A *WUSCHEL-LIKE HOMEOBOX* Gene Represses a *YABBY* Gene Expression Required for Rice Leaf Development[1[C][W]]

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YABBY and *WUSCHEL-LIKE HOMEOBOX* (*WOX*) genes have been shown to play important roles in lateral organ formation and meristem function. Here, we report the characterization of functional relationship between rice (*Oryza sativa*) *YAB3* and *WOX3* in rice leaf development. Rice *YAB3* is closely related to maize (*Zea mays*) *ZmYAB14* and Arabidopsis (*Arabidopsis thaliana*) *FILAMENTOUS FLOWER* (*FIL*), whereas rice *WOX3* is highly conserved with maize *narrow sheath1* (*NS1*) and *NS2* and Arabidopsis *PRESSED FLOWER* (*PRS*). In situ hybridization experiments revealed that the expression of both genes was excluded from the shoot apical meristem, but the transcripts were detected in leaf primordia, young leaves, and reproductive organs without any polar distribution. The function of the two genes was studied by both overexpression and RNA interference (RNAi) in transgenic rice. *YAB3* RNAi induced twisted and knotted leaves lacking specialized structures such as ligule and auricles, while no phenotypic change was observed in *YAB3* overexpression plants, suggesting that rice *YAB3* may be required for leaf cell growth and differentiation. Overexpression of *WOX3* repressed *YAB3* and showed a *YAB3* RNAi phenotype. The expression of class I *KNOTTED-LIKE HOMEOBOX* (*KNOX*) genes was ectopically induced in leaves of *YAB3* RNAi or *WOX3* overexpression plants. Data from inducible WOX3 expression and DNA-protein interaction assays suggested that WOX3 acted as a transcriptional repressor of *YAB3*. These data reveal a regulatory network involving *YAB3*, *WOX3*, and *KNOX* genes required for rice leaf development.

Most plant organs are formed during the postembryonic stages from the meristems. The shoot apical meristems (SAMs) are organized pools of undifferentiated or embryonic cells maintained by a dynamic balance between cell division and differentiation. The meristematic identity of cells in the SAM is correlated with the expression of specific regulatory genes. During the formation of leaf organ primordia, cells at the flanks of the SAM undergo a fundamental developmental transition from an indeterminate to a determinate cell fate as they are recruited into leaves. For the SAM to maintain its indeterminate function throughout the life of a plant, it is essential that cells recruited into lateral organs be constantly replenished. A growing list of transcription factor proteins are required to maintain, and possibly to establish, the SAM. *KNOTTED1* (*KN1*) defines the first homeobox gene family to be isolated in plants and was identified from maize (*Zea mays*) gain-of-function mutants that produced knots, or outgrowths, of indeterminate tissue on the leaf (Vollbrecht et al., 1991). *KN1*-like homeobox (*KNOX*) genes that share the highest degree of sequence similarity with *KN1* are expressed in overlapping domains within the SAMs of both monocot and dicot plants (for review, see Reiser et al., 2000; Hay et al., 2004). Loss-of-function mutation of *KN1* in maize or of the closely related gene *SHOOTMERISTEMLESS* in Arabidopsis (*Arabidopsis thaliana*) results in failure to maintain a SAM (Long et al., 1996; Vollbrecht et al., 2000). Down-regulation of *KNOX* expression in leaf founder cells within the meristem marks a change in cell fate from meristem to leaf (Smith et al., 1992). Exclusion of *KNOX* expression from leaves is important for leaf development, as ectopic expression confers indeterminate features to Arabidopsis leaves, including ectopic meristems and a dramatic change in leaf shape (Chuck et al., 1996). At least two mechanisms exist to repress *KNOX* expression in Arabidopsis leaves. The myb transcription factor ASYMMETRIC LEAVES1 and ASYMMETRIC LEAVES2, a member of the LATERAL ORGAN BOUNDARIES family of transcriptional regulators, act together to repress expression of *KNOX* genes in the leaf (Byrne et al., 2000; Ori et al., 2000). Activity of the YABBY family of putative transcription factors also contributes to the exclusion of *KNOX* gene expression from Arabidopsis leaves (Kumaran et al., 2002).

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The *YABBY* family transcription factors are characterized by a C_2C_2 zinc finger domain toward the amino terminus and a putative helix-loop-helix domain, also named YABBY, conserved in high mobility group transcription factors toward the carboxyl terminus (Bowman and Smyth, 1999; Golz and Hudson, 1999). There are at least six *YABBY* genes in Arabidopsis (*FILAMENTOUS FLOWER* [*FIL*] or *YAB1*, *YAB2*, *YAB3*; *INNER NO OUTER* [*INO*] or *YAB4*, *YAB5*; and *CRABS CLAW* [*CRC*]). All of these genes show a polar expression pattern and function to determine the abaxial cell fate of one or more above-ground lateral organs (Bowman, 2000). In addition to the repressive function of *KNOX* gene expression, Arabidopsis *YABBY* genes are mainly involved in the control of abaxial identity of lateral organs (Eshed et al., 1999; Siegfried et al., 1999). In contrast to Arabidopsis *YABBY* genes, *YABBY* gene family members that are closely related to *FIL* (*ZmYAB9*, *ZmYAB14*) are expressed on the adaxial side of incipient and developing leaf primordia (Juarez et al., 2004). These observations suggest that *YABBY* genes in monocots may have different functions in lateral organ formation compared to their homologs in Arabidopsis.

In rice (*Oryza sativa*), there are also at least six members in the *YABBY* gene family (Jang et al., 2004). Recently, the rice *YAB1* has been shown to play a role in stamen and carpel development (Jang et al., 2004). The *CRC*-related rice *YABBY* gene *DROOPING LEAF* (*DL*) whose mutation induces a *dl* phenotype is required for carpel specification and leaf midrib development (Yamaguchi et al., 2004). In contrast to Arabidopsis and maize *YABBY* members, rice *YAB1* and *DL* do not show any adaxial/abaxial polar expression pattern in lateral organs. Overexpression or mutation of these genes results in no adaxial/abaxial polarity change in lateral organs (Jang et al., 2004; Yamaguchi et al., 2004; Dai et al., 2007), suggesting at least these two rice *YABBY* members have different function from their Arabidopsis or maize homologs. However, the function of *FIL* or *ZmYAB14/9* closely related rice *YABBY* genes was not known.

Arabidopsis *WUSCHEL* (*WUS*) gene functions in a more restricted set of cells in the SAMs to promote stem cell fate and is regulated by *CLAVATA* genes in a negative feedback loop (Mayer et al., 1998). The *WUS* and *WUS*-like homeobox genes (*WOX*) contain a WUS box toward the amino terminus in addition to the homeobox toward the carboxyl-terminus end. It has been suggested that Arabidopsis *WOX* genes play an important role in region-specific transcription programs early during embryogenesis and lateral organ development (Haecker et al., 2004). For instance, *STIMPY/WOX9* is required for the growth of vegetative SAM in Arabidopsis (Wu et al., 2005), while *PRESSED FLOWER* (*PRS/WOX3*) is involved in the regulation of lateral axis-dependent sepal development as well as leaf stipules (Matsumoto and Okada, 2001; Nardmann et al., 2004). The maize duplicated *narrow sheath1* (*NS1*) and *NS2* genes, which are the immediate homologs of *PRS*, are suggested to function during recruitment of organ founder cells in a lateral domain of the SAM (Nardmann et al., 2004). Rice genome contains at least nine homologs of *WOX* genes, among which *quiescent center-specific homeobox* (*QHB*) has been found to function in root development (Kamiya et al., 2003).

To study transcriptional regulatory networks controlling rice shoot development, we underwent a research program to systematically study the expression and functional relationship of members of rice *YABBY*, *WOX*, and other putative transcription factor gene families in controlling shoot/leaf development. In this report, we present data on the expression and functional analysis of a rice *YABBY* gene (*YAB3*), which is closely related to *ZmYAB14/9* and to *FIL*, and a *NS1/2/PRS*-related rice *WOX* gene (*WOX3*). We show that both genes are coexpressed in most lateral organ primordia and young leaves without adaxial/abaxial polarity. Down-regulation of *YAB3* or overexpression of *WOX3* induced knotted outgrowth of leaves that lack clear separation between the leaf sheath and the leaf blade, along with ectopic expression of *KNOX*

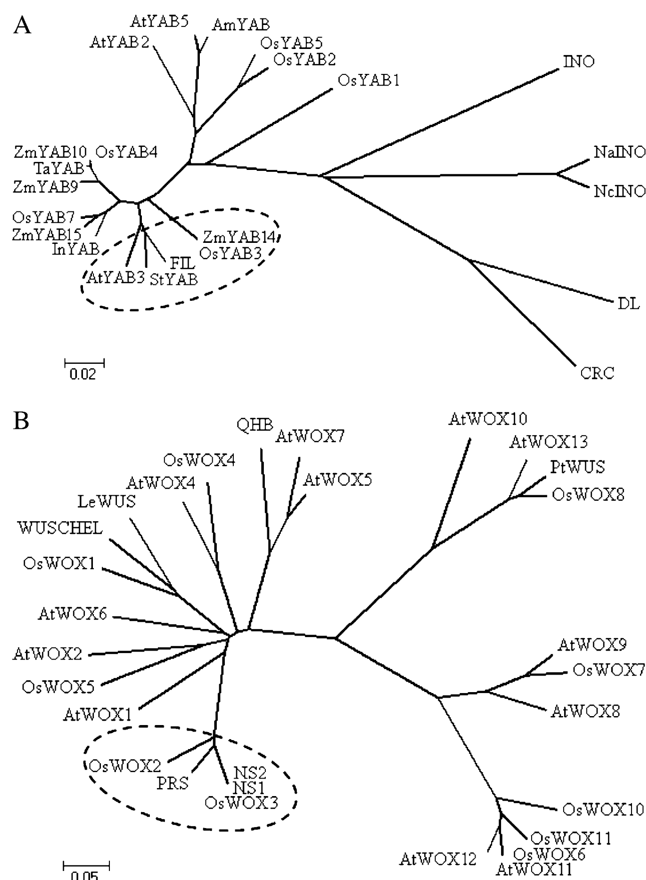

Figure 1. Phylogeny analysis of YABBY and WOX families. Neighbor-joining trees of YABBY (A) and WOX (B) proteins from rice (Os), Arabidopsis (At), *Antirrhinum majus* (Am), maize (Zm), *Triticum aestivum* (Ta), *Solanum tuberosum* (St), *Ipomoea nil* (In), *Nymphaea alba* (Na), *Nymphaea colorata* (Nc), and *Solanum lycopersicum* (Le). GenBank accession numbers are shown in Table I.

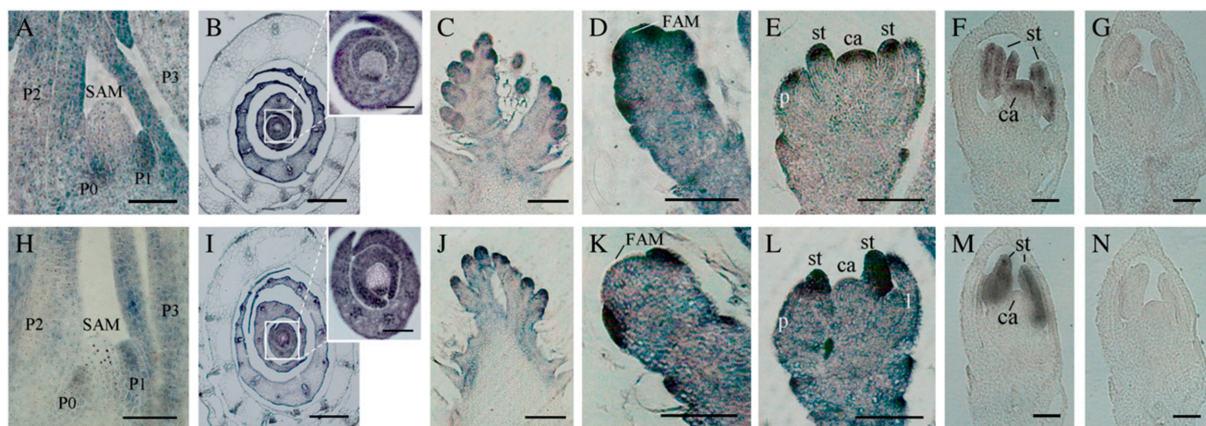

Figure 2. In situ detection of *YAB3* and *WOX3* transcripts. A to G, Sections hybridized with *YAB3* antisense (A–F) or sense (G) probes. H to N, Sections hybridized with *WOX3* antisense (H–M) or sense (N) probes. A and H, Longitudinal sections of SAM. B and I, Transverse sections of SAM; insets, enlarged views of the central areas of the apexes. C and J, Developing panicle longitudinal sections. D to F and K to M, Longitudinal sections of florets at different development stages. The midrib regions of leaf primordia (A and H) are designated by plastochron (P) numbers, such that the leaf incipient primordium is labeled P0, the next older leaf is labeled P1, and so on. FAM, Floral apical meristem; st, stamen; ca, carpel; l, lemma; p, palea. Bar = 100 μm.

genes in leaves of the transgenic plants. This phenotype suggests that rice *YAB3* is required for cell differentiation during leaf development. Expression and DNA-binding studies suggest that WOX3 functions as a transcriptional repressor of *YAB3*. Together, our data reveal a transcriptional regulatory hierarchy required for rice leaf development.

RESULTS

YAB3 Was Expressed in the Leaf and Floral Organ Primordia

Phylogenic analysis of YABBY proteins showed that rice YAB3 was highly conserved with ZmYAB14 (Juarez et al., 2004). The monocot proteins were mostly closely related to FIL among the Arabidopsis YABBY members (Fig. 1A). In situ hybridization experiments with a gene-specific region of the rice *YAB3* cDNA as the probe revealed that the *YAB3* transcripts were detected in leaf primordia and in young leaves but excluded from the SAM. In the young leaves, *YAB3* transcripts were homogenously distributed (Fig. 2). During panicle development, *YAB3* transcripts were detected initially in the floret meristems and then in the primordia of the floral organs, including palea, lemma, stamens, and carpel, and later were restricted to the stamens and carpel. Unlike Arabidopsis *YABBY* genes and maize *ZmYAB14/9*, rice *YAB3* did not show any polar expression patterns in the floral organs. This expression pattern suggested that rice *YAB3* may have a different function.

YAB3 Was Nucleus Localized

To study the subcellular localization of YAB3, the full-length *YAB3* coding sequence was translationally fused to the GFP-coding sequence under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The GFP alone controlled by the 35S promoter served as a control. The constructs were introduced into the onion (*Allium cepa*) cells using the particle bombardment method. The GFP expression was examined with confocal microscopy 36 h after bombardment. GFP alone was located throughout the cells (Fig. 3A), while the YAB3-GFP fusion protein was localized in the nuclei of the onion cells (Fig. 3B).

Down-Regulation of *YAB3* Induced Defects in Leaf and Flower Development

To study the developmental function of *YAB3*, we used a gene-specific region from the 3′ end of the cDNA (see ''Materials and Methods'') to construct a double-strand RNA producing or RNA interference (RNAi) vector. The construct was introduced into 'Zhonghua 11' by Agrobacterium-mediated transformation. More than 30 independent T0 transgenic plants were produced for each construct. Most of the transgenic plants produced aberrant twisted leaves displaying knotted outgrowth. The specialized rice leaf structures such as ligule, lamina joint, and auricles that are located at the junction between the leaf blade and the leaf sheath in the wild type were absent in the phenotypic transgenic plants (Fig. 4). Examination of cross sections revealed no alteration of the leaf adaxial/abaxial polarity (Supplemental Fig. S1). All the phenotypic plants showed a decrease of *YAB3* transcripts (Fig. 5A).

The twisted and knotted *YAB3* RNAi leaf phenotype was reminiscent of the knotted leaves in *kn1* mutants in maize, which is a dominant mutation causing ectopic expression of *KN1* in leaves. In addition, overexpression of rice class I *KNOX* genes also induces

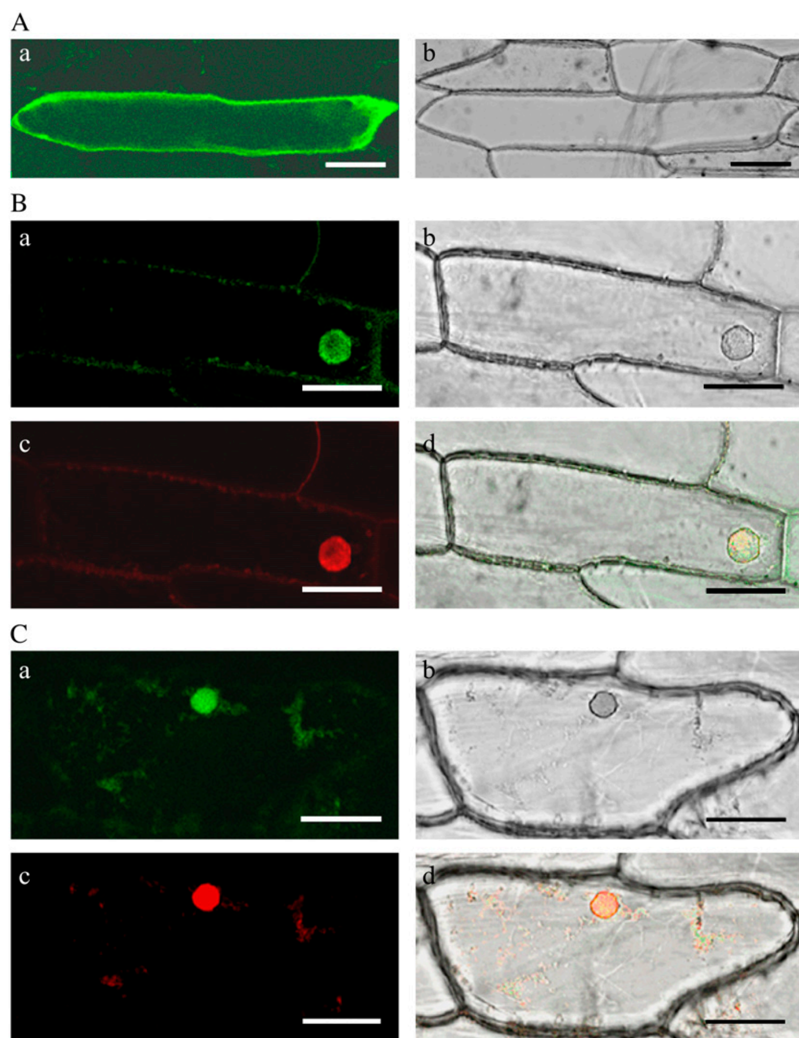

Figure 3. Nuclear localization of YAB3 and WOX3 GFP alone was localized in cytosol of onion skin cells (A). YAB3-GFP (B) and WOX3-GFP (C) are nucleus localized. a, GFP images. b, Transmission images. c, DAPI staining. d, Merged images. Bar = 50 μm.

knots on leaves and diffusion of the blade-sheath boundary (Sentoku et al., 2000). Reverse transcription (RT)-PCR analysis showed that two tested rice *KNOX* genes (*OSH1* and *OSH3*) were indeed ectopically expressed in leaves of *YAB3* RNAi plants (Fig. 5C), suggesting that *YAB3* had a function to repress *KNOX* genes in rice leaves.

The panicle development seemed normal. However, most florets were degenerated, but a few florets remained normal and eventually gave rise to seeds. Further investigation of the severely degenerated florets revealed that there was no recognizable anther structure and that ovules seemed not to exist (Supplemental Fig. S2). Florets with a less severe phenotype had smaller anthers than the wild type, which produced very few mature pollens (Supplemental Fig. S2).

To study whether the overexpression of *YAB3* had any effect on rice growth, we made a transgenic plant with a construct in which the maize ubiquitin promoter was used to direct the expression of the *YAB3* cDNA. Among more than 40 transgenic lines obtained, no visible phenotype was observed. Northern-blot analysis showed most tested transgenic plants overexpressed *YAB3* (Fig. 6A).

WOX3 Expression Pattern Overlapped with That of *YAB3*

Sequence analysis showed that rice *WOX3* was highly conserved with maize *NS1*/*NS2* genes that have been suggested to be orthologs of Arabidopsis *PRS* (Nardmann et al., 2004; Fig. 1). Both *NS1*/*NS2* and *PRS* are expressed in meristematic foci and in the margins of lateral organ primordia. However, in situ hybridization experiments revealed that *WOX3* had a different expression pattern than *NS1*/*NS2* and *PRS*, which was actually similar to that of *YAB3* in both vegetative shoots and in developing panicles, except in the carpel primordium where *WOX3* transcripts were not detected (Fig. 2). *WOX3* protein was also targeted into the nucleus of transiently transfected onion cells (Fig. 3C).

Overexpression of *WOX3* Repressed *YAB3*

Similarly, we intended to study the developmental function of *WOX3* by the transgenic approaches. Both

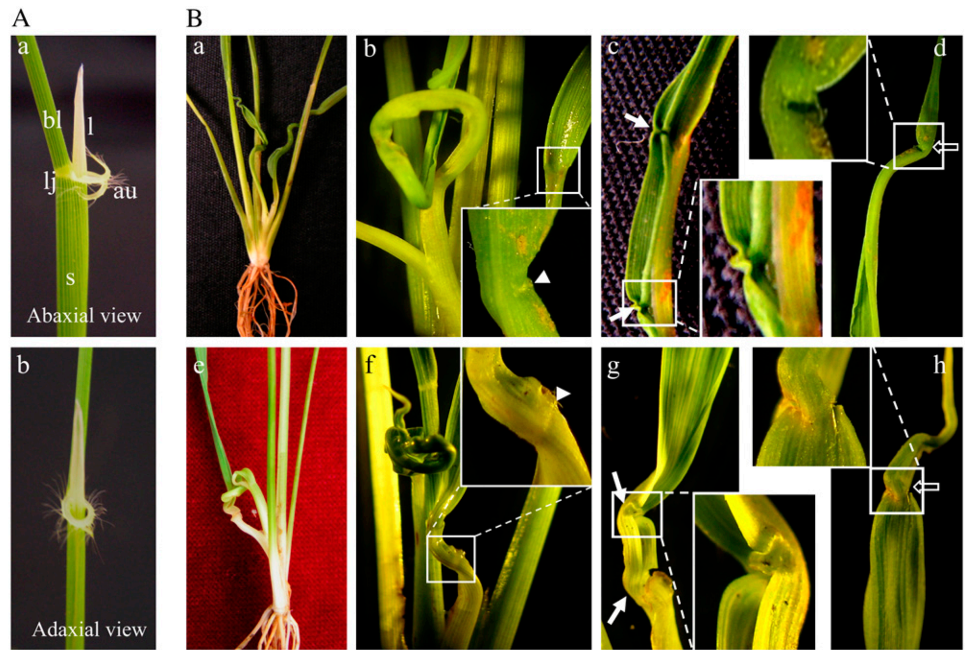

Figure 4. Shoot phenotypes induced by *YAB3* RNAi or by *WOX3* overexpression. A, Wild-type leaf at abaxial (a) and adaxial (b) views showing the structures of ligule (l), auricle (au), and lamina joint (lj). bl, Blade; s, sheath. B, Phenotypes in *YAB3* RNAi (a–d) and *WOX3* (e–h) overexpressing plants. a and e, Profile of the transgenic plants. b and f, Enlarged view of the parts with twisted leaves in transgenic plants. Insets, junctions between leaf blades and sheaths with ligules and auricles missing, indicated by triangles. c and g, Knots in the lower parts of some transgenic leaves; arrows indicate the position of the knots. d and h, Knots in the upper parts of transgenic leaves, arrows indicate the position of the knots. The knotted regions in c, d, g, and h are enlarged (insets).

overexpression and RNAi transgenic plants were obtained. Most of the *WOX3* overexpression plants showed a similar leaf phenotype as the *YAB3* RNAi plants (Fig. 4B). The *WOX3* overexpression plants also showed ectopic expression of *KNOX* genes *OSH1* and *OHS3* (Fig. 5). However, no phenotypic alteration was observed in the *WOX* RNAi populations (Fig. 6). The phenotypic resemblance induced by the two transgenes suggested that there might be a regulatory relationship between *WOX3* and *YAB3*. RT-PCR revealed that the expression of *YAB3* was repressed in leaves of the *WOX3* overexpression plants (Fig. 7A). The repression seemed to be specific to *YAB3*, because the expression of *YAB1*, *YAB2*, or *DL* was not altered (Fig. 7A). Down-regulation of *YAB3* by RNAi had no effect on the expression of *WOX3* or other *YABBY* genes (Fig. 7B). In situ hybridization experiments showed that overexpression of *WOX3* reduced the *YAB3* transcript levels in the shoot apexes and florets (Fig. 7C). Conversely, down-regulation of *WOX3* by RNAi induced the expression of *YAB3* in seedlings, inflorescences, and florets (Fig. 7D). However, there was no induction of *YAB3* in flag (mature) leaves where *YAB3* was not expressed (data not shown), suggesting that the silencing of *YAB3* in mature leaves involves additional mechanisms.

WOX3 Was Involved in Direct Repression of *YAB3*

To confirm whether WOX3 could directly repress the expression of *YAB3*, we used the glucocorticoid receptor (GR)-inducible system to artificially regulate the activity of WOX3 in vivo. The translational stop codon of *WOX3* was removed and replaced with a 288-amino acid segment of the mouse GR that contains the ligand-binding domain. The chimeric protein under the control of the double enhancers of the CaMV 35S promoter was introduced into rice plants by Agrobacterium-mediated transformation.

RT-PCR analysis showed that several analyzed transgenic plants overproduced the *WOX3-GR* mRNA (Fig. 8A). Three of the transgenic lines (WG3–WG5) were selected for further analysis. Siblings from each transgenic line were divided into three groups: one was treated with dexamethasone (DEX), the second was treated first with cycloheximide (CHX) for 1 h then with DEX, and the third had no treatment, together with the wild-type plants treated similarly. Leaves were harvested for RNA extraction and analyzed by quantitative real-time PCR to examine the transcript levels of *YAB3*. The results showed that the DEX treatment induced the repression of *YAB3* by 2- to 5-fold in the presence or absence of CHX, while the expression of *YAB3* in wild-type plants was not significantly affected by the treatments (Fig. 8B). These data indicated that the WOX3 was directly involved in the repression of *YAB3* transcription.

DISCUSSION

Function of *YABBY* Genes in Rice Leaf Development

In Arabidopsis, the function of *YABBY* genes has been well studied and shown to promote abaxial cell fate in the lateral organs (Bowman, 2000; Sieber et al., 2004; Lee et al., 2005; Meister et al., 2005). Arabidopsis *YABBY* gene transcripts are detectable in the abaxial domains of lateral organs when primordia emerge and begin to differentiate from the meristem (Bowman, 2000). However, maize *YABBY* members *ZmYAB14* and *ZmYAB9* are shown to be expressed in the adaxial

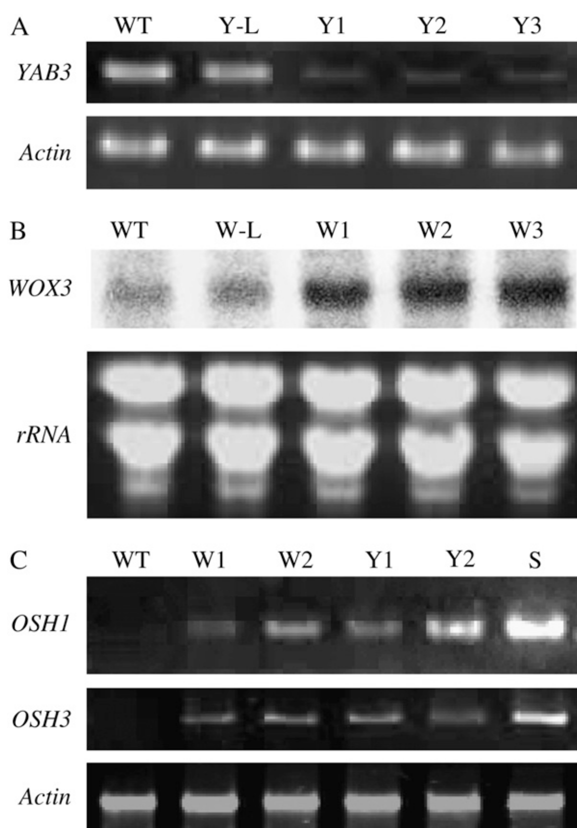

Figure 5. Expression analysis of *YAB3*, *WOX3*, and *KNOX* genes in *YAB3* RNAi and *WOX3* overexpression plants. A, *YAB3* transcript levels revealed by RT-PCR in wild-type (WT), a nonphenotypic (Y-L), and three phenotypic (Y1 to Y3) *YAB3 RNAi* transgenic plants. B, *WOX3* transcript levels revealed by northern blots in wild-type (WT), a nonphenotypic (W-L), and three phenotypic (W1–W3) *WOX3* overexpression transgenic plants. C, Transcript levels of rice *KNOX* genes *OSH1* and *OSH3* revealed by RT-PCR in leaves from wild-type (WT), phenotypic *YAB3* RNAi (Y1 and Y2), and *WOX3* overexpression plants (W1 and W2). Shoot apex mRNA was used as a positive control (S).

domain of leaves (Juarez et al., 2004). It is suggested that the maize YABBY genes play a role in lateral leaf outgrowth rather than specifying adaxial cell fate (Juarez et al., 2004). However, no loss-of-function phenotype of these genes is available to support the hypothesis. In this work, we report that the expression of rice *YAB3* that is closely related to *ZmYAB14* (Fig. 1A) did not show such an adaxial/abaxial polar expression pattern in leaves or reproductive organs (Fig. 2). Other studied rice *YABBY* genes including *YAB1* and *DL*, which are closely related to Arabidopsis *YAB2* and *CRC*, respectively, also have no polar expression in lateral organs (Jang et al., 2004; Yamaguchi et al., 2004; Dai et al., 2007). Likely, the function of rice *YABBY* genes in regulating leaf development may differ from its Arabidopsis and maize homologs. Analysis of RNAi transgenic plants revealed that rice *YAB3* had a function in leaf development. The *YAB3* RNAi plants do not show any abaxial-adaxial polarity change (Supplemental Fig. S1) but produce twisted and knotted leaves lacking differentiated tissues such as ligule, auricles, and distinction of the leaf sheath from the leaf blade, while the width of the leaf sheath and blade was not reduced (Fig. 4). The phenotypic alterations suggest that *YAB*3 is required for the promotion of differentiation during leaf organogenesis. This correlates with ectopic induction of class I *KNOX* genes *OSH1* and *OSH3* in *YAB3* RNAi leaves (Fig. 5), suggesting that the phenotype may be induced by the *KNOX* ectopic expression, as it has been shown that overexpression of rice *KNOX* genes induces similar phenotypes (Sentoku et al., 2000). These results also suggest that *YAB3* contributes to the exclusion of *KNOX* expression from the leaf tissues, as do Arabidopsis *FIL* and *YAB3*. This aspect of *YABBY* function seems to be conserved between rice *YAB3* and the Arabidopsis homologs. Loss-of-function mutation of another rice *YABBY* gene *DL* does not produce the *YAB3* RNAi phenotype (Yamaguchi et al., 2004), suggesting that the two rice *YABBY* genes have a distinct function in the regulation of *KNOX* gene expression and leaf development. In addition, overexpression of *YAB3* does not induce any morphological alteration (Fig. 6), which differs also from other rice *YABBY* genes. For instance, overexpression of rice *YAB1* induces defects in floral organ development (Jang et al., 2004), while *DL* ectopic expression plants produce curled leaf blades, forming a cylinder-like structure (Yamaguchi et al., 2004). These observations reinforce the suggestion that rice *YABBY* genes have distinct functions and that there is not much functional redundancy between them.

Rice *WOX3* Developmental Function

Rice *WOX3* is situated in the same branch as maize *NS1*/*NS2* genes in the phylogeny tree, which are the immediate homologs of Arabidopsis *PRS. PRS* is expressed at the lateral regions of lateral organs at very early stages (Matsumoto and Okada, 2001). The expression of *NS1*/*NS2* is also detected in meristematic foci and in the margins of lateral organ primordia (Nardmann et al., 2004). Loss-of-function mutations in the maize and Arabidopsis genes induce defects or deletion in leaf and sepal, respectively. Overexpression of *PRS* produced multicellular bulges with trichomes on the stem and on the peduncle (Matsumoto and Okada, 2001). It is suggested that there is a conserved function between *NS* and *PRS* during the recruitment of lateral founder cells from shoot meristems. During the formation of a maize leaf, founder cell recruitment begins on one flank of the SAM, which will form the central domain of the leaf, and proceeds toward the opposite flank, during which the function of *NS*/*PRS* initiates from lateral foci and recruits founder cells in a lateral meristematic domain (Nardmann et al., 2004). However, the rice *WOX3* transcripts were detected in relatively larger areas in leaf primordia, young leaves, and floral meristems (Fig. 2). This suggests that the function of rice *WOX3* might not be conserved with *NS1*/*NS2* or *PRS* in leaf development. The transgenic

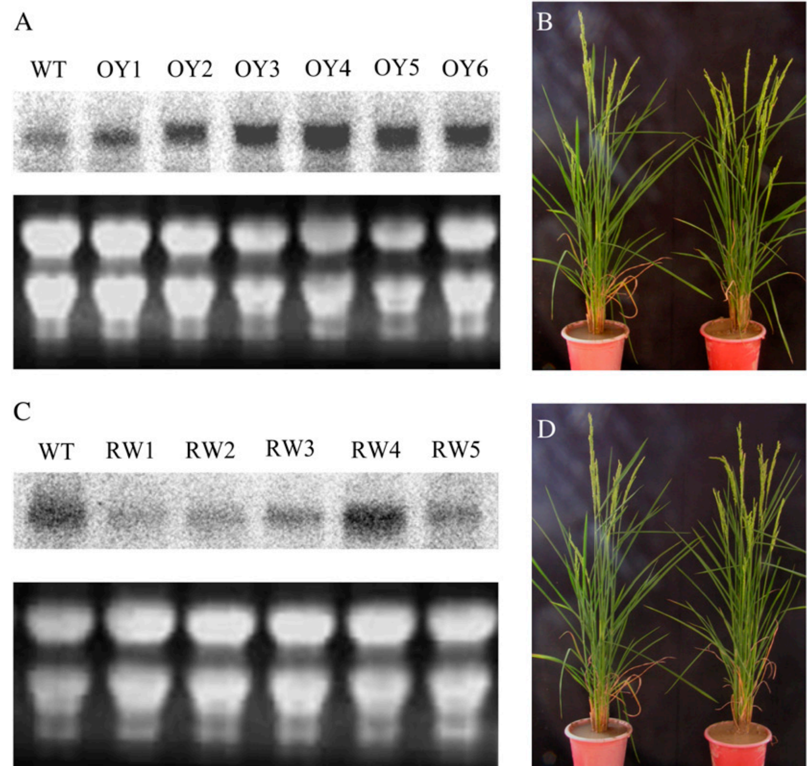

Figure 6. Analysis of *YAB3* overexpression and *WOX3* RNAi plants. A, Expression analysis of *YAB3* in wild-type (WT) and five independent *YAB3* overexpression transgenic lines (OY1–OY6). B, Comparison of wild type (left) to the *YAB1* overexpression transgenic line OY6 (right). C, Expression analysis of *WOX3* in wild-type (WT) and five independent *WOX3* RNAi transgenic plants (RW1–RW5). D, No phenotypic change observed in RW1 (right) compared to wild type (left). [See online article for color version of this figure.]

phenotypes support this hypothesis, as overexpression of *WOX3* induced deformed leaves in rice, while down-regulation of rice *WOX3* produced no visible phenotype, despite an induction of *YAB3* (Figs. 4 and 6). In addition, leaf founder cell recruitment is correlated with the repression of *KNOX* genes in simple leaf plants. Higher *NS1*/*NS2*/*PRS* activity would repress *KNOX* expression. However, rice *WOX3* overexpression induced ectopic expression of *KNOX* genes in transgenic leaves. This reinforces the hypothesis that rice *WOX3* is not involved in lateral founder cell recruitment during leaf formation.

No phenotype induced by down-regulation of *WOX3* suggests that other *WOX* genes (i.e. *WOX2* that is situated in the same branch as WOX3 in the tree; Fig. 1A) may act redundantly with *WOX3* to function independently of *YAB3*/*KNOX* regulation. Among the rice *WOX* genes, only *QHB* has been functionally characterized. *QHB* is expressed in the central cells of the quiescent center and is required for the root apical meristem function (Kamiya et al., 2003). However, ectopic expression of *QHB* also affects normal shoot and leaf development in rice (Kamiya et al., 2003). The malformed leaves induced by *QHB* overexpression are much like the leaves of *WOX3* overexpression or *YAB3* RNAi plants, suggesting that basic regulatory functions, such as DNA binding and transcriptional repression, of these WOX proteins might have been conserved during evolution, while their natural developmental functions diverge at least partially as a result of their differential expression patterns.

Regulatory Relationship between *WOX3* and *YAB3* in Rice Leaf Development

Although *WOX* and *YABBY* genes have been studied in different species, the functional relationship between the two families has not been established. Our data provide evidence that rice WOX3 functioned as a repressor of *YAB3*. Overexpression of *WOX3* represses *YAB3*, leading to similar phenotypic changes as induced by *YAB3* RNAi (Figs. 4 and 7). Conversely, repression of *WOX3* by RNAi induces the expression of *YAB3* (Fig. 7). Experiments with inducible WOX3 expression suggest that WOX3 directly regulated *YAB3* (Fig. 8). It has been determined that the target sequence of the WUS, the founding member of WOX, is TTAATGG in the intron of *AGAMOUS* gene (Lohmann et al., 2001). This sequence is also recognized by rice QHB (Kamiya et al., 2003), suggesting that the TTAATGG sequence may be the consensus WOX-binding site. Sequence analysis of *YAB3* locus identified a homologous sequence within the forth intron of the gene. Experiments with yeast (*Saccharomyces cerevisiae*) one-hybrid assays showed that WOX3 could interact with a segment of 230 bp of the *YAB3* gene compressing the putative WOX-binding site (Supplemental Fig. S3). In addition, the WOX3 protein produced in and purified from *Escherichia coli* cells could bind to the consensus-binding site of the *YAB3* gene in gel-shift assays (Supplemental Fig. S4). The WOX3-binding motif within *YAB3* is identical to the WUS site in *AGAMOUS*, while the flanking sequences are divergent. The WUS site

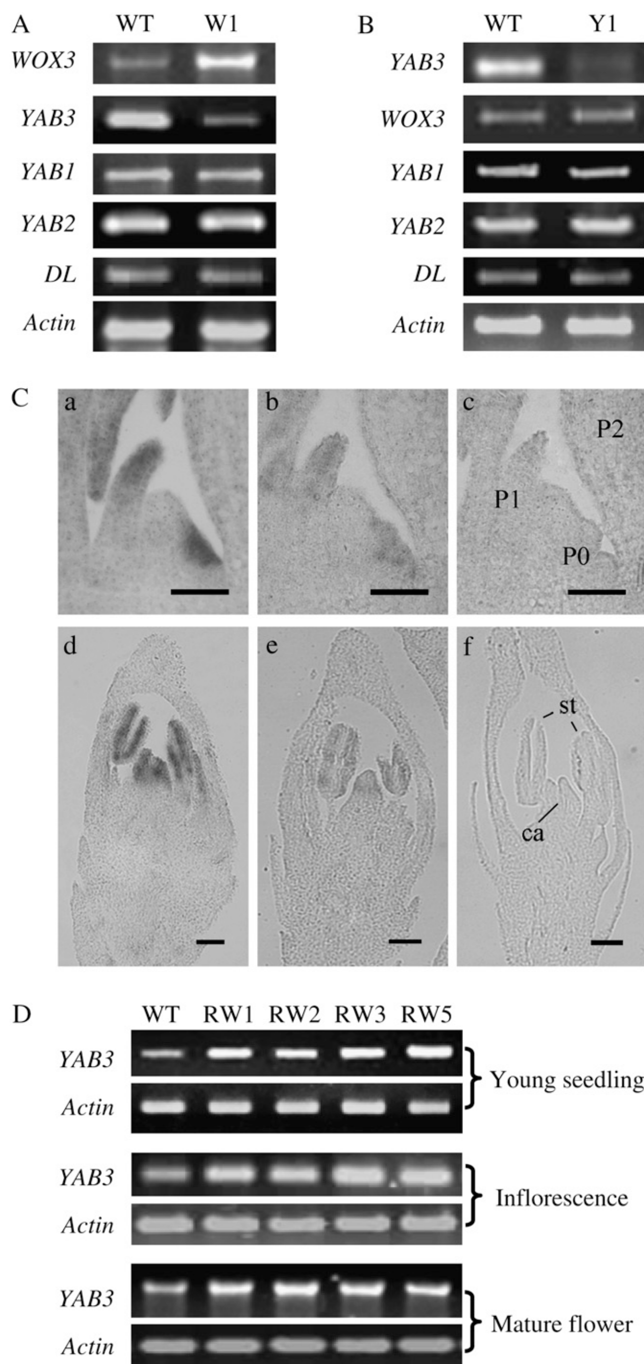

Figure 7. Expression analysis of *WOX3* and *YAB3* in the transgenic and wild-type plants. A, Semiquantitative RT-PCR analysis of four rice *YABBY* gene expression in *WOX3* overexpression line W1 compared to wild type. Rice *actin1* transcripts were measured as controls. B, Semiquantitative RT-PCR analysis of *WOX3* and the *YABBY* genes in the *YAB3* RNAi line Y1 compared to wild type. Rice *actin1* transcripts were used as control. C, In situ hybridization to detect *YAB3* transcripts in wild type (a and d) and *WOX3* overexpression (b and e) shoot apexes (top) and florets (bottom). A shoot apex and a floret hybridized with a sense probe of *YAB3* were used as controls (c and f). D, Expression analysis of *YAB3* in wild-type and *WOX3* RNAi 1-week-old seedlings, inflorescences, and florets. Rice *actin1* transcripts were measured and used as controls.

within the *AGAMOUS* is immediately flanked by the LEAFY-binding site and both of them are required to activate the gene (Lohmann et al., 2001). We speculate that additional cis-elements in the vicinity of the WOX3-binding site may exist in *YAB3* to define specificity of the regulation. The WOX3-binding motif is not found in the other rice *YABBY* genes, in agreement with the observation of no alteration of expression of the other *YABBY* genes, suggesting that WOX3 specifically regulates *YAB3* among the rice *YABBY* genes. However, whether the binding site within the *YAB3* gene was responsible for the repression by WOX3 awaits further analysis.

In vegetative shoots, the expression of the two genes overlaps. We speculate that a threshold of minimal expression level of *YAB3* is required to promote leaf cell differentiation and that *WOX3* has a function to control or limit the expression levels of *YAB3* in leaf tissues contributing to maintain the undifferentiated state of dividing cells. This regulatory relationship might be required to maintain the balance between cell division and cell differentiation during the leaf growth. No phenotypic alteration observed in *YAB3* overexpression or in *WOX3* RNAi plants suggest that additional factors are required for *YAB3* function in promoting leaf cell differentiation.

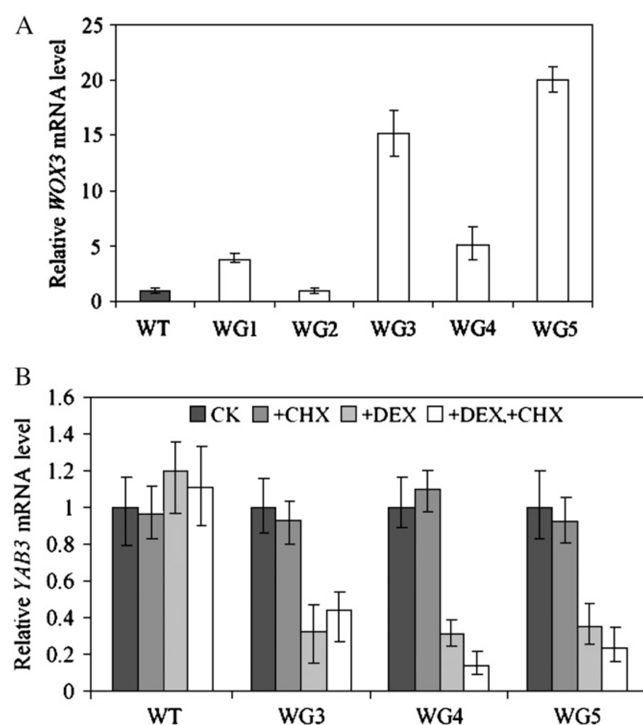

Figure 8. Activation of WOX3 directly repressed the expression of *YAB3*. A, Identification of transgenic lines expressing the *WOX3-GR* fusion by real-time RT-PCR. B, Relative expression levels of *YAB3* in wild-type and three *WOX3-GR* transgenic plants treated with or without DEX and/or CHX. The YAB3 transcript levels were normalized with the *actin1* mRNA levels, and that of the wild-type control was assessed as 1. Bars are SD ± three biological repeats.

Table I. *Accession numbers of YABBY and WOX proteins used for the construction of the phylogenic trees*

For Arabidopsis YABBY and WOX proteins, see Bowman (2000) and Haecker et al. (2004), respectively.

Accession Nos. of YABBY Proteins		Accession Nos. of WOX Proteins	
Name of Protein	Accession No.	Name of Protein	Accession No.
OsYAB1	BAF12697	OsWOX1	CAE04846
OsYAB2	ABF97910	OsWOX2	AAV44211
OsYAB3	BAF15337	OsWOX3	BAE48302
OsYAB4	BAF26935	OsWOX4	CAE04492
OsYAB5	BAF30318	OsWOX5	NP_915421
DL	ABF94636	OsWOX6	ABF95709
OsYAB7	BAF09473	OsWOX7	NP_916815
TaYAB	AAQ93323	OsWOX8	NP_915983
ZmYAB9	AAP79886	QHB	BAB84412
ZmYAB10	AAP79887	OsWOX10	BAD05582
ZmYAB14	AAP79884	OsWOX11	BAF22586
ZmYAB15	AAP79885	NS1	Q70UV1
AmYAB	AAS10178	NS2	Q6S3I3
StYAB	AAR87498	PtWUS	AAR83341
InYAB	CAG17551	LeWUS	Q84VT7
NaINO	BAC82106		
NcINO	BAC82107		

In summary, our data reveal distinct functions of rice *YAB3* and *WOX3* from their closely related homologs in Arabidopsis and in maize, which form a regulatory module of rice leaf development, in which *WOX3* negatively regulates *YAB3* that in turn represses *KNOX* genes in leaf tissues (Table I).

MATERIALS AND METHODS

Plant Materials

Rice (*Oryza sativa*) spp. *Japonica* variety 'Zhonghua 11' was used in this study.

Gene Cloning

The cDNA fragments of *WOX3* and *YAB3* were amplified by RT-PCR. The PCR primers were designed based on two cDNA clones, AB218893 and AK070205, respectively. The sequences of the primers for *WOX3* were FLWOX3-F (5′-GGTACCCTGAGGAGGATGCCTCAGAC-3′) and FLWOX3-R (5′-GGATCCATATTGGCAGTGGCACACAC-3′), and for *YAB3* were FLYAB3-F (5′-GGTACCAGGATACGCGCCATGATGTC-3′) and FLYAB3-R (5′-GGATCC-TGAGGCGTTAGAATGGAGTG-3′). The amplified *WOX3* and *YAB3* fragments were inserted into the T vector (Invitrogen) for sequencing.

Rice Transformation

The binary vector used in overexpression transformation was constructed based on pCAMIA1301 (CAMBIA) and pRTL2 (Mason et al., 1992; Kulakova et al., 1995). A *Hin*dIII fragment with a double CaMV 35S enhancer/promoter and the nopaline synthase A terminator from pRTL2 was inserted into pCAMIA1301. The new vector was named as p1301DS. For overexpression of *WOX3* and *YAB3*, the full-length coding sequences of the genes were inserted into p1301DS digested with *Bam*HI and *Kpn*I. The vector used in RNAi transformation is the vector pDS1301 (Chu et al., 2006). The gene-specific fragments were amplified from the cDNAs of the two genes by using the following primer pairs: for *WOX3*, WOX3RNAi-F (5′-GGGACTAGTCCAT-GGCTGGTTCCAGAACCACAAGG-3′) and WOX3RNAi-R (5′-GGGGA-GCTCCCTAGGCTGCAGCAATCTTCCTTGAG-3′); for *YAB3*, YAB3RNAi-F (5′-GGGACTAGTCCATGGTGCTCTTCAAGGACGGTCTC-3′) and YAB3RNAi-F (5′-GGGGAGCTCCCTAGGTATAAGAGGCAGCACGCACA-3′). The PCR fragments were sequenced and inserted into pDS1301 to produce double-strand RNA. To create WOX3-GR fusion protein, the full-length coding region of *WOX3* was amplified by PCR. The stop codon of *WOX3* was removed and replaced with a *Sal*I site. Another adaptor (*Kpn*I) was added to the end of the forward primer. The PCR product was inserted into the plasmid pSport1 (CLONTECH) in the *Sal*I and *Kpn*I sites. The primers used in PCR were WOX3 (GR)-F (5′-GGTACCGAGGATGCCTCAGACCCCTTCG-3′) and WOX3 (GR)-R (5′-GTCGACCAATTGGTGGAGGTGGAGCAAG-3′). In the same way, the DNA fragment that contains the steroid-binding domain of the mouse GR was amplified using the plasmid pBI-ΔGR (Lloyd et al., 1994) as template. The PCR primers were GR-F (5′-GTCGACAGATCCTGAAGCTCGAAAAAC-3′) with a *Sal*I adaptor and GR-R (5′-GGATCCACCGGCAACAGGATTCAATG-3′) with a *Bam*HI adaptor. The amplified GR fragment was inserted downstream to and in frame with *WOX3* in pSport1. The fused *WOX3-GR* sequence then was cut down from pSport1 and inserted into p1301DS with *Kpn*I and *Bam*HI.

RNA Isolation, RNA Gel Blot, and RT-PCR

For expression analysis of *YAB* genes, total RNA was extracted from seedlings of the wild-type and transgenic plants at four-leaf stage using an RNA extracting kit (TRIzol reagent, Invitrogen). For expression analysis of *KNOX1* genes, total RNA was extracted from leaves of wild-type and transgenic plants. For semiquantitative RT-PCR analysis, 4 μg total RNA was treated first with 2 units DNase I (Invitrogen) and then reverse transcribed in a total volume of 20 μL with 0.5 μg oligo(dT)$_{15}$, 0.75 mM dNTPs, 10 mM dithiothreitol, and 200 units SuperScripTM II RNase H^{-} reverse transcriptase (Invitrogen). The primers in RT-PCR were: YAB1-F (5′-CTTGCTCCTTTT-CACCAAGC-3′), YAB1-R (5′-ATGAGCCCAGTTCTTTGCAG-3′); YAB2-F (5′-CCCATGAGAGCAGCAAGAAG-3′), YAB2-R (5′-CGTCGACACTAGCT-GCATGT-3′); DL-F (5′-CAAATGAGGGTAGCCCAAGA-3′), DL-R (5′-CTG-AGCTGACGACGGTGATA-3′); OSH1-F (5′-CTACCTCGACTGCCAGAAGG-3′), OSH1-R (5′-CCATGTGCATCAATCTCAGG-3′); OSH3-F (5′- GCTGGAGCA-GAAGCAGATCAA-3′), OSH3-R (5′-CCAAGCTGACTTTCCCTTTGG-3′); for *WOX3* and *YAB3*, primers were the same ones used in gene cloning. Rice *actin1* gene was used as the internal control. The *actin1* primers were: Actin1-F (5′-TGCTATGTACGTCGCCATCCAG-3′) and Actin1-R (5′-AATGAGTAAC-CACGCTCCGTCA-3′). For northern-blotting analysis, 20 μg of total RNA was separated on 1.2% (w/v) denaturing agarose gel before being transferred to nylon membranes. *WOX3* fragment amplified with gene-specific primers 5′-CGGCGTCAGCGTCGGCAACT-3′ and 5′-ACGTGTGTGTGCCACTG-CCA-3′ and *YAB3* gene-specific fragment amplified by primers 5′-CAAAT-CACCCAGCTGAAAGC-3′ and 5′-AATGACGGCACCTCATCAAG-3′ were

labeled with ^{32}P-dCTP using Random Primer kit (Invitrogen) and hybridized to the RNA blots.

In Situ Hybridization

Plant materials were fixed in formaldehyde acetic acid (50% ethanol, 5% acetic acid, and 3.7% formaldehyde) overnight at 4°C, dehydrated through a concentration grade of ethanol, cleared through a xylene series, then infiltrated through a series of paraffin, and finally embedded in 100% paraffin melted at 52°C to 54°C. Then 8- to 10-μm-thick microtome sections were mounted on RNase free glass slides.

The hybridization and immunological detection were performed according to De Block and Debrouwer (1993). The *WOX3* probe was amplified with the primers used in RNAi, and *YAB3* probe was the fragment used in northern blotting. The PCR fragments were inserted into pGEM T-vector (Promega) for sequencing and RNA transcription. The digoxigenin-labeled sense and antisense RNA probes were produced by T7 and Sp6 transcriptase, respectively. The reagents used in the experiments were purchased from Roche.

GFP Imaging

The vector used for nuclear localization analysis was constructed based on the vector of pCAMBIA1391Xb (CAMBIA). The GUS fragment of pCAMBIA1391Xb was replaced by a CaMV 35S promoter-GFP cassette. The coding regions of *WOX3* and *YAB3* were amplified using the primer pairs: WOX3NLS-F (5′-GGTACCCTGAGGAGGATGCCTCAGAC-3′), WOX3NLS-R (5′-GGATCCATTGGTGGAGGTGGAGCAAG-3′); YAB3NLS-F (5′-GGTACCGGATCACCTAGCTAGGATAC-3′), YAB3NLS-R (5′-GGTACCGAATGGAGTGACACCCATGC-3′). The amplified fragment was inserted upstream to and in frame with GFP. The fusion plasmid (5 μg) was coprecipitated with 3 mg of gold particles. The particles were resuspended in ethanol in a total volume of 60 μL and divided into five aliquots for bombarding onion (*Allium cepa*) epidermal cells using the PDS-1000 system (Bio-Rad) at 1,100 psi helium pressure. The expression of the fusion protein of WOX3, YAB3, and GFP in the onion epidermal cells was observed by a confocal microscope (Leica) 36 h after bombardment.

Yeast One-Hybrid Analysis

Yeast (*Saccharomyces cerevisiae*) one-hybrid analysis was performed by using a CLONTECH system. The partial forth intron of *YAB3* amplified with primers was inserted into pHIS2. The full-length coding sequence of *WOX3* was amplified from the full-length cDNA clone using the primer pairs: WOX3CDS-F (5′-GAATTCCTGAGGAGGATGCCTCAGAC-3′) and WOX3CDS-R (5′-GGATCCATATTGGCAGTGGCACACAC-3′). The PCR product was inserted into the vector of pGADT7-Rec2 to fuse with the GAL4 activation domain. The two constructs were sequenced and used to transform the yeast strain Y187 for one-hybrid analysis.

Chemical Treatments and Quantitative RT-PCR

DEX (Sigma) was dissolved in ethanol to 30 mM and stored at −20°C before use. Before the experiment, DEX was diluted with growth medium to a final concentration of 10 μM. The regenerants from the same callus line were divided into three parts: one was treated with DEX, one was treated first with CHX for 1 h then treated with DEX, and the third had no treatment. For treatment of the whole plants, roots of the regenerants were submerged in growth medium with or without 10 μM DEX and CHX. After 12 h of treatment, the aerial part of the plantlets was harvested for RNA extraction, RT-PCR, and quantitative real-time PCR analysis.

Quantitative PCR was performed in a total volume of 25 μL with 1.5 μL of the RT reactions, 0.25 μM gene-specific primers, and 12.5 μL SYBR Green Master mix (Applied Biosystems) on a 7500 real-time PCR machine (Applied Biosystems) according to the manufacturer's instructions. The rice *actin1* gene was used as the internal control. All primers were annealed at 58°C and run 45 cycles. The relative expression level of each gene in transgenic plants was compared to that in wild-type ones, normalized using the *actin1* cDNA level, and averaged over three replicates. The primers used in real-time PCR were YAB3rq-F (5′-ATACAACG CATCAAGGCAAGC-3′), YAB3rq-R (5′-CATCAGGCCAAAATGGATGTG-3′) and WOX3rq-F (5′-GCCTCAAGGAAGATTGCTGCA-3′), and WOX3rq-R (5′-ACGCATATGGCC GCAGAAA-3′).

Histological Observation

Leaves and flowers were harvested from the wild-type and transgenic plants. The procedures of dehydration, clearing, infiltration, and embedding were carried out as mentioned above. The sections were deparaffinized in xylene, rehydrated through a graded ethanol series, and dried before staining with fast green (for leaves) and hematoxylin (for flowers).

Electrophoretic Mobility Shift Assay

To produce the WOX3 protein, the full-length *WOX3* cDNA was inserted into the pET-32a expression vector (Novagen) and expressed in Rosetta-gami (DE3) *Escherichia coli* cells (Novagen). The tagged protein was purified with B-PER 6× His Spin Purification kit (Pierce) and dosed by using the Bradford reagents. The *YAB3* intron DNA S1 (including the putative WUS-binding site, TTAATGG) was produced by annealing of two oligonucleotides: 5′-GAGCAACATTAATGGTCAGGTT-3′ and 5′-GTGCAACCTGACCATTAATGTT-3′. The S2 DNA, which is similar to S1 but lacks a WUS site, was generated by annealing of the oligonucleotides: 5′-GAGCAACATCAGGTT-3′ and 5′-GTGCAACCTGATGTT-3′. The double-stranded oligonucleotides were labeled with ^{32}P-dCTP using the Klenow fragment. DNA-binding reactions were performed at room temperature for 20 min in 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 1 mM MgCl2, 5% (v/v) glycerol, and 50 mg L^{-1} poly(dI-dC) poly(dI-dC) (Amersham Pharmacia Biotech) in the presence of about 50 ng of the WOX3 protein and 1 ng of the probes and separated on 6% polyacrylamide gels in the Tris-glycin (0.3% Tris, 1.88% glycin) buffer.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers BAF15337 (YAB3) and BAE48302 (WOX3).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Transverse-section of leaves from wild-type (A and B), *YAB3* RNAi (C and D), and *WOX3* overexpression (E and F) plants.

Supplemental Figure S2. Floral phenotypes in *YAB3* RNAi- and *WOX3*-overexpression plants.

Supplemental Figure S3. One-hybrid analysis of binding of WOX3 to *YAB3*.

Supplemental Figure S4. Gel-shift analysis of WOX3 binding to *YAB3* gene.

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