Characterization of a *half-pipe-like leaf*1 mutant that exhibits a curled leaf phenotype

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Leaf forms are diverse in angiosperms, and different types of cells are differentiated depending on the species. Rice leaves are composed of a leaf blade, a leaf sheath and the junction region between them. Cells with characteristic features, such as bulliform cells and sclerenchyma cells, are differentiated in the leaf blade, together with standard epidermal and mesophyll cells. To understand the genetic mechanism underlying leaf morphogenesis in rice, we focused on a mutant, *half-pipe-like leaf*1 (*hal1*), whose leaves are adaxially curled. Histological observation revealed that the bulliform cells, which are responsible for leaf rolling under dry conditions, were small in size and abnormal in shape in a semidominant *hal1-d* mutant. Bulliform cell files were often ambiguous in semi-transparent *hal1-d* leaves cleared by the TOMEI method, suggesting that the bulliform cells were undeveloped. Therefore, a reduction in the growth of the bulliform cells seemed to be a major cause of leaf curling in the *hal1-d* mutant. The *hal1-d* mutation also affected the size of the leaf blade and the spikelet.

Key words: bulliform cell, curled leaf, leaf development, rice (*Oryza sativa*)

Plants successively generate leaves that perceive sunlight for photosynthesis. Leaf primordia initiate at the peripheral region of the meristem, where the leaf founder cells are recruited from undifferentiated cells supplied from the stem cells (Aichinger et al., 2012). In the leaf primordia, three developmental axes (apical-basal, adaxial-abaxial and centrolateral axis) are established and specific cell types then differentiate along these axes (Kuhlemeier and Timmermans, 2016). At the peripheral region of the meristem, KNOX genes, such as *SHOOT MERISTEMLESS* (STM) of *Arabidopsis thaliana* and *OSH1* of *Oryza sativa* (rice), are down-regulated to specify the fate of leaf founder cells (Long et al., 1996; Sentoku et al., 1999). The HD-ZIPIII and *ETTIN* genes and small RNAs targeting these genes are involved in the establishment of adaxial-abaxial cell fate (Chitwood et al., 2007; Husbands et al., 2009). These initial developmental processes and the genes that regulate them in leaf development are likely to be conserved in eudicots and monocots.

In contrast to this conservation, the morphology and size of the leaves are highly diverse in angiosperms. Venation pattern also differs in eudicots and monocots. Therefore, developmental mechanisms after leaf primordium formation seem to be diversified in angiosperms. However, our understanding of gene function involved in the formation of diverse leaf forms or cell differentiation specific to each species is still poor.

In rice, like other grasses such as *Zea mays* (maize) and *Brachypodium distachyon*, the leaf is composed mainly of two parts, the leaf blade and leaf sheath. Between these two distinct structures, an auricle and a ligule are formed. Two types of vascular bundles, large and small, are formed in parallel in both the leaf blade and the leaf sheath. The two types are composed of partially different cell types. In the central region of the leaf blade, a strong structure, the midrib, is formed. The midrib develops from cells that proliferate in the central region of early leaf primordia (Yamaguchi et al., 2004; Ohmori et al., 2011). The *DROOPING LEAF* (*DL*) gene plays a central role in midrib formation by promoting cell proliferation (Yamaguchi et al., 2004; Ohmori et al., 2008). The midrib is indispensable for erection of the leaf blade, which is thin and very long in rice. In the *dl* mutant, the leaves fail to erect due to the lack of a midrib.

A number of genes are involved in the formation and elaboration of the leaf in rice. Failures in the establishment of the adaxial-abaxial axis generate abnormal leaves in mutants such as *shoot organization*2 and *wavy leaf*1 (Itoh et al., 2008; Abe et al., 2010). These abnormalities are often closely associated with partial defects.
in the shoot apical meristem. In contrast, slight defects in abaxial identity produce rolled leaves, in which other characteristics are largely normal (Yan et al., 2008; Zhang et al., 2009). Mutations in genes for auxin synthesis and for some transcription factors cause a narrow leaf phenotype, together with a reduction in the number of vascular bundles (Fujino et al., 2008; Cho et al., 2013; Ishiwata et al., 2013; Kubo et al., 2016). To understand the genetic mechanisms underlying rice leaf morphogenesis in more detail, we focused on the half-pipe-like leaf1 (hal1) mutant, which has curled leaves, in this paper.

We searched a rice mutant population, in which mutation had been induced by a chemical mutagen, N-methyl-N-nitrosourea (Satoh et al., 2010). hal1-d was found as a mutant showing an erect and curled leaf phenotype (Fig. 1A and 1B). Leaves were flat in wild type, whereas they were curled adaxially in hal1-d (Fig. 1B and 1C). The cross section of the hal1-d leaf looked like a half-pipe (Fig. 1C). Genetic analysis revealed that hal1-d is a semi-dominant trait; F1 plants between hal1-d and wild type (Kinmaze, the genetic background of hal1-d) showed a mildly curling leaf phenotype (Fig. 1C). F2 plants showing curled, mildly curled and flat leaves segregated 52:101:32, respectively (1:2:1; \( P = 0.053, \chi^2 = 5.89 \)). In this study, we used the mutant homozygous for hal1-d.

To describe the extent of curling, we measured the width of the leaf blade in its natural state (a) and in the forcibly flattened state (b) in the leaf, when the whole leaf blade had emerged from the older leaf sheath. The curling index (a/b) in wild type was about 1.0 (Fig. 1D). By contrast, it was approximately 0.5 in hal1-d, and this value was constant in all of the examined leaves (Fig. 1D). This result suggests that the extent of leaf curling is independent of the leaf position; that is, it was not affected by the developmental stage of rice plants.

To reveal the cause of leaf curling, we performed histological analysis. Bulliform cells are differentiated in the adaxial epidermis between the vascular bundles. In wild type, large teardrop-shaped bulliform cells were observed, and these cells are clearly distinguished from normal epidermal cells (Fig. 2A, 2C and 2E). By contrast, the bulliform cells were small and round in the hal1-d mutant (Fig. 2B, 2D and 2G). In some cases, peripheral bulliform cells were indistinguishable from normal epidermal cells in hal1-d (Fig. 2G). Although the bulliform cells between the large and small vascular bundles and those between small vascular bundles were reduced in size in hal1-d, the bulliform cells of the latter case seemed to be more profoundly affected (Fig. 2D and 2G). We measured the area of the bulliform cell clusters between the small veins using cross sections of the leaf (Fig. 2E and 2G). The area of the bulliform cell cluster was reduced to about 50% in hal1-d, as compared to wild type (Fig. 2K). Next, we examined bulliform cell files by making the leaf transparent using the TOMEI method (Hasegawa et al., 2016). The bulliform cell files were relatively wide and were clearly distinguished from other tissues in wild type (Fig. 2I). By contrast, the cell files were narrower in hal1-d than in wild type, and, in some cases, they were indistinguishable from other parts in hal1-d leaves.

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Fig. 1. Morphological characteristics of the hal1-d mutant. (A) Wild type and hal1-d plants at 108 days old. (B) The leaf blade. ab, abaxial side; ad, adaxial side. (C) Cross section of the leaf blade. (D) Curling index. Blue, wild type; light blue, hal1-d. ‘10th’, ‘14th’ and ‘last’ denote leaf position; the last leaf means the one formed immediately before the flag leaf. Numbers in parentheses indicate the number of leaves examined. Error bars indicate S.E. **P < 0.01; significance based on Student's t-test. Scale bars = 1 cm (B), 1 mm (C).
hal1-d mutant showing curled leaf phenotype

Fig. 2. Histological analysis of the leaf blade. (A and B) Transverse sections of the leaf blade. (C and D) The bulliform cells between large and small veins. (E and G) The bulliform cells between small veins. Closed black arrowheads indicate normal bulliform cells, and closed red arrowheads indicate small and abnormal-shaped bulliform cells. (F and H) Close-up view of sclerenchyma next to the small vascular bundle. Tissues were stained with toluidine blue in (A) to (H). (I and J) Segments of the leaf blade cleared by the TOMEI method. Open arrowheads indicate the position of the bulliform cell file, and red open arrowheads indicate narrower or unclear bulliform cell files. (K) The area of the bulliform cell cluster. (L) The width of the bulliform cell file. Error bars indicate S.E. **P < 0.01; significance based on Student’s t-test. Scale bars = 100 µm (A, B, I, J), 20 µm (C to H).

Size measurement showed that the width of the bulliform cell file was significantly smaller in hal1-d leaves than in wild type leaves (Fig. 2L). Taking these observations together, the size of the bulliform cells was reduced in hal1-d. Other tissues such as sclerenchyma were not obviously affected by the hal1-d mutation (Fig. 2F and 2H). Therefore, the reduction in bulliform cell size seems to be a major cause of leaf curling in hal1-d.

To further examine the hal1-d effect, we measured the size of the leaf blade. Each leaf was measured just after the whole leaf blade emerged from the older leaf sheath. The leaf size did not change substantially thereafter. In wild type, the last leaf (immediately before the flag leaf) was shorter than the 14th leaf (Fig. 3A); the length of the leaf blade varied depending on the leaf position. By contrast, the last leaf was longer than the 14th leaf in hal1-d. Thus, the changing pattern of leaf blade length during plant growth differed between wild type and hal1-d. The width of the leaf blade also exhibited a similar result (Fig. 3B). These observations suggest that the reduction in size of the last leaf in wild type was partially reversed in the hal1-d mutant. The last two leaves of hal1-d had more veins than those of wild type (Fig. 3C), consistent with the fact that hal1-d had broader last leaves than wild type (Fig. 3B).

The effect of hal1-d appeared weakly in the reproductive phase. The spikelet (lemma and palea) seemed to be slenderer in hal1-d, just before anthesis, than in wild type (Fig. 3D). Measurement of spikelet size showed a significant reduction in spikelet width (Fig. 3F). We often observed seeds in which the lemma and palea failed to close (Fig. 3E, upper), probably due to the reduced width of these organs. The hulled seeds of hal1-d were slightly narrower than those of wild type (Fig. 3E, lower).

In this paper, we characterized the phenotype of the rice hal1-d mutant. Leaves in the hal1-d mutant were curled adaxially and were slightly altered in their size. Histological observation revealed that bulliform cells were small and abnormally shaped in hal1-d leaves, as compared with wild-type leaves. In grasses, the bulliform cells are involved in leaf rolling because their volumes reduce under dry conditions, suggesting that these
cells are responsible for leaf flattening under normal conditions. Therefore, undeveloped small bulliform cells are likely to be a major cause of curled leaves in the hal1-d mutant. Because incompletely developed bulliform cells were formed in hal1-d, the HAL1 gene seems to be required for growth of bulliform cells, but not for differentiation.

A defect in a gene encoding a cellulose synthase-like protein (OsCSLD4) affects bulliform cell formation in rice, resulting in the generation of rolled and narrow leaf blades. This gene was identified independently and named SLENDER LEAF1 (SLE1) (Yoshikawa et al., 2013), NARROW AND ROLLED LEAF1 (NRL1) (Hu et al., 2010) or NARROW LEAF AND DWARF1 (ND1) (Li et al., 2009). Unlike hal1-d, mutation of this gene produces narrow leaves and a dwarf phenotype. Rolled leaves are also associated with a partial defect in the establishment of adaxial-abaxial polarity in the leaf. For example, a mutation in the ROLLED LEAF 9 (RL9)/SHALLOW-LIKE1 (SLL1) gene, a member of the KANADI gene family encoding a GRAS transcription factor, also brings about the rolled leaf phenotype (Yan et al., 2008; Zhang et al., 2009). In this case, the rolled leaf is related to incomplete development of sclerenchyma cells on the abaxial side. It will be interesting to know what protein is encoded by HAL1 and how it contributes to the growth of bulliform cells.

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REFERENCES

Abe, M., Yoshikawa, T., Nosaka, M., Sakakibara, H., Sato, Y., Nagato, Y., and Itoh, J. (2010) WAVY LEAF1, an ortholog of Arabidopsis HEN1, regulates shoot development by main-
Kubo, F. C., Yasui, Y., Kumamaru, T., Sato, Y., and Hirano, H.-Y. (2012) Plant stem cell niches. Annu. Rev. Plant Biol. 63, 615–636.

Chitwood, D. H., Guo, M., Nogueira, F. T., and Timmermans, M. C. P. (2007) Establishing leaf polarity: the role of small RNAs and positional signals in the shoot apex. Development 134, 813–823.

Cho, S. H., Yoo, S. C., Zhang, H., Pandeya, D., Koh, H. J., Hwang, J. Y., Kim, G. T., and Paek, N. C. (2013) The rice narrow leaf2 and narrow leaf3 loci encode WUSCHEL-related homeobox 3A (OsWOX3A) and function in leaf, spikelet, tiller and lateral root development. New Phytol. 198, 1071–1084.

Fujino, K., Matsuda, Y., Ozawa, K., Nishimura, T., Koshiba, T., Furuage, M. W., and Sekiguchi, H. (2008) NARROW LEAF 7 controls leaf shape mediated by auxin in rice. Mol. Genet. Genomes 279, 499–507.

Hasegawa, J., Sakamoto, Y., Nakagami, S., Aida, M., Sawada, S., and Matsunaga, S. (2016) Three-dimensional imaging of plant organs using a simple and rapid transparency technique. Plant Cell Physiol. 57, 462–472.

Hu, J., Zhu, L., Zhang, T., Gao, Z., Guo, L., Fang, Y., Zhang, G., Dong, G., Yan, M., Liu, J., et al. (2010) Identification and characterization of NARROW AND ROLLED LEAF 1, a novel gene regulating leaf morphology and plant architecture in rice. Plant Mol. Biol. 73, 283–292.

Husbands, A. Y., Chitwood, D. H., Plavskin, Y., and Timmermans, M. C. P. (2009) Signals and prepatterns: new insights into organ polarity in plants. Genes Dev. 23, 1986–1997.

Ishiwata, A., Ozawa, M., Nagasaka, H., Kato, M., Noda, Y., Yamaguchi, T., Nosaka, M., Shimizu-Sato, S., Nagasaka, A., Maekawa, M., et al. (2013) Two WUSCHEL-related homeobox genes, narrow leaf2 and narrow leaf3, control leaf width in rice. Plant Cell Physiol. 54, 779–792.

Itoh, J.-I., Sato, Y., and Nagato, Y. (2008) The SHOOT ORGANIZATION2 gene coordinates leaf domain development along the central-marginal axis in rice. Plant Cell Physiol. 49, 1226–1236.

Kubo, F. C., Yasui, Y., Kumamaru, T., Sato, Y., and Hirano, H.-Y. (2016) Genetic analysis of rice mutants responsible for narrow leaf phenotype and reduced vein number. Genes Genet. Syst. 91, 235–240.

Kuhlemeier, C., and Timmermans, M. C. P. (2016) The Sussex signal: insights into leaf dorsiventrality. Development 143, 3230–3237.

Li, M., Xiong, G., Li, R., Cui, J., Tang, D., Zhang, B., Pauly, M., Cheng, Z., and Zhou, Y. (2009) Rice cellulose synthase-like D4 is essential for normal cell-wall biosynthesis and plant growth. Plant J. 60, 1055–1069.

Long, J. A., Moan, E. I., Medford, J. I., and Barton, M. K. (1996) A member of the KNOTTED class of homeodomain proteins encoded by the STM gene of Arabidopsis. Nature 379, 66–69.

Ohmori, Y., Abiko, M., Horibata, A., and Hirano, H.-Y. (2008) A transposon, Ping, is integrated into intron 4 of the DROOPING LEAF gene of rice, weakly reducing its expression, and causing a mild drooping leaf phenotype. Plant Cell Physiol. 49, 1176–1184.

Ohmori, Y., Toriba, T., Nakamura, H., Ichikawa, H., and Hirano, H.-Y. (2011) Temporal and spatial regulation of DROOPING LEAF gene expression that promotes midrib formation in rice. Plant J. 65, 77–86.

Satoh, H., Matsusaka, H., and Kumamaru, T. (2010) Use of N-methyl-N-nitrosourea treatment of fertilized egg cells for saturation mutagenesis of rice. Breed. Sci. 60, 475–485.

Sentoku, N., Sato, Y., Kurata, N., Ito, Y., Kitano, H., and Matsuoka, M. (1999) Regional expression of the rice KN1-type homeobox gene family during embryo, shoot, and flower development. Plant Cell 11, 1651–1664.

Yamaguchi, T., Nagasawa, N., Kawasaki, S., Matsuoka, M., Nagato, Y., and Hirano, H.-Y. (2004) The YABBY gene DROOPING LEAF regulates carpel specification and midrib development in Oryza sativa. Plant Cell 16, 500–509.

Yan, S., Yan, C. J., Zeng, X. H., Yang, Y. C., Fang, Y. W., Tian, C. Y., Sun, Y. W., Cheng, Z. K., and Gu, M. H. (2008) ROLLED LEAF 9, encoding a GARP protein, regulates the leaf abaxial cell fate in rice. Plant Mol. Biol. 68, 239–250.

Yoshikawa, T., Eiguichi, M., Hibara, K., Ito, J., and Nagato, Y. (2013) Rice SLENDER LEAF 1 gene encodes cellulose synthase-like D4 and is specifically expressed in M-phase cells to regulate cell proliferation. J. Exp. Bot. 64, 2049–2061.

Zhang, G. H., Xu, Q., Zhu, X. D., Qian, Q., and Xue, H. W. (2009) SHALLOW-LIKE1 is a KANADI transcription factor that modulates rice leaf rolling by regulating leaf abaxial cell development. Plant Cell 21, 719–735.