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ORIGINAL ARTICLE

The SNF5-type protein BUSHY regulates seed germination via the gibberellin pathway and is dependent on HUB1 in Arabidopsis

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Abstract

Main conclusion **The SNF5-type protein BUSHY plays a role in the regulation of seed germination via the gibberellin pathway dependent on HUB1 in *Arabidopsis thaliana*.**

Abstract SWITCH/SUCROSE NONFERMENTING (SWI/SNF) complexes play diverse roles in plant development. Some components have roles in embryo development and seed maturation, however, whether the SNF5-type protein BUSHY (BSH), one of the components, plays a role in *Arabidopsis* seed related traits is presently unclear. In our study, we show that a loss-of-function mutation in BSH causes increased seed germination in *Arabidopsis*. *BSH* transcription was induced by the gibberellin (GA) inhibitor paclobutrazol (PAC) in the seed, and BSH regulates the expression of GA pathway genes, such as *Gibberellin 3-Oxidase 1* (*GA3OX1*), *Gibberellic Acid-Stimulated Arabidopsis 4* (*GASA4*), and *GASA6* during seed germination. A genetic analysis showed that seed germination was distinctly improved in the *bshga3ox1ga3ox2* triple mutant, indicating that BSH acts partially downstream of GA3OX1 and GA3OX2. Moreover, the regulation of seed germination by BSH in response to PAC is dependent on HUB1. These results provide new insights and clues to understand the mechanisms of phytohormones in the regulation of seed germination.

Keywords Chromatin remodeling · Gibberellic acid · Seed germination · Switch nonfermenting complexes

Abbreviations

BSH	SNF5-type protein BUSHY
GA	Gibberellin
HUB1	HISTONE MONOUBIQUITINATION1
PAC	Paclobutrazol
SWI/SNF	SWITCH/SUCROSE NONFERMENTING complex

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Introduction

Seed germination is a crucial process in the plant life cycle that allows the next generation to enter the ecosystem. In agriculture, uniform seed germination that occurs at the proper time is also necessary for high crop quality and yield. Many studies have shown that seed germination is a complicated process that is regulated by various genetic and environmental factors (Koornneef et al. 2002; Chen et al. 2008; Finkelstein et al. 2008; Bassel et al. 2011).

Previous studies have shown that gibberellin (GA) plays a pivotal role in promoting the seed germination and seedling establishment in *Arabidopsis* (Davies 1995; Hauvermale and Steber 2020). Application of exogenous paclobutrazol (PAC) and uniconazole, two GA synthesis inhibitors, represses seed germination, which suggests that newly synthesized GAs are required for radicle emergence (Nambara et al. 1991; Jacobsen and Olszewski 1993). This viewpoint is also supported by the fact that several GA-deficient mutants such as *ga1-3* and *ga2-1* show germination defects (Koornneef and Van der Veen 1980). Several GA-signaling components have been identified using

genetic approaches in *Arabidopsis*, and some of them play roles in seed germination, as shown by their loss-of-function mutant phenotypes (Olszewski et al. 2002). Among these, the DELLA proteins REPRESSOR OF GA (RGA), GIBBERELLIN INSENSITIVE (GAI), and RGA-LIKE 2 (RGL2) play negative roles in seed germination (Penfield et al. 2006; Finkelstein et al. 2008; Rajjou et al. 2012). The three GA receptors, GID1a, GID1b, and GID1c, positively regulate GA responses via GA/GID1-stimulated destruction of DELLA repressors. Genetic analysis showed the *gid1abc* triple mutant failed to germinate in the dark, and GID1c is a key downstream positive regulator of dark germination, which suggests that the three GID1 receptors display partial function differentiation in GA signaling (Ge and Steber 2018).

Endosperm cell separation plays a critical role in seed germination as a physical mechanism. Recently, a study showed that endosperm cell expansion is spatiotemporally controlled by the cell expansion gene *EXPANSIN 2* (*EXPA2*). The NAC transcription factors NAC25 and NAC1L function directly upstream of EXPA2. The DELLA protein RGL2 inhibits NAC-mediated transactivation and expression of activation of EXPA2, illuminating a key role of the GA/DELLA-NAC25/NAC1L-EXPA2 module in regulating endosperm cell expansion to mediate seed germination (Sanchez-Montesino et al. 2019). Furthermore, a cell wall-localized protein, Gibberellic Acid-Stimulated Arabidopsis 6 (AtGASA6) promotes seed germination and length of embryonic axis, which results from a promotion of cell elongation regulated by AtEXPA1. Intensive studies implied that AtGASA6 links the RGL2 and AtEXPA1 functions and exerts as an integrator of GA, abscisic acid (ABA), and glucose signaling to regulate seed germination through a promotion of cell elongation (Zhong et al. 2015). Furthermore, SPINDLY, a Ser/Thr O-linked N-acetylglucosamine transferase, also acts as a negative regulator of GA-dependent seed germination (Jacobsen and Olszewski 1993). SLEEPY1 (SLY1) is the F-box subunit of an SCF E3 ubiquitin ligase that degrades DELLA through polyubiquitination mediated by 26S proteasome and is thought to be a positive regulator of the GA pathway (Steber et al. 1998). Conversely, the application of exogenous ABA inhibits seed germination, and mutants defective in ABA biosynthesis or signaling, such as *aba1* and *aba2*, show enhanced germination efficiency (Finkelstein and Somerville 1990; Finkelstein and Lynch 2000). The first ABA biosynthesis mutants identified and isolated were initially screened as suppressors of the non-germinating GA-deficient mutants (Koornneef et al. 1982), illustrating the antagonism of ABA and GA in seed germination. The mechanisms by which GA, as a crucial positive regulator, regulate seed germination are largely understood; however, the upstream regulatory mechanisms of GA are not very clear.

Chromatin modifications have been shown to play roles in seed germination. Loss of function of the KRYPTONITE/SU(VAR)3-9 HOMOLOG4 (KYP/SUVH4), which encodes a histone methyltransferase required for histone 3 lysine 9 dimethylation (H3K9me2), results in decreased seed germination in response to ABA and the GA biosynthesis inhibitor PAC (Jackson et al. 2002; Zheng et al. 2012). After-ripened seeds of the *histone deacetylase 9* (*hda9*) mutant germinate faster than wild type, indicating a role for histone acetylation in seed germination (van Zanten et al. 2014). Moreover, histone deacetylation, mediated by SIN3-Like1 (SNL1) and SNL2, repress *AUXIN RESISTANT1* expression to regulate seed germination (Wang et al. 2016). Two histone arginine demethylases, JUMONJI DOMAIN-CONTAINING PROTEIN 20 (JMJ20) and JMJ22, regulate seed germination positively and redundantly by decreasing histone arginine methylation at *GA3OX1*/*GA3OX2* (Cho et al. 2012). HISTONE MONOUBIQUITINATION1 (HUB1), a C3HC4 RING finger protein, regulates seed dormancy positively through histone H2B monoubiquitination, but the detailed mechanism associated with HUB1 is not clear (Liu et al. 2007). SWITCH/SUCROSE NONFERMENTING (SWI/SNF) complexes play diverse and important roles in plant development by remodeling the structure and functions of chromatin (Amedeo et al. 2000; Noh and Amasino 2003; Tang et al. 2008). The minimal functional core of conserved SWI/SNF complexes consists of an SWI2/SNF2 ATPase, SNF5, SWP73, and a pair of SWI3 subunits (Phelan et al. 1999; Sudarsanam and Winston 2000). Mutations in ATSWI3A and ATSWI3B can cause arrested embryo development at the globular stage (Sarnowski et al. 2002). In contrast, mutations in ATSWI3C and ATSWI3D do not prevent embryonic development but affect the development of vegetative and reproductive organs (Sarnowski et al. 2002). In vivo and in vitro studies show that ATSWI3C can mediate GA perception and synthesis by interacting with DELLA proteins (Sarnowska et al. 2013). BRAHMA, an SNF2-like chromatin-remodeling ATPase protein, is involved in the repression of seed maturation associated with an SNF5-Like protein—BUSHY (BSH) (Tang et al. 2008). BRAHMA also can repress ABA responses by suppressing *ABI5* expression in Arabidopsis (Han et al. 2012). However, in contrast to the multiplicity of SNF2-like ATPase proteins and SWI3 homologs, Arabidopsis has only one copy of the SNF5-like gene *BSH* (Brzeski et al. 1999). BSH, the smallest SNF5-type protein so far identified, can complement the yeast *snf5* mutant. Transgenic Arabidopsis plants with decreased *BSH* transcript levels display a significant bushy growth phenotype and are defective in seed production (Brzeski et al. 1999). As shown above, SWI/SNF chromatin-remodeling complexes are involved in seed germination by mediating

phytohormone pathways, and also indicate the potential role of BSH in embryo or seed development. However, the biological role of BSH in seed germination is still unclear in Arabidopsis.

In this study, we report that BSH, a possible interactor of HUB1, plays an important role in seed germination by regulating the GA pathway. Loss of function of BSH increases the seed germination potential by regulating GA synthesis and the signaling pathway. Genetic analyses indicated that HUB1 displays epistatic to BSH in regulating seed germination. The results of our study shed more light on the complicated relationship between the GA pathway and epigenetic modifications in seed germination.

Materials and methods

Plant materials and growth conditions

All experiments were performed with *Arabidopsis thaliana* Columbia (Col-0) wild-type plants or mutants in the Col-0 background. The T-DNA insertion line *bsh-2* (SALK_073635) was obtained from the ABRC (Arabidopsis Biological Resource Center), and homozygous individuals were identified by PCR-based screening. Gene-specific primers were designed using the SIGNAL T-DNA verification primer design program and were used in combination with T-DNA left border primers. Reverse transcription-polymerase chain reaction (RT-PCR) with RNA isolated from leaves was performed to confirm the homozygous knockout lines. Semi-quantitative PCR was performed with 25 cycles of amplification for *ACTIN8* and 35 cycles for *BSH*. The PCR primers used here are listed in Suppl. Table S1. The mutants *hub1-5* and *ga3ox1ga3ox2* were described previously (Mitchum et al. 2006; Liu et al. 2007). The double mutant *bush-2hub1-5* and triple mutant *bsh-2ga3ox1ga3ox2* were obtained by standard crossing procedures. Seeds were sown in soil and grown in the greenhouse under a 16 h light/8 h dark photoperiod at 18–22 °C. Seeds sown on 0.5X Murashige and Skoog (MS) medium were first sterilized with 10% (v/v) NaClO. Plates were kept in the dark at 4 °C for 3 d to break dormancy (stratification) and were then moved into a climate chamber with a 16 h light/8 h dark (long-day) photoperiod at 22 °C.

Germination tests

Germination tests were performed as described by Alonso-Blanco et al. (2003). Germination was defined as radical emergence from the seed coat. All germination experiments were performed on filter paper in 6-cm Petri dishes. Filter papers were soaked with either control solution or solutions containing GA_{4+7}, the GA biosynthesis inhibitor PAC, or ABA. Each genotype had at least four seed pools or eight individual plants; 80–100 seeds per Petri dish from one individual plant or a seed pool were used in the seed germination tests. The average germination ratio was determined after 7 d of incubation in a climate room (25 °C, 16 h light with an intensity of 80–90 µmol m^{-2} s^{-1}). Seeds for each germination assay were collected from plants of different genotypes grown simultaneously and stored under identical conditions.

Constructs and plant transformation

The full genomic sequence of *BSH* was amplified with the primer pair BSH-GF/-GR (Suppl. Table S1) from DNA extracted from Arabidopsis Col young leaves using the Super Plant Genomic DNA Kit (Tiangen). The full *BSH* genomic fragment was then inserted into the *pBAR* vector between the HindIII and EcoRI restriction sites. Plants containing the *bsh-2* mutation were transformed with *Agrobacterium tumefaciens* strain GV3101 harboring the *BSH* genomic fragment using the floral dip method (Clough and Bent 1998). Transformants were selected based on their ability to survive for 7 days on MS medium containing 10 mg/L DL-phosphinothricin. Transformant lines segregating 3:1 were selected on MS medium containing 10 mg/L DL-phosphinothricin. Homozygous T_3-generation transgenic plants were used for phenotypic analyses. All of the DNA constructs used in this study were confirmed by sequencing.

RNA extraction and real-time PCR

Total RNA was extracted from seedlings or seeds using the RNAprep Pure Plant Plus Kit (Tiangen) according to the manufacturer's instructions. RNA was reversely transcribed into cDNA using the SuperScript IV One-Step RT-PCR System (Thermo Fisher). Quantitative real-time PCR (Q-PCR) was performed using SYBR Premix Ex Taq (TaKaRa). The expression value for each gene was quantified using a standard curve with a serial dilution of the plasmid of known concentration, and they were normalized to the expression of *ACTIN8*. Each experiment included at least three biological replicates. The sequences of the primers used are given in Suppl. Table S1. Mean expression values and standard errors were calculated from the three biological replicates and are shown in the figures.

Yeast two-hybrid assay

The *HUB1* and *BSH* cDNAs were amplified using Phusion® DNA Polymerase (NEB) with primer pairs *HUB1-F/-R* and *BSH-F/-R,* respectively (Suppl. Table S1). The PCR products were cloned into a Gateway Entry vector (Invitrogen), then fused in-frame with the GAL4 activation domain of the *pAD* (*pACT2-attR*) vector and the binding domain of the

pBD (*pAS2-attR*) vector to generate prey and bait plasmids, respectively (modified from Clontech). This pair of bait and prey plasmids were co-transformed into yeast AH109 cells using the lithium acetate method (Clontech) and analyzed for yeast growth on a selective medium lacking histidine, leucine, tryptophan, and adenine at 28 °C for 2 to 4 days. Positive and negative colonies were transferred to filter paper, permeabilized in liquid nitrogen, and assayed for expression of the *lacZ* reporter gene by staining for β-galactosidase activity in a solution containing the X-gal substrate. The plates containing the filter papers were incubated at room temperature for 3 h. The color of the colonies on the filter paper was monitored and colonies producing β-galactosidase turned blue (Agilent).

Histone extraction and protein gel blot analysis

Histones were isolated from seeds of the wild type, *bsh-2*, *hub1-5,* and *bush-2hub1-5* double mutants, which were collected from plants grown simultaneously and stored under identical conditions. Histone extraction was performed as described (Charron et al. 2009). The proteins were then separated by 12% (w/v) SDS-PAGE, transferred to polyvinylidene fluoride membranes, and probed with anti-H2Bub1 antibody (catalog number 05-1312; Millipore Sigma) and anti-H2B as the loading control (catalog number 07-371; Millipore Sigma). Then, the immunoblots were hybridized to peroxidase-conjugated secondary antibodies (Upstate; Sigma-Aldrich) and incubated with enhanced chemiluminescence reagents (Applygen Technologies, Beijing, China). The signals were detected by BioMax XAR film (Kodak) with exposure times selected to be in the linear range of detection.

Results

BSH is a potential interaction factor of HUB1 that negatively influences seed germination

Previous studies have shown that HUB1 regulates seed germination through histone H2B monoubiquitination, which plays roles in regulating the expression of genes related to the ABA and GA pathways (Liu et al. 2007). However, the molecular network of HUB1 is not well characterized. We identified several potential interacting factors including BSH, an SNF5-Like factor that is a component of the conserved SWI/SNF complexes, from the Arabidopsis Interactions Viewer database on the BAR website (http://bar.utoronto.ca/interactions/cgi-bin/arabidopsis_interactions_viewer.cgi), which is based on yeast two-hybrid screens or traditional biochemical methods. For further verification, we tested the interaction between HUB1 and BSH in a yeast two-hybrid assay. The HUB1 protein showed strong interaction with BSH on high stringency SD/-His/-Leu/-Trp/-Ade medium, which was confirmed by the chromogenic marker X-α-Gal using the Filter Lift Assay (Suppl. Fig. S1). Therefore, we hypothesize that BSH is also involved in seed germination. We determined the relative *BSH* transcript levels during different stages of seed development by real-time PCR. The results showed that *BSH* expresses at high level during seed maturation, with an expression peak occurring in developing seeds at 15 days after pollination (Fig. 1a and Suppl. Fig. S2c), indicating that BSH may play a role in seed dormancy initiation to control seed germination. We also obtained the *bsh-2* loss-of-function mutant (salk_073635) from the ABRC, and identified homozygous mutant plants

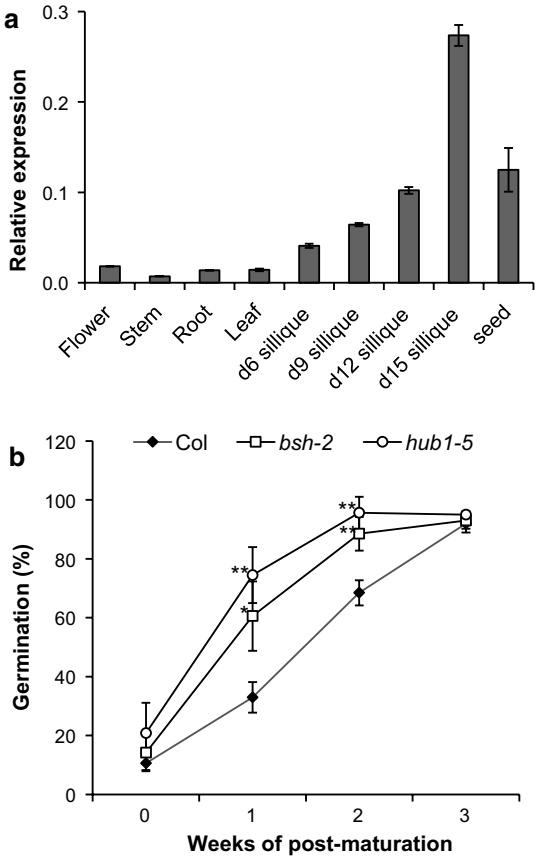

Fig. 1 BSH is expressed at high levels during seed development and is involved in seed germination. **a** qRT-PCR analysis of *BSH* in different plant tissues and developing siliques. The X-axis shows different plant tissues and siliques at several developmental stages after pollination. The mean values and SE were calculated from three biological replicates. *ACTIN8* was used as internal control for normalization of gene expression. D, days after pollination. **b** Germination of freshly harvested seeds from Col-0 (wild type) and the *hub1-5* and *bsh-2* mutants. Percentages of seed germination are the means ± SE based on at least eight individual plants for each genotype. The double asterisk and single asterisk indicate a significant difference compared to the WT as determined by Tukey's HSD test (**$P < 0.01$ and *$P < 0.05$)

using a PCR-based method (Suppl. Fig. S2a, b) (Tang et al. 2008). The T-DNA insertion is located in the 3' part of the *BSH* coding region, and it may only disrupt the C-terminal Box B, not the SNF5 domain (Tang et al. 2008). Seed germination was determined using seeds harvested at the same time. The *bsh-2* mutant showed significantly increased seed germination compared to Col (Fig. 1b). These results indicate that BSH is negatively involved in seed germination in *Arabidopsis thaliana*.

BSH is involved in the GA pathway to regulate seed germination

The balance between ABA and GA plays a pivotal role in regulating seed germination (Koornneef et al. 2002; Finkelstein et al. 2008). Therefore, seed germination of *bsh-2* mutant in response to ABA and PAC (as an inhibitor of GA biosynthesis) was tested. The results showed that *bsh-2* displayed significantly decreased sensitivity in response to PAC, but not to ABA. The addition of bioactive GA recovered seed germination of the *bsh-2* mutant in response to PAC (Fig. 2 and Suppl. Fig. S3), indicating that BSH is involved in the GA pathway to control seed germination. To verify the role of BSH in seed germination and the GA pathway, complementation lines were constructed. The genomic sequence of the *BSH* gene, including 1,200 bp upstream of the ATG, 2,279 bp of coding sequence, and 101 bp of the 3'-untranslated region (UTR), was introduced into the *bsh-2* mutant. The complementation lines (*Comp-1* and *Comp-2*) showed similar seed germination levels as wild type (Fig. 3a). The real-time PCR and semi-quantitative PCR assays showed that the levels of *BSH* transcripts in the seeds of the *Comp-1* and *Comp-2* were similar to the Col-0 control (Fig. 3b and Suppl. Fig. S4). Moreover, seed germination of *Comp-1* and *Comp-2* showed a similar sensitivity in response to PAC treatment as wild type (Fig. 3c).

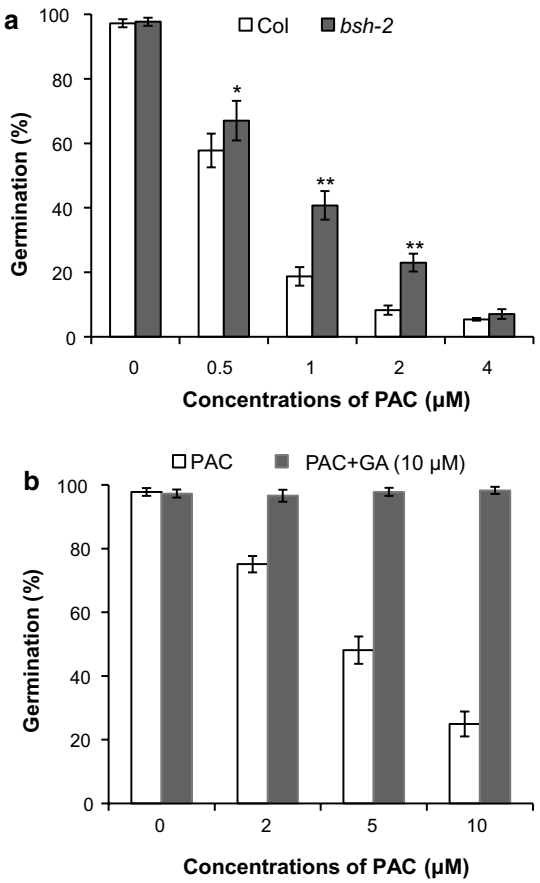

Fig. 2 Seed germination of *bsh-2* shows reduced sensitivity to paclobutrazol. **a** Germination phenotype of *bsh-2* and Col-0 in response to different concentrations of paclobutrazol (PAC). **b** Seed germination of *bsh-2* in 10 μM GA_{4+7} solution with different concentrations PAC. Percentages of seed germination are the means ± SD based on four seed pools for each genotype. The double asterisk and single asterisk indicate a significant difference compared to the WT as determined by Tukey's HSD test (**$P < 0.01$ and *$P < 0.05$)

Loss of function of BSH improves seed germination of GA-deficient mutants

To further address the relation between BSH and the GA pathway, real-time PCR was used to measure the transcript levels of several GA pathway-related genes in the *bsh-2* and complementation lines. The results showed that *Gibberellin 3-oxidase 1* (*GA3OX1*), a central gene in GA synthesis, was distinctly up-regulated, as were two important *GA-Stimulated in Arabidopsis* (*GASA*) genes, *GASA4* and *GASA6*. The transcript levels of these genes in the complementation lines *Comp-1* and *Comp-2* were similar to wild type (Fig. 4). To analyze the genetic relation of BSH with GA biosynthesis genes, the GA synthesis deficient double mutant *ga3ox1ga3ox2* was combined with *bsh-2*. The homozygous triple mutant *bsh-2ga3ox1ga3ox2* was identified and seed germination was measured. The results showed that *bsh-2ga3ox1ga3ox2* plants exhibited a clear increase in seed germination (18%) compared to the *ga3ox1ga3ox2* mutant (2%) without exogenous GA (Fig. 5a), indicating that the BSH-regulated GA pathway is only partially dependent on GA3OX1 and GA3OX2 for seed germination (Mitchum et al. 2006). To verify this, bioactive GA_{4+7} was used to quantify seed germination of the *bsh-2ga3ox1ga3ox2* and *ga3ox1ga3ox2* mutants. The results showed that *bsh-2ga3ox1ga3ox2* seeds germinated completely in the presence of 0.1 μM GA_{4+7}, while *ga3ox1ga3ox2* seeds require 10 μM bioactive GA for complete germination after 96 h (Fig. 5b). In our experiments, *bsh-2ga3ox1ga3ox2* always showed distinct germination improvement compared to *ga3ox1ga3ox2*, suggesting that

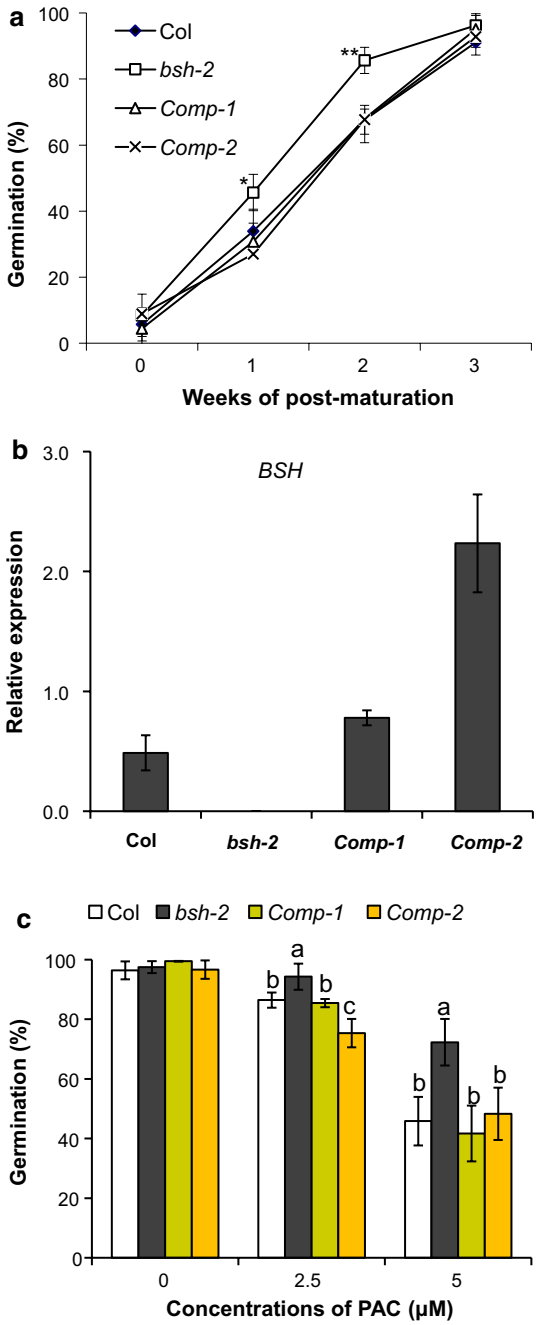

Fig. 3 Complementation of *bsh-2*. **a** Seed germination of two independent homozygous complementation lines *Comp-1* and *Comp-2*. Percentages of seed germination are the means ± SE based on at least eight individual plants for each genotype. The double asterisk and single asterisk indicate a significant difference compared to the WT and complementary lines as determined by Tukey's HSD test (**$P < 0.01$ and *$P < 0.05$). **b** *BSH* transcription in mature seeds of Col-0, *bsh-2,* and the complementation lines *Comp-1* and *Comp-2* determined by qRT-PCR. **c** Germination phenotypes of Col-0, *bsh-2*, *Comp-1,* and *Comp-2* in response to PAC treatment. Percentages of seed germination are the means ± SD based on four seed pools for each genotype. Different letters at the same PAC concentration indicate a significant difference as determined by Tukey's HSD test ($P < 0.05$)

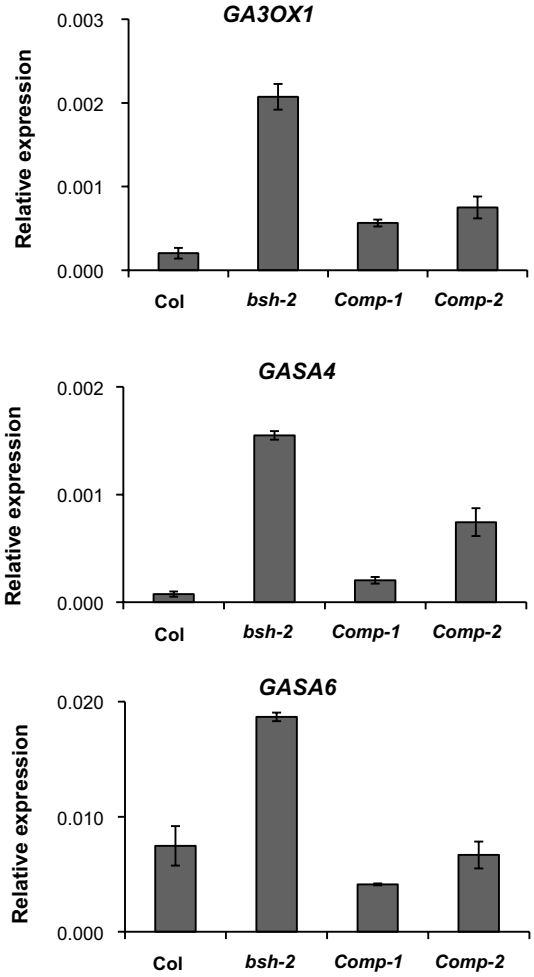

Fig. 4 Transcript levels of key genes associated with the GA pathway in Col-0, *bsh-2*, and the two complementation lines. qRT-PCR analysis of the expression of the GA pathway-related genes *GA3OX1*, *GASA4,* and *GASA6* in the *bsh-2* mutant and the complementation lines. Total RNA was extracted from freshly harvested seeds. The primers used are given in Suppl. Table S1. The mean values and SD were calculated from three biological replicates, and *ACTIN8* was used as the internal control

bsh-2ga3ox1ga3ox2 seeds require less GA to complete germination than *ga3ox 1ga3ox2* seeds (Fig. 5b).

BSH-mediated regulation of seed germination through the GA pathway is dependent on HUB1

We have demonstrated that there is a direct physical interaction between HUB1 and BSH using the yeast two-hybrid assay (Suppl. Fig. S1). To further uncover the relationship between these two genes, we created the *bush-2hub1-5* double mutant. The seed germination phenotype of the double mutant was very similar to that of *hub1-5* but not to *bsh-2* (Fig. 6a). After PAC treatment, the *hub1-5* and *bush-2hub1-5* mutants displayed similar seed germination phenotypes to

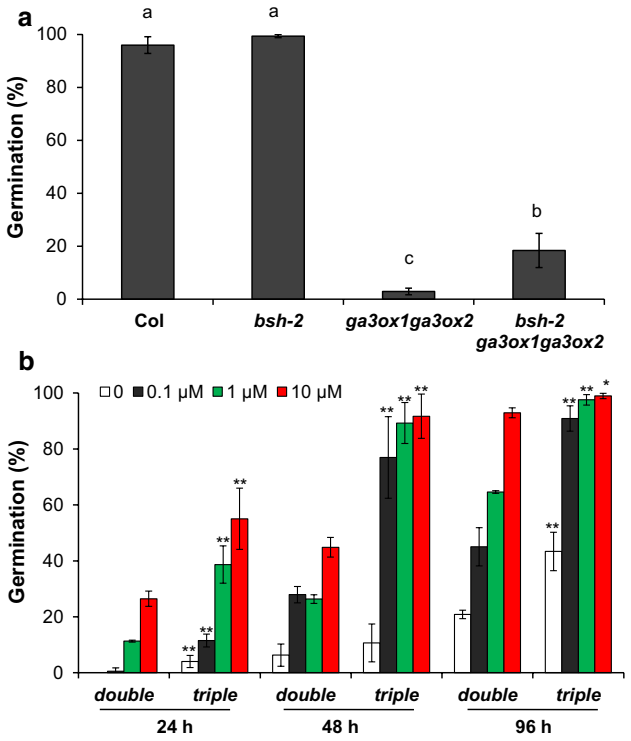

Fig. 5 *bsh-2* mutant improves seed germination of the GA-deficient double mutant *ga3ox1ga3ox2*. **a** Seed germination of Col, *bsh-2*, *ga3ox1ga3ox2* and *bsh-2ga3ox1ga3ox2*. Percentages of seed germination are the means ± SD based on at least four seed pools for each genotype. Different letters indicate a significant difference as determined by Tukey's HSD test ($P < 0.05$). **b** Seed germination of the *ga3ox1ga3ox2* double mutant and the *bsh-2ga3ox1ga3ox2* triple mutant in response to GA_{4+7}. Seed germination percentages were counted and analyzed at 24 h, 48 h, and 96 h. Percentages of seed germination are the means ± SD based on at least four seed pools for each genotype. Single and double asterisks indicate significant differences compared to the *ga3ox1ga3ox2* double mutant as determined by Tukey's HSD test (*$P < 0.05$ and **$P < 0.01$)

Col-0 but not to the *bsh-2* mutant (Fig. 6b). These results indicate that BSH requires HUB1 in its control of germination and for its role in the GA pathway. The regulation of seed germination by HUB1does not only involve the GA pathway but also other pathways (*e.g.*, *NCED9*, *CYP707A1*) (Liu et al. 2007), because the *hub1-5* mutant showed the same seed germination phenotype as Col-0 in response to PAC treatment as before (Peeters et al. 2002).

Discussion

HUB1 (RDO4) regulates seed germination by histone H2B monoubiquitination, which is an important histone modification with roles in DNA replication and gene activation (Hicke 2001; Liu et al. 2007). However, the direct interactors of HUB1 and their underlying mechanism remain unclear. Screening for protein–protein interactions using the yeast two-hybrid assay is a classic method to identify potential interacting factors. Using this technology, BSH was identified as a protein that interacts directly with HUB1 (http://bar.utoronto.ca/interactions/cgi-bin/arabidopsis_interactions_viewer.cgi), which was confirmed by our interaction studies (Suppl. Fig. S1). In the yeast two-hybrid assay, we also observed self-interaction of BSH (Suppl. Fig. S1), indicating that BSH may function as a dimer. However, we could not verify this interaction or self-interaction by bimolecular fluorescence complementation (BiFC). The reason for this could be that the fluorescent peptides fused with HUB1 and/or BSH might interfere with the protein interaction. Alternatively it is also possible that the complexity of the SNF complex inhibits the functional restructuring of the two parts of the fluorescent peptides.

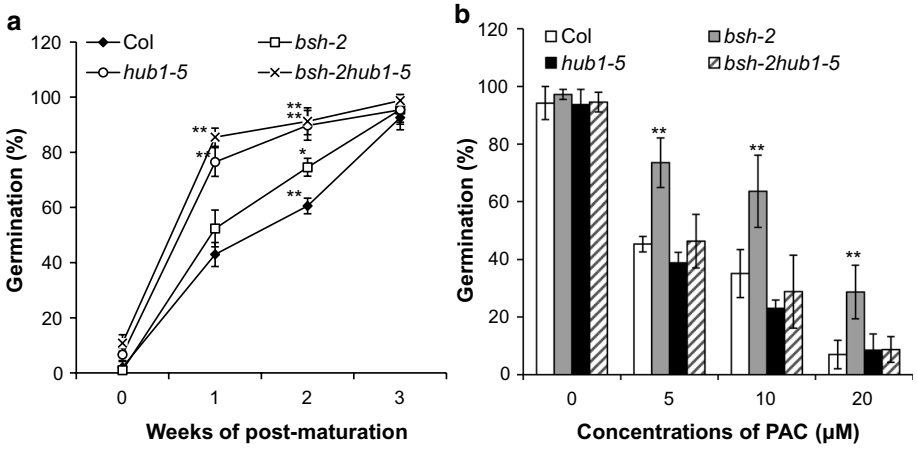

Fig. 6 BSH functions in seed germination via the GA pathway and depends on HUB1. **a** Seed germination of Col, *bsh-2*, *hub1-5* and *bsh-2hub1-5* at different seed storage times. **b** Seed germination of the mutants in response to PAC after stratification at 4 °C for three days. Values are the means ± SE based on at least eight individual plants for each genotype. Single and double asterisks indicate significant differences compared to Col-0 as determined by Tukey's HSD test (*$P < 0.05$ and **$P < 0.01$)

BSH is involved in the GA pathway to regulate seed germination

The interaction of BSH with HUB1 indicates that it might have a similar role in plant development. BSH is a nuclear-localized protein, but it does not show specific transcriptional activation in yeast when bound to DNA (Brzeski et al. 1999), indicating that BSH may function as a cofactor but not as an individual transcription factor. Its interaction with SWI3A and SWI3B supports this hypothesis (Sarnowski et al. 2002). Real-time PCR results showed that *BSH* transcripts accumulate during seed development and maturation, and reach a peak level in siliques at 15 days after pollination (Fig. 1a), suggesting that BSH might have a function in seed development. In accordance, the loss-of-function mutants of *BSH* showed increased seed germination ability in freshly harvested seeds, similar to the *hub1* mutant (Fig. 1b). We obtained evidence that BSH regulates seed germination through its involvement in the GA but not in the ABA pathway (Fig. 2 and Suppl. Fig. S3), which may be helpful to uncover the antagonism between GA and ABA in seed germination.

GA is a major positive factor in the promotion of seed germination (Carrera-Castaño et al. 2020). Loss of function of GA3OX1 and GA3OX2, two key factors in bioactive GA synthesis, results in a loss of seed germination ability, indicating the crucial role of GA in this process (Mitchum et al. 2006). Many GA related factors such as GASA4 and GASA6 have been identified to participate in seed germination. Over-expression of both *GASA4* and *GASA6* promotes seed germination in *Arabidopsis*, and the expression of both genes is induced by GA (Zhong et al. 2015; Qu et al. 2016). Furthermore, exogenous GAs are required for *GASA4* gene expression in the GA-deficient mutant *ga1-3* (Aubert et al. 1998). GA-dependent expression of cell wall-related hydrolase-encoding genes during germination has also been reported (Ren and Kermode 2000; Chen et al. 2002; Nonogaki 2008). However, the regulatory mechanism activating or repressing the GA signal is not fully understood. Here, we show that BSH, a chromatin remodeling complex factor, influences the GA pathway and consequently affects seed germination. The *bsh-2* mutant shows increased seed germination with or without seed stratification (Fig. 1b and Suppl. Fig. S5), implying that BSH is a negative regulator of seed germination. To verify this, transgenic complementation lines were constructed by introducing the full genomic sequence of *BSH* into the *bsh-2* mutant. Two independently obtained complementation lines, *Comp-1* and *Comp-2*, show similar germination percentages as the wild type for freshly harvested seeds and for stratified seeds in response to PAC treatments (Fig. 3a, c). Consistent with increased seed germination, the GA synthesis gene *GA3OX1* showed clearly increased transcription in *bsh-2* seeds (Fig. 4). Moreover, the two GA signal-related genes *GASA4* and *GASA6* also showed distinctly increased expression levels in the *bsh-2* mutant (Fig. 4). In a genetic study, the triple mutant *bsh-2ga3ox1ga3ox2* showed a higher seed germination rate than the *ga3ox1ga3ox2* double mutant (Fig. 5a), and the triple mutant required much less exogenous bioactive GA to complete germination than the *ga3ox1ga3ox2* mutant (Fig. 5b). In the *bsh* mutant, there is enhanced expression of GA biosynthesis genes suggesting that BSH influences GA biosynthesis. In the *ga3ox* mutant background, the *bsh* mutant still germinates better which suggests that the GA signaling pathway is also influenced by BSH. Therefore, BSH is probably involved in both the biosynthesis and signaling pathways of GA. GA still can promote seed germination in Col-0 and the *bsh-2* mutant, indicating that BSH may mediate different branches of the GA pathway (Suppl. Fig. S5). Interestingly, the application of exogenous PAC led to an increased expression of *BSH* in seeds (Suppl. Fig. S6). All of the above results strongly suggest that BSH plays an important role in the GA pathway to negatively regulate seed germination. It would be interesting to explore the direct targets of BSH in the GA pathway. Previous studies have shown that a DELLA protein interacts with SWI3C to modulate gibberellin responses in *Arabidopsis* (Sarnowska et al. 2013), suggesting that the GA pathway is regulated by the SNF complex. The BSH protein, a component of the SNF complex, can also interact with SWI3A and SWI3B physically (Sarnowski et al. 2002), which further supports the notion that BSH may be involved in GA pathway regulation via epigenetic modification.

The genetic relationship between HUB1 and BSH

HUB1 is involved in the regulation of seed germination and other plant developmental processes such as leaf color, flower opening and flowering time as well as biotic and abiotic stress tolerance by its regulation of the histone modification H2B monoubiquitination (Liu et al. 2007; Cao et al. 2008; Hu et al. 2014; Zhao et al. 2020; Zhou et al. 2017). Of these phenotypes, the bushiness of *hub1-2* plants is similar to that of transgenic plants over-expressing the reversed *BSH* cDNA. Furthermore, over-expressing the reversed *BSH* cDNA resulted in the production of abortive seeds (Brzeski et al. 1999), indicating that it is possible that BSH and HUB1 have a similar role in plant reproductive development. As a weak allele mutation, the *bsh-2* mutant does not display the abnormal bushy architecture and sterile flowers, which indicates that different domain of BSH protein may play various roles in plant development. To understand the genetic relationship between HUB1 and BSH, we constructed the *bush-2hub1-5* double mutant. Seed germination of homozygous *bush-2hub1-5* plants was similar to *hub1-5* with or without PAC treatment (Fig. 6), indicating that BSH requires HUB1

to regulate seed germination via the GA pathway. In addition, the *bush-2hub1-5* double mutant showed the same early flowering phenotype as *hub1-5* (Suppl. Fig. S7) (Cao et al. 2008). Altogether, these results indicate that BSH functions in the same pathway as HUB1 in seed germination regulation, and HUB1 may be involved in more biological pathways than BSH. This also explains how the loss of function of HUB1 results in more pleiotropic phenotypes than the loss of function of BSH (Fleury et al. 2007; Liu et al. 2007). We checked the levels of ubiquitinated H2B in the *bsh-2* and *bush-2hub1-5* mutant plants, and did not detect a difference between *bsh-2* and wild type, and between *hub1-5* and the *bush-2hub1-5* double mutant (Suppl. Fig. S8). This suggests that BSH does not regulate H2B ubiquitination, even though BSH interacts with HUB1 (Suppl. Fig. S1), and HUB1 functions independent of BSH in genetics (Fig. 6). We hypothesize that BSH might influence the expression of some specific targets such as GA pathway-related genes by interacting with HUB1 and other components of the SNF complex. It will be necessary to determine the biochemical function of the BSH protein in the future to clarify its role in the regulation of seed germination.

Taken together, the results of our study show that BSH regulates seed germination by mediating GA synthesis and signaling pathways, and this action is dependent on HUB1. This work provides important insights into the mechanisms by which phytohormones regulate seed germination in *Arabidopsis thaliana*.

Author contribution statement ZW and Y-XL designed the research. ZW, HC, CZ, and FC performed the experiments. ZW and Y-XL analyzed the data. ZW and Y-XL contributed reagents, materials, and analysis tools. ZW and Y-XL contributed to the discussion and wrote the manuscript. All authors read and approved the manuscript.

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