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HSP70-16 and VDAC3 jointly inhibit seed germination under cold stress in *Arabidopsis*

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Abstract

Abscisic acid (ABA) transport plays a crucial role in seed germination under unfavourable conditions such as cold stress. Both heat shock protein 70 (HSP70) and voltage-dependent anion channel (VDAC) protein are involved in cold stress responses in *Arabidopsis*. However, their roles in seed germination with regard to ABA signaling remain unknown. Here we demonstrated that *Arabidopsis HSP70-16* and *VDAC3* jointly suppress seed germination under cold stress conditions. At 4°C, both *HSP70-16* and *VDAC3* facilitated the efflux of ABA from the endosperm to the embryo and thus inhibited seed germination. HSP70-16 interacted with VDAC3 on the plasma membrane and in the nucleus, and the interplay between HSP70-16 and VDAC3 activated the opening of the VDAC3 ion channel. Our work established a novel function of *HSP70-16* in seed germination under cold stress and a possible association of VDAC3 activity with ABA transportation from endosperm to embryo under cold stress conditions. This study reveals that HSP70-16 interacts with VDAC3 and facilitates the opening of the VDAC3 ion channel, which influences ABA efflux from endosperm to embryo, thus negatively regulates seed germination under cold stress.

KEYWORDS
ABA transport, cold stress, heat shock protein, seed germination, voltage-dependent anion channel protein

1 | INTRODUCTION

Seed germination is a highly complex but well-coordinated process, in which abscisic acid (ABA) plays important regulatory roles (Machin and Bennett, 2020) by preventing seeds from germination under unfavourable conditions (Nakashima and Yamaguchi-Shinozaki, 2013). Two systems for ABA transport across plasma membrane (PM) from the site of its biosynthesis (i.e., the endosperm) to the site of its action (i.e., the embryo) for seed germination have been reported to be active under ambient temperature. One system is composed of four ABCG transporters, in which ABCG25 and ABCG31 export ABA from the endosperm while ABCG40 and ABCG30 import ABA into the embryo in *Arabidopsis* (Kang *et al*., 2015). The other system is the ABA importer OsPM1 (plasma membrane protein1) in rice (Yao *et al*., 2018). The mutants of *abcg25* (Kuromori *et al*., 2010) and *abcg40* (Kang *et al*., 2010) are hypersensitive to ABA, while *abcg30* and *abcg31* (Do *et al*., 2018) as well as the *OsPM1-RNAi* mutant (Yao *et al*., 2018) are hyposensitive to ABA in seed germination. The additionally identified ABA importer AIT1/NRT1.2/NPF4.6 (ABA-Importing Transporter1/Nitrate Transporter1.2/NPF4.6) (Kanno *et al*., 2012) and ABA exporter AtDTX50 (a member of the MATE family) (Zhang, 2014) function mainly in stomatal regulation and stress responses; however, *dtx50* (Zhang, 2014) and *ait1/nrt1.2/npf4.6* (Kanno *et al*., 2012) are hypersensitive to ABA in seed germination

under normal conditions. These findings indicate strong and active control of ABA transport in normal seed germination (Merilo *et al*., 2015). However, these systems are all inhibited under cold stress, and as such the identification of an ABA transport system that can function during seed germination under cold stress is as yet lacking (Kang *et al*., 2015; Park *et al*., 2017).

Voltage-dependent anion channels (VDACs), the most abundant mitochondrial outer membrane (MOM) channel proteins, mediate molecular trafficking of ions and metabolites between mitochondria and cytoplasm (Kanwar *et al*., 2020). Extra-mitochondrial VDACs are also reported in nucleus, endoplasmic reticulum (ER) and PM (Pendle *et al*., 2005; De Pinto *et al*., 2010; Robert *et al*., 2012; Takahashi and Tateda, 2013). VDACs form high and low conductance channels that open at low membrane potentials for trafficking of anions and close at high membrane potentials for trafficking of cations (Colombini, 2012; Kanwar *et al*., 2020). Nevertheless, regulation of VDAC gating is not yet fully understood, particularly in plants (Takahashi and Tateda, 2013). The four *Arabidopsis* VDACs (Tateda *et al*., 2011; Robert *et al*., 2012) are all dually localized to both mitochondria and PM, with VDAC3 being the most abundant on the PM (Robert *et al*., 2012). VDAC1, VDAC2 and VDAC4 function distinctly in mitochondria. However, unlike *vdac1*, *vdac2* and *vdac4* mutants, *vdac3* does not show a mitochondrial phenotype, implying that VDAC3 may play a more prominent role on the PM (Robert *et al*., 2012). Mammalian PM-localized VDACs usually function as scaffolds, functioning together with other PM proteins particularly when responding to exogenous signals (De Pinto *et al*., 2010). However, the role of PM-localized VDAC3 in plants remains as yet unknown.

In mammals, VDACs interact with various proteins and function in a suite of processes such as cell death, cell signaling, cell movement and cellular metabolism (Kanwar *et al*., 2020). Identified VDAC interactors include mitochondrial lipids, ions, metabolites and cytosolic proteins (Rostovtseva and Bezrukov, 2008), such as HSP70s (Hiyama *et al*., 2014). In mammals, HSP70s have been reported to act as chaperones, playing crucial roles in stress responses (Hageman *et al*., 2011), gene transcriptional regulation (Gao *et al*., 2015), intracellular trafficking (Prulière-Escabasse *et al*., 2007) and Ca^{2+} signal transduction (Krieger *et al*., 2006). In *Arabidopsis*, HSP70s are known to participate in diverse physiological processes, such as seed germination under both normal (Gallardo *et al*., 2001) and heat stress (Su and Li, 2008; Chen *et al*., 2019) conditions, yet their involvement in seed germination at low temperature remains elusive (Ray *et al*., 2016). In addition, while HSP70s are known to be involved in diverse signaling pathways including those dependent on ABA (Ray *et al*., 2016), little is known concerning the association of HSP70s and VDACs with ABA signaling in plants. In the current study, we report additional roles of *Arabidopsis HSP70-16* and *VDAC3* in the negative regulation of seed germination under cold stress conditions.

2 | MATERIALS AND METHODS

2.1 | Plant materials and growth conditions

All seeds used in this research were from *Arabidopsis thaliana* ecotype Columbia. Seeds of all TDNA-insertion mutants were obtained from the Salk institute while those of *hsp70-16* complementation line were generated ourselves (Chen *et al*., 2019). Seeds were sterilized, plated on ½ MS plates, and incubated at 4°C for 3 days in the dark. Following stratification plates with seeds were shifted to a growth chamber at 22°C, and 10 days later young seedlings were transferred from the plates to the soil. Plants were grown at 22/18°C under 16/8 h day/night with photon flux density of 100 μE m^{-2} s^{-1}. The plants of both wild-type and mutants were grown under the same condition, and seeds used in the experiments were collected at the same time from the same batch of plants. Seeds after harvesting were kept at 37°C for 3 days and then stored at 4°C.

2.2 | Seed germination

To conduct seed germination experiment, 15 seeds from each genotype were placed on a nylon membrane with a pore size of 0.45 μm. These nylon membranes were placed on ½ MS media plates. To analyse the effect of 4°C stratification, seeds were divided into 4 categories: non-stratified, 24 h stratified, 48 h stratified and completely incubated at stratification temperature of 4°C. Non-stratified seeds were directly incubated at 22°C, 24 h stratified were incubated at 22°C after 24 h stratification, and 48 h stratified seeds were incubated at 22°C after 48 h of stratification, while seeds completely incubated at stratification temperature were incubated at 4°C. The protrusion of radicle was considered as a standard for the germination of seeds. For exogenous ABA application, ABA was added into the ½ MS media, and plates of ½ MS with ABA were prepared from that media. Seeds were placed on nylon membranes, and the nylon membranes were placed on these ABA-complemented ½ MS plates.

2.3 | Protein expression and extraction

HSP70-16 and VDAC3 coding sequences were amplified from the cDNA of wild-type plant using the primers listed in Table S1. HSP70-16 and VDAC3 were ligated into pET28a and PMAL-C2X vectors, using the T4 DNA ligase (New England Bio-Labs) and In-Fusion HD Cloning system (Takara), respectively. The HSP70-16 vector was transformed into the *E.coli* strain Rosetta, and after incubation at 37°C for 3 h, bacteria containing HSP70-16 were supplemented with 0.3 mM IPTG and incubated at 18°C overnight. The VDAC3 containing vector was transformed into *E. coli* strain BL21 (DE3), and after incubation at 37°C for 3 h, bacteria were supplemented with 0.1 mM IPTG and incubated at 22°C for 6 h. Purification of VDAC3 and HSP70-16 proteins was performed according to the instructions of "New England Bio-Labs "and "Novagen" manual, respectively.

2.4 | qRT-PCR analysis

RNA was extracted from whole seeds, seed coats (endosperm plus testa), and embryos using Vazyme Fast-Pure Plant Total RNA Isolation Kit, according to the manual (Vazyme). Primescript RT reagent kit with

a genomic DNA eraser (Takara) was used to synthesize the cDNA from 1 μg of RNA for each sample. RT-qPCR was performed with a lightCycler system (Roche) using Super-Real Pre-Mix Plus (SYBR Green; Tiangen Biotech), according to the manufacturer's instructions. RT-qPCR profiles were generated as previously described by Yu *et al*. (2016). The normalization of data was performed as previously described (Livak and Schmittgen, 2001) using *EUKARYOTIC TRANSLATION INITIATION FACTOR 3K* (*EIF3K*, AT4G33250) as the reference gene. Expression levels of targeted genes were reported as relative level to that of EIF3K in the same tissue (Dekkers *et al*., 2012). Three technical replicates of three biological replicates were measured for each data point. Three F values were obtained from three technical repeats in each biological repeat, and the mean value from each biological repeat was calculated to form the three F values of one biological repeat. Bars indicated the variation of SD of means from each of the biological repeats. Primers used for RT-qPCR are listed in Table S1.

2.5 | Seed coat bedding assay

To perform seed coat bedding assay, one-month-old seeds were first imbibed in water for 1 h, and then seed coats were separated from embryo as previously described (Lee and Lopez-Molina, 2013). Embryos were dissected from *hsp70-16*, *vdac3*, *hsp70-16vdac3*, and wild-type seeds. To prepare seed coat beds 75 seed coats of the corresponding mutants and wild-type were placed on a nylon membrane having pores of 0.45 μm size. The nylon membrane containing these seed coat beds were then placed on ½ MS media complemented with 10 μM PAC to enhance the synthesis of ABA, while 15 embryos of the each corresponding mutant were placed on these seed coat beds.

2.6 | Quantification of ABA

To quantify ABA, seeds of each corresponding mutant and wild-type were imbibed in water for 30 min, and then embryos were separated from seed coats. A nylon membrane having 0.45 μm pore size was placed on 10 μM PAC complemented ½ MS. The seed coats were placed on that nylon membrane and incubated at 4°C for 6 h. Later after 6 h, these seed coats were shifted to 100 μl liquid ½ MS and incubated at 4°C for a further 72 h. The seed coats were separated from liquid ½ MS and washed with ice-cold ½ MS media. ABA was extracted with 80% methanol solution, vacuum dried, and the pellet was re-suspended with Tris-buffered saline buffer. ABA levels were determined using the ELISA Kit method (Jiang *et al*., 2012).

2.7 | Cell culture and transient transfection

Hela cells were cultured in DMEM (Gibco) with 10% FBS (Gibco). Twenty-four hours prior to transfection, the cells were seed into dishes. Plasmids encoding eGFP and VDAC3 channels were co-transfected with Lipofectamine 3000 reagent (Invitrogen) according to the manufacturer's instructions.

2.8 | Electrophysiological recording

To measure the currents of the VDAC3 channels expressed in Hela cells, standard whole-cell patch-clamping experiments were performed at room temperature (23–25°C) using EPC-10 amplifier and Patch Master Software (HEKA). Pipettes with resistance ranging from 2.0 to 5.0 MΩ were pulled using borosilicate glass capillaries (World Precision Instruments). Currents were sampled at 10 kHz and low-pass filtered at 2.0 kHz. The internal solution was as follows (in mM): 140 CsCl, 10 HEPES, 2 EGTA, and 1 NaATP, with pH adjusted to 7.4 by CsOH. The external solution contained (in mM) 140 NMDG-Cl, 10 HEPES and 10 Glucose, with pH adjusted to 7.4 by NaOH. The membrane potentials were held at 0 mV and then stepped to a pre-pulse from −100 to +100 mV with 20 mV increments for 1.5 s to elicit VDAC3 currents. Solutions were switched using a gravity-fed continuous focal perfusion system.

In vitro electrophysiological characterization of VDAC3 was performed by planar lipid bilayer membrane recording. The channel activity of VDAC3 was assayed by adding the purified protein to the bilayer chamber. Lipid bilayers formed from a solution of lipoid E80 (Lipoid GmbH) dissolved in *n*-decane (40 mg/ml) across a 0.2 mm in diameter hole in a delrin cup. The VDAC3 protein (100 ng) or protein buffer was added to the *cis* compartment. Protein was fused to lipoids by a salt gradient between the two chambers in asymmetric KCl solution (*cis/trans*, 300/100 mM). All solutions were buffered by 10 mM HEPES-KOH (pH 7.4). The channel currents were recorded in a voltage-clamp mode using a Warner BC-535 bilayer clamp amplifier (Warner Instruments) filtered at 1 kHz, digitized at 3 kHz. The currents were analysed with pClamp 10.4 software. The single-channel amplitude was determined by fitting to Gaussian functions. Opening times less than 0.5–1.0 ms were ignored. The membrane potential refers to the potential of the *trans* side.

2.9 | Yeast two hybrid

The coding sequences of HSP70-16 and VDAC3 were amplified and cloned into pGBKT7 or pGADT7 (Clontech) and then transformed into the yeast strain AH109. The yeast two-hybrid assays were performed according to the manufacturer's instructions (Clontech).

2.10 | Bimolecular fluorescence complementation (BiFC)

The coding region of HSP70-16 and VDAC3 were amplified and cloned into pXY106-nYFP and pXY104-cYFP plasmids, respectively. The recombinant vectors were co-transformed into *Agrobacterium*

tumefaciens GV3101. After centrifugation, the bacteria were collected and re-suspended in infection solution (10 mM MES, 10 mM MgCl2, and 200 μM acetosyringone) for infiltration (at A600 = 0.6). The prepared suspensions were infiltrated into *Nicotiana tabacum* leaves and grown in the dark for 2 days. Fluorescent eYFP signals were monitored using a Leica SP8 confocal microscope (Leica TCS SP8 X, excitation 514 nm; emission 522–555 nm).

2.11 | Transgenic construct and localization

To develop the transgenic line of HSP70-16-eGFP, a 4159 bp genomic DNA, including the full length *HSP70-16* gene and 870 bp upstream region before start codon as a promoter region was amplified using primers enlisted in Table S1. The amplified fragment was ligated into pCAMBIA-1301 with fused eGFP sequence at the C-terminal of HSP70-16 to make pHSP70-16::HSP70-16::eGFP construct. The vectors construction was confirmed by sequencing and the vectors were subsequently transformed into *Agrobacterium* strain GV3101.Transformation into Columbia wild-type plants was performed using the floral dip method (Zhang *et al*., 2006). Subsequently, positive transgenic plants were selected with Hygromycin B, and the presence of transgenes was also confirmed with PCR using specific primers. For transient expression, the coding sequence of HSP70-16 was amplified using primers reported in Table S1 and then ligated into pHB-35Spro-eGFP vector to make p35S::HSP70-16::eGFP construct. The resulting construct was sequenced and then transformed into *Arabidopsis* wild-type protoplast. For transient transformation, protoplasts were isolated from 6-week-old wild-type *Arabidopsis* leaves by Tape-*Arabidopsis* Sandwich method (Wu *et al*., 2009). For each transfection, 200 μl cells (2×10^5 protoplasts per ml) were incubated with 20 μg total DNA and 220 μl 40% (w/v) PEG4000 solution. Twenty hours after incubation under dim-light conditions, green (eGFP) fluorescence signals were observed and captured using a confocal microscope. Fluorescent signals of eGFP were monitored using a Leica SP8 confocal microscope excitation 488 nm, emission 500–550 nm. FM™ 4-64FX was used according to the manufacturer instructions (Invitrogen™ F34653) to stain the plasma membrane of the protoplast.

3 | RESULTS

3.1 | *HSP70-16* negatively regulates seed germination under cold stress

While exploring the role of *HSP70-16* in flower opening under normal and mild heat stress temperatures (Chen *et al*., 2019), we accidentally observed that *hsp70-16* seeds germinate earlier than wild-type seeds when incubated at 4°C and that HSP70-16 interacts with VDAC3 (see result below), a negative regulator of seed germination under cold stress (Yang *et al*., 2011). To explore the possible role of the interplay between HSP70-16 and VDAC3 in seed germination under cold stress, we incubated seeds of wild-type, *hsp70-16*, *vdac3*, *hsp70-16vdac3* and *hsp70-16* complemented by *HSP70-16* genomic sequence at 4°C. Seeds of *hsp70-16*, *vdac3*, and *hsp70-16vdac3* germinated significantly faster than wild-type seeds, and there were no differences in germination rates among *hsp70-16*, *vdac3*, and *hsp70-16vdac3* mutants (Figure 1a,b). Notably, seeds of *hsp70-16*

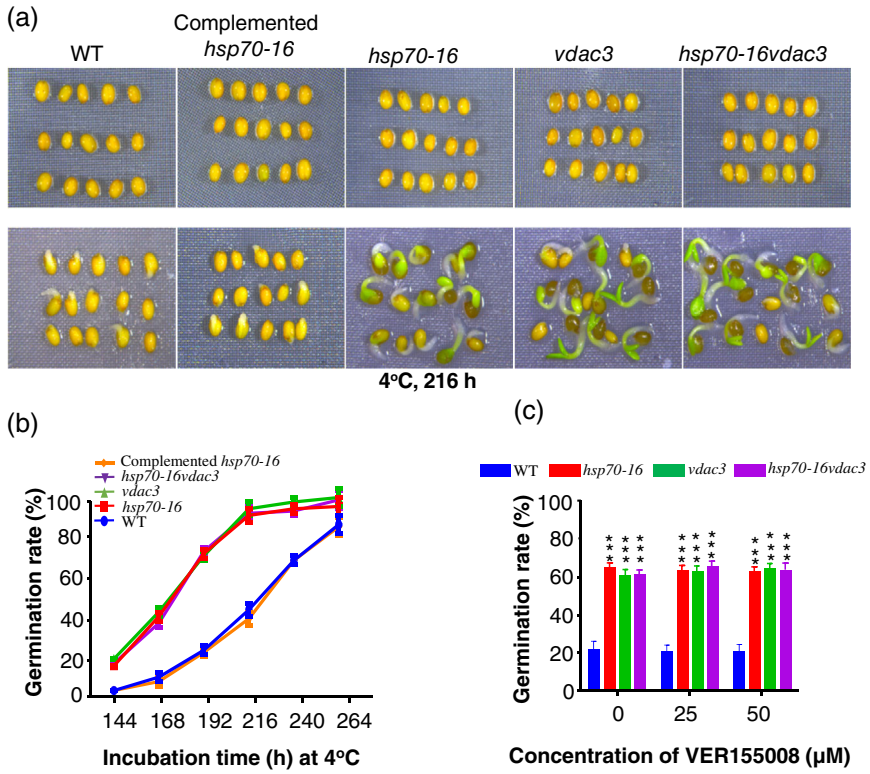

FIGURE 1 Germination patterns of *hsp70-16*, *vdac3*, *hsp70-16vdac3*, complemented *hsp70-16* (*hsp70-16* complemented by *HSP70-16* genomic DNA) and wild-type (WT) seeds at 4°C. (a) Seed images of wild-type, complemented *hsp70-16*, *hsp70-16*, *vdac3* and *hsp70-16vdac3*, before (upper) and after (lower) incubation at 4°C for 216 h. (b) Germination rates of seeds incubated at 4°C completely for a period of 264 h. (c) Germination rates of mutant and wild-type seeds incubated in ½ MS without or with 25 or 50 μM VER155008 at 4°C for 196 h. Seeds were placed on ½ MS plates, and data presented are mean ± SE of three independent experiments ($n = 3$, 3×80 seeds each)

complemented by *HSP70-16* genomic sequence recovered the phenotype of *hsp70-16*, germinating similarly to wild-type seeds (Figure 1a, b). To further analyse whether this effect is cold stress specific we compared germination rates of seeds of wild-type, *hsp70-16*, *vdac3*, and *hsp70-16vdac3* at 22°C with or without up to 48 h prior stratification at 4°C. Mutant seeds incubated at 22°C showed similar germination rates to wild-type seeds except those with prior 48 h stratification at 4°C (Figures S1,a–c). These results indicated that *HSP70-16* and *VDAC3* may function closely in negatively regulating seed germination under cold stress. Since HSP70-16 is a chaperone/ATPase (Figure S2), we further examined if the inhibitory effect of HSP70-16 on seed germination under cold stress is from its chaperone activity using a plant HSP70 ATPase activity inhibitor VER155008 (Zhang *et al*., 2015; Matsuoka *et al*., 2019). Neither mutant seeds (*hsp70-16*, *vdac3* and *hsp70-16vdac3*) nor wild-type seeds changed their earlier germination patterns under 4°C up to 196 h in the presence of VER155008 (25 μM or 50 μM) (Figure 1c), indicating that HSP70-16 negatively regulates seed germination under cold stress in a manner that is independent of its chaperone activity.

3.2 | *HSP70-16* and *VDAC3* genes are responsive to cold stress and highly expressed in the endosperm

We further performed expression analyses using quantitative reverse transcription PCR (qRT-PCR), which revealed that both *HSP70-16* and *VDAC3* are responsive to cold stress and that *VDAC3* has a relative stronger response (Figure 2a). Further studies using dissected endosperm (endosperm plus testa) and embryo tissues indicated that expression levels of both *HSP70-16* and *VDAC3* in the endosperm are higher than those in the whole seed, and much higher than those in the embryo (Figure 2b,c). This result corresponded well to the idea that embryonic suppression in seed germination is most likely driven by endosperm (Lee and Lopez-Molina, 2013).

3.3 | *HSP70-16* and *VDAC3* are involved in ABA signaling

The involvement of HSP70s in ABA signaling has been reported as young seedlings of double mutant *hsp70-1hsp70-4* and triple mutant *hsp70-2hsp70-4hsp70-5* in *Arabidopsis* are hyposensitive to ABA (Leng *et al*., 2017). In addition, seeds of *vdac2* are insensitive to ABA (Yan *et al*., 2009), while seeds of *cbl1* (mutant of the VDAC1 interactor CBL1) are hypersensitive to ABA (Albrecht *et al*., 2003). Notably, seeds of *vdac1* and *cbl1* germinated earlier as compared with wild-type (Li *et al*., 2013). With all these aspects taken into account, we examined the responses of mutant seeds to exogenous ABA under 4°C, to examine if HSP70-16 and VDAC3 mediated seed germniation under cold stress is ABA dependent. Exogenous ABA (0.3 and 0.7 μM) treatment abolished earlier germination phenotype of *hsp70-16*, *vdac3* and *hsp70-16vdac3* seeds, revealing that mutants seeds were

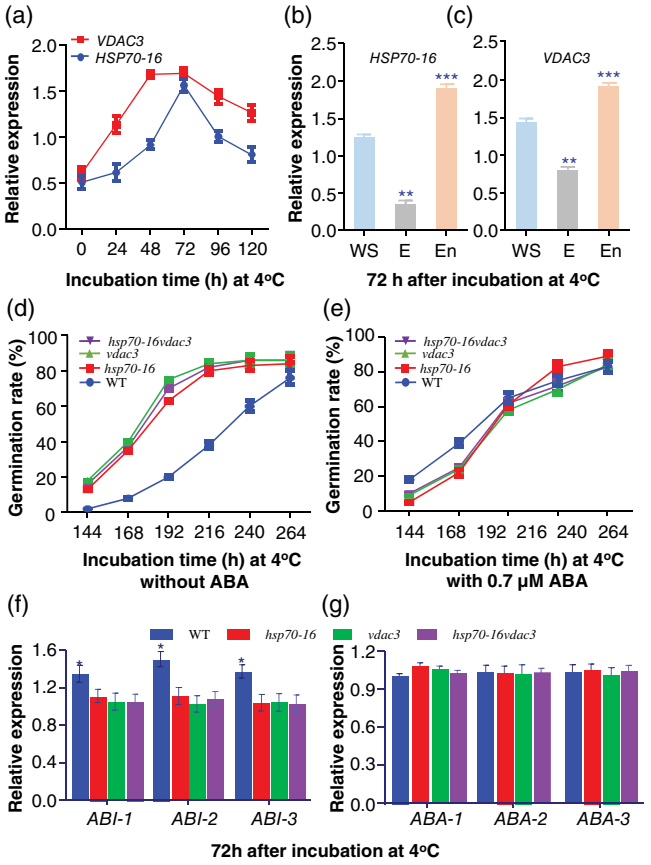

FIGURE 2 Expression patterns of *HSP70-16* and *VDAC3* and ABA responsiveness of their mutants. (a) Relative expression levels of *HSP70-16* and *VDAC3* in seeds incubated at 4°C revealed by qRT-PCR. (b,c) Relative expression levels of *HSP70-16* (b) and *VDAC3* (c) in whole seed (WS), dissected embryo (E), and dissected endosperm (En) (endosperm plus testa) incubated at 4°C for 72 h. (d,e) Germination rates of mutant and wild-type seeds in the absence (d) or presence (e) of 0.7 μM ABA in ½ MS plates incubated at 4°C. (f,g) qRT-PCR analysis of the expression of key genes involved in ABA signaling (f) or ABA synthesis (g) pathways in mutant and wild-type (WT) seeds incubated at 4°C for 72 h. Data were normalized using *AtEIF3K* as a reference gene, values represent mean ± SE of three biological repeats ($n = 3$)

hypersensitive to higher concentration (0.7 μM) of ABA as compared with wild-type seeds (Figures 2d,e and S3); such an ABA hypersensitive phenotype was also observed in the ABA exporter mutant *abcg25* seeds (Kuromori *et al*., 2010). To figure out whether HSP70-16 and VDAC3 are involved in ABA signaling or biosynthesis, we conducted gene expression analysis on ABA signaling and synthesis marker genes using qRT-PCR. The results showed that transcript levels of key genes involved in ABA signaling but not those involved in ABA biosynthesis were significantly reduced in *hsp70-16*, *vdac3* and *vdac3hsp70–16* seeds under 4°C, as compared with wild-type (Figure 2f,g), indicating that HSP70-16 and VDAC3 may regulate seed germination via their effects on ABA signaling.

3.4 | Seed coats of *hsp70-16* and *vdac3* retain more ABA and suppress less embryonic growth

Endosperm negatively regulates the embryonic growth under normal temperature by exporting its synthesized ABA to the embryo via ABCG transporters (Kang *et al*., 2015). To exam if HSP70-16 and VDAC3 participate in the export of ABA from the endosperm to the embryo under cold stress, we next quantified the amount of ABA released into liquid ½ MS and retained in the dissected seed coats of *hsp70-16*, *vdac3*, *hsp70-16vdac3* and wild-type incubated at 4°C for 72 h using an ABA ELISA Kit (Jiang *et al*., 2012). Wild-type and mutant seeds contained similar levels of ABA (Figure 3a), confirming that HSP70-16 and VDAC3 do not impact ABA biosynthesis. However, mutant seed coats retained substantially higher levels of ABA while secreted remarkable lower levels of ABA, as compared to wild-type (Figure 3b,c), clearly indicating that seed coats of *hsp70-16*, *vdac3* and *hsp70-16vdac3* mutants are defective in ABA export from the endosperm to the embryo. To validate this result, we subsequently performed a seed coat bedding assay (SCBA) developed recently to investigate the mechanistic role of seed coat on embryonic growth (Lee and Lopez-Molina, 2013). In which, four seed coat beds were prepared by placing dissected seed coats from *hsp70-16*, *vdac3*, *hsp70-16vdac3* and wild-type on ½ MS supplemented with GA inhibitor paclobutrazol (PAC) to maximize ABA synthesis and accumulation. Embryos from wild-type or from *vdac3*, *hsp70-16*, and *hsp70-16vdac3* were placed on each of these four endosperm beds, and their growth was monitored by measuring the length of radicle and angle between the cotyledon and radicle (Figure S4,a,b). Compared with those of wild-type, seed coats of *hsp70-16*, *vdac3*, and *hsp70-16vdac3* showed less inhibitory effects on the embryo growth derived from both wild-type (Figure 3d–f) and mutant (Figure S5,a–i) seeds. Results from both ABA quantification and seed coat bedding assay indicated that *HSP70-16* and *VDAC3* are both involved in the ABA export from the endosperm to the embryo in cold stressed *Arabidopsis* seeds to suppress embryonic growth.

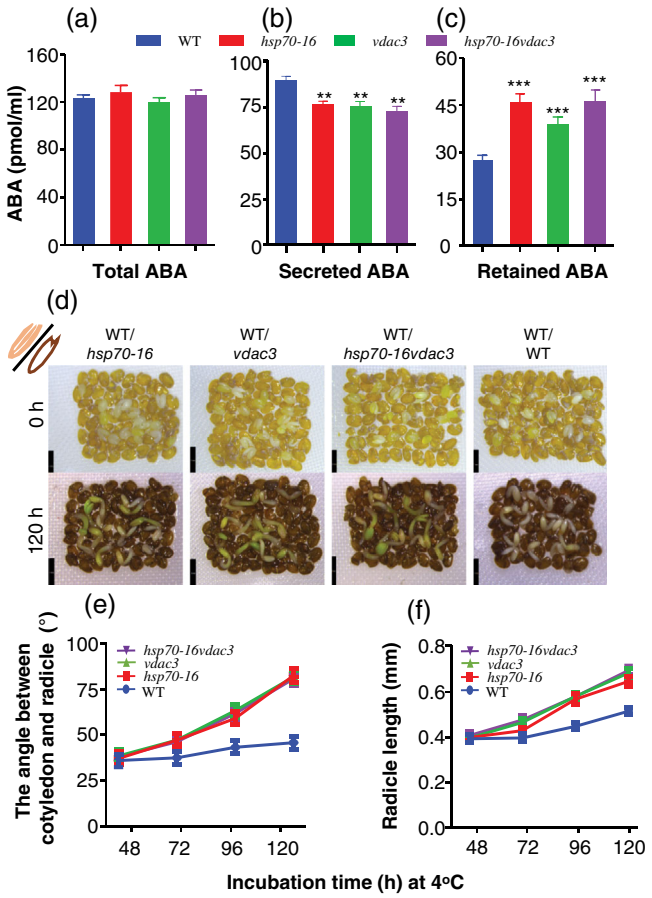

FIGURE 3 ABA transport and its consequence on wild-type (WT) embryonic growth in mutant (*hsp70-16*, *vdac3* and *hsp70-16vdac3*) seed coat beds at 4°C. (a–c) Levels of total ABA synthesized (a), secreted ABA from disected seed coats into the liquid ½ MS (b), and retained ABA in seed coats (c) of *hsp70-16*, *vdac3* and *hsp70-16vdac3* and wild-type incubated at 4°C for 72 h, respectively. Values represent means ± SE of three independent experiments ($N = 3$) (** $p < 0.01$; *** $p < 0.005$). (d) Images of embryonic growth of wild-type (WT) embryos on the seed coat beds made from mutants or wild-type (WT) when incubated at 4°C for 120 h. (e,f) Embryonic growth of wild-type (WT) on the mutant seed coat beds incubated at 4°C for 120 h in terms of the angle between the radicle and cotyledons (e) and the radicle length (f) in ½ MS plates complemented with 10 μM PAC. Scale bar, 1 mm. Each seed coat bed contained 75 seed coats and 15 embryos placed on it. Data represent mean ± SE for three independent experiments ($n = 3$, 3 × 15 each)

3.5 | HSP70-16 localizes to and interacts with VDAC3 in the plasma membrane and nucleus

To determine whether HSP70-16 and VDAC3 physically interact and to reveal the location of such interactions, we first investigated the subcellular localization of HSP70-16 using transgenic lines stably expressing the construct consisting of GFP fused to the C terminus of HSP70-16 genomic DNA including the 870-bp upstream region before the start codon. GFP signals were detected mainly in the PM and nucleus of mesophyll cells transformed with pHSP70-16::HSP70-16::eGFP (Figure 4a). However, GFP signals were detected solely in the PM of vascular tissues transformed with pHSP70-16::HSP70-16::eGFP (Figure S6a), likely due to the absence of a proper nucleus in vascular tissues (Davis *et al*., 1970; Lamoureux, 1975; Cayla *et al*., 2015). Transient expression of a p35S::HSP70-16::eGFP construct in protoplast from wild-type further confirmed the dual localization of HSP70-16 to the PM and nucleus, in which HSP70-16 co-localized with the PM marker FM4-64FX (Figure 4b). The PM localization of HSP70-16 was consistent with the report of a previous PM proteomic analysis (Mitra *et al*., 2009).

To further explore the function of HSP70-16 in addition to its function in sepal fusion (Chen *et al*., 2019) we screened a sepal cDNA library for its interacting partners, using the full cDNA sequence of HSP70-16 as a bait. Two potential interacting partners were isolated, including VDAC3 encoded by *At5G15090* and CaN2 encoded by *At2G40410*. Subsequent in vitro validation using yeast two-hybrid by growing yeast cells co-transformed with pGBKT7 plasmid containing HSP70-16 and pGADT7 plasmid containing VDAC3 or CaN2 on

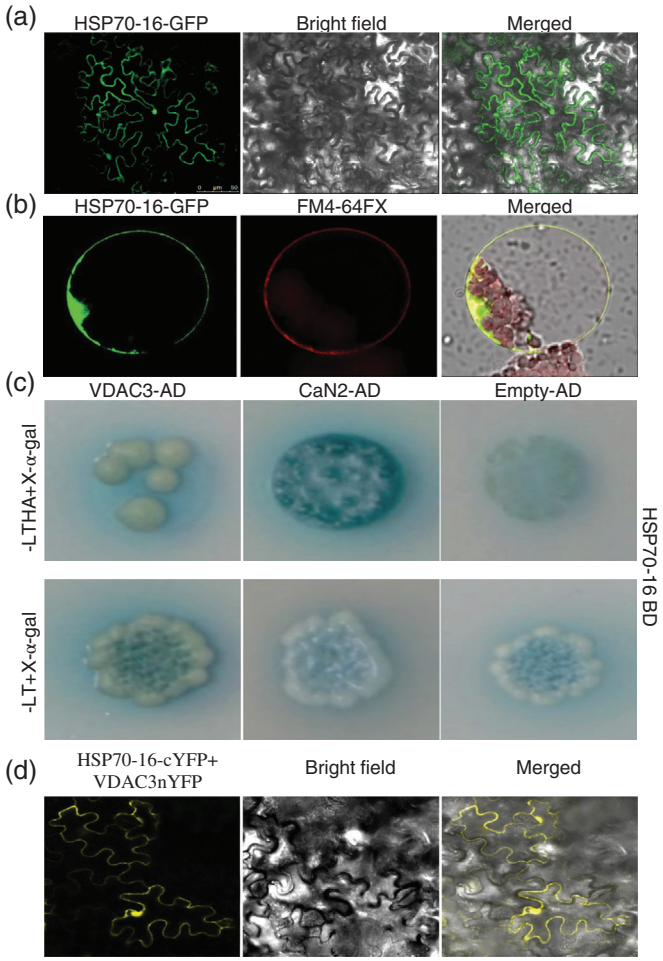

FIGURE 4 Subcellular localization of HSP70-16 and physical interaction between HSP70-16 and VDAC3. (a) Subcellular localization of HSP70-16 in transgenic *Arabidopsis* leaf mesophyll cells transformed with pHSP70-16::AtHSP70-16::eGFP. Panels from left to right represent eGFP fluorescence, bright field and merged image, respectively. (b) Subcellular localization of HSP70-16 in wild-type *Arabidopsis* protoplast transformed with p35S::HSP70-16::eGFP. Panels from left to right represent eGFP fluorescence, PM staining dye FM4-64FX fluorescence, and merged image, respectively. Scale bar, 10 μm. (c) Validation of interaction of HSP70-16 with VDAC3 and CaN2 using yeast two hybrid assay. (d) Validation of interaction of HSP70-16 with VDAC3 using bimolecular fluorescence complementation (BiFC) system in *Nicotiana tabacum* (tobacco) leaf. Panels from left to right represent YFP fluorescence, bright field and merged image, respectively

selective SD medium (−Leu/−Trp/−His/−Ade + X-a-Gal), corroborated that HSP70-16 interacts with both VDAC3 and CaN2 (Figure 4c). *In vivo* validation by BiFC (bimolecular fluorescence complementation) confirmed the interaction of HSP70-16 with VDAC3, in which YFP fluorescence was only detected in the PM and nucleus of tobacco leaves transformed with the construct pair nYFP-HSP70-16 and VDAC3-cYFP (Figure 4d), but not with the construct pair nYFP-HSP70-16 and CaN2-cYFP (Figure S6b). Both in vitro and in vivo results demonstrated that the localization of the two interactive proteins in the PM and nucleus fit well with their functions in ABA transport. VDACs have been reported to localized to locations other than mitochondria, such as the PM and nucleus (Schwarzer *et al*., 2002; Marmagne *et al*., 2004; Marmagne *et al*., 2007; Robert *et al*., 2012). Notably, VDAC3, together with two HSP70s (HSP70-2 and HSP70-12), is found to be in the *Arabidopsis* nucleolar proteome (Pendle *et al*., 2005).

3.6 | HSP70-16 regulates VDAC3 channel characteristics

In attempt to clarify the physiological functions of the interaction between HSP70-16 and VDAC3, electrophysiological techniques were used to characterize the effects of HSP70-16 on the electrophysiological properties of VDAC3 channel. In vivo, the VDAC3 channel displayed a closed state in the membrane of HeLa cells overexpressing VDAC3 at 0 mV membrane potential, which tended to switch to the open state (low to medium conductance state) at increased membrane potentials between −100 or + 100 mV with 20 mV increments per 1.5 s (Figure 5a). Upon the addition of HSP70-16, the VDAC3 channel became more open (high to the highest conductance state) under the same membrane potential increment regime (Figure 5b), indicating that HSP70-16 activates the opening of the VDAC3 channel. The voltage-dependent activation of VDAC3 conductance by HSP70-16 was clearly shown in the plot of the applied membrane potential (mV) with the conductance (pA) detected using HeLa cell system. In which, as the membrane potentials were increased, regardless of positive and negative directions, the conductance of VDAC3 was significantly increased, and the addition of HSP70-16 favoured the increase in the conductance of the VDAC3 channel (Figure 5c). The response of the VDAC3 to membrane potential was asymmetrical, with more increases in the conductance at positive potentials than at negative potentials, similar to a previously presented in vitro result using plasma membrane bilayer (Berrier *et al*., 2015). Interestingly, addition of HSP70-16 did not change this pattern (Figure 5a–c). These in vivo results indicate that HSP70-16 activates the electro-physiological properties of the VDAC3 channel in a manner that favours the opening of the VDAC3 channel.

In vitro, VDAC3 inserted in the planar lipid bilayer (PLB), whether in *cis* or *trans* compartment, exhibited an open state (high conductance state) in In/Ex 300/100 mM KCl at applied 0 mV membrane potential (voltage). Addition of HSP70-16, whether in *cis* (Figure 5d) or *trans* (Figure 5e) compartment, reduced significantly the conductance of VDAC3, resulting in a closed state, which was fully reversed to the open state by H^+ addition (Figure 5d,e). VDAC3 inserted in the *cis* compartment of the PLB displayed a closed state in In/Ex 100/100 mM KCl at applied 0 mV membrane potential (voltage). Increasing membrane potential from 0 to −30 mV shifted it to the open state (high conductance state), which was reversed back to the closed state by the addition of HSP70-16 in *cis* compartment (Figure 5f). However, when ABA was present together with VDAC3 in *cis*, adding of HSP70-16 failed to reverse the open state induced by the increase in membrane potential back to the closed state (Figure 5g). When membrane potential was increased to +30 mV in

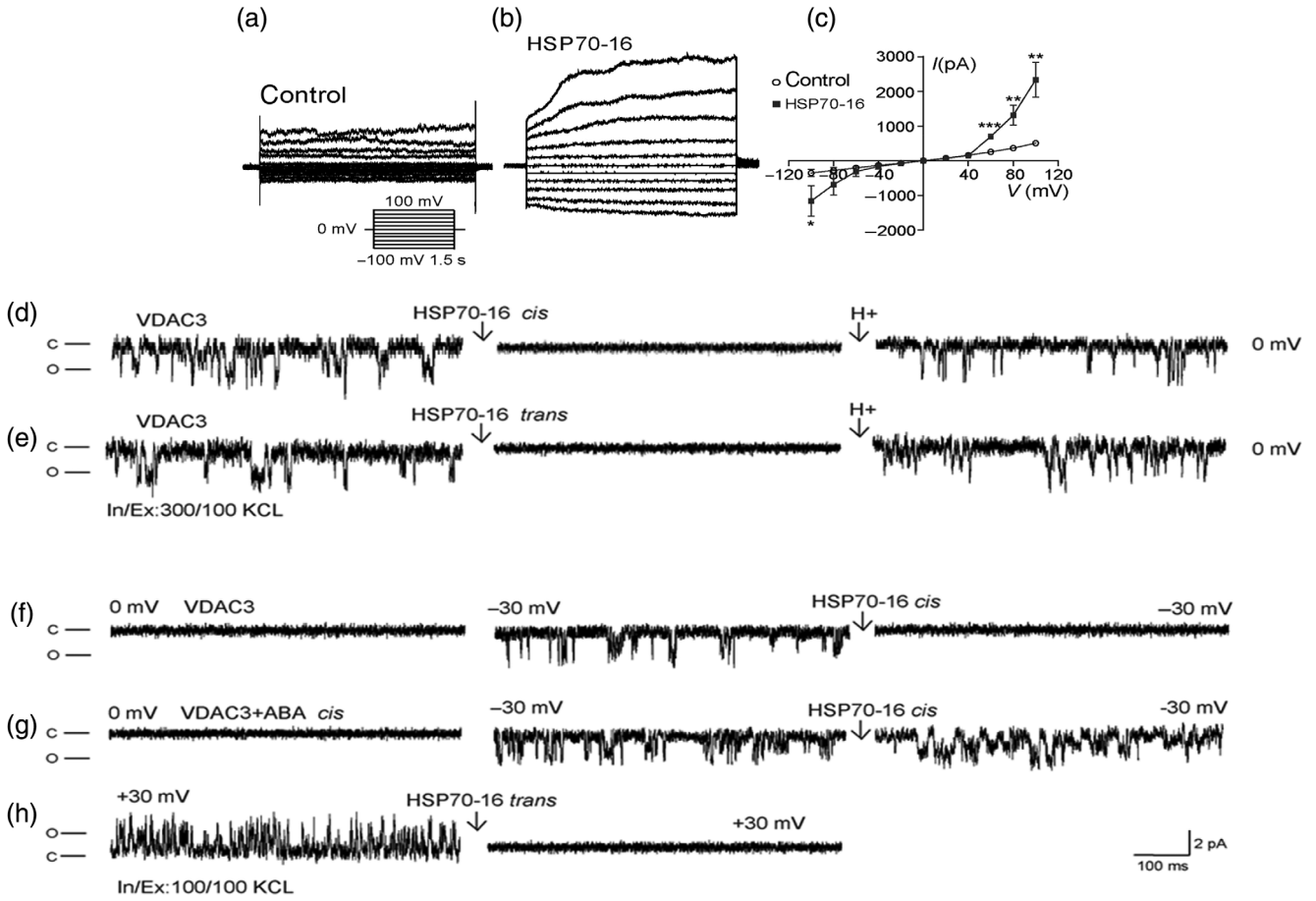

FIGURE 5 In vivo and in vitro activities of HSP70-16 on VDAC3 channel characteristics. (a–c), In vivo electrophysiological characteristics of VDAC3 recorded from Hela cells overexpressing the VDAC3 channel. (a) Typical whole-cell current traces recorded from Hela cells overexpressing the VDAC3 channel alone at voltage from −100 to +100 mV with 20 mV increments per 1.5 s. (b) Another version of typical whole-cell current traces after adding HSP70-16 on VDAC3 channel at voltage from −100 to +100 mV with 20 mV increments per 1.5 s. (c) The plot of voltage in the membrane at −100 to +100 mV and VDAC3 channel currents in the presence (solid squares) or absence (open circles) of HSP70-16. (d–h) In vitro electrophysiological characteristics of VDAC3 recorded from planar phospholipid bilayer system. (d,e) VDAC3 current recorded before (left) and after (middle) addition of HSP70-16 in both *cis* (d) and *trans* (e) sides, and after addition of H^+ (right in both). (f,g) VDAC3 current recorded before (left) and after (middle) the negative increase of potential from 0 to −30 Mv, and after adding HSP70-16 in *cis* side (right) in the absence (f) and in the presence of ABA (g) in *cis* side. (h) VDAC3 current recorded before (left) and after (right) adding HSP70-16 in *trans* side in the absence of ABA. The purified VDAC3 were incorporated into planer phospholipid bilayer and single channel currents were recorded in asymmetric KCl solutions (d,e) (In/Ex, 300/100 mM) and (f,h) (In/Ex, 100/100 mM), at the indicated voltages

trans compartment, VDAC3 exhibited an open state, while the addition of HSP70-16 in *trans* shifted the open state to a closed state in the absence of ABA (Figure 5h). In vitro results indicated that HSP70-16 affects the voltage-dependent electrophysiological characteristics of VDAC3 channel likely in an ABA dependent manner.

In sum, these results from both in vivo and in vitro experiments indicated that HSP70-16 activates the electro-physiological properties of VDAC3 channel under physiological conditions.

4 | DISCUSSION

Endosperm is the primary site for ABA synthesis in seeds (Lee and Lopez-Molina, 2013), and four ABCG transporters are responsible for the coordinated and successful delivery of ABA from the endosperm to the embryo during the process of seed germination under normal temperature. *ABCG25* and *ABCG31* are ABA exporter in the endosperm while *ABCG30* and *ABCG40* are ABA importers in the embryo. Remarkably, only non-stratified seeds of these *ABCG* mutants germinate earlier than wild-type seeds while stratification at 2–4°C abolishes germination phenotype of these mutants (Kang *et al*., 2015). The process of seed germination regulation regarding ABA transport under cold stress is unknown. In this study, we found that *VDAC3* and *HSP70-16* both negatively regulates the process of seed germination under cold stress. This is the first report on the functional and mechanistic characterization of a plant PM-localized VDAC3, which also involved an environmental and developmental cue responsive chaperone protein HSP70-16 (Chen *et al*., 2019). Based on our

results, *VDAC3* and *HSP70-16* are responsible for the successful delivery of ABA from endosperm to the embryo under cold stress conditions.

4.1 | Role of VDAC3 in the process of seed germination under cold stress

VDAC3 is the most abundant protein of the four *Arabidopsis* VDACs that localizes to the PM (Robert *et al*., 2012), and *VDAC3* was found to be highly expressed in the endosperm as compared with those in the embryo and whole seed (Figure 2c), which is consistent with those of known *ABCG* transporters responsible for ABA export (Kang *et al*., 2015). Seeds of *vdac3* germinated earlier than wild-type seeds under 4°C as previously reported (Yang *et al*., 2011), but this phenotype was apparent also at 22°C but only after at least 48 h cold treatment (Figure S1,a–c), showing a specific role of *VDAC3* in response to cold temperature during seed germination. In addition, *vdac3* seeds were hypersensitive to the application of exogenous ABA (Figures 2d, eand Figure S3), and its involvement in ABA signaling was also confirmed by the analysis of gene expression data (Figure 2f,g). Moreover, as compared with those of wild-type, *vdac3* seed coats retained more and released less ABA (Figure 3b,c) and exhibited less inhibitory effects on embryonic growth (Figures 3d–f and S5,a–i), pinpointing out its involvement in the ABA export from the endosperm in order to transfer it to the embryo. Previous studies indicated that plant MOM VDACs transport various ions (Cl^-, K^+, Na^+) (Colombini, 2012), metabolites (ATP, ADP) (Yang *et al*., 2011), nucleic acids (Salinas *et al*., 2006), tRNAs (Salinas *et al*., 2006) and even the proteins (cytochrome c) (Shimizu *et al*., 1999) across the membrane. Our major findings in this study indicate that the PM-localized VDAC3 likely involves in the transportation of the plant hormone ABA from endosperm to embryo under cold stress condition. ABA is a weak acid; under physiological conditions, it equilibrates the anionic (ABA-) and protonated (ABA-H) forms. In normal conditions, the acidic apoplast PM would be anticipated to favour ABA-H, resulting in ABA diffusion to cells. In stress conditions, the increase of apoplastic pH decreases diffusible ABA-H pool, thus, active ABA transporters are required for ABA export (Merilo *et al*., 2015). Given that VDACs are in essence anion channels with diverse transportation capacities (Shimizu *et al*., 1999), and that ABA is exported as an anion (Merilo *et al*., 2015), the role of VDAC3 as an ABA exporter under cold stress can be assumed. Nevertheless, currently, we do not know the exact mechanisms underlying the association of VDAC3 channel activity with the observed different ABA efflux between wild type and *vdac3* seeds. Further in-depth investigations are required to make it clear whether VDAC3 functions directly as an ABA exporter by employing radiolabeled ABA transport assay. Additional approaches are also required to examine whether VDAC3 functions indirectly on ABA transport; VDAC3 could influence an alternative ABA transport protein via affecting signaling processes that regulate an ABA-transporter protein or via a protein–protein interaction (Punzo *et al*., 2018).

4.2 | HSP70-16 is a novel interactor of VDAC3

The interaction of HSP70-16 and VDAC3 was first revealed by yeast two hybridization (Y2H)-based screening of a sepal cDNA library, which was confirmed later in vitro by Y2H (Figure 4c) and in vivo by BiFC (Figure 4d). The similarities, observed in rate of seed germination under 4°C (Figure 1a,b), responsiveness to cold stress (Figure 2a) and exogenous ABA treatment (Figures 2d,e and Figure S3), spatial expression pattern in seed (Figure 2b,c), seed coat property (Figures 3d–f and S5,a–i), and ABA export property (Figure 3a–c) among *hsp70-16*, *vdac3*, and *hsp70-16vdac3* mutants, strongly supported the interaction between these two proteins. VDAC3 and its previously characterized interactor kinesin-like protein 1 (KP1; At KIN14 h) both interact in the mitochondria and regulate aerobic respiration during seed germination under cold stress. Interestingly, while seeds of *KP1* overexpression lines germinate earlier, *kp1* seeds germinate similarly to wild-type seeds under cold stress (Yang *et al*., 2011). Thus, interaction between VDAC3 and KP1 was considered to regulate the aerobic respiration pathway possibly by regulating the ATP/ADP flux into the mitochondria via VDAC3 in concert with adenylate transporters of the inner mitochondrial membrane (Yang *et al*., 2011). Another study found that mitochondrial VDAC3 interacts with a chloroplast protein Trx m2 in mitochondria, causing the closure of VDAC3 under oxidizing conditions, and thus playing a role in oxidative stress responses (Zhang *et al*., 2015) These reports and our own findings together indicate that VDAC3 can be targeted by many proteins and plays diverse roles in plants. Our finding is different from abovementioned ones: first, VDAC3 interacts with HSP70-16 in the PM and nucleus, which is consistent with the PM localization of HSP70-16 (Figure 4a) and the mobilization of HSP70s in stress conditions (Gao *et al*., 2015; Ray *et al*., 2016). Second, single and double mutants of *HSP70-16* and *VDAC3* show many identical phenotypes, particularly in seed germination (Figure 1a,b) and ABA singling (Figure3f). Third, seed coats of *hsp70-16*, *vdac3* and *hsp70-16vdac3* release less while retained more ABA (Figure 3c), similar to previously reported ABA exporter mutants, *abcg25* and *abcg31* (Kuromori and Shinozaki, 2010; Kuromori *et al*., 2010; Kang *et al*., 2015). Thus, these findings establish a distinct function of the interplay between HSP70-16 and VDAC3 in the negative regulation of seed germination under cold stress via acting on the ABA export from endosperm to the embryo.

4.3 | HSP70-16 is a regulator of VDAC3

HSP70-16 activates the electrophysiological properties of the VDAC3 channel and favours its opening. The similar phenotypes of ABA levels (Figure 3a–c) and responses of *hsp70-16*, *vdac3* and *hsp70-16vdac3* mutants to exogenous ABA (Figures 2d,e and Figure S3) indicated that both HSP70-16 and VDAC3 might be involved in the same ABA export pathway. Our in vivo electrophysiological experiment in HeLa cells demonstrated the regulatory role of

HSP70-16 in the activation of the electrophysiological features of VDAC3 channel (Figure 5e–g), indicating that interplay between HSP70-16 and VDAC3 regulates seed germination under cold stress in *Arabidopsis* by controlling the gating of the VDAC3 channel.

The difference in both *in vivo* and *in vitro* experiments regarding behaviors of HSP70-16 and VDAC3 interaction can be explained. Planar lipid bilayer is a synthetic membrane system which contains only lipoid E80, and it is completely devoid of natural lipids, the direct regulators of VDACs channel characteristics (Rostovtseva and Bezrukov, 2008). Previous studies in seeds of *Phaseolus coccineus* showed that VDACs ion channel characteristics are sensitive not only to the ratio between lipid-sterols, but also to the types of lipids in the membrane (Rostovtseva *et al*., 2006; Mlayeh *et al*., 2010; Krammer *et al*., 2014; Barrero-Sicilia *et al*., 2017). Our results here implied that lipids could be candidate players to regulate VDAC3 ion channel characteristics together with ABA and HSP70-16, as lipid remodeling of the PM is the first initiative of a plant in response to cold stress (Barrero-Sicilia *et al*., 2017). Because ABA directly regulates the interaction between pyrabactin resistance-like (PYL) and phosphatase-type 2C (PP2C2/6) (Bai *et al*., 2013; Kai *et al*., 2019), it is necessary to deeply investigate into the interplays among HSP70-16, VDAC3, ABA and lipids regarding seed germination under cold stress.

Although various regulators of VDAC ion channels have been reported in animals and plants (Kanwar *et al*., 2020), HSP70-16 is the first reported plant HSP70 that regulates VDAC3 under cold stress condition. In mammals, a surface epithelium of the uterovaginal junction localized HSP70 controls VDAC2 functions by binding and strengthens sperm motility, which is accompanied by a significant increase in intracellular Ca^{2+} levels (Hiyama *et al*., 2014). The mammalian mitochondrial HSP70 (PBP74) interacts with VDAC1 (hVDAC1) to decrease the voltage dependency of the hVDAC1 channel (Schwarzer *et al*., 2002). Our data showed that the interplay between HSP70-16 and VDAC3 positively regulates the opening of VDAC3 channels (Figure 5a–c), indicating likely that HSP70-16 interacts with VDAC3 to facilitate the ABA efflux under cold stress.

4.4 | A proposed working model of the involvement of *HSP70-16* and *VDAC3* in the regulation of seed germination at 4°C

Cold stratification (in the dark at 4°C) used to break seed dormancy cycle (Milberg and Andersson, 1998) is, in itself, a cold stress condition that can lead to cell injury (Lyons, 1973). During cold stress, ATP homeostasis and ATPase activities are disrupted due to reduced permeability of membrane including PM (Jones, 1969), which inhibits activities of ATP dependent ABCG transporters for ABA transport. As reported, under normal temperature, four ABCG transporters cooperate to regulate ABA homeostasis and seed germination, therefore, seeds of *abcg25*, *abcg30*, *abcg31*, and *abcg40* show different germination phenotypes from wild-type only under normal temperatures (Kang *et al*., 2015). Under cold stress, when ATP is depleted and the activity of ABCG transporters is inhibited, how plants to control ABA homeostasis and seed germination is of particular significance for not only stress biology but also stress breeding. Our results indicate both HSP70-16 and VDAC3 ensure the successful export of ABA from the endosperm to the embryo under cold stress when ABCGs are not working. HSP70s have long been regarded as the major protective chaperones against various stresses functioning to re-establish cellular homeostasis (Ray *et al*., 2016). This is, however, the first report on the regulatory function of HSP70s in seed germination under cold stress in the context of VDAC channels in plants. Considering the fact that HSP70s, VDACs and ABA are all significantly induced by cold stress in plants (Ray *et al*., 2016; Chen *et al*., 2019), and that HSP70s play important roles in translocation of proteins under stress conditions (Rana *et al*., 2018), it is conceivable that such a machinery involves the action of all of them to ensure plant survival under cold stress. Further investigations will first focus on the elucidation of the entire regulatory machinery and subsequently the hierarchical organization of key players involved. The possible interaction of ABA with HSP70-16 in this process should also be investigated. Given that ABA binds to human HSP70 proteins including glucose-regulated protein (GPR78) and HSP70-2 (Kharenko *et al*., 2013), it will be important to examine whether such an interaction also occurs within plants.

In summary, our findings demonstrate that under cold stress, ABA produced in the endosperm is exported across the PM to the embryo, which is influenced by the VDAC3, HSP70-16 and the interaction between these two proteins. Our finding also indicate a function of PM VDAC3 and HSP70-16 in seed germination under cold stress, by which plant seeds re-establish a functional ABA transport capacity to replace those lost by cold-inhibition.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

J.S. designed and supervised all the research. M.A. designed and performed most of the experiments and analysed and interpreted data and wrote the manuscript. Q.M. carried out electrophysiological experiment and data analysis. X.R. helped in growing plants and sample collection. J.H. assisted in protein expression and extraction. L.S. helped for seed germination experiments. F.L. helped in seed coat bedding assay and RNA extraction. M.U. helped in yeast two hybrid and localization experiments. W.L. and A.F. participated in data analysis and discussion. J.S. and A.F. revised and finalized the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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