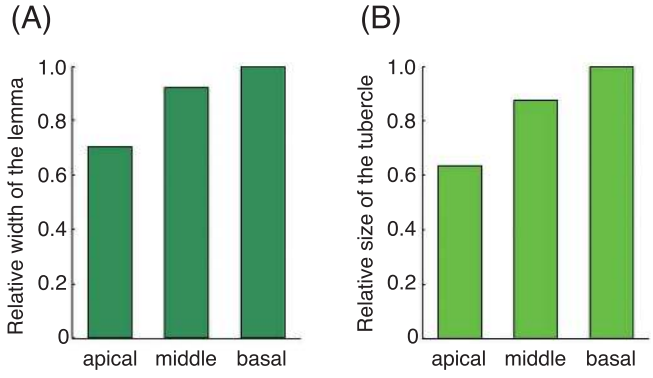

Fig. 8. Correlation between lemma width and tubercle size in *th1-6569*. Comparison of the relative width of the lemma (A) and the relative size of the tubercles (B) in each horizontal region of the *th1-6569* lemma. Values are relative to those of the basal region.

through a reduction in tubercle size, as was the case for the regular lemma. By contrast, *th1* had no significant effect on tubercle size or width of the homeotically transformed lemma in *g1*. The tubercle size of the transformed lemma in *g1* is smaller than that of the lemma in wild type and the extra lemma in *eg1* (Figs. 3H, 6E and 7E). It is therefore possible that the *th1* mutation is not effective in the reduction of tubercle size in the transformed lemma in *g1*. Alternatively, the difference in the *th1* effect may be related to the origin of the two ectopic organs. In the *eg1* mutant, the extra lemma is formed de novo and is independent of other organs. Therefore, the *th1* mutation may affect the extra lemma in a similar manner as it does the original lemma. By contrast, in the *g1* mutant, the sterile lemma is homeotically transformed into a lemma-like organ. In this case, residual activities of genes responsible for sterile lemma formation may weaken the effect of loss of *TH1* function.

Much progress has been made in our understanding of the molecular genetic regulation of spikelet development in rice (Hirano et al., 2014; Tanaka et al., 2014). Mutations in genes such as *LHS1*, *DP1*, *OPEN BEAK* (*OPB*)/*STAMENLESS1* (*SL1*), *MOSAIC FLORAL ORGAN1* (*MFO1*), *MULTI-FLORET SPIKELET1* (*MFS1*) and *RETARDED PALEA1* (*REP1*) (Jeon et al., 2000; Jin et al., 2011; Ohmori et al., 2009; Ren et al., 2013; Yuan et al., 2009) that regulate the development of non-floral spikelet organs cause morphological alterations in various spikelet organs, including the floral organs. In addition, *LHS1*, *MFO1* and *MFS1* seem to be associated with meristem function. By contrast, the effect of the *th1* mutation is small and independent of the meristem, consistent with the idea that *TH1* acts in the final stages of lemma and palea formation by controlling the size of the tubercles. Thus, *TH1* is likely to be involved in the fine-tuning of lemma/palea morphogenesis.

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