Leaves are the major photosynthetic organs in plants. The light-capture efficiency significantly differs depending on the leaf shapes, angles and arrangements. Thus, leaf morphology is critical for the survival of plant species. After the cell fate is determined in the shoot apical meristems (SAMs), leaf primordia grow in accordance with three axes; the proximal-distal, adaxial-abaxial, and medial-lateral directions (Moon and Hake 2011, Scarpella et al. 2010). The accurate developments along these axes ensure the morphogenesis of sophisticated leaf organs with high reproducibility.

The plant hormone auxin plays pivotal roles in the leaf development (Benjamins and Scheres 2008). Auxin is unique in its polar transportation due to the localized influx carriers and efflux carriers (Petrášek and Friml 2009). Once transported to SAMs, auxin flows to the leaf-primordium initiation sites through the epidermis layer L1 mediated by PIN-FROMED 1 (Petrášek and Friml 2009, Scarpella et al. 2010). Such auxin localization down-regulate class I KNOTTED1-like homeobox (KNOX) genes and promote the outgrowth of primordia, creating leaf tips, and the basipetal streams of auxin from the tip through the internal tissue induce the differentiation of vascular strand (Hay et al. 2004, Scarpella et al. 2006, Wenzel et al. 2007). Interestingly, similar auxin-mediated mechanisms also control the development of leaflet in the compound leaf (Barkoulas et al. 2008, Giacomo et al. 2013) and leaf serrations in Arabidopsis thaliana (Aloni et al. 2003, Bilsborough et al. 2011, Hay et al. 2006).

Soon after the initiation, the regions of leaf primordia facing the SAMs or away from the SAMs acquire the identity as the adaxial or abaxial side, respectively. Through the loss-of-function and/or gain-of-function analyses, the involvement of many genes in the establishment of adaxial-abaxial polarity has been revealed; in the case of A. thaliana, adaxial identity is regulated by class III HOMEODOMAIN-LEUCINE ZIPPER family genes and ASYMMETRIC LEAVES2 while abaxial identity is regulated by YABBY.
family genes, KANADI family genes, and AUXIN RESPONSE FACTOR family genes (Nakata and Okada 2013). The adaxial or abaxial specific expression of these genes is crucial for the establishment of the organ polarity, and adaxial/abaxial regulators are interacting antagonistically to maintain the expression regions (Nakata and Okada 2013, Scarpella et al. 2010). Other than gene interactions, small RNAs and auxin localization are also crucial for the establishment of the organ polarity (Heisler et al. 2005, Nakata and Okada 2013, Vernoux et al. 2010). The loss of adaxial-abaxial polarity induces the formation of narrow or needle leaves in A. thaliana (Sarojam et al. 2010, Stahle et al. 2009), suggesting that lamina growth is regulated downstream of the adaxial-abaxial polarity.

Many genetic approaches have been employed to reveal the lamina growth mechanisms, demonstrating that WUSCHEL-RELATED HOMEBOX (WOX) genes are critical for the development of leaf lateral domains. In maize, the loss-of-function mutations in both NARROW SHEATH1 (NS1) and NS2, which encode the duplicated WOX3 genes, result in the lack of marginal regions in leaves and floral organs as well as the shortened internode on the marginal side of the stem (Nardmann et al. 2004, Scanlon et al. 1996, Scanlon and Freeling 1998). The NS transcripts are accumulated in the marginal edges of leaf primordia, and ns1 ns2 double mutants fail to down-regulate KNOX proteins in the pre-marginal regions of leaf primordia, leading to the deletion of marginal region from the primordial stages (Nardmann et al. 2004, Scanlon et al. 1996). These results suggest that NS genes play pivotal roles in the recruitment of leaf founder-cells by down-regulating KNOX accumulation although the mechanism is still unclear (Scanlon 2000, Scanlon and Freeling 1997, Scanlon et al. 2000). Similar developmental defects in lateral domains were observed in the NS-orthologue mutants in rice (NARROW LEAF 2 [NAL2], NAL3; Cho et al. 2013, Ishiwata et al. 2013) and A. thaliana (PRESSED FLOWER1 [PRSI]; Matsumoto and Okada 2001, Nardmann et al. 2004), suggesting the conserved function of NS-related genes in the development of lateral organs.

WOX1 also plays a central role in lamina development. WOX1 is unique in that it belongs to the same clade of the WOX3/PRS family but seems to be absent in grasses (Haecker et al. 2004, Vandenbussche et al. 2009). The loss-of-function of WOX1 lead to severe defects in lamina outgrowth in petunia (MAEWEST; Vandenbussche et al. 2009), tobacco (LAM1; McHale and Marcotrigiano 1998), and Medicago truncatula (STENOFOLIA; Tadge et al. 2011). WOX1 genes are expressed in the middle mesophyll layers and at the leaf margin cells, similar to WOX3/PRS expression patterns, and wox1 prs double mutants exhibit not only the lost of leaf marginal tissues but also the confused adaxial-abaxial identity at leaf margin regions (Nakata et al. 2012, Vandenbussche et al. 2009). These results suggest that WOX1 and WOX3/PRS play pivotal role not only in lamina outgrowth but also in the formation of adaxial-abaxial boundaries at leaf margins.

Barley (Hordeum vulgare L.) is the fourth most-produced cereal in the world and is mainly utilized as animal feed and malts. Recently, barley attracts considerable attentions as healthy food rich in dietary fiber. The diploid nature makes barley a model crop of Triticeae. However, limited knowledge is available about developmental aspects of barley leaves. In the present study, we investigated barley narrow leafed dwarf1 (nld1) mutants whose phenotypes are thinner leaves accompanied by short stature. Detailed histological analysis indicated that narrowed leaf of nld1 was attributable to the lack of marginal regions. Map-based cloning revealed that NLD1 encodes a maize NS-related WOX3 protein, and we also found the marginal expression of NLD1. The results presented in this study indicate that NLD1 plays pivotal role in the increase of organ width and in the development of marginal tissues in lateral organs in barley.

### Materials and Methods

#### Plant materials

In the present study, we used two alleles of NLD1: nld1.a and nld1.b. The nld1.a was first isolated as a spontaneous mutant “Nagaoka Dwarf” from the F2 population of the cross Nagaoka × Marumi 16 (Takahashi et al. 1972), however, it was phenotypically quite similar to Nagaoka. Thus, this mutant was named as Nagaoka-dwarf, and its original cultivar was designated as Nagaoka here. Another independent mutant, nld1.b is a gamma-ray induced mutant derived from a line Kanto Nijo 29 (KN29). For the evaluation of mutant phenotypes, mutants and wild-type seeds were sown on soil and grown under natural conditions. To promote germination, seeds were kept at 15°C on wet paper for three days before sowing.

#### Epidermal cell observation

The second leaf blades of nld1 and wild-type were fixed with FAA (formaldehyde:glacial acetic acid:50% ethanol [2:1:17]) for 24 h at 4°C. They were then dehydrated in a graded ethanol series. Dehydrated samples were incubated at 96°C in chloralhydrate dissolved in 100% ethanol until they were cleared, and observed with a light microscope. The measurement of cell width was performed by image analysis with Image J (available at http://rsbweb.nih.gov/ij/).

#### Paraffin sectioning and histological analysis

Plant samples of nld1 and wild-type were fixed with FAA (formaldehyde:glacial acetic acid:50% ethanol [2:1:17]) for 24 h at 4°C for histological analysis, or fixed with PFA (4% w/v) paraformaldehyde and 1% Triton X in 0.1 M sodium phosphate buffer) for 48 h at 4°C for in situ hybridization. They were then dehydrated in a graded ethanol series, substituted with 1-butanol, and embedded in Paraplast® Plus (McCormick Scientific). The samples were sectioned at 8 μm thick using a rotary microtome. For the histological
analysis, sections were stained in haematoxylin or double-stained in safranin and fast green. After staining, sections were mounted with Poly-Mount® (Polysciences, Inc.) and observed with a light microscope. The measurement of leaf primordium width was performed by image analysis with Image J (available at http://rsbweb.nih.gov/ij/).

**In situ hybridization**

Paraffin sections were prepared as mentioned above. DIGoxigenin-labeled anti-sense and sense RNA probes were prepared from a 666-bp fragment of NLD1, which was amplified by PCR with forward primer (5'-AGCAGCTGATGATCCTGGAG-3') and reverse primer (5'-AGGTGGAGCAAGAGGGAGAC-3') using cDNA as a template. The amplified PCR product was cloned into pCR™-Blunt vector (invitrogen), followed by in vitro transcription using DIG RNA Labeling Kit (Roche). In situ hybridization and immunological detection with alkaline phosphatase were performed according to the methods of Kouchi and Hata (1993).

**Map-based cloning**

For the fine mapping of NLD1 gene, nld1.b plants were crossed with normal barley (NC117), and 105 F2 plants were used for mapping. The previous study reported that NLD1 is located about 21.6 cm proximal from the fragile stem 1 locus on chromosome 5HL (Hayashi et al. 1983, Takahashi et al. 1972). Thus, genotyping were performed using five markers (k09239, k03390, k01939, k04066, Bmag0337) (Sato et al. 2009, Varshney et al. 2007), which locate in the vicinity of predicted NLD1 locus, and the candidate region was further limited from k03390 (76.4 cM) to k01939 (80.7 cM). Since this region include WOX3 encoding MLOC_7772.1, an orthologous gene of maize NS1 and NS2 and rice NAL2/3, we compared the genomic sequence of the gene between nld1 mutants and wild-types.

**Results**

**Phenotypes of nld1 mutants in the vegetative phase**

In the present study, we used two alleles of NLD1: nld1.a and nld1.b (see Materials and Methods). The nld1.a is a spontaneous mutant isolated from a six-rowed cultivar Nagaoka, and nld1.b is a gamma-ray induced mutant derived from a line Kanto Nijo 29 (KN29). Thus, Nagaoka and KN29 were used as a wild-type of nld1.a and nld1.b, respectively, in all experiments (see Materials and Methods). (a and b) Seedlings at the second leaf stage of wild-type (KN29) (a), and nld1.b (b), (c) Leaf blades of the second leaf in wild-type (KN29) and nld1.b. (d and e) Comparison of leaf blade length (d) and width (e) between wild-type (Nagaoka and KN29) and nld1.b mutants. (f) and (g) Comparison of cell width (f) and number of cells (g) in the second leaf blades between wild-type (KN29) and nld1.b. Results are shown as means ± SE (n = 10) (d, e, f, g). Bars = 5 cm (a, b), 5 mm (c).

For the further analysis of leaf development, we performed histological analysis of leaf primordia in nld1 (Fig. 2a, 2b). In barley, leaf ridge development precedes leaf primordium development (Shewry 1992), and it is quite difficult to recognize the differentiation from leaf ridge to leaf primordium. Thus, in the present study, we defined the 6th leaf primordium as the P1 leaf primordium (the youngest primordial stage) in the 2nd leaf stage seedling (Supplemental Fig. 2). The comparison of leaf primordium width showed that nld1 already exhibited narrowed leaf phenotype at P1 primordial stage, and this trend was enhanced from P3 to P4 primordial stage (Fig. 2c, 2d). These results
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Supplemental Fig. surface of leaf-blades in mutants (Fig. 3a–3d). Since auricles are formed in the margins of lamina-joint, these lacks in nld1 indicate the possibility that nld1 lack the marginal region in the leaf. The histological analysis of the leaf primordium also showed the abnormal development of the marginal region (Fig. 3e, 3f). Occasionally, nld1 shows asymmetrical development of the leaf in medial-lateral directions (Supplemental Fig. 3). Detailed analysis of the leaf margins revealed the defective development of the sawtooth hairs and sclerenchymatous cells together with the thickening of leaf edges in nld1 mutants (Fig. 3g–3j). Thus, it was indicated that nld1 lack the marginal region of the leaf, which resulted in the significant reduction in leaf width. Interestingly, trichome developments were also impaired not only in the leaf edges but also in the surface of leaf-blades in mutants (Supplemental Fig. 4). The density of trichome, which is formed along with the longitudinal veins, is reduced in nld1 (Supplemental Fig. 4a–4c). Each trichome was smaller than those of wild-type (Supplemental Fig. 4d–4g). Occasionaly, quite narrow veins were formed in the leaf margins of nld1 mutants (Fig. 3i, 3j). The transparentization of leaf blades revealed that these marginal narrow veins are attributable to the looped commissural veins in the looped commissural veins toward leaf margins (Fig. 3k, 3l). The malformations of commissural veins, such as loop or interruption, were frequently observed in the leaf margin of nld1. These abnormal developments of commissural veins also indicate the abnormal development of leaf margins in the mutants.

The dwarf phenotype is another major characteristic of nld1 mutants (Fig. 4a). The comparison of the internode length indicated that the 1st and 2nd internode from the top showed the significant reduction in length (Fig. 4b). Occasionally, internodes exhibit abnormal winding development (Fig. 4c, 4d). This trend was more prominent in the upper internode. The cross section of the internode revealed the malformed development in nld1; the region where leaf margins are adjacent showed ill development in the internode. It was, therefore, considered that impaired development of internode is related to the defective development of leaf margins.
Phenotypes of nld1 mutants in the reproductive phase

The defective development of the marginal region is also appeared in the reproductive organs in the mutants. The lemmas, paleae, and empty glumes are apparently narrowed in nld1 (Fig. 5a). The histological analysis of spikelets revealed the marginal defect in lemmas and paleae in the mutants (Fig. 5b, 5c). The other organs such as stamens, pistils, and lodicules include no obvious malformations and the fertilities are comparable to those of the wild-types, suggesting that NLD1 is particularly involved in the development of lemmas and paleae in the reproductive organs. In matured spikes, the seeds are partly visible through gaps between lemmas and paleae in nld1 because of incomplete overlap between margins of these organs (Fig. 5d, 5e). These results indicated that NLD1 play pivotal roles in the marginal development in vegetative and reproductive lateral organs.

Map-based cloning of NLD1 gene

The previous study reported that NLD1 is located about 21.6 cM proximal from the fragile stem 1 locus on chromosome 5HL (Hayashi et al. 1983, Takahashi et al. 1972). The NLD1 locus was further limited within the region ranged from 76.4 cM (k03390) to 80.7 cM (k01939), which include WOX3 encoding MLOC_7772.1, an orthologous gene of maize NS1 and NS2 and rice NAL2/3. The ns1 ns2 and nal2/3 mutants exhibit narrowed leaves and spikelets due to the marginal defect, which seems to be similar to nld1 phenotypes. Thus, we performed the sequence analysis of MLOC_7772.1 in nld1 mutants and wild-types, and found mutant specific alterations in both nld1.a and nld1.b (Fig. 6a). It was revealed that nld1.a contained point mutation from G to A at 740 bp, which cause nonsense mutation in the middle of amino acid sequence, and that nld1.b deleted one nucleotide at 275 bp, which cause drastic alteration in the amino acid sequence due to the frame shift mutation. WOX3 proteins can be classified into two clades; one consists of maize NS-related proteins and the other rice DEPILOUS (DEP)-related proteins (Fig. 6b). It is likely that barley possess two WOX3 genes (HvNS/NLD1 and HvWOX3), but HvWOX3 is classified into DEP-related clade. Therefore, it is likely that NS-related WOX3 gene is only NLD1/HvNS, which can account for the phenotypic alteration in nld1 single mutant. The amino acid sequence alignment showed that NLD1-related proteins contain two conserved regions in N-terminal (the homeobox domain) and C-terminal (the WUSCHEL [WUS] box motif) (Fig. 6c). Both nld1.a and nld1.b possess intact homeobox domain but completely lost the WUS box motif (Fig. 6c). From these results, it was concluded that MLOC_7772.1 is identical to NLD1.
Expression analysis of *NLD1* gene

For the further understanding of the function of *NLD1* in barley development, we performed expression analysis of *NLD1* by *in situ* hybridization. The earliest expression in the course of leaf development was observed in the marginal edges of the initiating leaf ridge (Fig. 7a, 7b). These marginal expression were continually observed throughout the development of leaf ridge and leaf primordium, and were kept as late as P4 leaf primordium (Fig. 7c). These expression pattern indicate that *NLD1* plays pivotal role not only in the leaf-ridge generation from SAM but also in the development of leaf margins. Other than leaf edges, *NLD1* transcripts were also observed on the epidermal cells along with the longitudinal veins in leaf primordium later than P3 stage (Fig. 7c). While *NLD1* transcripts were continuously localized in leaf edges, those on the epidermal cells along with the longitudinal vein is interspersed (Supplemental Fig. 5), suggesting that *NLD1* plays different roles between these places. In reproductive organs, *NLD1* expression was observed in the marginal edges of lemmas, paleae, and empty glumes (Fig. 7d, 7e), which corresponded with the phenotypic alteration in *nld1* mutants (Fig. 5). From these results, we concluded that *NLD1* plays pivotal roles in the increase of organ width and in the development of marginal tissues by expressing in the marginal edge of lateral organs in barley.
Fig. 7. Expression pattern of the NLD1 gene. (a and b) Cross sections of shoot apex in wild-type (KN29) hybridized with NLD1 anti-sense probe (a) and sense probe (b). In (a), parts of the section enclosed with the rectangles are enlarged in (a′) and (a″). Seedlings at the 2nd leaf stage are used as the plant materials. Shoot apical meristem (SAM) and leaf primordial stages (P1–P4) are shown in the figures. (c) Cross section of leaf primordia in wild-type (KN29) hybridized with NLD1 anti-sense probe. Part of the section enclosed with the rectangle is enlarged in (c′). Seedling at the 2nd leaf stage is used as the plant material. Leaf primordial stages (P3 and P4) are shown in the figure. Arrow head in (c′) indicates the signal on the leaf epidermal cell. (d and e) Longitudinal section (d) and cross section (e) of the immature spikelets in wild-type (KN29) hybridized with NLD1 anti-sense probe. The paleae (Pa), lemmas (Le), and empty glume (EG) are shown in the figure. Bars = 200 μm (a, b, c), 400 μm (c), 50 μm (c′), 500 μm (d).

Discussion

In the present study, we showed that the narrowed leaf phenotype of nld1 mutants were attributable to the lack of the marginal regions in the leaves (Fig. 3g–3j). The defective development of margins are derived from the impaired development of leaf primordia (Fig. 2, Fig. 3e, 3f). Map-based cloning revealed that NLD1 encodes a NS-related WOX3 protein, whose expression was localized in the marginal edges of lateral organs (Fig. 7). From these results, we concluded that NLD1 plays pivotal role in the increase of organ width and in the development of marginal tissues in lateral organs.

Maize ns1 ns2 mutants and rice nal2/3 mutants exhibit significant reduction in the width of leaves and floral organs due to the defective marginal development (Cho et al. 2013, Ishiwata et al. 2013, Nardmann et al. 2004, Scanlon et al. 1996, Scanlon and Freeling 1998). In nsl ns2, additional features such as stem curvature and shortened internodes are appeared, which seem to be attributable to the uneven internode growth (Scanlon et al. 1996). These mutant phenotypes resemble those of nld1 mutants. In addition, the transcripts of NS and NAL2/3 genes are also accumulated to the marginal edges of lateral organs (Nardmann et al. 2004, 2007). These similarities in loss-of-function mutant phenotypes and gene expression patterns strongly indicate that molecular function of NS-related WOX3 genes is highly conserved across plant species.

The earliest NLD1 expression in the course of leaf development was observed in the marginal edges of the initiating leaf ridge (Fig. 7a). Maize NS genes are suggested to be involved in the recruitment of leaf founder-cells by down-regulating KNOX accumulation in the pre-marginal regions (Scanlon 2000, Scanlon and Freeling 1997, Scanlon et al. 2000). Thus, it is conceivable that NLD1 also plays critical roles in the establishment and maintenance of marginal regions. Leaf margin functions as an adaxial-abaxial boundary, where adaxial and abaxial regulators are down-regulated by WOX genes (Nakada and Okada 2013). This is well demonstrated by wox1 prs double mutants, which show confused adaxial-abaxial identity at leaf margins (Nakata et al. 2012, Vandenbussche et al. 2009). The lack of leaf-margin specific structures and the thickening of leaf margins in nld1 may indicate that leaf adaxial-abaxial boundary is compromised in the mutants (Fig. 3g–3j).

Other than leaf edges, NLD1 transcripts were also observed on the epidermal cells along with the longitudinal veins in leaf primordia later than P3 stage (Fig. 7c). Since such expressions have never been reported in other plants, this might be unique to barley. The signals were interspersed on the epidermal cells along with the longitudinal vein (Supplemental Fig. 5), which seem to be similar pattern to the development of the trichomes. In fact, nld1 show impaired trichome development such as the reduction in trichome density and size (Supplemental Fig. 4), although such phenotypes have never been reported in other plants. Further study is required to reveal the role of NLD1 gene in the development of leaf epidermal cells.

The development of leaf margin was impaired in nld1 mutants (Fig. 3e, 3f). Occasionally, nld1 shows asymmetrical development of the leaf in medial-lateral directions.
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(Supplemental Fig. 3). These mutant phenotypes suggest that NLD1 promote the expansion of lamina in the development of leaf primordium. However, the NLD1 transcripts were strictly limited within the few cells in leaf edges, implying that NLD1 functions non-cell-autonomously. This idea is supported by the lack of sclerenchymatous cells in nd1 lociating inner than epidermal layer, where NLD1 expression was not observed (Fig. 3i, 3j), or by the malformation of commissural veins in the leaf margin of the mutants (Fig. 3k, 3l). This contradiction could be explained by the migration of either NLD1 protein itself or the secondary signals derived from the marginal cells. Previous studies have shown that polar auxin transport plays an important role in determining vascular pattern in leaves (Sakaguchi et al. 2010, Scarpella et al. 2006), and loss-of-function of auxin biosynthesis or transport genes gave rise to reduction in leaf blades in rice (Fujino et al. 2008, Qi et al. 2008, Yoshikawa et al. 2014). Therefore, it is conceivable that nd1 includes some abnormalities in auxin transport. Cho et al. (2013) also referred to the possibility that nal2/3 phenotypes are partly attributable to the altered auxin transport. Thus, it is quite interesting whether auxin functions as the secondary signal of NS-related WOX3 genes.

In the present study, we identified barley NLD1 gene, which encodes NS-related WOX3 protein (HvNS). Database analysis revealed that barley possess another WOX3 gene (HvWOX3), which showed higher similarity to rice DEP gene than to NLD1/HvNS, suggesting that these two genes were differentiated earlier than the differentiation of rice and barley. While NS-related WOX3 genes have been widely studied, the information about DEP-related WOX3 genes is still limited (Angeles-Shim et al. 2012). Thus, further study of NLD1/HvNS and HvWOX3 will not only reveal the molecular mechanism of barley development but also provide new insight into the evolution of WOX3 gene families in plants.

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Literature Cited


Nardmann, J., J. Ji, W. Herr and M.J. Scanlon (2004) The maize duplicate genes narrow sheath1 and narrow sheath2 encode a conserved...


