Abscisic acid positively regulates L-arabinose metabolism to inhibit seed germination through ABSCISIC ACID INSENSITIVE4-mediated transcriptional promotions of *MUR4* in *Arabidopsis thaliana*

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Summary

- L-Arabinose (L-Ara) is a major monosaccharide in plant polysaccharides and glycoproteins, and functions in plant growth and development. However, the potential role of L-Ara during abscisic acid (ABA)-mediated seed germination has been largely ignored. Here, our results showed a function of L-Ara during ABA-mediated seed germination.
- ABA slowed down the reduction of L-Ara in seed cell wall, and exogenous L-Ara aggravated the inhibition of ABA on germination. We further found that *MUR4*, encoding URIDINE 5′-DIPHOSPHATE-D-XYLOSE 4-EPIMERASE 1, played a vital role in ABA-mediated germination. *MUR4* was highly expressed in embryo and induced by ABA in both seeds and seedlings. Overexpression of *MUR4* conferred hypersensitive seed germination and early postgermination growth to ABA.
- Further analysis revealed that ABSCISIC ACID INSENSITIVE4 (ABI4) positively modulated the *MUR4* expression by directly binding the Coupling Element1 motif of *MUR4* promoter. Consistently, *abi4-1* mutant had a lower L-Ara content in seed cell wall, while a higher L-Ara content in seed cell wall was observed in *ABI4* overexpressors. Genetic analysis suggested that overexpression of *MUR4* in *abi4-1* partly restored the ABA sensitivity of *abi4-1*.
- We established the link between ABA and L-Ara during ABA-mediated seed germination and cotyledon greening in *Arabidopsis* and revealed the potential molecular mechanism.

Introduction

The monosaccharide L-arabinose (L-Ara) is a key component of various cell-wall polymers, including pectic polysaccharides rhamnogalacturonan-I and rhamnogalacturonan-II, hemicellulosic polysaccharides xylan and xyloglucan, glycoproteins such as arabinogalactan proteins (AGPs), and extensions (Peña *et al.*, 2008; Scheller & Ulvskov, 2010; Bar-Peled & O'Neill, 2011; Schultink *et al.*, 2013; Showalter & Basu, 2016). These L-Ara-containing polymers play important roles in plant growth and development (Jones *et al.*, 2003; Peña & Carpita, 2004; Rautengarten *et al.*, 2011; Kotake *et al.*, 2016). Rautengarten *et al.* (2011) found that the cell-wall L-Ara-deficient mutants exhibited severe development defects in *Arabidopsis*. Pectic arabinan, a complex set of cell-wall polysaccharides in which the 1,5-arabinan backbones can be variously branched at O-2 or O-3 by short side chains or arabinosyl residues, have been implicated in stomatal function, cell–cell linkages, and cell adhesion (Jones *et al.*, 2003; Peña & Carpita, 2004). Gomez *et al.* (2009) showed that arabinans accumulated in embryo cell wall and disappeared during germination and seedlings establishment. The L-Ara released was finally incorporated into the growing seedling, which suggested that arabinans might serve as storage polysaccharides for seed development and germination (Gomez *et al.*, 2009). However, the potential role of L-Ara metabolism during seed germination is still largely unknown.

L-Ara is incorporated into those cell-wall polymers through the action of arabinosyltransferase using uridine 5′-diphosphate (UDP)-L-Ara as the precursor (Feingold & Avigad, 1980). UDP-L-Ara can be synthesized through *de novo* and salvage pathways. In the *de novo* pathway, UDP-L-Ara is synthesized from UDP-D-xylose via the UDP-D-xylose 4-epimerase encoding UDP-D-XYLOSE 4-EPIMERASE 1 (UXE1/MUR4) (Burget *et al.*, 2003). An alternate route is the salvage pathway, in which UDP-L-Ara is synthesized from free L-Ara by the sequential action of L-arabinokinase and UDP-Ara pyrophosphorylase (Burget & Reiter, 1999; Burget *et al.*, 2003). The *Arabidopsis* mutant *murus4* (*mur4*) has reduced L-Ara contents in aerial organs (Burget *et al.*, 2003). The L-Ara deficiency in *mur4* can be rescued by the presence of

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exogenous L-Ara in the growth media, presumably through the salvage pathway (Burget & Reiter, 1999).

The plant hormone abscisic acid (ABA) plays essential roles in regulating seed germination (Bewley, 1997). Several ABA signaling components, such as PYRABACTIN RESISTANCE (PYR)/PYR1-LIKE/REGULATORY COMPONENT OF ABSCISIC ACID RECEPTOR, SNF1-related protein kinase 2s, and type 2C protein phosphatases, have been identified in Arabidopsis (Leung *et al.*, 1997; Finkelstein *et al.*, 1998; Lopez-Molina *et al.*, 2002; Weiner *et al.*, 2010). Besides that, ABSCISIC ACID INSENSITIVE3 (ABI3), -4 and -5 act as key transcription regulators of ABA-mediated seed germination and postgermination. ABI4, an AP2-type transcription factor, can directly regulate target genes via binding Coupling Element1 (CE1) *cis*-elements (CACCG and CCAC motif) in their promoters to modulate seed germination (Niu *et al.*, 2002; Shu *et al.*, 2013, 2016; Huang *et al.*, 2017). ABI4 directly represses the expression of *CYP707A1* and *CYP707A2* to increase ABA content, thus modulating the primary seed dormancy (Shu *et al.*, 2013, 2016). ABI4 is also involved in ABA and cytokinin signaling by repressing the expression of type-A Arabidopsis response regulators to inhibit seed germination (Huang *et al.*, 2017). Additionally, ABI4 negatively regulates lipid mobilization and represses seed germination in the presence of ABA (Penfield *et al.*, 2006). Together, these studies demonstrate that ABI4 can modulate seed germination via diverse signaling pathways.

In the present study, we found that the presence of ABA slowed down the reduction of the L-Ara content in seed cell wall, and exogenous L-Ara aggravated the inhibition caused by ABA during germination. Overexpression of *MUR4* conferred hypersensitive seed germination to exogenous ABA. Further analysis revealed that ABI4 was involved in modulating the L-Ara content by directly binding the promoter of *MUR4*. Overexpression of *MUR4* in *abi4-1* mutant partly restored the ABA sensitivity in an *abi4-1* mutant. Together, these data indicate that ABI4 increases the expression of *MUR4* to inhibit seed germination by accumulating L-Ara in seed cell wall.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana (L.) Heynh. ecotype Columbia (Col-0), Landsberg *erecta* (Ler-0), *abi1-1* (CS22), *abi2-1* (CS23), *abi3-1* (CS24), *abi4-1* (CS8104), *abi5-8* (SALK_013163C), *mur4-2* (CS8569) and *mur4-3* (CS8570) mutants were obtained from the Arabidopsis Biological Resource Center (ABRC).

Seeds were sterilized and sown on solid medium containing ½ Murashige and Skoog (½MS) salts including vitamins and 1% (w/v) sucrose at 4°C for 2 d, and then grown in a growth chamber (22°C, 100–200 μmol m^{-2} s^{-1}, 14 h : 10 h, light : dark, 60% humidity).

Generation of transgenic plants

The full-length *MUR4* and *ABI4* were amplified by PCR using the specific primers (Supporting Information Table S1) and cloned into the pEarleyGate 101 vector using the BP and LR Clonase reaction (Invitrogen). The recombinant plasmid was sequenced and introduced to Columbia (Col-0) by *Agrobacterium tumefaciens* strain GV3101-mediated transformation. Positive transformants were selected on ½MS medium containing 25 μg ml^{-1} *basta* (Sigma-Aldrich). The resistant T_2 seedlings with 3 : 1 segregation of resistance were transferred to soil to obtain homozygous T_3 seeds from individual lines. The *OX-MUR4#2/abi4-1* double mutant was generated by genetic cross of *OX-MUR4#2* and *abi4-1* mutant.

Seed germination assays and cotyledon greening assays

More than 100 seeds harvested at the same time were sown on the ½MS medium containing 1% sucrose with or without different concentrations of exogenous ABA or L-Ara as indicated. The plates were stratified at 4°C for 2 d and placed at 22°C under light condition. Germination was scored for 0–3 d, when the radicle had emerged from the testa. Green cotyledons percentage was also scored.

Real-time PCR analysis

Total RNA from plant materials except germinating seeds and dry seeds was extracted using the RNeasy Plant Mini Kit (Qiagen) kit. Total RNA from dry seeds and germinating seeds was extracted as described by Oñate-Sánchez & Vicente-Carbajosa (2008). RNA was first treated with DNase I (Qiagen), and first-stand complementary DNA (cDNA) synthesis was performed using the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocol. Real-time PCR was performed using SYBR Select Master Mix (Applied Biosystems, Foster City, CA, USA) on diluted (five times) cDNA using the StepOne Plus Real-Time PCR System (Applied Biosystem 7500). The primers are listed in Table S1. Expression levels for all candidate genes were determined using the $2^{-\Delta\Delta C_T}$ method as described previously (Livak & Schmittgen, 2001).

MUR4 promoter::GUS construct and β-glucuronidase activity

A 2000 bp promoter region of *MUR4* was amplified by PCR using the specific primers listed in Table S1. The PCR product was cloned into the pGWB3 using the BP and LR Clonase reaction (Invitrogen). The recombinant plasmid was sequenced and introduced to Columbia (Col-0) by *A. tumefaciens* strain GV3101-mediated transformation. Positive transformants were selected on ½MS medium containing 50 mg ml^{-1} hygromycin (Omega Scientific, Mogadore, OH, USA). Germinating seeds were dissected into embryo and endosperm surrounded with seed coat. For histochemical staining of β-glucuronidase (GUS) activity, samples were submerged in 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (X-Gluc) buffer (50 mM sodium phosphate buffer, pH 7.0, 1 mM EDTA, 0.5 mg ml^{-1} X-Gluc, 0.4% Triton X-100, 100 mg ml^{-1} chloramphenicol, and 5 mM each of potassium ferri-/ferrocyanide) and incubated at 37°C for 3 h in the dark, followed by washing with 70% ethanol to remove Chl.

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Determination of ABA content

Briefly, seeds (100 mg) were collected and ground to a fine powder in liquid nitrogen, followed by extraction with 5 ml 80% methanol solution containing 1% (v/v) acetic acid and 19% (v/v) pure water. After centrifugation, the supernatant was collected and used for determination by ultra-performance liquid chromatography (Agilent, Santa Clara, CA, USA) as described previously (Ma *et al.*, 2016).

Cell-wall extracts preparation and analysis of cell wall

For measurement of L-Ara, germinating seeds of each genotype were harvested into 96% ethanol and incubated for 30 min at 100°C to inactivate cell-wall-degrading enzymes. The seeds were homogenized using a Retsch mixer mill and centrifuged. The pellet was washed with 100% ethanol and twice with a mixture of chloroform and methanol (2 : 1), followed by four successive washes with 100% (v/v) ethanol and acetone. The pellet was air-dried overnight. The starch in the samples was degraded with α-amylase, amyloglucosidase, and pullulanase (Megazyme, Wicklow, Ireland) as described previously (Fang *et al.*, 2016). The de-starched residue was referred to as alcohol-insoluble residue (AIR). Dried AIR (2 mg) was hydrolyzed in 2 M trifluoroacetic acid at 121°C for 1 h, and analyzed by high-performance anion-exchange chromatography on an ICS-5000 instrument (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a CarboPac PA20 (3 mm × 150 mm, Thermo Fisher Scientific) analytical anion-exchange column, PA20 guard column (3 mm × 30 mm), borate trap, and a 500 pulsed amperometric detector.

Yeast one-hybrid assay

Yeast one-hybrid (Y1H) assay was performed following the manufacturer's instructions of Matchmaker Gold Yeast One-Hybrid Library Screening System kit and the Yeastmaker Transformation System 2 kit (Clontech, Mountain View, CA, USA). The *MUR4* promoter region −700 to −1200 (translation start is + 1) was amplified by PCR using the specific primers listed in Table S1 and inserted into the vector pAbAi. The construct was linearized by *Bst*BI digestion and transformed into Y1HGold strain to generate the bait strain. Then, the full-length coding sequence of *ABI4* was cloned into the pGADT7 AD vector using the specific primers listed in Table S1. The construct pGADT7 AD-ABI4 or empty vector pGADT7 AD was transformed into the Y1H bait strain and cultured on a synthetic dropout/-Leu/-Ura plate and also containing 1000 ng ml^{-1} aureobasidin A (AbA; Clontech).

Chromatin immunoprecipitation–quantitative PCR assay

The chromatin immunoprecipitation (ChIP) assay was performed as reported previously (Huang *et al.*, 2017). *35S:ABI4-GFP-HA* transgenic plants (*OX-ABI4#2*) and wild-type, anti-hemagglutinin (anti-HA) antibodies produced in mouse (Sigma-Aldrich), and Imprint® Chromatin Immunoprecipitation Kit (Sigma-Aldrich) were used for ChIP experiments following the manufacturer's instructions. The enrichment of DNA fragments was quantified by quantitative PCR using specific primers (Table S1). A fragment of the *ACTIN 2* coding region was used as a reference gene. Enriched values were normalized with the level of input DNA.

Results

L-Ara plays a vital role in ABA-mediated inhibition of seed germination

To investigate the potential role of cell-wall monosaccharide L-Ara on seed germination in response to ABA, we first measured the cell-wall L-Ara content during seed germination. As shown in Fig. 1a, L-Ara content in seed cell wall significantly decreased during germination, which was consistent with a previous report (Gomez *et al.*, 2009). We then examined the change of L-Ara content in seed cell wall in response to exogenous ABA treatment, and our results showed that the presence of exogenous ABA obviously slowed down the reduction of the L-Ara content (Fig. 1b).

Several *abi* mutants have been reported to affect the ABA sensitivity (Leung *et al.*, 1997; Finkelstein *et al.*, 1998; Lopez-Molina *et al.*, 2002). So we wondered which components of the ABA signaling pathway act in the accumulation of L-Ara in the seed cell wall. As shown in Fig. 1c, there was a significant reduction of L-Ara level in *abi2-1* and *abi4-1* mutants compared with those in the wild-type, which implied that ABI2 and ABI4 might function together in regulating L-Ara accumulation. However, no obvious reduction was observed in *abi1-1*, *abi3-1* and *abi5-8* mutant seeds (Fig. 1c). Taken together, these results suggest that L-Ara plays a vital role in ABA-mediated inhibition of seed germination. ABI2 and ABI4 appear to be involved in this process.

Since L-Ara can be directly used by plants through the salvage pathway, the L-Ara-feeding experiment was conducted to explore the function of exogenous L-Ara on the ABA-mediated inhibition of seed germination (Burget & Reiter, 1999; Li *et al.*, 2007; Dugard *et al.*, 2016). Wild-type (Col-0) seeds were sown on ½MS medium without ABA or supplemented with 0.5 μM ABA and various concentrations of L-Ara (0, 30 mM, and 60 mM), and the germination rate and cotyledon greening rate were calculated. In the absence of ABA, exogenous L-Ara had no obvious influence on the germination and cotyledon greening rate. When grown on ½MS medium supplemented with 0.5 μM ABA, the presence of exogenous L-Ara significantly delayed seed germination and cotyledon greening rate (Fig. 2a–c). Meanwhile, we measured the L-Ara content in seed cell wall from germinating seeds and found that there was an increase in L-Ara content in the presence of ABA and L-Ara (Fig. 2d). To investigate whether exogenous L-Ara regulates seed germination by altering ABA content, we analyzed the ABA content in seeds exposed to L-Ara treatment. We found that there was no significant change in ABA content (Fig. S1). Moreover, we analyzed the expression of several key genes in the ABA biosynthesis pathway, such as *AAO3*, *ABA2*, *ABA3* and *NCED3*, and key ABA catabolic enzymes, such as *CYP707A2* and *CYP707A3*. Our results showed that all these genes had similar expression levels in both the presence and absence of exogenous L-Ara (Fig. S2). These results suggest that L-Ara does not affect the ABA metabolic pathway.

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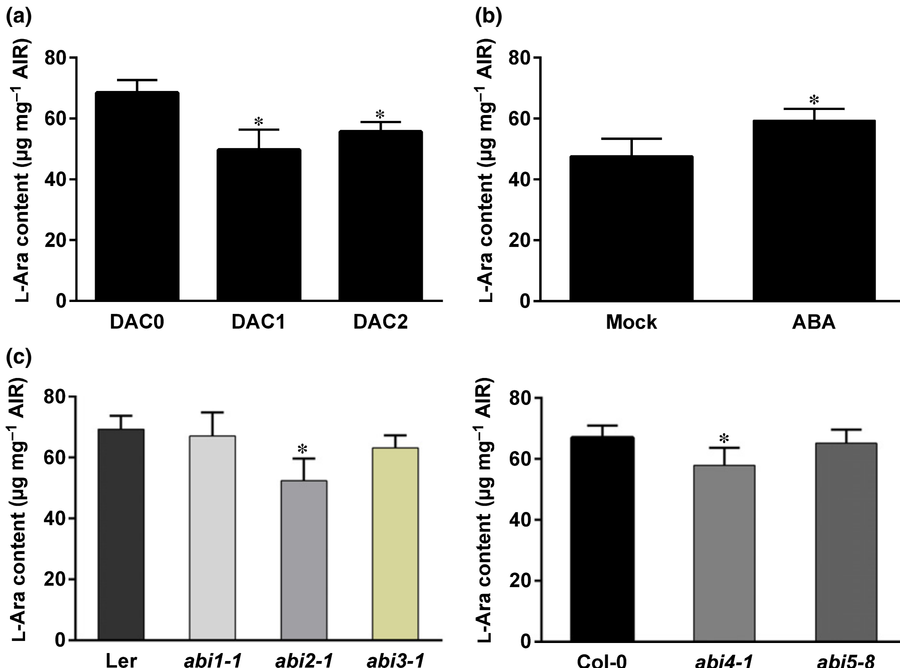

Fig. 1 L-Arabinose (L-Ara) plays a vital role in abscisic acid (ABA)-mediated inhibition of *Arabidopsis* seed germination. (a) L-Ara content in seed cell wall after germination for 0, 1 and 2 d. (b) L-Ara content in seed cell wall during seed germination in the presence of ABA. Seeds were germinated on ½ Murashige and Skoog medium supplemented with 0.5 μM ABA (ABA) or without ABA (Mock) for 1 d. DAC, days after cold stratification. (c) L-Ara content in seeds from wild-type and ABA-INSENSITIVE (*abi*) mutants. Seeds from different genotypes were imbibed at 4°C for 2 d, and alcohol-insoluble residue (AIR) was extracted and subjected to analysis for L-Ara content. Values show average ± SD ($n = 3$). The asterisk indicates a significant difference compared with the control using the unpaired Student's *t*-test (*, $P < 0.05$).

To further uncover the molecular networks of L-Ara in regulating ABA-mediated seed germination, we tested the expressions of several downstream ABA signaling pathway genes, such as *ABF4*, *EM1*, *EM6* and *RD29B*. Interestingly, our results showed that L-Ara positively regulated these genes only under the presence of ABA (Fig. S3). These results suggest that L-Ara plays a vital role in ABA-mediated inhibition of seed germination by modulating some downstream ABA signaling pathway genes.

MUR4 is highly expressed in the embryo

L-Ara can be synthesized *de novo* from UDP-D-xylose by the MUR4 through C-4 epimerization of UDP-xylose, and *mur4* mutant shows a 50% reduction in the L-Ara content in cotyledon (Burget *et al.*, 2003). We predicted that MUR4 might function in the ABA-mediated seed germination process, and the expression of *MUR4* should reflect this. The microarray results in the public domain (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) showed that *MUR4* transcripts accumulated at high levels specifically in dry seeds. Then, we extracted total RNA from various tissues and analyzed the expression level of *MUR4* using the real-time quantitative PCR assay. Our results demonstrated that *MUR4* was highly expressed in dry seeds and flowers (Fig. 3a), which is consistent with the microarray data. We also found that *MUR4* expression was reduced by 75% after stratification compared with that in dry seeds (Fig. 3b). To further analyze the expression of *MUR4* in the seed, we examined the activity of the GUS reporter fused to the *MUR4* promoter. The result showed that *MUR4* was expressed in the embryo only by dissection of seeds (Fig. 3c).

MUR4 is involved in ABA signaling

To investigate whether MUR4 is involved in ABA signaling, we first monitored the *MUR4* expression in response to ABA. In both seeds (Fig. 3d) and seedlings (Fig. S4), the transcript level of *MUR4* was highly induced by exogenous ABA. We then analyzed the expression of *MUR4* in wild-type and several *abi* mutants. As shown in Fig. 4a, the expression of *MUR4* in *abi4-1* mutant is much lower than that in wild-type (Col-0), but no difference was found in other *abi* mutants. These data suggest that MUR4 is involved in ABA signaling and may act downstream of ABI4.

ABI4 positively regulates *MUR4* expression in seeds

To explore the molecular link between MUR4 and ABI4, the expression of *ABI4* was also monitored. *ABI4* was highly expressed in dry seeds, and the expression level dropped significantly after germination (Fig. S5). Moreover, the expression of *ABI4* was also induced by ABA (Fig. S6). Thus, we analyzed the *MUR4* expression in *abi4-1* mutant in response to ABA. As shown in Fig. 4b, we observed that the ABA-induced *MUR4* expression was partly inhibited in *abi4-1* mutant. This clearly shows that ABI4 is a regulator of MUR4 in ABA signaling, and also implies that there are other regulators that function in this process.

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Fig. 2 Exogenous L-arabinose (L-Ara) delays abscisic acid (ABA)-mediated seed germination in *Arabidopsis*. (a) Seed germination rates of wild-type grown on ½ Murashige and Skoog (½MS) plates containing 0 (Mock) or 0.5 μM ABA (ABA) and different concentrations of exogenous L-Ara (0, 30 and 60 mM). (b) Photographs of seedlings grown on different media as indicated were taken after 8 d. (c) The percentages of green cotyledon grown on different media as indicated were calculated after 8 d. (d) L-Ara content in cell wall during seed germination in the presence of ABA and exogenous L-Ara. Seeds were germinated on ½MS medium containing no ABA (Mock) or 0.5 μM ABA (ABA) and different concentrations of exogenous L-Ara (0 and 60 mM) for 1 d, and alcohol-insoluble residue (AIR) was extracted and subjected to analysis for L-Ara content. Values show average ± SD ($n = 3$). The asterisks in (a), (c) and (d) indicate a significant difference compared with the control using the unpaired Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Next, we want to determine whether overexpression of *ABI4* affects the *MUR4* expression during germination. We generated *ABI4* transgenic plants overexpressing *ABI4-GFP-HA* driven by *35S* promoter. Two independent lines (*OX-ABI4#1* and *#2*) were confirmed by reverse transcription (RT)-PCR (Fig. S7). As expected, the expression level of *MUR4* was higher in the *ABI4* overexpressors than those in wild-type (Fig. 4c). The L-Ara content in *ABI4* overexpressors was also higher than in wild-type, which was consistent with the expression data (Fig. 4d). Together, these data suggest that ABI4 is a positive regulator of MUR4 in ABA-mediated inhibition of germination.

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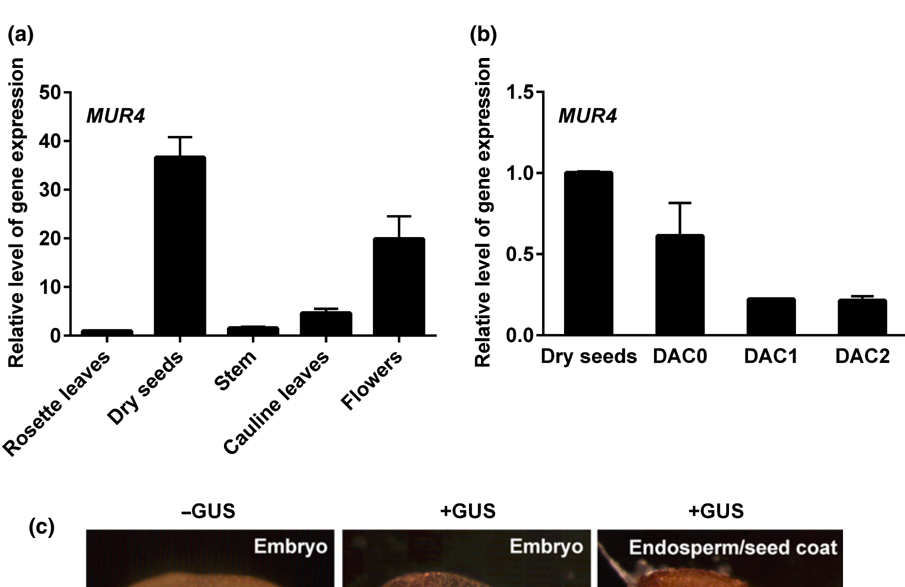

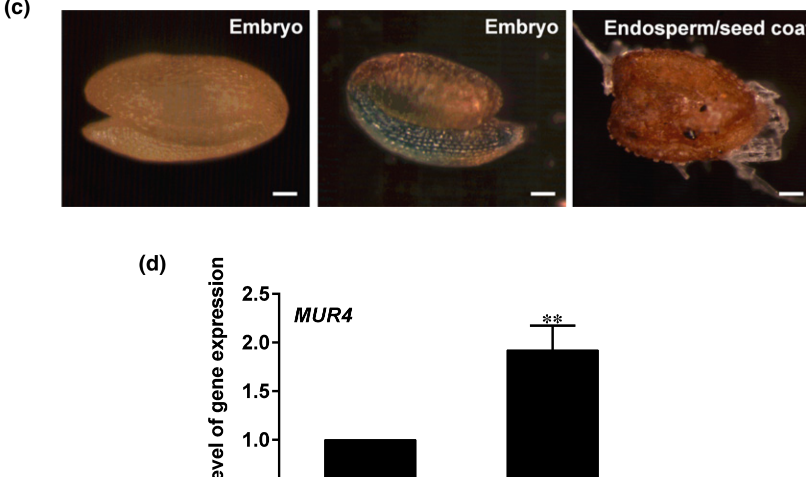

Fig. 3 Expression of *MUR4* in *Arabidopsis* seeds. (a) *MUR4* expression in rosette leaves, dry seeds, stem, cauline leaves, and flowers. (b) *MUR4* expression in dry seeds and germinating seeds. Wild-type seeds were stratified at 4°C for 2 d and then sown on ½ Murashige and Skoog (½MS) medium plates. Total RNA from different tissues was extracted and used to analyze the *MUR4* expression. DAC, days after cold stratification. (c) *MUR4 promoter::GUS* expression in embryo and endosperm/seed coat. Bars, 50 μm. (d) Induction of *MUR4* expression by exogenous abscisic acid (ABA) in seeds. Stratified seeds of wild-type were sown on ½MS medium containing 0.5 μM ABA and cultured for 1 d. *MUR4* expression was analyzed by real-time quantitative PCR. Values in (a), (b) and (d) show average ± SD ($n = 3$). Experiment in (c) was performed at least three times with similar results. The asterisks in (d) show a significant difference compared with the control using the unpaired Student's *t*-test (**, $P < 0.01$).

ABI4 directly binds to the *MUR4* promoter *in vivo* and *in vitro*

ABI4 regulates the expression of its target genes by recognizing CACCG (CE1 motif) elements (Yang *et al.*, 2011; Huang *et al.*, 2017). We searched putative ABI4 binding elements in the sequences 2000 bp upstream of the coding regions of the *MUR4* and identified two CE1 motifs in the promoter region of *MUR4* (Fig. 5a). To determine whether ABI4 directly binds to the promoter of *MUR4 in vivo*, a ChIP assay using *ABI4-GFP-HA* overexpressors was performed. The qRT-PCR assay showed that ABI4 protein could strongly bind to the P1 and P2, but not to the P3, which does not have a CE1 motif (Fig. 5b). This indicates that ABI4 directly binds to a specific CE1 motif of *MUR4* promoter *in vivo*.

To confirm this, a Y1H assay was performed. The interaction between ABI4 and a fragment (−700 to −1200) of *MUR4* promoter containing the CE1 motifs was tested by growing on medium lacking Leu and Ura and also supplemented with 1000 ng ml^{-1} AbA, which suppressed background activation. The assay shows that ABI4 can bind the promoter of *MUR4* in yeast (Fig. 5c). Together, these analyses suggest that ABI4 binds the promoter of *MUR4* both *in vivo* and *in vitro*.

Overexpression of *MUR4* enhances ABA sensitivity during seed germination

Our results showed that exogenous L-Ara delayed the ABA-mediated germination (Fig. 2) and that ABA significantly induced the *MUR4* expression (Figs 3d, S4), so we want to understand whether overexpression of *MUR4* enhances the ABA sensitivity. A previous study found that overexpression of *MUR4* increased the cell-wall L-Ara content in leaves, implying that MUR4 activity was rate limiting for the synthesis of arabinosylated glycans (Burget *et al.*, 2003). We generated *MUR4* transgenic plants overexpressing *MUR4-GFP-HA* driven by *35S* promoter. Two independent lines (*OX-MUR4#2* and *#4*) were confirmed by RT-PCR (Fig. 6a). The L-Ara content in seed cell wall from *MUR4* overexpressors was higher than those in wild-type (Fig. 6b). Wild-type (Col-0) and *MUR4* overexpressors' seeds

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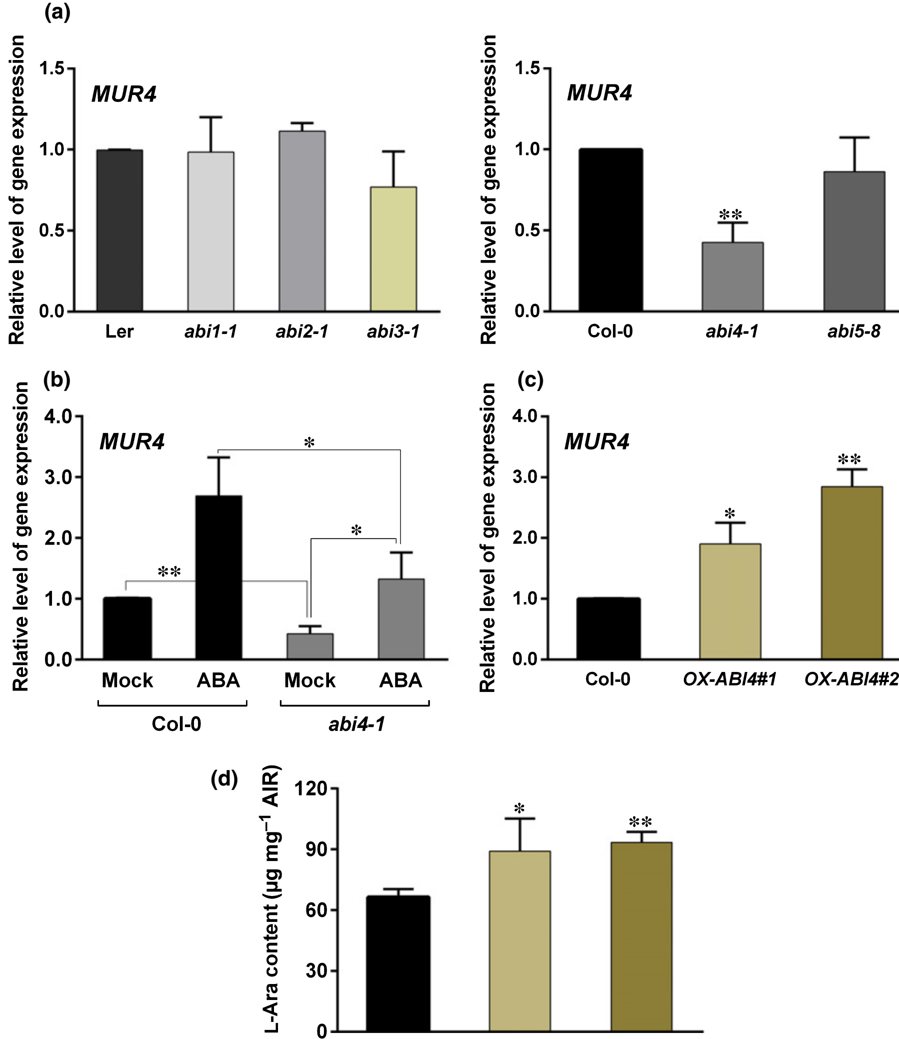

Fig. 4 ABSCISIC ACID INSENSITIVE4 (ABI4) positively regulates the *MUR4* expression in *Arabidopsis*. (a) Expression of *MUR4* in the seeds of wild-type and *abi* mutants. Col-0, Columbia; Ler, Landsberg *erecta*. (b) The expression of *MUR4* induced by abscisic acid (ABA) partly inhibited in *abi4-1* mutant. (c) *MUR4* expression in the seeds of wild-type and *ABI4* overexpressors. (d) L-Arabinose (L-Ara) content in seed cell wall from wild-type and *ABI4* overexpressors. Seeds from different genotypes in (a) and (c) were imbibed at 4°C for 2 d, and total RNA was extracted and subjected to real-time quantitative PCR assay. Stratified seeds of wild-type and *abi4-1* mutant in (b) were sown on ½ Murashige and Skoog medium containing 0.5 μM ABA and cultured for 1 d. Total RNA were extracted and then subjected to detect the *MUR4* expression. Alcohol-insoluble residue (AIR) was extracted from imbibed seeds in (d) and subjected to analysis for L-Ara content. Values show average ± SD ($n = 3$). The asterisks indicate a significant difference from the control using the unpaired Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$).

were sown on ½MS medium without ABA or supplemented with 0.5 μM ABA, and the germination rate and cotyledon greening rate were scored. We observed no obvious differences in germination and cotyledon greening rate between wild-type and *MUR4* overexpressors grown on ½MS medium (Fig. 6c–e). However, in the presence of exogenous ABA, *MUR4* overexpressors showed obviously delayed germination compared with wild-type (Fig. 6c). Meanwhile, the cotyledon greening rate of *MUR4* overexpressors was much lower than the wild-type after being grown on the ½MS medium containing 0.5 μM ABA (Fig. 6d, e). We also monitored the expressions of several downstream ABA signaling pathway genes, such as *ABF4*, *EM1*, *EM6* and *RD29B*, in the wild-type and *MUR4* overexpressor exposed to ABA treatment. The expression of these genes was induced by ABA in the wild-type, and this inducement by ABA was significantly upregulated in the *MUR4* overexpressor. However, in the absence of ABA, there was no significant difference in these genes' expressions between wild-type and *MUR4* overexpressor (Fig. S8). These results suggest that MUR4 acts as a positive regulator of ABA-mediated seed germination and postgermination growth.

mur4 mutants showed delayed germination in the absence or presence of exogenous ABA

A previous study showed that *mur4* mutants had a 50% reduction in the amount of cell wall L-Ara in cotyledons (Burget *et al.*, 2003). We monitored the ABA sensitivity of *mur4* mutants by using two *mur4* mutants. Surprisingly, *mur4* mutants showed delayed germination in the presence or absence of ABA (Fig. S9). This seems contradictory, but one possible reason may be that the L-Ara content in seed cell wall is too low to support the normal germination. To test this hypothesis, we scored the germination in the presence of exogenous ABA and L-Ara. We found that exogenous L-Ara could recover the slower germination in *mur4* mutants caused by low L-Ara level in seed cell wall (Fig. S10). Though exogenous L-Ara aggravated the delayed germination caused by ABA both in wild-type and *mur4* mutants, the germination rate of *mur4* in response to ABA was less inhibited by exogenous L-Ara treatment compared with those in wild-type (Fig. S10). Thus, it seems that there is a threshold of L-Ara content in seed cell wall that modulates the germination.

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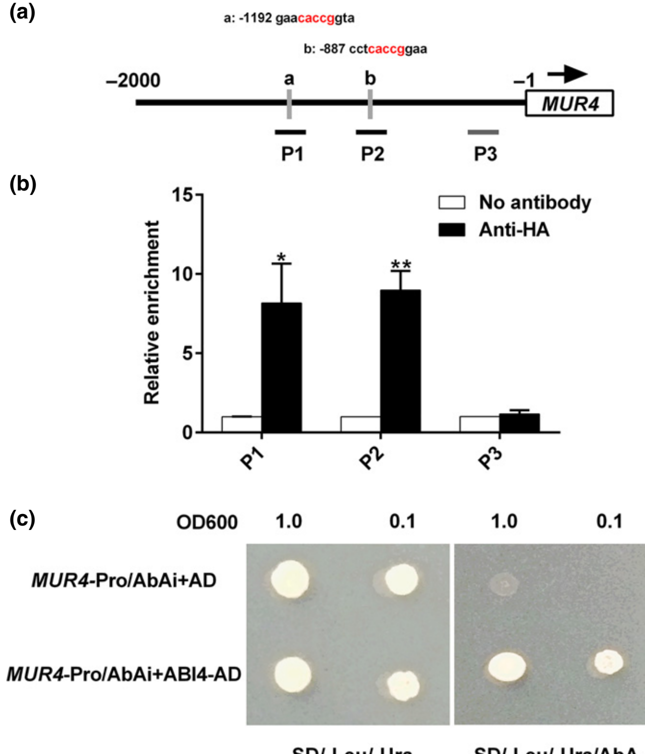

Fig. 5 ABSCISIC ACID INSENSITIVE4 (ABI4) directly binds the promoter of *MUR4 in vivo* and *in vitro*. (a) Schematic representation of putative ABI4 binding sites in the regions 2000 bp upstream of the start site in *MUR4* promoter. Gray lines indicate the CACCG motif. P1, P2 and P3 represent the fragments amplified in the chromatin immunoprecipitation (ChIP) assay. (b) ABI4 interacts with *MUR4 promoter* via ChIP–quantitative PCR assay. Chromatin was isolated from 3-d-old *Arabidopsis* seedlings of *ABI4* overexpressor plants. Chromatin was immunoprecipitated with hemagglutinin (HA) antibody produced in mouse (anti-HA, Sigma). The measurement values in control (no antibody) were set to 1 after normalization against *ACTIN 2* for quantitative PCR analysis. Values show average ± SD ($n = 3$). The asterisk shows a significant difference compared to the control using the unpaired Student's t-test (*, $P < 0.05$; **, $P < 0.01$). (c) ABI4 binds the *MUR4* promoter in yeast. Y1HGold strain co-transformed *MUR4-promoter* linked to the *Aureobasidin 1-C* (AbA^r) (*MUR4*-Pro/AbAi) and pGADT7 AD-ABI4 (ABI4-AD) or pGADT7 AD vector alone (AD) was grown on the SD/-Leu/-Ura with or without 1000 ng ml^{-1} AbA for 3 d. Numbers at the top represent the dilutions times of an optical density at 600 nm (OD600). Experiment in (c) was performed at least three times with similar results.

Overexpression of *MUR4* in *abi4-1* mutant can largely rescue the *abi4-1* mutant phenotype in response to exogenous ABA

Our results showed that *MUR4* overexpressors were hypersensitive to ABA during germination (Fig. 6), whereas *abi4-1* mutant was resistant to ABA (Finkelstein *et al.*, 1998). To further elucidate the genetic link between ABI4 and MUR4 in the ABA signaling, the double mutant *OX-MUR4#2/abi4-1* was obtained by crossing *OX-MUR4#2* with *abi4-1*. The expression of *MUR4* was confirmed by RT-PCR (Fig. S11). The L-Ara content in seed from *OX-MUR4#2/abi4-1* was partly restored compared with that in the *abi4-1* mutant, but it was still lower than that in the wild-type (Fig. S12). Seeds of wild-type and various genotypes were sown on the ½MS medium supplemented with or without exogenous ABA, and we observed that the germination rate and cotyledon greening rate in *OX-MUR4#2/abi4-1* were significantly repressed by ABA compared with those in *abi4-1* single mutant (Fig. 7). These results suggest that overexpression of *MUR4* in *abi4-1* mutant can at least partially recover the ABA-insensitive phenotype of *abi4-1* mutant.

Discussion

L-Ara is a major monosaccharide component in plant polysaccharides and glycoproteins, and the amount varies among different plant species and development stages, which functions in plant growth and development (Jones *et al.*, 2003; Peña & Carpita, 2004; Bar-Peled & O'Neill, 2011; Rautengarten *et al.*, 2011; Kotake *et al.*, 2016). A recent study showed that L-Ara metabolism was very important for salt stress tolerance (Zhao *et al.*, 2019). However, the contribution of L-Ara in seed germination is still poorly understood. Here, we established the link between ABA and L-Ara during ABA-mediated seed germination and revealed the potential molecular mechanism.

We found that L-Ara played a vital role in ABA-mediated inhibition of seed germination based on the following evidence. First, L-Ara content in seed cell wall significantly decreased during germination, and exogenous ABA slowed down the reduction of the L-Ara content in seed cell wall (Fig. 1). Second, exogenous L-Ara significantly delayed seed germination and cotyledon greening rate in the presence of ABA, but no effect was observed in the absence of ABA (Fig. 2). Third, L-Ara level was lower in the seed cell wall of *abi4-1* mutant than in wild-type, while an obvious induction in *ABI4* overexpressors in seed cell wall was observed (Figs 1c, 4d). Finally, overexpression of *MUR4*, which could generate L-Ara in cell wall, enhanced sensitivity to ABA treatment and partly restored the ABA-insensitive phenotype of *abi4-1* mutant (Figs 6, 7). L-Ara is involved in the decoration of a number of polymers and glycoproteins in the cell wall (Kotake *et al.*, 2016). Disruption of L-Ara metabolism would affect the formation of L-Ara-containing polymers. The L-Ara-containing polysaccharide arabinan is proposed to be the storage polysaccharides during seed development (Gomez *et al.*, 2009). Gimeno-Gilles *et al.* (2009) found that ABA-mediated inhibition of germination was related to the inhibition of several genes encoding cell-wall biosynthesis, including AGPs, in *Medicago truncatula*. Loss of function in *AGP30* resulted in a suppression of the ABA-induced delay in germination (Van Hengel *et al.*, 2002). Our results suggest that the L-Ara level in the seed cell wall is crucial for modulating the seed germination, and different types of L-Ara-rich polymers may have distinct roles in this process. Future work is needed to determine each polymer's specific role in ABA-mediated seed germination.

The AP2 transcription factor ABI4 is a key regulator in ABA-mediated germination (Söderman *et al.*, 2000; Penfield *et al.*, 2006; Wind *et al.*, 2013). Previous studies have shown that ABI4 mediated seed germination via regulating hormone biosynthesis and lipid metabolism (Penfield *et al.*, 2006; Shu *et al.*, 2013; Huang *et al.*, 2017). Our study showed that ABI4 transcription

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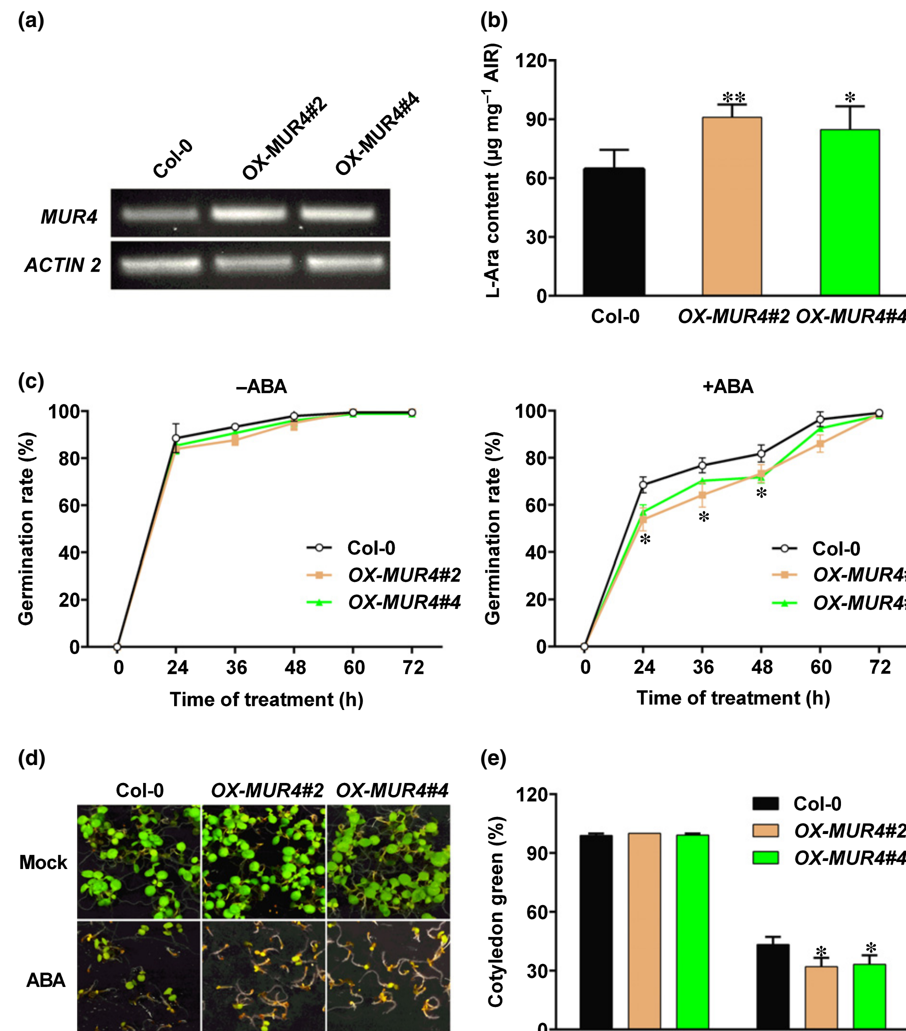

Fig. 6 *MUR4* overexpressors are sensitive to abscisic acid (ABA) during germination in *Arabidopsis*. (a) *MUR4* expression in *MUR4* overexpressors. Seeds from wild-type and *MUR4* overexpressors were imbibed at 4°C for 2 d, and total RNA was extracted and subjected to semi-quantitative PCR assay. (b) L-Arabinose (L-Ara) contents in seed cell wall from wild-type and *MUR4* overexpressors. Alcohol-insoluble residue (AIR) was extracted from stratified seeds and subjected to measurement for L-Ara content. (c) Seed germination rates of wild-type and *MUR4* overexpressors grown on ½ Murashige and Skoog plates without ABA or supplemented with 0.5 μM ABA. (d) Photographs of seedlings grown on different media as indicated were taken after 6 d. (e) The percentage of green cotyledon grown on different media as indicated was calculated after 6 d. Values in (b), (c) and (e) show average ± SD ($n = 3$). Experiment in (a) was performed at least three times with similar results. Col-0, Colombia. The asterisks show a significant difference between *MUR4* overexpressors and wild-type using the unpaired Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$).

factor modulated L-Ara level in seed cell wall (Figs 1c, 4d). The question is how ABI4 modulates the L-Ara content in seed cell wall during germination. L-Ara is generated as a form of UDP-L-arabinopyranose through C-4 epimerization of UDP-xylose in the *de novo* pathway catalyzed by MUR4 in the Golgi apparatus and by bifunctional UDP-glucose epimerase (UGE) in the cytosol. The *Arabidopsis mur4* mutant has a 50% reduction in cell wall L-Ara. However, in *uge1 uge3* double mutant, no obvious reduction was observed (Burget & Reiter, 1999; Burget *et al.*, 2003; Kotake *et al.*, 2009). Both *ABI4* and *MUR4* were highly expressed in the embryo and responsive to exogenous ABA. Their expression levels dropped during germination (Figs 3, S4, S5; Penfield *et al.*, 2006). The promotive effect of ABA on *MUR4* expression was largely impaired in the *abi4-1* mutant, and overexpression of *ABI4* upregulated the *MUR4* expression (Fig. 4). Furthermore, our results indicated that ABI4 directly bound to *MUR4* promoter via the CE1 elements, in agreement with previous reports (Fig. 5; Yang *et al.*, 2011; Huang *et al.*, 2017). Genetic analysis showed that the ABA insensitivity of *abi4-1* mutant was largely rescued by overexpressing *MUR4* in *abi4-1* mutant (Fig. 7). This indicates that ABI4 positively affects the L-Ara by directly regulating the *MUR4* expression during ABA-mediated seed germination. The L-Ara content in seed was partly restored by overexpressing *MUR4* in *abi4-1* mutant (Fig. S12). In addition, the expression levels of downstream genes (*ABF4*, *EM1*, *EM6* and *RD29B*) in *MUR4* overexpressor were lower than those in *ABI4* overexpressor exposed to ABA treatment. So, we speculate that genes that are yet to be identified should also be involved in this process.

Van Hengel *et al.* (2002) showed that lack of *AGP30* encoding an AGP affected the seed germination by modulating some ABA-regulated genes associated with ABA perception (*EM1* and *EM6*). Our results showed that the inducement of downstream genes in the ABA signaling pathway associated with seed germination (*ABF4*, *EM1*, *EM6* and *RD29B*) by exogenous ABA was significantly upregulated in the *MUR4* overexpressor or when exposed to exogenous L-Ara (Figs S3, S8), whereas L-Ara itself did not affect the ABA metabolism (Figs S1, S2, S13). We think it is possible that the L-Ara-containing polymers function in ABA-mediated seed germination by modulating the downstream ABA signaling pathway genes. In addition, our results showed that L-Ara only affected the ABA-meditated seed germination (Fig. 2). Moreover, we found that the inducement of L-Ara content by exogenous ABA was much higher than that by exogenous L-Ara

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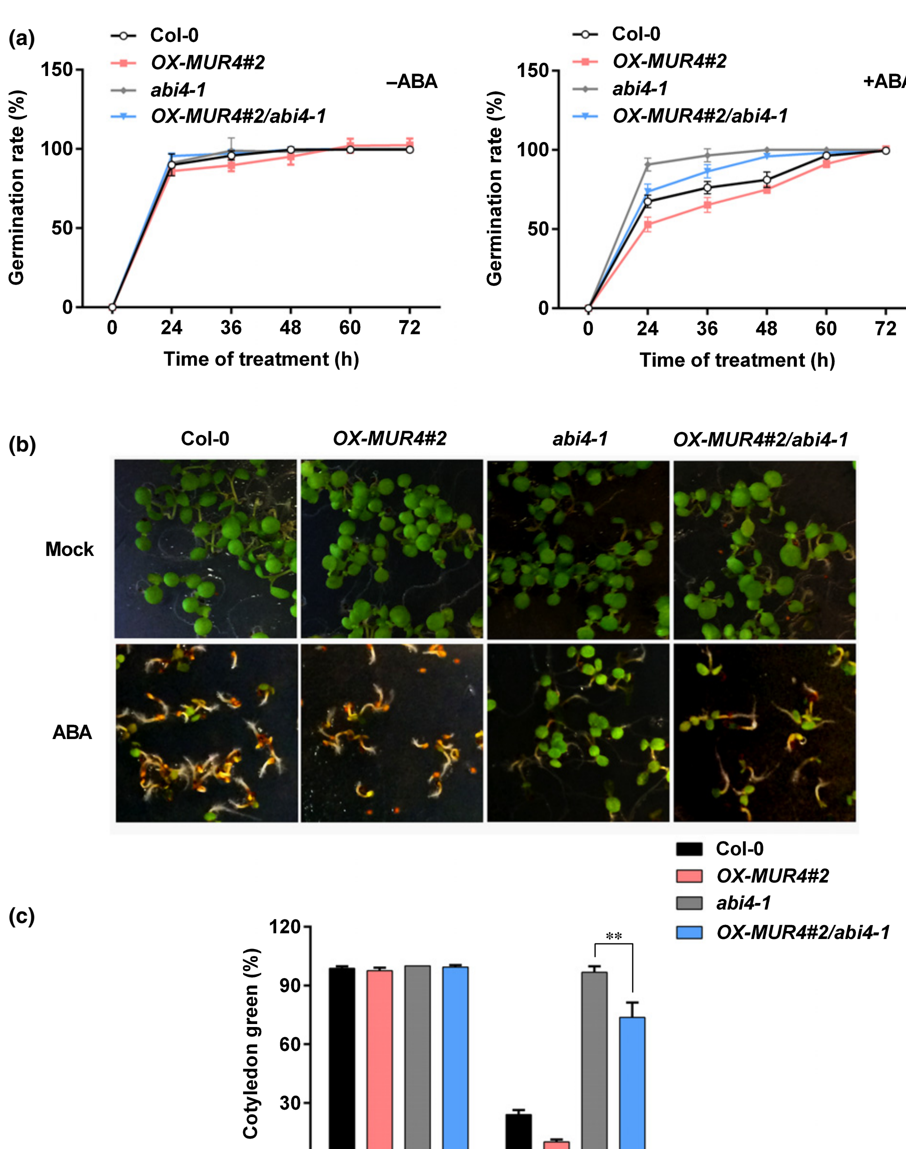

Fig. 7 Genetic analysis of ABSCISIC ACID INSENSITIVE4 (ABI4) and MUR4 in *Arabidopsis*. (a) Seed germination rates of Colombia (Col-0), *abi4-1*, *OE-MUR4#2* and *OE-MUR4#2/abi4-1* genotypes grown on ½ Murashige and Skoog (½MS) plates without abscisic acid (ABA) or supplemented with 0.5 μM ABA. (b) Photographs of various genotypes grown on ½MS medium without ABA or supplemented with 0.5 μM ABA were taken after 5 d. (c) The percentage of green cotyledon grown on ½MS medium without ABA or supplemented with 0.5 μM ABA was calculated after 5 d. Values in (a) and (c) show average ± SD ($n = 3$). The asterisks show a significant difference between *abi4-1* and *OE-MUR4#2/abi4-1* using the unpaired Student's *t*-test (**, $P < 0.01$).

treatment (Fig. 2d). Thus, we speculate that a minimal threshold of L-Ara content in seed cell wall is required to modulate ABA-mediated inhibition of seed germination.

Based on these results, a model is proposed to illustrate the link between ABA and L-Ara during ABA-mediated seed germination and cotyledon greening in *Arabidopsis*. During seed dormancy, a high level of ABA in embryo triggers the transcriptional activity of ABI4, thus inducing the expression of *MUR4* to synthesize the L-Ara-rich polymers in seed, resulting in ABA-inhibited seed germination. The endogenous ABA levels decrease after germination, promoting the L-Ara decreases by an ABI4-MUR4 transcriptional cascade. As a result, the ABA-inhibited seed germination is relieved.

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Author contributions

AZ conceived the project and designed the experiments. JY performed most of the experiments and analyzed the data; JY and AZ wrote the manuscript; LF helped to analyze the L-Ara content and revised the manuscript; LY analyzed the ABA content; LY, HH, YH and YL generated the transgenic lines.

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Supporting Information

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Fig. S1 ABA content in *Arabidopsis* seeds during seed germination exposed to exogenous L-Ara.

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Fig. S2 Expression levels of ABA biosynthetic and catabolic pathway genes during *Arabidopsis* seed germination in the presence of ABA and exogenous L-Ara.

Fig. S3 Expression levels of several downstream ABA-responsive genes during *Arabidopsis* seed germination in the presence of ABA and exogenous L-Ara.

Fig. S4 ABA induces the expression of *MUR4* in *Arabidopsis* seedlings.

Fig. S5 *ABI4* expression in dry seeds and germinating seeds of *Arabidopsis*.

Fig. S6 The expression of *ABI4* exposed to ABA in *Arabidopsis*.

Fig. S7 The expression level of *ABI4* in *ABI4* overexpressors.

Fig. S8 Expression levels of several downstream ABA signaling pathway genes in wild type, *ABI4* overexpressor and *MUR4* overexpressor exposed to ABA treatment.

Fig. S9 *mur4* mutants are sensitive to ABA during germination in *Arabidopsis*.

Fig. S10 The germination rate of *mur4* mutants in response to ABA is partly inhibited by exogenous L-Ara.

Fig. S11 *MUR4* expression in wild type, *OX-MUR4#2*, *abi4-1* and *OE-MUR4#2/ abi4-1*.

Fig. S12 L-Ara content in *Arabidopsis* seeds from wild type, *OX-MUR4#2*, *abi4-1* and *OE-MUR4#2/ abi4-1*.

Fig. S13 ABA content in dry seeds of wild type, *abi4-1*, *OX-ABI4#2*, *OX-MUR4#2* and *OX-MUR4#2/abi4-1* plants.

Table S1 PCR primers used.

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