MYB70 modulates seed germination and root system development in Arabidopsis

Highlights
- MYB70 regulates seed germination by enhancing ABA signaling via interaction with ABI5
- MYB70 activates the IAA conjugation process by upregulating GH3 genes expression
- MYB70 mediates root growth via repression of PER genes
- MYB70 negatively regulates suberin biosynthesis in roots

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MYB70 modulates seed germination and root system development in Arabidopsis

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SUMMARY
Crosstalk among ABA, auxin, and ROS plays critical roles in modulating seed germination, root growth, and suberization. However, the underlying molecular mechanisms remain largely elusive. Here, MYB70, a R2R3-MYB transcription factor was shown to be a key component of these processes in Arabidopsis thaliana. myb70 seeds displayed decreased sensitivity, while MYB70-overexpressing OX70 seeds showed increased sensitivity in germination in response to exogenous ABA through MYB70 physical interaction with ABI5 protein, leading to enhanced stabilization of ABI5. Furthermore, MYB70 modulates root system development (RSA) which is associated with increased conjugated IAA content and H2O2/O2− ratio but reduced root suberin deposition, consequently affecting nutrient uptake. In support of these data, MYB70 positively regulates the expression of auxin conjugation-related GH3, while negatively peroxidase-encoding and suberin biosynthesis-related genes. Our findings collectively revealed a previously uncharacterized component that modulates ABA and auxin signaling pathways, H2O2/O2− balance, and suberization, consequently regulating RSA and seed germination.

INTRODUCTION
Seed germination and root system architecture (RSA) are highly plastic in responses to environmental cues, and are fine-tuned by the crosstalk of different phytohormones and secondary signal molecules (Lata and Prasad, 2011; Lin et al., 2019; Signora et al., 2002). Abscisic acid (ABA) is a vital phytohormone, which plays key roles in modulating seed germination and various plant growth and developmental processes (Bu et al., 2009; Ding et al., 2015; Hirayama and Shinozaki, 2007; Zhang et al., 2019b). The basic leucine zipper (bZIP) transcription factor (TF), Abscisic Acid Insensitive 5 (ABI5), acts as a core regulator of the modulation of seed germination and seedling establishment (Finkelstein and Lynch, 2000). Many TFs or regulators modulate seed germination and seedling growth through regulation of ABI5 expression, or physical interaction with ABI5 to alter ABI5’s stabilization, or its regulatory effect on downstream target genes (Guan et al., 2014; Lim et al., 2013; Lopez-Molina et al., 2002; Zheng et al., 2012). For example, ABI3 modulates seed germination by directly inducing ABI5 expression (Lopez-Molina et al., 2002). Meanwhile, ABI3 and DELLA proteins interact with ABI5 to form a complex and subsequently improve ABI5 capacity to induce the expression of high temperature-responsive genes, such as SOMNUS (SOM), which encodes a CCCH-type zinc finger protein that negatively regulates light-dependent seed germination in Arabidopsis thaliana to inhibit seed germination under high temperature conditions (Kim et al., 2008; Lim et al., 2013).

The regulatory networks of seed germination and early seedling establishment involving ABA and associated TFs are extremely complex; and thus, a concerted effort is needed to identify and functionally characterize additional unknown members involved in these networks.

In Arabidopsis, early and quick auxin-responsive genes, including Auxin/Indole-3-Acetic Acid (Aux/IAA), Gretchen Hagen3 (GH3), Small Auxin-Up RNA (SAUR), and Auxin Response Factor (ARF), are key performers in auxin signal transduction (Hagen and Guilfoyle, 2002; Kieffer et al., 2009; Tiwari et al., 2003; Vanneste and Friml, 2009). Auxin-induced GH3 genes can mediate the inactivation of IAA by conjugation, which further attenuates auxin signal transduction (Staswick et al., 2005). Root system development is tightly modulated by various members of the auxin signaling (Vanneste and Friml, 2009). For example, auxin-inducible ARF7 and ARF19 regulate lateral root (LR) initiation and emergence by modulating the...
expression of Lateral Organ Boundaries Domain/Asymmetric Leaves 2-like (LBD/ASL) genes LBD18/ASL20, LBD16/ASL18 and LBD29/ASL16 (Lee et al., 2009; Okushima et al., 2007).

Reactive oxygen species (ROS), as a secondary signaling molecule, play critical roles in modulating different developmental processes (Manzano et al., 2014). ROS are majorly generated in chloroplasts, mitochondria, peroxisomes, and the apoplast (Mittler et al., 2011; Vahtera et al., 2014). Intracellular ROS equilibrium is fine-tuned by a series of reactions involving a set of enzymes, including NADPH/NADH oxidases, and antioxidant enzymes like catalases, peroxidases (PERs) and superoxide dismutases (Hossain et al., 2015; Mostofa et al., 2018). Many studies have shown that ROS are responsible for the alteration of RSA (Manzano et al., 2014; Tsukagoshi et al., 2010). The basic-helix-loop-helix (bHLH) Upbeat1 (UPB1) TF modulates the balance between differentiation and proliferation in the roots by regulating ROS levels via direct repression of several PER genes, thereby modulating root growth and development (Tsukagoshi et al., 2010). In addition to UPB1, MYB36 also showed a similar function in regulating lateral root primordium (LRP) development and ROS balance in Arabidopsis roots by modulating the transcription of a set of PER genes (Fernández-Marcos et al., 2017). Nevertheless, Mabuchi et al. (2018) found that MYB30 regulates primary root (PR) growth in an H2O2-dependent manner.

Cutin and suberin, the two types of lipid polyesters, act as transport barriers at the interface between plants and the environment (Holbein et al., 2021; Schreiber, 2010). Recent studies have shown the important roles of cuticle and suberin in modulating seed and root development (Barberon et al., 2016; Beisson et al., 2007; Coen et al., 2019). The Glycerol-3-Phosphate Acyltransferase 5 (GPAT5) positively regulates suberin biosynthesis in seed coats and roots (Beisson et al., 2007), while GPAT4 and GPAT8 functionally and redundantly regulate cuticle formation in root cap, and hence affect LR formation (Berhin et al., 2019). Several MYB TFs modulate the expression of genes involved in cuticle and suberin biosyntheses in plants. For example, in Arabidopsis, MYB96 positively regulates cuticular wax biosynthesis in an ABA-dependent manner (Seo et al., 2009), while MYB9 and MYB107 are responsible for suberin deposition in seed coats (Lashbrooke et al., 2016).

The Arabidopsis MYB44, MYB70, MYB73, and MYB77 genes, which share structural similarity and are the members of subfamily 22, are involved primarily in the regulation of plant growth and development, as well as plant responses to environmental stresses (Jung et al., 2008; Yang et al., 2019). MYB77 modulates LR growth and development through auxin signaling pathway by physically interacting with ARF7 (Shin et al., 2007; Zhao et al., 2014). The ABA receptor Pyrabactin Resistance 1-Like 8 (PYL8) mediates LR growth by physically interacting with and enhancing the activity of MYB77, as well as its homologs MYB44 and MYB73, to activate auxin signaling (Zhao et al., 2014). However, the functions of MYB70 in mediating plant growth and development have not yet been elucidated.

Here, we focused on characterizing the mechanisms of MYB70 involved in the processes of seed germination and root system development through the modulation of its dual transcriptional regulatory activities. myb70 seeds displayed decreased sensitivity, while MYB70-overexpressing OX70 seeds showed increased sensitivity in germination in response to exogenous ABA. We then used yeast two-hybrid (Y2H) assay to search for MYB70-interacting proteins or transcriptional regulators and identified the basic region/leucine zipper TF ABI5. Our results indicated that MYB70 is an important TF involved in ABI5-mediated seed germination. MYB70 also regulates root system growth and development by altering auxin signaling via its transactivation activity, while controlling ROS equilibrium via its transcriptional repression activity. MYB70 negatively regulates suberin deposition in the roots to modulate RSA via its transcriptional repression activity. Overall, our study showed that MYB70 integrates auxin, ROS and ABA signaling pathways to form a network regulating seed germination and root system development. These results enable us to gain further understanding on how MYB TFs may contribute to improving the roots’ ability in nutrient and water uptake to maintain normal plant growth in an era of climate change.

RESULTS

Determination of the developmental and tissue-specific expression of MYB70

The expression of the MYB70 gene was localized using a transgenic line in which β-glucuronidase (GUS) was fused to the 1.5-kb promoter region of MYB70. GUS activity was found in the micropyle region of seeds (Figure 1Aa), in the radicles (Figure 1Ab), and in the cotyledons (Figures 1Ab, 1Ac and 1Af). In addition, GUS activity was detected in the entire roots (Figures 1Ac–1Ae) and young leaves (Figure 1Af). In mature plants,
GUS activity was also detected in the flowers and siliques (Figure 1A). Additionally, MYB70 expression patterns were also examined in different tissues using qRT-PCR (Figure 1B). Since both auxin and ABA play important roles in modulating plant growth and development (Zhao et al., 2014), we also examined MYB70 expression in response to exogenous IAA and ABA treatments. The expression of MYB70 might be slightly induced and markedly upregulated in the whole seedlings in response to exogenous IAA and ABA, respectively, after 24 h of treatment (Figure 1C). When the expression patterns of MYB70 in response to exogenous ABA were studied separately in shoots and roots, the MYB70 transcription was more highly induced by ABA in shoots than in roots (Figure 1D). Additionally, the expression of MYB70 reached the highest levels in the roots and shoots after 6 and 24 h of treatment, respectively, after which its expression levels gradually declined (Figure 1D).

Figure 1. The gene expression analysis of MYB70 and seed germination in response to exogenous ABA in myb70 and abi5 mutants, ABIS-overexpressing ABISOX and MYB70-overexpressing OX70 transgenic plants, and their different combinations (A) Detection of MYB70 expression using the proMYB70:GUS transgenic line. GUS staining observed in seeds (bar, 500 µm) (a), 1-day-old seedling (bar, 500 µm) (b), 7-day-old seedling (bar, 500 µm) (c), lateral root (bar, 100 µm) (d), root tip of primary root (bar, 100 µm) (e), leaves of 15-day-old seedling (bar, 500 µm) (f) and flowers (bar, 500 µm) (g). (B) Relative expression of the MYB70 gene in Col-0 plants. (C) Relative expression of the MYB70 gene in 5-day-old Col-0 seedlings with or without 10 µM IAA or 10 µM ABA for 6 or 24 h. (D) Relative expression of the MYB70 gene in the shoots or roots of 5-day-old Col-0 seedlings with or without 10 µM ABA for 6, 12, 24 and 48 h. Results shown are means ± SD (n = 3). (E) Seeds of abi5 and myb70 mutants, and ABIS-overexpressing ABISOX and MYB70-overexpressing OX70 transgenic plants, and their different combinations after stratification at 4°C for 2 days were germinated on 1/2-strength MS medium with or without 0.5 µM ABA for an additional 7 days. (F) Seed germination was determined 2–5 days after stratification on 1/2-strength MS medium without or with 0.5 µM ABA or 1 µM abscisic acid (ABA). Results are means ± standard error (SEM) (n = 5, more than 120 seeds/genotype/repeat). Different letters show significantly different values at p < 0.05 according to a Tukey’s test, and asterisks show significant differences from the control (Student’s t-test, p < 0.05).
MYB70 negatively regulates seed germination in an ABA-dependent manner by physically interacting with AB15

We obtained two T-DNA insertion mutants, myb70-1 and myb70-2, from the European Arabidopsis Stock Centre (NASC), and also developed 35S:MYB70 (OX70-1, OX70-2, OX70-3, OX70-4, OX70-5 and OX70-6) transgenic Arabidopsis lines for in planta functional analysis of MYB70. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)-based analysis revealed that MYB70 expression was highly up-regulated in OX70 plants but was abolished in the two mutants (Figures S1A and S1B).

ABA plays an essential role in modulating seed germination (Guan et al., 2014). The expression of MYB70 was markedly induced by ABA (Figures 1C and 1D). We, thus, germinated seeds of ABA plays an essential role in modulating seed germination (Guang et al., 2014). The expression of OX70 and Col-0 plants on 1/2-strength Murashige and Skoog (MS) medium supplied with or without ABA to investigate the possible role of MYB70 in seed germination. When compared with Col-0 seeds in a seed germination assay as assessed by seed radicle appearance, myb70 seeds exhibited decreased sensitivity, while OX70 seeds exhibited increased sensitivity to ABA (Figures 1E and 1F). The result suggested that MYB70 negatively regulates seed germination in an ABA-dependent manner.

We then searched for MYB70-interacting proteins to identify potential regulators/coregulators, particularly those involved in ABA-mediated regulation of seed germination. Among the potential MYB70-interacting proteins, the B-group ABI protein, AB15, which plays a vital role in modulating seed germination (Zhao et al., 2018; Zhou et al., 2015), was identified. We then performed a Y2H assay using AB15 (fused to the binding domain of GAL4; BD-AB15) as bait and MYB70 (fused to the activation domain of GAL4; AD-MYB70) as prey. The result of the Y2H assay showed that AB15 indeed interacted with MYB70 in the yeast cells (Figure 2A). This interaction was also verified by an in vitro pull-down assay. As shown in Figure 2B, glutathione S-transferase (GST)-fused AB15 could retain MYB70-His, while GST alone could not. This physical interaction was also corroborated by the results of a coimmunoprecipitation (Co-IP) assay (Figure 2C). We further analyzed the interaction between the two proteins in plant cells using the bimolecular fluorescence complementation (BiFC) assay, in which the MYB70 and AB15 proteins were fused to the N-terminal part and the C-terminal part of the yellow fluorescent protein (YFP), respectively. A strong YFP fluorescence signal was detected in the nuclei, when AB15 and MYB70 fusions were examined together (Figure 2D). Taken together, these data indicated that AB15 interacts with MYB70 both in vivo and in vitro.

We then compared the expression of MYB70 and AB15 in both dry and germinating seeds. The expression patterns of MYB70 were similar to those of AB15 in both types of seeds (Figures 2E and 2F). These data collectively suggested that MYB70 may play a role in seed germination, perhaps through interaction with AB15. To elucidate the potential genetic interaction of MYB70 and AB15 in the mediation of ABA-induced repression of seed germination, we generated hybrid OX70 AB15OX, OX70 ab15, myb70 AB15OX, and myb70 ab15 lines by crossing AB15OX or ab15-8 (SALK_013163) with OX70 or myb70 lines. We then evaluated seed germination rates of AB15OX, ab15-8, OX70, myb70, OX70/AB15OX, myb70/AB15OX, myb70/ab15 and OX70/ab15 genotypes in response to ABA. The seeds of AB15OX were highly sensitive to ABA during germination, whereas those of ab15-8 were highly insensitive to ABA (Figure 1). Similar to the seeds of AB15OX plants, the seeds of hybrid OX70 AB15OX (Figure S2) and myb70 AB15OX lines were hypersensitive, and similar to those of the ab15-8 mutants, the seeds of hybrid OX70 ab15 and myb70 ab15 lines were insensitive to ABA during germination (Figures 1E and 1F). These results indicated that MYB70 functions as a negative regulator of seed germination by acting together with AB15 in modulation of ABA signaling where AB15 plays a more prominent role.

To confirm these results, we evaluated the expression of EM1 and EM6 genes, each of which is a direct target of AB15, in the germinating seeds of the double mutants. The transcript levels of both the EM1 and EM6 genes were higher in the hybrid OX70 AB15OX and myb70 AB15OX lines than in the Col-0 and myb70 mutant plants, while their transcript levels were similar in the ab15-8, OX70 ab15, and myb70 ab15 lines (Figures 3A and 3B). We also measured ABA levels in the OX70 plants and myb70 mutants, and found no difference between these lines (Figure 3C). These data demonstrated that MYB70 was involved in regulating ABA-mediated seed germination by enhancing ABA signaling via interaction with AB15, but not by increasing the ABA content.

We next investigated the role of the MYB70-AB15 interaction on the transcriptional regulation of the EM1 and EM6 genes that are the direct targets of AB15 (Finkelstein and Lynch, 2000). 35S:AB15 was cotransfected with 35S:MYB70 together with EM1-LUC or EM6-LUC reporter plasmids. Overexpression of AB15 or MYB70
increased the expression of the EM1 and EM6 genes, especially in the presence of ABA, with overexpression of ABI5 showing higher effect than that of MYB70 (Figures 3D and 3E). Cotransfection of 35S:ABI5 and 35S:MYB70 constructs stimulated EM1 and EM6 expression more than that of any single construct (Figures 3D and 3E), suggesting that the interaction of ABI5 with MYB70 improved ABI5’s ability to transcriptionally regulate its target genes.

**MYB70 interaction with ABI5 prevents ABI5 from 26S proteasome-dependent degradation**

Previous studies have demonstrated that ABA stabilized ABI5 protein, since ABI5 was degraded via 26S proteasome in the absence of exogenous ABA (Lopez-Molina et al., 2002; Seo et al., 2014). To determine whether MYB70 regulates ABI5 stabilization, the ABA-treated Col-0 and OX70 seeds were transferred to fresh 1/2 MS liquid medium without ABA for 8 and 12 h. As shown in Figure 3F, ABI5 was more abundant in OX70 seeds in the absence of ABA. After treatment with protein synthesis inhibitor cycloheximide (CHX), ABI5 degradation was also delayed in OX70 compared with Col-0 seeds (Figure 3G). However, treatment

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**Figure 2.** MYB70 interacts with ABI5 both in vitro and in vivo

(A) Yeast-two-hybrid (Y2H) analysis revealed an interaction between MYB70 and ABI5. Transformed yeast cells were grown on SD-Trp/-His/-Leu/-Ade medium.

(B) In vitro pull-down assay revealed the interaction between MYB70 and ABI5.

(C) Coimmunoprecipitation assay showing the interaction between MYB70 and ABI5.

(D) BiFC analysis of the interaction between MYB70 and ABI5. DAPI, 4',6-diamidino-2-phenylindole. Fluorescence that was resulted from complementation of the N-terminal region of YFP fused to MYB70 (MYB70-nYFP) with the C-terminal region of YFP fused to ABI5 (ABI5-cYFP) was observed in the nuclei of *N. benthamiana* leaves. No signal was observed in the negative controls. The blue and green fluorescence represent DAPI and GFP, respectively (bar, 50 μm).

(E and F) Relative expression of MYB70 (E) and ABI5 (F) in dry and germinating Col-0 seeds at different stages. The expression level of MYB70 and ABI5 in the dry Col-0 seeds was set to 1. Results shown are means ± SD (n = 3, more than 120 seeds/genotype/repeat). Different letters show significantly different values at p < 0.05 according to a Tukey’s test.
with proteasome inhibitor MG132 delayed ABI5 degradation in both Col-0 and OX70 seeds, indicating that ABI5 degradation was 26S proteasome-dependent (Figure 3H). Taken together, these results indicated that the higher abundance of ABI5 in OX70 was resulted from the reduced degradation of ABI5 due to the function of MYB70.

**MYB70 modulates root system architecture**

We analyzed the role of MYB70 in regulating RSA. As shown in Figures 4A and 4B, the PRs of the OX70 lines (e.g. OX70-3, OX70-4 and OX70-5) were shorter than those of Col-0, while the PRs of myb70 mutant and

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Figure 3. Interaction between MYB70 and ABI5

(A and B) Relative expression of the EM1 and EM6 genes in the myb70 abi5 double mutant and other related mutants and transgenic lines. The expression levels of the EM1 and EM6 genes in Col-0 were set to 1. Results shown are means ± SD (n = 3).

(C) ABA contents in myb70 mutant and MYB70-overexpressing OX70 transgenic plants. Results shown are means ± SD (n = 3, more than 260 seeds/genotype/repeat).

(D and E) Transient dual-LUC reporter assays indicated activation of EM1 and EM6 gene expression by MYB70, ABI5 or their combination in the presence or absence of 5 μM ABA. 62SK, ABI5 and MYB70 represent the empty pGreenII 62-SK, pGreenII 62-SK-ABI5 and pGreenII 62-SK-MYB70 vectors, respectively. EM1 and EM6 represent pGreenII 0800-pEM1::LUC and pGreenII 0800-pEM6::LUC, respectively. Results shown are means ± SD (n = 3). Different letters show significantly different values at p < 0.05 according to a Tukey’s test.

(F–H) MYB70 stabilized ABI5 protein. Seeds of Col-0 and OX70 plants were treated with 5 μM ABA in white light for 3 days and then harvested at 0, 8 and 12 h after the removal of ABA by either being washed out with liquid medium (F), after treatment with the protein synthesis inhibitor CHX (100 μM) (G), or after the proteasome inhibitor MG132 (50 μM) (H). RPN6, loading control. The experiments were repeated three times with similar results.
Col-0 plants showed no obvious phenotypic differences. To investigate the role of MYB70 in PR growth in more detail, we measured the length of the meristematic zone (MZ) and the elongation zone (EZ), MZ cell number and the length of six consecutive cells in the transition zone (G), total lateral root primordium (LRP) number (H), LRP density (I) and LRP number (J) were measured. Results are means ± standard error (SEM) (n = 3, more than 20 plants/genotype/repeat). Different letters show significantly different values at p<0.05 according to a Tukey's test.

Col-0 plants showed no obvious phenotypic differences. To investigate the role of MYB70 in PR growth in more detail, we measured the length of the meristematic zone (MZ) and the elongation zone (EZ), MZ cell number and the length of six consecutive cells in the transition zone. Both the MZ and EZ were significantly shorter (Figures 4C–4F), and the cells in the transition zone were more slowly elongated (Figure 4G) in OX70 transgenic plants than those in the Col-0 plants and the myb70-1 and myb70-2 mutants. To further explore the role of MYB70 on LR growth and development, we investigated the LRP initiation. The total number of LRPs was reduced; however, the LRP density was unaffected by overexpression of MYB70 (Figures 4H and 4I). Investigation of the four stages of LRPs indicated that overexpression of MYB70 reduced the number of LRPs in stages III and IV (Figure 4J). Taken together, these data indicated that MYB70 regulates RSA by affecting both PR growth and LR formation, perhaps in a negative manner.

**MYB70 plays a role in root system development by altering the auxin response by increasing the conjugated IAA level**

Next, we were interested in whether the reduced PR growth and LR formation observed in the OX70 lines occurred in an auxin-dependent manner. To address this question, we first investigated the effects of exogenous IAA on the root growth of the OX70, myb70 and Col-0 plants. Exogenous IAA inhibited PR growth and induced LR formation; however, overexpression of MYB70 resulted in reduced sensitivity to IAA, especially at high IAA concentrations (Figures 5A–5D). Specifically, in the presence of 75 nM IAA, the decrease
rates of PR length of OX70-3, OX70-4 and OX70-5 seedlings were 12.9%, 8.8% and 9.7%, respectively, lower than that of Col-0 (Figure 5B), while the increase rates of LR density of OX70-3, OX70-4 and OX70-5 seedlings were 69.5%, 53.5% and 57.5%, respectively, lesser than that of Col-0 (Figure 5D). Taken together, these data indicated that MYB70, at least in part, altered root growth by repressing the responsiveness to auxin.

To elucidate whether and how MYB70 affects auxin signaling, OX70 and myb70 lines were crossed with DR5:GUS and DR5:GFP reporter plants, respectively. Compared with the DR5:GFP and myb70/DR5:GFP seedlings, the OX70/DR5:GFP seedlings presented remarkably decreased GFP fluorescence in the root tips when grown in 1/2-strength MS medium (Figure 5E). Similarly, compared with the DR5:GUS and myb70/DR5:GUS seedlings, the OX70/DR5:GUS seedlings showed clearly decreased GUS activities in the root tips of PRs, LRs and cotyledons when they were grown in 1/2-strength MS medium (Figure 5F).

To verify these observations, we compared the free and conjugated IAA concentrations in the OX70 and Col-0 seedlings. There was no significant difference in the free IAA concentration between the OX70 and Col-0 plants; however, the OX70 plants showed 69.4% higher conjugated IAA concentration relative to that of the Col-0 plants (Figure 5G). These results collectively suggested that MYB70 affects auxin signaling by converting active IAA into inactive conjugated IAA.

Figure 5. Overexpression of MYB70 modulates root system growth by reducing the auxin response by increasing the conjugated IAA level. (A–D) Five-day-old Col-0, myb70 mutant and MYB70-overexpressing OX70 seedlings germinated on 1/2-strength MS medium were transplanted onto fresh medium supplemented with 0, 25, 50 or 75 nM indole-3-acetic acid (IAA) for 5 days, after which the primary root (PR) length (A), relative PR length (B), lateral root (LR) number (C) and LR density (D) were measured. Results are means ± standard error (SEM) (n = 3, more than 20 seeds/genotype/repeat). (E) Expression of DR5:GFP in the root tips of Col-0, myb70 mutant and OX70 seedlings (bar, 50 µm). (F) Expression of DR5:GUS in the PRs (bar, 50 µm), LRs (bar, 50 µm) and shoots (bar, 500 µm) of Col-0, myb70 mutant and OX70 seedlings. (G) Free and conjugated indole-3-acetic acid (IAA) levels in Col-0 and OX70 seedlings. Results are means ± standard error (SEM) (n = 3, more than 200 seeds/genotype/repeat). Different letters show significantly different values at p < 0.05 according to a Tukey’s test, and the asterisks show significant differences from the control (Student’s t-test, p < 0.05).
Comparative transcriptome analysis revealed molecular functions of MYB70 during root system development

To gain molecular insights into how MYB70 modulates RSA, we carried out a transcriptome analysis of OX70, myb70, and Col-0 plants using RNA-sequencing (RNA-seq) with three biological repeats. 974 DEGs were identified from the OX70/WT comparison (621 upregulated and 353 downregulated genes), while 237 DEGs were found from the myb70/WT comparison (208 upregulated and 29 downregulated genes) (log2 fold change ≥ 1 or ≤ –1; q ≤ 0.001) (Figure S3; Data S1 and S2).

We then performed a Gene Ontology (GO) enrichment analysis to investigate the molecular mechanisms underlying MYB70-mediated growth and development. ‘Peroxidase activity’, ‘Oxidoreductase activity, acting on peroxide as acceptor’ and ‘Antioxidant activity’ were among the significantly TOP20 enriched pathways of OX70-downregulated genes (Figure S4A). We then performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis according to the DEG results, OX70-downregulated 17%, 27%, and 4% of DEGs were enriched in ‘Phenylpropanoid biosynthesis’, ‘Biosynthesis of secondary metabolites’ and ‘cutin, suberin, and wax biosynthesis’, respectively (Figure S4B). These results suggested that MYB70 may modulate the ROS metabolic process and suberin biosynthesis.

MYB70 activates the auxin conjugation process by directly upregulating the expression of GH3 genes during root system development

The above results indicated that overexpression of MYB70 increased the levels of conjugated IAA (Figure 5G), and upregulated the expression of several auxin-responsive genes, including GH3.3 and GH3.5, in the OX70 compared with Col-0 plants (Figure 5J). GH3 genes encode IAA-conjugating enzymes that inactivate IAA (Park et al., 2007). MYB70 expression was markedly induced by ABA and slightly induced by IAA (Figure 1C); thus, we examined the effects of ABA and IAA on the expression of GH3 genes in OX70, myb70, and Col-0 plants. Exogenous ABA or IAA induced the expression of GH3.1, GH3.3, and GH3.5 both in roots and whole seedlings, with higher expression levels being observed in OX70 than Col-0 and myb70 plants (Figures 6A–6F, and 6A). These results indicated that MYB70-mediated auxin signaling was, at least in part, integrated into the ABA signaling pathway and that GH3 genes were involved in this process.

To investigate whether MYB70 could directly regulate the transcription of GH3 genes, we selected GH3.3, which can modulate root system development by increasing inactive conjugated IAA levels (Gutierrez et al., 2012), as a representative gene for a yeast-one-hybrid (Y1H) assay to examine the binding of MYB70 to its promoter, and found that MYB70 could bind to the tested promoter region (Figure S7). We then performed an electrophoretic mobility shift assay (EMSA) to test for possible physical interaction between MYB70 and the promoter sequence. Two R2R3-MYB TF-binding motifs, the MYB core sequence ‘YNGTTR’ and the AC element ‘ACCWAMY’, have been discovered in the promoter regions of MYB target genes (Kelemen et al., 2015). Analysis of the promoter of GH3.3 revealed several MYB-binding sites harboring AC element and MYB core sequences. We chose a 34-bp region containing two adjacent MYB core sequences (TAGTTTAGTTA) in the approximately −1,534- to −1501-bp upstream of the starting codon in the promoter region. EMSA revealed that MYB70 interacted with the fragment, but the interaction was prevented when unlabeled cold probe was added, indicating the specificity of the interaction (Figure 6G). To further confirm these results, we performed chromatin immunoprecipitation (ChIP)-qPCR against the GH3.3 gene using the 35S:MYB70-GFP transgenic plants. The transgenic plants showed an altered phenotype (different PR length and LR numbers), which was similar to that of the OX70 lines, demonstrating that the MYB70-GFP fusion protein retained its biological function (Figure S8). We subsequently designed three pairs of primers that contained the MYB core sequences for the ChIP-qPCR assays. As shown in Figure 6H, significant enrichment of MYB70-GFP-bound DNA fragments was observed in the three regions of the promoter of GH3.3.

To further confirm that MYB70 transcriptionally activated the expression of GH3.3, we cotransfected the 35S:MYB70 (pGreen II 62-SK-MYB70) plasmid along with the GH3.3-LUC (pGreen II 0800-promoterGH3.3-Luciferase) reporter construct. Cotransfection of MYB70 increased GH3.3 expression, especially under IAA treatment (Figure 6I), supporting the results of transcriptome and qRT-PCR analyses and indicated that MYB70 directly binds to the promoter of GH3.3 gene and upregulates its transcription. These results collectively suggested MYB70 modulates root system development by directly activating the auxin conjugation process through upregulating the expression of GH3 genes.
MYB70 modulates the ROS status in the roots by repressing the expression of PER genes independently of the UPB1 pathway

ROS play important roles in modulating root system development. The balance between O$_2$\textsuperscript{=} and H$_2$O$_2$ in root tips controls PR growth and differentiation independently of the auxin signaling pathway (Tsukagoshi et al., 2010). Our transcriptome, qRT-PCR and GO enrichment analyses revealed that MYB70 downregulated the expression of a set of PER genes and modulated the ROS metabolic process in the OX70 plants (Figures S4 A, S5 and S6). Several studies have demonstrated that overexpression of PER34 or PER57 resulted in longer PRs in overexpressor than in wild-type plants, whereas per33 per34 double mutant lines presented shorter PRs than wild-type control (Passardi et al., 2005; Tsukagoshi et al., 2010). We next

Figure 6. MYB70 positively regulates the expression of GH3.1, GH3.3 and GH3.5

(A–F) Relative expression of the GH3 genes in the roots of five-day-old Col-0, myb70 mutant and MYB70-overexpressing OX70 seedlings germinated on 1/2-strength MS medium and then transplanted to fresh medium supplemented with or without 10 μM abscisic acid (ABA) (A, B, C) or 10 μM indole-3-acetic acid (IAA) (D, E, F) for 24 h. Results shown are means ± SD (n = 3, more than 50 seedlings/genotype/repeat).

(G) EMSA detects the specific MYB70 binding to the GH3.3 promoter region harboring MYB70-binding sites.

(H) ChIP-qPCR assay of the MYB70-DNA complexes. The schematic of the primer design for the ChIP-qPCR of the GH3.3 promoter is shown at the top of the panel. The blue boxes on the black line represent the potential MYB70-binding sites, and the red lines mark sequences amplified by ChIP-qPCR. The promoter fragment enrichment assay following ChIP-qPCR was performed in the absence (IgG) or presence (anti-GFP) of anti-GFP antibody. Results shown are means ± SD (n = 3), and asterisks show significant differences from the control (IgG) (Student’s t-test, p < 0.05).

(I) Transient dual-luciferase reporter assays indicate that MYB70 transcriptionally activated GH3.3 expression without or with 5 μM ABA or 0.5 μM IAA. Results shown are means ± SD (n = 9). Different letters show significantly different values at p < 0.05 according to a Tukey’s test. 62SK represents empty pGreenII 62-SK vector. 62SK-MYB70 represents the pGreenII 62-SK-MYB70 vector. pGH3.3-LUC represents pGreenII 0800-pGH3.3-LUC vector.
measured ROS levels in OX70, myb70, and Col-0 root tips. Overexpression of MYB70 reduced \( \text{O}_2^- \) accumulation, especially in the root MZ, as indicated by the \( \text{O}_2^- \)-specific fluorescence probe dihydroethidium (DHE) (Figure 7A), whereas it increased \( \text{H}_2\text{O}_2 \) accumulation, especially in the EZ, as indicated by the \( \text{H}_2\text{O}_2 \)-specific fluorescence probe BES-\( \text{H}_2\text{O}_2 \)-AC (Figure 7B). Similarly, the diaminobenzene (DAB) staining (Figure S9) also showed that OX70 plants accumulated higher levels of \( \text{H}_2\text{O}_2 \) in the root tips as compared with myb70 mutants and Col-0 plants.

Figure 7. Overexpression of MYB70 modulates \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) balance in root tips by repressing the expression of PER genes

(A and B) Detection of endogenous \( \text{O}_2^- \) production (A) and \( \text{H}_2\text{O}_2 \) production in the root tips of five-day-old Col-0, myb70 mutant and OX70 seedlings (bar, 50 \( \mu \)m).

(C) Relative gene expression of the PER7, PER8, PER11, PER34 and PER57 genes in the roots of five-day-old Col-0, myb70 and OX70 seedlings. Results shown are means ± SD (n = 3, more than 50 seedlings/genotype/repeat).

(D) Specific binding of MYB70 to the PER57 promoter region harboring MYB70-binding sites.

(E) ChIP-qPCR assay of the MYB70-DNA complexes. The blue boxes on the black line represent the potential MYB70-binding sites in the PER57 promoter, and the red lines mark the sequences amplified by ChIP-qPCR. The promoter fragment enrichment assay following ChIP-qPCR was performed in the absence (IgG) or presence (anti-GFP) of anti-GFP antibody. Results shown are means ± SD (n = 3), and asterisks show significant differences from the control (IgG) (Student’s t-test, p< 0.05).

(F) Transient dual-luciferase reporter assay shows repression of PER57 expression by MYB70. Results shown are means ± SD (n = 9). 62SK, 62SK-MYB70 and pGreenII 62-SK-MYB70 represent empty pGreenII 62-SK, pGreenII 62-SK-MYB70 and pGreenII 0800-pPER57-LUC, respectively.

(G) Detection of the transcriptional repression activity of MYB70. Transcriptional activity assays in tobacco leaves (expressed in luciferase luminescence intensities) cotransfected with a pGreenII 0800-pUAS-35Smin-LUC reporter construct and one of the effector constructs fused with GAL4BD (schematic representation). The transcriptional activator VP16 was used as a positive control: MYB70-N (1–627), MYB70-C (628 to end, containing EAR motif); EAR (EAR motif-containing region 628 to 673); MYB70-C (ΔEAR) (MYB70-C without EAR motif, 673 to end). Results shown are means ± SD (n = 9). Asterisks show significant differences from the control (Student’s t-test, p< 0.05). Different letters show significantly different values at p< 0.05 according to a Tukey’s test.
We then evaluated the expression of five PER genes in both roots and whole seedlings. As shown in Figures 7C and 56B, compared with the Col-0 plants, the OX70 plants presented lower expression of these genes. Because overexpression of PER57 resulted in PR elongation (Passardi et al., 2005; Tsukagoshi et al., 2010), we selected PER57 as a representative gene to further test the direct relationship between MYB70 action and PER gene expression. First, we showed that MYB70 could directly bind to the promoter of PER57 in a Y1H assay (Figure S10). Second, EMSA showed that MYB70 interacted with a 28-bp fragment that contained two MYB core sequences (CAACTAAT and TTGTTA) in the approximately 667- to 639-bp upstream of the starting codon in the PER57 promoter region (Figure 7D). Third, ChIP-qPCR assay against PER57 involving 35S:MYB70-GFP transgenic plants confirmed the significant enrichment of MYB70-GFP-bound DNA fragments in the three regions of the promoter of PER57 that contain 1–3 MYB core sequences (Figure 7E).

The above results indicated that MYB70 can directly bind to the PER57 promoter, and the transcriptome and qRT-PCR results showed that OX70 plants presented lower expression of PER57 than the Col-0 plants did (Figures 7C–7E, S5, and S6). This finding is interesting as it suggests that MYB70 might also possess the transcriptional repression activity that could be attributed to the presence of a putative transcriptional repression motif, namely the ethylene-responsive element binding factor-associated amphiphilic repression (EAR) motif ‘LXLSL’ (Kagale et al., 2010; Persak and Pitzschke, 2014). To verify this hypothesis, we cotransfected the 35S:MYB70 plasmid and the PER57-LUC (pGreen II 0800-promoterPER57-Luciferase) reporter construct together in an LUC assay. Results of cotransfection shown in Figure 7F suggested that MYB70 indeed was able to repress the expression of PER57 gene. These results collectively indicated that MYB70-mediated downregulation of the expression of PER genes resulted in increased $\text{H}_2\text{O}_2/\text{O}_2^{\cdot-}$ ratio, and suggested that MYB70 is a key player in modulating ROS status in plants.

Next, a dual-luciferase reporter system with positive control VP16 was used to further confirm the transcriptional repression activity of MYB70 (Figure 7G). The VP16 exhibited high luciferase (LUC) activity, whereas VP16-MYB70 fusion protein presented much lower activity than VP16 (Figure 7G, Left). Moreover, both the full-length MYB70 and the truncated MYB70-C (628 to end, containing EAR motif) constructs displayed significantly lower LUC activities than the negative control (GAL4BD), while the EAR motif-containing region (628–673) also showed similar inhibitory effect to that of the full-length MYB70 and the truncated MYB70-C constructs (Figure 7G, Right). In contrast, MYB70-N (1–627) showed no significant difference in LUC activity when compared with GAL4BD control (Figure 7G). We found that the truncated MYB70-C without the EAR motif (673 to end) also showed significant inhibitory effect on the pGAL4-LUC expression, implying that other inhibitory region(s) existed in this region. Collectively, these results demonstrated that MYB70 acted as a transcriptional repressor and the EAR motif was required for the transcriptional repression activity.

The bHLH TF-encoding UPB1 plays a critical role in modulating the balance between differentiation and proliferation in the roots by regulating ROS equilibrium (Tsukagoshi et al., 2010). The above results (Figures 7A, 7B and S9) showed that the phenotype resulted from MYB70 overexpression was similar to that of UPB1-overexpressing plants (Tsukagoshi et al., 2010). Our RNA-seq, qRT-PCR, and Y2H analyses revealed that MYB70-mediated PER gene expression occurred independently of UPB1 (Figure S11; Data S1 and S2). Together, these results suggested that MYB70-mediated root growth via repression of PER genes is independent of the UPB1 pathway.

**MYB70 negatively regulates suberin biosynthesis in roots**

Another finding from the transcriptome analysis of particular interest was that the expression of several genes involved in suberin biosynthesis, such as GPAT5, GPAT7, CYP86A1, CYP86B1, CYP86B2, and FACT, and the expression of the cuticular wax biosynthesis-related gene WSD7 were downregulated in the OX70 plants (Figure S5), as also evidenced by the results of qRT-PCR analysis (Figures 8A and S6C). In comparison with the Col-0 plants, the OX70 plants presented lower expression levels of these six genes (Figures 8A and S6C), suggesting a negative feedback regulation of suberin and/or cuticular wax biosynthesis by MYB70.

To investigate whether MYB70 could directly regulate the expression of suberin biosynthesis-related gene(s), we performed the following assays with a representative gene, the GPAT5, whose loss-of-function presented decreased suberin deposition in their young roots and seed coats (Beisson et al., 2007). First, we
found that MYB70 bound to its promoter using a Y1H assay (Figure S12). Second, EMSA subsequently revealed that MYB70 interacted with a 32-bp fragment that contained two adjacent MYB core sequences (TAGTTTTGTTA) in the approximately 1,320- to 1,309-bp upstream of the starting codon in the promoter region of the GPAT5 (Figure 8B). Third, the physical interaction was also confirmed by the ChIP-qPCR assay against GPAT5 using the 35S:MYB70-GFP transgenic plants. As shown in Figure 8C, significant enrichment of MYB70-GFP-bound DNA fragments was detected in the two regions of the GPAT5 promoter, each of which contains 2 MYB core sequences. Finally, we examined the transcriptional repression activity of MYB70 using the dual-luciferase reporter system. As shown in Figure 8D, cotransfection of 35S:MYB70 with the reporter construct repressed LUC activity of pGreenII 0800-promoterGPAT5-LUC.

Since gpat5 loss-of-function mutants presented decreased suberin deposition in their young roots and seed coats (Beisson et al., 2007), cyp86A1 mutants showed reduced suberin composition in their roots (Höfer et al., 2008), and cyp86B1 mutants displayed a novel change in composition of suberin monomers (Compagnon et al., 2009). We, thus, suspected that compared with Col-0, OX70 might have a lower suberin deposition which could affect the growth and development of OX70, because suberin is a lipid-phenolic biopolyester that is present in cell walls and modulates root growth, water and ion uptake by the roots (Compagnon et al., 2009; Tylova et al., 2017). To test this hypothesis, we first investigated suberin deposition in the roots of the OX70, myb70, and Col-0 plants. Using the suberin histochemical lipophilic dye Sudan black B (Beisson et al., 2007), we found that compared with the myb70 and Col-0 roots, the OX70 roots presented less staining intensity (Figure 9A). The root suberization was then confirmed using fluorol yellow (FY) staining (Naseer et al., 2012). A striking reduction in suberization was observed in the OX70 roots.
To confirm these results, we then investigated whether a disruption of root suberization affected the uptake and transport of the fluorescent tracer fluorescein diacetate (FDA). After application of FDA, fluorescence was detected only slightly in the roots of the Col-0 and myb70 seedlings, whereas FDA accumulation was much higher in the roots of the OX70 seedlings (Figure 9C). These results suggested that MYB70 increased the uptake ability by repressing root suberization. To address this phenomenon, we next investigated the macronutrient and micronutrient contents in the roots and shoots of OX70, myb70, and Col-0. The myb70 mutant did not exhibit any significant changes in the contents of the measured elements in either roots or leaves (Figure S13). However, in the OX70 plants, the contents of manganese (Mn), iron (Fe), and copper (Cu) significantly increased in the roots (Figure S13A), and the contents of potassium (K) and Mn significantly increased in the leaves (Figure S13B), while the leaf Cu level significantly decreased (Figure S13B).

To further confirm that MYB70 affected root suberization, we detected suberin chemical composition in roots of OX70, myb70, and Col-0 plants using gas chromatography flame ionization detection (GC-FID). There were no significant differences in the contents of the total aliphatic suberin monomer between myb70 and Col-0 roots; however, the total aliphatic suberin monomer was 60.7% lower in OX70 roots than in Col-0 roots. This difference was due to a general decrease in almost all major suberin monomer constituents, including the significant decreases in C16:0, C20:0, C22:0, and C24:0 acids, C16:0, C18:1, C18:0, C20:0, C22:0, and C24:0 ω-OH acids, C16:0, C18:2, C18:1, C20:0, and C22:0 dioic acids, and C18:0 and...
C22:0 1-alcohols (Figure 9D). These results together indicated that the overexpression of MYB70 reduced suberin deposition in roots of the OX70 plants.

DISCUSSION

Elucidation of the crosstalk and balance among signaling molecules, like ABA, auxin, and ROS as well as their interactions in modulating plant growth and stress tolerance, has always been an important topic in botanical studies (Zhou et al., 2018). Here, analysis of the phenotypes of OX70 and myb70 plants at different developmental stages revealed various roles of MYB70 in responses to phytohormone signaling and developmental processes. In germinating seeds of various combinations of MYB70 and ABI5 overexpressor or mutant plants, the interaction of ABA-induced MYB70 and ABI5 improved ABI5’s ability to transcriptionally regulate its target genes by increasing ABI5 protein stabilization, thereby modulating seed germination in response to ABA. Furthermore, the underlying mechanisms involved direct regulation of the expression of GH3.3, PER57, and GPAT5 by MYB70’s dual transcriptional regulatory activities, which in turn modulate auxin signaling, ROS balance, and suberization in the roots, thereby affecting growth and development of the root system.

MYB70 negatively regulates seed germination in response to ABA by interacting with ABI5

Phenotypic analyses revealed that MYB70 negatively regulated seed germination in response to ABA (Figure 1). Moreover, ABA levels in OX70 and myb70 plants were unaltered (Figure 3C), suggesting that MYB70 modulates seed germination by regulating ABA signaling but not by affecting ABA biosynthesis. We thus searched for MYB70-interacting proteins or transcriptional regulators, particularly those participated in ABA-mediated regulation of seed germination, and identified ABI5. ABI5 acts as a central TF that is involved in ABA-mediated seed germination (Zhao et al., 2018; Zhou et al., 2015). Many studies have revealed ABI5-mediated signaling and regulatory mechanisms of ABI5-interacting proteins (Ju et al., 2019; Reeves et al., 2011). In recent years, with the continual discovery of ABI5-interacting proteins and the elucidation of their functions (Chang et al., 2019; Reeves et al., 2011; Zhao et al., 2018; Zhou et al., 2015), understanding of the molecular basis underlying the ABI5-mediated ABA transcriptional regulatory network has continually improved. In the present study, using Y2H, in vitro, Co-IP and BiFC assays, we identified the ABA-inducible R2R3 MYB TF MYB70 as a new ABI5-interacting protein (Figure 2). Subsequently, genetic analysis revealed that MYB70 additively regulated seed germination in response to ABA together with ABI5 (Figures 1 and 3). Results of the qRT-PCR and cotransfection assays indicated that MYB70 interacts with ABI5, resulting in improved ABI5’s ability to upregulate the expression of its target genes, EM1 and EM6 (Figures 3A, 3B, 3D and 3E). Furthermore, immunoblotting analysis showed that MYB70 increases ABI5 stabilization after the removal of ABA from germinating Arabidopsis seeds. Taken together, these data indicated that the interaction between MYB70 and ABI5 increases ABI5 protein stabilization; and thus helps in modulating ABI5-regulated seed germination in response to ABA signaling.

ABA-inducible MYB70 integrates auxin signaling to modulate root system development

The expression patterns of the members of R2R3 MYB subgroup S22, including MYB44, MYB70, MYB73, and MYB77, in response to ABA are similar in the roots (Figures 1C and 1D) (Persak and Pitzschke, 2014). Similar to those occurred in MYB44-overexpressing (OX44) (Jung et al., 2008) and MYB77-overexpressing (OX77) Arabidopsis plants (Shin et al., 2007), the PRs were shorter in OX70 plants than in Col-0 plants, while the knockout mutants (myb70, myb44 and myb77) exhibited no obvious phenotypic differences (Figures 4A and 4B) (Jung et al., 2008; Shin et al., 2007). Furthermore, in most of the assays, we observed that the phenotypic effects on the roots of myb70 plants were weak (Figure 4), suggesting that functional redundancy of R2R3 MYB subgroup S22 TFs occurs in the modulation of root growth and development (Lashbrooke et al., 2016).

Interestingly, we found that in contrast to OX77 plants that showed an increased auxin response, as indicated by the GUS staining of OX77/DR5:GUS plants (Shin et al., 2007), both the GUS staining of OX70/DR5:GUS plants and the GFP fluorescence of OX70/DR5:GFP plants showed decreased intensities of these two markers (Figures 5E and 5F). We thus examined free IAA levels and found that overexpression of MYB70 did not affect the free IAA levels in the OX70 plants (Figure 5G). However, our detailed examination indicated that overexpression of MYB70 increased the conjugated IAA levels in the OX70 plants (Figure 5G), suggesting that MYB70 may play a role in maintaining auxin homeostasis, and thus auxin signaling in plants. Subsequent transcriptome and qRT-PCR analyses revealed that MYB70 upregulated the expression
of several ABA-inducible GH3 genes, including GH3.1, GH3.3, and GH3.5 (Figures 6A–6F). Further analyses using Y1H, EMSA, and ChIP-qPCR assays indicated that MYB70 upregulated GH3.3 transcription by directly binding to its promoter (Figures 6G, 6H and S7), which was supported by a transcriptional activity assay using dual-luciferase reporter system (Figure 6I). The ABA-inducible GH3 genes encode IAA-conjugating enzymes whose activities result in IAA inactivation (Park et al., 2007). Growth of the root systems of GH3-overexpressing plants, such as GH3.5 OX plants, was shown to be reduced (Park et al., 2007; Seo et al., 2009), which is similar to the phenotype of OX70 plants (Figure 4). In support of our results, overexpression of the ABA-inducible MYB96 modulated RSA by upregulating the expression of GH3.3 and GH3.5 genes, and as a consequence increasing the conjugated IAA levels; however, it did not alter the free IAA levels in transgenic Arabidopsis OX96 plants (Seo et al., 2009). The stable levels of free IAA in OX70, OX77, and OX96 plants suggested a rigorous control of auxin homeostasis in plants to regulate root growth (Park et al., 2007; Seo et al., 2009).

In addition to PR growth, overexpression of MYB70 also markedly reduced LR formation, especially LR elongation, as indicated by the reduced number of LRP in stages III and IV (Figure 4J). These results support the hypothesis that MYB70 integrates ABA and auxin signaling to modulate root system growth and development through a negative feedback regulation of auxin homeostasis by upregulating ABA-inducible GH3 gene expression, and also indicate that there exist functional differences between MYB70 and MYB77 in modulating the auxin signaling pathway.

Involvement of MYB70 in modulating the $\text{H}_2\text{O}_2/\text{O}_2^{\cdot -}$ ratio in the root tips and subsequent root system development

Modulation of PER activities and ROS levels affects stem cell fate and the balance between differentiation and proliferation in plants (Tsukagoshi et al., 2010). Our transcriptome and qRT-PCR analyses indicated that MYB70 represses the expression of a set of PER genes (Figures 7C and S6B). Furthermore, Y1H, EMSA, and ChIP-qPCR analyses subsequently revealed that MYB70 could directly bind to the PER57 promoter, as a representative example, suggesting that PER genes are downstream target of MYB70 (Figures 7D, 7E and S10). Moreover, the transcriptional activity analysis revealed that MYB70 acts as a transcriptional repressor (Figure 7G), downregulating the expression of PER57 (Figure 7F). This result along with that described above for the transcriptional activity assay of the GH3.3 gene indicate that MYB70 has dual transcriptional activities, and can act as both activator and repressor to regulate the expression of its downstream genes. However, when the activation function and when the repression function act, this required further investigations. The dual functions of TFs in activation or repression of different target genes through direct physical interaction is an interesting phenomenon that has been reported previously, such as for AB14. AB14 modulates seed dormancy by directly repressing the transcription of ARR6, ARR7, and ARR15 (Huang et al., 2017), and reducing ABA degradation through direct repression of the expression of CYP707A1 and CYP707A2, while promoting GA degradation through direct activation of GA2ox7 expression (Shu et al., 2016a, 2016b). In addition, AB14 also modulates flowering by directly activating Flowering Locus C (FLC) expression (Shu et al., 2016b), while it modulates ROS levels by directly repressing Vitamin C Defective 2 (VTC2) expression in Arabidopsis (Yu et al., 2019). Results of this study, at least, suggest that both the activation and repression functions of MYB70 were activated in parallel for regulation of PR growth of Arabidopsis seedlings through the auxin and ROS signaling pathways (Figures 6 and 7). In addition, considering that MYB70 is a transcriptional repressor with a repression activity of EAR motif (Figure 7G), a co-activator might be required along with MYB70 to activate the expression of GH3 genes. This co-activator should also be able to overcome the repression activity of MYB70. It will then be interesting to discover detailed molecular mechanisms for the dual activities of MYB70 in regulation of plant growth and development in a spatiotemporal manner.

PERs regulate ROS status in two opposite ways, namely reduction of $\text{H}_2\text{O}_2$ by transferring electrons to donor molecules and formation of $\text{O}_2^{\cdot -}$ by catalyzing the hydroxyllic cycle (Passardi et al., 2005; Pitzschke et al., 2006; Tsukagoshi et al., 2010). In OX70 plants, repression of PER gene expression led to decreased $\text{O}_2^{\cdot -}$ and increased $\text{H}_2\text{O}_2$ accumulation in the roots, especially in the EZ (Figures 7A, 7B and S9). Although the phenotype of the PRs of OX70 was similar to that of 3SS:UPB1 (UPB1OX), our results revealed that the repression of PER gene expression by MYB70 occurred independently of UPB1 (Figure S11). These findings showed that various pathways are involved in the regulation of $\text{H}_2\text{O}_2/\text{O}_2^{\cdot -}$ ratio to maintain apical meristem activity in the root tips, and MYB70 pathway regulates ROS status at least independently of the UPB1 pathway.
In addition to modulating cell proliferation and differentiation, PER-mediated ROS status also plays a role in the modification of cell wall structure and initiation of cell expansion, thereby regulating root growth (Passardi et al., 2005; Tsukagoshi et al., 2010). Our transcriptome analysis revealed that in addition to PER genes, MYB70 also repressed the transcription of many other genes participated in modifying cell wall structure, such as Laccases (LACs), Xyloglucan Endotransglucosylase/Hydrolases (XTHs) and Extensins (EXTs) (Figure S5). Both PERs and LACs play roles in monolignol unit oxidation, which is responsible for the final step of lignin assembly, thereby modulating cell size and number (Passardi et al., 2004). In support of these data, OX70 plants showed reduced MZ cell number, the length of MZ and EZ, and the cell length in transition zones (Figures 4C–4G). Taken together, these results indicated that MYB70, at least in part, downregulated the expression of PER genes and cell wall structure-related genes, thereby altering root system growth.

**MYB70 and root suberization**

As a component of the plant secondary cell wall, suberin is deposited between the plasma membrane and primary cell wall to form lamellae (Barberon et al., 2016; Compagnon et al., 2009; Yadav et al., 2014). In the roots, suberin is deposited on the endodermal surface and Casparian strip to form an extracellular diffusion barrier to modulate water and ion transport (Andersen et al., 2015; Barberon et al., 2016). Therefore, modulation of suberin deposition in the roots alters root cell structure and physiology (Enstone and Peterson, 2003). Roots have evolved the capability to modulate their suberization to adapt to environmental cues (Yadav et al., 2014). Our results provided evidence that the reduced endodermal suberization in OX70 roots increased root uptake and transport abilities (Figures 9 and S13). Detection of nutrient contents revealed an increased abundance of Mn, Fe, and Cu in the roots (Figure S13A) and an increased abundance of K and Mn in the leaves of OX70 plants (Figure S13B), suggesting that the decreased suberin deposition in OX70 roots improved the uptake of these macronutrients and micronutrients by the roots. The result of ionomic profiling was also supported by the sharp increase in FDA fluorescent tracer in OX70 roots (Figure 9C). However, although overexpression of MYB70 increased the Cu content in the roots (Figure S13A), it decreased the Cu content in the leaves (Figure S13B), indicating reduced Cu transport from the roots to the leaves by the function of MYB70. These findings were also consistent with those published by previous reports that showed that disrupted endodermal suberization differentially affected element uptake and transport ability which, therefore, should be interesting for further elucidation (Barberon et al., 2016; Cohen et al., 2020).

Previous studies have demonstrated a correlation between ABA and suberization in plants (Barberon et al., 2016; Yadav et al., 2014). We showed here that ABA-induced MYB70 reduced suberin deposition in the roots by repressing the expression of genes involved in suberin biosynthesis (Figures 8 and 9). Further studies using Y1H, EMSA, and ChIP-qPCR analyses confirmed that MYB70 directly bound to the promoter of the suberin biosynthesis-related GPAT5 gene (Figures 8B, 8C and S12). In addition, the transcriptional activity assay using dual-luciferase reporter system revealed that MYB70 downregulated GPAT5 via its transcriptional repression activity (Figure 8D). Taken together, these results indicated that MYB70 acts as a negative regulator of suberization in an ABA-dependent manner.

Suberin biosynthesis follows a PER-mediated oxidative coupling process (Bernards et al., 2004; Passardi et al., 2004). Therefore, the altered expression of the PER genes by MYB70 also influences the modulation of suberin biosynthesis. Overall, MYB70 reduced suberization by downregulating the expression of genes encoding PERs and suberin biosynthesis-related genes, thereby altering root growth.

In summary, our findings revealed various functions of MYB70 in the modulation of plant growth and development. In seeds, MYB70 was shown to interact with ABI5 both in vitro and in vivo, and the interaction increased ABI5 stabilization; and thus, improved ABI5’s ability to transcriptionally modulate its target genes. In the roots, ABA-induced MYB70 modulated auxin signaling by increasing the content of conjugated IAA. In addition, MYB70 negatively regulated the expression of PER genes and suberin biosynthesis-related genes, thereby modulating ROS balance and decreasing suberization in the roots, which ultimately affected RSA (Figure 10). Our results might also open an avenue for creating new crop varieties with improved water and nutrient use efficiency through genetic engineering of MYB genes, which are suitable for sustainable agriculture in an era of climate change.

**Limitations of the study**

In seed germination assay, myb70 seeds displayed decreased sensitivity, while OX70 seeds showed increased sensitivity in response to exogenous ABA. However, in root system growth, the phenotypic
The interaction of ABA-induced MYB70 and ABI5 improves ABI5’s ability to transcriptionally regulate its target genes by increasing ABI5 protein stabilization, thereby inhibiting seed germination in response to ABA. Moreover, ABA-induced MYB70 represses auxin signaling by increasing inactive IAA levels through the direct upregulation of GH3.3 expression via interaction with an unknown transcriptional activator. MYB70 modulates $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ balance in the root tips via its transcriptional repression of $\text{PER57}$ gene expression. Increased MYB70 expression reduces root suberization by directly downregulating $\text{GPAT5}$ expression via its transcriptional repression activity, thereby ultimately inhibiting PR growth.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead contact
  - Materials availability
  - Data and code availability
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Arabidopsis thaliana
  - Bacterial strains
- **METHOD DETAILS**
  - Phenotypic analysis
  - Plasmid construction and transformation
  - Seed germination analysis
  - qRT-PCR and transcriptome analyses
  - *In vitro* pull-down, Y1H, and Y2H assays
  - Co-IP analysis
  - ChIP assays
  - BiFC assay
  - Immunoblotting analysis
Dual-luciferase (LUC) reporter assays
Quantification of endogenous ABA and IAA level
Fluorescence microscopy
Penetration assay
Histological analysis
Root suberin chemical composition analysis
Metal element content analysis
Accession numbers

QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.103228.

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AUTHOR CONTRIBUTIONS
J.X. designed and supervised the research. J.W. performed most experiments. R.W. performed Y1H and Y2H experiments. P.Z. and L.S. characterized the phenotypes and constructed vectors. S.L. and H.D. performed chemical composition analysis. Q.J. performed planting. J.W., L.-S.T., and J.X. analyzed the data and wrote the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Anti-HA tag antibody | Abcam  | CAT#AB13834; RRID:AB_443010 |
| Anti-Myc tag antibody| Abcam  | CAT#AB172; RRID:AB_302595 |
| Anti-His tag antibody | Abcam | CAT#AB14923; RRID:AB_443105 |
| Anti-GST tag antibody | Abcam  | CAT#AB19256; RRID:AB_444809 |
| Anti-ABI5 antibody | Abcam | CAT#AB98831; RRID:AB_10670685 |
| Anti-GFP tag antibody | Abcam | CAT#AB13970; RRID:AB_300798 |
| Anti-RPN6 antibody | Enzo  | CAT#BMI-PW8370; RRID:AB_10541615 |
| HRP-Goat anti-Rabbit IgG (H+L) secondary antibody | Thermo | CAT#31460; RRID:AB_228341 |
| **Bacterial and virus strains** |        |            |
| Agrobacterium tumefaciens GV3101 | Zhang et al., 2019a | N/A |
| Rosetta (DE3) chemically competent cells | Zhang et al., 2019a | CAT#J43270 |
| **Biological samples** |        |            |
| Y2HGold chemically competent cells | Clontech | N/A |
| Yeast strain AH109 | Clontech | N/A |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| Sudan black B | BBI | CAT#A602008 |
| Fluorescein diacetate | BBI | CAT#A600202 |
| 3,3'-diaminobenzidine | BBI | CAT#A690009 |
| Fluorol Yellow 088 | MKBio | CAT#MX4473 |
| BES-H$_2$O$_2$-Ac | WAKO | CAT#028-17811 |
| Isopropyl-β-D-thiogalactopyranoside | WAKO | CAT#090-05141 |
| Ni-NTA agarose | Qiagen | CAT#30210 |
| Dihydroethidium | Beyotime | CAT#50063 |
| 2-(4-Aminophenyl)-6-indolecarbamidine dihydrochloride | Beyotime | CAT#C1005 |
| Propidium iodide | Beyotime | CAT#ST51 |
| 3-indoleacetic acid | Sigma-Aldrich | CAT#I2886 |
| Abscisic acid | Sigma-Aldrich | CAT#90769 |
| MG132, a proteasome inhibitor | Sigma-Aldrich | CAT#M7449 |
| Cycloheximide, a protein synthesis inhibitor | Sigma-Aldrich | CAT#C7698 |
| AtMYB70-His | This study | N/A |
| GST-ABI5 | This study | N/A |
| AtMYB70-HA | This study | N/A |
| AtABI5-HA | This study | N/A |
| **Critical commercial assays** |        |            |
| Glutathione HiCap Matrix | Qiagen | CAT#30900 |
| Dual-Luciferase® Reporter Assay System | Promega | CAT#E1910 |
| RNAiso Plus Kit | Takara | CAT#9109 |
| PrimeScript™ RT reagent Kit with gDNA Eraser | Takara | CAT#RR047A |

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Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| UltraSYBR Mixture   | CWBIO  | CAT#CW0957M|
| Matchmaker® Gold Yeast One-Hybrid Library Screening System | Clontech | CAT#630491 |
| Matchmaker® Gold Yeast Two-Hybrid System | Clontech | CAT#630489 |
| Clarity Western ECL Substrate | Bio-Rad | CAT#1705060 |
| SurePAGE™, Bis-Tris, 10x8, 4-20% | Genscript | CAT#M00656 |
| Tris-MOPS-SDS Running Buffer Powder | Genscript | CAT#M00138 |
| Tanon™ High-sig ECL Western Blotting Substrate | Tanon | CAT#180-501 |
| Developer and Fixer Kit for Black and White Film and Paper | Beyotime | CAT#P0019 |
| SDS Lysis Buffer | Beyotime | CAT#P0013G |
| LightShift Chemiluminescent EMSA Kit | Beyotime | CAT#GS009 |

**Deposited data**

Raw RNA sequencing data

Experimental models: organisms/strains

| Arabidopsis: myb70-1 | NASC | SALK_208583 |
| Arabidopsis: myb70-2 | NASC | GK_350C10 |
| Arabidopsis: ab15-8 | Zheng et al., 2012 | SALK_013163 |
| Arabidopsis: ABI5OX | Bu et al., 2009 | Transgenic Col-0 |
| Arabidopsis: DR5:GUS | Ulmasov et al., 1997 | Transgenic Col-0 |
| Arabidopsis: DR5:GFP | Friml et al., 2003 | Transgenic Col-0 |
| Arabidopsis: upb1 | Tsukagoshi et al., 2010 | SALK_115536 |
| Arabidopsis: 35S:UPB1-3YFP | Tsukagoshi et al., 2010 | Transgenic Col-0 |
| Arabidopsis: MYB70OX-1 | This study | Transgenic Col-0 |
| Arabidopsis: MYB70OX-2 | This study | Transgenic Col-0 |
| Arabidopsis: MYB70OX-3 | This study | Transgenic Col-0 |
| Arabidopsis: MYB70OX-4 | This study | Transgenic Col-0 |
| Arabidopsis: MYB70OX-5 | This study | Transgenic Col-0 |
| Arabidopsis: MYB70OX-6 | This study | Transgenic Col-0 |
| Arabidopsis: proMYB70:GUS | This study | Transgenic Col-0 |
| Arabidopsis: proMYB70:MYB70-GFP | This study | Transgenic Col-0 |
| Arabidopsis: myb70-1/DR5:GFP | This study | Cross between DR5:GFP and myb70-1 |
| Arabidopsis: MYB70OX-3/DR5:GFP | This study | Cross between DR5:GFP and MYB70OX-3 |
| Arabidopsis: myb70-1/DR5:GUS | This study | Cross between DR5:GUS and myb70-1 |
| Arabidopsis: MYB70OX-3/DR5:GUS | This study | Cross between DR5:GUS and MYB70OX-3 |
| Arabidopsis: myb70-2/ab15-8 | This study | Cross between myb70-2 and ab15-8 |
| Arabidopsis: myb70-2/ABI5OX | This study | Cross between myb70-2 and ABI5OX |
| Arabidopsis: MYB70OX-3/ab15-8 | This study | Cross between MYB70OX3 and ab15-8 |
| Arabidopsis: MYB70OX-3/ABI5OX | This study | Cross between MYB70OX3 and ABI5OX |
| Tobacco: Nicotiana benthamiana | This study | N/A |

Oligonucleotides

| Primers used are shown in Table S1 | This study | N/A |

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RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Jin Xu (xujin@sxau.edu.cn; xujinsxau@163.com).

Materials availability
All materials generated in this study are available from the lead contact without restriction. This study did not generate new unique reagents.

Data and code availability
Raw RNA sequencing data derived from A. thaliana samples have been deposited in the Short Read Archive database of NCBI, and accession number PRJNA5086033 is listed in the key resources table.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.
EXPERIMENTAL MODEL AND SUBJECT DETAILS

Arabidopsis thaliana

Plants of the Columbia-0 (Col-0) wild-type genetic background and mutants were used in this study. Mutant myb70-1 (SALK_208583) and myb70-2 (GK_350C10) seeds were obtained from the European Arabidopsis Stock Centre (NASC). The upb1 and 35S::UPB1-3YFP (Tsukagoshi et al., 2010) mutant was kindly donated by Professor Philip N. Benfey (Duke University). The previously described T-DNA insertion abi5 mutant designated abi5-8 (SALK_013163) was used (Zheng et al., 2012). myb70/abi5-8, MYB70OX/abi5-8, myb70/ABI5-0X, MYB70OX/ABI5OX, myb70-1/DR5:GUS, MYB70OX-3/DR5:GUS, myb70-1/DR5:GFP, and MYB70OX-3/DR5:GFP double mutants were acquired by crossing myb70 or MYB70OX3 with abi5-8 or ABI5OX (Bu et al., 2009) lines, myb70-1 or MYB70OX-3 with DR5:GUS (Ulmasov et al., 1997) or DR5:GFP (Frimer et al., 2003) lines, respectively, and confirmed by PCR.

Arabidopsis seeds were surface-sterilized using 50% bleach (v/v), rinsed with sterile water five times, and sown on 1/2-strength MS medium comprising 1% sucrose (w/v) and 0.65% or 1% agar (w/v), with the pH adjusted to 5.75 via 1 M KOH or 3% HCl. The agar plates were stratified at 4°C for 2 days before being transferred to a growth chamber maintained at 22°C under a 16-h-light/8-h-dark photoperiod. Five-day-old seedlings were transplanted onto fresh agar medium that contained different chemicals for various indicated times.

Bacterial strains

Agrobacterium tumefaciens GV3101 was cultivated in Luria-Bertani (LB) medium supplemented with appropriate antibiotic in a shaker at 28°C; Rosetta (DE3) chemically competent cells was cultivated in LB medium supplemented with appropriate antibiotic in a shaker at 37°C.

METHOD DETAILS

Phenotypic analysis

After the seedlings were treated for the indicated time, images were taken by using an Epson Perfection V500 Photo scanner (Japan), after which the PR length, LR number and LR density were determined using ImageJ (v1.52a). With respect to LRP initiation, four stages were quantified (Zhang et al., 1999). For root MZ and EZ length analyses were determined (Casamitjana-Martinez et al., 2003; Yuan et al., 2013), the seedlings treated for the indicated times were mounted in 1 μg mL⁻¹ propidium iodide (PI) staining solution, after which images were taken by an LSM 710 confocal laser scanning microscope (Carl Zeiss, Germany). The lengths were subsequently determined using ImageJ.

Plasmid construction and transformation

The full-length MYB70 coding region was amplified from Arabidopsis cDNA, and MYB70 overexpression lines were subsequently generated by inserting MYB70 PCR products into a pCAMBIA1300 vector harboring the CaMV 35S promoter. To generate proMYB70:GUS constructs the 1.5-kb promoter sequence upstream of the ATG start codon of the MYB70 gene of Arabidopsis genomic DNA was amplified by gMYB70-forward (gMYB70-F) and gMYB70-reverse (gMYB70-R) primers and then inserted into a pCAMBIA1381 expression vector. With respect to the proMYB70:MYB70-GFP lines, the same primers were used to amplify the promoter containing a 1.5-kb sequence containing the MYB70 gene coding region from Arabidopsis genomic DNA, which was subsequently introduced into a pCAMBIA1302 vector. Through the Agrobacterium-mediated floral dip method, the above constructs were transformed into Col-0 to generate transgenic Arabidopsis materials.

Seed germination analysis

For seed germination rate analysis, sterilized seeds were stratified at 4°C for 2 days and then sown on 1/2-strength MS medium consisting of 0, 0.5 or 1 μM ABA. The numbers of seed radicle appearance were determined at indicated times.

qRT-PCR and transcriptome analyses

Col-0, myb70 mutant and OX70 seedlings grown in 1/2-strength MS medium for 5 days and then transplanted to fresh medium supplemented with 10 μM ABA or 10 μM IAA for 0, 12 and 24 h. Total RNA was isolated using RNAiso Plus (TaKaRa, Dalian, China). After the RNA concentration was determined, reverse
transcription was performed using a PrimeScript™ RT Reagent Kit in conjunction with gDNA Eraser (TaKaRa) and oligo (dT) 18 primers. qRT-PCR analyses were performed using 2× UltraSYBR mixture (CWBio, Beijing, China) on a LightCycler 480II platform (Roche, Switzerland). AC7IN8 was used as an internal control (Liu et al., 2016). A DNA melting curve with only one peak for the specific primers used was used in conjunction with qRT-PCR, and all tested genes were subjected to three technical repetitions and included three independent biological replications.

For transcriptome analyses, total RNA was extracted from 5-day-old Arabidopsis Col-0, myb70-2 mutant and MYB70X-3 seedlings using RNAiso Plus (TaKaRa), and cDNA was synthesized using a PrimeScript™ RT Reagent Kit in conjunction with gDNA Eraser (TaKaRa). The mRNA was purified by oligo (dT) magnetic bead adsorption and was fragmented randomly using fragmentation buffer. First- and second-stranded cDNAs were subsequently synthesized with 6-bp random hexamers as primers. The cDNA was then purified with AMPure XP beads, followed by end repair and the addition of an A tail. The cDNA was then linked to the sequencing adaptor and subjected to fragment size selection via AMPure XP beads. After PCR amplification and thermal denaturation of their products into single strand, a single-stranded circular DNA library was obtained by cycling the single-stranded DNA with a bridge primer. Comparative transcriptome analysis was performed using the BGISEQ-500 platform (BGI, Wuhan, China). The RNA sequencing data have been archived in the Short Read Archive database of the National Center for Biotechnology Information under the accession No. PRJNA506033. The RNA-seq data were trimmed using Trimmomatic (v0.36) program and mapped against A. thaliana annotated genes (TAIR10_Araport11) using Bowtie2 (v2.2.5). Gene expression levels were then calculated using RSEM (v1.2.8) program. DEGs were identified by a log2 fold change $\geq 1$ or $\leq -1$ and $q \leq 0.001$ thresholds using DESeq2 (Love et al., 2014).

In vitro pull-down, Y1H, and Y2H assays
In vitro pull-down assays, full-length coding DNA sequences (CDSs) of MYB70 and ABI5 were cloned into pET30a and pGEX4T-1 expression vectors, respectively. After induction by 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), the expression vector fused into Rosetta (DE3) chemically competent cells, and MYB70-His and GST-ABI5 proteins were expressed. Soluble GST or GST-ABI5 recombinant proteins were isolated and fixed by the glutathione HiCap matrix (Qiagen GmbH, Hilden, Germany). Using the fixed GST or GST-ABI5 proteins to incubate the MYB70-His protein, the interaction with anti-His antibodies (Sigma-Aldrich) was detected by western blot analysis. The details of the primers used are presented in Table S1.

Y1H assays were conducted using a Matchmaker Gold Yeast One-Hybrid Library Screening System (Clontech). The amplified promoter fragments of MYB70 target genes were fused into a pBait-AbAi vector to generate pBait-AbAi constructs. The constructs were then transferred into the yeast strain Y1HGold and subsequently selected. The coding regions of MYB70 were amplified from A. thaliana genomic DNA, ligated to the a pGADT7 prey vector to generate AD-MYB70, introduced into the strain Y1HGold pBait-AbAi, and subsequently selected on synthetic dropout (SD)/-Leu/-aureobasidin A (AbA) medium. The primers used are listed in Table S1.

The Y2H assays were conducted in accordance with the Matchmaker manufacturer’s protocol (Clontech, USA). The pGBK7T7 (BD) bait plasmid vector was used to introduce the full-length CDSs of ABI5 and UPB1, respectively, and a pGADT7 (AD) prey vector was used to clone the full-length CDS of MYB70. The constructs were then inserted into yeast strain Y2HG0ld cells. SD/-Leu/-His/-Ade/-Trp medium (Clontech, USA) supplemented with 40 μg mL⁻¹ 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-Gal) was used to select yeast transformants that were grown at 30°C for approximately 3 days, and afterward, the potential interactions between proteins were determined. The primers used are listed in Table S1.

Co-IP analysis
The full-length CDSs of MYB70 and ABI5 were amplified and then fused into tagging vectors with HA or Myc tags, respectively, harboring the CaMV 35S promoter. Next, transient expression mediated by agroinfiltration in N. benthamiana leaves was performed. The total protein extractions were used to immunoprecipitate with anti-HA antibodies, after which anti-Myc antibodies were used to coimmunoprecipitate ABI5-Myc. The specific primers used are presented in Table S1.
ChIP assays

For ChIP assays, approximately 2.5 g of proMYB70:MYB70-GFP seedlings were thoroughly rinsed with distilled water, after which they were flash frozen and finely ground with a mortar and pestle. Next, the chromatin DNA of the samples was isolated and subjected to ultrasonic interruptions, after which it was incubated in ChIP dilution buffer consisting of chromatin solution and protein G-agarose beads (Upstate Biotechnology, Lake Placid, NY). Anti-GFP (ab290, Abcam, Cambridge, United Kingdom) was used to immunoprecipitate the protein-DNA complex by incubating and gently rotating the mixture at 4°C overnight. After the samples were eluted and DNA purification, qRT-PCR was then conducted for GFP-specific enrichment of the fragments from the promoters of the GH3.3, PER57 and GPAT5 genes. The primers used are listed in Table S1.

BiFC assay

A pCAMBIA1300 vector was used to clone the amplified full-length CDSs of MYB70 and ABI5 and to construct C-terminal in-frame recombinants with C-YFP and N-terminal in-frame recombinants with N173 YFP. The generated plasmids were introduced into A. tumefaciens strain GV3101 (pSoup-p19), which were then coinjected into N. benthamiana leaves for approximately 72 h. The fluorescence in epidermal cells stained with 4’,6-diamidino-2-phenylindole (DAPI) was examined via an LSM 710 microscope (Carl Zeiss, Germany). Three independent experiments were carried out, five plants were used, and more than ten leaves were infected for each independent experiment. The primers used are presented in Table S1.

Immunoblotting analysis

Seeds of wild-type Col-0 and OX70-3, which were grown in the same growth chamber and harvested at the same day, were sown on MS medium containing 5 μM ABA and cultivated in a growth chamber under continuous white light for 3 d. After being washed twice with liquid MS medium, the seeds were transferred to fresh liquid MS medium supplemented with or without 50 μM MG132 (Sigma-Aldrich) or 100 μM CHX (Sigma-Aldrich), and harvested at the indicated time points. Subsequently, total proteins were extracted from the samples using SDS lysis buffer (Beyotime) and accurately quantified. After mixing with 5 × SDS-loading buffer, the proteins were boiled for 10 min, isolated on a 10% SDS-PAGE gel, and subsequently transferred to polyvinylidene fluoride (PVDF) membrane and incubated in blocking buffer (20mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, 5% nonfat milk powder) for 1 h at room temperature, then with 1:2000 anti-ABI5 (Abcam) or 1:1,000 anti-RPN6 (Enzo) for 12 h at 4°C, and followed with HRP-conjugated goat anti-rabbit IgG (Thermo) for 1 h. Finally, the PVDF membrane was overlaid on high-signal ECL western blotting substrate (Tanon) for 5 min and imaged using a CHEMI DOC™XRS (Bio-rad).

Dual-luciferase (LUC) reporter assays

The full-length ABI5 and MYB70 coding regions were amplified and then transferred into a pGreen II 62-SK vector, after which pGreenII 0800-LUC double-reporter vector was fused to the promoter fragments of EM1 (2070 bp) and EM6 (1,364 bp). The above constructs were then transferred into A. tumefaciens strain GV3101 (pSoup-p19), and pro3SS:MYB70, pro3SS:ABI5, proEM1:LUC and proEM6:LUC recombinant strains were generated. The pro3SS:MYB70 and/or pro3SS:ABI5 with proEM1:LUC as well as the pro3SS:MYB70 and/or pro3SS:ABI5 with proEM6:LUC strains were subsequently coinfiltrated into N. benthamiana leaves and cultivated for 3 days in a growth chamber supplemented in the presence or absence of 5 μM ABA; proEM1:LUC or proEM6:LUC was used as an internal control. The promoters of GH3.3 (1,556 bp), PER57 (1,443 bp) and GPAT5 (1,559 bp) were amplified and cloned into pGreenII 0800-LUC. The constructs were transferred into A. tumefaciens strain GV3101 (pSoup-p19), and proGH3.3:LUC, proPER57:LUC and proGPAT5:LUC recombinant strains were generated. The pro3SS:MYB70 with proGH3.3:LUC or proPER57:LUC or proGPAT5:LUC were subsequently coinfiltrated into N. benthamiana leaves (TF-encoding gene fused to 3SS promoter/promoter-LUC fusions in pGreenII 0800-LUC ratio = 9/1, v/v) (Hellens et al., 2005) and cultivated for 72 h in a growth chamber supplemented in the presence or absence of 5 μM ABA or 0.5 μM IAA.

For the transcriptional activity analyses of MYB70, the full-length MYB70 coding region and the truncated MYB70 fragments, including the MYB70-C (628 to end, containing EAR motif), the EAR motif containing region (628–673), the MYB70-N (1–627) and the truncated MYB70-C without EAR motif (673 to end), were PCR amplified and were then fused to the GAL4 DNA binding domain (BD), driven by the 3SS promoter,
respectively. VP16 is a transcriptional activator that was used as a positive control and GAL4-BD was used as a negative control. These constructs were transferred into A. tumefaciens strain GV3101 (pSoup-p19), respectively, and then transiently expressed in N. benthamiana leaves.

After inoculation and a transient incubation of 72 h, a dual-LUC reporter assay system (Promega), which included firefly LUC and Renilla (REN) LUC, was used to measure the relative LUC activity. Two cm² leaf discs were sampled and fine-ground in 500 µL of Passive Lysis Buffer, and then 8 µL of rude extract were added to 40 µL of Luciferase Assay Buffer (Hellens et al., 2005). Finally, the activity of the promoter was determined by the LUC/REN value using a luminometer (Modulus™, Promega). Three independent biological experiments were conducted. The specific primers used are listed in Table S1.

**Electrophoretic mobility shift assay.** Electrophoretic mobility shift assay (EMSA) was conducted using a LightShift Chemiluminescent EMSA Kit (GS009, Beyotime, China) according to Zhang et al. (2019a). After induction by IPTG, MYB70-His and GST-ABI5 proteins were purified by Ni-NTA agarose (Qiagen) and a glutathione HiCap matrix, respectively. The wild-type and mutant probe sequences used are listed in Table S1.

**Quantification of endogenous ABA and IAA level**

Five-day-old Arabidopsis seedlings were used for the quantification of IAA and ABA. Approximately 0.5 g (fresh weight) samples were collected and frozen in liquid nitrogen. The frozen tissues were finely ground with a mortar and pestle and extracted using 80% acetonitrile, and the crude extracts were purified by a sequence of steps, including filtration and partitioning, followed by methylation by a diazomethane gas stream and resuspension in 100 mL of ethyl acetate. The endogenous ABA and IAA levels were ultimately detected separately by GC-MS (5975C-7890A, Agilent Technologies, USA).

**Fluorescence microscopy**

For GFP visualization, 5-day-old DR5:GFP, myb70b-1/DR5:GFP, MYB70OX/DR5:GFP and 35S:MYB70-GFP marker lines were subjected to confocal laser microscopy (LSM 710, Carl Zeiss, Germany) at an excitation wavelength of 488 nm and an emission wavelength of 509 nm.

**Penetration assay**

The apoplastic tracers PI and FDA were used to measure root uptake ability (Cohen et al., 2020). In brief, 9-day-old seedlings exhibiting three different phenotypes were incubated in 10 mg mL⁻¹ FDA solution for 30 s in the dark and subsequently counterstained with 50 mg mL⁻¹ PI solution. The FDA and PI fluorences were then immediately captured for 2–10 min at 4-min intervals using an LSM 710 confocal microscope (Carl Zeiss, Germany).

**Histological analysis**

For GUS staining analysis, proMYB70:GUS seeds and seedlings at different developmental stages, 9-day-old DR5:GUS, myb70b-1/DR5:GUS, and MYB70OX/DR5:GUS were used. The samples were immersed in GUS staining solution consisting of 1 mM 5-bromo-4-chloro-3-indolyl-b-D-GlcA cyclohexyl-ammonium (Sigma-Aldrich) for 1 h at 37°C. After the samples were washed and placed in 75% (w/v) ethanol, a Carl Zeiss imaging system (Axio Imager A2/HM525 NX UV) was used to collect images.

For in vivo ROS detection, the O₂⁻-specific probe DHE and the H₂O₂ probe BES-H₂O₂-Ac were used to examine O₂⁻ and H₂O₂, respectively. The seedlings were incubated in 10 µM DHE or 50 µM BES-H₂O₂-Ac for 1 h at 37°C in the dark, rinsed briefly in water, and then imaged using an LSM 710 instrument. To detect H₂O₂ using DAB staining, the seedlings were immersed in a 1 mg mL⁻¹ solution for 3 h, washed five times and then imaged.

To analyze the lipid polyesters in the roots, Sudan black staining was performed. The roots were incubated in a solution consisting of 1% (w/v) Sudan black in 75% ethanol for 1 h. After rinsed with water, the roots were imaged.

For suberin lamella analysis, FY staining was conducted. Suberin lamellae of 9-day-old Arabidopsis roots were examined. After staining with a solution consisting of 0.01% fluorescein yellow O88 (w/v, lactic acid) for
30 min at 70°C in the dark, the samples were gently rinsed with water and then imaged using an LSM 710 instrument.

**Root suberin chemical composition analysis**

The roots of 9-day-old seedlings grown 1/2-strength MS medium were harvested for suberin analysis. After a sequence of tissue delipidation and depolymerization, the samples were derivatized for free hydroxyl and carboxyl groups. Subsequently, 100 µL pyridine and 100 µL acetic anhydride were added to the methylation tube followed by cooling and evaporating under nitrogen gas. Finally, the samples were redissolved in 100 µL heptane:toluene (1:1 v:v) and the aliphatic suberin composition was determined using gas chromatography flame ionization detection (GC-FID).

**Metal element content analysis**

Nine-day-old seedlings were transplanted to 1/2-strength MS liquid medium for 3 weeks, at which point their roots and leaves were harvested. 1 mM EDTA solutions were used to chelate surface ions of the harvested tissues for 30 min, after which the samples were fixed in an oven at 105°C for 1 h and then oven-dried at 70°C until they reached a constant weight. Concentrated nitric acid was subsequently used to wet wash the samples for 72 h followed by boiling for 2 h. Finally, the following mineral elements were quantified by using inductively coupled plasma atomic emission spectroscopy (ICP-AES; iCAP6300, Thermo Fisher Scientific, Waltham, MA, USA): K, calcium (Ca), Fe, Mn, zinc (Zn) and Cu. Each experiment was repeated three times.

**Accession numbers**

The sequence data for genes analyzed in this study could be found in Arabidopsis Genome Initiative as followings: At2g36270 (ABI5), At1g49240 (ACTIN8), At3g51810 (EM1), At2g40170 (EM6), At5g63560 (FACT), At5g58860 (CYP86A1), At5g23190 (CYP86B1), At2g14960 (GH3.1), At2g23170 (GH3.3), At4g27260 (GH3.5), At3g11430 (GPAT5), At5g06090 (GPAT7), At2g23290 (MYB70), At1g05250 (PER2), At1g30870 (PER7), At1g34510 (PER8), At1g68850 (PER11), At2g35380 (PER20), At3g49120 (PER34), At5g14130 (PER55), At5g17820 (PER57), At5g47000 (PER65), At5g12420 (WSD7) and At2g47270 (UPB1).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

All the details of the statistics presented in this study are provided in the Method Details section. Student’s t test or one-way analysis of variance (ANOVA) followed by Tukey’s test were performed in IBM SPSS Statistics v20 software, together with asterisk symbols (*) and different lowercases were shown as significant difference at p< 0.05, respectively.