

# The endoplasmic reticulum-associated degradation is necessary for plant salt tolerance

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**Eukaryotic organisms have quality-control mechanisms that allow misfolded or unassembled proteins to be retained in the endoplasmic reticulum (ER) and subsequently degraded by ER-associated degradation (ERAD). The ERAD pathway is well studied in yeast and mammals; however, the biological functions of plant ERAD have not been reported. Through molecular and cellular biological approaches, we found that ERAD is necessary for plants to overcome salt stress. Upon salt treatment ubiquitinated proteins increased in plant cells, especially unfolded proteins that quickly accumulated in the ER and subsequently induced ER stress responses. Defect in HRD3A of the HRD1/HRD3 complex of the ERAD pathway resulted in alteration of the unfolded protein response (UPR), increased plant sensitivity to salt, and retention of ERAD substrates in plant cells. Furthermore, we demonstrated that Ca<sup>2+</sup> release from the ER is involved in the elevation of UPR and reactive oxygen species (ROS) participates the ERAD-related plant salt response pathway.**

**Keywords:** salt stress; ERAD; UPR; Ca<sup>2+</sup>; ROS

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## Introduction

In eukaryotic cells, both luminal and integral membrane proteins entering the secretory pathway are modified and folded in the endoplasmic reticulum (ER) to achieve their native structures. About one-third of newly synthesized proteins are misfolded [1]. Misfolded proteins are retained in the ER until they are either further folded with the help of ER chaperones, or ultimately destroyed by ER-associated degradation (ERAD) [2]. The accumulation of unfolded proteins in the ER can be induced by impairment of protein folding derived from mutation, perturbation of protein-protein interactions, or different biotic and abiotic stress stimuli. Accumulated unfolded proteins then cause ER stress [3]. The cell has evolved several mechanisms to recover under ER stress conditions. The misfolded proteins are cleared by the

ERAD system. Meanwhile, protein translation is halted to decrease ER burden and a mechanism termed the unfolded protein response (UPR) is activated to increase the expression of ER chaperones and ERAD components to help protein folding and to stimulate degradation of unfolded proteins. If all these processes still cannot restore cell homeostasis within a certain time period, programmed cell death (apoptosis) would be initiated [4]. ERAD is a specific ubiquitin/proteasome system associated with the ER. Similar to the general ubiquitination/degradation systems, it requires ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), ubiquitin ligase (E3), and 26S proteasome, as well as other associated proteins [5]. Because the process occurs in the ER, there are specific complexes involved, including at least some specific E2s and E3 and other associated proteins [6]. It is known that there are two different ERAD complexes in yeast, the HRD1/HRD3 complex and the DOA10 complex. The key components of the HRD1/HRD3 complex are E2s, including UBC1 and UBC7, RING finger type E3 ligase HRD1, and HRD3 that recruits the unfolded proteins for degradation. The key components of the DOA10 complex are E2s, includ-

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ing UBC6 and UBC7, and RING finger type E3 ligase DOA10 [7]. Deletion of these genes would affect the ability of the organism to scavenge unfolded proteins [8, 9].

To date, most of the knowledge about ERAD is obtained from yeast and mammals. It has been shown to be involved in a number of human diseases [10-12] and in yeast, ERAD has been shown to be related with various stresses. The defect of UBC7 and UBC1 or the high expression of HRD1 affects yeast responses to  $\text{Cd}^{2+}$ , a toxic ion to organisms [13]; *doa10* and *ubc7* mutants are more sensitive to cold than wild type (WT), perhaps through effects on sterol biosynthesis [9]; and overexpression of UBC1, UBC6, and UBC7 increases yeast tolerance to several stresses, including heat,  $\text{H}_2\text{O}_2$ , and canavanine [14].

Plants are sessile organisms that must cope with multiple environmental stresses throughout their lives, such as dramatic temperature changes, drought, high salinity, and pathogenic infections [15, 16]. Among them, salt stress is a serious and global abiotic factor, since high  $\text{Na}^+$  ions in soil are toxic to plants. Under salt stress the activities of many essential enzymes are inhibited, reactive oxygen species (ROS) accumulate, cell division and expansion are blocked, cellular membrane is disorganized, and the osmotic balance is destroyed, all of which finally lead to growth inhibition [17]. Many genes have been identified to be involved in the plant response to salt stress. An excellent example is the set of SOS genes described by Zhu and coworkers [32]. Salt stress induces the accumulation of  $\text{Ca}^{2+}$  in the cytoplasm, which is sensed by calcium sensors such as SOS3, which then interacts with SOS2, a protein kinase. The SOS3-SOS2 complex phosphorylates SOS1, a plasma membrane  $\text{Na}^+$ / $\text{H}^+$  anti-porter, which effuses excess  $\text{Na}^+$  ions out of cell to reduce the salt damage to plant cells. Previous studies have shown that UPR is induced upon salt stress [18] and that many stress-related genes are upregulated by tunicamycin (TM) and dithiothreitol (DTT), two ER stress inducers [19, 20]. Some proteins that have functions in both ER quality control (ERQC) and stress responses also have been identified [21-23]. These studies suggest that ER stress and UPR are involved in plant responses to environmental stresses. While studies with a number of substrate proteins have demonstrated that the ERAD pathway exists in plants [24-27], the relationship between ERAD and environmental stress responses has not been explored in plant.

In this study, we identified an ERAD component, HRD3A, in *Arabidopsis*, which is the homolog of yeast HRD3. By genetic, molecular, and cell biology approaches, we discovered that HRD3A is an active com-

ponent of plant ERAD and is involved in plant UPR. By mutational analysis, we found that the intact plant ERAD pathway was necessary for the plants' tolerance to salt stress. Defects of ERAD affected the plant response to salt, and ROS was found to be involved in this process. Furthermore, we found that UPR induced by accumulation of unfolded proteins upon salt and TM treatment was affected by  $\text{Ca}^{2+}$  release from the ER.

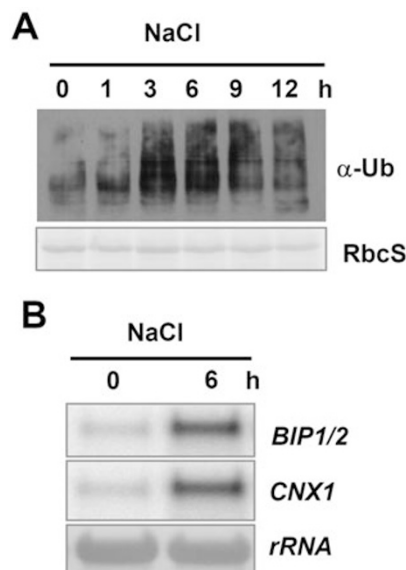
## Results

### *Salt treatment quickly induces the ER stress response*

It is known that in mammals and yeast, the ubiquitin/26S proteasome system (UPS) and especially ERAD are involved in degrading the misfolded proteins induced by different physical and biological invasions [3]. In plants, the UPS has been increasingly implicated in plant responses to environmental stresses, and there is some indirect evidence indicating that ERAD indeed occurs in plants [24, 26], but whether the ERAD pathway is involved in plant tolerance to abiotic stresses is unclear. To gain further insight into the relationship between ERAD and abiotic stresses in plants, 2-week-old WT seedlings were treated with salt, and protein extracts were prepared for western blot analysis. The level of total ubiquitinated proteins was upregulated by salt, especially at 3 and 6 h after treatment (Figure 1A), indicating that a number of proteins were quickly ubiquitinated and perhaps degraded through UPS. We then analyzed the expression of ER stress marker genes that reflects the accumulation of unfolded proteins in the ER. The expression levels of *BIP1/2* as well as *CNX1* are largely increased by NaCl treatment (Figure 1B). We conclude that, salt treatment altered the levels of total ubiquitinated proteins and promoted the UPR in the plant. These results suggest that plant ERAD may function in salt stress responses.

### *HRD3A is the only functional homolog of yeast HRD3 in Arabidopsis*

To define the relationship between ERAD and the plant salt response, genes encoding protein homologs to yeast ERAD components were identified by Blast analysis. Candidate genes (Supplementary information, Figure S1A) were further analyzed by reverse genetics to determine whether they participate in the plant ERAD and salt stress response. In this study, we explored in detail the function of the plant ERAD component HRD3. Through protein alignments with yeast HRD3, two putative HRD3 homologs, named HRD3A (at1g18260) and HRD3B (at1g73570), were identified in *Arabidopsis* (Supplementary information, Figure S1D). The T-DNA insertion mutant lines of these two genes were obtained from the



**Figure 1** Salt-induced UPR in plants. The 2-week-old seedlings were treated in MS liquid medium containing 300 mM NaCl, and samples were collected at different time points. Equal amounts of total proteins were separated by SDS-PAGE and analyzed by immunoblots. Ponceau S staining of RbcS served as a loading control. For RNA analysis, 15  $\mu$ g of total RNA from each sample were applied for Northern blotting. **(A)** Western blot analysis by using an ubiquitin-specific antibody. **(B)** Expression of ER stress chaperone genes were detected with  $^{32}$ P-labeled *BIP1/2* and *CNX1* probes. The bottom panel shows methylene blue-stained 28S rRNA as a loading control.

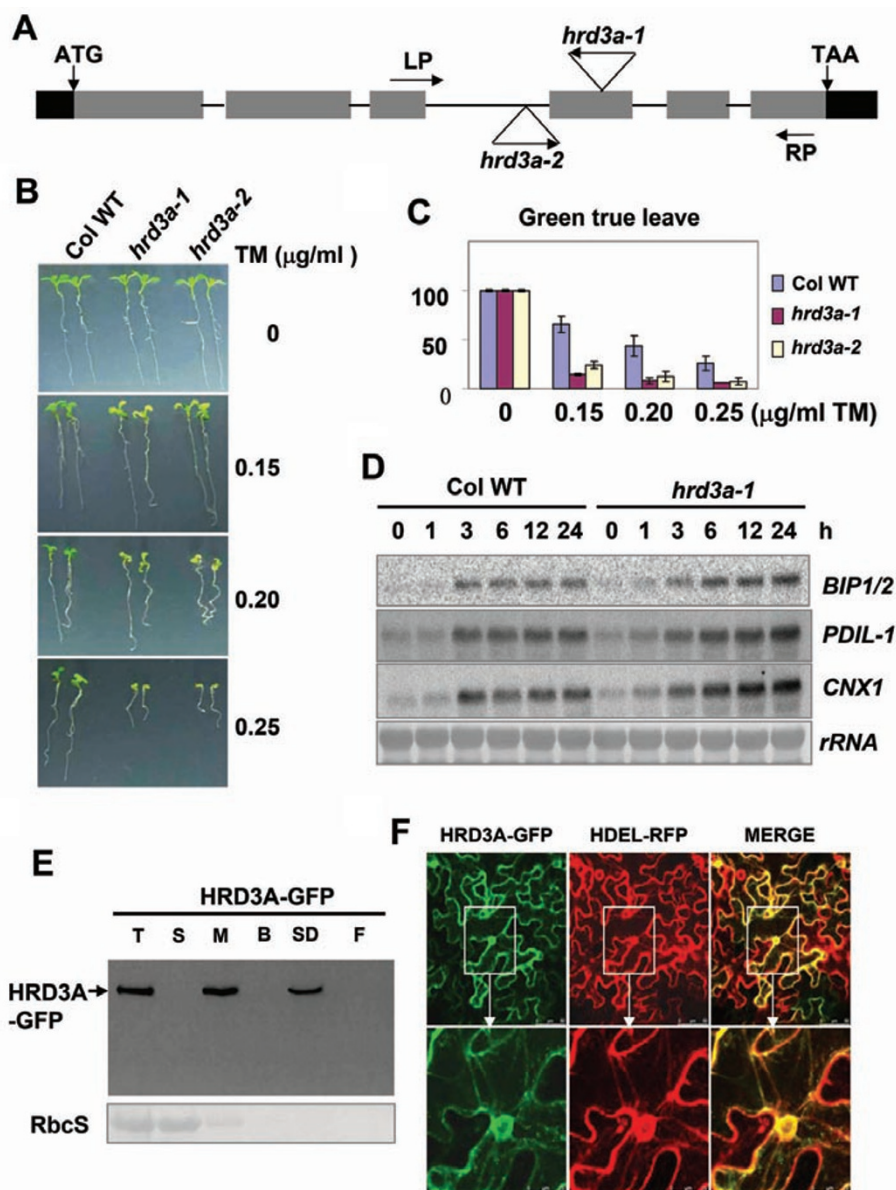
*Arabidopsis* Biological Resource Center (ABRC), stock center. Homozygous mutant lines of the two genes were isolated and identified as *hrd3a-1* (SALK\_109430) and *hrd3b-1* (SALK\_054396) (Figure 2A and Supplementary information, Figure S2A). Loss of specific RNA expression in the mutants was confirmed by RT-PCR analysis (Supplementary information, Figure S1B and S1C). To examine whether both HRD3A and HRD3B are active components of the ERAD complex in *Arabidopsis*, *hrd3a-1*, and *hrd3b-1* mutants were grown on medium containing 0.2  $\mu$ g/ml TM, an ER stress inducer through inhibiting protein glycosylation [28]. Surprisingly, only the growth of *hrd3a-1*, but not *hrd3b-1*, was sensitive to TM (Supplementary information, Figure S2B). To determine the reason for the insensitivity of *hrd3b-1* to TM, the *HRD3B* cDNA was cloned from WT young seedlings and sequenced, but the result was not consistent with the CDS predicted by TAIR (<http://www.arabidopsis.org>). In fact, the second intron was retained in the cDNA, causing the protein to be terminated prematurely (Supplementary information, Figures S1D and S2A). To investigate whether the *HRD3B* transcript is alternatively

spliced, RT-PCR analysis was performed with primers designed nearby or in the second intron (Supplementary information, Figure S2A), by using RNA isolated under different treatments and RNA from different tissues, including cauline leaves, rosette leaves, stems, roots, siliques, and flowers. We found that the second intron of *HRD3B* was present in transcripts from all tissues and transcripts under different treatments (Supplementary information, Figure S2C and S2D). Combining the genetic and molecular data, we conclude that *HRD3B* may be a pseudogene or perhaps encodes only a truncated protein, and *HRD3A* is likely the only known functional homolog of yeast *HRD3* in *Arabidopsis*.

#### *HRD3A* is involved in plant tolerance to ER stress and UPR

Since there is only one functional HRD3 in *Arabidopsis*, HRD3A, we then focused on exploring its function. To ensure that the phenotype of *hrd3a-1* is indeed the result of a null mutation of *HRD3A*, we identified the other allele, *hrd3a-2* (SALK\_050640). The expression of the *HRD3A* gene was also abolished in this line (Figure 2A and Supplementary information, Figure S1B). On Murashige and Skoog (MS) standard growth medium, no obvious differences were observed between the WT and both *hrd3a* mutants. However, on plates containing different concentrations of TM, both *hrd3a-1* and *hrd3a-2* showed more sensitivity than the WT in a dosage-dependent manner (Figure 2B and 2C). On 0.15  $\mu$ g/ml TM plates, more than 60% of the WT seedlings grew well. By contrast, the percentages in mutants were less than 25%. On 0.25  $\mu$ g/ml TM plates, the growth of almost all seedlings of both mutants was blocked, while about 25% of the WT seedlings still had green true leaves. This result indicates that the *hrd3a* mutants are sensitive to TM. Since the *hrd3a-1* and *hrd3a-2* mutants had similar phenotypes under all stresses tested (Figures 2B, 4B, 6B and Supplementary information, Figure S5), we simply chose *hrd3a-1* for subsequent in-depth molecular analyses.

The ER stress would trigger UPR [5, 29]. A sign of induced UPR is the high expression of ER chaperone genes, such as *BIP1/2*, *CNX*, and *PDIL1-1*. The 2-week-old seedlings of the WT and *hrd3a-1* mutant were treated with 5  $\mu$ g/ml TM, and total RNA was extracted for northern analysis. ER chaperone gene expression was induced in the WT, which is consistent with a previous study [18], and in *hrd3a-1* the induced level was much higher than in the WT with prolonged increases for all the three marker genes analyzed (Figure 2D). Therefore, we propose that the defect in the putative ERAD component HRD3A in plants enhances UPR under ER stress.



**Figure 2** The mutant plant of ER membrane protein HRD3A is sensitive to ER stress inducer, tunicamycin. **(A)** Schematic diagram of the *HRD3A* gene structure and primers for RT-PCR. Closed boxes represented exons; lines between closed boxes represent introns; and black boxes represent the 5'- and 3'-UTR. The two null mutants, *hrd3a-1* and *hrd3a-2*, were identified. LP, forward primer; RP, reverse primer. **(B)** Growth of WT and mutant plants on MS medium containing a range of concentrations of TM (0, 0.15, 0.20, and 0.25  $\mu\text{g/ml}$ ). Seeds were germinated and grown for 8 days on plates with or without TM, and representative plants are shown. Percentages are means ( $n = 60$  each) of three independent repeats. **(C)** Quantitative analysis of *hrd3a* mutants and WT seedlings with green true leaves on plates containing a range of concentrations (0, 0.15, 0.2, and 0.25  $\mu\text{g/ml}$ ) of TM. **(D)** Expression patterns of transcripts of ER chaperones in response to TM treatment. The 2-week-old seedlings were treated with MS media containing 5  $\mu\text{g/ml}$  TM for different times. Total RNA (15  $\mu\text{g}$ ) from each sample was hybridized with  $^{32}\text{P}$ -labeled *BIP1/2*, *PDIL-1*, and *CNX1* probes. The bottom panel shows methylene blue-stained 28S rRNA as a loading control. **(E)** Cell fractionation assays of HRD3A. 35S:HRD3A-GFP was infiltrated into tobacco leaves and the samples were collected after 3 d. HRD3A-GFP was detected using an anti-GFP antibody (top panel). Ponceau S staining of the RbcS is shown as a loading control (bottom panel). B, buffer-extracted fraction; F, final membrane fraction; M, membrane fraction; S, soluble fraction; SD, SDS-extracted fraction; T, total extract. **(F)** Co-localization of HRD3A-GFP with HDEL-RFP. Tobacco leaves were co-infiltrated with 35S:HRD3A-GFP and 35S:HDEL-RFP. Signals were observed directly under a confocal microscope after 3 d. The photographs in the bottom panel are the enlargements of the nuclear area of corresponding frames as indicated by the arrows.

### *HRD3A is an ER-localized protein*

HRD3A is the homolog of yeast HRD3, an ER-localized membrane protein. By using the SMART program, we could not identify a typical membrane localization sequence in HRD3A. Therefore, cell fractionation and HRD3A-GFP fusion analysis were performed to detect HRD3A localization. First, we constructed an *HRD3A-GFP* fusion cassette under the control of a cauliflower mosaic virus 35S promoter. This construct was transiently expressed in *Nicotiana benthamiana* leaf cells by agrobacteria infiltration [30]. Cell fractionation and protein gel blot assays showed that HRD3A-GFP was present in the microsomal fraction, but not in the soluble fraction (Figure 2E). The association of HRD3A-GFP with the membranes was further confirmed by resuspension of the membrane fraction in detergent-free and detergent-containing buffers for extensive cell fractionation analysis. HRD3A-GFP was only removed from the microsomal fractions by detergent treatment (Figure 2E). Therefore, we conclude that HRD3A is associated with the intracellular membranes.

The subcellular localization of HRD3A was further examined by microscopy. After 3 days of incubation, expression of the *HRD3A-GFP* gene was examined under a confocal laser scanning microscope. As shown in Figure 2F and Supplementary information, Figure S3, HRD3A-GFP exhibited typical ER localization. The ER localization of HRD3A-GFP was further confirmed by co-expression with the ER marker, HDEL-RFP. Co-localization of HRD3A and the ER marker was observed, as indicated by the perfect overlay of the RFP and GFP fluorescent signals (Figure 2F). Both the biochemical and cellular analyses indicate HRD3A is an ER-localized protein.

### *HRD3A is an active component of ERAD in plants*

Recent studies revealed that two mutated BR receptors, *bri1-5* and *bri1-9*, are ERAD substrates in *Arabidopsis* [25, 26]. To further explore the function of HRD3A in ERAD, we examined the effect of the *hrd3a* mutation on *bri1-5* and *bri1-9*. The *hrd3a-1 bri1-5* double mutants have larger rosette leaves with noticeable petioles than that of the *bri1-5* single mutant (Figure 3A). Meanwhile, the *hrd3a-1 bri1-9* double mutant also suppressed the *bri1-9* small rosette phenotype (Figure 3B). Consistent with the morphological phenotypes, the two double mutants regained partial sensitivity to epi-brassinolide (eBL), an active form of BR (Figure 3C and 3D), as measured at the biochemical level by BL-induced BES1 dephosphorylation assays [31].

It had been shown that accumulated *bri1-5* and *bri1-9* proteins in the ER may saturate the ERQC machinery,

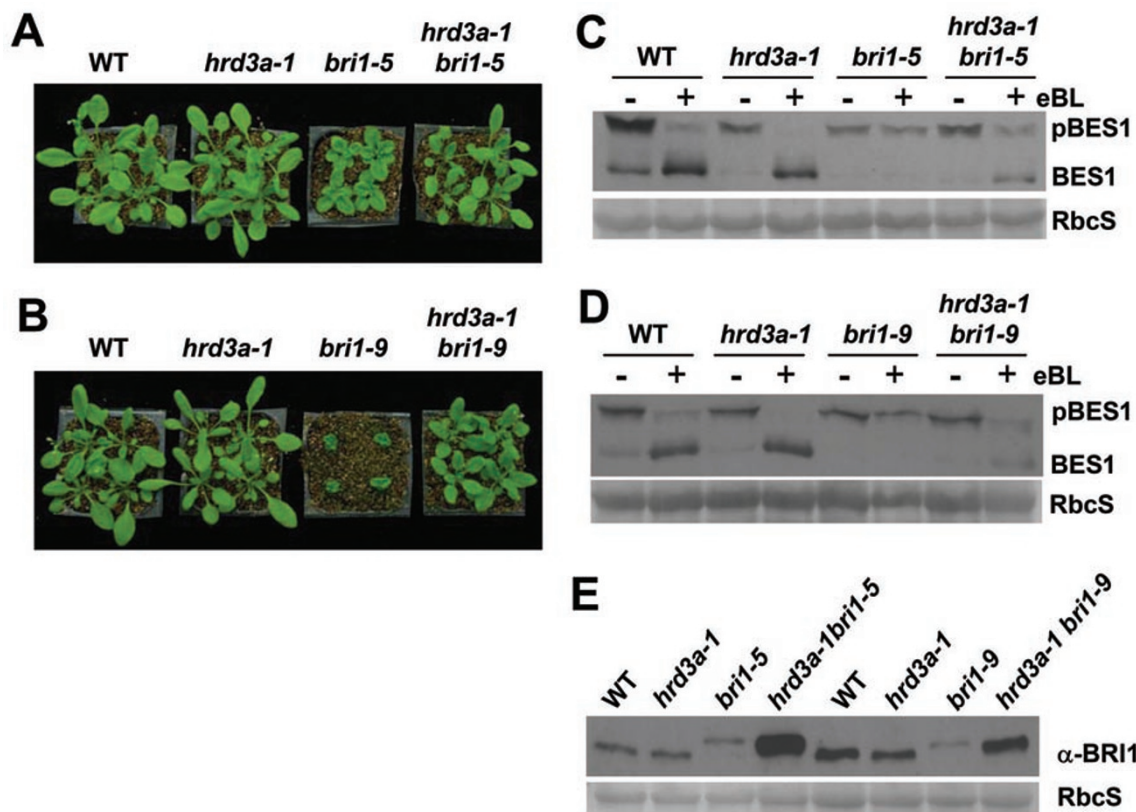
and those proteins would leak out of the ER to the cell surface where they can receive BR signals [25, 26]. Therefore, the protein levels of *bri1-5* and *bri1-9* were examined in both double mutants. Both *bri1-5* and *bri1-9* proteins were accumulated at higher levels in double mutants compared with that in their single mutants, WT and in the *hrd3a-1* mutant (Figure 3E).

In addition, we tested the effect of HRD3A on another known ERAD substrate MLO-1, which is a mutant form of Barley powdery mildew resistance o (MLO) [24]. *35S:Myc-MLO-1* and *35S:GFP* plasmids were co-transfected into *Arabidopsis* mesophyll protoplasts prepared from WT or *hrd3a-1* lines. Total proteins were extracted from the protoplasts 1 day after transfection and analyzed by SDS-PAGE. As shown in Supplementary information, Figure S4, the level of Myc-MLO-1 proteins was increased dramatically in the *hrd3a-1* mutant compared with that in the WT, whereas the level of the *Myc-MLO-1* mRNA was not different between the two samples. Collectively, our results indicate that HRD3A is an active component of ERAD in *Arabidopsis*.

### *ERAD defects increase the plant sensitivity to salt*

Our results thus far indicate that HRD3A is an important component of the *Arabidopsis* ERAD complex. Defects in ERAD are related to different stress responses in yeast [12, 14] and important diseases in mammals [11, 12]. Our current observations of salt treatment in *Arabidopsis* indicated that ERAD was involved in plant salt stress response. As HRD3A is an active component of the ERAD complex, it is expected that the *hrd3a* mutant plants would have altered responses to high-salt conditions. Therefore, seedlings grown on 125 mM NaCl plates for 2 weeks were examined. Seedlings of both *hrd3a* mutants were smaller and weaker than the WT seedlings (Figure 4A). The green seedlings of the WT were about 55% while less than 25% of the seedlings of both mutants were green (Figure 4B). Similar results were observed when the plants were grown on medium with 125 mM KCl, while no obvious differences were detected when 250 mM mannitol (equal to the same osmotic effect of 125 mM NaCl or KCl) was added to the medium (Supplementary information, Figure S5). The results above suggest that the HRD3-dependent stress response is salt ion specific rather than a result of osmotic stress.

It has been shown that ubiquitinated proteins increase upon salt treatment in this work. We then examined the status of ubiquitinated proteins in the *hrd3a-1* mutant with or without salt treatment. The total level of ubiquitinated proteins was already higher in the *hrd3a-1* mutant than the WT without salt treatment. While the ubiquitinated proteins accumulated in both lines upon salt treat-



**Figure 3** The *hrd3a-1* mutant suppressed the *bri1-5* and *bri1-9* phenotypes by affecting their protein accumulation. **(A)** The 4-week-old soil-grown plants of sibling WT, *hrd3a-1*, *bri1-5*, and *hrd3a-1 bri1-5* double mutant. **(B)** The 4-week-old soil-grown plants of sibling WT, *hrd3a-1*, *bri1-9*, and *hrd3a-1 bri1-9* double mutant. **(C, D)** Immunoblot analysis of the BES1 phosphorylation status in seedlings treated with or without 10  $\mu$ M epi-brassinolide (eBL). Total proteins were extracted from the 2-week-old seedlings treated with or without 10  $\mu$ M eBL for 1 h in liquid MS, separated by 10% SDS-PAGE, and analyzed by immunoblotting with anti-BES1 antibody. pBES1 is the phosphorylated form of BES1. Coomassie blue staining of RbcS served as the loading control. **(E)** Immunoblot analysis of the abundance of BRI1 and its mutant forms in plants are shown in **(A)** and **(B)**. Equal amounts of total proteins extracted were separated by SDS-PAGE and analyzed by immunoblots using an anti-BRI1 antibody. Coomassie blue staining of RbcS served as a loading control.

ment, the level was higher in the *hrd3a-1* mutant than in the WT (Figure 4C). This result suggests that there may be more unfolded proteins in *hrd3a* mutants than in WT plants upon salt treatment. High accumulation of unfolded proteins in the cell would induce UPR. Thus, total RNA was extracted from salt-treated seedlings and blots were probed with ER chaperone genes. The expression of ER chaperone genes was induced in both *hrd3a-1* and WT, while the induction levels of all three chaperones in *hrd3a-1* was much higher and lasted for a longer period of time than in the WT (Figure 4D). The fact that more unfolded proteins accumulated in the *hrd3a* mutant may explain the sensitive phenotype of *hrd3a* mutants to salt.

#### *Ca<sup>2+</sup> release from the ER is involved in the elevation of UPR*

Next, we sought to explore the signaling pathway

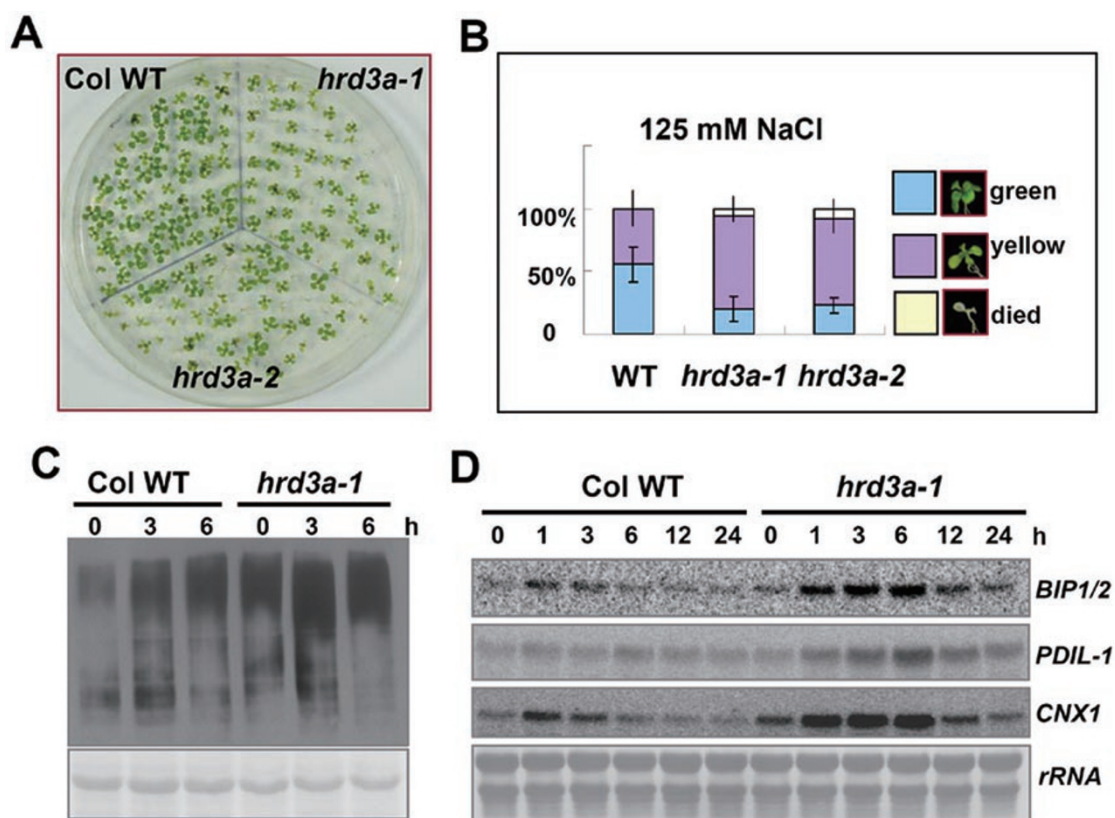
related to the function of HRD3A in the salt response. Previous studies have shown that  $Ca^{2+}$ , as a second messenger, is released from the ER under salt treatment to induce salt responses in plant cells [32, 33]. In our study, we found the expression of ER chaperone genes was highly induced in the *hrd3a* mutant upon salt treatment (Figure 4E). To address whether the high-level induction of the UPR was also affected by  $Ca^{2+}$  release from ER, we conducted the  $Ca^{2+}$  release inhibition experiment. Two-week-old seedlings of both the WT and *hrd3a-1* mutant were treated with salt with or without 100  $\mu$ M 2-aminoethoxydiphenyl borate (2-APB), an inhibitor of  $InsP_3$ -induced calcium release [34]. The RNA gel blot analysis showed that 2-APB blocked the induction of the *BIP1/2* gene upon salt treatment in both WT and *hrd3a-1* samples. The expression of the *BIP1/2* gene upon treatment with both NaCl and 2-APB was reduced to almost

the same level between the WT and *hrd3a-1* samples (Figure 5A). This indicates that NaCl indeed induced UPR through the release of  $\text{Ca}^{2+}$  from the ER. The expression of *BIP1/2* was blocked by the inhibitor of  $\text{Ca}^{2+}$  release for two possible reasons. Either unfolded proteins had accumulated in the ER upon salt treatment due to  $\text{Ca}^{2+}$  release from the ER, as previous studies had shown that reduced  $\text{Ca}^{2+}$  in the ER induces ER stress, or the ER stress signal could not be transmitted to the nucleus to stimulate the UPR when the release of  $\text{Ca}^{2+}$  from the ER is inhibited. To test these hypotheses, we treated seedlings with TM, a direct ER stress inducer by inhibiting protein glycosylation and inducing accumulation of unfolded proteins. Similar to salt treatment, 2-APB also blocked the induction of the *BIP1/2* gene in both the WT and *hrd3a-1* samples (Figure 5B). Thus, the induction of

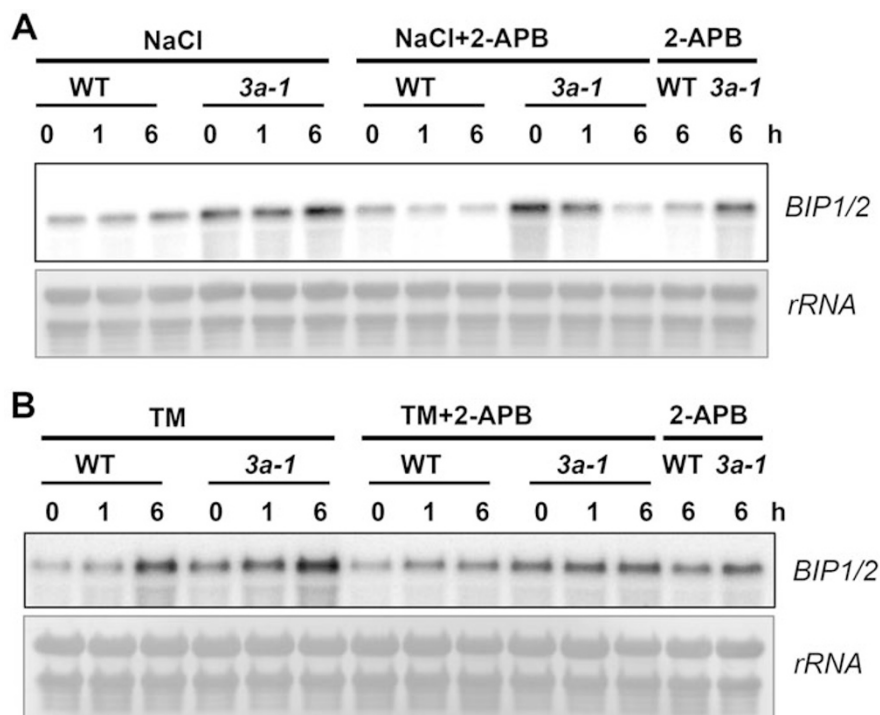
UPR under salt-induced ER stress requires  $\text{Ca}^{2+}$  release from the ER.

#### *ROS is involved in the ERAD-related plant salt response pathway*

When a plant faces a high-salt environment, its growth is arrested and prolonged salt treatment also leads to plant death. It is known that salt stress generates ROS in plants [35, 36], and over-production of ROS in animals results in oxidative stress and accumulation of unfolded proteins [37, 38]. To determine whether the production of ROS in ERAD deficient plant is different from that of WT plants upon salt treatment, the  $\text{H}_2\text{O}_2$  accumulation in the *hrd3a-1* mutant upon salt treatment was visualized by 3,3-diaminobenzidine (DAB), a chemical which converts to dark-brown polymers in the presence of  $\text{H}_2\text{O}_2$



**Figure 4** The inhibition of ERAD seriously affected plant responses to NaCl. **(A)** Growth of different lines of plants on MS medium plates containing 125 mM NaCl. Seeds were germinated and grown for 2 weeks. **(B)** Quantitative analysis of different plant phenotypes of *hrd3a* mutants and WT as shown in **(A)**; percentages are means ( $n = 60$  each) of three independent repeats. **(C)** Western blot analysis of *hrd3a-1* mutant and WT treated with 300 mM NaCl for different time points using anti-ubiquitin antibody. Plant was grown for 2 weeks, and then treated in MS liquid medium containing 300 mM NaCl. Proteins were extracted and immunoblotted with anti-ubiquitin antibody. **(D)** Expression patterns of transcripts of ER chaperones in response to NaCl treatment. Plants were grown for 2 weeks, and then treated with medium containing 300 mM NaCl for different time points. Total RNA (15  $\mu\text{g}$ ) from each sample was hybridized with  $^{32}\text{P}$ -labeled *BIP1/2*, *PDIL-1*, and *CNX1* probes. The bottom panel shows methylene blue-stained 28S rRNA as a loading control.



**Figure 5** Ca<sup>2+</sup> release from the ER is a signal to initiate UPR. Expression patterns of ER chaperone *BIP1/2* in response to NaCl and TM treatments. The 2-week-old plants were subjected to different treatments and samples were collected at different time points. Total RNA (15 μg) from each sample was hybridized with <sup>32</sup>P-labeled *BIP1/2* probe. The bottom panels show methylene blue-stained 28S rRNA as a loading control. **(A)** Treatment of 300 mM NaCl with or without 100 μM 2-APB. **(B)** Treatment of 5 μg/ml TM with or without 100 μM 2-APB.

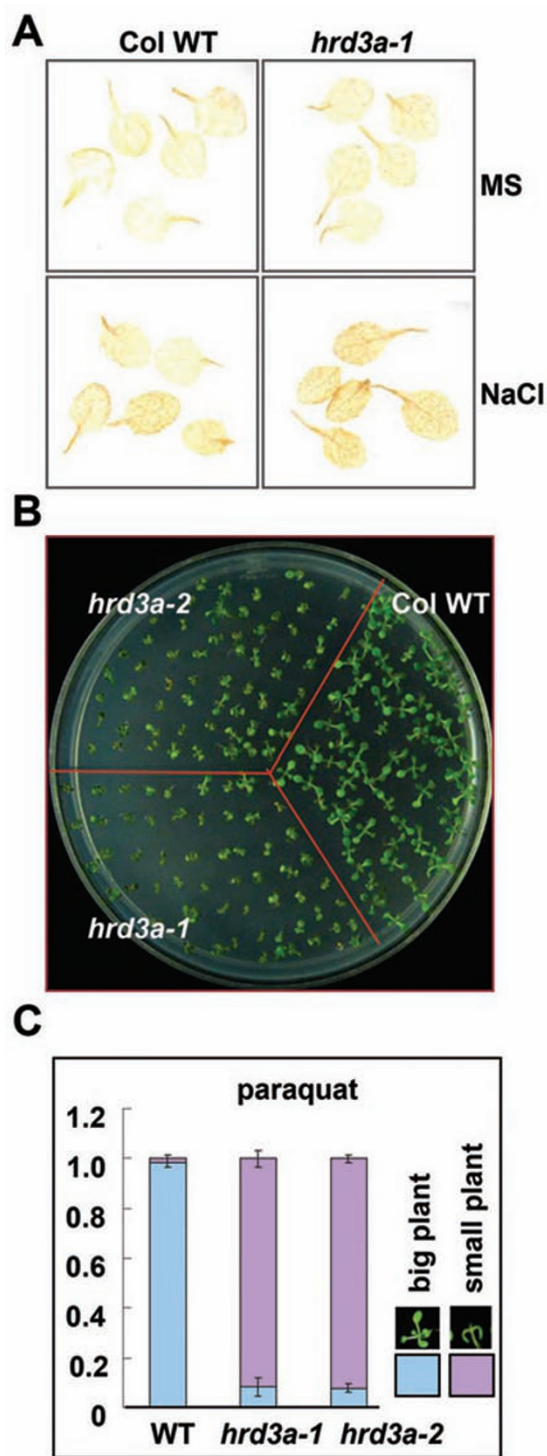
[39]. The H<sub>2</sub>O<sub>2</sub> level in *hrd3a-1* was higher than in WT both with and without salt treatment (Figure 6A). The salt treatment promoted H<sub>2</sub>O<sub>2</sub> accumulation, especially in *hrd3a* plants. This result indicates that HRD3A is at least important for removing the source of ROS production in plant cells, and perhaps this is the reason why the *hrd3a* plants showed arrested growth and died more quickly under the salt treatment condition. The chemical paraquat, an inducer of oxidative stress, can induce cell death in both plants and animals [40, 41]. To determine whether paraquat can be more harmful to *hrd3a* mutant plants, *hrd3a-1*, *hrd3a-2*, and WT seeds were plated and grown on MS medium containing 0.2 μM paraquat. After being grown for 2 weeks, the *hrd3a-1* and *hrd3a-2* mutants each showed an obvious sensitive phenotype to paraquat, as most of the mutant seedlings were small, and the leaves were bent (Figure 6B). The vast majority of the WT seedlings (98%) grew well, but less than 10% of the seedlings of the two mutants were larger plants (Figure 6C). Continued treatment also led to faster death of those *hrd3a* mutant plants, indicating that ERAD was necessary for clearing ROS-induced accumulation of unfolded proteins.

Taking these findings altogether, we conclude that salt stress activated ROS signals and induced the accumulation of unfolded proteins in the ER. The removal of those unfolded proteins by the ERAD is important for plant tolerance to salt. When a key component of the ERAD, HRD3A, was defective in the plant, accumulation of unfolded proteins strongly induced UPR that required Ca<sup>2+</sup> release from the ER.

## Discussion

To date, knowledge of ERAD has mainly been obtained from studies of yeast and mammalian systems, but little is known about plant ERAD components and their effects on plant development and growth. Genome sequence and several microarray experiments have revealed that ERAD components similar to yeast and mammals are present in plants [19, 20]. We discovered that HRD3A is the only active HRD3 homolog in *Arabidopsis*; however, we could not detect the complementation of yeast *hrd3* mutant by HRD3A (data not shown), which may be due to the low similarity between these two proteins (29.6%). HRD3A is an ER-localized protein





**Figure 6** ROS is involved in the ERAD-related plant salt response pathway. **(A)** 3,3-diaminobenzidine staining of the  $H_2O_2$  levels in WT and *hrd3a-1* mutant plants with or without NaCl treatment. For NaCl treatment, 3-week-old seedlings were treated with MS medium containing 300 mM NaCl for 6 h. **(B)** Growth phenotype of WT and *hrd3a* mutants germinated and grown for 2 weeks on MS medium containing 0.2  $\mu$ M Paraquat. **(C)** Quantitative analysis of the different phenotypes of **(B)**. Percentages are means ( $n = 60$  each) of three independent repeats.

and the stability of several ERAD substrates was increased in *hrd3a* mutant. In the case of the BR receptor, however, we could not detect the cytoplasmic form of the BRI1 mutants in two double mutants, *hrd3a-1 bri1-5* and *hrd3a-1 bri1-9* by Endo H digestion. This result could be due to the low amounts of the mutant proteins, or the different effect of ERAD component deficiency in comparison to the changes in upstream components affecting glycosylation [25].

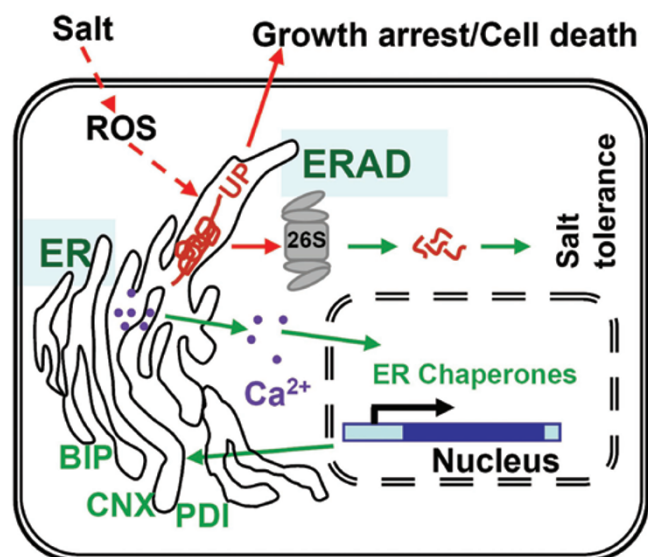
Salt stress is one of the most serious environmental stresses to plants. ERAD has been shown to be involved in a number of human diseases and stress responses in yeast. We demonstrated, here, that ERAD is involved in the plant response to salt. In mammals, under ER stress BIP protein is occupied by the accumulated misfolded proteins in ER, and the interaction of BIP and PERK is disturbed. PERK is then activated to inhibit the translation of most proteins [29]. The biological effect is the cell-cycle arrest. If this pathway is conserved in plant, it might be able to explain the smaller phenotype of *hrd3a* under salt stress. Our results showed that the total ubiquitinated proteins accumulated to a higher level in *hrd3a* than in WT upon salt treatment, this may be because of the fact that proteins ubiquitinated by ERAD only contribute to part of total ubiquitinated proteins or the result of the compensation by other ERAD complex, such as uncharacterized DOA10 complex in plant. The other explanation is that HRD3A mutation may not affect the ubiquitination of misfolded proteins, but only their degradation by ERAD.

Signal transduction under ER stress to induce UPR has been well studied in yeast and mammalian cells. In yeast, UPR is activated through the IRE1-HAC1 pathway [2], and compared with yeast, the induction of UPR in the mammalian system is a more complicated event. There are at least three ER stress sensors, activating transcription factor 6, IRE1a, or IRE1b, and PERK that activate UPR through distinct ways [29]. The bZIP28 and bZIP60 proteins have also been shown to function as ER stress sensors in plants [21, 42-44]. Whether plants utilize all those sensor systems will need to be addressed in the future. Here, we identified  $Ca^{2+}$ , an important second messenger in cells, as essential for the induction of UPR upon salt treatment in plants, especially when ERAD is deficient. However, whether the induction of UPR through  $Ca^{2+}$  signals and bZIP28 or bZIP60 signals are related to each other or belong to completely different pathways remains to be determined. Indeed,  $Ca^{2+}$  signals are known to be involved in other types of biotic and abiotic stresses, such as drought, cold, heat, immersion, and several different infectious diseases. Although UPR-related diseases in plants have been documented [45],

whether ERAD is involved in all those stress signals are not yet known.

ROS are generated immediately when plants are under environmental stress. They can both function as a signal to activate stress-related responses and cause oxidative damage to cells [46]. High salinity in soil is a serious environmental stress to plants and induction of ROS accumulation in plants has also been observed [35, 47]. However, how ROS accumulation causes plant growth inhibition and cell death under salt stress has not been well defined. Our results provide evidence that the damage to plants may be partially through ROS-induced accumulation of misfolded proteins in cells. It is known that oxidative stress enhances ROS production through the oxidation and reduction of disulfide bonds [48]. It is well known that the formation of disulfide bonds is important for correct protein folding. Protein folding in the ER is a dynamic process, and proteins require reducing capacity to allow refolding when disulfide bonds form incorrectly. The accumulation of ROS is also known to occupy most NADPH in cells, resulting in the accumulation of misfolded proteins. When these misfolded proteins are not dealt with properly, growth inhibition and cell death occur.

All current evidence indicates that an ERAD mechanism is important for plants to survive under high-salt conditions. According to our study and previous work, we propose the following model in plants (Figure 7). The misfolded proteins accumulate in the ER upon salt



**Figure 7** Proposed model for the role of ERAD in the salt signaling pathway in plants. UP: unfolded protein.

treatment, which may be mediated through ROS signaling pathways. Those misfolded proteins are recognized by ERAD and finally degraded by the 26S proteasome. Plants can also alleviate salt stress by inducing the expression of ER chaperones to help the misfolded proteins to refold. If ERAD is deficient under salt stress, the plant response to salt is changed. A much stronger UPR would arise to induce additional ER chaperones, and this process requires the release of  $Ca^{2+}$  from the ER. Consequently, plant growth is inhibited, which may be the result of inhibition of translation of essential proteins in the cells, and prolonged salt treatment would also cause plant cell death by activating apoptosis-like programmed cell death.

## Materials and Methods

### Plant materials and growth conditions

The *Arabidopsis thaliana* Columbia ecotype was used for this study. Seeds were surface sterilized with 10% bleach for 15 min and washed five times with sterile water. Sterile seeds were suspended in 0.2% agarose and plated on MS medium (the MS medium used in this study is actually 1/2 MS). These plates were stratified in darkness for 3 days at 4 °C, after that transferred to a tissue culture room at 22 °C under a 16-h-light/8-h-dark photoperiod. The 2-week-old seedlings were potted in soil and placed in a growth chamber at 22 °C and 70% RH under a 16-h-light/8-h-dark photoperiod. For stress treatments, the MS medium was supplemented with TM, NaCl, KCl, paraquat, or Mannitol as necessary.

### Vector constructions

For 35S:Myc-MLO-1, the MLO gene fragment was first cloned into pEAZY-Blunt vector. After site-directed mutating the 484-nucleotide T to C, the MLO mutant fragment (named MLO-1) was cloned into the *Xba*I/*Asc*I site of pCambia1300221-Myc vector. To produce 35S:HRD3A-GFP, the *Kpn*I/*Sac*I GFP fragment derived from pGFP2 plasmid was inserted into pCambia1300221-Myc, digested with *Xba*I/*Asc*I, to generate pCambia1300221-GFP. Then a 2 031-bp *Kpn*I/*Kpn*I fragment containing the HRD3A CDS (the *Arabidopsis* Information Resource locus At1g18260) was cloned into the vector pCambia1300221-GFP. The 35S:HDEL-RFP was a gift from Dr Zhen Zhu of IGDB, CAS. The CDS of HRD3B from young seedling was cloned into pEAZY-Blunt vector for sequence.

### Verification of the T-DNA insertion mutant

The *hrd3a-1* (SALK\_10943), *hrd3a-2* (SALK\_050640), and *hrd3b-1* (SALK\_054396) seeds were obtained from the ABRC (Ohio State University, Columbus, USA). Homozygous mutants were identified by PCR from genomic DNA. For *hrd3a-1* and *hrd3a-2*, using HRD3A gene-specific primer (LP, 5'-TGCATTACCTTCTTCAAACACC-3'; RP, 5'-ATGCTGGTCAACCAAAGGCT-3') and T-DNA left border primers (LBb1, 5'-GCGTGGACCGCTTGCTGCAACT-3'). For *hrd3b-1*, using forward primer (LP3, 5'-GCCTCTGAACAGG-GAAACG-3'), T-DNA left border primers (LBb1), and HRD3B gene-specific reverse primer (RP2, 5'-TGTGCCACGTTAGCAC-CAA-3').

### RT-PCR amplification

To examine the expression of *HRD3A* and *HRD3B* by RT-PCR, DNase I-treated total RNA (2 µg) was denatured and subjected to reverse transcription reaction at 42 °C for 1 h. To determine the changes of *MLO-1* expression in *hrd3a-1*, RNA was extracted with Rneasy plant mini kit (NO.74904 QIAGEN) from 200 µl protoplast. After being treated with DNase I, total RNA was denatured and subjected to reverse transcription reaction at 42 °C for 1 h. For *HRD3A* gene, PCR amplification was performed using *HRD3A*-specific forward and reverse primers (the same as LP, RP mentioned above for T-DNA verification). For *HRD3B*, PCR amplification was performed using *HRD3B*-specific primers (the same as LP3, RP2 mentioned above for T-DNA verification). Expression levels of *Actin1* were monitored with forward and reverse primers (forward, 5'-CATCAGGAAGGACTTGTACGG-3'; reverse, 5'-GATGGACCTGACTCGTCATAC-3') to serve as an internal control. To find out whether *HRD3B* has alternative splicing form, PCR amplification was performed using primers (LP1, 5'-CTGAGCCAGTTAGAATCCAC-3'; LP2, 5'-GGAGAGCCTATGTCAATGGAAC-3'; RP1, 5'-CATCCTTCTTGACTC-CAGTG-3') near or in the putative second intron. For detecting the expression of *MOL-1*, PCR amplification was performed using *MLO*-specific primers (F, 5'-GCATTTTGTGTGGACAGTGG-3'; R, 5'-TCGTTAGGATTTAGTCTGTGC-3'). GFP was amplified with primers (F, 5'-ATGGGTAAAGGAGAAGAAC-3'; R, 5'-TTATTTGTATAGTTCATCCATG-3') as an internal control.

### Gene expression analysis

The 2-week-old seedlings grown on MS plates were treated with TM, NaCl, and NaCl or TM plus 2-APB for corresponding times. Total RNA was isolated by LiCl precipitation [49]. Totally 15 µg for each sample was separated on 1.2% (w/v) agarose formaldehyde gels and transferred to Hybond-N nylon membranes (Amersham Pharmacia Biotech). Blots were probed with PCR fragments labeled with ( $\alpha$ -<sup>32</sup>P) dCTP using a Ready-Primed labeling kit (Amersham International). The transcripts of ER chaperone were detected by hybridization to the following segments of the coding sequences: *BIP1/2*, nucleotides 1-554; *CNX1*, nucleotides 150-910; and *PDIL1*, nucleotides 444-1 291.

### Protein extraction and immunoblot analysis

The 2-week-old seedlings were ground in liquid nitrogen and resuspended in 2× SDS buffer (200 mM Tris-HCl PH6.8, 4% SDS, 0.2% bromphenol, 20% glycerol, and 0.2 M DTT) by vortex. Total extract was boiled for 10 min and centrifuged at 10 000× g at 4 °C for 10 min. Supernatant was subjected to protein gel blots. Antibodies and the dilutions used in these experiments were as follows: anti-Myc antibody (sc-40 Santa Cruz, 1:500), anti-GFP antibody (NO. 1814460 Roche, 1:1 000), anti-ubiquitin monoclonal antibody (raised in our laboratory) and goat anti-mouse HRP-conjugated antibody (00001-1 Proteintech, 1:2 500), anti-BRI1 antibody was a gift from Zhiyong Wang of Carnegie Institution.

### Cell fractionation assay

For cell fractionation analysis, *HRD3A*-GFP was transiently expressed in *Nicotina benthamiana* leaf cells. The mixture of agrobacteria containing the 35S: *HRD3A*-GFP and agrobacteria containing 35S:*p19* was injected into *N. benthamiana* leaves as described [30]. After incubation for 3 d, the infiltrated parts of leaves

were subjected to protein extraction followed by cell fractionation. Buffers and the procedure were as described [50, 51].

### Subcellular localization

For *HRD3A* localization, the agrobacteria containing the 35S:*HRD3A*-GFP or the mixture of agrobacteria containing the 35S:*HRD3A*-GFP and agrobacteria containing 35S:*HDEL*-RFP was injected into *N. benthamiana* leaves as described [30]. Agrobacteria containing 35S:*p19* was co-infiltrated to promote protein expression. Around 3 days later, GFP and RFP signals were observed and images were acquired using confocal laser scanning microscope.

### Protoplast transformation

Rosette leaves of 4–6-week-old soil-grown *Arabidopsis* were used for Mesophyll protoplasts isolation. Protoplast transfected with plasmid based on a previously described protocol [52], except that the leaves were first sterilized in 70% ethanol for 1 min. Transfected cells were kept in the dark at 22 °C and the protoplasts were collected after 24 h. For detection of the Myc-*MLO-1* protein level in the *hrd3a-1* mutant, the 35S:*Myc-MLO-1* plasmid was transformed into protoplasts of WT and *hrd3a-1* plants. The pGFP2 plasmid was transfected alongside for an internal control.

### In vivo detection of H<sub>2</sub>O<sub>2</sub> in plants

The accumulation of H<sub>2</sub>O<sub>2</sub> in plant upon salt treatment was visually detected by using DAB. The 3-week-old seedlings of WT and *hrd3a-1* were first treated with 300 mM NaCl. Then the leaves were cut off and immersed in 1 mg/ml solution of DAB, pH 3.8, for 8 h under light at 25 °C. The reaction was terminated by immersion of the leaves in destaining solution (mixture of 20% acetic acid, 20% glycerol and 60% ethanol) and boiled for 10 min. After cooling, the leaves were kept in fresh destaining solution and photographed.

### Chemicals and stresses treatment

To find out whether *HRD3B* gene was undergoing alternative splicing under different conditions, 2-week-old seedlings grown on MS plates were treated with different kinds of stresses and chemicals for 5 h. The concentrations of chemicals were as follows: 5 µg/ml TM, 5 mM AZC, 2 mM DTT, 150 mM NaCl, 7% glucose, 300 mM Mannitol, 50 µM ABA, 2 µM IAA, 0.5 µM SA, and 50 µM MeJA. For drought treatment, seedlings were transferred onto a filter paper in a covered Petri dish and subjected to drought treatment in an environment of 70% RH. For hot and cold treatment the temperature was 42 °C and −2 °C, respectively.

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