Genetic analysis of rice mutants responsible for narrow leaf phenotype and reduced vein number

Fumika Clara Kubo¹, Yukiko Yasui¹, Toshihiro Kumamaru², Yutaka Sato³ and Hiro-Yuki Hirano¹*

¹Department of Biological Sciences, Graduate School of Science, The University of Tokyo, Bunkyo-ku, Tokyo 113-8654, Japan
²Faculty of Agriculture, Kyushu University, Hakozaki 6-10-1, Fukuoka 812-8581, Japan
³National Institute of Genetics, Mishima, 411-8540, Japan

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Leaves are a major site for photosynthesis and a key determinant of plant architecture. Rice produces thin and slender leaves, which consist of the leaf blade and leaf sheath separated by the lamina joint. Two types of vasculature, the large and small vascular bundles, run in parallel, together with a strong structure, the midrib. In this paper, we examined the function of four genes that regulate the width of the leaf blade and the vein number: NARROW LEAF1 (NAL1), NAL2, NAL3 and NAL7. We backcrossed original mutants of these genes with the standard wild-type rice, Taichung 65. We then compared the effect of each mutation on similar genetic backgrounds and examined genetic interactions of these genes. The nal1 single mutation and the nal2 nal3 double mutation showed a severe effect on leaf width, resulting in very narrow leaves. Although vein number was also reduced in the nal1 and nal2 nal3 mutants, the small vein number was more strongly reduced than the large vein number. In contrast, the nal7 mutation showed a milder effect on leaf width and vein number, and both the large and small veins were similarly affected. Thus, the genes responsible for narrow leaf phenotype seem to play distinct roles. The nal7 mutation showed additive effects on both leaf width and vein number, when combined with the nal1 single or the nal2 nal3 double mutation. In addition, observations of inner tissues revealed that cell differentiation was partially compromised in the nal2 nal3 nal7 mutant, consistent with the severe reduction in leaf width in this triple mutant.

Key words: leaf development, narrow leaf, rice (Oryza sativa), vasculature, vein number

Angiosperms produce diverse shapes of leaves from simple leaves to various types of compound leaves such as palmate and pinnate leaves (Bar and Ori, 2014; Tsukaya, 2014). Their leaves also show various venation patterns, which are roughly divided into two types, reticulate and parallel vein patterns. The veins consist of a vascular bundle and associated tissues. The vascular bundles act as the pathway of water and nutrients and as the mechanical support for this thin organ. Grass species such as rice (Oryza sativa) and maize (Zea mays) generate leaves with parallel veins, as do other monocots (Nelson and Dengler, 1997; Itoh et al., 2005). Grass leaves are composed of three parts: the leaf blade, leaf sheath and a region connecting these parts, called the lamina joint. There are two types of vasculature, large and small vascular bundles, in the leaf blade and leaf sheath of rice, and these transverse veins are connected with small commissural veins (Sakaguchi and Fukuda, 2008; Sakaguchi et al., 2010). The midrib, which contains several vascular bundles, is formed in the central region of the leaf blade. The YABBY gene DROOPING LEAF (DL) plays a crucial role in initiating midrib development, and its activity is associated with the size of the midrib (Yamaguchi et al., 2004; Ohmori et al., 2008, 2011).

Several genes that regulate leaf size have been identified in rice. Although each single mutant of narrow leaf2 (nal2) and nal3 shows no obvious phenotype, the nal2 nal3 double mutant produces very narrow leaves with a reduced number of veins (Cho et al., 2013; Ishiwata et al., 2013). These two genes belong to the WUSCHEL-RELATED HOMEOBOX (WOX) gene family and are orthologous to the PRESSSED FLOWER (PRS)/WOX3 family.
gene in *Arabidopsis thaliana* and the *NARROW SHEATH* genes in maize (Matsumoto and Okada, 2001; Nardmann et al., 2004). The *NAL2* and *NAL3* genes are extremely similar to each other, probably because of a recent segmental duplication of 2–3 Mb in chromosome 11 and 12 (Ishiwata et al., 2013). The *nal1* mutant displays a reduction in the width of the leaf blade and the number of crown roots (Qi et al., 2008; Cho et al., 2014; Jiang et al., 2015). *NAL1* function is also associated with some characteristics found by quantitative trait analysis, such as spikelet number and photosynthesis (Fujino et al., 2013; Takai et al., 2013), suggesting that *NAL1* is involved in various biological activities. *NAL1* encodes a putative trypsin-like serine/cysteine protease, although its molecular function is still unknown (Qi et al., 2008; Cho et al., 2014). A mutation in *NARROW LEAF7* (*NAL7*) also causes a narrow leaf phenotype. *NAL7* encodes a YUCCA enzyme responsible for auxin biosynthesis, suggesting an involvement of auxin in the regulation of leaf size (Fujino et al., 2008). *narrow leaf and dwarf1* (*nd1*)/*narrow and rolled leaf1* (*sle1*) mutants exhibit a narrow and rolled leaf phenotype, together with reduced plant height (Li et al., 2009; Hu et al., 2010; Yoshikawa et al., 2013). *ND1/NRL1/SLE1* encodes cellulose synthase-like protein D4. Interestingly, *SLE1* is specifically expressed in M-phase cells, and cell-cycle regulation is disturbed in the *sle1* mutant (Yoshikawa et al., 2013). Thus, various genes are involved in regulating the width of the leaf blade. However, these mutants are isolated from strains with different genetic backgrounds. In this paper, we examined the effect of *nal2 nal3*, *nal1* and *nal7* mutations on leaf morphology and their genetic interactions, by introducing the mutated loci into a single genetic background (Taichung 65 (T65), a *japonica* subspecies).

To investigate the function and genetic interactions of genes responsible for the narrow leaf phenotype, we used *nal1* and *nal7* single mutants and the *nal2 nal3* double mutant. The *nal7* mutant and the *nal2 nal3* double mutant were described previously (Fujino et al., 2008; Cho et al., 2013; Ishiwata et al., 2013). We focused on a new mutant line, CM1054, which also showed a narrow leaf phenotype. Rough mapping using an F2 population between CM1054 and the Kasalath line indicated that this mutation mapped to a region encompassing the *NAL1* locus on chromosome 4 (Qi et al., 2008; Cho et al., 2014; Jiang et al., 2015). Sequence analysis revealed that CM1054 had a missense mutation in the second exon of the *NAL1* gene, which causes an amino acid substitution from Gly to Asp at position 202 of the *NAL1* protein. Thus, CM1054 was found to be an allele of the *NAL1* gene. We named this mutant *nal1-1054*.

The *nal1-1054* and *nal7* mutants were backcrossed twice with the standard wild-type strain T65, and *nal1-1054* (BC2) and *nal7* (BC2) were obtained by self-pollination and genotyping of each gene. The effect of the *nal7* mutation on leaf width was partially relieved by introducing the mutation into the genetic background of T65 (Fig. 1, C and F). *nal1-1054* (BC1) and *nal7* (BC1) were crossed with each other and the *nal1-1054 nal7* double mutant was selected from the F2 plants by genotyping (Fig. 1, D). This *nal1-1054 nal7* double mutant, which had partial T65 genetic background, was compared with *nal1-1054* (BC2) and *nal7* (BC2). Hereafter, these mutants are described without ‘BC’.

The vein pattern varies along the proximodistal axis in the leaf blade. New veins arise in the distal and middle parts of the leaf blade, whereas they disappear from the leaf lamina by being incorporated into the midrib region.

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**Fig. 1.** Phenotypes of leaf blades around the incorporation point. (A) Wild type (T65), (B) *nal1-1054* (BC2), (C) *nal7* (BC2), (D) *nal1-1054* *nal7*, (E) *nal2* *nal3* (BC2), (F) *nal7*, (G) *nal2* *nal3* *nal7*. Part of the leaf blade just before the flag leaf is shown. An example of the incorporation point is shown by the black arrow in (A). Bars = 5 mm.
in the middle part of the leaf blade. For example, the incorporation of a large vein was clearly observed in T65 (Fig. 1, A). To analyze quantitatively, we compared vein numbers at the position, named the ‘incorporation point’, where the large vein first fused with the midrib.

Although the number of small and large veins was reduced in both nal1-1054 and nal7, the degree of reduction differed between the two mutants (Fig. 2, B and C). The number of both small and large veins was moderately reduced in nal7, and the degree of reduction was similar (30% for the small vein, 26% for the large vein). By contrast, the small vein number was greatly reduced in

![Fig. 2. Comparison of leaf blade width and the numbers of small and large veins at the incorporation point. (A and D) Leaf blade width. (B and E) Small vein number. (C and F) Large vein number. nal1, nal1-1054; nal1 +7, nal1-1054 nal7; nal2 +3, nal2 nal3; nal2 +3 +7, nal2 nal3 nal7. Results shown are means ± S.E. Statistical significance was calculated by Student’s t-test. *P < 0.05, **P < 0.01, ***P < 0.001. ns, not significant. Smaller asterisks beside the bars indicate the significance of differences between wild type (T65) and each mutant.](image-url)
nal1 (64% reduction), whereas the large vein number was not significantly affected (19% reduction). Thus, the nal1 mutation seems to predominantly affect the generation of small veins. In the nal1-1054 nal7 double mutant, leaves were narrower than in either single mutant (Fig. 1, B–D; Fig. 2, A). The numbers of small and large veins were also reduced in nal1-1054 nal7 (Fig. 2, B and C). These results indicated that the nal1-1054 and nal7 mutations had additive effects on the leaf width and the number of both veins.

Next, we examined the genetic interaction of narrow leaf mutants in a different combination. We crossed the nal7 mutant with the nal2 nal3 double mutant (BC2 for T65). To isolate triple mutants, we first chose plants homozygous for nal7 from the F2 plants by genotyping. It is very difficult to find the nal2 nal3 double mutation by genotyping, because NAL2 and NAL3 are highly similar to each other in nucleotide sequence, and the NAL3 gene is entirely deleted in the original nal2 nal3 mutant (Ishiwata et al., 2013). However, the nal2 nal3 double mutation can be identified by phenotypes such as narrow leaves and number of tillers, because neither of the single mutations show any phenotype (Cho et al., 2013; Ishiwata et al., 2013). Therefore, we screened the plant that showed the narrowest leaf phenotype and multiple tillers from the nal7 homozygote (59 plants) and used it as a nal2 nal3 nal7 triple mutant (Fig. 1, G).

Leaf width and vein number were reduced in nal2 nal3 (Fig. 1, E; Fig. 2, D–F). Small vein number was much reduced in nal2 nal3 (Fig. 2, E), compared to large vein number, as in nal1-1054. The combination of the nal2 nal3 mutation with the nal7 mutation caused a severe reduction in leaf width (Fig. 1, C, E and G; Fig. 2, D). The number of both small and large veins was significantly reduced in the nal2 nal3 nal7 mutant, compared with the nal7 and nal2 nal3 mutants (Fig. 2, E and F). Thus, the nal2 nal3 mutation and the nal7 mutation acted additively on leaf width and vein number.

Since the leaves of the nal2 nal3 nal7 triple mutant were extremely narrow (more than 70% reduction in

![Fig. 3. Transverse sections of the leaf blade.](image_url)
Genetic interaction of narrow leaf mutants

width compared to wild type), it is likely that the internal structure of the leaf blades was affected. To confirm this, we observed transverse sections of the leaf blades of the nal2 nal3, nal7 and nal2 nal3 nal7 mutants. Leaf segments about 1 cm long around the incorporation point of the leaf blade were sampled, and two sections, 7–8 mm apart, were independently observed under a microscope after toluidine blue staining.

Mesophyll cells, which are well stained with toluidine blue, were densely packed in both the nal7 and nal2 nal3 mutants (Fig. 3, A and B). We observed abnormal cells with no staining in internal tissue of the nal2 nal3 nal7 triple mutant (Fig. 3, C, D, F and G). These abnormal cells appeared to be vacuolated. We examined six sections (three leaf segments) of nal2 nal3 nal7 leaf blades and found a total of 32 abnormal cells. By contrast, we failed to find such abnormal cells in the nal2 nal3 mutant (six sections examined) and the nal7 mutants (four sections examined). Therefore, these vacuolated abnormal cells seem to be a consequence of the triple mutation of nal2 nal3 nal7. Bulliform cells, which are likely to act in preventing the leaf blade from curling, are differentiated in the adaxial epidermis between veins. Because the bulliform cells are large and teardrop-shaped, they are clearly distinguished from the standard epidermal cells, which are small and round. The bulliform cells were differentiated in epidermal regions between veins in the nal2 nal3 mutant and the nal7 mutant, with no exception (Fig. 3, A and B). By contrast, undeveloped bulliform cells were sometimes observed in the nal2 nal3 nal7 mutant (Fig. 3, D and E). In some severe cases, bulliform cells were indistinguishable from the epidermal cells. In other cases, the bulliform cells were small and round. Thus, the nal2 nal3 nal7 mutation seems to affect the differentiation or growth of the bulliform cells. Furthermore, in rare cases, we observed a bulge which protruded from the abaxial side of the leaf blade (Fig. 3, E and H). Interestingly, a vascular bundle was formed in this bulge. However, the orientation of the xylem and phloem in this bulge was altered (Fig. 3, H). These observations indicated that the nal2 nal3 nal7 mutation affected the differentiation of both mesophyll and epidermal cells, in addition to the regulation of leaf width and vein number.

In this paper, we analyzed genetic interactions of genes responsible for aspects of leaf morphology, namely leaf width and vein pattern. Both the NAL1 and NAL2 NAL3 genes seem to have a distinct role in controlling vein number: these genes have a predominant role in the formation of small veins, compared with large veins. Our preliminary analysis suggests that the mechanism underlying vascular development is different between the small and large veins. That is, a new small vein is likely to appear between older veins, whereas a large vein is likely to be formed by the divergence of a preexisting large vein. Thus, it is possible that specific contributions of these genes to the formation of each type of vein are associated with this difference. In contrast to the NAL1 and NAL2 NAL3 genes, the mutation in NAL7 affected evenly the number of small and large veins, and the effect of the nal7 mutation is milder than that of either the nal1 or the nal2 nal3 mutation. This suggests that NAL7 has a general role in vascular bundle differentiation, the general effect of the nal7 mutation on the number of small and large veins is thus consistent with its role in auxin biosynthesis. The nal7 mutation had an additive effect on the nal1 single mutation and the nal2 nal3 double mutation: leaf characteristics such as small and large vein numbers and leaf width were similarly reduced in the nal1 nal7 and nal2 nal3 nal7 mutants. This result also supports the general role of NAL7.

Although the molecular function of NAL1 has not yet been revealed, Jiang et al. (2015) have shown that NAL1 has a role in cell cycle progression and cell proliferation. Overexpression of NAL2/3 produces a wider leaf (Ishiwata et al., 2013), suggesting that these genes are also involved in cell proliferation across the leaf width. An involvement of NAL1 and NAL2/3 in cell proliferation is consistent with the narrower leaf formation in the corresponding mutants. The nal2 nal3 nal7 triple mutation had a severe effect on leaf width: the growth of the leaf blade along the centrolateral axis was strongly inhibited, suggesting that cell division was profoundly affected by these mutations. In addition, cell differentiation was also partially compromised in the triple mutant. Differentiation and growth of the bulliform cells were partially inhibited in the epidermis, and a larger vacuolated cell was occasionally formed among mesophyll cells in the inner tissues. These defects may be associated with a strong reduction of cell division in nal2 nal3 nal7. In rare cases, a small bulge was detected in the abaxial side of the leaf blade in nal2 nal3 nal7. In this bulge, a vascular bundle was ectopically formed. The xylem and phloem are aligned along the adaxial-abaxial axis in the normal leaf, whereas the orientation of the ectopic vascular bundle was disturbed in the ectopic bulge. It is well known that ectopic protrusions are formed in plants which are defective in the determination of adaxial-abaxial polarity (Waites and Hudson, 1995; Nakata et al., 2012). Therefore, the formation of this bulge and the altered orientation of the xylem and phloem imply that adaxial-abaxial polarity is weakly compromised in the nal2 nal3 nal7 triple mutant.

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