The rice narrow leaf2 and narrow leaf3 loci encode WUSCHEL-related homeobox 3A (OsWOX3A) and function in leaf, spikelet, tiller and lateral root development

Sung-Hwan Cho1*, Soo-Cheul Yoo1*, Haitao Zhang1*, Devendra Pandeya1, Hee-Jong Koh1, Ji-Young Hwang2, Gyung-Tae Kim2 and Nam-Chon Paek1

1Department of Plant Science, Plant Genomics and Breeding Institute, Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul, 151-921, Korea; 2Department of Molecular Biotechnology, Dong-A University, Busan, 604-714, Korea

Summary

- In order to understand the molecular genetic mechanisms of rice (Oryza sativa) organ development, we studied the narrow leaf2 narrow leaf3 (nal2 nal3; hereafter nal2/3) double mutant, which produces narrow-curly leaves, more tillers, fewer lateral roots, opened spikelets and narrow-thin grains.
- We found that narrow-curly leaves resulted mainly from reduced lateral-axis outgrowth with fewer longitudinal veins and more, larger bulliform cells. Opened spikelets, possibly caused by marginal deformity in the lemma, gave rise to narrow-thin grains.
- Map-based cloning revealed that NAL2 and NAL3 are paralogs that encode an identical OsWOX3A (OsNS) transcriptional activator, homologous to NARROW SHEATH1 (NS1) and NS2 in maize and PRESSED FLOWER in Arabidopsis. OsWOX3A is expressed in the vascular tissues of various organs, where nal2/3 mutant phenotypes were displayed. Expression levels of several leaf development-associated genes were altered in nal2/3, and auxin transport-related genes were significantly changed, leading to pin mutant-like phenotypes such as more tillers and fewer lateral roots.
- OsWOX3A is involved in organ development in rice, lateral-axis outgrowth and vascular patterning in leaves, lemma and palea morphogenesis in spikelets, and development of tillers and lateral roots.

Introduction

Lateral organs of higher plants are initiated at the periphery of the shoot apical meristem (SAM) by recruitment of a group of founder cells (Poethig & Szymkowiak, 1995; Dolan & Poethig, 1998). SAM maintenance and meristematic cell fate in organ primordia are controlled by the interplay of homeodomain transcription factors, including WUSCHEL (WUS), SHOOT-MERISTEMLESS (STM) and CLAVATA (Clark et al., 1996; Gallois et al., 2002; Lenhard et al., 2002).

Leaf primordia develop in a radial pattern at the periphery of the SAM (Reinhardt et al., 2000), and leaf protrusion occurs as a result of increased cell division and cell expansion. During leaf vascular formation in rice, provascular strands including the midrib extend longitudinally (Itoh et al., 2005). Thereafter, small longitudinal and transverse provascular strands form (Nishimura et al., 2002), and bulliform cells differentiate on the adaxial surface. The adaxial–abaxial polarity of leaves is established by asymmetrical distribution of cell types. ASYMMETRIC LEAVES2 (AS2) and HD-ZIPIII family members act as adaxial-specific regulators (McConnell et al., 2001; Iwakawa et al., 2002), whereas YABBY (YAB) and KANADI genes specify abaxial cell fate (Siegfried et al., 1999; Kerstetter et al., 2001; Emery et al., 2003). These genes exhibit polar expression patterns and determine the abaxial cell fate of aboveground lateral organs (Bowman, 2000). Recently, YAB genes were reported to have a wide function not only in adaxial–abaxial polarity of leaves, but also in shoot development (Sarojam et al., 2010).

In rice, YAB1 functions in stamen and carpel development (Jang et al., 2004). DROOPING LEAF (DL), a CRABS CLAW-related gene and a member of the YAB family, is required for carpel speciﬁgeneciation and leaf midrib development (Yamaguchi et al., 2004). YAB5/TONGARI-BOUSHI1 (TOBI) is involved in lateral organ development and maintenance of meristem organization for spikelet development (Tanaka et al., 2012). Unlike the YAB members in Arabidopsis and maize, rice YAB1, YAB5 and DL do not show adaxial/abaxial-specific expression in lateral organs (Yamada et al., 2011).

WUS-related homeobox (WOX) proteins regulate diverse aspects of Arabidopsis development (Haecker et al., 2004). For...
instance, WOX2 and WOX8 regulate cell fate in the apical and basal lineage of the proembryo (Breuninger et al., 2008). WOX3/ PRESS FLOWER (PRS) acts in lateral-axis expansion of lateral organs (Matsumoto & Okada, 2001). WOX1 acts redundantly with WOX3/PRS for cell proliferation in the blade outgrowth and margin development downstream of adaxial/abaxial polarity establishment in Arabidopsis leaves (Nakata et al., 2012). WOX5 functions in the maintenance of stem cells in shoot and root meristems (Sarkar et al., 2007; Nardmann et al., 2009). WOX8/ PRETTY FEW SEEDS2 regulates ovule development (Park et al., 2005). WOX9/STENOFOLIA is required for early embryonic growth, and the maintenance of cell division and growth in shoot and root apices (Wu et al., 2005). Such WOX functions have been also reported in other plant species. In petunia, the WOX8/9 homologue EVERGREEN regulates cytosolic inflorescence development (Rebocho et al., 2008), and the WOX1 homologue MAEWEST is required for laminar growth (Vandenbussche et al., 2009). In Medicago truncatula, the WOX1 homologue STENOFOlia regulates leaf blade outgrowth and vascular patterning (Tadege et al., 2011).

In monocots, maize narrow sheath1 narrow sheath2 (ns1 ns2) double mutants display a narrow leaf sheath and margin-deleted phenotype in the lower portion of leaf blades. Positional cloning revealed that ns1 and ns2 (hereafter ns) are redundant, duplicated WOX3 genes, required to recruit founder cells from lateral domains of the SAM and form lateral and marginal regions of leaves (Scanlon et al., 1996; Nardmann et al., 2004). PRS, an Arabidopsis NS homologue, recruits founder cells during lateral organ development to form lateral domains of vegetative and floral organs (Matsumoto & Okada, 2001; Shimizu et al., 2009). In prs mutants, lateral stipules at the base of cauline leaves, sepals and stamens are deleted because of failure to recruit lateral founder cells. The functions of PRS and NS are conserved to form marginal regions of lateral organs through the recruitment of founder cells from SAM lateral domains. The rice genome contains three WOX3 genes, the duplicated OsWOX3A genes (also termed OsNs; Nardmann et al., 2007; GenBank accession number: AB218893) on the short arms of chromosome 11 and 12, which are putative orthologues of ns1 and ns2 in maize and PRS in Arabidopsis, and OsWOX3B (LOC_Os05 g02730; GenBank: AM490244) on chromosome 5. OsWOX3A acts as a transcriptional repressor of YAB3 and OsWOX3A-overexpressing plants exhibit twisted and knotted leaves. However, RNAi-mediated downregulation of OsWOX3A produced no visible phenotype (Dai et al., 2007). OsWOX3B/DEP has been identified recently by positional cloning and functions in the regulation of trichome formation in leaves and glumes (Angeles-Shim et al., 2012).

The plant hormone auxin (indole-3-acetic acid, IAA) plays critical roles in cell division, cell elongation, organ initiation, vascular differentiation and root initiation (Reinhardt et al., 2000; Benkova et al., 2003; Friml et al., 2003). Auxin is synthesized in the young shoot apex and transported to maturing tissues. Directional transport of auxin results from the asymmetric distribution of different auxin membrane carriers, that is, the influx carrier AUX1 family and the efflux carriers in the PIN-FORMED (PIN) family (Bennett et al., 1996; Galweiler et al., 1998; Xu et al., 2005). The rice narrow leaf7 (nal7) mutant, a mutation in YUCCA8 (YUC8), involved in auxin synthesis, produces narrow and curly leaves throughout development (Fujino et al., 2008). The narrow and rolled leaf3 (nrl3), encoding the cellulose synthase-like protein D4 (OsCslD4), regulates plant architecture and development of leaf veins and bulliform cells (Hu et al., 2010). Interestingly, NAL7/YUC8 levels are upregulated in nrl1 mutants, suggesting the association of the nrl1 phenotype with auxin biosynthesis. Auxin maxima and polar transport trigger development of leaves and lateral roots from the initiation of primordia to the formation of the mature organ (Benkova et al., 2003). The narrow leaf1 (nal1) mutation affects polar auxin transport and vascular patterning (Qi et al., 2008). The Arabidopsis pin mutants exhibit auxin transport defects and alterations in shoot, flower and root development (Galweiler et al., 1998; Benkova et al., 2003; Reinhardt et al., 2003). Rice OsPIN1 and OsPIN2 are also involved in tiller and root development (Xu et al., 2005; Chen et al., 2012). Although auxin functions in leaf development, vascular patterning and root initiation have been reported (Reinhardt et al., 2003; Scarcella et al., 2006; Bilsborough et al., 2011), auxin function in plant organ development has not been fully elucidated.

In this study, we analysed the rice pleiotropic nal2 nal3 (hereafter nal2/3) double mutant, which produces narrow-curly leaves, opened spikelets, narrow-thin grains, more tillers and fewer lateral roots. Map-based cloning revealed that the nal2 and nal3 loci encode an identical OsWOX3A/OsNS5 protein. Our histological and molecular analyses in nal2/3 demonstrate a conserved role for OsWOX3A/NS/PRS in the regulation of lateral-axis outgrowth and margin development in leaves. In addition, our identification of OsWOX3A functions in the development of spikelet, tiller and root organs will inform further studies of the WOX family.

Materials and Methods

Plant materials and growth conditions

The nal2 nal3 (nal2/3) double recessive mutant of Oryza sativa L. japonica rice was previously obtained from Kyushu University, Japan, and has been maintained by the Rural Development Administration, Korea. The nal2/3 mutant was backcrossed twice with a Japanese japonica rice cv ‘Kinmaze’ and progressed for several generations. Kinmaze was used as the parental wild-type plant in this study. The growth chamber conditions were 12-h light (500 μmol m−2 s−1) at 30°C and 12-h dark at 20°C.

Histological analysis of leaves and spikelets

Samples were fixed in 3.7% formaldehyde, 5% acetic acid and 50% ethanol overnight at 4°C, and dehydrated in a gradient series of ethanol, cleared through a xylene series, then infiltrated through a series of Paraplast (Sigma) and finally embedded in 100% Paraplast at 55–60°C or in Technovit 7100 resin (Heraeus Kulzer GmbH, Frankfurt, Germany) for thin sections. Then, 4–12 μm-thick microtome sections were mounted on glass slides.
Genetic and physical mapping of *nal2* and *nal3*

For map-based cloning of the *nal2* and *nal3* loci, an F₂ mapping population was generated by a cross of the *nal2/3* mutant (*japonica*) and a tongil-type cv ‘Milyang23’ which has a closer genetic makeup to *indica* (Yoo et al., 2009). SSR markers were obtained from information in GRAMENE, the Arizona Genomics Institute, and the Rice Genome Research Program. STS markers on chromosomes 11 and 12 were identified by nucleotide sequence alignments between *japonica* and *indica* in the BAC clones AL51300, BX000505, BX000500, BX000494 and BX000496 for high-resolution physical mapping. PCR primer sequences for SSR and STS markers are listed in Supporting Information Table S1.

Rice transformation and complementation

The recombinant pC1300intC binary vector (GenBank: AF294978) was prepared by inserting a 3831-bp genomic DNA segment (2141-bp 5'-upstream, 612-bp ORF, and 1078-bp 3'-downstream sequences of OsWOX3A) for the complementation test with *nal2/3*. The recombinant plasmid in an *Agrobacterium* strain, LBA4404, was introduced into the calli of mature embryos from *nal2/3* mutant seeds (Jeon et al., 2000b). Transformants were confirmed by PCR with primers in the pC1300intC vector (TC1) and OsWOX3A genomic fragment (TC2; Table S1).

RNA extraction, reverse transcription and quantitative real-time PCR (qRT-PCR)

Total RNAs were extracted independently from wild-type and *nal2/3* using the RNA extraction kit (iNtRON Biotechnology, Seoul, Korea), and were treated with RNase-free DNase (Ambion) to remove possible contaminating genomic DNA as per the manufacturer’s instructions. For qRT-PCR, 2 μg of total RNA was reverse-transcribed using the M-MLV reverse transcription kit (Promega). First-strand cDNAs equivalent to 50 ng of total RNA were used for qRT-PCR. Each RT product was analysed using a LightCycler480 (Roche), with Universal SYBR Green Master Mix (Roche), and data analysis was conducted using the Roche Optical System software (v1.5). The qRT-PCR conditions were as follows: activation at 95°C for 2 min, 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 60 s, followed by melt analysis ramping to 95°C. For the efficiency of qRT-PCR, we calculated the amplification efficiency by comparing the slope of the linear regression of Ct and log₁₀ of gene copies. The expression of each gene was normalized against *Ubiquitin*. The relative expression of each gene in WT and *nal2/3* plants was analysed using the 2⁻ΔΔCt method as described (Livak & Schmittgen, 2001), and Student’s t-test was used to determine statistically significant differences. The qRT-PCR primer information is given in Table S1.

Membrane protein extraction and immunoblot analysis

Membrane proteins were extracted from 2-wk-old rice seedlings using the ProteoExtract kit (M-PEK; Calbiochem, Darmstadt, Germany). Equal amounts of protein were subjected to SDS-PAGE and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Amersham). The proteins were detected by immunoblotting with anti-AtPIN1 (Santa Cruz Biotech, Santa Cruz, CA, USA), anti-AtPIN2 (Agrisera, Vännäs, Sweden) or anti-Lhcb1 (Agrisera) antibodies, and then stained with Coomassie Brilliant Blue (Sigma) for loading control. Quantification of band intensity on the immunoblots was performed using Image J (v1.36) software according to the instructions (http://rsb.info.nih.gov/ij/docs/menus/analyze.html#gels).

Histochemical analysis of OsWOX3A expression

For GUS (β-glucuronidase) assays, a 2.1-kb 5'-upstream promoter region of *OsWOX3A* was amplified by genomic PCR (Table S1) and then subcloned into the pCAMBA1301 plasmids, fusing the *NAL2/3* promoter to the *GUS* coding sequence (*Pro*OsWOX3A-*GUS*). The construct was transformed into rice calli as described (Jeon et al., 2000b). Nine independent transgenic lines were obtained after screening, and GUS activity was detected histochemically as described (Jefferson et al., 1987).

Subcellular localization of the OsWOX3-GFP fusion protein in protoplasts

The ORF of *OsWOX3*, except for the stop codon, was amplified using the primers ORF1 and ORF2 (Table S1), and subcloned into pCAMLA-GFP, resulting in the 35S::*OsWOX3-GFP*. Maize mesophyll protoplasts were transfected by PEG transformation (Sheen, 2001; Yoo et al., 2007). At 20 h after transformation, GFP signals were observed by confocal microscopy (MRC-1024; BioRad).

Transcriptional activation in the yeast GAL4 system

The *OsWOX3A* ORF was divided into two parts: N-terminal (1–333 bp) and C-terminal (316–612 bp). Full-length (primers: Y-F and Y-R), N-terminal (Y-F and Y-R333) and C-terminal (Y-F316 and Y-R) fragments were amplified from the cDNAs of wild-type, *nal2*, and *nal3* (Table S1). Each cDNA fragment was digested with EcoRI and BamHI, and then inserted into the pGBK7 vector as bait (Clontech, Mountain View, CA, USA). Bait clones were transformed into yeast strain AH109 with the empty prey vector pGADT7. The yeast cells containing the pGBK7-p53 and pGADT7-T plasmids were used as a positive control. The negative control AH109 cells were obtained by co-transforming the empty pGBK7 and pGADT7 plasmids. Cotransformed cells were identified by spotting on SD/-Leu/-Trp medium, then replica-plated on SD/-Adel/-His/-Leu/-Trp medium. Transactivation activity was determined according to cell survival on SD/-Adel/-His/-Leu/-Trp medium.
Effect of IAA on lateral root development

Surface-sterilized rice seeds were germinated on $10^{-7}$ M IAA 0.5X MS medium with 0.8% phytoagar and grown vertically in a growth chamber. At 14 d after germination, the length and number of lateral roots were measured.

Scanning electron microscopy

In order to examine the adaxial and abaxial surfaces of leaf blades, 1.5 mm transverse sections of the middle region of a fully expanded third leaf blade were sampled. Sections were fixed in primary fixative (2% paraformaldehyde, 2% glutaraldehyde), post-fixed with 1% osmium tetroxide, and dehydrated in a series of ethanol and propylene oxide, then finally embedded in Spurr’s resin. After polymerization, sections were observed with a scanning electron microscope (JSM 5410LV; JEOL, Tokyo, Japan).

Results

Phenotypic characterization of rice nal2 and nal3 (nal2/3) double mutants

The nal2/3 double mutant was named for its phenotype: the production of narrow leaf blades throughout development (Fig. 1a–c). These loci were also termed curly leaf2 (cul2) and cul3, respectively, in the GRAMENE database (http://www.gramene.org/) because the leaf blades exhibited both narrow and curly phenotypes. The single nal2 or nal3 mutants did not show any defect in leaf morphology. Compared with the parental wild-type cv ‘Kinmaze’, the widths of leaf blades in nal2/3 were consistently narrow from early seedling to fully mature stages (Fig. 1a, b; Table S2). In addition, the nal2/3 leaves curled upward (Fig. 1b). The number of large veins (LVs) in the leaf blades was slightly reduced to c. 80% of wild-type (Fig. 1c; Table S2), and the number of small veins (SVs) between adjacent LVs was reduced to almost a half of wild-type. The number of SVs between LVs was quite irregular in each leaf blade, and the vein distribution on the left and right side of the midrib was different. Sawtooth hairs at the leaf margins were significantly reduced (Fig. 1d). Ligule, auricle, leaf sheath and stems were also narrower and thinner (Fig. 1e,f; Table S2). Despite the reduction in leaf width, the lengths of leaf blades and leaf sheaths, and plant height were similar to wild-type (Table S2). These results indicate that NAL2/3 function is mainly associated with vein patterning during leaf development and lateral-axis expansion during shoot organogenesis.

Defects in lateral-axis outgrowth, margin development and vascular patterning during leaf development

We performed a histological analysis to define the effects of nal2/3 on leaf organ development in more detail. Cross-sections of mature leaf blades clearly showed reduced SV numbers between LVs in nal2/3 (Fig. 2b). The size of vascular bundles (VBs) was reduced and simplified (Fig. 2c,e). The organization of xylem and phloem were also altered (Fig. 2g,h). This result indicates that NAL2/3 acts in vascular patterning during leaf development.

Bulliform cells, located between adjacent veins on the adaxial side of leaf blades, were more abundant and bigger, and were irregular in shape, compared with their spherical form in wild-type (Fig. 2a–f). In addition, there were significantly fewer longitudinal furrows (the zone of bulliform cells) on the adaxial side due to fused SVs (Fig. S1c). Therefore, the upward curling during leaf elongation appears to be associated with abnormal development of bulliform cells. Moreover, the mesophyll cell regions between veins and bulliform cells were narrower and thicker (Fig. 2d,f). Interestingly, nal2/3 clearly lacked leaf margin...
structures; the edges of leaf blades were blunt (Fig. 2g,h) and sawtooth hairs at the margins of leaf blades were nearly absent or poorly developed (Fig. 1d), indicating that adaxial–abaxial patterning was compromised in \( \text{nal2/3} \) mutants. \( \text{nal2/3} \) mutants have fewer cell files in the margins of leaf blades without a change in cell size (Fig. 2g,h), indicating that cell proliferation of the margin of leaf blades was reduced. Defective leaf margin structures were also observed in the leaf sheaths. The overlapped margins of the leaf sheaths were much shorter, the leaf sheaths were narrower, and there were fewer vasculatures in each leaf sheath in \( \text{nal2/3} \) (Fig. 2i,j). Taken together, these results indicate that \( \text{NAL2/3} \) is involved in lateral domain development at early leaf developmental stages, and the reduced size of SAM in \( \text{nal2/3} \) is probably associated with the narrow leaf production from the leaf primordial stage.

Defective lemma and palea morphogenesis during spikelet development in \( \text{nal2/3} \)

In rice, the spikelets are normally opened at fertilization stage (Fig. S3) and closed after fertilization and throughout grain filling to produce normally shaped grains. However, the spikelets of \( \text{nal2/3} \) were not closed after fertilization and remained open during grain filling, resulting in narrow-thin grains (Fig. 3a,b; Table S2). Transverse sections of spikelets in \( \text{nal2/3} \) showed that the widths of lemma and palea were significantly reduced to 58% and 51%, respectively, compared with those of wild-type (Fig. 3c–f; Table S2). Noticeably, the number of VBs in all lemmas was increased in \( \text{nal2/3} \) because two more VBs were located at the end of the lemma margins (Fig. 3e,f). In addition, the lemma and palea margins were not fully developed (Fig. 3c,
increased in both active and maximum tillering stages in nal2/3 plants (Table S4; Fig. S4), whereas the number of panicles per plant was significantly reduced in the mutant (Table S4), indicating that the production of unproductive tillers increases in nal2/3. Taken together, our phenotypic analysis of the field-grown nal2/3 plants suggests that nal2/3 mutation negatively affects both vegetative and reproductive organ development with a severe loss of grain yield.

Map-based cloning of nal2 and nal3 loci

In order to identify the nal2 and nal3 loci, genetic mapping was performed using an F2 mapping population generated from a cross of nal2/3 (japonica) and the wild-type indica cv ‘Milyang23’. The segregation ratio of 10,000 F2 individuals was consistent with the expected ratio of 15 (9388 wild-type): 1 (612 mutant), a typical Mendelian segregation ratio for unlinked duplicate genes. Using 612 F2 individuals displaying the mutant phenotype, we found that nal2 was closely linked to an SSR marker RM286 on the short arm of chromosome 11 (Fig. 4a). The 384-kb candidate region was annotated by the Rice Genome Research Program (http://rgp.dna.afrc.go.jp/). Among the annotated genes in the first BAC clone BX072548, one highly likely candidate gene, OsWOX3A/OsNS (Os11g01130), was found (Fig. 5a). Next, the nal3 locus was mapped onto the short arm of chromosome 12 using the SSR markers RM19, RM247 and RM277 (Fig. 4b). Using six sequence tagged site (STS) markers (Table S1), the nal3 locus was further narrowed down to a 149-kb interval from STS 6 to the end of the chromosome. In the two BAC clones in this interval (GenBank: BX000503 and BX000496), we found an identical OsWOX3A gene (Os11g01120) that has the same nucleotide sequence as the candidate gene for nal2 on chromosome 11. This finding was not surprising because the first 3 Mb on the short-arm ends of chromosomes 11 and 12 are completely duplicated (The Rice Chromosomes 11 and 12 Sequencing Consortia, 2005). OsWOX3A has a single 612-bp exon and encodes a 203-amino acid (aa) protein containing a WUS homeodomain at the N-terminus (4–68 aa) and a WUS-box domain at the C-terminus (172–179 aa; Haecker et al., 2004; Fig. S5b). Phylogenetic analysis revealed that OsWOX3A/OsNS belongs to the same clade as ZmNS1, ZmNS2 and AtWOX3/PRS (Fig. S6; Table S5). However, OsWOX3A shares only 66% aa sequence identity with maize NS1 and NS2 (hereafter NS), and 42% with Arabidopsis WOX3/PRS, mainly because the middle sequences of OsWOX3A are quite dissimilar to those of NS and PRS.

In order to determine the lesions in nal2/3, we sequenced 20 RT-PCR products from nal2/3 mutant leaves. We found two classes of cDNAs with different OsWOX3A mutations, which we expected to represent the two different loci, nal2 and nal3 (Figs 4c, 5a). Seven cDNAs had a change from G to A at the 66th base of the open reading frame (ORF), causing a single amino acid substitution from Met (ATG) to Ile (ATA) (designated nal2). In the other 13 cDNAs, several deletion, insertion and point mutations occurred (designated nal3). To confirm that these OsWOX3A mutations were responsible for the nal2/3

---

**Fig. 3** nal2/3 exhibits opened spikelets due to abnormal development of palea and lemma, giving rise to narrow-thin grains. (a, b) Comparison of rice (Oryza sativa) wild-type (WT) and nal2/3 spikelets and grains. (c, e) Magnified junction zones between lemma and palea (c, e), showing one (d) and two (f) vascular bundles in (c) and (e), respectively. (c–f) Asterisks indicate vascular bundles. (d, f) Black arrowheads indicate the junction of lemma and palea. L, lemma; P, palea; G, grain; NSC, nonsilicified cell; SC, silicified cell; SPC, spongy parenchymatous cell; FS, fibrous sclerenchyma. Bars: (a, b) 3 mm; (c, e) 100 μm; (d, f) 50 μm.
phenotype, we next complemented the mutant phenotype. For complementation, the 3831-bp genomic DNA of OsWOX3A (see the Materials and Methods section) was expressed in nal2/3 plants. All transgenic lines produced normally shaped leaves (Fig. S7) and spikelets (data not shown) in the nal2/3 background, showing that the introduced genomic OsWOX3A fragment complemented the nal2/3 mutation.

OsWOX3A is a nuclear protein that is expressed in the vasculatures of various organs

The subcellular localization of an OsWOX3A-GFP fusion was examined in maize leaf protoplasts, which revealed that OsWOX3A is a nuclear protein (Fig. 5a,b). We also used qRT-PCR to measure organ/tissue-specific expression of OsWOX3A. OsWOX3A was ubiquitously expressed, and expressed at high levels in the shoot base (SB; including the SAM) and young panicle (Fig. 5c). Unexpectedly, it was also expressed in roots at similar levels to leaf blades. To examine the spatial expression of OsWOX3A in each organ, we stained for β-glucuronidase (GUS) activity in the transgenic plants containing the ProOsWOX3AGUS reporter gene (see the Materials and Methods section). In agreement with the qRT-PCR results, OsWOX3A was expressed in both large and small VBs of the leaf blade and leaf sheath, especially phloem tissues, and the shoot base and the vascular cylinder of roots (Fig. 5d–i). GUS activity was also observed in the longitudinal veins of spikelets in young panicles at heading stage (Fig. 5j). These results support the hypothesis, based on the nal2/3 phenotype, that OsWOX3A is mainly expressed in vasculatures for the rice organ development.

Altered expression of leaf development-associated genes in nal2/3

Leaf development is regulated by several groups of genes; for example, auxin synthesis-related genes such as YUC genes (Fujino et al., 2008), auxin transport-associated genes (Benkova et al., 2003), and YAB genes (Dai et al., 2007). To further understand the regulatory functions of OsWOX3A in leaf organogenesis, we measured mRNA levels of leaf development-associated genes in the SB region by qRT-PCR (Fig. 6a). We measured auxin signal transduction, transport and biosynthesis-related genes, such as AUXIN RESPONSE FACTOR (ARF), PIN-FORMED (PIN) and YUC family genes, respectively, as well as leaf development-
related genes, such as \textit{NAL1}, \textit{NRL1} and \textit{YAB} family genes. We found that in \textit{nal2}/3, \textit{ARF1}, \textit{ARF4}, \textit{YAB4}, \textit{YAB7}, \textit{YUC8}/\textit{NAL7} and \textit{PIN1} were significantly downregulated, and that \textit{YAB1}/3, \textit{YUC1}, \textit{YUC4} and \textit{PIN2} were upregulated. Expression levels of \textit{YAB5}, \textit{NRL1} and \textit{PIN3} were slightly reduced. However, the expression levels of \textit{ARF2}/3, \textit{ARF5}, \textit{YAB6}, \textit{YUC2}/3, \textit{YUC6}/7, \textit{YUC9} and \textit{NAL1} were not altered (Fig. 6a). The qRT-PCR results suggest that \textit{OsWOX3A} modulates the transcription levels of several auxin- and leaf development-related genes.

The expression levels of \textit{ARF} genes are affected by the amount of free IAA (Waller \textit{et al.}, 2002; Xing \textit{et al.}, 2011). \textit{YUC} genes, encoding flavin monooxygenase-like enzymes, function in the auxin biosynthesis pathway (Yamamoto \textit{et al.}, 2007; Fujino \textit{et al.}, 2008). Thus, we examined endogenous free auxin concentrations in wild-type and \textit{nal2}/3 seedlings with an IAA-ELISA kit (see Methods S1) and found no significant difference in endogenous free IAA concentrations (Fig. S8), indicating that the altered expression of some of \textit{ARF} and \textit{YUC} genes did not affect the endogenous free IAA content in \textit{nal2}/3. Because IAA concentrations were not altered in \textit{nal2}/3 mutant, we hypothesized that compromised auxin transport caused the mutant phenotype. Immunoblot analysis using the anti-PIN1 antibody (\textit{\alpha}-PIN1) and \textit{\alpha}-PIN2 revealed that PIN1 was significantly reduced whereas the PIN2 concentration was slightly increased in \textit{nal2}/3 (Fig. 6b), consistent with their expression levels. These results suggest that the \textit{nal2}/3 phenotype may be related to altered auxin transport in addition to the transcriptional activity of leaf development-related genes.

\textbf{Altered expression of \textit{OsPIN1} and \textit{OsPIN2} may reduce lateral root numbers in \textit{nal2}/3}

Overexpression of \textit{OsPIN1} significantly increased the lateral root number (LR) in the transgenic rice (Xu \textit{et al.}, 2005), whereas \textit{OsPIN2}-overexpressing plants displayed reduced LR density (Chen \textit{et al.}, 2012). Because the expression levels of \textit{OsPIN1} and \textit{OsPIN2} were altered in \textit{nal2}/3, we further characterized the \textit{pin} mutant-associated phenotypes such as the number of lateral or adventitious roots. Consistent with \textit{OsPIN} expression, LR number was significantly reduced in \textit{nal2}/3 (Fig. 7c,d,g). However, adventitious root number was not different between wild-type and \textit{nal2}/3 (Fig. 7g). The results indicate that altered expression of \textit{OsPIN1} and \textit{OsPIN2} negatively affects LR development in \textit{nal2}/3.

A previous report indicated that in \textit{OsPIN1}-RNAi rice, polar auxin distribution was compromised, and this was partially complemented by exogenous IAA (NA; Xu \textit{et al.}, 2005). To further test whether reduced number of LRs in \textit{nal2}/3 was caused by
failure of endogenous IAA distribution, we grew nal2/3 plants in the MS medium containing 10^{-7} M IAA for 2 wk. As expected, exogenous IAA treatment partially rescued the reduced LR number in the mutant, to c. 90% of wild-type (Fig. 7b,e–g), suggesting that altered expression of OsPIN1 and OsPIN2 in nal2/3 compromised the precise distribution of endogenous IAA, as previously observed in other OsPIN mutants (Xu et al., 2005; Chen et al., 2012).

In order to further understand OsWOX3A function in LR formation, we analysed OsWOX3A expression during LR development histochemically using transgenic rice containing the ProOsWOX3A:GUS transgene (Fig. 8). OsWOX3A was expressed at the base region of LR from primordial to early developmental stages (Fig. 8a–c). After emergence of LR primordia, OsWOX3A expression was detected only in the vascular cylinder during LR elongation (Fig. 8d,e). This result supports the idea that OsWOX3A is involved in development of LR primordia.

**OsWOX3A acts as a transcriptional activator**

The qRT-PCR results suggested that in addition to the repression of YAB3 (Dai et al., 2007), OsWOX3A may also be involved in the transcriptional upregulation of several leaf development-associated genes (Fig. 6a). To test whether OsWOX3A can act as a transcriptional activator, we used the yeast GAL4 system in which a transactivating protein fused to the GAL4 DNA binding domain activates a HIS reporter gene, enabling recombinant yeast to survive on His-deficient medium. We found that the yeast cells expressing OsWOX3A fused to a DNA-binding domain (DB:OsWOX3A) grew vigorously on the His-deficient medium, indicating that OsWOX3A has transactivation activity. However, the mutated nal2 (DB:nal2) and nal3 (DB:nal3) proteins lost the activity completely (Fig. 9, first panel). Both N-terminal and C-terminal fragments of OsWOX3A also showed transcriptional activation in yeast. However, the C-terminal fragment of nal2 and the N- or C-terminal fragments of nal3 did not activate the reporter gene (Fig. 9, second and third panels), suggesting that both the N-terminal WOX3 homeodomain and the C-terminal WUS-box domain are required for the transactivation activity of OsWOX3A.

**Discussion**

Here, we examined the pleiotropic phenotype of nal2/3 double mutant rice, and used map-based cloning to identify NAL2 and NAL3 on chromosomes 11 and 12, respectively. They encode an identical OsWOX3A/OsNS protein that is homologous to NS of maize and PRS of Arabidopsis, all of which belong to the same WOX3 subfamily (Figs S5b, S6; Zhang et al., 2008). Both NS and PRS play an important role in the recruitment of founder cells for margin development of lateral organ primordia in monocots and eudicots, respectively (Nardmann et al., 2004). However, the functions of NS/PRS homologues in other plant species remain unknown. Our histological and molecular studies show that in rice, OsWOX3A is involved in differentiation of lemma and palea in spikelets, and tiller and LR production, in addition to lateral-axis expansion in leaves.

**OsWOX3A in lamina outgrowth and vascular patterning**

The nal2/3 mutation has a wide range of morphological defects in development of various organs in rice (Figs 1–3, 7; Tables S2, S4). Especially, it profoundly reduces leaf width, similar to nal1, nal7, and nrl1 mutants in rice (Fujino et al., 2008; Qi et al., 2008; Hu et al., 2010). The nal2/3 mutant produces narrow leaf blades with upward curling (Fig. 1b,c), and the widths of all leaf blades are consistently narrower, starting from the primordial stage (Fig. S2). In addition, nal2/3 has reduced leaf margin structures (Fig. 1d) and defects in distribution and arrangement of LVs and SVs (Figs 1c, 2a–h). In rice, SVs are formed later than...
LVs during leaf development (Itoh et al., 2005). The short interval between LVs in nal2/3 (Fig. 2b) suggests that there is not enough space to generate as many SVs as in wild-type. In addition, narrower and thicker mesophyll cell regions between SVs and bulliform cells (Fig. 2b,f) indicate that there is a defect in lateral cell proliferation between veins in nal2/3. To compensate for the reduction of lateral cell number in nal2/3, newly divided mesophyll cells may be deposed vertically during leaf elongation, resulting in increased leaf thickness (Tsukaya, 2008). During leaf initiation, leaf primordia are also narrower and thicker (Fig. S2), suggesting that there are fewer leaf founder cells in nal2/3. This result indicates that NAL2/3 genes are required for cell proliferation in lateral region, which promote outgrowth of leaf blade, from the leaf primordial stage. In addition, nal2/3 clearly lacked leaf margin structures; blunt leaf blades (Fig. 2g,h) and fewer sawtooth hairs at the margins of leaf blades (Fig. 1d), indicating that adaxial–abaxial patterning was compromised in nal2/3 mutants. Similar defective phenotypes were observed in Arabidopsis prs and wox1 double mutants in which leaf blade outgrowth was compromised due to the reduced cell proliferation in lateral region, resulting in production of narrow and thick leaves (Vandenbussche et al., 2009; Nakata et al., 2012). The double mutant also showed defects in margin-specific tissues and adaxial/abaxial patterning, which are caused by loss-of-function mutation of middle domain-specific WOX genes in early-developing leaves (Nakata et al., 2012). These results suggest that NAL2/3 may work in the similar manner with Arabidopsis WOX genes for leaf development. In maize, ns mutant has narrow leaf sheaths with a margin-deleted phenotype in the lower portion of the leaf blades (Scanlon et al., 1996). Although NS and OsWOX3A have similar functions in lateral-axis development of leaf primordia, the phenotypic defects in their mature leaves are quite different, mainly due to their structural differences in the upper and lower portions in leaf blades. In addition, SAM size was also reduced in nal2/3, as observed in sequential transverse sections (Fig. S2), suggesting that NAL2/3 genes may be required for the maintenance of SAM size. The Arabidopsis yab quadruple mutants showed defects in apical dominance, reduced SAM as well as loss of leaf expansion with polarity defects (Sarojam et al., 2010). These results indicate that YAB gene activity is required for both shoot development and marginal domain establishment in leaf blade, and altered expression of YAB genes in nal2/3 may have caused defects in development of SAM as well as leaf expansion.

Recently, OsWOX3B/DEP, a homologue of OsWOX3A, has been reported to regulate the formation of bristle-type trichomes in the leaves and glumes (Angeles-Shim et al., 2012). Although they belong to the same subfamily of OsWOX proteins (Fig. S6), the biological function of OsWOX3B/DEP was considerably different from that of OsWOX3A, suggesting that rice WOX3 genes within the same subfamily may have diversified to have separate biological functions. Together, our results suggest that OsWOX3A plays an important role in promoting cell proliferation in leaf width during leaf initiation and outgrowth, which is a conserved function of WOX3 among species. This can be explained by the specific expression of OsWOX3A/OsNS in the lateral margins of leaf primordia as described previously (Nardmann et al., 2007).

OsWOX3A regulates lemma and palea development in spikelets

Defects in seed development were not reported for maize ns mutants or Arabidopsis prs mutants; however, the rice nal2/3 mutant produces abnormally narrow-thin grains with opened spikelets. Lemma and palea in the outmost layer of the spikelet
OsWOX3A expression during lateral root formation. Two-week-old transgenic rice (Oryza sativa) plants expressing ProOsWOX3A::GUS were stained to observe OsWOX3A expression at various developmental stages. (a–c) GUS expression in lateral root (LR) initiation stages (a) and early developmental stages (b, c). Note that ProOsWOX3A::GUS was highly expressed at the base of lateral root primordia. Arrowheads indicate GUS signals detected at the base of LR. (d, e) GUS expression in later stages of LR development. Note that ProOsWOX3A::GUS signals were only detected in the vasculatures. Asterisk indicates the absence of GUS signal at the base of LR. Bars, 50 μm.

OsWOX3A is involved in formation of tillers and LRs, possibly by regulating OsPIN1 and OsPIN2 expression

Among the leaf development-associated genes, OsWOX3A probably acts as a negative regulator of YAB1, YAB2, YAB3, YUC1, YUC4 and OsPIN2 (Dai et al., 2007). However, it appears that OsWOX3A also acts as a positive regulator of several leaf development-associated genes, especially those involved in auxin synthesis and transport, such as ARF1, ARF4, YUC8/NAL7 and OsPIN1 (Fig. 6a), although it remains to be determined whether OsWOX3A binds to their promoter regions directly as a transcriptional activator (Fig. 9).

Auxin plays important roles in controlling cell identity, cell division, initiation of lateral organs, and formation of leaf margins and vasculature (Scanlon, 2003; Scarpella et al., 2006; Grieneisen et al., 2007). The ARFs are all transcription factors that bind to auxin-response elements in the promoters of early auxin-responsive genes (Tiwari et al., 2003). Although YUC and ARF transcript levels were altered, endogenous concentrations of free IAA were not significantly changed in nal2/3 (Fig. 8). The auxin efflux carrier PIN1 facilitates polar transport of auxin in Arabidopsis (Paponov et al., 2005; Kleine-Vehn et al., 2009) and in rice (Xu et al., 2005; Qi et al., 2008). Nonpolarized localization of PIN1 causes abnormal distribution of the auxin maxima required for vascular networking in Arabidopsis (Shirakawa et al., 2009). In rice, repression of OsPIN1 by RNAi caused defects in adventitious roots and increased the number of tillers. Overexpression of OsPIN1 increased LR number (Xu et al., 2005) and overexpression of OsPIN2 increased tiller number and reduced LR number (Chen et al., 2012). In nal2/3, tiller number increased (Fig. 5) and LR number decreased, possibly due to both downregulation of OsPIN1 and upregulation of OsPIN2. Furthermore, the reduced LR number in nal2/3 was rescued by exogenous IAA treatment, similar to the case of...
OsPIN1-RNAi plants (Xu et al., 2005). This strongly suggests that increased tillers and reduced LRs in nal2/3 seem to be attributable to altered expression of OsPIN1 and OsPIN2. In Arabidopsis, AtPIN1 is expressed in the inner layer cells of LR primordia; however, AtPIN2 is detected in the outer cells only after primordium emergence (Benkova et al., 2003). Precise regulation of spatial expressions of AtPIN1 and AtPIN2 is crucial in LR development, which is based on balanced auxin supply to the tip and its PIN2-dependent retrieval (Benkova et al., 2003). Notably, OsWOX3A expression was detected at the base region of LR primordia during LR emergence (Fig. 8). Therefore, it can be speculated that OsWOX3A modulates LR organogenesis by regulating OsPIN1 and OsPIN2.

The YUC genes, whose expression was altered in nal2/3 (Fig. 6a), are involved in auxin biosynthesis and thereby control leaf blade outgrowth and margin development (Wang et al., 2011). The PRS and WOX1 are required for promoting cell proliferation in outgrowth of leaf blade, maybe due to activated cell division via auxin and cytokinin (Nakata et al., 2012). In the ns mutants of maize, microarray data showed that TIR1 and ARF-GAP, auxin signalling- and transport-related genes are downregulated (Zhang et al., 2007). In addition, STF of Medicago truncatula and LAM1 of tobacco, the Arabidopsis WOX1 orthologues, also affect auxin concentrations (Tadege et al., 2011), strongly suggesting that the WOX family acts in the regulation of auxin biosynthesis, signalling or transport.

Our physiological and molecular data suggest that OsWOX3A regulates the transcription of genes involved in auxin synthesis, signalling and/or polar transport for lateral cell proliferation during vegetative and reproductive organ development (Table S2, Table S4). Further identification of as-yet-unknown regulatory genes downstream of OsWOX3A will provide further insights into the function of the WOX3 subfamily.

Acknowledgements

This work was supported by a grant from the Next-Generation BioGreen 21 Program (Plant Molecular Breeding Center No. PJ008128), Rural Development Administration, Republic of Korea.

References


Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Scanning electron microscopy of the adaxial and abaxial surfaces of nal2/3 leaves.

Fig. S2 The shoot apex region of the nal2/3 mutants displayed reduced size of the shoot apical meristem (SAM) and defective marginal structure in leaf primordia.

Fig. S3 nal2/3 displayed normal morphology in pistil and stamen development.

Fig. S4 nal2/3 showed a significantly increased number of tillers at both active and maximum tillering stages.

Fig. S5 Characterization of nal2 and nal3 mutant proteins and OsWOX3A homologues.

Fig. S6 Phylogenetic tree of the WOX families.

Fig. S7 Complementation of nal2/3 by OsWOX3A.

Fig. S8 Endogenous free-IAA concentrations in the whole plants of 2-wk-old wild-type (WT) and nal2/3 using IAA ELISA analysis.

Table S1 Primers used in this study

Table S2 Morphological characteristics of nal2/3

Table S3 Cell size and cell number in the marginal region of lemma of wild-type and nal2/3 plants

Table S4 Agronomic traits in nal2/3

Table S5 Accession numbers of WOX proteins in the phylogenetic tree (Fig. S6)

Methods S1 Quantification of endogenous IAA.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the New Phytologist Central Office.