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ORIGINAL ARTICLE

WOX2 functions redundantly with *WOX1* and *WOX4* to positively regulate seed germination in *Arabidopsis*

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Abstract

Main conclusion **WOX family gene *WOX2* is highly expressed during seed development, which functions redundantly with *WOX1* and *WOX4* to positively regulate seed germination.**

Abstract WOX (WUSCHEL-related homeobox) is a family of transcription factors in plants. They play essential roles in the regulation of plant growth and development, but their function in seed germination is not well understood. In this report, we show that WOX1, WOX2, and WOX4 are close homologues in *Arabidopsis*. WOX2 has a redundant function with WOX1 and WOX4, respectively, in seed germination. *WOX2* is highly expressed during seed development, from the globular embryonic stage to mature dry seeds, and its expression is decreased after germination. Loss of function single mutant *wox2*, and double mutants *wox1 wox2* and *wox2 wox4-1* show decreased germination speed. WOX2 and WOX4 are essential for hypocotyl–radicle zone elongation during germination, potentially by promoting the expression of cell wall-related genes. We also found that WOX2 and WOX4 regulate germination through the gibberellin (GA) pathway. These results suggest that WOX2 and WOX4 integrate the GA pathway and downstream cell wall-related genes during germination.

Keywords *Arabidopsis* · Gibberellin · Seed germination · WOX · XTH

Abbreviations

DAP	Days after pollination
GA	Gibberellin
GO	Gene Ontology
GUS	β-Glucuronidase
PAC	Paclobutrazol
WOX	WUSCHEL-related homeobox
XTH	Xyloglucan endotransglucosylase/hydrolase

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Introduction

The WUSCHEL (WUS)-related homeobox (WOX) family is a group of plant-specific transcription factors. Its members share a highly conserved DNA-binding domain composed of about 60 amino acids, known as the homeodomain (van der Graaff et al. 2009). In *Arabidopsis thaliana*, the WOX family contains 15 members (Tvorogova et al. 2021). They can be divided into three distinct clades based on phylogenetic analysis: the ancient clade (WOX10, WOX13, and WOX14), the intermediate clade (WOX8, WOX9, WOX11, and WOX12), and the modern clade (WOX1-7 and WUS) (Wu et al. 2019).

WOXs play important roles in many aspects of developmental processes. The *WUS* gene of the modern clade has a wide range of functions (Jha et al. 2020). It regulates the maintenance of shoot meristems, flower development, and somatic embryogenesis (Mayer et al. 1998; Zuo et al. 2002; Cao et al. 2015; Sun et al. 2019). The WOX1 and WOX3/

PRESSED FLOWER (PRS) act redundantly to control leaf blade development and adaxial–abaxial leaf polarity (Nakata et al. 2012). In *Medicago truncatula*, when *WOX1* ortholog, the *STENOFOLIA* (*STF*) gene is loss of function, the mutant displays multiple defects in leaf lamina, leaf vasculature, and flower development (Tadege et al. 2011). *WOX2*, *WOX8*, and *WOX9* regulate early embryogenesis (Ueda et al. 2011; Palovaara et al. 2016). After fertilization and the first zygotic division, *WOX2* expression is restricted to the apical cell, and *WOX8* is active exclusively in the basal cell. The differential expression patterns of *WOX2* and *WOX8* mediate apical-basal embryo polarity formation through localized auxin responses (Breuninger et al. 2008). Furthermore, WOX2 and WOX8 function redundantly to enhance the expression of the *CUP-SHAPED COTYLEDON* (*CUC*) genes, influencing the development of the cotyledon boundary (Lie et al. 2012). Previous studies have shown that *WOX4* plays versatile roles in plant growth and development (Kucukoglu et al. 2017; Yasui et al. 2018). WOX4 works redundantly with WOX14 downstream of the PHLOEM INTERCALATED WITH XYLEM (PXY) signaling pathway, promoting vascular cell proliferation (Etchells et al. 2013). In addition, *WOX4* is directly suppressed by AUXIN RESPONSE FACTOR 5 (ARF5), which participates in auxin-mediated vascular cambium development (Brackmann et al. 2018). Studies in *Oryza sativa* show that *OsWOX4* also has an important role in early leaf development and shoot apical meristem maintenance (Ohmori et al. 2013). *WOX5* is a central regulator of stem cell activity in the root system (Sarkar et al. 2007). *WOX5*, which is specifically expressed in the root quiescent center (QC), restrains the division of QC cells and represses the differentiation of stem cells (Kong et al. 2015). The molecular mechanisms underlying the *WOX5* regulating root system are complicated but have been comprehensively studied (Zhang et al. 2015; Zhou et al. 2015; Burkart et al. 2022). WOX5 inhibits the activity of D-type cyclins (CYCD) in QC, establishing quiescence (Forzani et al. 2014). Meanwhile, WOX5 protein transfers from QC into the columella stem cells to inhibit the expression of *CYCLING DOF FACTOR 4* (*CDF4*) and then maintains stem cell state. TOPLESS/TOPLESS-RELATED (TPL/TPR) co-repressors and the HISTONE DEACETYLASE 19 (HDA19) are involved in the inhibition of WOX5 on *CDF4* (Pi et al. 2015). The main function of *WOX6* is to regulate the development of ovules (Park et al. 2005; Pillitteri et al. 2007). In addition, *WOX6* enhances cold tolerance in *Arabidopsis* (Zhu et al. 2004). *WOX7* mediates the inhibition of sugar on lateral root formation. WOX7 binds directly to the promoter of the cell cycle gene *CYCLIND6;1* (*CYCD6;1*) and represses the expression of *CYCD6;1* (Kong et al. 2016). *WOX9* is essential for stimulating cell division and preventing premature differentiation during embryogenesis and post-embryonic development (Wu et al. 2005, 2007). *WOX11/12* participates in callus and adventitious root formation. With the increase of auxin levels in explants, upregulated *WOX11/12* promotes the expression of *LATERAL ORGAN BOUNDARIES DOMAIN16* (*LBD16*) and *LBD29*, resulting in the first-step cell fate transition (Liu et al. 2014). WOX11/12 directly upregulates *WOX5/7*, which promotes root primordia initiation (Hu and Xu 2016). *WOX11/12* has been found to regulate seed dormancy downstream of the phytochrome B (PHYB) signal pathway in *Arabidopsis* (Liao et al. 2022). *WOX13* and *WOX14*, from the ancient clade, are found to participate in the regulation of the development of flowers, fruits, and vascular tissues (Deveaux et al. 2008; Romera-Branchat et al. 2013; Petzold et al. 2018; Smit et al. 2020). Collectively, the WOXs are involved in a wide variety of aspects of plant development by interacting with their targets, regulators, and partners.

Seed dormancy and germination are important stages in the plant life cycle (Penfield 2017). Dormancy can ensure that plants germinate under suitable environmental conditions and complete the whole life cycle. Germination is the initiation of a seedling establishment, which is a vital feature for the propagation of plant species (Bassel 2016). Therefore, it is of great theoretical and biological significance to reveal the mechanisms of seed dormancy and germination. WOX members are involved in the regulation of plant growth and development, but their roles in dormancy and germination remain largely unknown in *Arabidopsis*.

In this work, we identified the important functions of WOX1/2/4 in seed germination. Thus, we hypothesize that they act a redundant function in promoting the growth of the hypocotyl–radicle zone through the gibberellin (GA) pathway and the downstream cell wall-related genes. Our results offer more insights into the effects of embryonic growth on seed germination and the functions of the *WOX* gene family.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia-0 (Col) was used as a wild-type plant. All of the *Arabidopsis* T-DNA insertion mutant lines *wox1* (SALK_148070), *wox2* (SALK_114607), *wox2-3* (SALK_050488C), and *wox4-1* (SALK_210239) were obtained from the Arabidopsis Biological Resource Center (ABRC). Homozygous mutants were identified using PCR-based screening. Gene-specific primers were designed using the SIGnAL T-DNA primer design tool and were used in combination with T-DNA left border primers (http://signal.salk.edu/tdnaprimers.2.html). The PCR primers for mutant screening are listed in Supplementary Table S1. The double mutants *wox1 wox2* and *wox2 wox4-1* were generated by crossing. Homozygous lines were confirmed by PCR

and utilized for phenotyping. Seeds were imbibed at 4 °C in darkness for 3 days for stratification and then sown into the soil. Plants were grown in a growth chamber at 20 ± 2 °C, 50–60% humidity, and a 16-h light/8-h dark cycle.

Germination assays

The seeds used for all germination comparison assays in this work were harvested in the same batch of plants. For seed dormancy tests, freshly harvested seeds were immediately used for experiments or stored at room temperature for after-ripening treatment. About 50 to 100 seeds harvested from individual plants were plated onto filter paper saturated with distilled water in Petri dishes and incubated in a growth chamber (16-h light/8-h dark, 22 °C). After 7 days, germination was scored as radicle emergence. Six to eight individual plants of each genotype were used in the dormancy phenotyping.

For ABA and paclobutrazol (PAC) sensitivity tests, seeds were stored for 3 months to be fully released from dormancy and sown on filter paper moistened with water containing different concentrations of ABA (0.5, 1.5, and 3 μM) or PAC (0.5 and 1 μM). The seeds were stratified for 3 days at 4 °C in darkness and then transferred to a growth chamber at 22 °C. The germination was recorded at different time points of incubation. For GA treatment, freshly harvested seeds were imbibed with water containing 2.5, 5, and 10 μM GA_{4+7} and incubated at 22 °C in a growth chamber. Each germination test was done with at least three replicates from eight independent plants. Stock solutions of ABA, PAC, and GA_{4+7} were dissolved in ethanol. When imbibed with chemicals, the control (0 μM) corresponds to 0.01% (v/v) ethanol in water.

Hypocotyl and radicle lengths analysis

The freshly harvested seeds were stored at room temperature for 3 months to break dormancy. Seeds were imbibed in distilled water at 4 °C for 3 days for stratification and then transferred to a growth chamber (16-h light/8-h dark, 22 °C). The seeds germinated at different time points and were photographed by stereo microscope (Nikon 80i). The lengths of hypocotyl and radicle and the transition zone were quantified with ImageJ software (https://imagej.net/ij/index.html).

Cotyledon phenotype analysis

The seeds of wild type (Col) and *wox2 wox4-1* were sown on half-strength Murashige and Skoog (1/2 MS) medium containing 0.8% (m/v) agar and 1% (m/v) sucrose. Subsequently, the seeds were stratified at 4 °C in the dark for 3 days and then incubated in a growth chamber (16-h light/8-h dark, 22 °C) for 6 days. The images of seedlings were taken by a Nikon 80i digital camera (https://www.nikon.com).

Construction of transgenic lines

A 2000 bp (− 2000/0) promoter region of *WOX2* (AT5G59340) was fused to the *β-glucuronidase* (*GUS*) reporter gene. PCR was performed using DNA extracted from *Arabidopsis* Col young leaves as a template, and primer pairs proWOX2-F/-R (Supplementary Table S1) were used to amplify the *WOX2* promoter fragment. The forward primer contained a *Hin*dIII site, and the reverse primer contained a *Sal*I site. The fragment was purified and then inserted into the pBI101 vector between the *Hin*dIII and *Sal*I restriction sites. Sequencing confirmed all of the DNA constructs used in this study. *WOX2pro::GUS* fusion was introduced into *Arabidopsis* wild-type Col using *Agrobacterium*-mediated transformation (Clough and Bent 1998). The seeds of transformants were collected, surface sterilized, and then selected based on their ability to survive for 7 days on 1/2 MS medium containing 25 mg L^{-1} hygromycin. Transgenic lines with a single copy insertion of a T-DNA cassette were confirmed by segregation ratio analysis. The homozygous T_3 generation lines were used for subsequent analysis.

RNA extraction and RT-qPCR

Total RNA was extracted from dry seeds or seeds with different times of stratification or germination with Plant Total RNA Purification Kit (GeneMark, Taiwan, China) according to the manufacturer's instructions and then reverse transcribed using the FastKing RT Kit with gDNA Remover (TIANGEN, Beijing, China). The qPCR was performed on an Eppendorf Mastercycler RealPlex real-time system using Taq Pro Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China). The qPCR primer pairs were designed based on the qPCR Primer Database (https://biodb.swu.edu.cn/qprimerdb/) and are listed in Supplementary Table S1. The gene expression data were analyzed using the $2^{-\Delta\Delta CT}$ or $2^{-\Delta CT}$ method using the *Arabidopsis ACTIN8* and *UBIQUITIN5* as reference genes (Gutierrez et al. 2008). Each analysis had at least three biological replicates.

RNA-seq analysis

The after-ripened seeds of Col and *wox2 wox4-1* were cold stratified for 3 days and germinated at 22 °C. Total RNA was extracted from seeds that germinated for 18 h. There were three biological replicates of the experiment. The RNA samples were sequenced using an Illumina HiSeq 2000 by BGI in Shenzhen, China. Total RNA-seq reads were mapped to the *Arabidopsis* Information Resource (TAIR) 10 genome. Genes with fold change > 1 and false

discovery rate (FDR)-adjusted P values < 0.05 were assigned as differentially expressed compared with the wild-type Col. Heatmap was plotted by https://www.bioinformatics.com.cn (last accessed on July 10, 2023), an online platform for data analysis and visualization.

GUS analysis

T_3 transgenic homozygous plants containing the *WOX2pro::GUS* construct were selected for GUS analysis. During embryogenesis, immature embryos were dissected from green siliques 3, 4, 5, and 10 days after pollination (DAP) under a dissecting microscope using a fine forceps and a tungsten tip needle. Silique was collected at 14 DAP. After the seeds were mature, embryos were dissected from freshly harvested dry seeds, and the seeds were stratified for 24 h and 3 days. Cotyledons were from seedlings that grew for 36 h. The shoot apical meristem and the first true leaf were from 5- and 10-day-old seedlings, respectively. Inflorescence, shoot, lateral root, and primary root were collected from 20-day-old plants. The samples at different developmental stages were incubated in the GUS staining solution (50 mM sodium phosphate buffer, pH 7.0, 1 mM EDTA, 0.1% [v/v] Triton X-100, 1 mg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 2 mM ferricyanide, and 2 mM ferrocyanide) at 37 °C in the dark for 12 h. Samples were transferred to 70% (v/v) ethanol and then washed several times to remove chlorophyll. Photographs were taken using a Nikon 80i stereomicroscope.

Statistical analysis

For data analysis, the results are expressed as the means ± standard error (SE). Student's t-test was performed to see if there were significant differences between the samples using SPSS version 26 (Statistical Product and Service Solutions, IBM). The difference was considered statistically significant when $P < 0.05$.

Accession numbers

Sequence data from this article can be found in TAIR under the following accession numbers: *WOX1* (AT3G18010), *WOX2* (AT5G59340), *WOX3* (AT2G28610), *WOX4* (AT1G46480), *WOX5* (AT3G11260), *WOX6* (AT2G01500), *WOX7* (AT5G05770), *WOX8* (AT5G45980), *WOX9* (AT2G33880), *WOX10* (AT1G20710), *WOX11* (AT3G03660), *WOX12* (AT5G17810), *WOX13* (AT4G35550), *WOX14* (AT1G20700), *WUSCHEL* (AT2G17950), *XTH7* (AT4G37800), *XTH24* (AT4G30270), *XTH33* (AT1G10550), *ARF2* (AT5G62000), *IAA9* (AT5G65670), *KAO2* (AT2G32440), *FCLY* (AT5G63910), *NAC1* (AT1G56010), *ACTIN8* (AT1G49240), and *UBIQUITIN5* (AT3G62250).

Results

Identification of candidate *WOXs* genes involved in seed germination

We previously found that two histone deacetylase-binding factors, SWI-INDEPENDENT3 (SIN3)-LIKE1 (SNL1) and SNL2, negatively regulate radicle protrusion during seed germination (Wang et al. 2016). Transcriptome analysis showed that the expression of several *WOX* family members, such as *WOX2/3/5/11/12* and *WUS*, were identified significantly changed in the imbibed seeds of the *snl1 snl2* double mutant (Wang et al. 2016). We therefore speculate that some WOXs may be involved in seed germination regulation. To investigate the expression pattern of *WOX* genes in seed, we performed the RT-qPCR test on dry seeds, cold stratified seeds, germinating seeds, and seedlings of ecotype Col at seven different time periods.

The results showed that four *WOX* genes (*WOX4*, *WOX6*, *WOX7*, and *WOX8*) were expressed at relatively low levels at all the tested stages (Fig. 1a–d; Fig. S1a–c); *WOX5*, *WOX9*, *WOX11*, *WOX12*, and *WOX13* transcripts were highly accumulated during stratification and then decreased to a quite low level after germination (Fig. 1e–i; Fig. S1d–h); the expression levels of *WOX1*, *WOX2*, *WOX10*, and *WOX14* were high in dry seeds and decreased to a much lower level after stratification and germination (Fig. 1j–m; Fig. S1i–l); *WOX3* and *WUS* were undetectable throughout all the time points when *ACTIN8* and *UBIQUITIN5* were used as control, while *WOX8* was not detected when *UBIQUITIN5* as control. The expression pattern of *WOX2* in seeds is much like that of *SNL1/SNL2*. Furthermore, the expression of *WOX2* was altered in the germinating seeds of the *snl1 snl2* mutant (Wang et al. 2016). These results suggest a possible role for *WOX2* in seed germination.

WOX2 is highly expressed in seed embryos

To further monitor *WOX2* expression pattern in different tissues, we generated stable transgenic lines in the Col background by introducing a *WOX2pro::GUS* construct. The histochemical GUS staining showed that *WOX2* is highly expressed during embryonic stages, such as the globular stage at 3 DAP under our growth conditions (Fig. 2a), the transitional stage from the globular stage to the heart stage at 4 DAP (Fig. 2b), and the heart stage at 5 DAP (Fig. 2c). GUS signals were subsequently observed in embryos at the mature green stage at 10 DAP (Fig. 2d) and the post-mature green stage at 14 DAP in the silique (Fig. 2e). When the

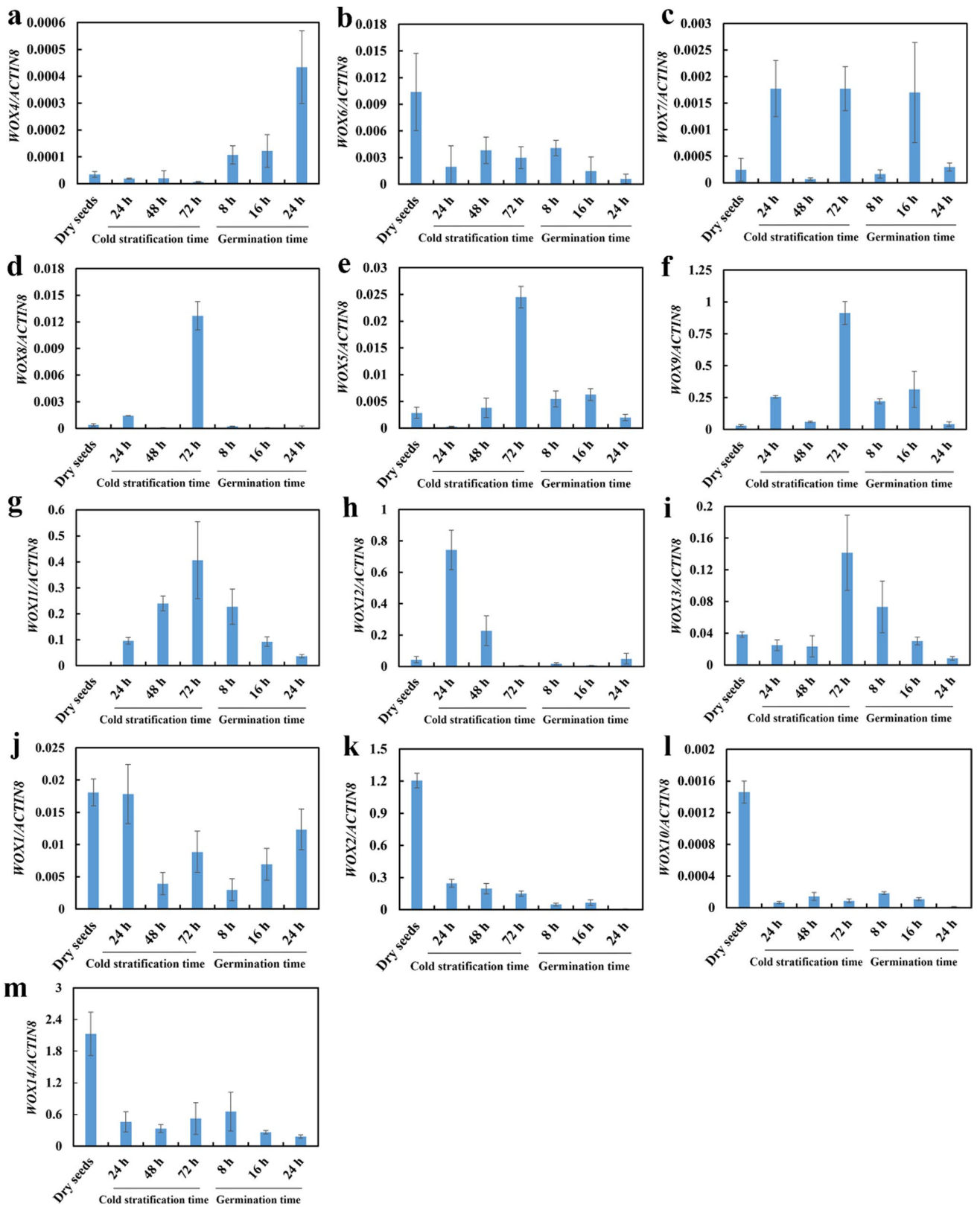

Fig. 1 Relative transcript abundance of the *WOX* family genes in dry, stratified, and germinating seeds and seedlings. **a**–**m** The expression levels of *WOX4*, *WOX6*, *WOX7*, *WOX8*, *WOX5*, *WOX9*, *WOX11*, *WOX12*, *WOX13*, *WOX1*, *WOX2*, *WOX10*, and *WOX14* in dry, stratified, and germinating seeds, and seedlings. *ACTIN8* was used as an internal control for the normalization of gene expression. Values are the means of three biological replicates. The error bars represent SE. Dry seeds, seeds stratified for 24 h, 48 h, and 72 h, and seeds germinated for 8 h, 16 h, and seedlings germinated for 24 h, were collected for total RNA extraction

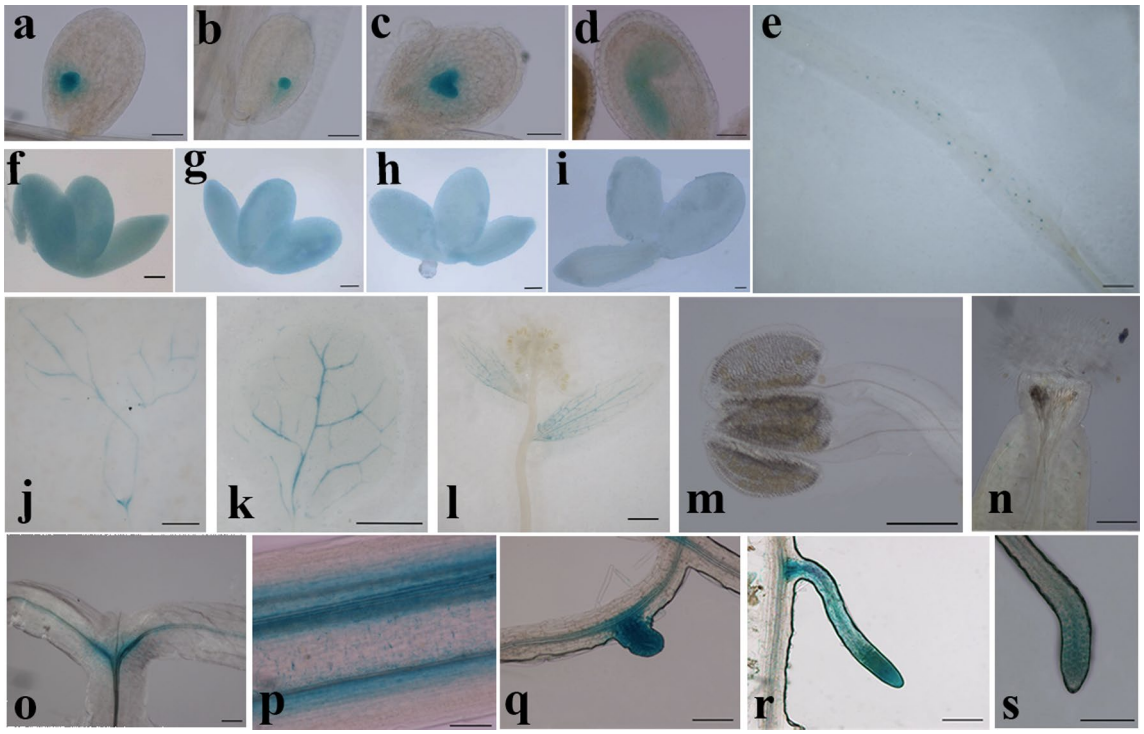

Fig. 2 *WOX2* promoter-driven *GUS* expression during seed development, seed germination, and in other various tissues. **a** Globular stage of embryonic development at 3 days after pollination (DAP). **b** Transitional stage from globular to heart stage at 4 DAP. **c** Heart stage at 5 DAP. **d** Post-embryonic stage at 10 DAP. **e** Silique at 14 DAP. **f** Freshly harvested dry seed. **g**, **h** Seed cold stratified for 24 h (**g**) and 3 days (**h**). **i** Seedling germinated for 24 h. **j** Cotyledons. **k** First true leaf of the 10-day-old seedling. **l** Leaves and inflorescence of 20-day-old plants. **m** Anthers. **n** Stigma. **o** Shoot apical meristem of 5-day-old seedling. **p** Shoot of 20-day-old plants. **q** Lateral root primordium of 20-day-old plants. **r** Lateral root of 20-day-old plants. **s** Primary root of 20-day-old plants. Experiments were repeated three times, and representative images were displayed. Bars = 100 μm (**a**–**d**, **f**–**j**, **o**–**q**, **s**), 200 μm (**m**, **n**, **r**), 1 mm (**e**, **k**, **l**)

seed became mature and desiccated, GUS expression was detected with strong signals in the whole embryo (Fig. 2f). The GUS signals decreased in embryos from seed stratified for 24 h to that stratified for 3 days (Fig. 2g, h) and showed weaker staining toward germination (Fig. 2i). In addition, GUS staining signals were also observed in other tissues, including the veins of the cotyledons (Fig. 2j), the first true leaves and mature leaves (Fig. 2k, l). By contrast, no GUS signals were detected in inflorescence (Fig. 2l), anthers (Fig. 2m), and stigma (Fig. 2n). GUS signals were further found in the shoot apical meristem (Fig. 2o), shoot (Fig. 2p), lateral root primordia (Fig. 2q), lateral root (Fig. 2r), and primary root (Fig. 2s). The *WOX2* promoter activity in embryos indicates that WOX2 may have a function in seed development or germination.

WOX2 acts redundantly with *WOX1* and *WOX4* to positively regulate seed germination

The phylogenetic analysis of the *Arabidopsis* WOX family showed that WOX2 is closely related to WOX1 and WOX4 (Fig. S2), implying that they have similar functions. To investigate the molecular function of the three members, we ordered T-DNA insertion mutant lines *wox1*, *wox2*, *wox2-3*, and *wox4-1* from the ABRC. By genome PCR assay, we found T-DNA is inserted in the third intron of *WOX1* as for *wox1* mutant, and the second exons of both *WOX2* and *WOX4* as for the *wox2, wox2-3*, and *wox4-1* mutants, respectively (Fig. 3a). To investigate whether WOX1/2/4 is involved in seed germination regulation, we first examined the dormancy phenotype of these single null mutants. The results showed that freshly harvested *wox2* and *wox2-3* single mutant seeds showed enhanced seed dormancy (Fig. S3a), whereas the loss function of *WOX1* or *WOX4* did not affect seed dormancy (Fig. S3b, c). We then analyzed the germination speed of these single mutants using after-ripened (dormancy-released) seeds. As shown in Fig. 3b, *wox2* and *wox2-3* seeds germinated slightly more slowly than the wild-type Col after cold stratification. In contrast, there was no significant difference in germination rates between the single mutant *wox1* and Col (Fig. 3c), *wox4-1* and Col (Fig. 3d). Because WOX1, WOX2, and WOX4 are closely related and their transcripts were all detected in seeds (Fig. S2; Fig. 1), we tested their redundancy by constructing two double mutants, *wox1 wox2* and *wox2 wox4-1*. The homozygous

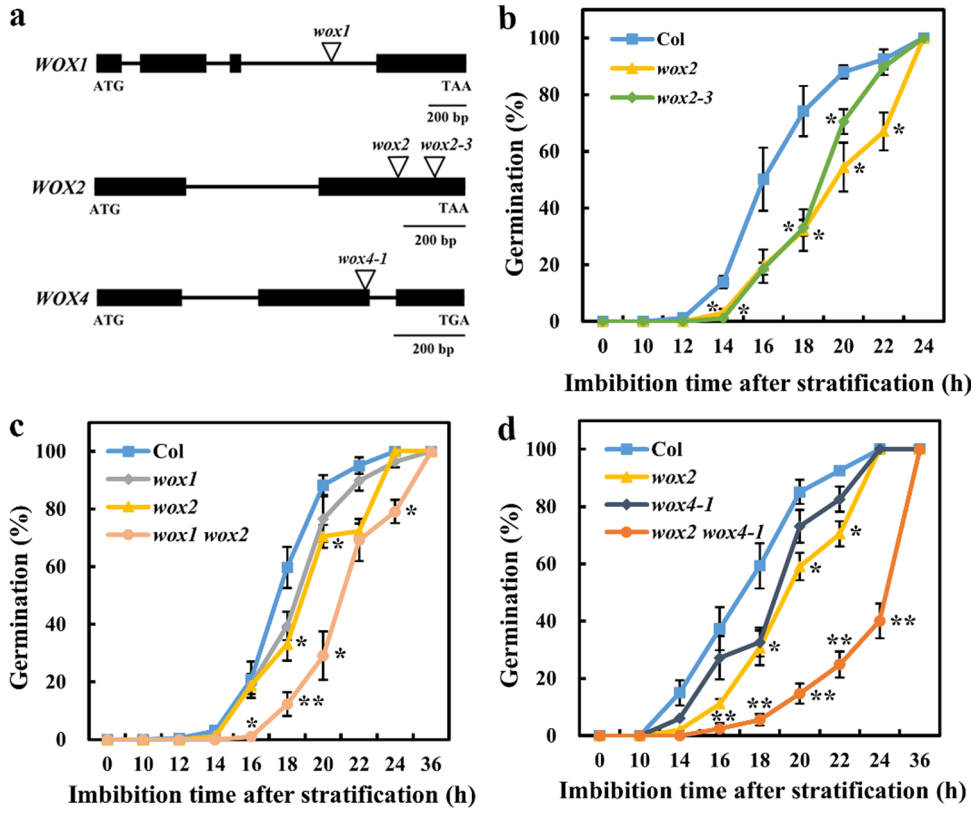

Fig. 3 Loss of function of *WOX2* and *WOX4* results in slower germination in *Arabidopsis*. **a** Schematic representation of the T-DNA insertion in *wox1*, *wox2*, *wox2-3*, and *wox4-1* mutants. The black box represents exons, the horizontal line represents introns, and the inverted triangle represents insertion sites. ATG indicates the start codon, and TAA (or TGA) indicates the stop codon. **b** Germination phenotypes of wild type (Col), *wox2*, and *wox2-3*. **c** Germination phenotypes of Col, *wox1*, *wox2*, and *wox1 wox2*. **d** Germination phenotypes of Col, *wox2*, *wox4-1*, and *wox2 wox4-1*. In **b** to **d**, seeds were stratified for 3 days at 4 °C before the germination assays. Percentages of seed germination are the means ± SE based on the seeds from six to eight individual plants for each genotype. Asterisks indicate significant differences compared to the wild type as determined by Student's *t*-test (**$P < 0.01$ and *$P < 0.05$)

double mutants were confirmed by genome PCR before phenotypic analysis. As shown in Fig. S3b, c, both *wox1 wox2* and *wox2 wox4-1* double mutants showed enhanced seed dormancy compared to the wild type. Furthermore, both double mutants exhibited strong phenotypes of reduced germination speed (Fig. 3c, d). At 20 h following stratification, seeds of the wild type germinated over 80%, whereas *wox1 wox2* and *wox2 wox4-1* seeds germinated about 30% and 10%, respectively (Fig. 3c, d). Interestingly, we also found that the double mutant *wox2 wox4-1* displayed defects in cotyledon initiation (Fig. S4), but *wox1 wox2* had no difference from the wild type. The results of germination assays indicate that WOX2 functions redundantly with WOX1 and WOX4 in the positive regulation of seed germination.

The hypocotyl and radicle growth of *wox2 wox4-1* was weakened

As GUS activity driven by the *WOX2* promoter was observed in hypocotyl and radicle in stratified seeds, germinating seeds, and seedlings (Fig. 2g–i), we hypothesized that the retarded germination of the *wox2 wox4-1* double mutant is caused by hypocotyl and radicle development. To verify this hypothesis, we measured the hypocotyl and radicle lengths of the wild-type and *wox2 wox4-1* seeds during germination. As shown in Fig. 4a, b, the hypocotyl and radicle of *wox2 wox4-1* were smaller than those of the wild type when imbibed for 5 h (before radicle emergence). When seeds were imbibed in water for 5 h, 12 h, and 24 h, the hypocotyl and radicle lengths of the *wox2 wox4-1* double mutant were significantly shorter than those of Col, while they showed no difference at 8 h (Fig. 4c). Similarly, the *wox2 wox4-1* mutant also showed a significantly shorter hypocotyl–radicle transition zone in seeds imbibed for 0 h, 5 h, and 12 h, and seedlings germinated for 24 h (Fig. 4d). These results supported that the hypocotyl and radicle combinatorial parts are smaller in the *wox2 wox4-1* double mutant, implying that WOX2 and WOX4 accelerate seed germination by promoting the hypocotyl elongation and radicle protrusion.

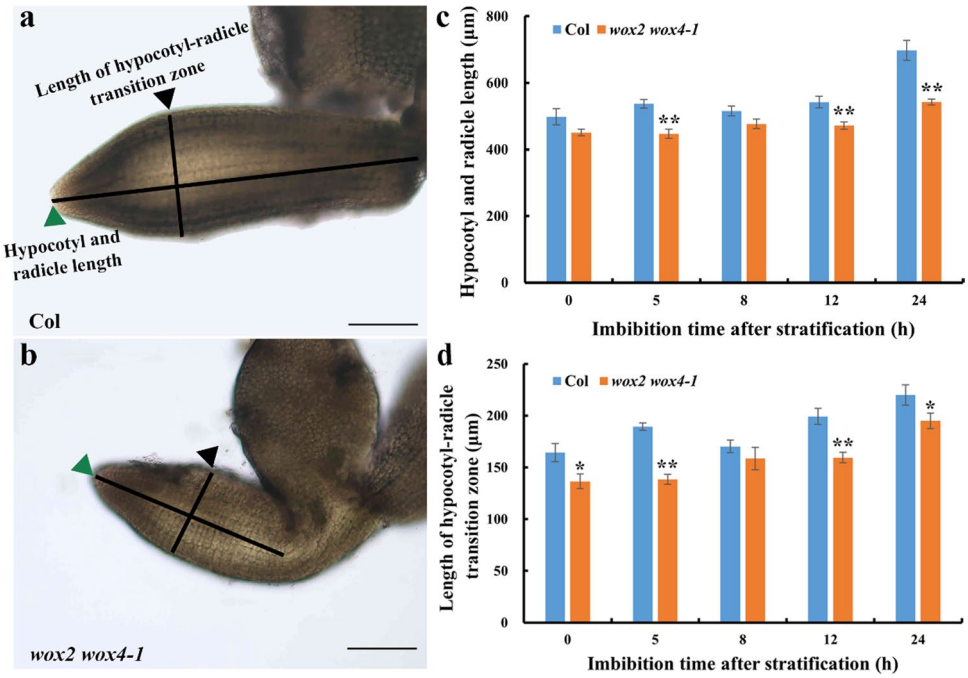

Fig. 4 The growth of hypocotyl and radicle of *wox2 wox4-1* is weakened. **a**, **b** The hypocotyl and radicle morphology of wild type (Col) (**a**) and *wox2 wox4-1* (**b**) at 5 h after imbibition. Black triangles indicate the length of the hypocotyl–radicle transition zone. Green triangles indicate the hypocotyl and radicle length. Bars = 100 μm. **c**, **d** Lengths of hypocotyl and radicle (**c**) and hypocotyl–radicle transition zone (**d**) of Col and *wox2 wox4-1* at different imbibition time points, respectively. Seeds were stratified for 3 days at 4 °C before the germination assays. Values are the means ± SE based on 12 seeds from eight individual plants for each genotype. The double asterisk and single asterisk indicate significant differences from the wild type using Student's *t*-test (**$P < 0.01$ and *$P < 0.05$)

Transcriptome analysis of *wox2 wox4-1*

To further understand the downstream pathways affected by *WOX2* and *WOX4* mutations during seed germination, we performed RNA-seq. We used wild-type Col and *wox2 wox4-1* seeds imbibed at 25 °C for 18 h when the two materials showed a significant difference in germination (Fig. S5). A total of 514 differentially expressed genes (DEGs) were identified (Supplementary Table S2). Among them, 410 DEGs were upregulated, and 104 DEGs were downregulated. Gene Ontology (GO) enrichment analysis showed that these genes were enriched in cell wall biogenesis, and organization, and response to phytohormones (Fig. 5a). By analyzing the DEGs in the highlighted pathways in Fig. 5a, we found that the *Xyloglucan endotransglucosylase/hydrolase* (*XTH*) family genes were significantly enriched in processes such as cell wall biogenesis, and xyloglucan metabolic process, and xyloglucosyl transferase activity (Fig. S6). The heatmap showed that the expression of seven *XTH* genes decreased in *wox2 wox4-1* (Fig. 5b). In addition, the expression of some genes involved in phytohormone signaling pathways was also altered (Fig. S6).

Furthermore, we examined the DEGs by RT-qPCR using dry seeds, stratified seeds, and germinating seeds. Eight genes involved in the cell wall, phytohormones, and root growth were selected. As shown in Fig. 6a, expression of *XTH7* was decreased in the double mutant seeds compared with the wild type when stratified for 72 h and germinated for 18 h. *XTH24* transcript level decreased in dry seeds and 24 h stratified seeds, while it showed no differences in seeds germinated for 5 h and 18 h (Fig. 6b). *XTH33* was upregulated in both dry and 72-h stratified seeds but downregulated in seeds germinated for 18 h, in the double mutant compared to that of the wild type (Fig. 6c). In addition, the expression levels of some phytohormone-related genes were also affected by *WOX2* and *WOX4* mutations, including auxin signaling pathway-related genes *ARF2* and *Indole-3-acetic acid 9* (*IAA9*) (Fig. 6d, e), GA synthesis gene *Ent-kaurenoic acid oxidase 2* (*KAO2*) (Fig. 6f), and ABA signaling pathway gene *Farnesylcysteine lyase* (*FCLY*) (Fig. 6g). We also found that the NAC family transcription factor gene *NAC1*, involved in root development regulation (Xie et al. 2000), was upregulated in *wox2 wox4-1* mutant seeds germinated for 5 h and 18 h (Fig. 6h). These results suggest that WOX2 and WOX4 regulate seed germination through the phytohormone pathway and then the downstream XTH family genes.

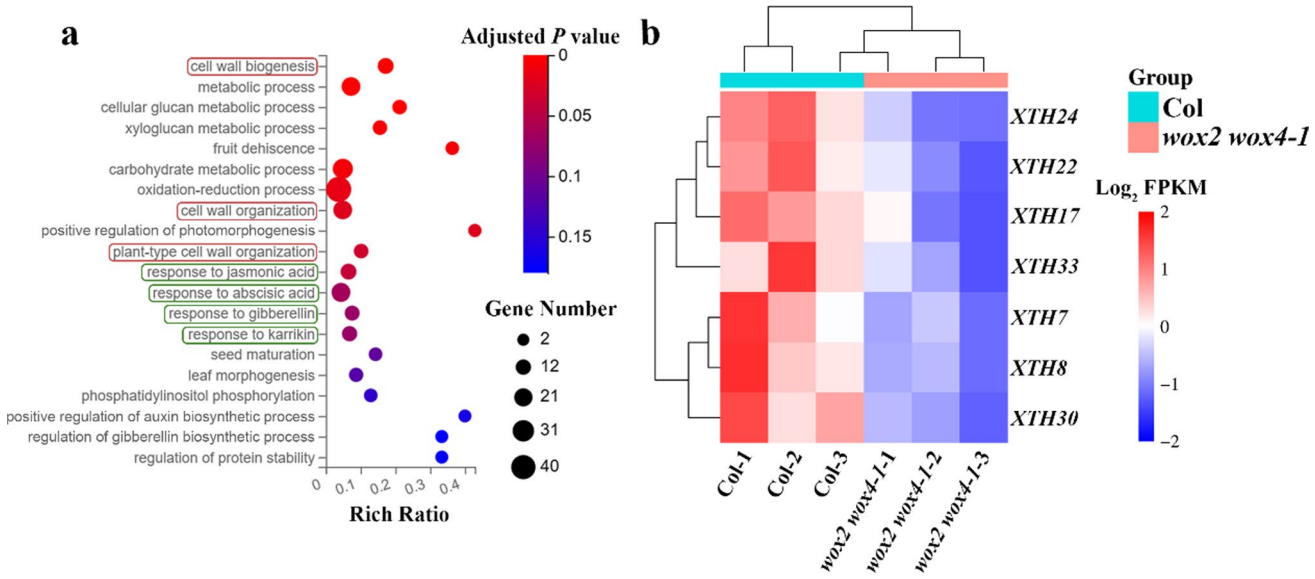

Fig. 5 Transcriptomic analysis of gene expression profiles in wild-type (Col) and *wox2 wox4-1* germinating seeds. **a** Gene Ontology (GO) enrichment analysis of all differentially expressed genes in *wox2 wox4-1* versus wild type (Col). The GO category biological processes are shown. Processes related to the cell wall are highlighted with red boxes. Phytohormone-related pathways are marked with green boxes. The dot size is proportional to the gene number. The color scale indicates the significance level. **b** The expression heatmap of the *XTH* family genes analyzed by RNA-seq. The color scale indicates the $\log_2$ FPKM value

Seed germination sensitivity of *wox2 wox4-1* to ABA, PAC, and GA_{4+7}

The balance between ABA and GA plays a pivotal role in regulating seed germination (Shu et al. 2016). Our transcriptome data showed that the expression of GA and ABA-related genes was affected (Fig. 5a). We, therefore, hypothesized that the retarded germination of *wox2 wox4-1* might be caused by an enhanced ABA pathway or an attenuated GA pathway. We then examined the seed germination in response to ABA, GA_{4+7}, and PAC (an inhibitor of GA biosynthesis). In the presence of PAC, fully after-ripened seeds of *wox2 wox4-1* showed enhanced sensitivity to PAC compared with the wild type. The results indicate that the GA pathway is affected in *wox2 wox4-1* (Fig. 7a). GA_{4+7} treatment could partially restore the weakened seed germination phenotype of *wox2 wox4-1* (Fig. 7b). However, *wox2 wox4-1* seeds showed a similar sensitivity to ABA as the wild type, suggesting that the ABA signaling pathway is not affected in *wox2 wox4-1* (Fig. 7c). These results suggest that WOX2 and WOX4 are involved in the GA pathway to control seed germination.

Discussion

In this study, we showed that the WOX family gene *WOX2* is highly expressed during seed development and acts redundantly with WOX1 and WOX4 to positively regulate seed germination. We hypothesize that WOX2 and WOX4 regulate seed germination by stimulating cell elongation in the hypocotyl–radicle region via the GA pathway and downstream cell wall-related genes (Fig. 8).

The mature *Arabidopsis* seed consists of two parts: the embryo and the surrounding tissues (single-cell endosperm layer and testa). When the seed dormancy release is complete and the external environment is suitable for germination, the seed will enter the germination state (Holdsworth et al. 2008). The completion of seed germination depends on the balance of two opposing forces: the growth potential of the embryo and the restraint of the enclosing structures. In *Arabidopsis*, the hypocotyl–radicle transition zone and lower hypocotyl, as growing tissues, increase the embryo growth potential to exceed the restraint and then complete seed germination (Sliwinska et al. 2009; Steinbrecher and Leubner-Metzger 2017). The molecular genetic networks that regulate seed germination have been gradually unraveled (Rajjou et al. 2012). In this study, we preliminarily investigate the function of *WOX* genes on seed germination. The double mutant *wox2 wox4-1* showed reduced seed germination, and its growth of the hypocotyl–radicle zone was weakened (Figs. 3, 4), which led us to speculate that *WOX2* and *WOX4* may promote seed germination by increasing the embryo growth potential. The GA signaling pathway plays an important role during seed germination. In *Arabidopsis*, root apical meristem is an important site for GA production. GA signals moved from the radicle to the hypocotyl, promoting the elongation of

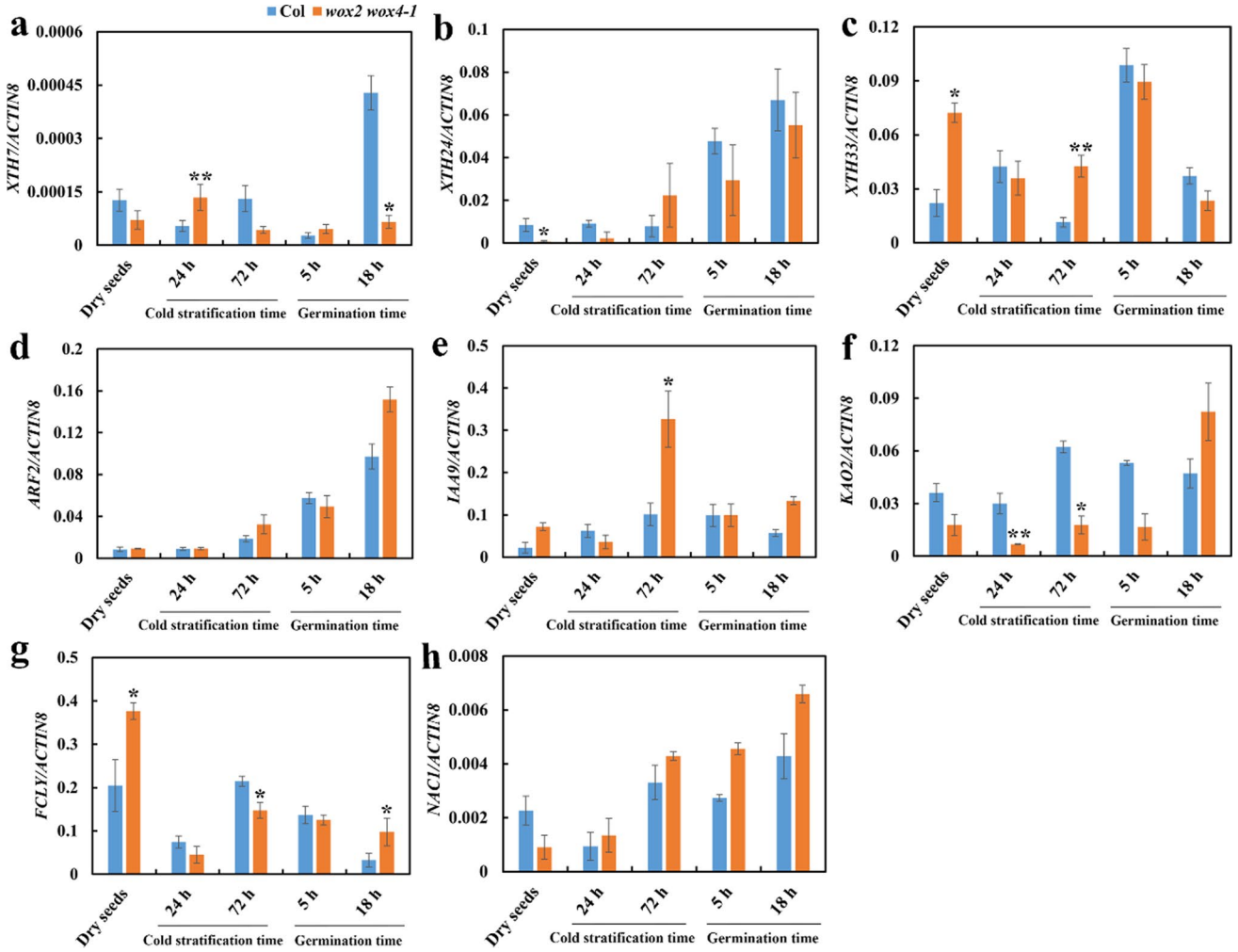

Fig. 6 Relative expression of *XTHs*, phytohormones-related genes, and *NAC1* in dry seeds, cold stratified seeds, and germinating seeds for wild-type Col and *wox2 wox4-1* mutant. **a–c** RT-qPCR validation of the expression levels of *XTH7*, *XTH24*, and *XTH33*. **d–g** RT-qPCR analysis of the expression levels of *ARF2*, *IAA9*, *KAO2*, and *FCLY*. **h** RT-qPCR analysis of the expression level of root development-related gene *NAC1*. Total RNAs were isolated from freshly harvested dry seeds, 24-h and 72-h cold stratified seeds, and 5-h and 18-h germinating seeds. Expression levels were normalized to *ACTIN8*. Values are means of three biological replicates. The error bars represent SE. Asterisks indicate significant differences compared to the wild type as determined by Student's *t*-test (**$P < 0.01$ and *$P < 0.05$)

the hypocotyl–radicle zone by regulating the expression of downstream genes, including the *XTH* genes *XTH9/19* and the expansin genes *EXPA1/8/15* (Becnel et al. 2006; Bassel et al. 2014). The GA biosynthetic mutant *ga1-3* could not germinate. After exogenous application of GA, the hypocotyl cells can elongate normally, and the mutant seeds can eventually germinate (Sun et al. 1992; Bassel et al. 2014). Therefore, GA signals are crucial for hypocotyl–radicle zone elongation. In this study, GO enrichment analysis of the DEGs revealed that "response to gibberellin" is one of the significantly enriched GO terms (Fig. 5; Fig. S6). Seed germination sensitivity to PAC and GA_{4+7} indicated that the GA pathway was affected by *WOX2* and *WOX4* mutations (Fig. 7a, b). RT-qPCR assay also confirmed that the GA biosynthesis gene *KAO2* was downregulated in *wox2 wox4-1* (Fig. 6f). These results suggest that WOX2 and WOX4 may regulate seed germination through the GA pathway.

The structure of the hypocotyl in *Arabidopsis* is relatively simple. Normally, the number of hypocotyl cells in *Arabidopsis* is determined during embryogenesis (Gendreau et al. 1997). Previous studies have shown that hypocotyl–radicle zone elongation is the result of cell expansion rather than cell division (Sliwinska et al. 2009). The prerequisite for cell expansion is cell wall loosening (Cosgrove 2005). In *Arabidopsis*, the XTH family contains 33 members (Becnel et al. 2006). *XTHs* encode cell wall modification enzymes, which can catalyze the hydrolysis or transfer of xyloglucan molecules, thereby loosening the structure of the cell wall (Van Sandt et al. 2007; Maris et al. 2009; Miedes et al.

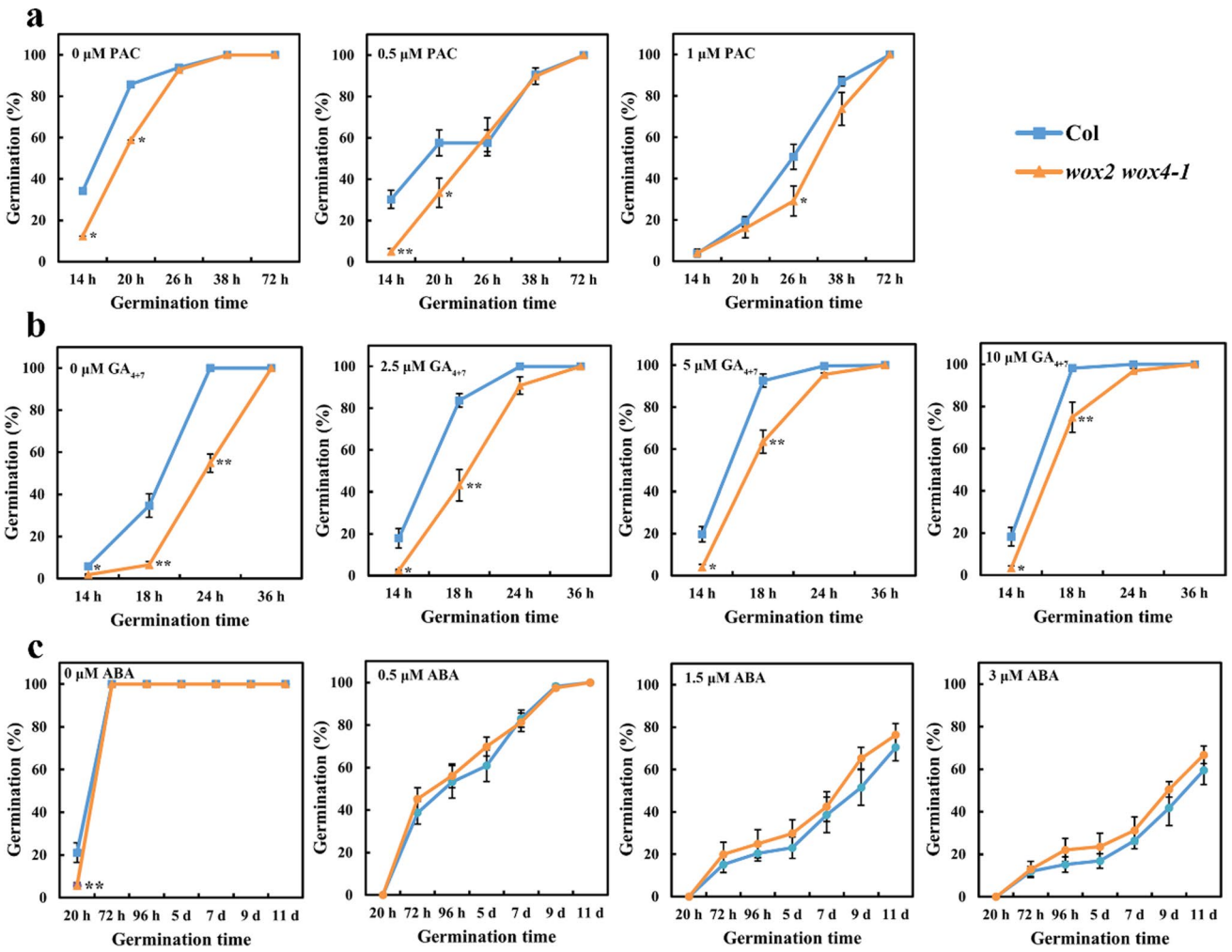

Fig. 7 Germination of wild-type and *wox2 wox4-1* mutant seeds in the presence of different concentrations of PAC, ABA, and GA_{4+7}. **a** Germination of wild-type Col and *wox2 wox4-1* mutant seeds treated with different concentrations of PAC (0, 0.5, and 1 μM). **b** Germination of Col and *wox2 wox4-1* mutant seeds treated with different concentrations of GA_{4+7} (0, 2.5, 5, and 10 μM). **c** Germination of Col and *wox2 wox4-1* mutant seeds treated with different concentrations of ABA (0, 0.5, 1.5, and 3 μM). Seeds were cold stratified for 3 days before being treated with PAC or ABA. Freshly harvested seeds were used for GA_{4+7} treatment. At least 50 seeds for each genotype were used in three independent biological replicates. Data are means (±SE). Significant differences compared with the wild type were determined using Student's *t*-test (**$P < 0.01$ and *$P < 0.05$)

2013). Research has shown that XTH is involved in multiple processes of plant growth and development, such as root elongation, leaf vein differentiation, fruit ripening, and petal senescence (Matsui et al. 2005; Liu et al. 2007; Singh et al. 2011; Tsuchiya et al. 2015). XTH7 was reported to regulate hypocotyl elongation in *Arabidopsis* by ethylene and brassinosteroid signaling pathways (Liu et al. 2018). XTH24 works downstream of SHORT-ROOT (SHR) to regulate hypocotyl cell elongation (Dhar et al. 2022). XTH33 is involved in root growth in response to ethylene (Kong et al. 2018). Furthermore, they were also expressed in the hypocotyl, cotyledon, or elongation zone, which highly overlapped with the expression pattern of *WOX2* (Becnel et al. 2006; Fig. 2). These data suggest that *XTH7*, *XTH24*, and *XTH33* are the downstream genes of WOX2 and WOX4. We hypothesize that the two transcription factors may act redundantly in hypocotyl–radicle cells to promote the expression of *XTH* genes, increasing cell wall extensibility, cell elongation, and, eventually, seed germination. The induction of WOX2 and WOX4 on *XTH* genes requires further confirmation by a transcriptional activation assay. Whether the cell expansion in the *wox2 wox4-1* hypocotyl–radicle zone is retarded still needs further cell morphological analysis.

In conclusion, our research identified a novel function of WOX2 and WOX4 in seed germination. Genetic and transcriptomic analyses provide a framework for how WOX2 and WOX4 regulate seed germination. We suggest that WOX2 and WOX4 increase the growth of the

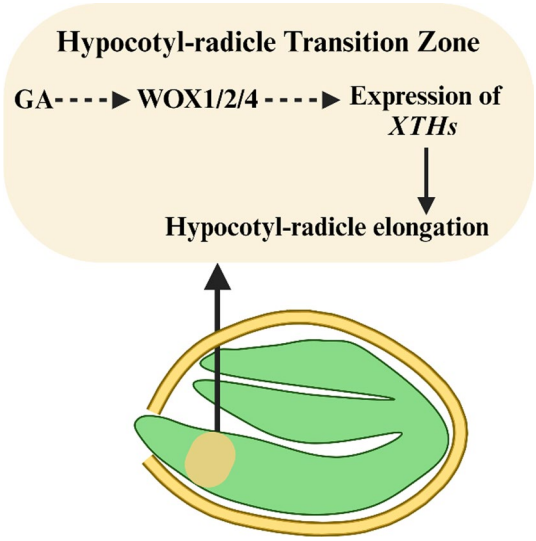

Fig. 8 A model for the roles of WOX1/2/4 in the control of seed germination. Under favorable conditions, GA is synthesized in seeds, which may induce or stabilize WOX1/2/4 proteins. WOXs positively regulate the expression of *XTH* genes (directly or indirectly), and then promote cell elongation of the hypocotyl–radicle region. Consequently, seed germination is facilitated. Arrows indicate positive effects, and the dashed line indicates indirect or unknown regulation. The graphic was created with BioRender.com

hypocotyl–radicle zone through the downstream *XTH* genes (*XTH7/24/33*) during germination, hence increasing germination potential. Our results provide insights into the regulatory mechanism of *WOX* genes in seed germination.

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Author contributions YXL and HC planned and designed the research. YY, ZR, and LL conducted the experiments. YL and YH contributed materials and analysis tools. YY, ZR, YXL, and HC analyzed the data. YY and HC wrote the manuscript. YXL and HC revised the manuscript. All authors read and approved the manuscript.

Data availability All data in this study are provided in this manuscript and supplementary data files. It will be provided upon a reasonable request.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest about the work described in this manuscript.

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