Specific ER quality control components required for biogenesis of the plant innate immune receptor EFR

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Plant innate immunity depends in part on recognition of pathogen-associated molecular patterns (PAMPs), such as bacterial flagellin, EF-Tu, and fungal chitin. Recognition is mediated by pattern-recognition receptors (PRRs) and results in PAMP-triggered immunity. EF-Tu and flagellin, and the derived peptides elf18 and flg22, are recognized in *Arabidopsis* by the leucine-rich repeat receptor kinases (LRR-RK), EFR and FLS2, respectively. To gain insights into the molecular mechanisms underlying PTI, we investigated EFR-mediated PTI using genetics. A forward-genetic screen for *Arabidopsis* elf18-insensitive (*elfin*) mutants revealed multiple alleles of calreticulin3 (*CRT3*), UDP-glucose glycoprotein glucosyl transferase (*UGGT*), and an HDEL receptor family member (ERD2b), potentially involved in endoplasmic reticulum quality control (ER-QC). Strikingly, FLS2-mediated responses were not impaired in *crt3*, *uggt*, and *erd2b* null mutants, revealing that the identified mutations are specific to EFR. A *crt3* null mutant did not accumulate EFR protein, suggesting that EFR is a substrate for CRT3. Interestingly, *Erd2b* did not accumulate CRT3 protein, although they accumulate wild-type levels of other ER proteins. ERD2B seems therefore to be a specific HDEL receptor for CRT3 that allows its retro-translocation from the Golgi to the ER. These data reveal a previously unsuspected role of a specific subset of ER-QC machinery components for PRR accumulation in plant innate immunity.

endoplasmic reticulum | innate immunity | receptor kinase

Plant innate immunity involves three main processes: Recognition of conserved pathogen-associated molecular patterns (PAMPs) leading to PAMP-triggered immunity (PTI), suppression of defense by pathogen effectors, and recognition of specific effectors by cytoplasmic host proteins resulting in effector-triggered immunity (ETI) (1–4). Three pattern recognition receptors (PRRs) that can initiate PTI are known in the plant model *Arabidopsis thaliana*. Bacterial flagellin, and its peptide surrogate flg22 are recognized by the leucine-rich repeat receptor kinase (LRR-RK) FLS2 (5), bacterial elongation factor (EF)-Tu, and its surrogate peptide elf18 are recognized by the related LRR-RK EFR (6), while recognition of fungal chitin and unknown bacterial PAMP(s) depend on CERK1, a LysM domain RK (7–9).

EFR and FLS2 are glycosylated transmembrane proteins (6, 10) and therefore need to enter the secretory pathway to mature and to reach their final plasma membrane destination. The endoplasmic reticulum (ER) is the first organelle of the secretory pathway and is responsible for the proper folding and assembly of polypeptides that are then directed to the Golgi. After translocation in the ER, newly synthesized polypeptides interact with different chaperones that will assist them to fold properly and to avoid aggregation in a process called ER quality control (ER-QC) (11). Misfolded proteins are directed to ER-associated degradation, leading to their clearance by the ubiquitin-proteasome in the cytosol (12). Most of our knowledge on ER-QC is based on studies in yeast and mammals, while plant ER-QC mechanisms are still not well characterized (13). Studies in mammals and yeast have defined three main systems in the ER-QC (14). The first one relies on the retention of misfolded proteins by the luminal binding protein BiP, an ER member of the Hsp70 family of chaperone. In this system, the ER Hsp40 protein ERdj3 first binds directly to unfolded proteins. ERdj3 then recruits BiP and activates BiP's ATPase activity present in its N terminus, leading to interaction of the C-terminal region of BiP with the substrate and the release of ERdj3b (15, 16). The second involves recognition of free thiol groups and leads to the formation of disulfide bonds in non-native proteins by protein disulfide isomerases (PDIs) and other thiol oxidoreductases (17–19). Finally, the best studied system is specific to glycoproteins and relies on the so-called calnexin/calreticulin (CNX/CRT) cycle (20). CNX and CRT are lectins that interact with glycoproteins bearing monoglucosylated high-mannose type oligosaccharides via polypeptide based interactions (21). The enzyme UDP-glucose: glycoprotein glucosyltransferase (UGGT) serves as a "folding sensor" (22, 23). In this system, client glycoproteins are delivered to UGGT after the trimming of their innermost glucose residue by glucosidase II, which releases them from the lectin-chaperones CNX and CRT. UGGT is inactive against folded proteins, allowing them to proceed to the Golgi apparatus for further processing to complex- or hybrid-type glycoforms. On the other hand, this enzyme efficiently glucosylates incompletely folded glycoproteins to monoglucosylated structures, providing them with an opportunity to interact with CNX/CRT.

We report here on three elf18-insensitive (*ELFIN*) genes that are specifically required for EFR function, all of which encode potential components of the ER-QC pathway. Surprisingly, although FLS2 and EFR belong to the same subfamily of LRR-RK (LRR-XII) and induce similar responses (6), the reported *elfin* mutations do not impair FLS2 function. We conclude that a dedicated subset of ER-QC components is specifically required for the proper accumulation of a subset of PRRs in plant innate immunity.

Results

Identification of *CRT3* Mutant Alleles that Compromise EFR Signaling. To better understand PTI, we screened 137,500 EMS-mutagenized M2 *Arabidopsis* Col-0 seeds for elf18-insensitive (*elfin*) mutants that

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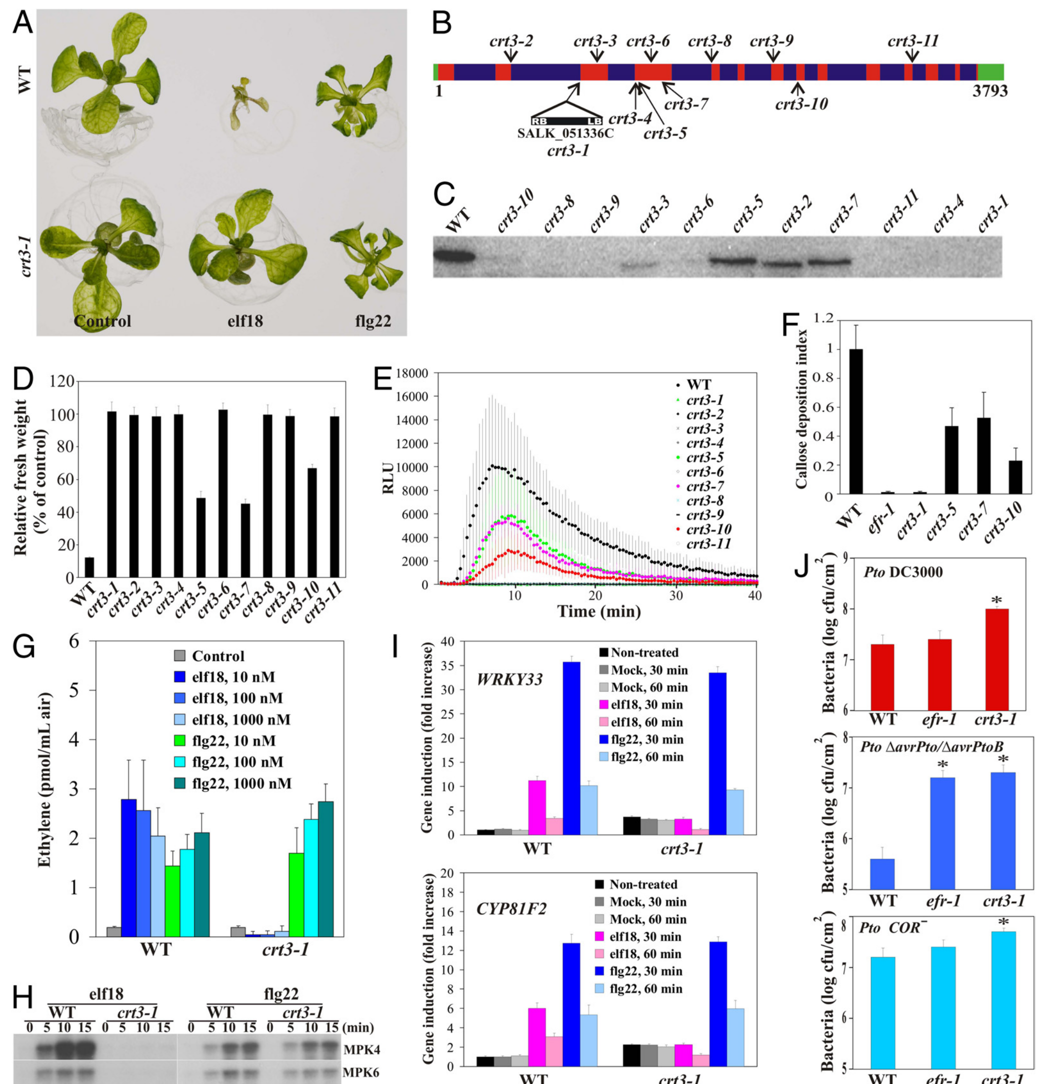

Fig. 1. CRT3 is required for EFR-mediated responses. (*A*) Growth inhibition is completely blocked by elf18 but not by flg22 in *crt3–1* mutants. WT (Col-0) and *crt3–1* seedlings were grown for 9 days in the presence of 50 nM elf18 and 100 nM flg22, respectively. (*B*) Summary of *crt3* alleles. The corresponding nucleotides in WT and mutants are indicated by their positions in the *CRT3* gene, red or green letters, respectively. Green, red, and blue boxes indicate UTRs, exons, and introns, respectively. (*C*) Western blot on mutant lines using a CRT3-specific antibody. (*D*) Seedling growth of WT and *crt3* mutants after treatment with 50 nM elf18. Treated and control seedlings were weighed 7 days after transfer to the peptide solution. Results shown are means ± SD (n = 8). (*E*) Oxidative burst induced by 100 nM elf18, and measured in relative light units (RLU) in leaf discs. Results are means ± SD (n = 12). (*F*) Callose deposition triggered by 100 nM elf18. Leaf samples were taken at 18 h after infiltration and stained with aniline blue for visualization of callose. The amount of callose was quantified with the program IMAGEJ. The data were normalized against WT. Results shown are means ± SD (n = 8). (*G*) Ethylene release in response to elf18 and flg22. Results shown are means ± SD (n = 4). (*H*) MAP kinase activation. Two-week-old *Arabidopsis* seedlings in liquid MS 1% were treated with 100 nM elf18 or flg22. MPK4 and 6 were affinity-purified using specific antibodies and used for in vitro kinase reactions performed with myelin basic protein (MBP) as a substrate in the presence of [γ-^{32}P]ATP. (*I*) Induction of defense marker genes *WRKY33* and *CYP81F2* by 100 nM elf18 or flg22 as determined by RT-qPCR. (*J*) Bacterial susceptibility assays in *crt3–1* and WT. Plants were infected by spraying with suspensions of indicated strains (OD_{600} = 0.02). *In planta* grown bacteria were extracted from leaves at 3 dpi. Asterisks indicate significant difference ($P < 0.05$) from WT control as determined by Student's *t test*. Results are means ± SE (n = 4). All experiments were independently performed at least two times with similar results.

lost seedling growth inhibition (SGI) in response to elf18 (24). *Elfin5–4* was mapped to At1g08450, which encodes calreticulin 3 (CRT3) (Fig. S1 *A* and *B*). Sequencing of *CRT3* in 103 *elfin* mutants revealed another nine alleles (Fig. 1 *A* and *B* and Fig. S1*B*). A SALK T-DNA insertion line in *CRT3* (SALK_051336C: *crt3–1*; Fig. 1*B* and Fig. S1*B*) also showed loss of SGI in response to elf18 (Fig. 1*A*), confirming that CRT3 is required for elf18-triggered SGI. We raised a specific anti-CRT3 antibody and assessed CRT3 protein accumulation in the *crt3* alleles; all showed reduced or abolished accumulation of CRT3 protein (Fig. 1*C*).

***crt3* Mutants Are Compromised in EFR but Not FLS2 Signaling.** The *crt3* alleles were compared for SGI induced by elf18 or flg22. Interestingly, all alleles showed reduced or abolished SGI by elf18 (Fig. 1*D*), but were wild-type (WT) in their SGI after flg22 treatment (Fig. S2*A*). We next compared *crt3* alleles for elf18-triggered oxidative burst; all showed reduced or abolished oxidative burst by elf18 (Fig. 1*E*), but were WT in their response to flg22 (Fig. S2*B*). The partial loss in elf18-triggered SGI, oxidative burst, and callose deposition in the *crt3–5*, −*7*, and −*10* alleles, correlated with a partial reduction in CRT3 protein (Fig. 1 *C–F*). The null *crt3–1* T-DNA allele (Fig. 1*C*) was completely insensitive to elf18, as measured by SGI, oxidative burst, callose deposition, ethylene production, MAP kinase activation, and defense gene induction (Fig. 1 *D–I*). Chitin responses were also not compromised in *crt3–1* mutants (Fig. S2 *C* and *D*). Thus, CRT3 is required for EFR, but not FLS2 or CERK1, function.

***crt3* Mutants Are More Susceptible to Phytopathogenic Bacteria than *efr* Mutants.** We tested whether *crt3–1* shows enhanced susceptibility to infection by the virulent strain *Pseudomonas syringae* pv tomato DC3000 (*Pto* DC3000) and to isogenic hypovirulent strains deleted for effectors AvrPto and AvrPtoB (*Pto* DC3000 Δ*avrPto*/Δ*avrPtoB*) (25) or for synthesis of the phytotoxin coronatine (*Pto* DC3000 *COR*⁻) (26). AvrPto, AvrPtoB, and coronatine are virulence factors that suppress early PTI signaling; the use of mutant strains is therefore more likely to reveal phenotypes linked to PTI defects. While the *efr-1* mutant showed significantly enhanced disease sensitivity only to *Pto* DC3000 Δ*avrPto*/Δ*avrPtoB*, the *crt3–1* mutant was clearly more susceptible to all three *Pto* DC3000 strains (Fig. 1*J*). This suggests that EFR is not the only PRR whose function is compromised by CRT3 mutations.

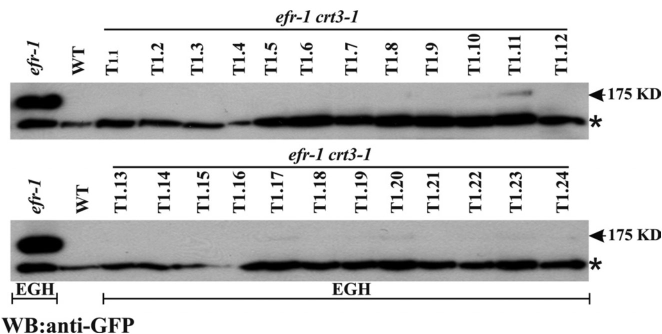

Fig. 2. CRT3 is required for EFR accumulation. Accumulation of EFR-GFP-HA (EGH) fusion protein in *efr-1* or *efr-1 crt3–1* genetic backgrounds. Total proteins were extracted from primary transformants in WT nontransformed (lane 2, *A* and *B*), *efr-1* (left lane, *A* and *B*), or *efr-1 crt3–1*. The EFR-GFP-HA (EGH) fusion protein was detected by an anti-GFP antiserum. The nonspecific band indicated by an asterisk provides a loading control.

CRT1 and CRT2 Play a Minor Role in PTI. In addition to *CRT3*, *Arabidopsis* carries the closely related *CRT1* and *CRT2* genes (27), two related calnexin genes *CNX1* and *CNX2* (Fig. S3 *A* and *B*). Null Salk T-DNA insertion lines in *CRT1* or *CRT2* (Fig. S4 *A–C*) retained elf18-triggered SGI (Fig. S4*F*), but a *crt1–1 crt2–1* double mutant showed a partial reduction in SGI by elf18 (Fig. S4 *F–H*). Furthermore, the *crt1–1 crt2–1* double mutant showed partial reduction in oxidative burst (Fig. S4*G*), but not in defense gene induction (Fig. S4*H*), after elf18 treatment. CRT3 accumulation was not impaired in *crt1 crt2* (Fig. S4*C*). However, a double mutant in two, more distantly related, calnexin genes, *CNX1* and *CNX2* (Fig. S4 *D* and *E*), showed no impairment in elf18-triggered SGI, oxidative burst, or defense gene induction (Fig. S4 *F* and *G*). Thus, loss of CRT1 together with CRT2 compromises EFR function to a certain extent, while loss of CRT3 alone abrogates EFR function completely.

CRT3 Is an ER-Localized Protein Required for EFR Protein Accumulation. CRT1, 2, and 3 carry a signal peptide (SP) and a C-terminal HDEL sequence, which is classically associated with protein retention of soluble proteins in the ER (28). To confirm the CRT3 subcellular localization *in planta*, we engineered a *CRT3*-promoter *SP-YFP-CRT3* fusion construct, in which a yellow fluorescent protein (YFP) tag was introduced one amino acid after the predicted SP cleavage site (Fig. S5*A*). This construct was transiently coexpressed in *Nicotiana benthamiana* with a control ER marker construct, ER-CK (29). Both proteins colocalized, suggesting that CRT3 is localized in the ER (Fig. S5*B*).

We tested if the elf18-insensitivity of the *crt3* mutant could be due to a defect in EFR protein localization or accumulation. We first tested whether EFR protein accumulation was reduced in the absence of CRT3 function. An *EFR*-promoter *EFR-GFP-HA* fusion construct (EGH) was transformed into a double mutant *Arabidopsis* line *efr-1 crt3–1* or into a control *efr-1* line. Multiple transgenic lines in the *efr-1 crt3–1* background accumulated no or very little EGH protein (Fig. 2), whereas transgenic lines in the *efr-1* background showed a strong signal in most lines as detected by anti-GFP immunoblots (Fig. 3, left lanes, and Fig. S6*A*). Transcript levels of the *EFR-GFP-HA* construct were, however, still detectable in *efr-1 crt3–1* lines that do not accumulate EGH protein (Fig. S6*B*; the EGH transformant in *efr-1* in Fig. 2 corresponds to line 4.1 in Fig. S6*A*). Thus, CRT3 is an ER-localized protein required for full EFR protein accumulation.

Identification of *UGGT* Mutant Alleles that Compromise EFR but Not FLS2 Signaling. We hypothesized that additional components of the calnexin/calreticulin cycle might be identified in the *elfin* screen. *Arabidopsis* UGGT is encoded by a single gene, At1g71220, and is ER localized (30). We found that *elfin21–2* mapped to this region (Fig. S7*A*), and sequencing revealed a point mutation corresponding to allele *uggt-3* (Fig. 3*A* and Fig. S7*B*). DNA sequencing of At1g71220 in additional *elfin* mutants revealed an additional two EMS-induced *UGGT* alleles (Fig. 3*A* and Fig. S7*B*). The allele *uggt-3*, and a null insertion line (*uggt-4*) (Fig. 3*A* and Fig. S7*B* and Fig. S8*B*) also showed elf18 insensitivity (Fig. 3 *B* and *C*) confirming that UGGT is required for EFR function. Like *crt3* mutants, *uggt* mutants were unaltered in SGI and oxidative burst triggered by flg22 (Fig. 3 *B* and *C*). UGGT protein levels were unaltered in

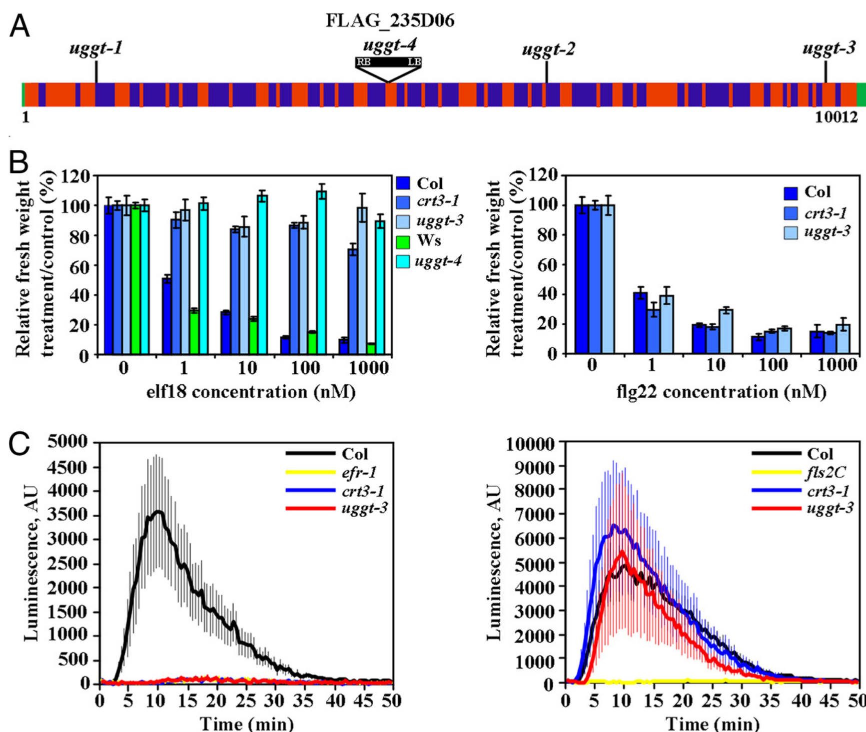

Fig. 3. *UGGT* is required for elf18 but not flg22 responses. (*A*) Summary of *uggt* alleles. Schematic representation of the *UGGT* gene with UTRs (green), exons (red), and introns (blue). See Fig. S7*B* for details of each allele. (*B*) Seedling growth inhibition of WT and mutants after treatment with a series of elf18 and flg22 concentration. Treated and control seedlings were weighed seven days after transfer to the peptide solution. Results shown are means ± SD ($n = 12$). Due to natural deficiency in flagellin perception in Ws ecotype, seedling growth inhibition in presence of flg22 was not tested for *uggt4*. (*C*) Oxidative burst induced in *uggt* mutants by 100 nM elf18 and flg22, and measured in RLU in leaf discs. Results are means ± SD ($n = 12$). Three independent experiments show similar data.

crt3–1 mutants, and CRT3 protein levels were unaltered in *uggt* mutants (Fig. S8 *A* and *B*).

Identification of *ERD2b* Mutant Alleles that Compromise EFR but Not FLS2 Signaling. We investigated a third mutant, *elfin5–3*, and found that it contained a G to A transition (Fig. S9 *B–D*) in the gene At3g25040 (ERD2b) that corresponds to one of two *Arabidopsis* homologs (Fig. S10) of the yeast ER retention receptor ERD2 (31). We therefore renamed *elfin5–3* as *erd2b-1*. ERD2 recognizes the C-terminal H/KDEL motif of certain soluble ER proteins to ensure their retrograde transport from the Golgi to the ER (32). While ERD2a was previously shown to be a functional ER retention receptor that complements the yeast *erd2* mutant (33), the function of ERd2b is still unknown. Further analyses revealed four additional *erd2b* alleles in the *elfin* mutant collection (Fig. S9 *B* and *C*). All five *erd2b* alleles were strongly reduced in their responsiveness to elf18, but remained fully sensitive to flg22 and chitin (Fig. 4*A* and *B*). Similarly to CRT3 and UGGT, ERD2b is therefore specifically required for EFR function.

ERD2b Is Golgi-Localized. We raised a specific anti-ERD2b antibody and showed that ERD2b protein levels are higher in the microsomal fraction in comparison to the soluble fraction isolated from WT plants (Fig. 5*A*), suggesting that ERD2b is a membrane protein. In addition, *erd2b-1* and *erd2b-2* mutants exhibit substantially reduced ERD2b protein levels compared with the WT (Fig. 5*A*). ERD2a, the closest paralog of ERD2b, is widely used as a Golgi marker for protein localization studies, although it cycles continuously between the ER and the Golgi (34). To determine ERD2b sublocalization, we generated an *SP-EYFP-ERD2b* fusion construct driven by the *ERD2b* native promoter (Fig. 5*B*) and transiently coexpressed this construct in *N. benthamiana* with a control Golgi marker construct, G-CK (29). Both proteins colocalized (Fig. 5*B*), suggesting that ERD2b is a Golgi-localized membrane protein consistently to its potential function as a receptor for ER lumen proteins that escaped the ER.

ERD2b Is Required for CRT3 Protein Accumulation. To test if ERD2b regulates CRT3 levels via retro-transport of CRT3 from the Golgi to the ER, we assessed CRT3 protein levels in the five *erd2b* alleles. Strikingly, all *erd2b* alleles show impaired CRT3 protein accumulation (Fig. 5*D*). This observation suggests that the elf18-insensitivity of the *erd2b* mutants is caused by CRT3 deficiency. Our previous data suggest that CRT1 and CRT2 might also contribute to EFR signaling, while calnexins are not required (Fig. S2 *F* and *G*). Interestingly, their protein levels were not affected in *erd2b* plants (Fig. 5*D*). Furthermore, we tested the levels of ER-resident luminal binding proteins BIPs belonging to the Hsp70 family of chaperones. Although BIP1 and BIP2 carry the HDEL motif (35), they accumulate in *erd2b* mutants to similar levels as in WT (Fig. 5*D*). These results suggest that CRT3 is a specific substrate of ERD2b.

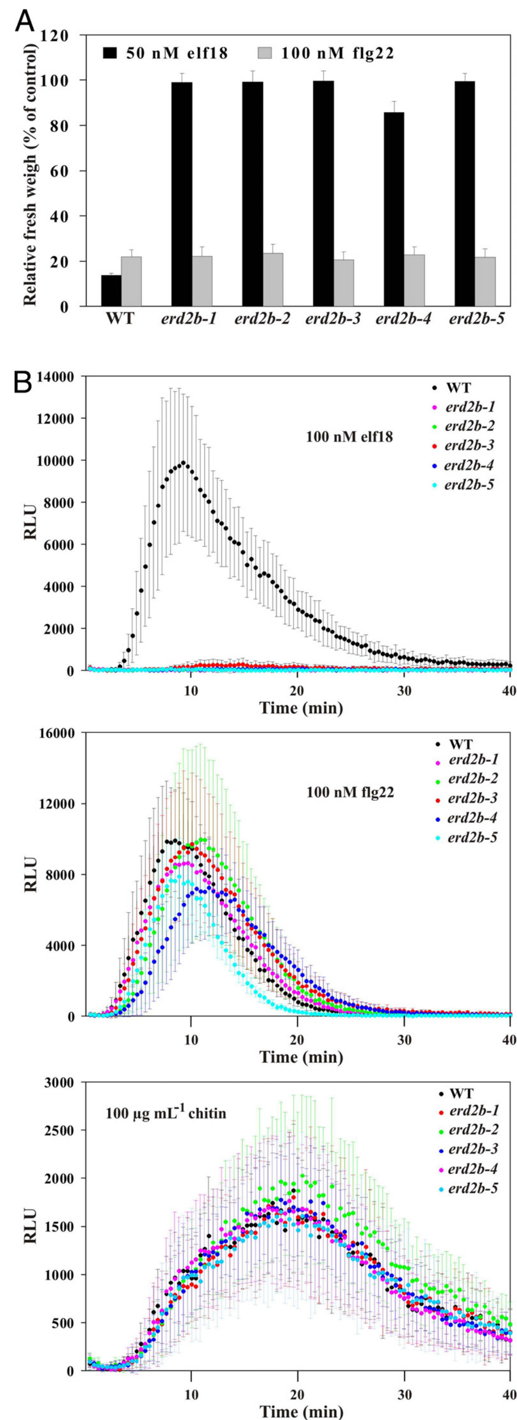

Fig. 4. *ERD2b* is required for elf18 responses. (*A*) Seedling growth of WT and *erd2b* mutants after treatments with 50 nM elf18 or 100 nM flg22. Treated and control seedlings were weighed 7 days after transfer to the peptide solution. Results shown are means ± SD ($n = 8$). (*B*) Oxidative burst induced in *erd2b* mutants by 100 nM elf18 and flg22 or 100 μg/mL chitin and measured in RLU in leaf discs. Results are means ± SD ($n = 12$). All experiments were independently performed at least three times with similar results.

Discussion

Despite the major contribution of PTI to plant innate immunity, our knowledge of the molecular events underlying PRR biogenesis, PAMP perception by PRRs, and downstream signaling is limited. We report here on three proteins (CRT3, UGGT, and ERD2b) that are required for the proper accumulation of the PRR EFR.

CRT3, together with CRT1 and CRT2, are the *Arabidopsis* orthologs of the mammalian soluble luminal lectin CRT involved in the folding of glycoproteins in the ER-QC (27, 36). Our finding that EFR does not accumulate in a *crt3* null mutant, reveals that *Arabidopsis* CRT3 plays a similar function as its mammalian counterpart in ER-QC and that EFR is a client of CRT3 in vivo. *Crt3* mutants were completely insensitive to elf18, while *crt1 crt2* double mutants show reduced sensitivity to elf18. This shows that CRT1 and CRT2 are not able to complement for the loss of CRT3, but that CRT3 might complement for the loss of CRT1 and CRT2. We hypothesize that CRT proteins are part of an ER protein complex (37) in which CRT3 is required for EFR maturation but in which CRT3 can partially compensate for loss of CRT1 and CRT2. Although FLS2 function is not impaired, *crt3* mutant plants are

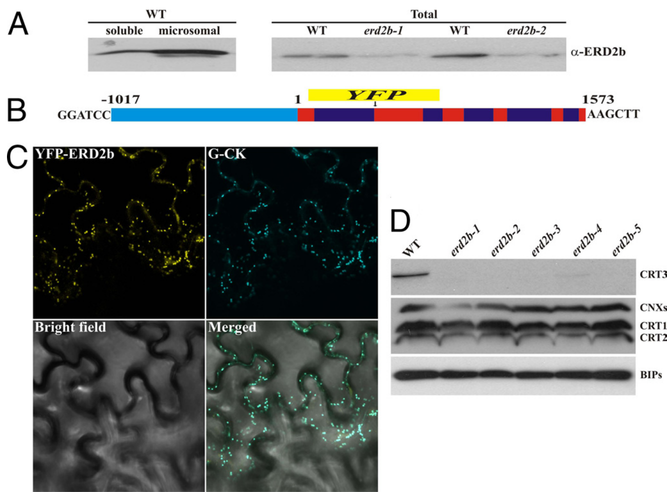

Fig. 5. ERD2b is Golgi-localized and is required for CRT3 accumulation. (*A*) Western blot analysis of ERD2b expression in WT (*Left*) or in *erd2b* mutants in comparison to WT (*Right*) with an anti-rabbit ERD2b antibody. (*B*) Schematic representation of *ERD2b-YFP* fusion construct driven by *ERD2b* native promoter (cyan). An *YFP* DNA fragment was introduced into the second exon (red), five nucleotides after the splicing acceptor site of the first intron (blue). (*C*) Subcellular localisation pattern of ERD2b. The YFP-ERD2b fusion proteins and the G-CK marker (29) were transiently coexpressed using *Agrobacterium* in *N. benthamiana*. (*D*) Proteins of leaf crude extracts were detected with specific antibodies against the ER chaperons including CRTs, CNXs, and BIPs.

more susceptible than *efr* mutant plants to bacterial infection, suggesting that CRT3 may be required for the accumulation of additional, yet unknown PRRs mediating bacterial recognition, or conceivably, that it regulates other aspects of plant defense. Given that *crt3* mutants are not impaired in FLS2-dependent responses, CRT3 is not required for BAK1 accumulation or function, a LRR-RK that positively regulates FLS2 function (38, 39). Our finding that *uggt* and *crt3* mutants are insensitive to elf18 and that *crt3* mutants do not accumulate any detectable levels of EFR proteins show that in the absence of CRT3 or UGGT, EFR protein is probably misfolded and therefore targeted to ERAD. EFR is therefore a client for CRT3/UGGT-mediated ER-QC.

The retention of soluble ER protein relies mainly of the recognition of a C-terminal sorting signal (i.e., HDEL and KDEL) by the ER-lumen protein-retaining receptor, ERD2 (32, 40). ERD2 binds the ER-escaped proteins and retrieves them back to the ER. The ERD2b protein is highly homologous to the yeast HDEL receptor and shows very high sequence similarity with ERD2a, which has been shown to complement the lethal phenotype of the yeast *erd2* mutant (33). BLASTP analysis identified five additional, more distantly related, *ERD2* paralogs (ERD2-like proteins, or ERPs) in *Arabidopsis* (Fig. S10). Interestingly, ERPs seem only present in plants (31), suggesting that they might play a role in a plant-specific biological process. Strikingly, ERD2a, ERD2b, and the ERPs show highly conserved gene structures (Fig. S10). In particular, exon lengths are invariant between *ERD2a* and *ERD2b* (Fig. S10), indicating very recent functional divergence. Given the presence of several ERD2 homologs in *Arabidopsis*, it was suggested that different retention signal (e.g., HDEL vs. KDEL) could be recognized by different ERD2 isoforms (31). However, our results show that the *erd2b* mutation specifically affects CRT3, but not CRT1 and CRT2, although they all carry a C-terminal HDEL signal, making this hypothesis unlikely. Our data, however, clearly demonstrate that ERD2b is essential for CRT3 accumulation, suggesting that the elf18-insensitive phenotype of *erd2b* mutants is due to lack of CRT3 protein accumulation that itself results in lack of EFR protein accumulation. This also suggests that CRT3 is a likely substrate for ERD2b. This is in agreement with the hypothesis that CRT might be degraded in a post-ER compartment after ER export if ERD2-mediated retrieval fails (41).

We have recently demonstrated the requirement of the soluble luminal proteins SDF2 and the Hsp40 ERdj3B for elf18 responses (24). SDF2 and ERdj3B form a complex with BiP in vivo, in which ERdj3B acts a bridge between SDF2 and BiP. *Sdf2* mutants are strongly impaired in EFR protein accumulation, demonstrating that EFR biogenesis also requires the SDF2/ERdj3B/BiP complex, in addition to ER-QC mediated by CRT3 and UGGT. Interestingly, *sdf2* or *erdj3b* mutants are not completely insensitive to elf18, suggesting that BiP retention is less critical than CRT-based ER-QC for EFR proper folding and protein accumulation. Because EFR contains two pairs of conserved Cys residues flanking the LRR ectodomain, it will be interesting to see if thiol reduction is also involved in EFR ER-QC. BiP and CRT exist in an abundant large complex in tobacco (37, 42). CRT3, SDF2, ERdj3B, BIP, and potentially UGGT may therefore exist in the same complex to regulate proper EFR folding. Such large chaperone complexes have been reported in mammals (43). So far, we failed to detect CRT3 or UGGT in the SDF2 immuno-complex. It is known that BiP-CRT heterodimers cannot be detected with BiP antibodies (37). Further work is therefore required to investigate the existence of such large complex.

Our studies provide a clear demonstration of a physiological requirement for the ER-QC in the control of transmembrane receptor in plants. The transmembrane LRR-RK BRI1 is the receptor for brassinosteroid class of plant hormone (44). Mutations in ER-QC components have been reported to specifically suppress the phenotype of the weak *Arabidopsis bri1–9* and *bri1–5* alleles by relaxing ER-QC, so that partially misfolded, but yet functional proteins can escape the ER and reach the plasma membrane (30, 45). We examined suppression of *bri1–9* in *crt3–1* mutants and found that like *UGGT* mutations (30), *crt3–1* specifically suppresses the phenotype of *bri1–9* but not *bri1–301* that carries a mutation in the kinase domain (46) (Fig. S8). In all of these examples, the function of the WT BRI1 receptor was never affected by mutations in ER-QC components (30, 45), demonstrating that WT BRI1 is not a physiological substrate for ER-QC.

Together with another study (24), our findings reveal a subset of previously uncharacterized ER-QC components, including CRT3, UGGT, SDF2, the Hsp40 ERDj3B, and the Hsp70 BiPs, that are specifically required for the biogenesis of EFR and likely other PRRs in plant PTI. The specificity of mutations in these components clearly argues against a general defect in protein secretion. The existence of an ER chaperone complex involved in PTI mirrors the requirement of a cytosolic chaperone complex involving SGT1, RAR, Hsc70, and Hsp90 for the accumulation of several nucleotide-binding domain and leucine-rich repeat-containing (NLR) proteins involved in plant ETI (47).

Why would EFR require ER quality control components that appear to be dispensable for FLS2 function? Although both FLS2 and EFR belong to the subfamily XII of LRR-RKs, they have clear differences in their protein structures, including different number and position of putative glycosylation sites (5, 6). We speculate that since EFR is only found in the *Brassicaceae* (6) whereas FLS2 has been identified in several dicots and monocots (5, 48–50), EFR may have evolved more recently than FLS2, and thus its amino acid sequence is less capable of folding properly in the absence of these components. It is conceivable that evolution of recognition proteins may result in proteins that detect novel ligands but that have not been selected for high protein stability and thus require extra “buffering” (51). Regardless of this speculation, additional defense components must depend on CRT3 function, since *crt3* mutants are more susceptible to bacteria than *efr* mutants. Thus, the ER-QC system is likely to be required for the function of additional pattern recognition receptors and/or defense components whose identity will be interesting to investigate in future experiments. The molecular mechanisms underlying the differential requirement of EFR

and FLS2 for ER-QC components will need to be identified in the future.

Materials and Methods

Plant Materials and Growth Conditions. *Arabidopsis* plant growth conditions were described in ref. 52 with the exception of germination medium (Murashige-Skoog medium containing 1% sucrose and 1% agar). *N. benthamiana* plants used for the transient expression assay were grown as one plant per pot at 20–23 °C with an 8-h photoperiod for 5–6 weeks. The isolation of elfin mutants was performed as described in Reference 24.

Bioassays and Infections. Assays for seedling growth inhibition, oxidative burst, ethylene evolution, and protein kinase activity were performed as described (6, 53–56). Spray inoculations on *Arabidopsis* leaves were performed as described in ref. 55 with a bacterial suspension at $OD_{600} = 0.02$, plants were covered during the whole experiment and bacteria extracted at 3 days postinoculation (dpi).

CRT3 and ERD2b Protein Detection. Rabbit antibodies raised against the CRT3-specific peptide TAGKWPGDPDNKG and the ERD2b-specific peptide YHKAVHR-TYDREQDT were generated by Eurogentec and used to detect CRT3 and ERD2b in plant extracts as described in *SI Methods*.

Generation of Transgenic Plants. *Efr-1* and *efr-1 crt3–1* mutant plants were transformed via the floral dipping method (57) with the *epiGreenB*(*EFRp*::*EFR-eGFP-HA*) construct (24). The BASTA-resistant transformants were further selected by PCR with GFP specific primers 5′-GTGAG-CAAGGGCGAGGAGC-3′ and 5′-GATGTTGTGGCGGATCTTGAAG-3′.

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