AtSec62 is critical for plant development and is involved in ER-phagy in Arabidopsis thaliana

Shuai Hu1*, Hao Ye1, Yong Cui1 and Liwen Jiang1,2*

1. Centre for Cell & Developmental Biology and State Key Laboratory of Agrobiotechnology, School of Life Sciences, The Chinese University of Hong Kong, Hong Kong, China
2. CUHK Shenzhen Research Institute, The Chinese University of Hong Kong, Shenzhen 518057, China

*Correspondences: Shuai Hu (hushuailuck@gmail.com); Liwen Jiang (ljiang@cuhk.edu.hk; Dr. Jiang is fully responsible for the distribution of all materials associated with this article)
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Abstract The endoplasmic reticulum (ER) is the major site for protein folding in eukaryotic cells. ER homeostasis is essential for the development of an organism, whereby the unfolded protein response (UPR) within the ER is precisely regulated. ER-phagy is a newly identified selective autophagic pathway for removal of misfolded or unfolded proteins within the ER in mammalian cells. Sec62, a component of the translocon complex, was recently characterized as an ER-phagy receptor during the ER stress recovery phase in mammals. In this study, we demonstrated that the Arabidopsis Sec62 (AtSec62) is required for plant development and might function as an ER-phagy receptor in plants. We showed that AtSec62 is an ER-localized membrane protein with three transmembrane domains (TMDs) with its C-terminus facing to the ER lumen. AtSec62 is required for plant development because atsec62 mutants display impaired vegetative growth, abnormal pollen and decreased fertility. atsec62 mutants are sensitive towards tunicamycin (TM)-induced ER stress, whereas overexpression of AtSec62 subsequently enhances stress tolerance during the ER stress recovery phase. Moreover, YFP-AtSec62 colocalizes with the autophagosome marker mCh-Atg8e in ring-like structures upon ER stress induction. Taken together, these data provide evidence for the pivotal roles of AtSec62 in plant development and ER-phagy.

INTRODUCTION

The endoplasmic reticulum (ER) is one of the most prominent membrane-bound organelles in the endomembrane system and functions as a major site of protein synthesis, assembly and export in all eukaryotic cells (Dufey et al. 2014). Unfolded polypeptides synthesized on cytosolic ribosomes are transported into or across the ER membrane for proper processing and folding, which is assisted by sets of chaperones. Even though the protein folding quality control system is very elegant in higher eukaryotes, protein folding is an error-prone process, especially under extreme environmental conditions. To maintain a healthy cellular environment, multiple ER quality control (ERQC) systems, including the ER retention and retrieval pathways, have been evolved in higher eukaryotes (Ellgaard and Helenius 2003; Wang et al. 2018a). Under extreme environmental conditions, the demand for protein folding would go beyond the ERQC causing a certain amount of misfolded or unfolded proteins to accumulate in the ER lumen, leading to a potentially lethal condition, known as ER stress (Wan and Jiang 2016). To alleviate ER stress, an ER-associated degradation (ERAD) system is activated to accelerate degradation of the misfolded or unfolded proteins (Li et al. 2017; Tian et al. 2018). In addition, to avoid such serious consequences, the unfolded protein response (UPR) can be triggered to...
restore protein homeostasis by upregulating the degradative capacity and protein-folding machinery of the ER (Wan and Jiang 2016).

Unfolded protein response is a set of signaling mechanisms that can enhance protein-folding capacity and prevent accumulation of misfolded or unfolded proteins in the ER (Walter and Ron 2011). In mammals, three mechanisms are involved in UPR, (i) the inositol-requiring enzyme 1 (Ire1), which has a dual-function in protein kinase and ribonuclease activities with corresponding kinase and ribonuclease domains, splices the mRNA of transcription factor X-box binding protein 1 (XBP1) to mediate stress response genes expression; (ii) the ER membrane-associated activating transcription factor 6 (ATF6) regulates the expression of stress response related genes; and (iii) the protein kinase RNA-like ER kinase (PERK) is involved in the regulation of the translation progression pathway. These three mechanisms or pathways either work together or individually to activate UPR related gene expression to increase the protein-folding capacity in the ER or to decrease the load of proteins entering the ER (Walter and Ron 2011).

So far only two UPR pathways have been identified in Arabidopsis: the basic-leucine zipper transcription factor family protein 28 (bZip28) (the ATF6 counterpart in plants) and the Ire1 pathways (Nagashima et al. 2011). Upon ER stress, ER-localized bZip28 is transported from the ER to the Golgi by a sub-population of COPII vesicles and is then cleaved by two proteases, Site-1 protease (S1P) and Site-2 protease (S2P) in the Golgi apparatus (Liu et al. 2007). The transcription factor domain of the cleaved bZip28 then enters the nucleus to upregulate the expression of UPR related genes. Distinct from the bZip28 pathway, pre-mRNA of bZip60, the XBP1/Hac1 counterpart in plants, is spliced by Ire1b and translated to activate the ER chaperones, such as Bip3, and then triggers the downstream genes expression of UPR in order to regulate ER stress responses (ERSR) (Nagashima et al. 2011).

Autophagy is an evolutionarily conserved self-eating cellular process to deliver cytoplasmic materials or organelles into the lysosome/vacuole for degradation by forming a double-membrane structure termed the “autophagosome” (Zhang and Zhang 2016). A series of autophagy-related (ATG) proteins conserved in eukaryotes have been shown to play essential roles in this process (Mizushima et al. 2011). Even though ER stress can be alleviated via UPR, abnormal morphology and dysfunction of the ER can also be caused by over-accumulation of misfolded or unfolded proteins. To resolve ER stress and restore ER homeostasis, a selective autophagic pathway for removal of misfolded or unfolded proteins accumulating in the ER has recently been defined as ER-phagy (Grumati et al. 2018). Similarly, the autophagy receptors, which act as a bridge between autophagosome formation and autophagic cargoes selection, are also required in ER-phagy (Rogov et al. 2014). In recent years, several ER-phagy receptors have been identified and characterized, including ATG39 and ATG40 in yeast; FAM134B, CCPG1, RTN3, and ATL3 in mammals (Fregno and Molinari 2018). In plants, autophagy can also be dramatically induced by treatments with ER stress agents including tunicamycin (TM) and dithiothreitol (DTT), that prevent proper folding of proteins in the ER (Liu et al. 2012). Under such ER stress conditions, misfolded or unfolded proteins and fragments of ER can be delivered into the vacuole for degradation via ER-phagy (Liu et al. 2012), indicating the possible involvement of ER-phagy receptors in plants.

A recent study showed that a protein from the Sec translocon system is also involved in the ER stress response (Fumagalli et al. 2016). Sec62 is normally associated with Sec63 and Sec61 and participates in protein post-translational translocation (Meyer et al. 2000). Interestingly, during the ER stress recovery phase, Sec62 functions as an ER-phagy receptor via interaction with microtubule-associated protein 1A/1B-light chain 3 (LC3-1) to initiate LC3-1 anchoring to the membrane of the phagophore, thus promoting delivery of misfolded or unfolded proteins and selected ER subdomains to autolysosomes for clearance (Fumagalli et al. 2016). Furthermore, in sec62 knockdown cell line, the calnexin (CNX) labelled ER could not be delivered into autolysosomes under ER stress conditions, indicating the essential role of Sec62 in UPR of mammalian cells (Fumagalli et al. 2016). However, the functions of Sec62 in plants remain largely unknown, especially in the ER stress response.

In this study, we used multiple approaches to study the roles of Sec62 in plant development and ER-phagy in Arabidopsis. We showed that AtSec62 is an ER-localized membrane protein with three transmembrane domains (TMDs) and its C-terminus faces towards the ER lumen. This topology is different to its yeast and mammalian counterparts. We also demonstrated that AtSec62 is required for plant development because
atsec2 mutants display impaired vegetative growth, abnormal pollen, and decreased fertility. In addition, these atsec2 mutants are more sensitive towards both TM- and salt-induced ER stress, whereas overexpression of AtSec62 subsequently enhances stress tolerance during the ER stress recovery phase. Moreover, under ER stress conditions, YFP-AtSec62 colocalizes with the autophagosome marker mCh-Atg8e in ring-like structures. Lastly, the AtSec62-mediated delivery of misfolded or unfolded proteins to the vacuole for degradation is dependent upon the core autophagic machineries. Thus, AtSec62 may function as an ER-phagy receptor during ER stress in Arabidopsis.

RESULTS

AtSec62 is an integral membrane protein with its C-terminus facing the lumen

Sec61-α, Sec62, and Sec63, integral membrane proteins with multiple TMDs, are the three major components of the Sec translocon complex in different organisms (Gething and Sambrook 1992). Interestingly, AtSec62 in Arabidopsis was predicted to have a third TMD in its C-terminus region (Schweiger and Schwenkert 2013), which would mean having a different protein topology compared to its counterparts in yeast and mammalian, and thus perhaps having a unique function in Arabidopsis.

To study the precise topology of AtSec62 in Arabidopsis, a topology prediction (TMHMM server 2.0) and amino acid sequence alignment between different organisms were first performed. As seen, AtSec62 is predicted to contain three TMDs and two ATG8-family interacting motifs (AIMs) (Figures 1A, S1).

To find out whether AtSec62 is a membrane protein, we performed a western blot analysis. Total proteins were first extracted from Arabidopsis protoplasts expressing YFP-AtSec62, followed by ultracentrifugation to separate the total proteins into soluble and cell membrane fractions for subsequent immunoblotting analysis using GFP antibodies. As shown in Figure 1B, AtSec62 was detected in the membrane fraction, but not in the soluble fraction (Figure 1B, lanes 1 and 2), indicating the intrinsic membrane protein nature of AtSec62. To further determine whether AtSec62 is an integral or peripheral membrane protein, the membrane fraction was then further subjected to treatments of high salt (1 M KCl), high pH (0.1 M Na2CO3), and detergent washes (1% sodium dodecyl sulfate (SDS) and 1% Triton X-100) prior to immunoblotting with GFP antibodies where the antivacuolar sorting receptor (VSR) was used as a marker for integral membrane protein (Paris et al. 1997). Indeed, the panels of anti-GFP and anti-VSR showed the same band patterns (Figure 1B, lanes 3 to 10), revealing that AtSec62 is an integral membrane protein in Arabidopsis.

To determine which terminus of AtSec62 faces the cytosol, a protease protection assay was performed using microsomes isolated from Arabidopsis cells co-expressing YFP-AtSec62 and AtSec62-YFP, followed by immunoblotting with GFP antibodies. In the presence of the protease trypsin with or without Triton X-100, no band was detected for YFP-AtSec62, indicating that the N-terminus of AtSec62 together with YFP is facing the cytosol and can be digested by trypsin (Figure 1C, lanes 1 to 3). As expected, the C-terminus of AtSec62-YFP was protected in microsomal vesicles as a protein band was detected in the presence of trypsin without Triton X-100 (Figure 1C, lane 5). Taken together, these results indicate that AtSec62 is an integral membrane protein with its C-terminus facing the lumen.

AtSec62 is an ER membrane protein

To study the subcellular localization of AtSec62, YFP-AtSec62 was transiently co-expressed with various organelle markers including the ER membrane marker CNX1-RFP, the ER lumen protein marker RFP-HDEL as well as other two translocon components RFP-Sec61-α1, RFP-Sec63-1 in Arabidopsis protoplasts. Confocal laser-scanning microscopy (CLSM) analysis revealed that YFP-AtSec62 fully co-localizes with the ER markers CNX1-RFP, RFP-HDEL, and translocon component RFP-Sec61-α1, RFP-Sec63-1 in Arabidopsis protoplasts (Figures 1D, S1B, C). To further verify the results obtained from transient expression in Arabidopsis protoplasts, we generated double transgenic Arabidopsis plants co-expressing YFP-AtSec62 with CNX1-RFP or RFP-HDEL under the control of the ubiquitin-10 gene promoter (UBQ10). Confocal laser-scanning microscopy data indicated that YFP-AtSec62 fully co-localizes with CNX1-RFP, showing a typical ER network distribution pattern (Figure 1E). Interestingly, YFP-AtSec62 does not co-localize with the ER-body marked by RFP-HDEL (Figure S1C), which may be due to the fact that RFP-HDEL is an ER luminal protein.
marker (Tolley et al. 2008), whereas AtSec62 is an ER membrane protein in Arabidopsis.

AtSec62 is required for plant vegetative growth
To illustrate the possible functions of AtSec62 in plant growth and development, an atsec62 T-DNA insertional mutant line was ordered from the Arabidopsis information resource (TAIR). Genotyping PCR was used to identify the atsec62 T-DNA homozygous mutants. Because the T-DNA insertion site was located at the first exon of AtSec62 (indicated as GK_871A06 in Figure 2A), two sets of reverse transcription polymerase chain reaction (RT-PCR) primers were used to double confirm the absence of AtSec62 transcription in the atsec62 T-DNA insertional mutant (Figure 2A). Consistently, the transcription of AtSec62 in atsec62

Figure 1. AtSec62 is an endoplasmic reticulum (ER)-localized membrane protein in Arabidopsis
(A) Predicted protein topology of the Arabidopsis AtSec62 using TMHMM server 2.0. TMD, transmembrane domain. AIM, ATG8-family interacting motif. (B) YFP-AtSec62 is an integral membrane protein. Immunoblot analysis upon different treatments as indicated, showing that AtSec62 is a transmembrane protein. Total soluble cytosolic fractions were first isolated from Arabidopsis protoplasts expressing YFP-AtSec62 via centrifugation for 30 min at 16,000 g. The soluble fractions were then further subjected to ultracentrifugation at 100,000 g for 1 h to isolate the S and P microsome fractions respectively. The P fractions were treated with 1 M KCl, 0.1 M Na2CO3, 1% SDS, or 1% Triton X-100, followed by immunoblot analysis using various antibodies as indicated. VSR, vacuolar sorting receptor (as integral membrane protein marker); cFBPase, cytosolic fructose-1,6-bisphosphatase (cytoplasm marker); P, Pellet; S, Supernatant. (C) Protease protection assay to verify the predicted topology of AtSec62. Microsomes were isolated from Arabidopsis protoplasts expressing YFP-AtSec62 or AtSec62-YFP, followed by trypsin digestion with or without 1% Triton X-100 as indicated, and subsequent protein extraction and immunoblot analysis using GFP antibodies. (D) YFP-AtSec62 is localized at the endoplasmic reticulum (ER) in Arabidopsis protoplasts. YFP-AtSec62 was transiently co-expressed with the ER membrane marker CNX1-RFP in Arabidopsis protoplasts, followed by confocal images collection at 12 to 14 h after transfection. CNX1, Calnexin 1; UBQ10, ubiquitin-10 gene promoter; NOS ter, Nopaline synthase terminator. Bar = 25 μm. (E) YFP-AtSec62 is localized at the ER in transgenic Arabidopsis plant. Root cells of 5-d-old Arabidopsis transgenic plant co-expressing YFP-AtSec62 and CNX1-RFP were subjected to confocal imaging. Bar = 10 μm.
T-DNA mutant was undetectable in RT-PCR analysis using P1-P2 and P3-P4 primers (Figure 2A). Since only one T-DNA insertional mutant line was identified, we also generated artificial micro RNAi (amiRNAi) knockdown transgenic lines (Figure 2B) according to previous guidelines (Schwab et al. 2006). In total, we isolated two amiRNAi-atsec62 knockdown mutant lines, amiRNAi-atsec62-1# and amiRNAi-atsec62-2#. To verify the efficiency of amiRNAi-atsec62 against AtSec62, we generated YFP-AtSec62/amiRNAi-atsec62 double transgenic plants. Confocal laser-scanning microscopy analysis showed that the GFP signal in the YFP-AtSec62/amiRNAi-atsec62 double transgenic plant was significantly reduced as compared to the YFP-AtSec62 single transgenic plant (Figure 2C). In addition, western blot analysis using GFP antibodies failed to detect the YFP band in these double transgenic plants (Figure 2D), confirming that the amiRNAi-atsec62 is specific against AtSec62 and knockdowns the expression of AtSec62 in plants. Interestingly, in

Figure 2. Generation and analysis of Arabidopsis atsec62 mutants

(A) T-DNA insertional mutant of atsec62, insertion site, genotyping PCR and reverse transcription polymerase chain reaction (RT-PCR) analysis used indicated primers. (B) Generation of artificial micro RNAi (amiRNAi) lines: the amiRNAi-atsec62 target sequence was indicated in red. (C) Confocal imaging analysis of YFP-AtSec62 signals in 5-d-old root cells of transgenic Arabidopsis plants expressing YFP-AtSec62 with or without amiRNAi-atsec62. Bar = 10μm. (D) Western blot analysis for the efficiency of amiRNAi-atsec62 against YFP-AtSec62 in Arabidopsis transgenic plants. Total proteins were extracted from 5-d-old seedlings of YFP-AtSec62 and YFP-AtSec62/amiRNAi-atsec62 lines, followed by western blot analysis using GFP antibodies. cFBPase was used as the loading control. (E) Phenotyping of 7-d-old seedlings of the atsec62 T-DNA insertional mutant line and two amiRNAi-atsec62 knockdown mutant lines. Bar = 1cm. (F) Phenotypic analysis of 4-week-old atsec62 mutants vs. WT in soils. WT, wild type. Bar = 1cm.
comparison to wild type (WT), no obvious phenotypes were observed among the 7-d-old seedlings of atsec62 T-DNA insertion mutant and two amiRNAi-atsec62 knockdown lines (Figure 2E). However, at the vegetative growth stage in soil, the 4-week-old plants of the atsec62 mutants show a much smaller and weaker phenotype than the WT, indicating that AtSec62 is required for plant vegetative growth (Figure 2F).

**AtSec62 is important for pollen development and fertility**

To further confirm the functional roles of AtSec62 in the vegetative growth, the overall morphology of atsec62 mutants were observed at their mature stage. Eight-week-old plants of mutants exhibited dwarf and a smaller number of siliques compared to the WT on soil (Figure 3A). A silique-by-silique comparison with the WT showed that all siliques of atsec62 mutants are much shorter than those of WT. We also found that many aborted and with a high percentage of empty siliques in the matured atsec62 T-DNA mutant line compared to the WT. Although abnormal seeds could be observed in the two amiRNAi-atsec62 mutants, the seed formation in siliques shows no more severe defects than that in the T-DNA mutant (Figure 3B). To gain further insight into the possible causes of the weak fertility of mutants, we observed the morphology of pollen by scanning electron microscopy (SEM). As shown in Figure 3C, the structures of pollen are severely altered in both atsec62 T-DNA and amiRNAi- atsec62 mutants, as smaller and round depressed pollen were observed in these mutants vs. the WT (Figure 3C). Therefore, AtSec62 depletion general causes defects in pollen development which may affect their proper pollination.

**The atsec62 mutant is sensitive towards TM- and salt-induced ER stress**

HsSec62 was recently shown to be a crucial regulator in the maintenance and recovery of ER homeostasis during ER stress recovery phase in mammals (Fumagalli et al. 2016). To investigate the regulatory role of AtSec62 under ER stress, we conducted a high-concentration of TM treatment assay accordingly (Liu et al. 2012; Yang et al. 2016). Both the autophagy related protein ATG5 and ATG7 have been shown to play essential roles in autophagosome formation and ER stress repression, whereas both atg5 and atg7 mutants exhibited the hypersensitive phenotypes under ER stress condition (Yang et al. 2016; Zheng et al. 2019). Therefore, in this study, we also included the atg5 and atg7 mutants (Zhuang et al. 2017) as positive controls for phenotypic responses to TM-induced ER stress. The seeds of WT, atg5 mutant, atg7 mutant, atsec62 T-DNA mutant and YFP-AtSec62 were plated on half-strength Murashige and Skoog (MS) medium with or without 100 ng/mL TM, and the percentage of surviving seedlings were quantified after 12 d incubation. Seedlings with two green cotyledons and two green true leaves were defined as survival ones, while seedlings with gray leaves or cotyledons were defined as dead ones (Yang et al. 2016) (Figure 4C). In the control group, atg5, atg7 and atsec62 mutant seedlings have a similar phenotype as compared to WT and YFP-AtSec62 (Figure 4A). Nevertheless, on MS medium containing 100 ng/mL TM, the atg5, atg7 and atsec62 mutant seedlings revealed much weaker growth and a significantly lower survival percentage compared to the WT and YFP-AtSec62 (Figure 4A, D). Only 35% of atsec62 mutant seedlings can survive under ER stress conditions (Figure 4D). In addition, to know whether AtSec62 is involved in the salt-induced ER stress response, the seeds of WT, atsec62 T-DNA and amiRNAI mutants were germinated on half MS medium with 150 mM NaCl for 14 d (Li et al. 2017), followed by phenotypic observation and quantification. The seedlings with white or fully expanded cotyledon were considered as “bleached” or “green,” respectively (Figure S3). As the results indicated in Figure S3, many more bleached seedlings could be observed in atsec62 T-DNA and amiRNAI mutants vs. WT (Figure S3). Thus, AtSec62 might be involved in TM- and salt-induced ER stress responses. To investigate the protein expression level of AtSec62 upon ER stress, total proteins were extracted from the same biomass YFP-AtSec62 seedlings of TM-treated and untreated groups for immunoblotting analysis with anti-GFP antibodies. As expected, the protein amount of AtSec62 was much higher in the TM treatment group versus the control group (Figure 4B). These results indicated that AtSec62 may contribute to the ER stress tolerance and that the expression of AtSec62 may be induced by ER stress agents.

**Phenotype of atsec62 mutants could not recover from TM-induced ER stress**

HsSec62 was shown to function as an ER-phagy receptor to mediate the engulfment of misfolded or
unfolded proteins and their delivery into autophagosomes during the ER stress recovery phase in mammalian cells (Fumagalli et al. 2016). In Arabidopsis, the atsec62 mutant is sensitive towards ER stress (Figure 4A). To further illustrate the roles of AtSec62 in the ER stress recovery phase, the seeds of WT, amiRNAi-atsec62 mutants and AtSec62 overexpression transgenic lines (OE-AtSec62) were germinated on MS

**Figure 3.** The atsec62 mutants exhibit dwarf phenotype, shorter siliques, and abnormal pollen morphology

(A) The 8-week-old plants of atsec62 T-DNA mutant and two amiRNAi-atsec62 knockdown mutant lines showed dwarf phenotype compared with WT. Bar = 3 cm. (B) Observation of shorter siliques from atsec62 T-DNA and amiRNAi-atsec62 knockdown mutant lines vs. WT. Bar = 5 mm. (C) Scanning electron microscopy (SEM) analysis of pollen grains from both atsec62 T-DNA and amiRNAi-atsec62 knockdown mutant lines (showing abnormal pollen morphology) vs. the WT. Bar = 10 μm.
medium with a high concentration of TM (300 ng/mL) for 6 d, and then transferred to normal half MS medium without TM for 6 d and 10 d, respectively. We then quantified the root lengths at indicated days after recovery. As shown in Figure 5, both WT and OE-AtSec62 seedlings could recover from ER stress as demonstrated by the quantification of germination and root length (Figure 5). In contrast, the recovery phenotypes of the two amiRNAi-atsec62 mutants were substantially weaker than those in the WT and OE-AtSec62. Briefly, the root lengths of the two individual amiRNAi-atsec62 knockdown lines were 0.37 cm, 0.31 cm after recovery for 6 d and 0.45 cm and 0.46 cm after recovery for 10 d, respectively, which

Figure 4. The atsec62 mutants are sensitive to TM-induced ER stress

(A) The 12-d-old seedlings of WT, atsec62, YFP-AtSec62, atg5 and atg7 were observed on half-strength MS medium with or without 100ng/mL TM (to induce ER stress). DMSO was used as a solvent control for all experiments. MS, murashige and skoog; DMSO, dimethyl sulfoxide. Bar = 1cm. (B) Western blot analysis of YFP-AtSec62 under ER stress. Total proteins were extracted from the YFP-AtSec62 seedlings with or without TM-induced ER stress as indicted in (A), followed by SDS-PAGE and western blot analysis using GFP antibodies. cFBPase was used as a loading control. (C) Five times enlargement of the section indicated in (A), showing the surviving seedlings (examples as indicated by the white arrowheads) and the dead seedlings (as indicated by the white arrows). (D) Quantification analysis of the survival rate using three replicates with 50 seedlings of each genotype per replicate. Asterisk indicated significant different (Student’s t-test, P < 0.05).
were significantly shorter than those of WT and OE-AtSec62 (Figure 5B, C). Therefore, AtSec62 plays an important role during ER stress recovery phase in Arabidopsis.

**AtSec62 is involved in ER-phagy during ER stress**

Atg8e is widely used as an autophagosome marker in Arabidopsis (Contento et al. 2005). To study the possible interplay between AtSec62 and autophagy under...
ER stress, we generated double transgenic Arabidopsis plants co-expressing YFP-AtSec62 and mCh-Atg8e. Five-d-old YFP-AtSec62/mCh-Atg8e double transgenic plants were first subjected to TM and DTT treatments to induce ER stress, followed by confocal observations. As shown in Figure 6, upon treatment with TM and DTT for 8 h and 4 h respectively, mCh-Atg8e positive autophagosomes were detected in transgenic plants, but such structures were rarely seen in control plants (Figure 6A, panels 1 to 3, middle), indicating that autophagy is induced by both TM- and DTT-induced ER stress (Liu et al. 2012). In addition, large numbers of YFP-AtSec62 positive ring-like structures were observed, which were distributed throughout the ER network upon TM and DTT treatments (Figure 6A, panels 1 to 3, left), demonstrating that YFP-AtSec62 not only shows a normal ER network pattern but is also enriched in ER subdomains and forms AtSec62 positive ring-like structures under ER stress conditions. However, such structures were not observed in the control plants (Figure 6A, panel 1).

Importantly, these ring-like structures could co-localize with the autophagosomal marker mCh-Atg8e (Figure 6A, panels 2 and 3, right). To further find out whether mCh-Atg8e were fully co-localized with all of the YFP-AtSec62 positive ring-like structures under ER stress agents, the Z-Stack projection images were collected and the co-localization percentage of mCh-Atg8e with YFP-AtSec62 positive ring-like structures was quantified (Figure S4, panels 2 and 3). About 78% and 66% of mCh-Atg8e positive autophagosomes co-localize with YFP-AtSec62 positive ring-like structures under TM and DTT treatment conditions, which are significantly higher than that in control group (Figure 6B).

The increased co-localization percentage of YFP-AtSec62 positive ring-like structures with mCh-Atg8e under ER stress could be due to either (i) the AtSec62-labelled ER network acts as a membrane source for the formation of autophagosomes (Zhuang et al. 2017), or (ii) the misfolded or unfolded proteins may trigger the interaction between AtSec62 and Atg8e that assists the engulfment of misfolded or unfolded ER proteins as well as ER subdomains into autophagosomes during ER-phagy (Chen et al. 2018). Previous studies have demonstrated that exogenous benzo thiadiazole (BTH) or starvation can trigger autophagosome formation in plants (Zhuang et al. 2013) and misfolded or unfolded proteins caused ER stress can induce ER-phagy (Liu et al. 2012). To distinguish between these two possibilities, we next performed autophagosome induction and GFP core turn over assays.

Upon BTH and starvation (minus sucrose or nitrogen) treatments, many more mCh-Atg8e labelled red dots and ring-like structures appeared in root cells, whereas few YFP-AtSec62 positive ring-like structures were observed during these autophagy induction conditions (Figure 6A, panels 4 to 6), indicating that YFP-AtSec62 does not respond to these conventional autophagosome induction treatments. Quantitative analysis from Z-stack projections (Figure S4, panels 4 and 5) further confirmed this conclusion (Figure 6B).

One possible role of autophagy during ER stress is to degrade misfolded or unfolded proteins via ER-phagy. Since two AIMS were found in AtSec62 protein (Figures 1A, S1A), we then firstly investigated the possible relationship between AtSec62 and Atg8e during ER-phagy. Various AIMP motif mutant forms of AtSec62, including YFP-AtSec62-ΔAIM1, YFP-AtSec62-ΔAIM2, and YFP-AtSec62-ΔAIM1/2, were transiently co-expressed with mCh-Atg8e in Arabidopsis protoplasts, followed by TM treatment at 5 μg/mL for 6 h (Bao et al. 2018). Total proteins were then extracted from the protoplasts and used for co-immunoprecipitation (Co-IP) using GFP-trap agarose beads, followed by immunoblotting with GFP- and RFP-antibodies. As shown in Figure 6C, the wild type form AtSec62 could interact with Atg8e, but the single AIMP motif mutated form of AtSec62 reduced its binding ability to Atg8e, whereas the double AIMS mutated form of AtSec62 almost abolished its interaction with Atg8e (Figure 6C). Taken together, AIMS are indispensable for AtSec62 interaction with Atg8e during ER-phagy. To further find out the possible relationship between AtSec62 and Atg8e-mediated ER-phagy, a GFP turnover assay reflecting the delivery of autophagosome to vacuole was performed. Total proteins extracted from YFP-AtSec62 plants upon TM, DTT, BTH, and starvation treatments were then subjected to immunoblotting analysis with GFP antibodies. Consistent with the data from confocal analysis, much more YFP core was detected in YFP-AtSec62 after TM and DTT treatments (Figure 6D, lane 1), supporting the notion that AtSec62 and Atg8e may assist the engulfment of misfolded or unfolded proteins as well as ER subdomains into autophagosomes for subsequent delivery to vacuole during ER-phagy. Taken together, these results demonstrate that the formation of AtSec62 positive ring-like...
AtSec62 regulates plant development and ER-phagy
structures may be involved in the degradation of misfolded or unfolded proteins during ER-phagy induced by ER stress and that AtSec62 can mediate the delivery of misfolded or unfolded proteins into autophagosomes by interacting with Atg8e during ER-phagy.

The interplay between AtSec62 and autophagy in ER-phagy requires core autophagic machinery
Autophagosome initiation and completion require two core autophagic machinery genes, ATG5 and ATG7. ATG7 activates ATG5 to form a complex with ATG12 and ATG16L1 that promotes the extension of the phagophore membrane in autophagic vesicles (Zhuang et al. 2013). To determine the possible relationship between AtSec62 and core autophagic machinery in ER-phagy, we transformed YFP-AtSec62 into ATG5- and ATG7-deficient mutants to investigate their depletion effects on AtSec62 subcellular localization under ER stress. We hypothesized that the proper function of AtSec62 in ER-phagy depends on the autophagic machinery, where depletion of ATG5 and/or ATG7 would affect its function in ER-phagy under ER stress (Figure 7A). As shown in Figure 7B, YFP-AtSec62 positive ring-like structures were clearly observed in WT root cells upon TM and DTT treatments (indicated by arrows in Figure 7B, panels 2 and 3, left). However, in both atg5 and atg7 mutant background transgenic plants, no obvious ring-like structures were observed for YFP-AtSec62 under identical ER stress treatments (Figure 7B, panels 2 and 3, right). Additionally, no YFP-AtSec62 positive ring-like structures appeared in WT, atg5 and atg7 mutant background transgenic lines after the conventional autophagy induction treatments (BTH, -C, and -N) (Figure 7B, panels 4 to 6). These results demonstrated that both ATG5 and ATG7 are essential for ER-phagy in plants.

Moreover, a GFP turnover assay was carried out to further confirm the essential roles of ATG5 and ATG7 in AtSec62-mediated autophagy in ER-phagy under ER stress conditions. Total proteins extracted from YFP-AtSec62/atg5 or atg7 transgenic plants after various treatments (i.e., TM, DTT, BTH, -C, and -N) were subjected to immunoblotting analysis with GFP antibodies. Consistent with our hypothesis and the confocal data, little YFP core was detected in YFP-AtSec62/atg5 or atg7 after these treatments (Figure 6D, lanes 2 and 3), demonstrating that ATG5 and ATG7 deficiency impair the formation of YFP-AtSec62 positive ring-like structures and their subsequent delivery to vacuole via the autophagosome pathway during ER-phagy (Figure 7A, B). Taken together, AtSec62-mediated autophagy in ER-phagy requires these two core autophagic machineries.

**DISCUSSION**

AtSec62 has a unique topology distinct from yeast and mammals
The Sec translocation system, consisting of a central pore Sec61α and two auxiliary proteins Sec62 and Sec63, has been well-characterized in yeast and mammals. All three Sec proteins contain multiple TMDs and localize to the ER membrane (Meyer et al. 2000). In the
Arabidopsis genome, the counterparts of the central pore component Sec61-α has three isoforms, Sec63 has two isoforms whereas only one Sec62 isoform has been identified in Arabidopsis (Yamamoto et al. 2008; Schweiger and Schwenkert 2013). The Sec62 protein mainly plays a role in the post-translational translocation process (Lang et al. 2012). It has been suggested that although AtSec62 shows less homology to

Figure 7. AtSec62-mediated ER-phagy depends on the core autophagic machineries
(A) Possible roles of AtSec62 in ER-phagy and relationship with the autophagic machinery under experiments. Upon TM and DTT treatments, AtSec62 together with ATG5 and ATG7 would mediate ER-phagy to lead the degradation of misfolded proteins. However, in both atg5 and atg7 mutants, AtSec62 could not mediate misfolded proteins into the vacuole during ER stress conditions. (B) The YFP-AtSec62 positive ring-like autophagosome structures are induced by both TM and DTT treatments in WT seedlings, but atg5 and atg7 mutants prevented the formation of these structures (panels 2 and 3). Five-d-old YFP-AtSec62 transgenic Arabidopsis seedlings in WT, atg5 and atg7 mutants were transferred to liquid MS medium with dimethyl sulfoxide (DMSO), 5,000 ng/mL TM, 2 mM DTT, 100 μM BTH or without sucrose (-C), nitrogen (-N) for indicated times, followed by confocal imaging analysis. Bar = 10 μm.
the counterparts in yeast and mammals, it still contains the same translocon domains among all organisms (Schweiger and Schwenkert 2013). Indeed, in this study, we found that only 12% and 15% sequence identity exist between AtSec62 in Arabidopsis and its orthologues in yeast and mammalian, respectively (Figure S1A). Interestingly, and distinct from its yeast and mammalian homologs, AtSec62 is predicted to contain a third TMD in the C terminus which faces the lumen (Figure 1A, S1). This unique topology indicates that the AtSec62 may harbor additional plant-specific function in Arabidopsis.

AtSec62 is required for plant development
In this study, both 7-d-old seedlings of atsec62 T-DNA and amiRNAi-atsec62 knockdown mutants showed no obvious phenotype, which may be due to pre-existing storage compounds and nutrition in seeds which would be sufficient to support the early stage seedlings development during which the ER import activity is at low level. Interestingly, at late stages, we found that atsec62 mutants display multiple growth defective phenotypes including abnormal pollen, short and empty siliques, and inhibited vegetative growth and dwarf phenotype in soil (Figures 2, 3). Thus, the atsec62 mutant caused a Sec translocon complex deficiency that could indeed result in plant developmental defects.

Sec63 is a J domain-containing protein, which function as a Sec translocon component during protein post-translational translocation across the ER membrane in mammals and yeast (Yamamoto et al. 2008). Two isoforms of Sec63, orthologues of mammalian ERdj2 and yeast Sec63, have been identified in Arabidopsis (Yamamoto et al. 2008). They are AtERdj2A and AtERdj2B. Yamamoto et al. (2008) reported that mutation of AtERdj2A could cause a lethal phenotype in the aterdj2a homozygote mutant but defects in pollen germination in the aterdj2a heterozygote mutant (Yamamoto et al. 2008). It has been reported that protein secretion is indispensable for pollen development. Inhibitions of fertilization, pollen germination and tube elongation have been reported when the protein secretory pathway is blocked (Fragkostefanakis et al. 2016). For example, Sec24A is a key component of the COPII vesicle coat, which plays an essential role in the process of ER-to-Golgi protein transport. Loss-of-function mutation of AtSec24A can cause defects in male transmission during fertilization (Conger et al. 2011). Rab2, a small GTPase, is involved in ER-to-Golgi protein transport. The pollen germination and pollen tube growth inhibitions can be observed when a dominant-negative mutant of Rab2 is expressed in tobacco pollen, in which proteins secretion is impaired (Cheung et al. 2002). Thus, the pollen germination defect of aterdj2a probably reflects protein translocation and secretion involvement of AtERdj2A in Arabidopsis. Both AtSec62 and AtERdj2A are the key components of Sec translocon during protein post-translational translocation in Arabidopsis. In this study, severely altered pollen morphology and low infertility phenotypes were observed in both T-DNA and amiRNAi mutants of atsec62 (Figure 3B, C), indicating that fertilization, pollen germination or pollen tube elongation process may be inhibited or disturbed owing to the deficiency of protein translocation and secretion in atsec62 mutants. Recent studies showed that overexpression of AtGet3a, a guided entry of tail-anchored proteins (GET) pathway related protein, in the atget1 mutant exhibited similar but more severe vegetative growth defects in comparison with atsec62 (Srivastava et al. 2017; Xing et al. 2017). Since both AtGet3 and AtGet1 are involved in the process of tail-anchored proteins to ER import, it was hypothesized that overexpression of AtGet3a in atget1 mutant background may result in trapping of tail-anchored proteins in the cytosol, thus disturbing AtSec62-mediated post-translational translocation of proteins (Xing et al. 2017), resulting in reduced seeds number, shorter sique and dwarf phenotype both in these mutant plants and atsec62 mutants. Moreover, immunoglobulin-binding proteins (Bips) have been demonstrated to play an essential role in pollen development and fertilization, indicating that not only protein quality control, protein folding and protein translocation in the ER require Bips, but also male gametogenesis and pollen tube elongation need Bips participation in Arabidopsis (Maruyama et al. 2014). Taken together, Sec62 and Sec63, as Sec translocon components, are required for plant development, especially in pollen development and male fertility.

AtSec62 is involved in UPR
Unfolded protein response activated by the accumulation of misfolded or unfolded proteins in the ER is a critical cellular stress response in ER stress. ER stress not only can be induced by external stresses such as the elevated temperature (ET) but also by internal developmental and physiological conditions (Bernales et al. 2006). For example, the ER-folding machinery related components and
UPRs are found to be activated and upregulated at specific stages of pollen development via proteome and transcriptome analyses, supporting the elevated demands for protein secretion during this process (Fragkostefanakis et al. 2016). In plants, two UPR pathways have been identified (Nagashima et al. 2011), (i) The membrane-associated RNA splicing factor-Ire1; (ii) The ER membrane-associated transcription factor-bZIP28/bZIP17. As one branch of UPR, Ire1 is essential for regulation of ER homeostasis upon ER stress.

In mammals, Ire1 is essential for mouse development because inactivation of the Ire1a gene could lead to the lethality phenotype of mouse due to severe placental dysfunction (Iwawaki et al. 2009). In Arabidopsis, Ire1 has two isoforms, AtIre1a and AtIre1b (Walter and Ron 2011), whereas Liu et al. (2012) and Bao et al. (2018) reported that AtIre1b is the major component of Ire1 pathway that involved in the ER stress response (Liu et al. 2012; Bao et al. 2018). In field plants, heat shock stress is the major ER stress inducer (Nadeem et al. 2018). Upon ET, the atire1a atire1b double mutant shows sensitivity to ET with defects in silique development. Upon TM treatment, the atire1a atire1b double mutant showed over-sensitivity phenotype compared with atire1a or atire1b single mutants and WT (Deng et al. 2016), showing that the AtIre1 pathway is essential for UPR in Arabidopsis under extreme conditions. In addition, Yan et al. (2019) reported that knockout of bZIP17 has profound effects on vegetative development in the atire1a atire1b atbZIP17 triple mutant background under normal condition (Bao et al. 2019), revealing the UPR not only participates in development of Arabidopsis at a low active level under normal condition but is also highly regulated upon ER stress. Thus, UPR pathways are essential for plant reproductive and vegetative growth. Interestingly, in our study, we found that atsec62 mutants are much more sensitive towards TM-induced ER stress as shown by reduced surviving percentage and defective phenotype in ER stress recovery phase (Figures 4 and 5). Also, atsec62 mutants showed decreased fertility and inhibited vegetative growth in soil compared to WT under normal condition (Figure 3). These phenotypes are consistent with those found in atire1a atire1b double and atire1a atire1b atbZIP17 triple mutants, thus we hypothesize that AtSec62 plays important function in UPR.

Li et al. (2017) reported that ER chaperones and endoplasmic-reticulum-associated protein degradation (ERAD) related proteins, Hrd3a and Hrd1b, could be specifically regulated by the UPR transcription factors (TFs) AtbZIP60, AtbZIP17, and AtbZIP28 under salt-induced ER stress condition. For example, over-expression of these three bZIPs TFs in the ERAD deficiency mutant hrd3a-2, could restore its tolerance to salt-induced ER stress, suggesting a possible interplay mechanism between ERAD and UPR in Arabidopsis (Li et al. 2017). Interestingly, both T-DNA and amiRNAi mutants of atsec62 showed hypersensitive phenotype towards salt treatment, in which many more bleached and dead seedlings of atsec62 mutants were observed vs. WT (Figure S3). We thus hypothesize that AtSec62 may also be involved in the ERAD pathway under salt-induced ER stress even though the underlying mechanism of AtSec62 function in the ERAD pathway remains elusive.

AtSec62 may act as an ER-phagy receptor in plants

Generally, in order to cope with the accumulation of misfolded or unfolded proteins in the ER caused by ER stress, cells elicit UPR and ER-phagy (Bernales et al. 2006; Liu et al. 2012; Yang et al. 2016; Grumati et al. 2018). To maintain ER homeostasis under ER stress, misfolded or unfolded proteins must be rapidly recognized by ER-phagy receptor and transported to the lysosome/vacuole for degradation via autophagosomes. An ER-phagy receptor, acting as a bridge between autophagosomes formation and autophagic cargoes in the autophagic pathway, should have at least three characters, (i) contains at least one LC3 interacting region (LIR) motif (AIM motif in plants); (ii) has ER membrane localization pattern; and (iii) function in mediating misfolded or unfolded proteins to the lysosome/vacuole (Grumati et al. 2018). Up to now, several ER-phagy receptors have been identified in yeast and mammals, including ATG39, ATG40, FAM134B, CCPG1, RTN3, and ATL3, with all of them showing ER membrane localization pattern and containing at least one LIR motif (Fregno and Molinari 2018). The mammalian Sec62, a constituent of the translocon complex, was recently shown to be an ER-phagy receptor to maintain ER homeostasis during ER stress recovery phase (Fumagalli et al. 2016).

In this study, we showed that the ER network distribution pattern of YFP-Sec62 can co-localize with the autophagosomal marker mCh-Atg8e in ring-like structures upon ER stress agents in plants. However, these YFP-AtSec62 positive ring-like structures were rarely formed under conventional autophagic induction conditions (induced by BTH treatment and nutrition...
starvation) and the percentage of co-localization between mCh-Atg8e and YFP-AtSec62 was dramatically lower than those under ER stress condition induced by TM or DTT (Figure 6A, B). Co-immunoprecipitation analysis data further demonstrated that AIM motifs in AtSec62 are indispensable for the interaction between AtSec62 and Atg8e, especially the second AIM motif (Figures 1A, 6C, S1A). In addition, we investigated the subcellular behavior of YFP-AtSec62 in atg5 or atg7 mutant backgrounds during ER-phagy induced by ER-stress, whereas the AtSec62-positive ring-like structures were hardly observable, indicating that AtSec62-mediated ER-phagy is ATG core machinery dependent. Taken together, since AtSec62 is an ER membrane-localized protein with two AIM motifs and specifically responds to ER stress at the subcellular level, these features tend to meet the criteria of AtSec62 being an ER-phagy receptor, albeit that the specific cargoes (misfolded or unfolded proteins) of AtSec62 during ER-phagy remain to be identified. Moreover, the root length and seedlings phenotype of AtSec62 over-expression lines recovered from ER stress for 10 d even slightly better than WT (Figure 5), suggesting that AtSec62 may also have a similar function in recovery ER-phagy (recovER-phagy) as HsSec62 in mammals.

In conclusion, consistent with our findings in this study, an independent work on AtSec62 recently showed that loss of AtSec62 led to impaired growth and drastically reduced male fertility and that the C-terminus of AtSec62 was indispensable for its function (Mitterreiter et al. 2019). In this study, we have also provided the first set of evidence to demonstrate the roles of AtSec62 in ER-phagy under ER stress and its likely function as an ER-phagy receptor in plants. Thus, being an ER-phagy receptor in UPR, misfolded or unfolded proteins under TM- or DTT-induced ER stress would be recognized by the C-terminus of AtSec62 and subsequently promote the interaction with Atg8e via AIM motifs for vacuolar degradation via ER-phagy to maintain ER homeostasis (Figure 8).

MATERIALS AND METHODS

Plant materials and growth and treatment conditions
Arabidopsis thaliana ecotype Columbia-0 was used as wild-type control and background. Arabidopsis PSBD cell line was also used for transient expression. Artificial microRNA interference (amiRNAi) lines were generated according to previous methods (Li et al. 2013). Primers are listed in Table S1. Fluorescent tag lines and amiRNAi lines were generated and selected by Agrobacterium flower dipping. Arabidopsis T-DNA insertional mutant atsec62 (GK_871A06) was obtained from the TAIR. Seeds were surface sterilized and sown on half MS medium with 1% sucrose and 0.8% agar. The plates were kept at 4°C for 1 d before being moved to the growth chamber (22°C, 16 h light/8 h dark cycle). Five-d-old seedlings were transferred in liquid MS with 100 μM BTH or 5,000 ng/mL TM for 8 h, or 2 mM DTT for 4 h before observation. For starvation treatment, 5-d-old seedlings grown on half MS plates were then transferred to liquid half MS medium without sucrose or nitrogen for an additional 16 h in the dark. For phenotypic analysis, seeds were grown on the half MS plates or half MS plates containing 300ng/ml TM until further analysis as indicated in results.

RNA extraction and RT-PCR analysis
Total RNA was extracted from 5-d-old seedlings using the RNeasy (Plant) Mini Kit (Qiagen, Valencia, CA) according to manufacturer’s instructions. The first strand for each complementary DNA was synthesized by SuperScript II reverse transcriptase (Invitrogen, Thermo Fisher Scientific, CA). Oligonucleotides for RT-PCR were listed in Table S1.

Confocal microscopy
Transient expression of fluorescent protein in Arabidopsis PSBD cells were performed as described previously (Miao and Jiang 2007). Five-d-old Arabidopsis seedlings were collected and imaged with Leica TCS SP8 confocal laser-scanning microscope. 63× (NA 1.20) water immersion objective was used for imaging. We used emission laser at the wavelength of 488 nm for YFP, and 552 nm for mCh/RFP. YFP and mCh/RFP channels were used sequential scanning to collect fluorescent signals. The same microscope settings were used for both the control groups and the mutant or treated groups. At least three biological and technical replicates were performed in this experiment.

Protein extraction
To extract protein from protoplasts, transformed protoplasts were first washed with 250 mM NaCl and followed by centrifugation at 100 g for 10 min to
collect cells. Cells were resuspended in ice cold lysis buffer containing 50 mM Tris‐HCl (pH 7.4), 150 mM NaCl, 0.5 mM EDTA, 5% glycerol, 0.5% Triton X-100, and 1× Complete Protease Inhibitor Cocktail (Roche Diagnostics, CA). The total cell extracts were centrifuged at 20,000 g for 30 min at 4°C. For the protein from transgenic plants, 5-d-old Arabidopsis seedlings were grinded in lysis buffer, followed by brief centrifugation at 600 g for 5 min to remove cell debris. The supernatant was then centrifuged at 16,000 g for 30 min at 4°C. All experiments related to protein extraction were done on ice or at 4°C before further analysis.

Topology analysis and protease protection assay
Total proteins were extracted from protoplasts expressing YFP-AtSec62 without the addition of detergent as described previously (Zhuang et al. 2017). Total protein extracts were divided into soluble and membrane fractions by centrifugation at 16,000 g for 30 min. Membrane pellets were then divided into equal fraction and incubated with 1 M KCl, 0.1 M Na₂CO₃, 1% Triton X-100 or 1% SDS for 30 min, followed by ultracentrifugation at 100,000 g for 30 min to separate into soluble and membrane fractions again. Fractions were then subjected to SDS-PAGE and immunoblot analysis using anti-GFP, anti-VSR and anti-cFPBase antibodies as described previously (Chung et al. 2018). For protease protection assay, microsomes isolated from protoplasts expressing YFP-AtSec62 were subjected to trypsin digestion as described previously (Chung et al. 2018). GFP antibodies were used for immunoblotting analysis.

Immunoprecipitation and immunoblotting
The supernatant was incubated with GFP-Trap agarose beads (ChromoTek GmbH, Martinsried, Germany) for 4 h in immunoprecipitation (IP) buffer (50 mM Tris- HCl, pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 5% glycerol, 2% Nonidet P-40, and 1× Complete Protease Inhibitor Cocktail). Then the beads were washed five times with ice-cold washing buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 5% glycerol, and 0.05% Nonidet P-40) and boiled in SDS sample loading buffer for 10 min at 100°C. The samples were then separated on 12% SDS-polyacrylamide gel (SDS-PAGE) and transferred to nitrocellulose membranes (Bio-Rad, Richmond, CA) followed by blocking in PBS-T (0.05% Tween-20) with 5% milk powder and antibody.

Figure 8. Working model of AtSec62 function in ER-phagy
Both TM and DTT treatments caused the accumulation of misfolded or unfolded proteins in the ER lumen, resulting in ER stress. To maintain ER homeostasis, AtSec62 may function as an ER-phagy receptor and interacts with Atg8e via AIM motifs, that would enable the engulfment of misfolded proteins and parts of the ER membranes into the autophagosomes for subsequent delivery to the vacuole for degradation.
incubation (4 μg/mL) (Wang et al. 2018b; Yu et al. 2018). Horseradish peroxidase-conjugated secondary antibodies were used, and signal was detected by the ECL Western Blotting system (GE Healthcare, Pasadena, CA, USA).

Scanning electron microscopy
Pollen grains were collected from freshly dehisced anthers of 4-week-old plants. Then the samples were mounted on SEM stubs. The pollen grains were dried in the air and then coated with palladium-gold in a sputter coater (Edwards Coater S150B, Edwards, London, UK) and examined by SEM (S-3400N, Hitachi, Tokyo, Japan) at an acceleration voltage of 10 kV.

Sequence analysis
The topology cartoon of Sec62 were defined based on predictions from TMHMM2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The AIM motifs were identified using in silico identification of functional LC3 interacting region motifs (http://repeat.biol.ucy.ac.cy/cgi-bin/iLIR/iLIR_cgi). Amino acid sequence alignments were generated using MEGA-X and Clustal-W.

Statistical analysis
Root length and co-localization percentage analysis were addressed using Student’s t-test (two-sided, equal variance) in SPSS 22.0. The significance threshold was set at P ≤ 0.05.

Accession numbers
Sequence data for the proteins analyzed used in this study can be found in the NCBI data libraries under following accession numbers: At1g29310 (AtSec61), At3g20920 (AtSec62), At5g61790 (CNX1), At1g79940 (AtSec63), At5g61790 (CNX1), XP_008681002 (Zea mays Sec62), XP_015627309 (Oryza sativa Sec62), XP_003573202.1 (Brachypodium distachyon Sec62), XP_009406043.1 (Physcomitrella patens Sec62), XP_001701717 (Chlamydomonas reinhardtii Sec62), NP_081292 (Mus musculus Sec62), NP_003253 (Homo sapiens Sec62), and NP_015231 (Saccharomyces cerevisiae Sec62).

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AUTHOR CONTRIBUTIONS
S.H. and L.J. conceived and designed the experiments. S.H. and H.Y. performed the experiments. S.H., H.Y., and Y.C. wrote the article. S.H., H.Y., Y.C., and L.J. edited the manuscript. All authors read and approved the final manuscript.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the supporting information tab for this article: http://onlinelibrary.wiley.com/doi/10.1111/jipb.12872/supinfo

**Figure S1.** AtSec62 is conserved in eukaryotic organisms (A) Amino acid sequence alignment of AtSec62 with homologues from O.ativa, Z.mays, B.distachyon, M.acuminata, P.patens, C.reinhardtii, M.musculus, H.sapiens, S.cerevisiae. AIM motifs were highlighted in red. Conserved sequences were shaded in black/grey. (B) Topology models of Sec62 protein from yeast and human.

**Figure S2.** AtSec62 co-localizes with ER marker and two translocon components, Sec61-α1 and Sec63-1 (A) Constructs used in transient expression. (B) The constructs of two translocon complex components Sec61-α1 and Sec63-1 were transiently co-expressed with AtSec62 in Arabidopsis protoplasts for 12-14 h before confocal imaging (panels 1 and 2). Bar = 25 μm. (C) Colocalization of YFP-AtSec62 with the ER marker RFP-HDEL in Arabidopsis protoplasts (panel 3) and root cells of 5-day-old transgenic seedlings (panel 4). Bar = 25 μm.

**Figure S3.** atsec62 mutants are sensitive to salt stress (A) The seeds of atsec62 mutants and WT were cultured on the half MS medium with 150 mM NaCl for 14 d prior to phenotypic observation. (B) Quantification of the green and bleached seedlings based on results in (A). Seedlings with white cotyledon were considered as “bleached”. Seedlings with fully expanded cotyledon were considered as “green”.

**Figure S4.** AtSec62 colocalized with the autophagosome marker mCh-Atg8e in ring-like structures in transgenic Arabidopsis seedlings treated with TM and DTT Z-stack projections for the 5-day-old transgenic plant roots expressing YFP-AtSec62 and mCh-Atg8e upon TM, DTT, BTH, -C and -N treatments were collected via confocal image analysis. Bar = 20 μm.

**Table S1.** The primers used in the experiments