**Arabidopsis** ABI5 plays a role in regulating ROS homeostasis by activating CATALASE 1 transcription in seed germination

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**Abstract**

It has been known that ABA INSENSITIVE 5 (ABI5) plays a vital role in regulating seed germination. In the present study, we showed that inhibition of the catalase activity with 3-amino-1,2,4-triazole (3-AT) inhibits seed germination of Col-0, abi5 mutants and ABI5-overexpression transgenic lines. Compared with Col-0, the seeds of abi5 mutants showed more sensitive to 3-AT during seed germination, while the seeds of ABI5-overexpression transgenic lines showed more insensitive. H$_2$O$_2$ showed the same effect on seed germination of Col-0, abi5 mutants and ABI5-overexpression transgenic lines as 3-AT. These results suggest that ROS is involved in the seed germination mediated by ABI5. Further, we observed that T-DNA insertion mutants of the three catalase members in Arabidopsis displayed 3-AT-insensitive or -hypersensitive phenotypes during seed germination, suggesting that these catalase members regulate ROS homeostasis in a highly complex way. ABI5 affects reactive oxygen species (ROS) homeostasis by affecting CATALASE expression and catalase activity. Furthermore, we showed that ABI5 directly binds to the CAT1 promoter and activates CAT1 expression. Genetic evidence supports the idea that CAT1 functions downstream of ABI5 in ROS signaling during seed germination. RNA-sequencing analysis indicates that the transcription of the genes involved in ROS metabolic process or genes responsive to ROS stress is impaired in abi5-1 seeds. Additionally, expression changes in some genes correlative to seed germination were showed due to the change in ABI5 expression under 3-AT treatment. Together, all the findings suggest that ABI5 regulates seed germination at least partly by affecting ROS homeostasis.

**Keywords** Arabidopsis thaliana · ABI5 · Catalase · ROS homeostasis · Seed germination

**Abbreviations**

ABRC  Arabidopsis Biological Resource Center
CAT  Catalase
ROS  Reactive oxygen species
3-AT  3-aminotriazol

**Introduction**

Seeds are important for sustainable agriculture production and landscape biodiversity. As the first phase transition in the life cycle of higher plants, seed germination is a complex process affected by many factors. However, molecular mechanisms of seed germination are largely unknown.

Reactive oxygen species (ROS), including superoxide ("O$_2^−$"), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical ("HO"), and singlet oxygen ("O$_2$"), has been believed to be connected with many biotic and abiotic stress, such as oxidative stress, drought stress, salt stress, and pathogen stress (Dat et al. 2000; Apel and Hirt 2004; Laloi et al. 2004; Torres and Dangl 2005; Miller et al. 2008). New evidence suggests that ROS is not only a cytotoxic molecule, but also emerges as a key regulator in seed physiology where ROS mediates seed germination (Bailly 2004; El-Maarouf-Bouteau et al. 2007; Bailly et al. 2008; Ye et al. 2012). An optimal range of ROS level is required for successful germination.

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Disruption of the ROS homeostasis will reduce the ability of seed germination (Bailly et al. 2008). The balance between ROS production and scavenging should be under elaborate and strict control for seed germination.

H$_2$O$_2$, a major ROS, is more stable than other ROS and is capable of long-distance diffusion across membranes, which is also admitted as the most likely ROS messenger (Möller and Sweetlove 2010). H$_2$O$_2$ promotes seed germination (Fontaine et al. 1994; Wang et al. 1998; Ogawa and Iwabuchi 2001) and affects the aleurone programmed cell death (PCD) of cereal grains during germination and seedling establishment (Fath et al. 2001). Arabidopsis catalase (CAT) forms a highly conserved enzyme family consisting of three members, CAT1 (At1g20630), CAT2 (At4g35090) and CAT3 (At1g20620), which are involved in catalyzing the H$_2$O$_2$ decomposition to water and oxygen (Chevalier et al. 1992; Frugoli et al. 1996). CAT provides the cell with an extremely efficient mechanism of removing H$_2$O$_2$ because they decompose H$_2$O$_2$ without consuming the cellular reducing equivalents (Smykowski et al. 2010). These CATALASE genes exhibit different spatial and temporal expression patterns throughout the plant life cycle. Whereas CAT1 is primarily expressed in the reproductive tissues and seeds, CAT2 is strongly expressed in the photosynthetic tissue and CAT3 is ubiquitously expressed, especially in roots and young leaves (Zimmermann et al. 2006; Du et al. 2008; Mhamdi et al. 2010). It has been shown that CAT controls the concentration of ROS in cells (Foyer and Noctor 2000; Mhamdi et al. 2010). CAT activity is also influenced by other factors, such as salicylic acid and nitric oxide (Chen et al. 1993; Durner and Klessing 1996; Clark et al. 2000).

Previous studies focused mainly on CAT function in plant leaves, especially in regulating the onset of leaf senescence (Smykowski et al. 2010), but whether CAT regulates seed germination is largely unknown in Arabidopsis.

Abscisic acid is a kind of important stress hormones, which functions in many physiological processes, including seed maturation, seed dormancy, growth and developmental regulation, and response to environmental stresses (Zeevaart and Creelman 1988; Hoffmann-Benning and Kende 1992; Wang et al. 2013). In Arabidopsis, ABSCIC ACID-INSENSITIVE MUTANT 5 (ABI5), a bZIP transcription factor, plays a vital role in mediating ABA signaling during seed maturation (Finkelstein and Lynch 2000). ABI5 interacts with ABA responsive cis-regulatory elements of some genes, such as EMI and EM6 (a class I Late Embryogenesis Abundant protein), to regulate seed maturing and seed germination (Carles et al. 2002).

It has been reported that the maize gene CAT1 promoter contains a G-box or ABA responsive element (ABRE) and antioxidant responsive element (ARE), which indicates the important protective role of CAT in response to oxidative stresses (Polidoros and Scandalios 1999; Guan et al. 2000).

GBF1, a G-box binding factor, is involved in the regulation of CAT2 expression and intracellular H$_2$O$_2$ content, which directly binds to the CAT2 promoter both in vitro and in vivo (Smykowski et al. 2010). However, the mechanism by which the CAT1 gene transcription is regulated remains unknown so far. Could ABI5, a G-box binding protein, bind to the G-box element of CAT1 promoter and regulate CAT1 expression? Does ABI5 mediate the seed germination partially depending on the ROS signaling? These questions concerning the possible interrelationships between ABI5 and CAT1-mediated ROS signal are of importance but currently remain open.

In the present experiments, we showed that the catalases play a critical role in the ABI5-mediated seed germination regulation, in which ABI5 regulates CAT1 expression directly. Furthermore, ABI5 regulates seed germination at least partly by affecting ROS homeostasis. These findings help to understand regulatory mechanisms of seed germination mediated by ABI5.

**Results**

**Compared with Col-0, the seeds of abi5 mutants showed more sensitive to 3-AT during seed germination, while the seeds of ABI5-overexpression transgenic lines showed more insensitive**

We assessed the possible role of H$_2$O$_2$, a major kind of ROS, in ABI5-mediated seed germination in Arabidopsis. 3-amino-1,2,4-triazole (3-AT), a CAT activity inhibitor (Margoliash et al. 1960), affects ROS homeostasis by promoting H$_2$O$_2$ accumulation. The mutants abi5-1 and abi5-7, loss-of-function mutants of ABI5, were used (Finkelstein 1994a, b; Nambara et al. 2002; Tamura et al. 2006; Albertos et al. 2015). When treated by different concentrations of 3-AT, including 3, 5 and 10 mM, seed germination rate of the wild-type seeds decreased. Furthermore, with the increasing of the concentration of 3-AT, the inhibition of 3-AT to seed germination were more and more significant. This result showed that 3-AT is a seed germination inhibitor (Fig. 1). In the absence of 3-AT, Col-0, abi5 mutants and ABI5-overexpression lines (ABI5OE), including ABI5OE-GFP and ABI5OE-Myc, had the same germination rates. In the presence of 3-AT, we found that compared with Col-0, the seed germination of abi5-1 or abi5-7 was much slower, revealing that abi5-1 and abi5-7 were hypersensitive to 3-AT, but the seed germination of ABI5-overexpression lines, was much faster, revealing that ABI5OE was insensitive to 3-AT (Fig. 1). Because 3-AT is connected with catalase, we suggested that catalase probably participated in the seed germination mediated by ABI5.
It is well known that catalase is a vital kind of enzymes removing H$_2$O$_2$. Inhibition of catalase activity by using 3-AT will result in the change in H$_2$O$_2$ content. So we explored the effect of H$_2$O$_2$ on seed germination of Col-0, $abi5$ mutants and ABI5-overexpression lines. When treated by different concentrations of H$_2$O$_2$, including 3, 4 and 5 mM, the wild-type seeds showed delayed seed germination, revealing that H$_2$O$_2$ could inhibit seed germination (Fig. 2). As same as the results of 3-AT treatment, with the increasing of the concentration of H$_2$O$_2$, the inhibition of H$_2$O$_2$ to seed germination was more and more significant. In the absence of H$_2$O$_2$, Col-0, $abi5$ mutants and ABI5-overexpression lines, had the same germination rates. In the presence of H$_2$O$_2$, we found that compared with Col-0, the seed germination of $abi5$-1 or $abi5$-7 was much slower, revealing that $abi5$-1 and $abi5$-7 were hypersensitive to H$_2$O$_2$. As for ABI5-overexpression lines, we found that seed germination were insensitive to different concentrations of H$_2$O$_2$ (Fig. 2). These results showed that H$_2$O$_2$ was involved in the seed germination mediated by ABI5.

### CAT mutants show either insensitive or hypersensitive to the catalase activity inhibitor 3-AT

To furtherly explore the function of catalase in seed germination mediated by ABI5, we examined the phenotypes of catalase members with 3-AT treatment. Considering the composition of CAT family, we identified CAT1, CAT2 and CAT3 mutants from the ABRC stock, including $cat1$-1, $cat1$-3, $cat2$-2, $cat2$-3, $cat3$-1 and $cat3$-2 (Fig. 3a). To characterize these mutants, we analyzed the CAT expression using quantitative RT-PCR and protein gel blot analysis. CAT antibodies were used to recognize three CAT members synchronously. Because CAT1 and CAT2, with the high homology and similar molecular weight, appeared in the same band in the protein gel (Hu et al. 2010; Supplementary Fig. S1), we could not distinguish CAT1 and CAT2. RT-PCR results showed that $cat1$-1, $cat1$-3, $cat3$-1 and $cat3$-2 were knockout mutants, while $cat2$-2 and $cat2$-3 were knockout mutants (Fig. 3b). The protein gel blot analysis showed that there was no obvious change
in CAT protein level in cat1-1 and cat1-3 mutant seeds, although CAT1 showed remarkably decrease at RNA level (Fig. 3c). We did not detect the CAT1 and CAT2 proteins in cat2-2 and cat2-3 mutants, but we observed dominant increase of CAT3 expression. In cat3-1 and cat3-2 mutants, the increase of CAT1 and CAT2 expression was remarkable. These indicated that different CAT members had the feedback effect on other member expression.

Although CAT members had different changes in these mutants, we found that they all showed the same germination rate under normal condition. But under 5 mM 3-AT treatment, these mutants showed different sensitivities to 3-AT. The seed germination rates of cat1-1, cat1-3, cat2-2 and cat2-3 were much higher than that of wild-type seeds, but cat3-1 and cat3-2 seeds had lower germination rates compared with wild-type seeds, revealing that the seed germination of cat1-1, cat1-3, cat2-2 and cat2-3 was insensitive to 3-AT, while the seed germination of cat3-1 and cat3-2 was hypersensitive to 3-AT (Fig. 3d). These different phenotypes of seed germination suggested that CAT members may play different roles in ROS signaling during seed germination.
Change in AB15 expression negatively affects CAT protein level, while 3-AT does not affect CAT protein level in the different genotypes

Because the capacity of catalase decomposing H2O2 is decided by catalase activities and CAT protein levels, H2O2 content should be affected by the two factors. Firstly, we checked the expressions of CAT at RNA and protein levels in seeds of wild-type Col-0, abi5-1 and AB15OE-Myc, respectively. We found that the expression of CAT1 and CAT3 changed significantly at RNA level in abi5-1 seeds without 3-AT treatment (Fig. 4a–c). CAT1 expression was down-regulated, while CAT3 expression was up-regulated. Interestingly, expression of CAT3 also showed up-regulated in AB15OE-Myc seeds. As for CAT2, there was no obvious changes in abi5-1 and AB15OE-Myc compared with Col-0. 3-AT treatment had the different effect on expression of CAT members in Col-0, abi5-1 and AB15OE-Myc. It inhibited CAT1 expression in Col-0 and AB15OE-Myc, while induced CAT2 expression in Col-0, abi5-1 and AB15OE-Myc, and CAT3 expression in Col-0 and abi5-1. It seemed that the change in AB15 expression could regulate the expression of CAT members with complex mechanism.

We also detected CAT proteins in seeds of Col-0, abi5-1 and AB15OE-Myc. Compared with Col-0, CAT protein level increased in the seeds of abi5-1, while decreased in the seeds of AB15OE-Myc without 3-AT treatment (Fig. 4d), indicating that AB15 mutation affects the CAT protein level. On the other hand, we examined the protein levels of the CAT members under 5mM 3-AT treatment in the seeds of Col-0, abi5-1 and AB15OE-Myc, and found that 3-AT did not affect the CAT protein levels (Fig. 4d). Here we found that expression changes in CAT members were not synchronous completely between RNA and protein level. For example, compared with Col-0, CAT1 showed down-regulated and CAT2 showed no changes in abi5-1 at RNA level; whereas the sum quantity of CAT1 and CAT2 showed a significant increase at protein level. There should be a complicated mechanism on maintaining the balance of CAT members at post-transcription and translation level.

Although 3-AT had no obvious effects on the expression of CAT members at protein level, the difference of their expressions among Col-0, abi5-1 and AB15OE-Myc should be responsible for the corresponding phenotypes with 3-AT treatment.

3-AT inhibits catalase activities dependently of AB15 expression

It has been reported that 3-AT is a catalase inhibitor, so we assayed catalase activities of different genotypes in the presence or absence of 3-AT. In the absence of 3-AT, catalase activity of abi5-1 seeds was lower than that of Col-0 and AB15OE-Myc (Fig. 5a, b), but there was no difference of the phenotype in seed germination among them (Figs. 1, 2). This suggested that although catalase protein and activity were different in Col-0, abi5-1 and AB15OE-Myc seeds without 3-AT treatment, these differences were not enough to affect ROS homeostasis which can influence seed germination. Under 3-AT treatment, the catalase activity in Col-0, abi5-1 and AB15OE-Myc seeds were all obviously inhibited, showing that 3-AT could function as the catalase activity inhibitor (Fig. 5a, b). Furthermore, we found that the inhibition of catalase activity in abi5-1 seeds was the most serious (Fig. 5a, b). We suggested that the most...
serious inhibition of catalase activity was connected with the postponed germination of abi5-1 seeds.

3-AT affects H$_2$O$_2$ content in seeds of wild-type, abi5-1 and ABI5OE-Myc plants in a different way

The capacity of catalase scavenging H$_2$O$_2$ is correlated with the catalase protein and catalase activity, which code-determine the H$_2$O$_2$ content. The lower catalase activity can induce H$_2$O$_2$ accumulation, and higher CAT protein accumulation can promote H$_2$O$_2$ decomposition. The final H$_2$O$_2$ content should be decided by which effect was dominant. We measured H$_2$O$_2$ content in seeds of Col-0, abi5-1 and ABI5OE-Myc. Without 3-AT treatment, H$_2$O$_2$ content in both abi5-1 and ABI5OE-Myc seeds were lower than that in Col-0 seeds (Fig. 5c, d). Although the fundamental H$_2$O$_2$ content was different in Col-0, abi5-1 and ABI5OE-Myc seeds in the absence of 3-AT, the difference did not influence seed germination (Figs. 1, 2). When using 3-AT treatment, the CAT protein level was not influenced, but the catalase activity was inhibited obviously. We conjectured that there would be H$_2$O$_2$ accumulation in the seeds of Col-0, ABI5OE-Myc and abi5-1. The vertical axis represents the relative change in the absolute value of H$_2$O$_2$ content of Col-0, ABI5OE-Myc and abi5-1 with 5 mM 3-AT treatment in relative to that without 3-AT treatment, respectively. The absolute values of H$_2$O$_2$ content in the seeds of Col-0, ABI5OE-Myc and abi5-1 without 3-AT treatment are taken as a control (100%), respectively. Each value is the mean±SE of three independent biological experiments, and different letters indicate significant differences at P<0.05 (Duncan’s multiple range test) when comparing values within the same 3-AT concentration.
relative decrease of H$_2$O$_2$ content in ABI5OE-Myc seeds was more remarkable than that in Col-0 seeds (Fig. 5c, d). So we suggested that the relative changes in H$_2$O$_2$ content with 3-AT treatment were connected with the different phenotypes of seed germination in Col-0, abi5-1 and ABI5OE-Myc.

**ABI5 binds to the promoter of CAT1**

How does ABI5 affect ROS homeostasis? Previous studies have demonstrated that ABI5 is a versatile transcription factor, binding to the G-box (ACGT) motif to promote the expression of EM1 and EM6 (Carles et al. 2002). Using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) to analyze the sequence of AtCAT1 promoter, we found two G-box elements within the 442 bp sequence upstream of the AtCAT1 start codon. Combined with previous evidence on that CAT1 expression was down-regulated in abi5-1 at RNA level (Fig. 4a), we were interested in whether ABI5 could affect ROS homeostasis by regulating CAT1 transcription. We detected interaction of the ABI5 with the promoter of CAT1 in a yeast one-hybrid system. Yeast cells which were co-transformed with both a ABI5-encoding cDNA and CAT1 promoter could grow in a selection SD medium (lacking Trp, Leu and His nutrients, and containing 3-AT), indicating a potential interaction between the ABI5 and CAT1 promoter (Fig. 6a). As a negative control, yeast cell which were co-transformed with both control vector (p53) and CAT2 promoter could not grow in this medium (Fig. 6a). These data suggested that ABI5 could potentially bind to the promoter of CAT1 (Fig. 6a).

To investigate whether ABI5 could directly regulate CAT1 expression, we used electrophoretic gel mobility shift assay (EMSA) with the purified recombinant ABI5 protein. The full length ABI5 protein was overexpressed with a C-terminal 6 residue histidine fusion in E. coli. Most of the recombinant proteins were expressed in the soluble fraction and purified by nickel ion chelate chromatography. We analyzed the CAT1 promoter described in Fig. 6b and found two G-box in its promoter region. The probe fragment of pCAT1, which is 189 bp long (−296/−108) and contains two G-box, named box1 and box2, was chosen (Fig. 6b). The mutant probe fragments included box1 mutation and box2 mutation, respectively. The EMSA results indicated that ABI5 binds specially to the pCAT1 probe. Both box1 mutation and box2 mutation could weaken the binding between ABI5 protein and CAT1 promoter (Fig. 6c).

Furthermore, we assayed the interaction of ABI5 with the same CAT1 promoter fragment as EMSA assay by ChIP analysis combined with quantitative real-time PCR. We observed that ABI5 can bind the CAT1 promoter (Fig. 6d). This result was consistent with the result of the above-described EMSA assay (Fig. 6c), and revealed that ABI5 can interact with the CAT1 promoter in vivo.

**ABI5 activates CAT1 transcription in vivo**

The evidence from yeast one-hybrid, EMSA and ChIP analysis indicated that ABI5 could bind to the CAT1 promoter. Thus in vivo luciferase complementation imaging assay (LCI) was employed to investigate whether ABI5 could active CAT1 expression in vivo. By analyzing the promoter sequences, the reporter plasmid Pro-CAT1-LUC and the effector plasmid pCAMBIA-ABI5-Myc were constructed separately. When Pro-CAT1-LUC was transfected into the N. benthamiana tobacco leaves together with pCAMBIA-ABI5-Myc, strongly LUC activity was detected (Fig. 6e, f). However, when the pCAMBIA-ABI5-Myc was substituted by the equal amount of pCAMBIA-GFP or pCAMBIA-GUS control vector, the LUC activity decreased obviously (Fig. 6e, f). These results revealed that ABI5 can active CAT1 transcription in vivo.

**The CAT1 mutation suppresses 3-AT-hypersensitive phenotype of the loss-of-function mutant abi5-1 in seed germination**

Above evidence had showed that ABI5 can bind to the promoter of CAT1 and active its transcription (Fig. 6). Subsequently, we explored the genetic function of CAT1 in ROS signaling mediated by ABI5. As shown in Figs. 1, 3d, abi5 mutants showed hypersensitive to 3-AT during seed germination, while cat1 mutants showed insensitive to 3-AT. So we suggested that ABI5 and CAT1 played opposite roles in seed germination, respectively. Based on above reason, we generated double mutants of cat1-1abi5-1 by crossing. When cat1 mutation was introduced into abi5-1 mutant, the hypersensitive phenotype in seed germination of abi5-1 was recovered (Fig. 7). These data provided the genetic evidence that CAT1, as a direct target of ABI5, functioned downstream of ABI5 in ROS signaling. Besides, we also noticed that cat1-1 abi5-1 did not show the same phenotype as cat1-1 did under 3-AT treatment, which meant that CAT1 mutation could not completely block the pathway mediated by ABI5, we suggested that CAT1 was one of the targets of ABI5 during seed germination regulated by ROS.

**Disruption of ABI5 alters the expression of genes related to ROS metabolism and ROS signaling in seed germination**

To further confirm that ABI5 functioned as a ROS regulator during seed germination, we analyzed the effect of the abi5 mutation on the expression of ROS-responsive genes in seed germination. Total RNAs were extracted from
the seeds of wild-type and abi5-1. We performed RNA-sequencing (RNA-seq) analyses on wild-type and abi5-1. The differently expressed genes in Col-0 and abi5-1 were analyzed. A total of 16,561 genes were found in Col-0 seeds, and of 16,116 genes in abi5-1 seeds (Supplemental Fig. S2A). Using the twofold change and the false discovery rate (FDR), 0.05 as the p-value cutoff for selecting the differentially expressed transcripts, lots of differentially expressed genes (DEGs) were found (Fig. 8a; Supplemental Fig. S2b). Among of these DEGs, there were 616 genes which were induced and 1303 genes which were repressed in abi5-1 seeds (Supplemental Tables S9, S10).

In this study, we focused on the genes that are responsible for ROS metabolism or in response to ROS. The Gene
some genes in response to oxidative species metabolic process, there were 25 genes involved in among 34 DEGs related to oxygen and reactive oxygen nase (AT5G09660), and so on (Table 1). Furthermore, (AT1G80840), an peroxisomal NAD-malate dehydroge-

obviously down-regulation, including AT WRKY40 and ABA signaling, changed significantly, showing that the disruption of ROS regulators (Table 1). Besides, due to the mutation impairing ROS signaling by feedback due to abi5

Fig. 6 AB15 can directly bind to the promoter of CAT1 in vitro and activate CAT1 expression in vivo. a Yeast one-hybrid assay. The prey vector harboring AB15 (indicated by pGADT7-AB15) and the bait vector pHIS2 harboring CAT1 promoter (indicated by pHIS2-CAT1) were used to co-transform yeast cells. The transformation with vectors pHIS2-53 and pHIS2-CAT2 was taken as a negative control. The experiments were repeated three times with the same results. b The promoter structure of CAT1 gene. The box1 and box2 denote two G-box from left to right with sequence sites relative to the star code. The fragment from −296 to −108 indicates the sequence used in electrophoretic mobility shift assay described in the panels. c Electrophoretic retic mobility shift assay shows that AB15 can bind the box1 site and box2 site. The 6His tag peptide (6His), which was fused to AB15 protein for AB15 protein production. “-“ represents that 6His was served as a negative control. Box1 mutation is ACGT→TTTT, and box2 mutation is ACGT→TTTT (see Methods section). Wild type probe, not mutation labeled probe; box1 mutation, box1 mutation labeled probe; box2 mutation, box2 mutation labeled probe; 20x, 20-fold unlabeled probes as competitors. Black arrow means shift band. These experiments were repeated six times substantially with the same results. d ChIP-qPCR assays were performed using the specific primer corresponding to the CAT1 promoter region. The ACTIN promoter was used as a negative control. Primers used in the ChIP-qPCR assays are indicated by arrows and are presented in Fig. 6b. Each data bar represents mean±SE (n>3). Similar results were obtained from four independent experiments. e Test of the interactions of AB15 with the CAT1 promoter in vivo in tobacco leaves. The tobacco leaves were transformed with the following constructs all together with ProCAT1::LUC. The control constructs harbor GFP and GUS encoding open reading frame, called “35S::GFP” and “35S::GUS” respectively. Co-expressed the constructs of “35S::AB15” with ProCAT1::LUC can active the activity of ProCAT1::LUC, but controls cannot. f Quantitative analyses of luminescence intensity are shown in e. The panels show the corresponding quantitative data corresponding to fluorescent images. Each value is the mean±SE with five independent determinations. The experiments were performed three biological repeats and obtained the similar trend.

Ontology (GO) analysis indicated that among these down-regulation DEGs, there were many genes which participate in oxygen and ROS metabolic process and regulation of H2O2 metabolic process (Fig. 8b). About 10% of the DEGs involved in oxygen and ROS metabolic process showed obviously down-regulation, including AT WRKY40 (AT1G80840), an peroxisomal NAD-malate dehydrogenase (AT5G09660), and so on (Table 1). Furthermore, among 34 DEGs related to oxygen and reactive oxygen species metabolic process, there were 25 genes involved in H2O2 metabolic process. Some genes in response to oxidative stress were up-regulated in ab15-1, which might be the feedback due to ab15 mutation impairing ROS signaling by inactivating ROS regulators (Table 1). Besides, due to the disruption of AB15, expressions of some genes, participating in GA metabolism, GA signaling, ABA metabolism and ABA signaling, changed significantly, showing that they were involved in the process of AB15 regulating seed germination (Table 2).

Surprisingly, we did not find CAT1 transcript in the DEGs. It seemed to contradict with the result of RT-PCR (Fig. 4a). It has been well known that, because of the technical biases in RNA-seq library generation and sequencing, false positive and false negative results will be produced in RNA-Seq data (Langmead et al. 2009; Zheng and Moriyama 2013). On the other hand, absence of CAT1 transcript in DEGs could be connected with possible differences in sampling between the RT-PCR and RNA-Seq. To ensure the reliability of our RNA-Seq data, we analyzed the expressions of AB15 target genes in RNA-Seq data, including EM1 and EM6 (Carles et al. 2002). Both EM1 and EM6 showed down-regulation in ab15-1, which suggested that our RNA-Seq results were reliable (Supplemental Tables S11).

Also, in the RT-PCR analyses of the expression of EM1 and EM6 in ab15-1, we showed that their expression were down-regulated due to the disruption of AB15 (Fig. 9).

3-AT treatment alters expressions of genes correlative to seed germination in Col-0, AB15OE-Myc and ab15-1 seeds

By analyzing RNA-Seq data, we found that many vital genes involved in seed germination expressed differentially between Col-0 and ab15-1 mutant (Table 2). To exploring the effects of 3-AT on gene expressions, we analyzed the transcript levels of EM1, EM6, DOG1, GA3ox, NCED9 and PYL7 using quantitative RT-PCR analysis in the seeds of Col-0, AB15OE-Myc and ab15-1 which were treated with or without 5 mM 3-AT. The results of RT-PCR showed the same change trend of these genes as that shown in RNA-Seq data without 3-AT treatment in Col-0 and ab15-1 (Fig. 9). This was also the important evidence of reliability about the RNA-Seq data.

EM1 and EM6 are ABA-responsive genes, which function in seed development (Gaubier et al. 1993; Carles et al. 2002). DOG1 (DELAY OF GERMINATION 1) was identified as a major regulator of dormancy in various species (Bentsink et al. 2006; Graeber et al. 2010). Here, we found that the change in AB15 expression had effects on these gene expressions, especially in ab15-1 seeds. When treated by 5 mM 3-AT, EM1 and EM6, which functions in promoting seed germination, were dominantly down-regulated in Col-0, which could partly explain the inhibition of 3-AT on seed germination of Col-0. Contrarily, DOG1, which functions in delaying seed germination, was up-regulated in Col-0 and abi5-1, while down-regulated in AB15OE-Myc under 3-AT treatment (Fig. 9). The different effects of 3-AT on gene expressions in Col-0, AB15OE-Myc and ab15-1 could be responsible for their different phenotypes during seed germination.

In addition, we found that 3-AT can affect the expression of genes involved ABA and GA metabolism differently in Col-0, AB15OE-Myc and ab15-1. Genetic and physiological evidence supports the importance of two hormones,
abscisic acid (ABA) and gibberellins (GAs), in seed dormancy and germination (Koornneef et al. 2009; Finkelstein et al. 2000). NCED9 regulates a key step of ABA biosynthesis (Lefebvre et al. 2006), while GA3ox is an vital enzyme in GA biosynthesis (Yamaguchi 2008). Without 3-AT treatment, NCED9 and GA3ox showed up-regulated and down-regulated in abi5-1, respectively. Combined with the expression of EM1, EM6 and DOG1, their expression changes tended to inhibit seed germination of abi5-1 in the presence of 3-AT treatment. However, there was no difference on the seed germination of Col-0, ABI5OE-Myc and abi5-1 without 3-AT treatment. It seemed that there was delicate and complicated balance among genes correlative to seed germination. Due to 3-AT treatment, the balance was disrupted, which resulted in different 3-AT phenotypes of Col-0, ABI5OE-Myc and abi5-1.

PYL7 is a member of ABA receptors (Park et al. 2009). The change in ABI5 expression resulted in increasing of PYL7 expression in Col-0, ABI5OE-Myc and abi5-1 under 3-AT treatment (Fig. 9). This suggested that 3-AT can affect seed germination mediated by ABI5 partly depending on ABA signaling.

Discussion

It is well known that ABI5 is a positive regulator in ABA signaling (Finkelstein 1994a, b). Lots of evidence shows that ABI5 regulates seed germination by regulating ABA signaling or the expression of genes involved in seed development. For instance, it binds to EMs promoter and affects their expression (Carles et al. 2002). Here we found that 3-AT, a kind of catalase activity inhibitor, could affect seed germination mediated by ABI5. In the absence of 3-AT, seed germination of abi5 mutants and ABI5OE lines showed the same germination rates as that of Col-0. In the presence of 3-AT, seed germination of abi5 mutants and ABI5OE lines showed the same germination rates as that of Col-0. In the presence of 3-AT, seed germination of abi5-1 and abi5-7 showed strong 3-AT-hypersensitive phenotypes compared with wild-type seeds. Meanwhile, the seeds of ABI5OE lines, including ABI5OE-Myc and ABI5OE-GFP, showed a significantly 3-AT-insensitive phenotype (Fig. 9). This suggested that 3-AT can affect seed germination mediated by ABI5 partly depending on ABA signaling.
exogenous H₂O₂ was similar to that in different concentrations of 3-AT medium, which further confirmed that ABI5 regulated seed germination partly depending on the ROS signaling pathways.

CAT members are the vital enzymes scavenging H₂O₂, but their functions in seed germination have not been clearly identified in *Arabidopsis*. Previous results suggested that the *CAT1* gene is mainly expressed in pollen

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**Table 1** Go analysis of differentially expressed genes in Col-0 and *abi5-1*

| Gene set name(no. genes) | Description | Gene number in overlap | Fold change  | Gene names |
|--------------------------|-------------|------------------------|--------------|------------|
| OXYGEN_AND_REACTIVE_OXYGEN_SPECIES_METABOLIC_PROCESS(347) | GO:0006800 oxygen and reactive oxygen species metabolic process, GOslim:biological process | 34 | Down-regulation | AT5G02790 AT3G55800 AT1G80840 AT4G13510 AT1G19670 AT4G16190 AT5G09660 AT1G33590 AT4G33560 AT2G20570 AT5G24530 AT3G09940 AT2G36470 AT2G26400 AT1G32060 AT1G59870 AT1G77120 AT1G74710 AT4G33070 AT1G02220 AT5G20020 AT5G20480 AT2G28200 AT5G66570 AT5G21090 AT3G28930 AT3G14150 AT5G60360 AT2G44490 AT1G68520 AT1G54410 AT1G66880 AT1G18570 AT1G66200 |
| REGULATION_OF_HYDROGEN_PEROXIDE_METABOLIC_PROCESS(187) | GO:0010310 regulation of hydrogen peroxide metabolic process, GOslim:biological process | 25 | Down-regulation | AT1G80840 AT1G59870 AT3G28930 AT1G32060 AT2G44490 AT5G24530 AT1G20020 AT4G33560 AT5G20480 AT1G18570 AT5G09660 AT5G66570 AT2G20570 AT4G33070 AT1G66880 AT1G33590 AT1G74710 AT1G19670 AT2G36470 AT1G54410 AT4G13510 AT1G77120 AT3G55800 AT2G26400 AT1G68520 |
| RESPONSE_TO_OXIDATIVE_STRESS (582) | GO:0006979 response to oxidative stress, GOslim:biological process | 22 | Up-regulation | AT1G60970 AT2G31570 AT5G64510 AT5G16960 AT2G39800 AT2G40880 AT1G71000 AT2G22080 AT5G39610 AT1G14200 AT4G33940 AT4G08770 AT1G13340 AT1G69270 AT3G60980 AT1G35720 AT5G40390 AT5G16990 AT4G34890 AT1G50290 AT2G47180 AT1G09080 |

P-value < 0.05; Differentially expressed genes twofold-down-expression change or twofold-up-expression change

**Table 2** Differentially expressed genes related to seed germination and ROS metabolism in Col-0 and *abi5-1*

| Gene_ID | Gene names | Fold change  | Up/Down | Gene description | Pathway |
|---------|------------|--------------|---------|------------------|---------|
| AT1G15550 | GA3OX1     | 0.158792     | Down    | Gibberellin 3-oxidase 1 | GA biosynthesis |
| AT1G66350 | RGL1       | 0.394985     | Down    | RGA-like 1       | GA signaling |
| AT5G45830 | DOG1       | 8.766422     | Up      | Delay of germination 1 | Seed development |
| AT3G51810 | EM1        | 0.169394     | Down    | Stress induced protein | |
| AT2G40170 | EM6        | 0.477625     | Down    | Stress induced protein | |
| AT1G78390 | NCED9      | 2.70285      | Up      | Nine-cis-epoxycarotenoid dioxygenase 9 | ABA metabolism |
| AT2G40330 | PYL6       | 0.450936     | Down    | PYR1-like 6       | ABA signaling |
| AT2G26040 | PYL2       | 0.493138     | Down    | PYR1-like 2       | ABA signaling |
| AT2G38310 | PYL4       | 0.410952     | Down    | PYR1-like 4       | ABA signaling |
| AT4G01026 | PYL7       | 5.927423     | Up      | PYR1-like 7       | ABA signaling |
| AT3G19290 | ABF4       | 3.243769     | Up      | ABRE binding factor 4 | |
| AT5G57050 | ABI2       | 2.09312      | Up      | Protein phosphatase 2C family protein | |
| AT2G31570 | GPX2       | 3.094566     | Up      | Glutathione peroxidase 2 | ROS metabolism |
| AT4G11600 | GPX6       | 0.483308     | Down    | Glutathione peroxidase 6 | |
| AT1G77490 | APXT       | 2.813915     | Up      | Thylakoidal ascorbate peroxidase | |
| AT2G35690 | ACX1.2     | 4.115625     | Up      | Acyl-CoA oxidase 5 | |

P-value < 0.05; Differentially expressed genes twofold-down-expression change or twofold-up-expression change
and seeds, CAT2 in photosynthetic tissues but also in roots and seeds, while CAT3 is associated with vascular tissues but also leaves (Du et al. 2008). This means that they will function in different tissues and development process. The further evidence showed that different CAT members may play different roles during the same process. Previous studies indicated that ABA has an opposite effect on the transcription of CAT1 and CAT2 in barley aleurone, which suggests that they play the opposing roles (Fath et al. 2001). In our study, CAT mutants differed in their 3-AT phenotypes with regard to seed germination. Phenotypes of CAT1 and CAT2 mutants were completely opposite to those of CAT3 mutants (Fig. 3d). By examining the RNA and protein expression of three CAT members, we found that there was a difference at RNA and protein level in different CAT mutants without 3-AT treatment. At the RNA level, the expression changes in any members can induce the expression changes in other members more or less (Fig. 3b). But at the protein level, in addition to the CAT1 mutation, both CAT2 and CAT3 mutations could affect the expression of CAT family obviously (Fig. 3c). There may be an unknown mechanism on the difference of CAT expression at RNA and protein level. Moreover, there is a delicate balance among the CAT members during seed germination in response to 3-AT treatment. The final status of the balance between the three CAT genes determines the germination phenotype under 3-AT treatment.

We examined the CAT protein and catalase activity in seeds of Col-0, abi5-1 and ABI5OE-Myc with or without 3-AT treatment. The abi5 mutation or ABI5 overexpression could result in the changes in CAT protein and catalase activity (Figs. 4, 5). Furthermore, 3-AT treatment could significantly affect the catalase activity differently in Col-0, abi5-1 and ABI5OE-Myc seeds (Fig. 5c, d), but 3-AT treatment did not obviously change the CAT protein levels (Fig. 4d). We thought that the difference of CAT protein levels in Col-0, abi5-1 and ABI5OE-Myc was a vital reason resulting in the difference of catalase activity in response to 3-AT treatment. For example, because of CAT protein accumulation in abi5-1, 3-AT had a more obvious inhibition on catalase activity in abi5-1. The balance of CAT protein levels and catalase activity decided the final 

Fig. 9 3-AT alters the expressions of genes related to seed germination due to ABI5 disruption and overexpression. Seeds of Col-0, ABI5OE-Myc and abi5-1, were treated with or without 5 mM 3-AT and grown under 16-h light/8-h dark conditions for 24 h after stratification. The transcript levels of EM1, EM6, DOG1, NCED9, GA3ox and PYL7 were examined using quantitative RT-PCR analysis. Each value is the mean ± SE of three independent experiments.
Based on above results, it was shown that ROS played a vital role in the seed germination mediated by ABI5.

Furthermore, RNA-Seq data supported that disruption of ABI5 could alter expressions of genes participating in ROS metabolism, especially H$_2$O$_2$ metabolism, and ROS signaling (Table 1). Combining with RNA-Seq data, we used RT-PCR to examine the effects of 3-AT treatment on the DEGs involved in seed germination in Col-0, abi5-1 and ABI5OE-Myc. The results showed that due to different expression of ABI5, 3-AT had different effects on expressions of these vital genes, which were involved in seed development, ROS metabolism, GA metabolism, GA signaling, ABA metabolism, ABA signaling and so on (Fig. 9; Table 2).

In addition, biochemical and physiological evidence supported that ABI5 could affect the balance of three CAT members by regulating the CAT1 expression directly (Fig. 6). Previous evidence has shown that plants have evolved the complex defense systems to regulate the homeostasis of the intracellular H$_2$O$_2$ level, which plays a vital role during the plant evolution process (Mhamdi et al. 2010). H$_2$O$_2$ is a major ROS, and the balance of the CAT protein and catalase activity, which jointly determines the H$_2$O$_2$ content, should be the key factor in charge of ROS homeostasis. Previous research on ABI5 regulating seed germination is focused on ABA signaling. Here we proposed that ABI5 affects seed germination partly dependent on ROS. Much evidence has shown the crosstalk of ROS signaling, abscisic acid and ethylene signaling (Bueso et al. 2007; El-Maarouf-Bouteau et al. 2015). Our data illustrated a new crosstalk among different signaling during the seed germination mediated by ABI5. Taken together, a new mechanism of ABI5 regulating the process of seed germination was demonstrated, in which CAT acted as a link between the ABI5 and ROS homeostasis. Moreover, the different roles of CAT members in mediating ROS signaling in seeds were demonstrated.

Materials and methods

Plant materials and growth conditions

The mutant Arabidopsis lines cat1-1 (SAIL_525_C10), cat1-3 (SALK_208924), cat2-2 (SALK_057998), cat2-3 (SALK_144919), cat3-1 (SALK_092911) and cat3-2 (SALK_088601) were obtained from the Arabidopsis Biological Resource Center (Columbus, OH, USA). The homozygous mutants were identified by PCR using gene-specific primers (Supplemental Table S1). The mutated ABI5 gene in the abi5-1 mutant (ABRC Stock Number CS8105; named abi5-1 in this report) was transferred from its background Wassilewskija (Ws) ecotype into Col-0 ecotype by backcrossing (Liu et al. 2012). For the generation of the transgenic lines of ABI5OE-GFP, the open reading frame (ORF) sequences of ABI5 was amplified by PCR and cloned into the binary vector pCAMBIA-1300-221 (http://www.cambia.org) with a green fluorescent protein (GFP) tag which contains the cauliflower mosaic virus 35S promoter. The specific primers used for generating the ABI5OE-GFP are listed in Supplemental Table S7. Some of plant materials used in this study were previously described: abi5-7 and ABI5OE-Myc (Bu et al. 2009). We examined ABI5 expression in abi5-1, abi5-7, ABI5OE-Myc and ABI5OE-GFP by RT-PCR (Supplemental Fig. S3).

Arabidopsis seeds were disinfected and plated on MS medium (Phyto Technology Laboratories, USA, product No. M519) supplemented with 3% sucrose and 0.8% agar (pH 5.9), chilled for 3 days at 4°C and transferred to a growth chamber at 80 μmol photons m$^{-2}$ s$^{-1}$ or to compost soil at 120 μmol photons m$^{-2}$ s$^{-1}$ using cool white fluorescent lamps under a 16 h-light/8 h-dark photoperiod and 60% relative humidity.

Phenotypic analysis

Phenotypic analysis was performed as described previously (Wu et al. 2009; Shang et al. 2010). For germination assays, ~100 seeds were sterilized and plated in triplicate on MS medium (Phyto Technology Laboratories, USA, product No. M519). The medium contained 3% sucrose and 0.8% agar (pH 5.9), supplemented with or without different concentrations of 3-AT (3-amino-1,2,4-triazole; Sigma, USA, product No. A8056) or H$_2$O$_2$. The seeds were chilled at 4°C for 3 days before being placed at 22°C under light conditions (16 h light/8 h dark), and germination (emergence of radicals) was scored at the indicated times.

Quantitative RT-PCR analysis of mRNA expression

For quantitative RT-PCR (qRT-PCR) analysis, total RNA was isolated from imbibing seeds, which were chilled for 3 days at 4°C to stimulate germination and then placed at 22°C under a 16-h light/8-h dark photoperiod for 24 h. Total RNA was isolated using a seed RNA Extraction Kit (BioTeke, China, product No. RP1201), treated with RNase-free DNase I (NEB, USA, M0303) at 37°C for 30 min to degrade genomic DNA, and purified by using an RNA Purification Kit (BioTeke, China, Product No. RP1801).

RNA (2 μg) was subjected to first-strand cDNA synthesis using a kit according to the manufacturer’s instructions (Roche Applied Science, USA, product No. 095014379012001). The primers used for qPCR are listed in Table S1 in the Supporting Information. Analysis
was performed using the Bio-Rad Real-Time System CFX96TM C1000 Thermal Cycler (Bio-Rad, USA). All experiments were repeated at least three times. The qRT-PCR analysis was performed using the SYBR Green kit (Takara, Japan). PCR conditions were as follows: 10 min of denaturation at 95 °C, followed by 45 cycles of 10 s of denaturation at 95 °C, 5 s of annealing at 55 °C and a 10 s extension at 72 °C. The mRNA levels were measured relative to the constitutive ACTIN 2/8 mRNA (Wallström et al. 2012). All the gene-specific primers for real-time PCR analysis were shown in Supplemental Table S8.

**Protein gel blot analysis**

Protein extraction of Arabidopsis seeds was performed essentially according to previously described procedures (Wu et al. 2009; Shang et al. 2010). The seeds were frozen in liquid nitrogen, ground in a pre-chilled mortar with a pestle to a fine powder and transferred to a 1.5 mL tube. The extraction buffer consisted of 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.1% (v/v) Triton X-100, 10% (v/v) glycerol and a protein inhibitor cocktail (Roche). The extraction buffer was added to the tube (20 mL buffer/g sample) and was placed on ice for 2 h. The mixture was centrifuged for 10 min at 12,000 rpm at 4 °C to remove insoluble material, and the supernatant was transferred to a defatted column (PEXBIO, China, product No. A020208C). After centrifugation for 2 min at 12,000 rpm at 4 °C, the sample in the collection column was ready for use.

SDS–PAGE and protein gel blot analysis were performed essentially according to previously described procedures (Wu et al. 2009; Shang et al. 2010). Specific antibody against CAT was purchased from Agrisera (Stockholm, Sweden; website: http://www.agrisera.com; product No. AS09501).

**Catalase Activity Assays**

Protein was extracted from imbibing seeds, which were chilled for 3 days at 4 °C to stimulate germination and then placed at 22 °C under a 16-h light/8-h dark photoperiod for 24 h with different treatment. The supernatant was used as the crude extract and catalase activity was tested with the Catalase Activity Assay Kit (Beyotime, China, product No. S0038). The imbibition seeds which were chilled for 3 days at 4 °C to stimulate germination and then placed at 22 °C under a 16-h light/8-h dark photoperiod for 24 h were ground into powders. Then the seed powder was added to the tube by 20 mL lysis buffer of Hydrogen Peroxide/g sample. The samples were centrifuged at 4 °C, 12,000×g for 5 min to gather the supernatants for the following tests. A 50 μL volume of each supernatant was then transferred to a 96-well microtiter plate. A 100 μL volume detection solutions of Hydrogen Peroxide was added to each well and thoroughly mixed with the supernatant. After incubation at room temperature for 30 min, the plates were read using a spectrophotometer (Synergy™ H1, Biotek, USA) at a wavelength of 560 nm. The concentration of H2O2 was calculated according to standard concentration curve originated from standard solutions upon the identical experiments. All the operations were carried out on ice.

**Determination of H2O2 content**

H2O2 in seeds was quantified using the Hydrogen Peroxide Assay Kit according to the manufacturer’s instructions (Beyotime, China, product No. S0038). The imbibition seeds which were chilled for 3 days at 4 °C to stimulate germination and then placed at 22 °C under a 16-h light/8-h dark photoperiod for 24 h were ground into powders. As defined as the quantity of enzyme catalyzing the decomposition of 1 mM H2O2 per minute.

**Yeast one-hybrid assay**

Yeast one-hybrid assay was performed essentially as previously described (Shang et al. 2010) with the kit provided by Clontech (Matchmaker™ One-Hybrid Library Construction & Screening Kit CATALOG No. 630304) using the Y187 yeast strain according to the manufacturer’s instructions. The related cDNAs or promoter DNAs were amplified by PCR using the primer pairs as supplemental Table 3. The promoter fragments of CAT1 and CAT2 were sub-cloned into the Smal/MluI sites of pHIS2 vector. ABI5 cDNA was sub-cloned into the EcoRI/BamHI sites of pGADT7 vector. Yeast cells were co-transformed with pHIS2 bait vector that harbored promoter of target genes and pGADT7 prey vector that contained ORF of ABI5. As negative controls, the yeast cells were transformed with pGADT7-ABI5 vector with pHIS2-p53 (Wei et al. 2006) or pHIS2-CAT2 harboring the corresponding promoter. Transformed yeast cells were grown in the tryptophan (Trp) and leucine (Leu)-deficient SD medium (SD–Trp–Leu) to ensure that the yeast cells were successfully co-transformed, and then the yeast cells were grown on the plates of the tryptophan (Trp), leucine (Leu) and histidine (His)-deficient SD medium (SD–Trp–Leu–His) supplemented with 3-AT (Sigma) at 0, 10, 20, and 50 mM. The plates were then incubated at 30°C for 3 days for investigations.
Electrophoretic mobility shift assay (EMSA)

EMSA was performed essentially as previously described (Shang et al. 2010) using the recombinant His-ABI5 protein purified from E. coli strain BL21 (DE3) as mentioned above. ABI5 cDNA was sub-cloned into the SalI/EcoRI sites of pMAL-c5X vector (NEB). The biotin-labeled probes used for this EMSAs were amplified by PCR using the following primer pairs as supplemental Table 4. The site-specific mutations of the G-box, named box1 mutation and box2 mutation, were introduced into the CATI promoter sequences by the independent PCR.

Then the labeled probes (20 fmol) were incubated with the recombinant His–ABI5 proteins (1 µg) in a binding buffer solution (25 mM HEPES, 40 mM KCl, 5 mM MgCl2, 1 mM DTT, 1 mM EDTA, and 8% glycerol, pH 8.0) in the presence of 1 µg/µL of poly (deoxyinosinic–deoxycytidylic) sodium salt [poly(dI-dC)] (Sigma, USA, product No. P4929) for 30 min at room temperature. The DNA–protein reaction mixtures were separated on a 5% non-denaturing polyacrylamide gel (19:1 acrylamide:bisacrylamide) in 0.25 mM Tris-borate-EDTA at 4°C, transferred onto a nylon membrane for 1 h at 4°C, exposed under ultra-violet light to cross-link the samples to the membrane for 2 min. All the following procedures were performed by using the Light Shift Chemiluminescent EMSA kit (Thermo Scientific, product No. 89880) according to the manufacturer’s instructions. The mutation probes were verified by sequence analysis. Competition experiments were performed using 20-fold unlabelled fragments.

Chromatin coimmunoprecipitation (ChIP) analysis

The experiment was performed as described previously (Shang et al. 2010), using the seedlings of the 2-week-old ABI5OE-Myc transgenic plants and the wild-type (Col-0) plants for the ChIP assay. Immunoprecipitation was performed by using Rabbit anti-Myc-tag mAb-Magnetic beads (MBL, product No. M047-11). To determine quantitatively ABI5 binding to the CATI promoter, real-time PCR analysis was performed according to a procedure described previously with the Actin 2 3′-untranslated region sequence as the negative control (Shang et al. 2010). The primers used for real-time PCR analysis were listed in Supplementary Table S6. The experiment was repeated for three biological repeats with similar results.

Cis-activation of CAT1 promoter activity by ABI5 in tobacco leaves

This assay was performed essentially according to the previously described procedures (Shang et al. 2010). ABI5 was used for the effector construct. The promoter of the CATI gene was amplified by PCR using the following primer pairs as supplemental Table S5. Then the reporter construct was composed of the CATI promoter linked to LUC. The cDNA of the ABI5 gene was fused to pCAMBIA1300-Myc vector downstream of the CaMV 35 S promoter and Myc tag. The cDNA of GUS and GFP were fused to pCAMBIA1300-Myc vector downstream of the CaMV 35 S promoter, which was used as an internal control. The constructs were mobilized into A. tumefaciens strain GV3101. Bacterial suspensions were infiltrated into young but fully expanded leaves of the 7-week old N. benthamiana plants using a needleless syringe. It is noteworthy that the amounts of the constructs were the same among treatments and controls for each group of assay. After infiltration, plants were grown firstly under dark for 24 h and then with 16 h light/day for 60 h at room temperature. The LUC activity was observed with a CCD imaging apparatus (Andor iXon, Andor, UK).

RNA sequencing and bioinformatics analysis of RNA-Seq data

For RNA sequencing (RNA-Seq) analysis, total RNA was isolated from imbibed seeds of wild type Col-0 and abi5-1 plants, which had been chilled for 3 days at 4°C to stimulate germination and then placed at 22°C under a 16-h light/8-h dark photoperiod for 24 h. Total RNA was isolated using a seed RNA Extraction Kit (BioTeke, China, product No. RP1201), treated with RNase-free DNase I (NEB, USA, product No. M0303) at 37°C for 30 min to degrade genomic DNA, and purified by using an RNA Purification Kit (BioTeke, China, product No. RP1801). The construction of RNA libraries, RNA sequencing and bioinformatics analysis were performed at Bionova company (Beijing, China). For each sample, three biological replicates were sequenced. The raw sequence reads have been deposited into the GEO database under accession number GSE90004.

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Author contributions X-FW initiated the research and prepared the article with input from all the authors. CB designed and conducted all the experiments. Other authors offered experimental instructions.

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Compliance with ethical standards

Disclosures  Xiao-Fang Wang initiated the research and prepared the article with input from all the authors. Chao Bi designed and conducted all the experiments. Other authors offered experimental instructions.

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