Characterization of a *half-pipe-like leaf1* mutant that exhibits a curled leaf phenotype

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(Received 27 March 2017, accepted 4 May 2017; J-STAGE Advance published date: 30 June 2017)

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 leaf1* (*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 forma-

tion 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 organization2* and *wavy leaf1* (Itoh et al., 2008; Abe et al., 2010). These abnormalities are often closely associated with partial defects

Edited by Koji Murai

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DOI: <http://doi.org/10.1266/ggs.17-00013>

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 (Sato 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 forcedly 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

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).

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).

(Fig. 2J). 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

Fig. 3. Size of the leaf blade and the spikelet. (A) The length of the leaf blade. (B) The width of the leaf blade. Blue, wild type; light blue, *hal1-d*. (C) The number of small veins in the last two leaves. (D) The spikelet of wild type and *hal1-d*. (E) Mature seeds of wild type and *hal1-d*. Typical unclosed seeds of *hal1-d* are shown. Representative hulled seeds are displayed in the lower panels. (F) The width of the spikelet. 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 mm.

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*)/*SHALLOT-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.

We thank A. Takahashi for technical assistance. We also thank Dr. N. Nonomura and Mr. M. Eiguchi at the National Institute of Genetics and technicians at the Institute for Sustainable Agro-Ecosystem Services at the University of Tokyo for cultivating the rice used in our study. This research was supported in part by Grants-in-Aid for Scientific Research from MEXT (25113008 to H.-Y. H.) and NIG-JOINT (2015-A1-60, 2016-A1-64).

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