Supporting Information

Su et al. 10.1073/pnas.1013251108

SI Experimental Procedures

Plasmid Construction and Generation of Transgenic Plants. A 4.5-kb genomic fragment of *At1g73570* (*AtSel1B*) was amplified from the bacterial artificial clones F6D5 and subsequently cloned into *pPZP222* (1) to make the *pPZP222-gAtSel1b.* Site-directed mutagenesis with the QuikChange XL site-directed mutagenesis kit (Stratagene) was performed on the *pPZP222-gEBS5* and *pPZP222-gAtSel1b* plasmids to generate an AgeI restriction site near the predicted start codon of each annotated gene. The *pPZP222-pEBS5:AtSel1b* and *pPZP222 pAtSel1b:EBS5* plasmids were constructed by swapping the smaller promoter-containing KpnI–AgeI restriction fragments of the *pPZP222-gEBS5* and *pPZP222-gAtSel1b* plasmids. All of the transgenic plasmids were individually sequenced and transformed into *ebs5-1 bri1-9* mutant by the vacuum-infiltration method (2).

Transient Expression and Confocal Microscopic Analysis of the GFP-Tagged EBS5 (EBS5-GFP) Fusion Protein in Tobacco Leaves. The genomic DNA of *EBS5* lacking the annotated stop codon and 3′-untranscribed/untranslated region was amplified from the *pPZP222-gEBS5* plasmid and cloned into the *pENTR/D-TOPO* vector (Invitrogen). The resulting *pENTR-gEBS5* plasmid was then mobilized with the Gateway LR Clonase enzyme mixture (Invitrogen) into a gateway destination *pMDC107* vector (3) following the manufacturer's suggested protocol to create a *pMDC107-gEBS5-GFP* plasmid. The plasmid *pSITE03-RFP-HDEL* (4) was a gift from T. Tzfira (University of Michigan). They were cotransformed into leaves of 3-wk-old tobacco (*Nicotiana benthamiana*) plants via an *Agrobacterium*-mediated infiltration method (5). The localization patterns of EBS5-GFP and red fluorescent protein (RFP) tagged with the C-terminal ER retrieval motif His-Asp-Glu-Leu (HDEL) (RFP-HDEL) in the coinfiltrated leaves were examined by using a Leica confocal laser-scanning microscope (TCS SP5 DM6000B) with an HCX PL APO CS 63× 1.30 glycerin lens and LAS AF software (Leica Microsystems). GFP and RFP were excited by using 488- and 543-nm laser light, respectively.

Expression of Fusion Proteins and Generation of Antibodies. A 900-bp cDNA fragment encoding the N-terminal 300 aa of EBS5 was cloned into *pGEX-KG* (6) or *pMAL-c2* (New England Biolabs) and subsequently transformed into *BL21* competent cells (Novagen). The protein induction and the subsequent purification of the GST fusion proteins on glutathione sepharose 4B beads (GE Healthcare) and the maltose-binding protein (MBP) fusion proteins on amylose resin (New England Biolabs) were carried out according to the manufacturers' recommended protocols. The purified GST-EBS5 protein was sent to Pacific Immunology for custom antibody production, and the resulting antiserum was affinity-purified with the MBP-EBS5 fusion protein that was covalently immobilized to the Aminolink Plus coupling resin (Thermo Scientific) by the manufacturer's suggested procedures.

RNA Isolation and RT-PCR. Total RNAs were isolated from 3-wk-old seedlings grown on 1/2 MS medium as described previously (7). For RT-PCR, 2 μg of total RNAs were reverse-transcribed following the manufacturer's suggested protocol (SuperScript First-Strand Synthesis System for RT-PCR; Invitrogen). To analyze the *hrd1a hrd1b* double mutation in the *bri1-9* background, 0.5 μL of the first-strand cDNA reaction was used as a template for PCR amplification with the *Hrd1aRT* and *Hrd1bRT* primer sets (see Table S1). The transcript of *β-Tubulin* was amplified by using the βTubulinRT primer set for 25 cycles as a control. Amplified PCR products were separated by 1% agarose gel and visualized by ethidium bromide staining.

1. Hajdukiewicz P, Svab Z, Maliga P (1994) The small, versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. *Plant Mol Biol* 25:989–994.
2. Bechtold N, Pelletier G (1998) In planta *Agrobacterium*-mediated transformation of adult *Arabidopsis thaliana* plants by vacuum infiltration. *Methods Mol Biol* 82:259–266.
3. Curtis MD, Grossniklaus U (2003) A gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physiol* 133:462–469.
4. Chakrabarty R, et al. (2007) PSITE vectors for stable integration or transient expression of autofluorescent protein fusions in plants: Probing *Nicotiana benthamiana*-virus interactions. *Mol Plant Microbe Interact* 20:740–750.
5. Voinnet O, Rivas S, Mestre P, Baulcombe D (2003) An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *Plant J* 33:949–956.
6. Guan KL, Dixon JE (1991) Eukaryotic proteins expressed in *Escherichia coli*: An improved thrombin cleavage and purification procedure of fusion proteins with glutathione *S*-transferase. *Anal Biochem* 192:262–267.
7. Li J, Nam KH, Vafeados D, Chory J (2001) BIN2, a new brassinosteroid-insensitive locus in *Arabidopsis*. *Plant Physiol* 127:14–22.

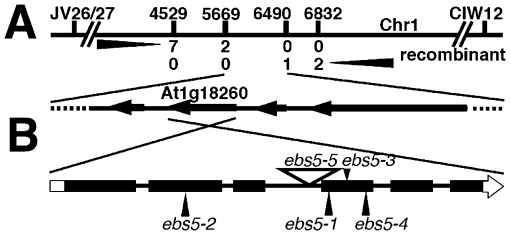

Fig. S1. Positional cloning of the *Arabidopsis* EBS5 gene. (*A*) *EBS5* was mapped to an 820-kb genomic region between markers 5669 and 6490 on the top of chromosome I. The line represents genomic DNA, and markers and numbers of recombinants are shown above and below the line, respectively. Shown below the recombination numbers is a diagram of four annotated genes (indicated by black arrows), including *At1g18260* and its three neighbors. (*B*) *EBS5* contains six exons (black bar) and five introns (line). White boxes denote untranslated regions, arrows indicate positions of *ebs5* mutations, and triangle denotes the transfer DNA (T-DNA) insertion in *ebs5-5*.

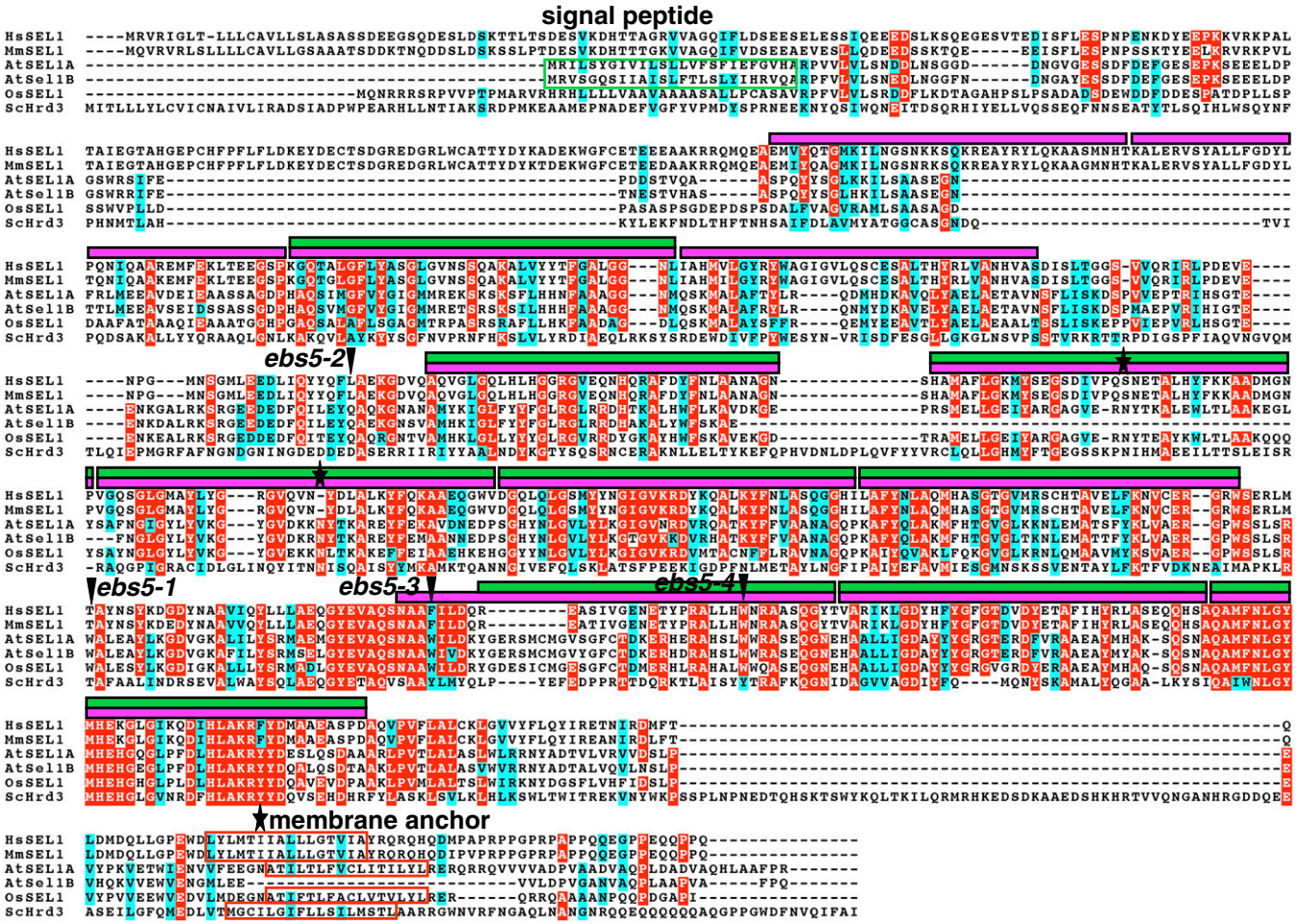

Fig. S2. Sequence alignment of EBS5 with the yeast Hrd3/mammalian Sel1L and their homologs. The predicted amino acid sequence of EBS5 (accession no. NP_564049) was aligned with the yeast Hrd3 (ScHrd3, NP_013308), the human Sel1 protein (HsSel1, NP_005056), the other *Arabidopsis* Sel1 homolog (AtSel1B, NP_177498), and Sel1 homologs from rice (OsSel1, NP_001049611) and mouse (MmSel1, NP_001034178) by using the ClustalW program. The aligned sequences were subsequently shaded by the Boxshade program at the Mobyle portal (http://mobyle.pasteur.fr) with identical amino acids in ≥4 sequences shaded in red and similar residues shaded in cyan. Each Sel1-like repeat is indicated by a color bar (pink for human/mouse Sel1L and green for EBS5 and AtSel1B). The predicted signal peptides of EBS5 and AtSel1B are marked by green boxes, and the predicted C-terminal transmembrane anchors are indicated by red boxes. Black arrows show the positions of amino acids changed to stop codon in four EMS-generated *ebs5* alleles, and the black stars mark the predicted N-glycosylation sites of the EBS5 protein.

Fig. S3. A T-DNA insertional mutation of *At1g18260* suppresses the dwarf phenotype of both *bri1-9* and *bri1-5* mutants. Shown, from left to right, are 3-wk-old soil-grown plants of wild type (*Upper*, col-0 ecotype; *Lower*, Ws-2 ecotype), *bri1-9* (*Upper*) or *bri1-5* (*Lower*), and *ebs5-5 bri1-9* (*Upper*) or *ebs5-5 bri1-5* (*Lower*).

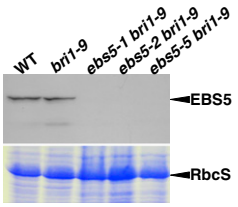

Fig. S4. Immunoblot analysis of EBS5 in wild-type, *bri1-9*, and three allelic *ebs5 bri1-9* mutants. Total proteins extracted from 2-wk-old seedlings were separated by two duplicate 10% SDS/PAGE gels. One of them was analyzed by immunoblotting with an immunoaffinity-purified anti-EBS5 antibody, and the other was stained with Coomassie blue to reveal the relative abundance of Rubisco (RbcS) used as a loading control.

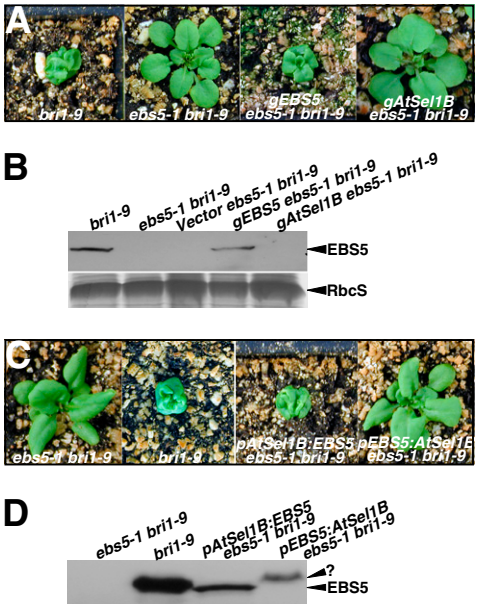

Fig. S5. AtSel1B does not play a role in the endoplasmic reticulum (ER)–associated degradation (ERAD) of two mutated BR receptors. (*A*) Shown here are pictures of 3-wk-old soil-grown seedlings of *bri1-9*, *ebs5-1 bri1-9*, a representative *ebs5-1 bri1-9* transgenic line expressing a genomic *EBS5* transgene, and a representative *ebs5-1 bri1-9* transgenic line carrying a genomic *AtSel1B* transgene. (*B*) Immunoblot analysis of EBS5. (*C*) Shown here, from left to right, are 3-wk-old soil-grown plants of *ebs5 bri1-9*, *bri1-9*, and a representative *ebs5-1 bri1-9* line expressing a *pAtSel1B:EBS5* or *pEBS5:AtSel1B* chimeric genomic transgene. (*D*) Immunoblot analysis of the EBS5 abundance in plants shown in *C*. For *B* and *D*, equal amounts of total proteins extracted from 3-wk-old seedlings were separated by SDS/PAGE and analyzed by immunoblotting with affinity purified anti-EBS5 antibody. Coomassie blue staining of RbcS serves as a loading control.

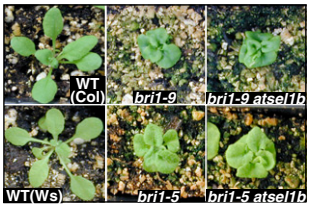

Fig. S6. Elimination of AtSel1B has no effect on the *bri1-9* or *bri1-5* mutant. *Upper* shows pictures of 3-wk-old soil-grown plants of wild type (Col-0 ecotype), *bri1-9*, and *atsel1b bri1-9* double mutant, and *Lower* shows the pictures of 3-wk-old soil-grown plants of wild-type control (Ws-2 ecotype), *bri1-5*, and *atsel1b bri1-5* double mutant.

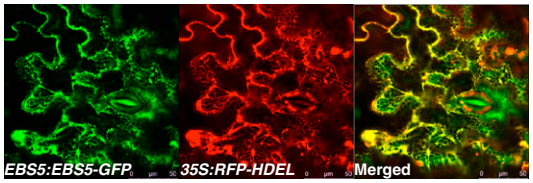

Fig. S7. EBS5 is an ER-localized protein. Shown here are confocal microscopic images of agro-infiltrated tobacco leaves coexpressing an ER-localized RFP-HDEL (*Center*) and EBS5-GFP (*Left*). (*Right*) Merged picture of GFP and RFP images.

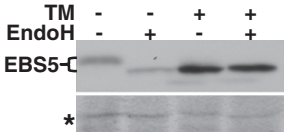

Fig. S8. EBS5 is a glycosylated protein that is induced by the unfolded protein response. Two-week-old wild-type seedlings were submerged in 1/2 MS medium supplemented with or without 5 μg/mL tunicamycin (TM) for 24 h. Equal amount of total proteins extracted from submerged seedlings were treated with or without Endo H, separated by 10% SDS/PAGE, and analyzed by immunoblotting with the anti-EBS5 antibody. Coomassie blue staining of RbcS was used as a loading control.

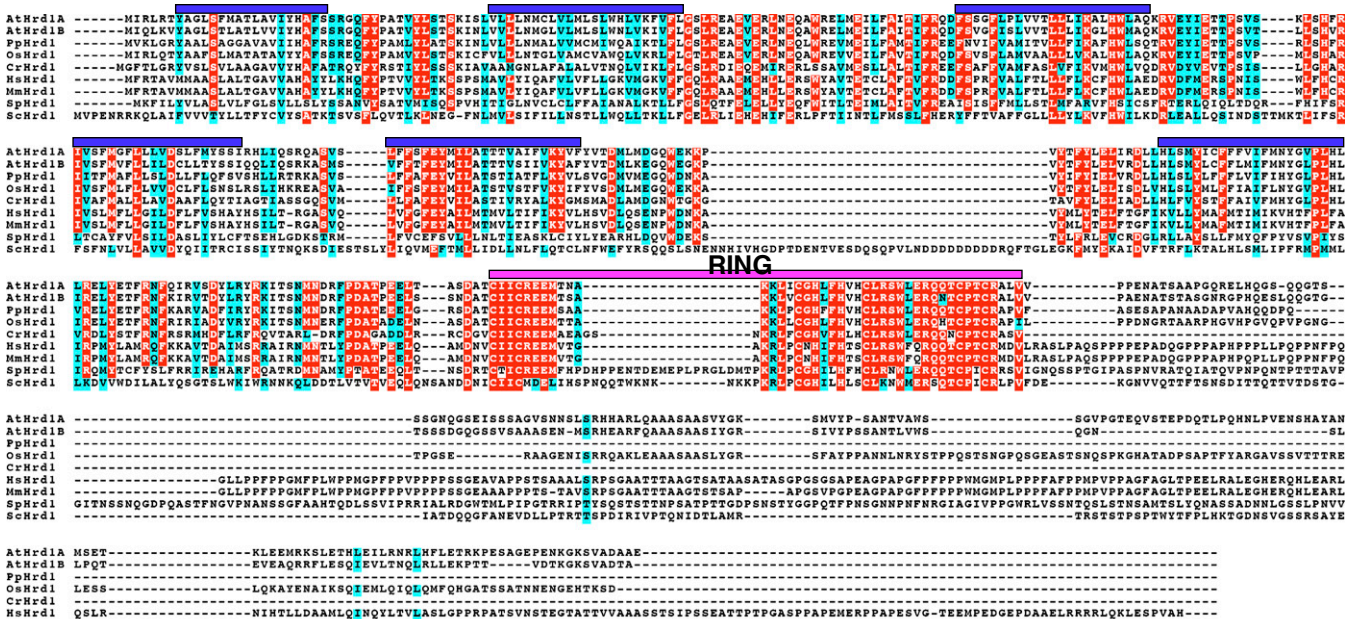

Fig. S9. Sequence alignment of the yeast Hrd1 E3 ligase with its homologs from other eukaryotic organisms. The Hrd1 protein/homologs from *Arabidopsis* (AtHrd1A, NP_188230 and AtHrd1B, NP_849843), *Physcomitrella patens* (PpHrd1, XP_001778650), *Oryza sativa* (OsHrd1, NP_001057454), *Chlamydomonas reinhardtii* (CrHrd1, XP_001701276), human (HsHrd1, NP_115807), mouse (MmHrd1, NP_083045), *Schizosaccharomyces pombe* (SpHrd1, NP_596376), and *Saccharomyces cerevisiae* (ScHrd1, NP_014630) were aligned with the ClustalW program. The aligned amino acid sequences were shaded by the Boxshade program at the Mobyle portal (http://mobyle.pasteur.fr). Residues identical in ≥7 sequences are shaded in red, and similar residues are shaded in cyan. Each Hrd1 sequence contains six transmembrane segments (marked by blue bars) and a highly conserved $Cys_3His_2Cys_3$-type RING domain (marked by a pink bar).

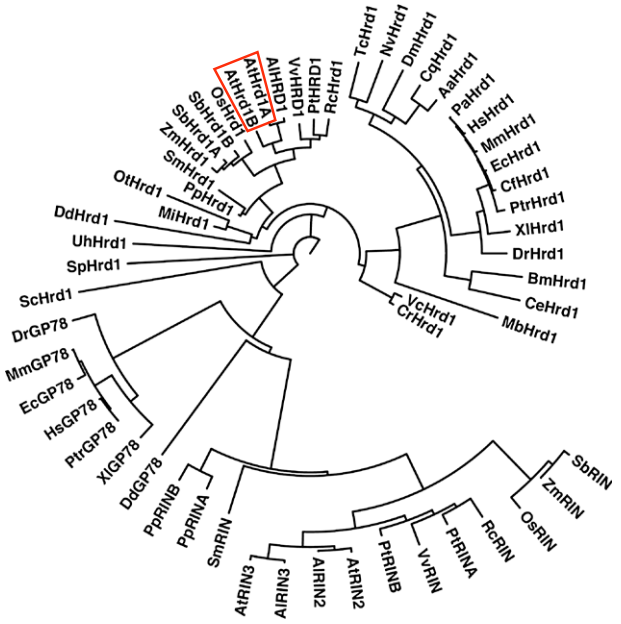

Fig. S10. Phylogenetic analysis of the two *Arabidopsis* Hrd1 proteins, the yeast and human Hrd1, and Hrd1 homologs from other eukaryotic organisms. The sequence alignment was carried out with the ClustalW program at the Mobyle portal (http://mobyle.pasteur.fr). The protdist and neighbor-joining programs of the Phylip package were used to construct the phylogenetic tree. Names and accession nos. of the analyzed Hrd1 sequences used for the analysis are as follows: AaHrd1 (Aa, *Aedes aegypti*, XP_001649607), AtHrd1A (At, *Arabidopsis thaliana*, NP_188230), AtHrd1B (NP_849843), AlHrd1 (Al, *Arabidopsis lyrata*, XP_002882977), BmHrd1 (Bm, *Brugia malayi,* XP_001897069), CeHrd1 (Ce, *Caenorhabditis elegans*, NP_505969), CfHrd1 (Cf, *Canis familiaris*, XP_540867), CqHrd1 (Cq, *Culex quinquefasciatus*, XP_001845016), CrHrd1 (Cr, *Chlamydomonas reinhardtii*, XP_001701276), DdHrd1 (Dd, *Dictyostelium discoideum* AX4, XP_637096), DmHrd1 (Dm, *Drosophila melanogaster*, NP_651894), DrHrd1 (Dr, *Danio rerio*, Q803I8), EcHrd1 (Ec, *Equus caballus*, XP_001492211), HsHrd1 (Hs, *Homo sapiens*, NP_115807), MbHrd1 (Mb, *Monosiga brevicollis* MX1, XP_001744385), MiHrd1 (Mi, *Micromonas sp. RCC299*, XP_002503557), MmHrd1 (Mm, *Mus musculus*, NP_083045), NvHrd1 (Nv, *Nasonia vitripennis*, XP_001600783), OsHrd1 (Os, *Oryza sativa*, NP_001057454), OtHrd1 (Ot, *Ostreococcus tauri*, CAL56130), PaHrd1 (Pa, *Pongo abelii*, XP_002821577), PpHrd1 (Pp, *Physcomitrella patens* XP_001778650), PtHrd1 (Pt, *Populus trichocarpa* XP_002319894), PtrHrd1 (Ptr, *Pan troglodytes* XP_522059), RcHrd1 (Rc, *Ricinus Communis,* XP_002520020), SbHrd1A (Sb, *Sorghum bicolor*, XP_002449013), SbHrd1B (XP_002438282), ScHrd1 (Sc, *Saccharomyces cerevisiae*, NP_014630), SmHrd1 (Sm, *Selaginella moellendorffii*, XP_002981293), SpHrd1 (Sp, *Schizosaccharomyces pombe*, NP_596376), TcHrd1 (Tc, *Tribolium castaneum*, XP_974421), UhHrd1 (Uh, *Ustilago hordei*, CAJ41969), VcHrd1 (Vc, *Volvox carteri f. nagariensis*, EFJ42202), VvHRD1 (Vv, *Vitis vinifera* XP_002274331), XlHrd1 (Xl, *Xenopus laevis*, NP_001084825), and ZmHrd1 (Zm, *Zea mays*, NP_001137060). The two *Arabidopsis* Hrd1 homologs are marked with red box. Also included in this analysis are two additional Hrd1 homologs from *Arabidopsis*, AtRIN2 (NP_194253) and AtRIN3 (NP_568760) (1), and the other human Hrd1 homolog, HsGP78 (NP_001135) (2), plus their homologs from other plant species and vertebrates. These include AlRIN2 (XP_002869683), AlRIN3 (XP_002864111), DdGP78 (XP_641804), DrGP78 (NP_998328), EcGP78 (XP_001915522), MmGP78 (Q9R049), OsRIN (NP_001059649), PpRINA (XP_001752735), PpRINB (XP_001765176), PtRINa (XP_002303501), PtRINb (XP_002329924), PtrGP78 (XP_001135064), RcRIN (XP_002510949), SbRIN (XP_002462866), SmRIN (XP_002988953), VvRIN (XP_002266334), XlGP78 (NP_001085387), and ZmRIN (NP_001151203).

1. Kawasaki T, et al. (2005) A duplicated pair of *Arabidopsis* RING-finger E3 ligases contribute to the RPM1- and RPS2-mediated hypersensitive response. *Plant J* 44:258–270.
2. Chen B, et al. (2006) The activity of a human endoplasmic reticulum-associated degradation E3, gp78, requires its Cue domain, RING finger, and an E2-binding site. *Proc Natl Acad Sci USA* 103:341–346.

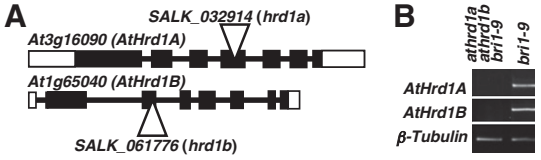

Fig. S11. Identification of T-DNA insertional mutants of the two *Arabidopsis* homologs of the yeast *Hrd1* gene. (*A*) Diagram of the T-DNA insertion sites of the two *hrd1* mutants. (*B*) RT-PCR analysis of the expression of two *Arabidopsis Hrd1* homologous genes in 2-wk-old seedlings of *bri1-9* and *hrd1a hrd1b bri1-9* mutants. The transcript of *β-Tubulin* was amplified with the βTubulinRT primer set as control.

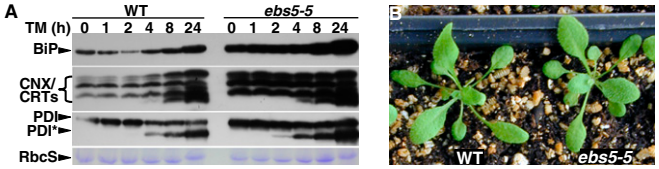

Fig. S12. Elimination of EBS5 has little effect on the plant morphology but activates the *Arabidopsis* unfolded protein response. (*A*) Three-week-old seedlings of wild type or *ebs5-5* (in *BRI1*$^{+}$ background) were removed from 1/2 MS agar medium and submerged into liquid 1/2 MS medium supplemented with 5 μg/mL tunicamycin (TM). Equal amounts of seedlings were removed at different time points to extract total proteins, which were separated by 10% SDS/PAGE and analyzed by immunoblotting with antibodies against immunoglobulin binding protein (BiP), the maize calreticulin (CRT), and the *Arabidopsis* protein-disulfide isomerase (PDI) as previously described (1). Coomassie blue staining of RbcS serves as a loading control. (*B*) Shown here are 3-wk-old soil-grown plants of wild type and the T-DNA insertional *ebs5-5* mutant.

1. Jin H, Yan Z, Nam KH, Li J (2007) Allele-specific suppression of a defective brassinosteroid receptor reveals a physiological role of UGGT in ER quality control. *Mol Cell* 26:821–830.

Table S1. Oligonucleotides used in this study

Name	Sequence (5′ to 3′)	Usage
JV26/27	CAAGAGATTGCAACATCCACA	SSLP
	AAGCTCCTTGGATCCGATTT	Ws-2 < Col-0
ciw12	AGGTTTTATTGCTTTTCACA	SSLP
	CTTTCAAAAGCACATCACA	Ws-2 < Col-0
4529	CCTTGACTCGACCAGAATCTTGAAG	dCAPS
	TCGCCGTCCGCCGCGTAATCGAATT	EcoRI cuts Col-0 DNA
5669	GTACACGCTTGAAGGGCCATTTAC	dCAPS
	TGGTCCATGGTACATCCAAGAAGCT	HindIII cuts Col-0 DNA
6490	TAGATGCTAAAACATGTTGAAA	dCAPS
	CTTTAAGATATATACAATGACT	HinfI cuts Col-0 DNA
6832	TCACAGAGATAACAACCCATCGATTG	dCAPS
	CATGTCCGTTACAGGAAGACCTGCA	PstI cuts Col-0 DNA
gEBS5	CG**GGTACC**GATCTTCGTTATTGGTTGCTCCT	Generation of the *EBS5* genomic transgene
	GC**CCCGGG***G*TACGTATCACATGGAGTATTAGTG	
gAtSel1b	CG**GGTACC**CACGAATTAGTGCTAACCATTGG	Generation of the *AtSel1b* genomic transgene
	GC**CCCGGG**CCACAAGTTATGGAGAGAATTTGTG	
EBS5-N	GT**CCATGG**CAATGAGAATATTAAGCTACGG	Expression of a fusion protein in *E. coli*
	C**GAGCTC**TTAGAGACCTTCTTTTGCAG	
Hrd1aRT	CATGCATTCAGCAGCAGAGGC	RT-PCR analysis of *AtHrd1a* expression
	AGCAACAGTCGTTGTCGCCAG	
Hrd1bRT	ATGATTCAGCTAAAGGTTTACGCG	RT-PCR analysis of *AtHrd1b* expression
	CGTGAAGAAGACTGACATTGAAGC	
βTubulinRT	TTCCAGGTTTGTCACTCGTTG	RT-PCR analysis of *β-Tubulin* expression
	ATGAAGAAGTGAAGACGGG	

Boldface indicates added restriction sites. Italic indicates gene names. SSLP, simple sequence length polymorphism; dCAPS, derived cleaved amplified polymorphic sequences.