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RESEARCH PAPER

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 GA_3 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 GA_{20} and bioactive GA_1 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 GA_3 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,

2003). 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 (Matsuo, 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 Genschik, 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 geranylgeranyl diphosphate, which is converted to *ent*-kaurene by *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS) (Aach *et al.*, 1997; Helliwell *et al.*, 2001). *ent*-Kaurene is thereafter converted to GA_{12} by two cytochrome P450 enzymes, *ent*-kaurene oxidase (KO) and *ent*-kaurenoic acid oxidase (KAO). Then, GA_{12} is converted to the bioactive GA_1 form through the precursors GA_{53}, GA_{44}, GA_{19}, and GA_{20} in the 13-hydroxylation pathway; GA_{12} is also converted to the bioactive GA_4 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 *WOX3* subclade are involved in the regulation of lateral organ development. In *Arabidopsis*, *WOX3*/*PRESSED FLOWER* (*PRS*) is involved in the development of lateral-axis expansion of sepals 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 *Ubiquitin5* (*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 paraplast 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^{-6} M GA_3 (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 GA_3 or 10 μM paclobutrazol and harvested at different time points for RT-qPCR analysis. For expression analysis of *GA20ox2* and *GA3ox2*, WT and *OsWOX3A*-OX plants were sprayed with 50 μM GA_3 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, *nal2/3* 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 $2^{-\Delta\Delta C}{}_{T}$ methods 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 *nal2/3* 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, *nal2/3* 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−$^2H_2$]GA_1, −GA_8, −GA_{19}, −GA_{20}, −GA_{29}, −GA_{44}, and −GA_{53}). 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 (GA_1), 594/596 (GA_8), 434/436 (GA_{19}), 418/420 (GA_{20}), 506/508 (GA_{29}), 432/434 (GA_{44}), and 448/450 (GA_{53}).

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 plasmid, 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 pLacZi vectors (Clontech). These constructs were used to transform the yeast strain YM4571. All the procedures followed the manufacturer's manual (Yeast Protocols Handbook PT3024–1; http://www.clontech.com/).

Electrophoretic mobility shift assay

To produce the His-tagged OsWOX3A protein, the full-length *OsWOX3A* cDNA was inserted into the *Bam*HI and *Eco*RI sites of the expression vector pRSET-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 OD_{600} 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 mg purified proteins, 2 μl biotin-labelled annealed oligonucleotides, 2 μl of 10 × 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 M KCl, 1 μl of 100 mM $MgCl_2$, 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 mM Tris, 45 mM boric acid, and 1 mM EDTA (TBE), pH 8.3. The gel was sandwiched and transferred to N^+ nylon membrane (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.

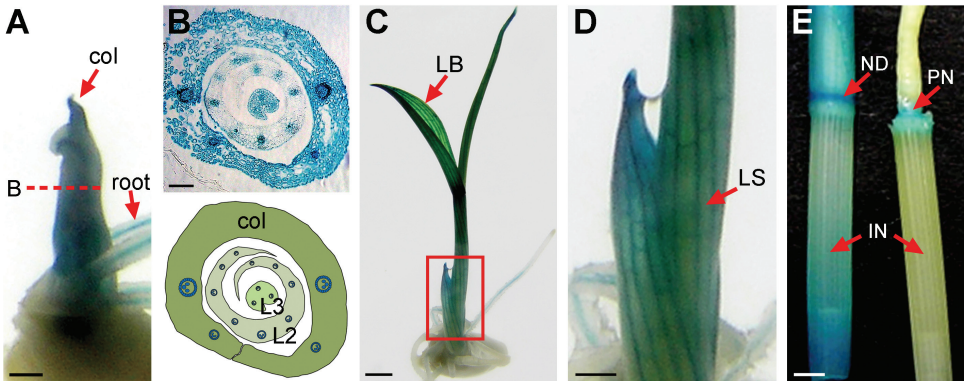

Fig. 1. Expression pattern of *OsWOX3A*. (**A, C**) Detection of GUS expression under the control of the *OsWOX3A* promoter (*ProOsWOX3A::GUS*) in 3-day-old elongating shoot in (A) and 1-week-old young seedling in (C). (**B**) Cross section of elongating shoot showing GUS expression in coleoptile, leaf primordia, and vasculature. (**D**) Magnified view of the bottom of leaf sheath in red rectangle in (C). (**E**) GUS expression in a 2-month-old node, elongating node (left) and young panicle node (right). col, coleoptile; IN, internode; L2, 2nd leaf; L3, 3rd leaf; LB, leaf blade; LS, leaf sheath; ND, node; PN, panicle node;. Scale bars = 1 mm (A), 0.2 mm (B), 4 mm (C), 2 mm (D, E). These experiments were repeated more than twice with similar results.

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 T_0 transgenic plants were obtained from the transgenic calli (Supplementary Fig. S1). 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*(2×)*::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 GA_3 treatment rescued the dwarfism phenotype of OsWOX3A-*OX plants*

GA, 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 GA_3 by spraying with 10^{-6} M or 10^{-8} M GA_3 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 GA_3 (data not shown). In response to treatment with 10^{-6} M GA_3, 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*

GA_1 is 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 GA_1 metabolic pathway were measured (Fig. 4A) using GC-MS. In *OsWOX3A*-OX plants, the bioactive GA_1 level decreased to about 20% of the WT level. Furthermore, the levels of GA_{20} (precursor of GA_1), GA_8 (deactivated form of GA_1), GA_{53} (upstream precursor), and GA_{19} (upstream precursor) were significantly lower, whereas the level of GA_{29} (deactivated form of GA_{20}) 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.

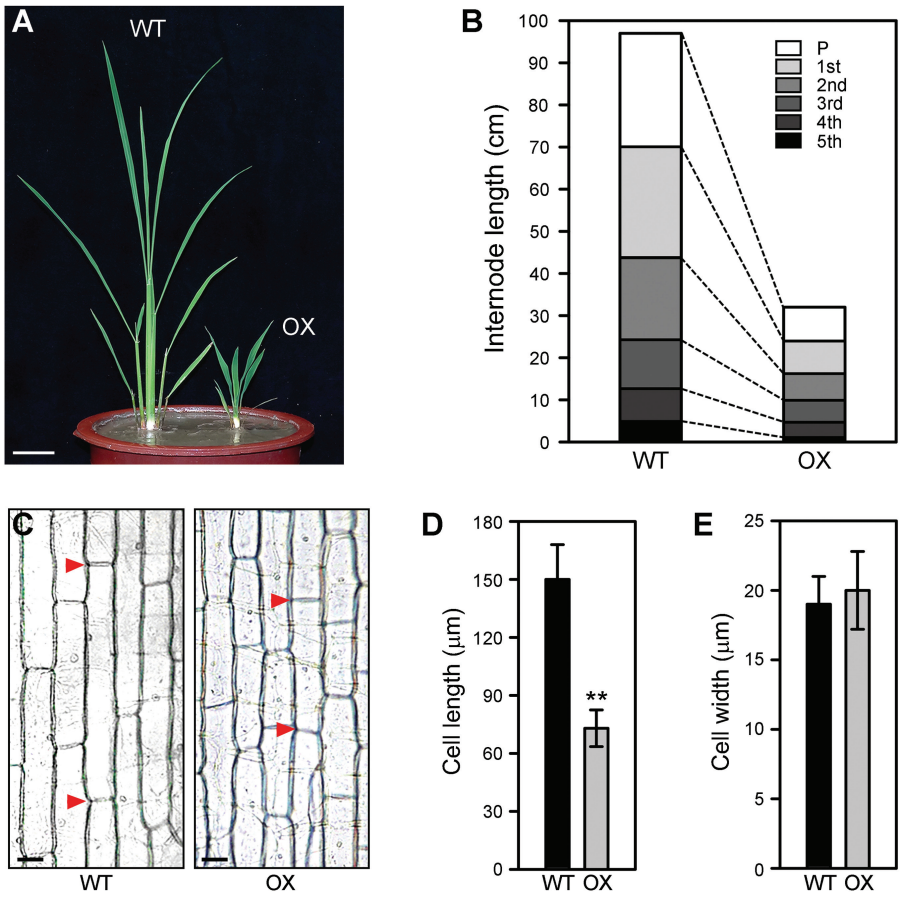

Fig. 2. *OsWOX3A*-OX induces severe dwarfism. (**A**) The 1-month-old plants grown in the paddy field. WT, wild type. OX, *OsWOX3A*-OX. **(B)** Internode lengths of plants at 2 weeks after heading. The average values were calculated from measurement of at least 15 plants. P, panicle; 1st to 5th nodes, respectively. (C-E) Length of epidermal cells of second leaf sheath in 1-month-old plants. (**C**) Red arrows indicate the upper or lower limits of epidermal cells. (**D, E**) Quantitative measurement of the cell lengths (D) and widths (E) of second leaf sheath. Data are mean ± SD from at least 15 plants. Significant difference was determined by Student's *t*-test (***P* < 0.01). Scale bars = 4 cm (A), 25 mm (C). These experiments were repeated more than twice with similar results.

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*, *KAO*, *GA20ox2*, *GA3ox2*, *GA2ox1*, and *GA2ox3* were compared between WT and *OsWOX3A*-OX plants (Fig. 4C). Interestingly, the expression of *KAO*, 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 GA_3-treated WT and *OsWOX3A*-OX plants. The exogenous GA_3 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 *KAO* 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 GA_{53} and GA_{19} levels, and decreases in GA_{20} and GA_8 levels, compared with WT (Fig. 5B). Overall, bioactive GA_1 slightly increased in *nal2/3* mutants (Fig. 5B). The changes in GA contents are consistent with the expression levels of *KAO* and *GA20ox2* in *nal2/3* mutants. The increased expression of *GA20ox2*, *GA3ox2*, *GA2ox1*, and *GA2ox3* in *OsWOX3A*-OX plants

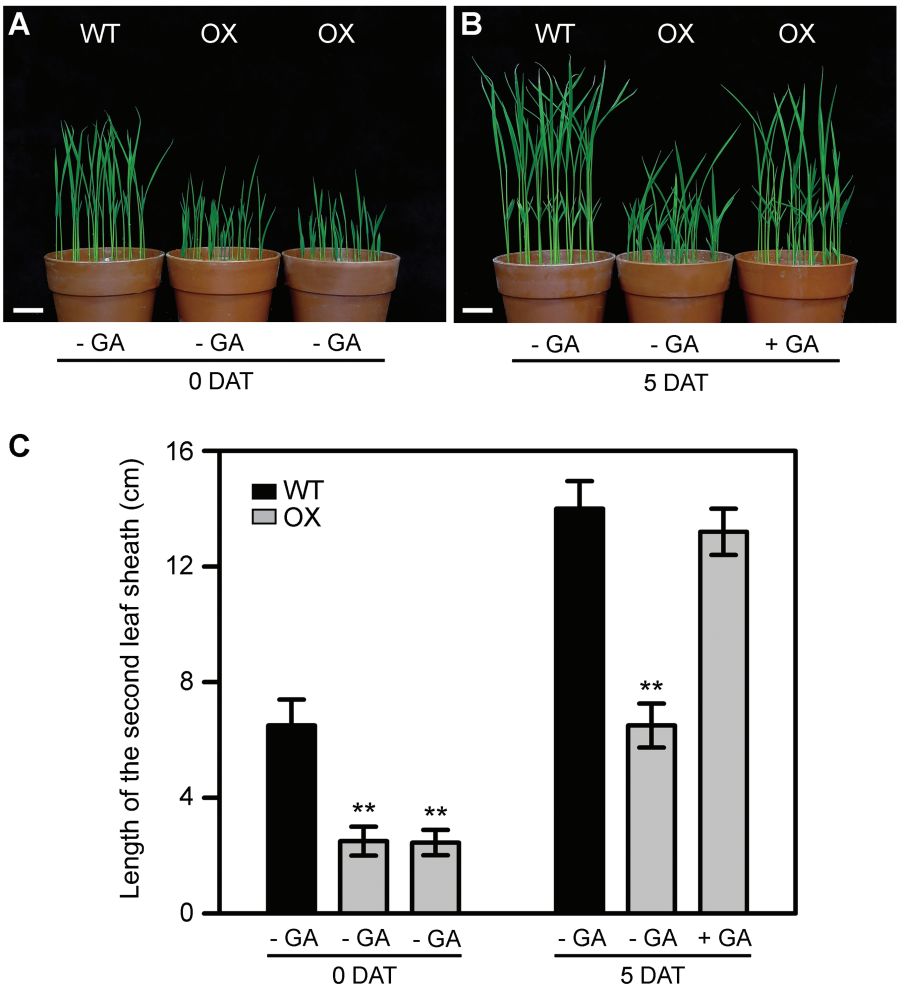

Fig. 3. Rescue of dwarf phenotype by exogenous GA_3 treatment. (**A, B**) Plants were grown in soil for 14 days (A) and then sprayed with 10^{-6} M GA_3 in 100 ml water and photographed after 5 days. DAT, day(s) after treatment; OX, *OsWOX3A*-OX. The lengths of the second leaf sheath are rescued in the *OsWOX3A*-OX plants (right) in B. (**C**) Quantitative measurement of the length of the second leaf sheath. Data are mean ± SD from at least 15 plants. Significant differences compared with WT were determined by Student's *t*-test (**$P < 0.01$). Scale bars = 2 cm (A, B). These experiments were repeated more than twice with similar results.

(Fig. 4B) and decreased expression of *GA20ox2* (Fig. 5A) in *nal2/3* 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 ~1 kb promoter region of *KAO* (W2), but not to the promoters of *GA20ox2* or other tested genes (Fig. 6B). The W4 promoter region from −2 kb to −1 kb 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, 2 kb 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

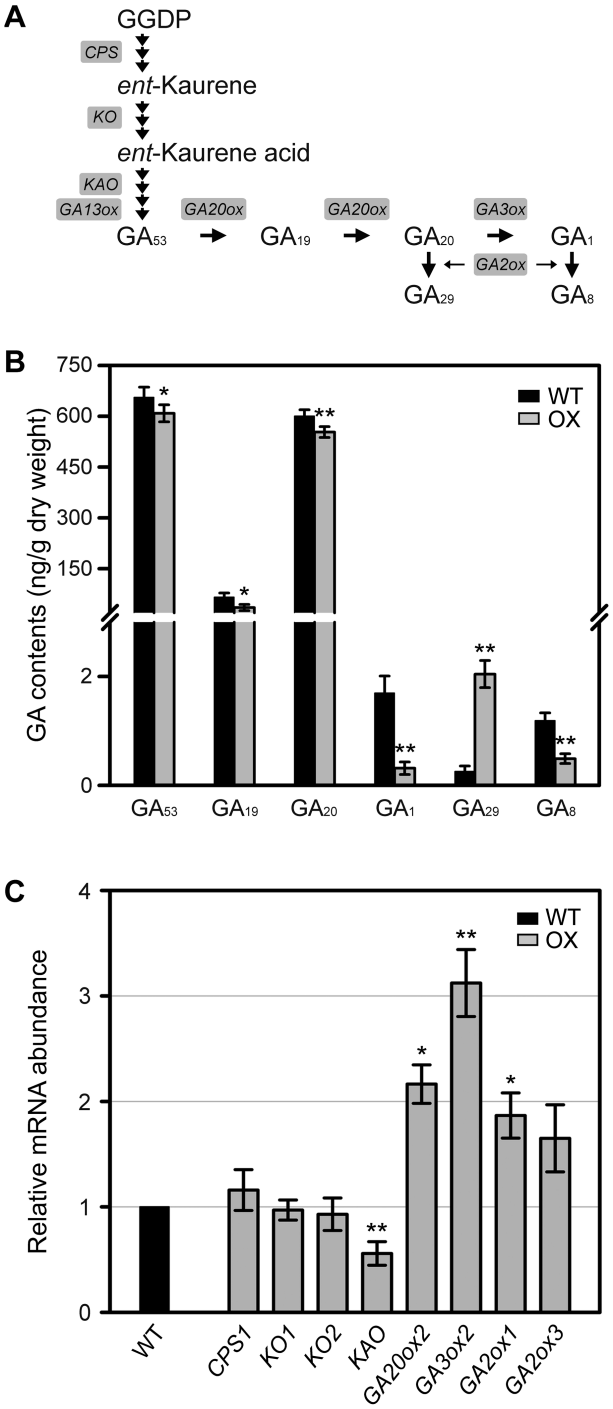

Fig. 4. *OsWOX3A*-OX altered GA accumulation and expression levels of GA metabolic genes. (**A**) Schematic representation of GA biosynthetic genes. (**B**) Quantification of 13-hydroxylated GA intermediates in 4-week-old seedlings. GA_{12} had undetectably low abundance. Data are means ± SD from three biological samples (ng/g dry weight). Significant difference was determined by Student's *t*-test (*$P < 0.05$, **$P < 0.01$). (**C**) RT-qPCR analysis of eight genes in the GA metabolic pathway in 4-week-old *OsWOX3A-OX* plants. Relative mRNA levels of each gene were normalized to the mRNA levels of *Ub5* (Os01g0328400). Expression levels for each gene in *OsWOX3A*-OX 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 (*$P < 0.05$, **$P < 0.01$). These experiments were repeated more than twice with similar results.

protein did bind to the consensus W1 sequence but not to the M1 sequence (Fig. 6C); in addition, OsWOX3A failed to interact with the repeated CAAT motif (W2) (Fig. 6D). 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_3 treatment alters the expression of *OsWOX3A*. To this end, 2-week-old WT seedlings were sprayed once with 50 µM GA_3, 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 with10 µM paclobutrazol, 2-week-old WT plants were harvested at the same time points as for the GA_3 treatment. Application of paclobutrazol and GA_3 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 *NS1/2* 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 *ns1/2* 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 GA_{53}, GA_{20}, and GA_1 (Sakamoto *et al.*, 2004). This study showed that *OsWOX3A*-OX downregulates *KAO* expression, which causes significantly reduced levels of GA_{53}, GA_{20}, and, finally, bioactive GA_1 (Fig. 4). Loss-of-function *nal2/3* mutants showed increased expression of *KAO* (Fig. 5).

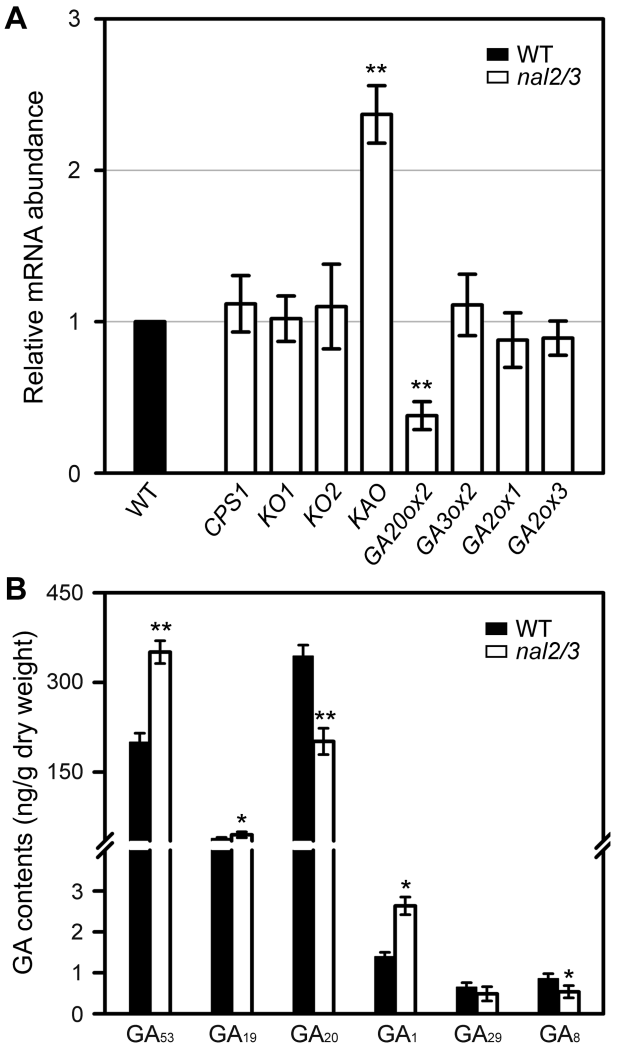

Fig. 5. Loss of *OsWOX3A* activity altered GA accumulation and expression of GA metabolic genes. (**A**) RT-qPCR analysis of the eight genes in the GA metabolic pathway in 4-week-old *nal2/3* mutants. Relative mRNA levels of each gene are normalized to the mRNA levels of *Ub5* (Os01g0328400). Expression 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. GA_{12} 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 GA_3 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 GA_1. 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 GA_1 accumulation (Fig. 5). Interestingly, *nal2/3* mutants showed decreased *GA20ox2* expression, which might be associated with lower levels of GA_{20} (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 GA_1. 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 GA_{29} from the GA_{20} intermediate in *OsWOX3A*-OX plants, whereas these plants have reduced levels of GA_8 caused by low levels of GA_1 (Fig. 4). The *nal2/3* mutation did not affect the expression of *GA2ox* genes, probably owing to a slight increase of GA_1 levels. Moreover, reduced expression of *GA20ox2* and a slight accumulation of more bioactive GA_1 (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 GA_1 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

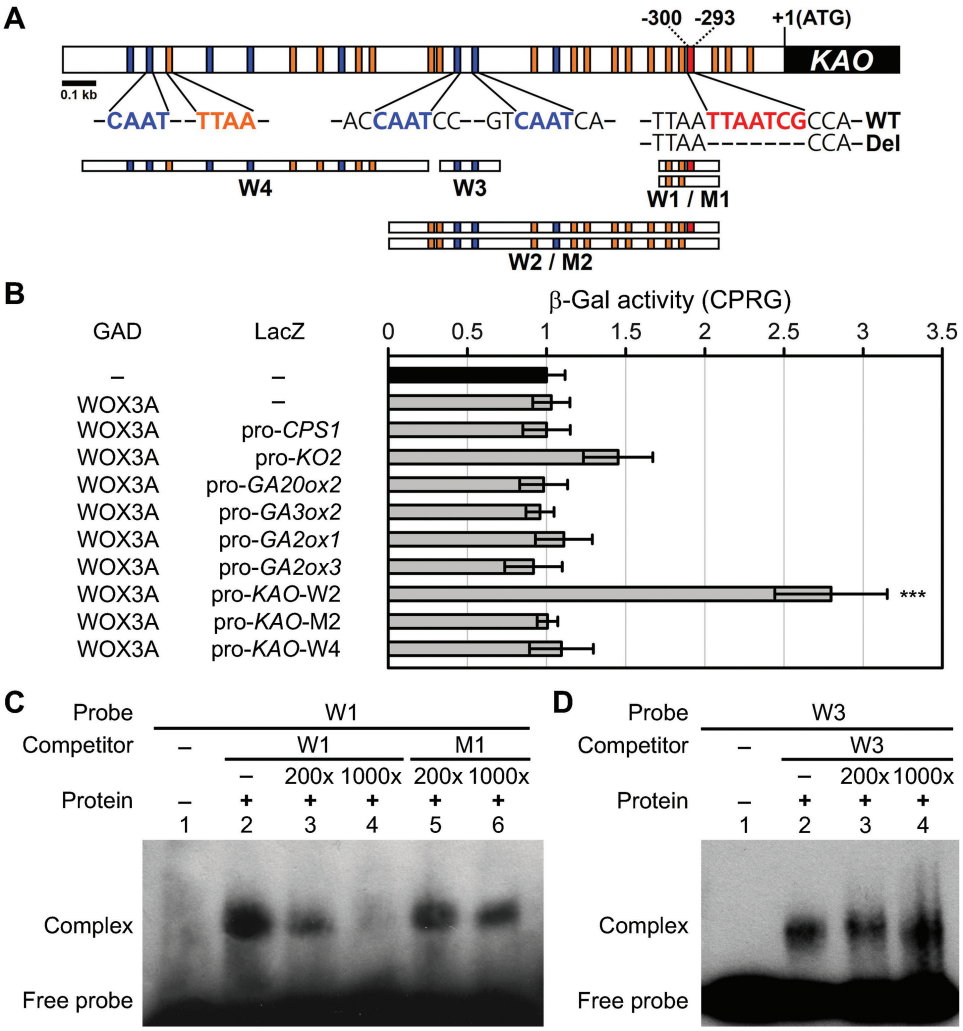

Fig. 6. OsWOX3A protein directly binds to the promoter region of *KAO*. (**A**) The locations of the W1, M1, W2, M2, W3, and W4 probes within the promoter region of *KAO*. The W1 probe includes the TTAATCG sequence, which is shown in red letters and by a red box. Reported core binding elements for WUS are indicated in blue and orange and by blue and orange boxes. (**B**) Analysis of OsWOX3A binding to the promoters of GA metabolic genes (*CPS1*, *KO2*, *KAO*, *GA20ox2*, *GA3ox2*, *GA2ox1*, and *GA2ox3*) using yeast one-hybrid assays. β-Galactosidase (β-Gal) activity was measured by liquid assay using chlorophenol red-β-D-galactopyranoside (CPRG). Each promoter binding activity is shown relative to the CPRG unit (10^4 ml^{-1} min^{-1}) of the negative control that contains empty bait and prey plasmids (–), which is set as 1. Data are means ± SD from six independent colonies. Significant difference was determined by Student's *t*-test (***$P < 0.001$). (**C, D**) EMSA showing His-OsWOX3A fusion protein binding to 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.

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 (Fig. 6; Supplementary Fig. S4–S6). A previous study reported that OsWOX3A acts as a transcriptional repressor of *YABBY3* during leaf development (Dai *et al.*, 2007a). However, in contrast to its role as a repressor, it has been reported that OsWOX3A may act as a transcriptional activator of leaf development and auxin-related genes (Cho *et al.*, 2013; Ishiwata *et al.*, 2013). Ikeda *et al.* (2009) found that WUS is a bifunctional transcriptional factor that acts as a repressor but also acts as a direct activator of the expression of the *AGAMOUS* gene. Taken together, these results indicate

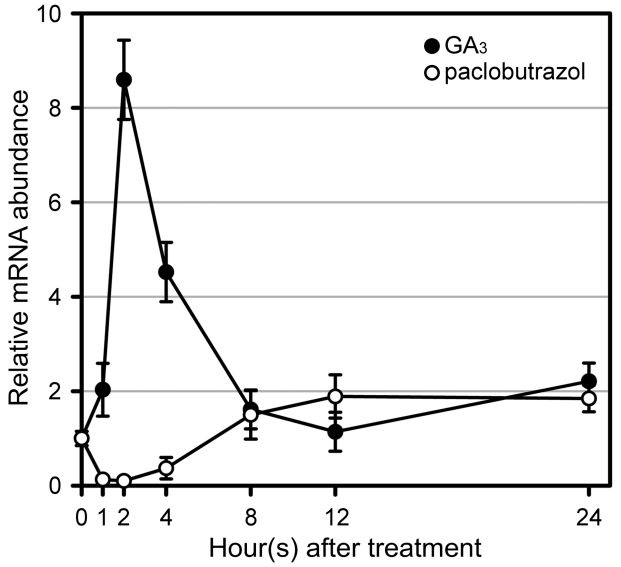

Fig. 7. Transient, rapid alteration of *OsWOX3A* expression by GA_3 and paclobutrazol. RT-qPCR analysis of *OsWOX3A* mRNA levels in 4-week-old WT seedlings treated with 50 μM GA_3 or 10 μM paclobutrazol. Relative mRNA levels normalized to the mRNA levels of *Ub5* (Os01g0328400). Expression levels of *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.

that OsWOX3A might act as a transcriptional repressor rather than an activator in the GA pathway. Furthermore, our physiological study revealed that severe dwarfism of *OsWOX3A*-OX plants can be rescued by exogenous GA_3 treatment (Fig. 2). Taking these observations together, this study indicates that OsWOX3A is involved in the negative feedback regulation of GA homeostasis during growth and development in rice.

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 GA_3 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. coil*.

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

Table S1. Primers used in this study.

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