OsWOX3A is involved in negative feedback regulation of the gibberellic acid biosynthetic pathway in rice (Oryza sativa)

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Abstract

The plant-specific WUSCHEL-related homeobox (WOX) nuclear proteins have important roles in the transcriptional regulation of many developmental processes. Among the rice (Oryza sativa) WOX proteins, a loss of OsWOX3A function in narrow leaf2 (nal2) nal3 double mutants (termed nal2/3) causes pleiotropic effects, such as narrow and curly leaves, opened spikelets, narrow grains, more tillers, and fewer lateral roots, but almost normal plant height. To examine OsWOX3A function in more detail, transgenic rice overexpressing OsWOX3A (OsWOX3A-OX) were generated; unexpectedly, all of them consistently exhibited severe dwarfism with very short and wide leaves, a phenotype that resembles that of gibberellic acid (GA)-deficient or GA-insensitive mutants. Exogenous GA3 treatment fully rescued the developmental defects of OsWOX3A-OX plants, suggesting that constitutive overexpression of OsWOX3A downregulates GA biosynthesis. Quantitative analysis of GA intermediates revealed significantly reduced levels of GA20 and bioactive GA1 in OsWOX3A-OX, possibly due to downregulation of the expression of KAO, which encodes ent-kaurenoic acid oxidase, a GA biosynthetic enzyme. Yeast one-hybrid and electrophoretic mobility shift assays revealed that OsWOX3A directly interacts with the KAO promoter. OsWOX3A expression is drastically and temporarily upregulated by GA3 and downregulated by paclobutrazol, a blocker of GA biosynthesis. These data indicate that OsWOX3A is a GA-responsive gene and functions in the negative feedback regulation of the GA biosynthetic pathway for GA homeostasis to maintain the threshold levels of endogenous GA intermediates throughout development.

Key words: Dwarfism, electrophoretic mobility shift assay, gibberellic acid, KAO, negative feedback regulation, OsWOX3A, rice.

Introduction

Semi-dwarfism is one of the most attractive and useful traits in cereal crop breeding because semi-dwarf plants show more resistance to lodging damage in unfavourable environments, such as wind and flood. Semi-dwarf plants also often show improved grain production owing to increased nitrogen-use efficiency (Borlaug, 1983; Evans, 1993; Khush, 1999; Hedden,
Semi-dwarf variants of many crop plants have been identified and shown to enhance agronomic values. Indeed, at least 70 dwarf mutants have been reported in rice (*Oryza sativa*), and several of them have been characterized as gibberellic acid (GA)-deficient or GA-insensitive mutants (Matsumo, 1997; Itoh et al., 2001; Sakamoto et al., 2004; Asano et al., 2009; Li et al., 2011; Zhang et al., 2014).

The essential phytohormone GA has pivotal roles in many developmental processes, such as seed germination, shoot and stem elongation, leaf expansion, flowering, and seed development (Achard and Geschik, 2009; Swain and Singh, 2005). In particular, GA is a major factor in determining plant height (Sakamoto and Matsuoka, 2004). GA metabolic pathways have been intensively analysed in plants (Hedden and Phillips, 2000; Olszewski et al., 2002). GA intermediates are synthesized through several steps from germinal copalyl diphosphate, which is converted into GA 3-oxoglutarate synthase (CPS) and GA-kaurene synthase (KS) (Aach et al., 1997; Helliwell et al., 2001). GA-kaurene is thereafter converted to GA$_2$, by two cytochrome P450 enzymes, en-kaurene oxidase (KO) and en-kaurenoic acid oxidase (KAO). Then, GA$_2$ is converted to the bioactive GA$_4$ form through the precursors GA$_3$, GA$_4$, GA$_9$, and GA$_{20}$ in the 13-hydroxylation pathway; GA$_{12}$ is also converted to the bioactive GA$_1$ form via GA$_{15}$, GA$_{24}$, and GA$_9$ in the non-13-hydroxylation pathway (Sakamoto et al., 2004; Yamaguchi, 2008). The overall rates of GA biosynthesis and deactivation maintain the levels of the bioactive forms of GA in plants (Hedden and Phillips, 2000). The flux of bioactive GA intermediates (i.e., GA$_1$ and GA$_4$) is regulated by three dioxygenases, GA 20-oxidase (GA20ox), GA 3-oxidase (GA3ox), and GA 2-oxidase (GA2ox). These enzymes have an important role in GA homeostasis (Hedden and Phillips, 2000). Both GA20ox and GA3ox catalyse the conversion of GA intermediates into bioactive forms, while GA2ox catalyses the conversion of bioactive GA intermediates into inactive catabolites (Yamaguchi, 2008).

In rice, several genes involved in GA metabolic pathways, including D1, D18, D35, SD1, EUI, and BC12/GDD1, affect plant height (Ueguchi-Tanaka et al., 2000; Itoh et al., 2001; Spielmeyer et al., 2002; Itoh et al., 2004; Zhu et al., 2006; Li et al., 2011). For example, loss-of-function mutations of GA3ox2 (d18) and GA20ox2 (sd1) resulted in dwarf plants (Sakamoto et al., 2004). In particular, GA3ox and GA20ox are expressed in rapidly growing organs, including leaf primordia, young leaves, elongating internodes, and developing anthers and embryos (Kaneko et al., 2003; Sakamoto et al., 2004), whereas CPS1, KS1, KO2, KAO, and GA2ox are broadly expressed in many organs and tissues (Sakamoto et al., 2004).

WUSCHEL (WUS)-related homeobox (WOX) nuclear proteins play key roles in coordinating transcription of many genes in various developmental processes (Haecker et al., 2004). In particular, members of the WOX subclade are involved in the regulation of lateral organ development. In *Arabidopsis*, WOX3/RESSED FLOWER (PRS) is involved in the development of lateral-axis expansion of sepal and stipules (Matsumoto and Okada, 2001). Maize (*Zea mays*) WOX3 protein, encoded by the duplicated genes *NARROW SHEATH1* (NS1) and NS2 (termed NS1/2), regulates shoot apical meristem and leaf development (Nardmann et al., 2004).

In rice, OsWOX3A protein, encoded by the duplicated genes *NARROW LEAF2* (NAL2) and *NAL3* (termed NAL2/3), plays important roles in lateral-axis outgrowth and vascular patterning in leaves and spikelets, development of tillers, and the formation of lateral roots and root hairs (Cho et al., 2013; Yoo et al., 2013). OsWOX3B protein, encoded by *DEPILOUS* (DEP), is required for trichome formation in leaves and glumes (Angeles-Shim et al., 2012). Interestingly, transgenic rice plants overexpressing OsWOX3A (OsWOX3A-OX) exhibited a severe dwarf phenotype with wider leaves than wild type (Ishiwata et al., 2013). Although recent work reported the possible functions of WOX8/9 genes in the GA metabolic pathway (Wang et al., 2014), the function of WOX genes in the GA metabolic pathway has not been fully elucidated.

This study showed that the severe dwarfism of OsWOX3A-OX plants was fully rescued by application of exogenous GA$_3$. Quantification of endogenous GA intermediates revealed decreased levels of GA$_{20}$ and GA$_1$ in OsWOX3A-OX plants, because the expression of GA synthetic genes is altered by overexpression of OsWOX3A. Notably, OsWOX3A interacts directly with the KAO promoter to repress KAO expression. These results indicate that OsWOX3A is involved in the negative feedback regulation of GA biosynthesis for GA homeostasis throughout development in rice.

**Materials and methods**

**Plant materials and growth conditions**

The Korean *japonica* rice cultivar ‘Dongjinbye’ (hereafter termed wild type; WT) was used in this study. The full-length cDNA of OsWOX3A (accession no. AB218893) was isolated from WT. The rice mutant of OsWOX3A, nal2/3, was obtained from Kyushu University, as previously reported (Cho et al., 2013). Plants were grown in the paddy field (Seoul National University Farm, Suwon, Korea) or in the growth chamber (12-h light at 30°C/12-h dark at 20°C).

**Vector construction and rice transformation**

A 612-bp full-length *OsWOX3A* cDNA was amplified by reverse transcription PCR (RT-PCR) (primers listed in Supplementary Table S1). The cDNAs were cloned into pCR8/GW/TOPO (Invitrogen), followed by recombination into the binary vector pMDC32 (TAIR accession: 1009003741), a plant transformation vector containing a double cauliflower mosaic virus (CaMV) 35S promoter (Curtis and Grossniklaus, 2003). The recombinant plasmid was transformed into *Agrobacterium* strain EHA105 and introduced into the calli of mature embryos of WT (Jeon et al., 2000). Transgenic plants developed from the calli were grown in Murashige and Skoog medium for 1 month, and confirmed by PCR with primers in the pMDC32 vector (35STC-F) and OsWOX3A fragment (TC-R) (Supplementary Table S1). To examine the expression levels of OsWOX3A in the transgenic rice plants, reverse transcription and quantitative real-time PCR (RT-qPCR) were conducted as previously described (Yoo et al., 2009). The primers used for the OsWOX3A and *UbiquitinS* (Ub5) (GenBank accession no. AK061988; Os01g0328400) genes are listed in Supplementary Table S1.
Histochemical analysis of OsWOX3A expression
For β-glucuronidase (GUS) assays, transgenic rice plants containing the ProOsWOX3A::GUS transgene, which have previously been reported, were used (Cho et al., 2013). GUS activity was detected histochemically as previously described (Jefferson et al., 1987).

Histological observation
To detect GUS activity in the elongating shoot in the ProOsWOX3A::GUS transgenic plants, 2-day-old seedlings were fixed in fixation solution (3.7% formaldehyde, 5% acetic acid, and 50% ethanol) overnight at 4°C, and dehydrated through a gradient series of ethanol, cleared in a xylene series, then infiltrated through a paraffin series (Sigma) for sections. The microtome sections (10–15 μm) were mounted on glass slides for imaging.

Growth chemical treatments
The 2-week-old WT and OsWOX3A-OX seedlings were sprayed with 100 ml of 10^{-4} M GA3 (in water) at 4–6 h after dawn every day for 5 days. The length of the second leaf sheath was measured at 5 days of treatment. For OsWOX3A expression analysis, 2-week-old WT seedlings were sprayed with 50 μM GA3, or 10 μM paclobutrazol and harvested at different time points for RT-qPCR analysis. For expression analysis of G2Aox2 and G3ox2, WT and OsWOX3A-OX plants were sprayed with 50 μM GA3 and harvested after 3 h for RT-qPCR analysis.

RNA extraction and quantitative real-time PCR
For expression analysis of OsWOX3A and GA biosynthetic genes, the WT, na2l3 mutants (Cho et al., 2014), and OsWOX3A-OX plants were grown in the growth chamber and leaf samples were harvested and homogenized in liquid nitrogen. Total RNA was extracted using an RNA extraction kit (RNeasy Plant Mini Kit, QiAGEN). Then, RT-qPCR was performed as previously described (Cho et al., 2014). RT products equivalent to 50 ng of total RNA and GoTaq qPCR Master Mix (Promega) were used in 50 μl reactions using the Light Cycler 480 (Roche). Roche Optical System software was used to calculate threshold cycle values. Ub5 was used as an internal control. The relative expression of each gene was calculated using the 2^{-ΔΔCt} method as previously described (Livak and Schmittgen, 2001). The primers used for qPCR are listed in Supplementary Table S1.

GA quantification
GA quantification was carried out as previously described (Foster and Morgan, 1995; Lee et al., 1998). For accurate quantification, OsWOX3A-OX and na2l3 plants were planted on opposite sides of WT plants in the same pot to minimize environmental effects. The 4-week-old seedlings of OsWOX3A-OX, na2l3 mutants, and WT plants were harvested for quantitative GA analysis. After harvesting, the samples were immediately frozen in liquid nitrogen, freeze-dried, and ground into fine powder using a mortar and pestle. After extraction with methanol, GA intermediates were purified using a combination of preparatory column chromatography, solvent partitioning, and reverse-phase HPLC (Foster and Morgan, 1995). Deuterated internal standards were added (20 ng each of [17,17,17-3H]GA1, −GA3, −GA5, −GA6, −GA7, −GA8, −GA9, and −GA10). GC-MS analysis was performed using a Hewlett-Packard model 6890 (Chemstation, USA). Gibberellin levels were calculated as the peak area ratios of endogenous (non-deuterated, sample) to deuterated GA intermediates, after correcting for any contribution from the deuterated standard to non-deuterated GA. The peak-area ratios of the following ion pairs, in the appropriate HPLC fractions, and having a retention time similar to that of the corresponding GA intermediates, were determined to calculate the concentrations of endogenous GA intermediates by reference to calibration curves: 506/508 (GA1), 594/596 (GA3), 434/436 (GA5), 418/420 (GA20), 506/508 (GA29), 432/434 (GA14), and 448/450 (GA33).

Yeast one-hybrid assay
The full-length coding sequence of OsWOX3A was amplified by PCR using the full-length cDNA (primers listed in Supplementary Table S1). The PCR product was inserted into the pGAD424 vector (Clontech) to fuse it with the GAL4 activation domain. To generate the reporter plasmids, fragments of the CPS1, KO2, KAO, GA20ox2, GA3ox2, GA2ox1, and GA2ox3 promoters were amplified by PCR using genomic DNA with specific primers (Supplementary Table S1) that spanned from −1 to about −1000 in each promoter and PCR products were inserted into the p LacZi vectors (Clontech). These constructs were used to transform the yeast strain YM4571. All the procedures followed the manufacturer’s manual (Yeast Protocols Handbook PT302–1; http://www.clontech.com/).

Electrophoretic mobility shift assay
To produce the His-tagged OsWOX3A protein, the full-length OsWOX3A cDNA was inserted into the BamHI and EcoRI sites of the expression vector pSET-A (Invitrogen). The His-tagged construct was transformed into Escherichia coli BL21 (DE3). Cells were grown at 38°C and induced by the addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 1 mM when the OD600 of the culture was 0.4 to 0.6. The fusion protein was purified with Ni-NTA His-bind Superflow beads (Novagen). Nucleotide sequences of the double-strand oligonucleotides for KAO used for EMSA are listed in Supplementary Table S1. The oligonucleotides were synthesized and labelled with biotin by Macrogen (Seoul, Korea). The DNA-binding reactions were performed at room temperature for 20 min in 20 μl standard reaction mixtures [2 μg purified proteins, 2 μl biotin-labelled annealed oligonucleotides, 2 μl of 10 μM binding buffer (100 mM Tris, 500 mM KCl, and 10 mM DTT, pH 7.5), 1 μl of 50% glycerol, 1 μl of 1% Nonidet P-40, 1 μl of 1 mM KCl, 1 μl of 100 mM MgCl2, 1 μl of 200 mM EDTA, 1 μl of 1 mg/ml poly(deoxyguanylic-deoxycytidylic) acid, and 8 μl of water]. The samples were loaded onto 10% native polyacrylamide gel containing 45 μM Tris, 45 mM borate, and 1 mM EDTA (TBE), pH 8.3. The gel was sandwiched and transferred to Nitrocellulose membranes (Millipore) in 0.5 × TBE buffer at 380 mA and 4°C for 1 h. Biotin-labelled DNA was detected by the LightShift Chemi-luminescent EMSA kit ( Pierce) following the manufacturer’s manual.

Results
OsWOX3A is expressed in rapidly growing organs
OsWOX3A is expressed in almost all tissues of rice plants, including leaf blades, leaf sheaths, and roots; in particular, it is highly expressed in the shoot base and the developing young panicles (Cho et al., 2013). In situ hybridization experiments detected OsWOX3A transcripts in the vegetative shoots and in young leaves (Ishiwata et al., 2013). Especially in the vegetative shoots, OsWOX3A transcripts were detected at the marginal edges of leaf primordia but not in the shoot apical meristem. In more detail, the ProOsWOX3A::GUS transgenic rice showed GUS expression in the coleoptile and vascular bundles of elongating shoots (Fig. 1A, B). The expression of OsWOX3A in young seedlings was detected in whole leaf blades and sheaths (Fig. 1C, D); GUS expression in leaf sheaths was mostly detected in vascular bundles and epidermal cells. Moreover, GUS expression was detected in elongating internodes, nodes, and panicle nodes (Fig. 1E). Therefore, OsWOX3A was broadly expressed in many different tissues, mostly in rapidly growing organs.
Overexpression of OsWOX3A resulted in a severe dwarf phenotype

To study the function of OsWOX3A, OsWOX3A-OX plants constitutively expressing OsWOX3A under the control of the double CaMV35S promoters were generated. To this end, the Pro35S(2x)::OsWOX3A construct was introduced into the calli derived from the mature embryos of japonica WT cultivar ‘Dongjinbyeo’ by Agrobacterium-mediated transformation and seven independent T0 transgenic plants were obtained from the transgenic calli (Supplementary Fig. S1A). All transgenic plants displayed a severe dwarf phenotype with dark green and much wider leaf blade compared with WT (Fig. 2A; Supplementary Fig. S1A–C). Ishiwata et al. (2013) reported that the ProACTIN1::OsWOX3A transgenic rice showed a very similar phenotype to the Pro35S(2x)::OsWOX3A transgenic rice plants produced here. The height of OsWOX3A-OX plants was approximately one-quarter that of WT (Fig. 2A; Supplementary Fig. S1A). After heading, the panicle and internodes were shorter than WT (Fig. 2B). In addition, the epidermal cells of the second leaf sheath were much shorter (Fig. 2C), indicating that cell elongation becomes markedly reduced in OsWOX3A-OX plants (Fig. 2C, D). However, the width of epidermal cells in the second leaf sheath was not altered (Fig. 2E). OsWOX3A-OX panicles were much shorter than WT and thus the panicles had many fewer spikelets; no alteration of spikelet shape was observed, but grains of the transgenic plants were slightly shorter than WT (Supplementary Fig. S2A). In addition, the OsWOX3A-OX plants had more lateral roots but slightly fewer adventitious roots than WT (Supplementary Fig. S2B). The number of lateral roots in the same region of the primary root did not significantly differ between WT and OsWOX3A-OX (Supplementary Fig. S2B). Interestingly, the OsWOX3A-OX lateral roots were considerably shorter than WT lateral roots (Supplementary Fig. S2C). Thus, in addition to its previously reported functions (Cho et al., 2013; Yoo et al., 2013), OsWOX3A also can function in the inhibition of the longitudinal elongation of cells in both vegetative and reproductive organs during rice development.

Exogenous GA3 treatment rescued the dwarfism phenotype of OsWOX3A-OX plants

GA3, the most important hormone regulating the longitudinal growth and elongation of plant cells, plays a major role in determining plant height (Sakamoto and Matsuoka, 2004). To identify whether the severe dwarf phenotype of OsWOX3A-OX plants was caused by GA deficiency or GA insensitivity, the 2-week-old OsWOX3A-OX plants were treated with GA3 by spraying with 10⁻⁶ M or 10⁻⁸ M GA3 for 5 days and measuring their heights. The lengths of the second leaf sheath of both WT and OsWOX3A-OX plants did not change in response to treatment with 10⁻⁸ M GA3 (data not shown). In response to treatment with 10⁻⁶ M GA3, the sheath length of OsWOX3A-OX was fully rescued, becoming similar to WT (Fig. 3). This result suggests that OsWOX3A-OX plants are possibly deficient in bioactive GA. This observation is consistent with the GA-deficient cells that exhibit impairment or retardation of cell elongation throughout development in rice (Dai et al., 2007b; Li et al., 2011).

Endogenous levels of GA in OsWOX3A-OX plants

GA1, the major bioactive GA that regulates longitudinal elongation of vegetative organs in rice (Kobayashi, 1988). To determine endogenous levels of GA, the levels of different 13-hydroxylated GA intermediates within the GA1 metabolic pathway were measured (Fig. 4A) using GC-MS. In OsWOX3A-OX plants, the bioactive GA1 level decreased to about 20% of the WT level. Furthermore, the levels of GA₂₀ (precursor of GA1), GA₈ (deactivated form of GA1), GA₃₃ (upstream precursor), and GA₁₀ (upstream precursor) were significantly lower, whereas the level of GA₅₃ (deactivated form of GA₃₃) was about 8-fold higher in OsWOX3A-OX plants compared with WT (Fig. 4B). This result strongly suggests that ectopic and constitutive overexpression of OsWOX3A negatively affects the GA biosynthetic pathway throughout development.
OsWOX3A-OX alters the expression of GA biosynthetic genes

Changes of the GA intermediate levels in OsWOX3A-OX plants may be caused by altered expression of genes encoding GA metabolic enzymes. Thus, the expression levels of GA biosynthesis genes, such as CPS, KO1, KO2, KO3, GA20ox2, GA3ox2, GA2ox1, and GA2ox3 were compared between WT and OsWOX3A-OX plants (Fig. 4C). Interestingly, the expression of KO3, whose product catalyses the oxidation of ent-kaurenoic acid, was drastically downregulated in OsWOX3A-OX plants (Fig. 4C). However, the expression of CPS1, KO1, and KO2 showed no significant difference between WT and OsWOX3A-OX plants. Unusually, in OsWOX3A-OX plants, expression levels of GA20ox2, GA3ox2, GA2ox1, and GA2ox3 were upregulated to about 2–3-fold higher than in WT. GA20ox2 and GA3ox2 are the major negative feedback regulators that maintain the threshold levels of endogenous GA (Itoh et al., 2001; Itoh et al., 2002). To further understand the upregulation of GA20ox2 and GA3ox2 expression in OsWOX3A-OX plants, the expression patterns of these genes were compared between GA3-treated WT and OsWOX3A-OX plants. The exogenous GA3 greatly downregulated their expression in both WT and OsWOX3A-OX plants (Supplementary Fig. S3), demonstrating that GA deficiency causes the dwarfism of OsWOX3A-OX plants (Fig. 4A).

Alteration of GA biosynthetic gene expression and endogenous GA levels in nal2/3 mutants

To further investigate the function of OsWOX3A in the GA biosynthetic pathway, the expression levels of GA biosynthetic genes were examined in nal2/3 mutants (Cho et al., 2013). RT-qPCR showed about a 2-fold increase in KO3 expression compared with WT (Fig. 5A). The expression of GA20ox2 was downregulated in nal2/3 mutants. However, the expression levels of GA3ox2, GA2ox1, and GA2ox3 were not altered. Analysis of 13-hydroxylated GA intermediates in nal2/3 mutants showed significant increases in GA20 and GA3 levels, and decreases in GA20 and GA3 levels, compared with WT (Fig. 5B). Overall, bioactive GA1 slightly increased in nal2/3 mutants (Fig. 5B). The changes in GA contents are consistent with the expression levels of KO3 and GA20ox2 in nal2/3 mutants. The increased expression of GA20ox2, GA3ox2, GA2ox1, and GA2ox3 in OsWOX3A-OX plants
Cho et al. (Fig. 4B) and decreased expression of GA20ox2 (Fig. 5A) in ntl2l3 mutants might be achieved by indirect mechanisms (e.g., altered auxin distribution). Thus, it can be speculated that OsWOX3A directly downregulates the expression of KAO as a trans-repressor or upregulates the expression of GA20ox2 as a trans-activator.

OsWOX3A protein interacts with the KAO promoter to repress its expression

To test whether the OsWOX3A protein directly interacts with the promoters of KAO or GA20ox2, yeast one-hybrid assays were used to test the promoter regions of KAO, GA20ox2, and other genes in the GA synthetic pathway. The assays showed that OsWOX3A only binds to the ~1kb promoter region of KAO (W2), but not to the promoters of GA20ox2 or other tested genes (Fig. 6B). The W4 promoter region from -2kb to -1kb of KAO was also tested by yeast one-hybrid assay, which showed that OsWOX3A does not bind to this region of KAO (Fig. 6A, B).

To see whether the promoter of KAO contains reported target motifs for WOX binding, 2kb of sequence in the KAO promoter region was examined. This sequence analysis revealed that the KAO promoter region has consensus sequences for the motifs CAAT (eight occurrences), TTAA (19 occurrences), and TTAATCG (one occurrence) (Fig. 6; Supplementary Fig. S4), which have been reported as target binding motifs for WOX proteins (Lohmann et al., 2001; Busch et al., 2010; Franco-Zorrilla et al., 2014). In spite of the many CAAT and TTAA sequences, OsWOX3A failed to bind to the W4 promoter region. To examine the importance of the TTAATCG motif, a construct with a deleted TTAATCG sequence (M2) was tested with a yeast one-hybrid assay, which showed that OsWOX3A does not bind to the deleted M2 probe from the promoter of KAO (Fig. 6B). These results suggest that OsWOX3A may bind to the TTAATCG motif in the promoter of KAO. To confirm if binding of OsWOX3A requires the consensus sequence of the KAO promoter, EMSA was carried out using the His-fusion OsWOX3A (His-OsWOX3A) produced in E. coli (Supplementary Fig. S5). The consensus binding sequence of KAO (W1) and deleted sequence lacking the TTAATCG (M1) were used as probes (Fig. 6A). The EMSA revealed that the His-OsWOX3A

![Image](image_url)
Taken together, these results indicate a direct involvement of OsWOX3A in downregulating the expression of KAO.

Exogenous GA upregulates OsWOX3A expression

The expression patterns of OsWOX3A in shoot base or elongating stem were quite similar to those of GA biosynthetic genes (Fig. 1; Cho et al., 2013; Kaneko et al., 2003). Thus, this study tested whether exogenous GA treatment alters the expression of OsWOX3A. To this end, 2-week-old WT seedlings were sprayed once with 50 μM GA3, and then the aerial parts were harvested at 0 to 24 h. RT-qPCR analysis showed that OsWOX3A expression rapidly increased almost 9-fold at 2 h after treatment and then decreased to control levels at 8 h after treatment (Fig. 7). Furthermore, the effect of a well-known inhibitor of GA biosynthesis, paclobutrazol, on OsWOX3A expression was examined. After treatment with 10 μM paclobutrazol, 2-week-old WT plants were harvested at the same time points as for the GA3 treatment. Application of paclobutrazol and GA3 treatment caused opposite changes in the expression of OsWOX3A (Fig. 7). These observations strongly suggest that the temporal increase of endogenous GA levels rapidly induces the expression of OsWOX3A, possibly decreasing the rate of GA biosynthesis and affecting GA homeostasis.

Discussion

The phenotypic and molecular genetic analysis of nal2/3 mutants in rice demonstrated that OsWOX3A has a conserved role similar to those of NSI/2 of maize and PRS of Arabidopsis in the regulation of lateral-axis outgrowth and margin development in founder cells and lateral organ primordia (Nardmann et al., 2004; Cho et al., 2013; Ishiwata et al., 2013). Interestingly, unlike nsi/2 and prs mutants, mutation of WOX3A has a pleiotropic effect in rice (Cho et al., 2013). However, other functions of OsWOX3A throughout development have remained unknown. The spatial expression of OsWOX3A overlaps with that of the GA biosynthetic genes, which are expressed in several organs, including vegetative shoot base, leaf sheaths, leaf blades, and elongating stems (Fig. 1; Sakamoto et al., 2004; Cho et al., 2013). This study provides evidence that OsWOX3A functions in the negative feedback regulation of the GA biosynthetic pathway for GA homeostasis throughout development.

OsWOX3A has a negative role in GA biosynthesis at the transcriptional level

To date, only one study has reported a regulatory role of a WOX protein in GA signalling: DWARF TILLER1, a homolog of Arabidopsis WOX8 or WOX9, is required for tiller and shoot growth through GA signalling (Wang et al., 2014). The rice mutant of KAO (oskao-1) has low levels of GA53, GA20, GA30, GA32, GA33, and GA1 (Sakamoto et al., 2004). This study showed that OsWOX3A-OX downregulates KAO expression, which causes significantly reduced levels of GA53, GA20, GA30, GA32, and, finally, bioactive GA1 (Fig. 4). Loss-of-function nal2/3 mutants showed increased expression of KAO (Fig. 5).
However, nal2/3 mutants did not show decreased expression of GA3ox2 (Fig. 5), which helps explain why the nal2/3 mutants showed a slight increase of GA1 accumulation (Fig. 5). Interestingly, nal2/3 mutants showed decreased GA20ox2 expression, which might be associated with lower levels of GA20 (Fig. 5). In this scenario, a reduction of GA20ox2 expression in the nal2/3 mutants might be caused by negative feedback regulation in response to the increase of bioactive GA1. In addition, the promoter region of GA20ox2 does not have an OsWOX3A binding motif, strongly suggesting that OsWOX3A is indirectly involved in GA20ox2 expression. Likewise, OsWOX3A indirectly upregulates GA2ox3 or GA2ox1, consistent with higher accumulation of GA29 from the GA20 intermediate in OsWOX3A-OX plants, whereas these plants have reduced levels of GA9 caused by low levels of GA1 (Fig. 4). The nal2/3 mutation did not affect the expression of GA2ox genes, probably owing to a slight increase of GA1 levels. Moreover, reduced expression of GA20ox2 and a slight accumulation of more bioactive GA1 (Fig. 5) did not increase the height of nal2/3 mutant plants (Cho et al., 2013). Interestingly, it has also been reported that auxin controls the expression of GA metabolic genes and altered auxin distribution plays a role in regulating the GA biosynthetic genes (Desgagne-Penix and Sponsel, 2008; Frigerio et al., 2006). Thus, the increase of GA1 in nal2/3 mutants may not be sufficient to affect cell elongation.

Previous work reported that OsWOX3A is involved in the formation of lateral roots, possibly by regulating auxin-related genes (Cho et al., 2013). GA intermediates negatively affect lateral root formation by inhibiting lateral root primordium initiation via modification of polar auxin transport (Gou et al., 2010). Therefore, although more physiological and biochemical studies are needed, the expression pattern of OsWOX3A in roots and the alteration of formation and elongation in the lateral roots in OsWOX3A-OX plants suggest that OsWOX3A may function in roots through the crosstalk between GA-related and auxin-related pathways (Fig. 1A; Supplementary Fig. S2B, C).

OsWOX3A is involved in negative feedback regulation of the GA biosynthetic pathway for GA homeostasis

In plants, the balance between GA biosynthesis and degradation tightly controls the levels of bioactive GA intermediates. In particular, GA biosynthesis is controlled by feedback regulation through the activity of the GA-responsive pathway, because plant growth and development require precise GA homeostasis. The expression of OsWOX3A is GA-responsive, because it is rapidly but temporarily activated by exogenous GA treatment and suppressed by the GA biosynthesis inhibitor paclobutrazol (Fig. 7). This suggests that, at least in part, OsWOX3A might be involved in the regulation of a feedback pathway in GA biosynthesis. OsWOX3A binds to the promoter of KAO, and thus may be closely associated with the GA activity-dependent downregulation of KAO expression (Fig. 6; Supplementary Fig. S4, S5). In this model, rapid activation of OsWOX3A by GA suppresses the expression of KAO and consequently decreases endogenous levels of

**Fig. 5.** Loss of OsWOX3A activity altered GA accumulation and expression of GA metabolic genes. **(A)** RT-qPCR analysis of the eight genes in the GA metabolic pathway in 4-week-old nal2/3 mutants. Relative mRNA levels of each gene are normalized to the mRNA levels of Ub5 (Os01g0328400). Expression levels for each gene in nal2/3 mutants are shown relative to the expression in WT, which is set as 1. Data are means ± SD from three biological repeats. Significant difference was determined by Student’s t-test (**P < 0.01**). **(B)** Quantification of 13-hydroxylated GA intermediates in 4-week-old seedlings. GA1 had undetectably low abundance. Data are means ± SD from three biological repeats (ng/g dry weight). Significant difference was determined by Student’s t-test (**P < 0.05, **P < 0.01**). These experiments were repeated more than twice with similar results.

Notably, OsWOX3A interacts with a WOX-binding motif, TTAATCG, in the KAO promoter (Fig. 6). These results suggest that OsWOX3A is involved in the negative feedback regulation of KAO expression for GA homeostasis.

GA biosynthesis is controlled by negative feedback regulation through bioactive GA intermediates, and GA20ox2 and GA3ox2 act as major negative feedback regulators in rice (Itoh et al., 2001; Itoh et al., 2002). Exogenous GA1 treatment of WT and OsWOX3A-OX plants markedly downregulated the expression of both GA20ox2 and GA3ox2 (Supplementary Fig. S3). Therefore, upregulation of GA20ox2 and GA3ox2 expression in OsWOX3A-OX plants might be caused by negative feedback regulation under low levels of bioactive GA1.

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bioactive GA intermediates, which later leads to downregulation of OsWOX3A expression.

OsWOX3A acts as a transcriptional repressor

The target sequence of WUS, TTAAT(G/C)(G/C), occurs in the intron of AGAMOUS in Arabidopsis (Lohmann et al., 2001). Similarly, rice QHB, WOX3, and WOX11 proteins also bind to the TTAATGG sequence (Kamiya et al., 2003; Dai et al., 2007a; Zhao et al., 2009). In addition, WUS protein specifically recognized the sequence CACGTG (Busch et al., 2010), and two binding core sequences for WOX13 (CAAT and TTAA) have been identified (Franco-Zorrilla et al., 2014). These studies suggest that CACGTG, CAAT, TTAA, and TTAAT(G/C)(G/C) are the consensus sequences for WUS-binding and WOX-binding motifs. Here, analysis of the promoters of GA biosynthetic genes revealed that only the KAO promoter (~2kb) contains a WOX-binding motif (Fig. 6A; Supplementary Fig. S4). Yeast one-hybrid assays and EMSA supported the idea that OsWOX3A can interact with the WOX-binding motif of the KAO promoter. Oligonucleotides containing W1 (the KAO promoter binding site) or W3 (the KAO promoter with reported binding element for WUS), were used as the biotin-labelled probes. The W1 probe is shown in red letters in (A) and the M1 probe is shown with a dotted line in Del in (A). The negative control is indicated in Lane 1 in (C, D). Biotin-unlabelled W1, M1 (deleted version of W1), and W3 were used as the unlabelled competitors. The (+) presence or (−) absence of His-OsWOX3A fusion protein is indicated. These experiments were repeated more than three times with similar results.
OsWOX3A does not bind to the M1 promoter

Expression of recombinant OsWOX3A fusion protein in E. coli. 

Fig. S3. Effect of exogenous GA3 treatment on the relative expression of GA20ox2 and GA3ox2 in 4-week-old wild type (WT) and OsWOX3A-OX (OX) plants.

Fig. S4. Analysis of OsWOX3A-binding motifs in the promoter of KAO.

Fig. S5. Expression of recombinant OsWOX3A fusion protein in E. coli.

Fig. S6. OsWOX3A does not bind to the M1 promoter region of KAO.

Table S1. Primers used in this study.

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Fig. 7. Transient, rapid alteration of OsWOX3A expression by GA3 and paclobutrazol. RT-qPCR analysis of OsWOX3A mRNA levels in 4-week-old WT seedlings treated with 50 μM GA3 or 10 μM paclobutrazol. Relative mRNA levels normalized to the mRNA levels of Ubi5 (Os01g0328400). Expression levels of OsWOX3A are relative to the expression at time point zero (control; no treatment), which is set as 1. Data are means ± SD from three biological repeats. These experiments were repeated more than twice with similar results.

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Phenotypic characteristics of OsWOX3A-OX (OX) plants.

Fig. S2. Multiple developmental defects in OsWOX3A-OX (OX) plants.

Fig. S3. Effect of exogenous GA3 treatment on the relative expression of GA20ox2 and GA3ox2 in 4-week-old wild type (WT) and OsWOX3A-OX (OX) plants.

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