WUSCHEL-related homeobox family genes in rice control lateral root primordium size

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Published December 30, 2021.

Edited by Luis Herrera-Estrella, Department of Plant and Soil Science, Texas Tech University, Lubbock, TX; received January 29, 2021; accepted November 3, 2021

The development of a plastic root system is essential for stable crop production under variable environments. Rice plants have two types of lateral roots (LRs): S-type (short and thin) and L-type (long, thick, and capable of further branching). LR types are determined at the primordium stage, with a larger primordium size in L-types than S-types. Despite the importance of LR types for rice adaptability to variable water conditions, molecular mechanisms underlying the primordium size control of LRs are unknown. Here, we show that two WUSCHEL-related homeobox (WOX) genes have opposing roles in controlling LR primordium (LRP) size in rice. Root tip excision on seminal roots induced L-type LR formation with wider primordia formed from an early developmental stage. QHB/OsWOX5 was isolated as a causative gene of a mutant that is defective in S-type LR formation but produces more L-type LRs than wild-type (WT) plants following root tip excision. A transcriptome analysis revealed that OsWOX10 is highly up-regulated in L-type LRP. OsWOX10 overexpression in LRP increased the LR diameter in an expression-dependent manner. Conversely, the mutation in OsWOX10 decreased the L-type LR diameter under mild drought conditions. The qhb mutants had higher OsWOX10 expression than WT after root tip excision. A yeast one-hybrid assay revealed that the transcriptional repressive activity of QHB was lost in qhb mutants. An electrophoresis mobility shift assay revealed that OsWOX10 is a potential target of QHB. These data suggest that QHB represses LR diameter increase, repressing OsWOX10. Our findings could help improve root system plasticity under variable environments.

Significance

In recent years, phenotypic plasticity has received attention for improving plant adaptability to variable environments. For more than half a century, it has been known that rice and cereal plants develop different types of lateral roots (LRs), unlike the dicot model plant Arabidopsis. Despite the importance of plastic LR development under variable water conditions, the molecular mechanisms regulating LR types are unknown. Here, we report the regulatory mechanism of LR primordium size in rice, an important determinant of LR type. We identified two WUSCHEL-related homeobox (WOX) transcription factors that opposingly regulate LR primordium size. Our findings form the basis for improving root phenotypic plasticity for sustainable crop production under variable environments.

Author contributions: T. Kawai, M.N., Y.S., A.T., Y.C., K.H.M.S., A.Y., and Y.I. designed research; T. Kawai, K.S., R.A., N.L.-A., M.K.-N., R.R.S., and M.N. performed research; T. Kawai, K.S., R.A., and Y.I. analyzed data; and T. Kawai and Y.I. wrote the paper.

The authors declare no competing interest.

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PNAS 2022 Vol. 119 No. 1 e2101846119

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indicator of sink strength (19), and LR diameter is positively correlated with LR length (18). Recently, we isolated a rice mutant with promoted L-type LR formation, and the analysis of the mutant suggested the involvement of auxin in L-type LR formation (20). However, the molecular mechanisms underlying primordium size control of LRs are unclear.

The WUSCHEL-related homeobox (WOX) genes encode plant-specific homeodomain transcription factors. WOX transcription factors have important roles in determining cell fate and plant tissue development. In rice, 13 WOX genes were found in its genome (21). QHB/OsWOX5 is the first characterized rice WOX gene involved in the specification and maintenance of stem cells in the root apical meristem (RAM) (22). OsWOX11 positively regulates CR emergence and growth by the modulating of plant hormone signaling (23, 24). Other rice WOX genes are involved in maintaining shoot apical meristem and leaf and tiller development (25–27).

In a previous study, we established a method of root tip excision in SR, which promotes L-type LR formation in the remaining proximal portions of wild-type (WT) rice (“Taichung 65”) (18). In this study, the mechanism of LR primordium (LRP) size control is studied through both forward and reverse genetics approaches with the root tip excision method. Root tip excision to determine the cause of reduction in S-type LRs was enhanced in the mutants, including the T3-7–1 mutant. We hypothesized that enhanced production of L-type LRs in the T3-7–1 mutant with promoted L-type LR formation, and the analysis of the mutant suggested the involvement of auxin in L-type LR formation (20).

The LRP then underwent anticlinal and periclinal divisions, and the mutant produced more L-type LRs than the WT following root tip excision to determine the cause of reduction in S-type LRs in response to root tip excision. In addition, a transcriptome analysis between S- and L-type LRs revealed that OsWOX10 is highly up-regulated in L-type LRs. Further analysis suggested that these two WOX genes play opposing roles in controlling LRP size in rice.

Results
Identification of a Mutant with a Defect in S-Type but Promoted L-Type LR Formation Following Root Tip Excision. A method of root tip excision in SR, which promotes L-type LR formation in the remaining proximal portions of WT rice (“Taichung 65”) (Fig. 1 A–C and ref. 18), was applied to an N-methyl-N-nitrosourea (MNU)-mutagenized population to screen mutants with altered LR formation. A recessive mutant, T3-7–1, was isolated; this mutant developed fewer LRs, but new LRs emerged following root tip excision (Fig. 1 D–G). Interestingly, this recovery was observed in L-type LRs (≥ 150 μm in diameter) but not S-type LRs (<100 μm) or the intermediate type (Fig. 1 H–J). Notably, the mutant produced more L-type LRs than the WT following root tip excision, with increases in regions distant from the cut site (Fig. 1 J and K). This suggests that the cause of the T3-7–1 mutant represses the increase in LR diameter in regions distant from the cut site. Second-order LRs did not emerge from L-type LRs induced by root tip excision in the mutant; however, this defect in second-order branching was rescued by root tip excision in L-type LRs (SI Appendix, Fig. S1 A–C). Additionally, the mutant produced fewer CRs than the WT (SI Appendix, Fig. S1 D and E). Neither the length nor the gravitropic response in SRs changed in the T3-7–1 mutant (SI Appendix, Fig. S1 F–J). Thus, a causative gene of the T3-7–1 mutant was assumed to play a major role in developing CRs and S-type LRs, including second-order LRs, and repressing LR meristem enlargement in response to root tip excision (Fig. 1 L).

We hypothesized that enhanced production of L-type LRs after root tip excision in the mutant was due to fewer CRs or LRs. Therefore, SRs were subjected to root tip excision in crl1 and Osaa13 mutants, which produce fewer CRs and LRs, respectively (SI Appendix, Fig. S2 A and B and refs. 28 and 29). Consequently, both mutants produced similar numbers of L-type LRs compared with the WT after root tip excision (SI Appendix, Fig. S2 C and D). However, elongation of emerging LRs was enhanced in the mutants, including the T3-7–1 mutant (SI Appendix, Fig. S2E). Thus, the presence of fewer LRs and CRs may not be responsible for the increased number of L-type LRs in the T3-7–1 mutant.

Isolation of the Causative Gene of the T3-7–1 Mutant. We performed a linkage analysis of simple-sequence repeat markers to identify the causative gene of the T3-7–1 mutant using the 1,370 F2 plants derived from a cross between the mutant and “Kasalath.” The root phenotype of the mutant segregated in a 3:1 WT:mutant ratio. Map-based cloning revealed that the locus was located on chromosome 1 between RM11876 and RM3783 (~426 kb; Fig. 2A). This region contains about 40 genes including QHB/OsWOX5 (Quiescent center specific homeobox; Os01g0854500; herein QHB). Comparing the WT and mutant sequences revealed a single-nucleotide substitution, from G to A, at the end of the intron in QHB (Fig. 2B). As the mutated site was likely to be important for splicing, the messenger RNA (mRNA) sequence was compared by synthesizing complementary DNA from the WT and mutant. The first nucleotide in the second exon was deleted in the mutant mRNA, resulting in a stop codon at the beginning of this exon (Fig. 2C). The introduction of a 4.7-kb genomic DNA fragment containing the entire QHB gene into the mutant complemented the mutant phenotype (SI Appendix, Fig. S3A). Thus, we concluded that the mutation in the QHB gene causes the mutant phenotype. Herein, the mutant is referred to as the qhb mutant. QHB is the first isolated Wox-type homeobox gene specifically expressed in the RAM of rice (22). AtWOX5, an orthologous gene in Arabidopsis, contains a WUS-box and an EAR motif in the C-terminal region, which confer repressive transcription factor activity (30). Both the WUS-box (TLE/QLFP) and EAR motif (P/LLE/DLRL) are well conserved among QHB orthologous genes from various plant species (SI Appendix, Fig. S3B). In the qhb mutant, a stop codon is present in front of the WUS-box and EAR motif (SI Appendix, Fig. S3B), suggesting that qhb may be a loss-of-function mutant.

Analysis of S- and L-Type LR Development in the WT and qhb Mutant. We analyzed the development of L-type LRs without root tip excision to determine the cause of reduction in S-type LRs in the qhb mutant. In the WT, the first anticlinal cell division was observed in pericycle cells ~5 mm from the SR tip (Fig. 2D). The LRP then underwent anticlinal and periclinal divisions, and the meristem was formed 24 h after initiation (Fig. 2E and SI Appendix, Fig. S4 B–F). Thereafter, S-type LRs continued to grow, emerging from the SRs within 36 h of initiation (Fig. 2E and SI Appendix, Fig. S4 G and H). No meristem formation was observed in the qhb mutant; smaller and deformed LRs were observed 24 h after initiation (SI Appendix, Fig. S4Q). Among the LRs observed 7 d after initiation in the qhb mutant, 20% stopped cell division after one round of anticlinal and periclinal divisions with irregular-shaped cells (Fig. 2 F, Left), and the remaining 80% had additional cell divisions with disturbed cellular organization (Fig. 2 F, Right) (n = 20 LRs from five independent SRs). Furthermore, the LRs emerging from SRs were often short with a disorganized apical meristem (SI Appendix, Fig. S4R). Similar to S-type LR development, deformed CR primordia (CRPs) were found in mutant stems (SI Appendix, Fig. S5 A–C), consistent with QHB expression in CRPs (22).

Next, we analyzed L-type LR development in the WT and qhb mutant. The SR was cut 5 mm behind the root tip where the first anticlinal division was observed in the pericycle cell (Fig. 2D). A total of 6 h after root tip excision, LRP underwent an additional anticlinal division followed by periclinal division in pericycle cells and grew wider than the S-type with expanded cells (Fig. 2G). The expanded cells underwent anticlinal divisions in the L-type LRP 12 h after root tip excision (Fig. 2G), while pericycle cells in S-type underwent additional periclinal
Then, LRP underwent rounds of anticlinal and periclinal divisions, and the meristem formed 30 h after root tip excision (Fig. 2G and SI Appendix, Fig. S4L–N), which was 6 h later than in S-type (Fig. 2E). The meristem was larger in L-type LRP with more cell layers in the ground tissue and stele than S-type (Fig. 2E and G). In the qhb mutant, L-type LR meristems with organized cell layers were established 30 h after root tip excision, similar to that in the WT (Fig. 2H). The sum of deformed LRPs and emerged LRs in the mutant was comparable with the number of LRs in the WT both without and with root tip excision (Fig. 2L). This suggests that QHB functions in meristem development but not in the initiation, and root tip excision recovers meristem formation of the initiated LRs of qhb mutants. Furthermore, the sum of CRPs and emerged CRs in the mutant was comparable to that in the WT (SI Appendix, Fig. S5D), suggesting that the function of QHB in meristem formation is conserved between S-type LRs and CRs.

We next analyzed QHB expression during S- and L-type LR development. QHB expression was first detected in pericycle cells at the initiation stage (Fig. 2I and SI Appendix, Fig. S6A). Thereafter, QHB expression was detected in whole LRP in both S- and L-types, with broader expression in the pericycle.
cells of SRs with root tip excision (Fig. 2 J and K and SI Appendix, Fig. S6 B, C, E, and F). In S-type LRP, QHB expression was restricted to the quiescent center (QC) as meristem formation progressed (Fig. 2 J and SI Appendix, Fig. S6I). Expression was similar in second-order LR formation on L-type LRs induced by root tip excision (SI Appendix, Fig. S6 H–K). In L-type LRP, QHB was expressed in late metaxylem (LMX) precursor cells and the QC after meristem formation (Fig. 2K and SI Appendix, Fig. S6G), similar to that in SRs (SI Appendix, Fig. S6L and ref. 31).

Fig. 2. Map-based cloning of the causative gene of the T3-7-1 mutant and its function in LR development. (A) High-resolution linkage and physical map of the causative gene locus on chromosome 1. The vertical bars represent molecular markers, and the numbers of recombinant plants are indicated below the linkage map. (B) Structure of the QHB/OsWOX5 gene (Os01g0854500). Black and gray boxes indicate exons and untranslated regions, respectively. The arrowhead represents a single-nucleotide mutation in the intron at position 488 bp in the open reading frame. (C) The mutation site at the intron and resulting changes in splicing junction and amino acid sequence in the mutant. (Left) Sanger sequencing analysis around the splicing junction of QHB complementary DNA in the WT and qhb mutant. (Right) mRNA structures and amino acid sequences in WT and qhb mutant. (D–H) LR development in SR without (+ cut) (E and F) or with (+ cut) root tip excision (G and H) in the WT (D, E, and G) and qhb mutant (F and H) plants. The first anticlinal division in pericycle cells was observed 5 mm behind the SR tip before root tip excision in the WT (D). Time after root tip excision is shown. (Scale bars, 50 μm.) Detailed development of WT plants is shown in SI Appendix, Fig. S4. (I–K), Expression of the QHB gene without (+ cut) (J) or with (+ cut) root tip excision (K) during LR development observed in a cross-section of SR of pQHB-GUS in a WT background. Expression in the region 5 mm behind the root tip before root tip excision (I). (Scale bars, 50 μm.) (L) Comparison of the numbers of LRs and LR primordia in a region 0 to 25 mm from the cut site 7 d after root tip excision in the WT and qhb mutant. Values represent mean ± SD (n = 5 independent biological replicates). Different lowercase letters indicate significant differences among groups (P < 0.05, multiple-comparison Tukey’s test).
Identification of the Genes Differentially Expressed in S- and L-Type LRP. We next performed an RNA sequencing (RNA-seq) analysis of L-type LRP from isolated LRPs from SRs after 12 h of root tip excision and S-type LRPs of a similar developmental stage isolated from intact SRs in the WT, respectively. In total, 531 genes were up-regulated (259 genes) or down-regulated (272 genes) by more than twofold (adjusted P < 0.05) in L-type LRP than S-type (Fig. 3A and B and Datasets S1 and S2). Gene ontology (GO) analysis revealed the enrichment of genes with common and different functions between up-regulated and down-regulated genes (Fig. 3C, SI Appendix, Figs. S7 and S8, and Datasets S3 and S4). In both groups, genes with “transcription regulator activity” were enriched (Fig. 3C). Enriched GO terms of “molecular function” were greater in up-regulated than down-regulated genes and included several terms related to oxidative stress (Fig. 3C), suggesting that the root tip excision triggers the wounding stress response in L-type LRP. Because L-type LRs are induced by auxin treatment (20), we explored the response of 259 up-regulated genes in response to auxin (32). A total of 73 of the 259 up-regulated genes in L-type LRP were auxin inducible in roots (P < 0.05, Fold change > 2, at least one time point after indole-3-acetic acid treatment compared with no treatment in the “root gene expression profile in response to auxin” microarray dataset [identification: RXP-1008, Dataset S1]), which was significantly enriched among all genes (P = 1.9E-15, χ² test). The expression of OsIAA9 and 20, which are highly auxin-inducible AUX/IAA genes (33), were up-regulated in L-type LRP, which was confirmed by qRT-PCR (SI Appendix, Fig. S9). The most plausible hypothesis is that auxin accumulates in the proximal portions after root tip excision, causing L-type LR formation. To test this hypothesis, an SR after root tip excision was treated with a polar auxin transport inhibitor, N-1-naphthylphthalamic acid (NPA), in the WT. The NPA treatment resulted in a decrease in LR number

![Fig. 3. Transcriptome analysis in S- and L-type LRPs. S- and L-type LRPs were isolated from SRs without and with 12 h of root tip excision in the WT, respectively. (A and B) Logarithm fold changes in 259 up-regulated (A) and 272 down-regulated genes (B) presenting more than twofold changes in L-type LRP than S-type in the WT. (C) Enriched GO terms of “molecular function” in up-regulated and down-regulated genes in L-type LRP compared with S-type. (D) Phylogenetic tree constructed using the full-length amino acid sequences of WOX family proteins in rice and Arabidopsis using MEGA7. Numbers on branches are the 500 bootstrap values. Accession numbers of proteins are shown in SI Appendix, Table S4. (E) Relative expression levels of QHB/OsWOX5 and OsWOX10 in S- and L-type LRPs. Expression levels were quantified in S- and L-type LRPs isolated from SRs without and with 12 h of root tip excision, respectively. Values represent mean ± SD (n = 3 technical replicates, repeated with two biological replicates with similar results). Asterisks indicate significant differences between treatments (**P < 0.01, two-tailed Student’s t test). (F) Promoter activity of OsWOX10 visualized in T3 transgenic plants containing pooswox10-NLS-3xVenus. Expression in S- and L-type LRP developed in crown roots without (∼ cut) or with (∼ cut) root tip excision, respectively. Green dots and blue color indicate OsWOX10 expression and autofluorescence of cell walls, respectively. (Scale bars, 50 μm.)

https://doi.org/10.1073/pnas.2101846119
in intact SRs in a dose-dependent manner; however, after both root tip excision and the treatment, a decrease in L-type LRs was not observed (SI Appendix, Fig. S10 A and B). In Arabidopsis, de novo auxin biosynthesis and auxin transport cooperatively increase LR number after root cutting (34). The application of an auxin biosynthesis inhibitor 5-(4-chlorophenyl)-4H-1, 2, 4-triazole-3-thio (Yucasin; ref 35) did not affect the L-type LR increase by root tip excision (SI Appendix, Fig. S10D), but the cotreatment of NPA and Yucasin suppressed L-type LR formation (SI Appendix, Fig. S10F), suggesting the synergistic regulation of L-type LR formation after root tip excision by auxin biosynthesis and transport.

Among 73 auxin-inducible genes up-regulated in L-type LRP, OsWOX10 (Os08g0242400), a WOX domain transcription factor, was up-regulated to the greatest extent in L-type LRP (Fig. 3A). Notably, OsWOX11 (Os07g0864900) and OsWOX6 (Os03g0325600), homologous genes of OsWOX10 clustered in the same clade among 13 rice WOX family proteins (Fig. 3D). Western blot analysis showed slight up-regulation of OsQHB/OsWOX5 in L-type LRP; however, the increment was much higher in OsWOX10 (Fig. 3E). Expression analysis with pOsWOX10-NLS:3xVENUS revealed that OsWOX10 is expressed in the basal part of LRP from the early developmental stage in L-types and in the epidermal cell layer after meristem formation in both S- and L-types (Fig. 3F). Furthermore, several auxin-inducible genes encoding transcription factors that function as regulators of root development such as the LATERAL ORGAN BOUNDARIES DOMAIN (LBD) genes were also up-regulated in L-type LRP (SI Appendix, Fig. S9). Among them, OsLBD2-1 (also named as OsLOB16; ref. 36) is the closest rice orthologous gene to AtLBD16, which is directly regulated by AtWOX11 and ARF7/19 and functions in LR formation (37, 38). Thus, OsWOX10 and its homologous genes might play a major role in L-type LRP development.

OsWOX10 Positively Affects LR Diameter Increase. To analyze the functions of OsWOX10 in LRP development, OsWOX10 was overexpressed under the control of the QHB promoter to express it in the LRPWs of intact SRs. QHB was expressed in S-type LRPs in intact SRs as well as L-types emerged after root tip excision (Fig. 2 J and K), while OsWOX10 was not expressed in S-types at an early developmental stage (Fig. 3F). In a WT background, the number of L-type LRs in intact roots increased, while S-types and total LRs decreased under high OsWOX10 expression (Fig. 4 A–D and SI Appendix, Fig. S11 A and B). Interestingly, the diameter of LRs increased in transgenic plants with increasing OsWOX10 expression (Fig. 4E and SI Appendix, Fig. S11C). Notably, pQHB-OsWOX10 did not rescue the reduced number of LRs in the intact roots of the qhb mutant (SI Appendix, Fig. S11 D–F). To verify the role of OsWOX10 in the control of LRP size, Oswox10-mutant

![Fig. 4. Functional analysis of OsWOX10 in LR formation. (A) LR formation on CRs in T<sub>0</sub> transgenic plants containing pQHB-OsWOX10 fusion DNA fragment in a WT background. (Scale bars, 1 cm.) (B–E) Relative expression levels of OsWOX10 in CR tips (B) (n = 3 technical replicates), density of S- (C) and L-type LRs (D), and maximum LR diameter (E) (n = 4 independent biological replicates) in each transgenic line shown in A. VC, Vector control. D., LR diameter. Bar plots show mean values ± SD. Different lowercase letters indicate significant differences among groups (P < 0.05, multiple-comparison Tukey’s test). (F and G) LR formation on CRs in the WT (F) and Oswox10 mutant (G) grown in soils under mild drought stress condition. (Right) Enlarged views of the boxed areas. Arrow heads indicate second-order LRs. (Scale bars, 5 mm.) (H) Diameter of LRs with second-order LRs in F and G. Boxes show the first quartile, median, and third quartile. Whiskers show the range of nonoutlier values. Individual points indicate outlier values less than the first quartile or greater than the third quartile by 1.5 times the interquartile range. P value derived from two-tailed Student’s t test (n = 52, 58 LRs from three independent CRs in WT and Oswox10, respectively).](https://doi.org/10.1073/pnas.2101846119)
Taichung 65 plants were generated using CRIPSR/Cas9 (SI Appendix, Fig. S11G). Under mild drought conditions, the WT produced LRs with higher-order branching, distinct from S-types in diameter and length (Fig. 4F). Oswox10 mutants also produced LRs with higher-order branching; however, these LRs were significantly thinner than the WT (Fig. 4 G and H), verifying that OsWOX10 positively affects LR diameter.

As the qhb mutant produced more L-type LRs after root tip excision than WT (Fig. 1 J and K), we examined whether OsWOX10 is differentially regulated in WT and the mutant. qRT-PCR revealed that the increased OsWOX10 expression, caused by root tip excision, gradually weakened with distance from the cut site in the WT (Fig. 5A), similar to the change in L-type LR number (Fig. 1K). qhb mutants had higher OsWOX10 expression than the WT near the cut site, which was maintained at higher levels even in distant regions (Fig. 5A and SI Appendix, Fig. SI1H), consistent with the formation of L-type LRs in distant regions in the mutant (Fig. 1K). QHB expression increased following root tip excision in the WT (Fig. 5B and SI Appendix, Fig. SI1J); however, the mutant-type QHB might not be functional, resulting in a greater increase in OsWOX10 expression in the mutant. As orthologous genes of both OsWOX10 and QHB are up-regulated after wounding in leaf explants in Arabidopsis (39), it was assumed that the higher expression of OsWOX10 in the qhb mutant was due to higher sensitivity to wounding stress in the mutant. The expression patterns of wounding stress marker genes, up-regulated in L-type LRP, were analyzed to test this hypothesis. As a result, no major differences between the WT and qhb mutant were detected in the tested genes (SI Appendix, Fig. S12), suggesting that wounding signaling level does not differ between the WT and qhb mutant.

Next, we tested the hypothesis that QHB negatively regulates OsWOX10 expression, and thus, OsWOX10 expression is higher in the qhb mutant than the WT after root tip excision. We first analyzed the transcriptional repression activity of QHB, as the WUS-box and EAR motif, which confers repressive activity, are well conserved in QHB (SI Appendix, Fig. S3B). In the yeast one-hybrid assay, QHB suppressed the transcriptional activity of VP16 (Fig. 5C), suggesting the transcriptional repression of downstream genes. The mutant-type QHB protein, which lacks the WUS-box and EAR motif, did not suppress the activity of VP16 (Fig. 5C), suggesting that mutant-type QHB does not function as a transcriptional repressor. Next, we examined whether QHB directly binds to OsWOX10 promoter regions. In the 1-kb upstream region of OsWOX10, we found two WOX-binding sequences (TATAATGC/C; refs. 22, 23, and 36) and nine core sequences (TAAT or its tandem repeat; refs. 30, 40, and 41). Among the nine core sequences, three were located near each other (from −530 to −283 base pair [bp] from the
QHB (translation start site); thus, it is more likely that QHB binds to this region. To test the binding of QHB to these candidate sequences, we performed an electrophoresis mobility shift assay (EMSA) with recombinant QHB proteins and Cy5-labeled DNA probes (P1–3, SI Appendix, Fig. S13A). The QHB bound to P2, containing three “TAAT” core sequences, but not to other probes (SI Appendix, Fig. S13B). As the shifted band in P2 was positioned higher than that in the positive control (introm of Arabidopsis AGAMOUS gene including “TTAA TGG”; ref. 22), it was assumed that QHB could target several “TAAT” core sequences in the P2 region. To verify which core sequences QHB binds to, we conducted EMSA with additional probes containing mutation(s) in the core sequences (Fig. 5D). As a result, shifted bands were not observed when Core 1 or 3 was mutated (Fig. 5D); thus, QHB binds to Core 1 and 3. These results suggest that OsWOX10 is a potential target of QHB and negatively regulated by QHB.

To examine the regulation of LR diameter by QHB and OsWOX10 under soil conditions, the WT and qhb mutant seedlings were grown in soils with different water conditions, waterlogged and mild drought. Under mild drought, L-type LR number increased compared with waterlogged condition in the WT, with increased OsWOX10 expression (Fig. 5 F–H). In the qhb mutant, OsWOX10 expression and L-type LR number were higher than in WT under mild drought (Fig. 5 F–H). These observations suggest that QHB and OsWOX10 also control plastic LR development induced by mild drought in soil conditions.

Discussion

Based on this study’s results, we provide models for primordium size control of LRs (Fig. 5E) and regulatory mechanisms for developing different LR types (SI Appendix, Fig. S14) in which QHB/OsWOX5 and OsWOX10 play different roles. Interestingly, the root tip excision method identified QHB/OsWOX5 as a key regulator of root development in rice (Figs. 1 and 2 A–C). The qhb mutant was defective in developing S-type LRs, including second-order LRs, and CRs (Fig. 1 and SI Appendix, Fig. S1). In Arabidopsis, a mutation in AtWOX5, the orthologous gene of QHB, decreases LR density (42), but the detailed LRP phenotype of the Atwox5 mutant has not been reported. During LR formation in Arabidopsis, AtWOX5 expression is first detected in QC cells at the same time as QC is specified in the outer layer–derived cells (43), which is later than that in rice QHB detected just after LR initiation (Fig. 2 I–K). Therefore, QHB and AtWOX5 might be involved in different steps of LR formation. The qhb mutant produced similar numbers of LRPCs and CRPs to WT; however, cellular organization was disturbed in the primordia, decreasing the number of emerged S-type LRs and CRs (Fig. 2 F and L and SI Appendix, Fig. S5). In Arabidopsis, adventitious roots regenerated from leaf explants decreased in the Atwox5 Atwox7 double mutant (Atwox7 is the homologous gene of AtWOX5) with root primordia formed but defective in cellular organization (39). Expressions of AtWOX5 and AtWOX7 were detected in the primordia after the transition of root founder cells to primordia in leaf explants of Arabidopsis (39). In rice CRs, QHB expression is first detected in the outer cell layer at the two-layer stage (22), which is as early as AtWOX5 and AtWOX7 expressions during adventitious rooting from leaf explants in Arabidopsis (39) and LR formation in rice (Fig. 2 I–K). These observations suggest that QHB and AtWOX5 function in the cellular organization of root primordia in the root types in which they are expressed from the early developmental stage.

L-type LR development was recovered by root tip excision in the qhb mutant (Fig. 1), although QHB was expressed in both S- and L-type LRPCs after initiation (Fig. 2 I–K). These data suggest that unknown factors might redundantly function in meristem formation of L-type LRs induced by root tip excision, but not S-type LRs. Notably, OsWOX10 expression in LRPC driven by the QHB promoter did not recover the defect in meristem formation in the qhb mutant (SI Appendix, Fig. S11 D–F); thus, other mechanisms might contribute to meristem formation of L-type L Rs after root tip excision in the mutant (SI Appendix, Fig. S14). After meristem formation, QHB was expressed in the precursor cells of LMX and QC in L-type LRP (Fig. 2K). In SRs, decreased expression of QHB in LMX cells by the overexpression or exogenous treatment of FON2-LIKE CLE PROTEIN2 (FCP2p) impairs LMX development (31). QHB expression in LMX precursor cells of L-type LRPCs may confer a more developed vascular structure of L-type LRs accompanied by an LMX and a larger number of proto xylems than S-types (10).

We revealed the opposing roles of two WOX transcription factors, QHB/OsWOX5 and OsWOX10, in the control of LR size in rice (Fig. 5E). OsWOX10 expression was positively correlated with LR diameter. In the WT, OsWOX10 up-regulation by root tip excision gradually decreased with distance from the cut site (Fig. 5A) accompanied by a smaller L-type LR number in distant regions (Fig. 1K). Conversely, OsWOX10 up-regulation was maintained in regions farthest from the cut site in qhb mutants (Fig. 5A), possibly increasing L-type LRs after root tip excision (Fig. 1 J and K). EMSA revealed that OsWOX10 is a potential target of QHB (Fig. 5D), and thus, QHB may directly repress OsWOX10 expression and the increase of LR diameter. Notably. OsWOX10 expression was not up-regulated in the qhb mutant than WT in intact SRs (SI Appendix, Fig. S11H). Since OsWOX10 was not expressed in S-type LRPCs before the meristem formation in the WT (Fig. 3F), it seems that up-regulation by a transcriptional activator is needed for OsWOX10 expression in LRPCs. As OsWOX10 is an auxin-inducible gene and the auxin signaling is up-regulated in L-type LRPCs than S-types (SI Appendix, Fig. S9), it is assumed that activation of auxin signaling and thus ARF transcription activity triggers OsWOX10 expression in L-type LRPCs. Both QHB and OsWOX10 were induced in the SR sections after root tip excision (Fig. 5 A and B); however, the up-regulation of OsWOX10 was much stronger than that of QHB in L-type LRPCs (Fig. 5E). Thus, the mutant qhb up-regulation after root tip excision may not be strong enough to completely repress OsWOX10 expression near the cut site, resulting in OsWOX10 up-regulation in L-type LRPCs. It is noteworthy that S-type LRPCs at early developmental stages can increase their LR size and become L-types before the meristem is established (16, 18). The root cutting at different root positions revealed that early stage LRPCs, which grow as S-type LRPCs, increase their diameter after root cutting and become L-type LR (16, 18). Further analyses are needed to clarify the function of QHB and OsWOX10 in this flexible regulation of LR size.

The identified regulation of LR diameter by QHB and OsWOX10 would be involved in plastic LR development in soil conditions. OsWOX10 expression increased with mild drought and possibly confers the increased L-type LRPCs under the condition in the WT (Fig. 5 F–H). In the qhb mutant, OsWOX10 expression, hence L-type LR number, was higher than the WT under mild drought (Fig. 5 F–H). It is possible that the unidentified factor for meristem formation (discussed in the third paragraph) is absent in waterlogged conditions; thus, L-type LR number did not increase despite the higher OsWOX10 expression in the qhb mutant than the WT under the condition. The mutation in OsWOX10 decreased L-type LR diameter (LRs with second-order LRPCs) grown under mild drought conditions (Fig. 4 F–H). Interestingly, LRPCs in Oswox10 plants grown under mild drought conditions produced higher-order branching despite the LR diameter decreasing (Fig. 4 F–H). This indicates that the
thickness and higher branching ability of LRs, the characteristics used to determine LR types, are regulated by independent mechanisms. In Arabidopsis, the Atwox11 Atwox12 double mutant produces LRs with fewer higher-order branches in soil than the WT (37), suggesting that WOX11/12 proteins differentially regulate the plastic LR development between rice and Arabidopsis.

In conclusion, our study reveals genetic mechanisms underlying primordium size control of LRs in rice. As the LR type determined by its primordium size is an important phenotypic trait for the growth maintenance of rice plants under variable water environments, our study provides important insights for improving plant resilience to environmental stresses through plastic root development.

Materials and Methods

Detailed information on plant material and growth conditions, root cutting treatments, chemical treatments, morphological characterization, histological characterization, map-based cloning, plasmid constructs, plant transformation, expression analysis, laser microdissection, RNA-seq analysis, yeast one-hybrid assay, electrophoresis mobility shift assay, and statistical analysis is provided in SI Appendix, Materials and Methods.

Data Availability. Transcriptome data have been deposited in DDBJ (DNA Data Bank of Japan; https://www.ddbj.nig.ac.jp/dbget/index-e.html) (PRJDB11157, SAMD000276461-SAMD000276468). Gene expression profile was obtained at RiceXpro version 3.0 (https://ricexpro.dna.affrc.go.jp/), accessed on 4 October 2019.

Acknowledgments. We thank Kinya Inukai, Eiko Murakami, and Sakai Nishiiuchi for their valuable technical support; Masafumi Mikami, Seiichi Toki, and Masaki Endo (National Agriculture and Food Research Organization) for providing the CRISPR–Cas9 vectors; Kotaro Miura for providing the screening lines for rice mutants; and Keisuke Nagai and Tokunori Hobo for providing the material for the yeast one-hybrid assay. This work was supported by the PRESTO program of the Japan Science and Technology Agency (JST) (15H04435), the SATREPS program (JPMJSA1706) of the JST and Japan International Cooperation Agency, and MEXT/JSPS KAKENHI (18H02174, 18JJ21800).

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