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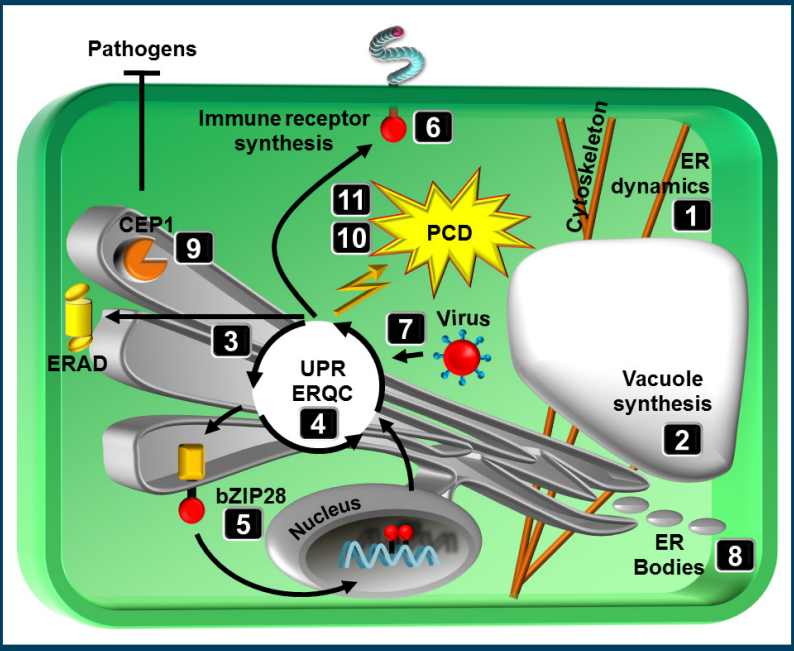

ENDOPLASMIC RETICULUM – SHAPE AND FUNCTION IN STRESS TRANSLATION

Topic Editors
Lorenzo Frigerio, Federica Brandizzi, Stephen H. Howell and Patrick Schäfer

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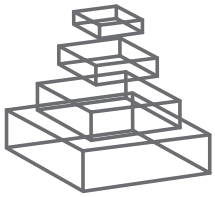

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ENDOPLASMIC RETICULUM - SHAPE AND FUNCTION IN STRESS TRANSLATION

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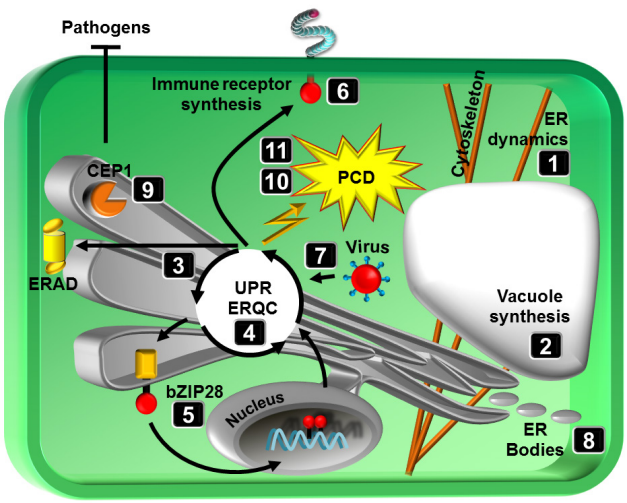

Schematic representation of topics covered in the Research Topic. Numbers correspond to articles as follows: (1) Griffing et al. (2014), ER network dynamics are differentially controlled by myosins XI-K, XI-C, XI-E, XI-I, XI-1, and XI-2, (2) Viotti (2014), ER and vacuoles: never been closer, (3) Li and Liu (2014), Endoplasmic reticulum-mediated protein quality control in Arabidopsis, (4) Ruberti and Brandizzi (2014), Conserved and plant-unique strategies for overcoming endoplasmic reticulum stress, (5) Srivastava et al. (2014), Stress sensing in plants by an ER stress sensor/transducer, bZIP28, (6) Tintor and Saijo (2014), ER-mediated control for abundance, quality, and signaling of transmembrane immune receptors in plants, (7) Verchot (2014), The ER quality control and ER associated degradation machineries are vital for viral pathogenesis, (8) Nakano et al. (2014), ER bodies in plants of the Brassicales order: biogenesis and association with innate immunity, (9) Höwing et al. (2014), Endoplasmic reticulum KDEL-tailed cysteine endopeptidase 1 of Arabidopsis (AtCEP1) is involved in pathogen defense, (10) Williams et al. (2014), When supply does not meet demand-ER stress and plant programmed cell death, (11) Cai et al. (2014), Endoplasmic reticulum stress-induced PCD and caspase-like activities involved.

The endoplasmic reticulum (ER) is a manufacturing unit in eukaryotic cells required for the synthesis of proteins, lipids, metabolites and hormones. Besides supporting cellular signalling networks by its anabolic function, the ER on its own or in communication with other organelles directly initiates signalling processes of physiological significance. Based on the intimate and immediate involvement in stress signalling the ER is considered as sensory organelle on which cells strongly rely to effectively translate environmental cues into adaptive stress responses. The transcellular distribution of the ER providing comprehensive cell-to-cell connections in multicellular organisms probably allows a concerted action of cell alliances and tissue areas towards environmental constraints. At the cellular level, stress adaptation correlates with the capability of the ER machinery to synthesise proteins participating in stress signalling as well as in the activation of ER membrane localised proteins to start cell-protective signalling processes. Importantly, depending on the stress insult, the ER either supports protective strategies or initiates cell death programmes. Recent, genetic, molecular and cell biological studies have drawn an initial picture of underlying signalling events activated by ER membrane localised proteins. In this Research Topic, we provided a platform for articles describing research on ER morphology and metabolism with a focus on stress translation. The Research Topic is sub-divided into the following sections:

1. ER in stress signalling and adaptation
2. ER structure and biosynthetic functions
3. Regulation of protein processing
4. Regulation of programmed cell death

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Endoplasmic reticulum—shape and function in stress translation

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Keywords: unfolded protein response (UPR), ER associated degradation (ERAD), ER stress, bZIP transcription factors, myosins, caspases, ER bodies, cysteine endopeptidase, programmed cell death

The endoplasmic reticulum (ER) is a very versatile organelle. Besides its major role as the gateway to the secretory pathway the ER is central to adaptation against abiotic and biotic stress. Here, we summarize the current knowledge on ER dynamics and architecture, the association and interaction of the ER with other organelles as well as its role in stress translation and adaptation (**Figure 1**).

The ER is a dynamic network of membrane tubules and sheets. Movement of the ER along the actin cytoskeleton has a significant influence on both its architecture and dynamics. Members of class XI myosin motor proteins have recently been shown to control the movement of the ER and other organelles such as Golgi bodies, peroxisomes and mitochondria. Griffing et al. (2014) explore a subset of class XI myosins with a focus on their role in regulating ER network remodeling by affecting ER tubulation, sheet formation and the persistence of these structures. In addition to the importance of these ER network dynamics for organelle communication, the ER may further directly provide the source membrane for vacuole biogenesis, as hypothesized by Viotti (2014). The majority of soluble vacuolar proteins travel from the ER to the Golgi complex. A growing number of reports however indicate that some proteins, including some vacuolar membrane proteins appear to reach the vacuole without visiting intermediate compartments of the endomembrane system.

An essential function of the ER is the synthesis of secreted proteins. To insure that only correctly folded proteins are exported from the ER, eukaryotes have evolved ER quality control (ERQC) mechanisms, which supervise the folding process. Protein folding is monitored through the stepwise modification of oligosaccharide side chains on glycoproteins, and proteins that fail to fold correctly are extracted from the folding process and subjected to ER associated degradation (ERAD). Li and Liu (2014) describe the protein folding process and how client proteins are chosen for ERAD. Proper function of the ER is particular relevant under stress where the demand for secreted proteins exceed the ER working load capacity. Under such conditions, ER homeostasis requires cellular communication between the ER and the nucleus. Therefore, eukaryotes have signaling proteins on the ER membrane that sense impaired ER function (ER stress) through the accumulation of misfolded proteins in the ER and that employ different strategies to signal the nucleus. Some of these communication strategies are common to all eukaryotes, while others are unique to plants. Ruberti and Brandizzi (2014) compare the ER stress signal pathways between yeast, plants and animals and review the responses in plants, which vary from adaptive measures to cell death. The bZIP transcription factor bZIP28 represents an ER stress signaling factor that is tethered

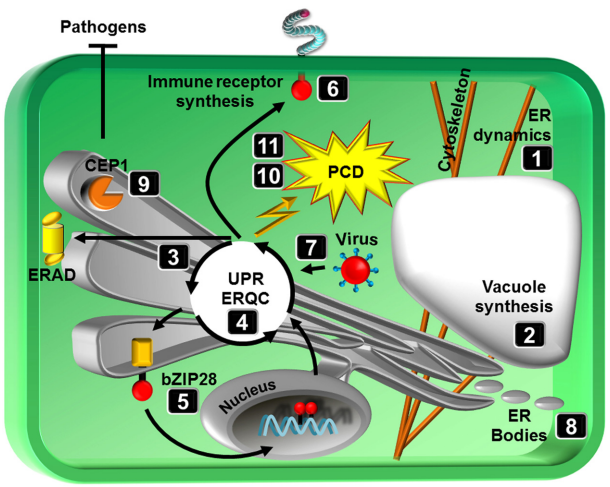

FIGURE 1 | Schematic representation of topics covered in the special issue. Numbers correspond to review articles as follows: (1) Griffing et al. (2014), ER network dynamics are differentially controlled by myosins XI-K, XI-C, XI-E, XI-I, XI-1, and XI-2, (2) Viotti (2014), ER and vacuoles: never been closer, (3) Li and Liu (2014), Endoplasmic reticulum-mediated protein quality control in Arabidopsis, (4) Ruberti and Brandizzi (2014), Conserved and plant-unique strategies for overcoming endoplasmic reticulum stress, (5) Srivastava et al. (2014), Stress sensing in plants by an ER stress sensor/transducer, bZIP28, (6) Tintor and Saijo (2014), ER-mediated control for abundance, quality, and signaling of transmembrane immune receptors in plants, (7) Verchot (2014), The ER quality control and ER associated degradation machineries are vital for viral pathogenesis, (8) Nakano et al. (2014), ER bodies in plants of the Brassicales order: biogenesis and association with innate immunity, (9) Höwing et al. (2014), Endoplasmic reticulum KDEL-tailed cysteine endopeptidase 1 of Arabidopsis (AtCEP1) is involved in pathogen defense, (10) Williams et al. (2014), When supply does not meet demand-ER stress and plant programmed cell death, (11) Cai et al. (2014), Endoplasmic reticulum stress-induced PCD and caspase-like activities involved.

to the ER membrane under unstressed conditions but is mobilized and transferred to the nucleus upon stress. Srivastava et al. (2014) describe the structure of bZIP28 and underlying principles of bZIP28 mobilization in response to stress.

ER stress activates the unfolded proteins response (UPR) and the ERAD system to eliminate misfolded proteins, which is vital for the establishment of an effective immune system. Tintor and Saijo (2014) introduce the significance of the ERQC in the synthesis of immune receptors and provide further insights into the causalities of ER function (UPR) and plant immunity. Despite its role in immunity, the ER has been hijacked by viruses to promote viral pathogenesis. As reviewed by Verchot (2014), viruses have developed sophisticated strategies to overload the ER protein folding machinery with viral encoded proteins in infected cells. Interestingly, this activates the UPR and up-regulates cellular chaperones that further aid in virus protein folding. The overall significance of the ER in stress adaption is however apparent and underlined by the review of Nakano et al. (2014) which, in addition to the biogenesis and evolution, discusses the putative function of ER bodies in abiotic and biotic stress. As ER-derived compartments ER bodies contain stress-associated proteins (e.g., various β-glucosidases of the PYK10 family) that are thought to enhance stress resilience by activating glucosinolates and glucosylated phytohormones. The study of Höwing et al. (2014) further identified an immune-active function of the ER-localized cysteine endopeptidase AtCEP1. In addition to its function in developmental processes, AtCEP1 is expressed and localized to the ER network, which condenses around haustoria during powdery mildew infection. Knockout of AtCEP1 results in enhanced susceptibility, suggesting that AtCEP1 is involved in restricting fungal infection, possibly by controlling a defense-related programmed cell death (PCD) at the late stages of the interaction.

Cells mount adaptive responses (e.g., UPR) to mitigate the damage caused by ER stress. However, when stress becomes so excessive that the ERQC system cannot meet the demands, then PCD ensues. Williams et al. (2014) review the possible links between the UPR and PCD and discuss the involvement of calcium signaling and N-rich proteins in promoting PCD and the role of ER chaperones in limiting it as well as the connection of pathogens to ER-mediated cell death. Cai et al. (2014) finally compare the activation and/or regulation of ER stress-induced PCD in animals and plants and highlight the significance of caspase and caspase-like activities in underlying PCD processes.

The articles published in this e-book summarize our current knowledge of the multi-functionality of the ER in stress adaptation. By providing a stable microenvironment for the synthesis of metabolites and secreted proteins, the ER functions as an intracellular stress sensory organelle and, accordingly, initiates and regulates adaptive responses to environmental stress. Understanding the molecular basis of these processes and the role of ER architectural dynamics therein is of high relevance for sustainable crop production. We hope our e-book will stimulate research in the field to further enhance our knowledge of ER biology in plants.

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ER network dynamics are differentially controlled by myosins XI-K, XI-C, XI-E, XI-I, XI-1, and XI-2

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The endoplasmic reticulum (ER) of higher plants is a complex network of tubules and cisternae. Some of the tubules and cisternae are relatively persistent, while others are dynamically moving and remodeling through growth and shrinkage, cycles of tubule elongation and retraction, and cisternal expansion and diminution. Previous work showed that transient expression in tobacco leaves of the motor-less, truncated tail of myosin XI-K increases the relative area of both persistent cisternae and tubules in the ER. Likewise, transient expression of XI-K tail diminishes the movement of organelles such as Golgi and peroxisomes. To examine whether other class XI myosins are involved in the remodeling and movement of the ER, other myosin XIs implicated in organelle movement, XI-1 (MYA1),XI-2 (MYA2), XI-C, XI-E, XI-I, and one not, XI-A, were expressed as motor-less tail constructs and their effect on ER persistent structures determined. Here, we indicate a differential effect on ER dynamics whereby certain class XI myosins may have more influence over controlling cisternalization rather than tubulation.

Keywords: endoplasmic reticulum, movement, myosin, persistency mapping, dynamics, remodeling

INTRODUCTION

The endoplasmic reticulum (ER) is a polygonal network that rapidly interconverts between tubular and cisternal forms, with tubules also growing, shrinking, and undergoing lateral sliding to form junctions, and cisternae undergoing expansion and shrinkage (Griffing, 2010; Sparkes et al., 2011). Whilst ER network dynamics are mainly controlled by actin and myosin (Quader et al., 1987; Liebe and Menzel, 1995; Sparkes et al., 2009a; Ueda et al., 2010), ER morphology and structure are also governed by additional factors such as reticulons and RHD3 implicated in ER membrane curvature and homotypic fusion (Sparkes et al., 2011; Zhang and Hu, 2013). Quantifying network dynamics is challenging because there are at least two levels at which movement takes place; (1) flow of protein within the lumen and on the surface of ER tubules and cisternae, as well as (2) remodeling through translocation and transformation of the tubular and cisternal elements themselves. Note, membrane flow can occur even when there is no remodeling (Sparkes et al., 2009a). The former movement, flow within and on the membrane is measured with fluorescent recovery after photobleaching (FRAP) and photoactivation (Sparkes et al., 2009a) as well as with optical flow image processing (Ueda et al., 2010; Stefano et al., 2014). But it is the latter form of movement, ER remodeling, which is analyzed here, starting from the perspective that there are relatively persistent features of the ER upon which change agents act. Hence, an approach to visualize and measure the relatively static or persistent regions in the network has been developed, persistency mapping (Sparkes et al., 2009a). Persistency mapping is an image processing technique that includes morphometric processing to separate tubules from cisternae and visualizes the relative movement and change of these ER elements as the ER remodels. The less-moving, more persistent fraction of the ER tubules and cisternae is then quantified. It is tempting to assign functions of some of the persistent structures of the ER, such as the potential anchor sites (Sparkes et al., 2009a,b) which may be regions where traffic between the plasma membrane and the ER can take place or/and could act as anchors to tether the ER required for spread throughout the cortex. A role in tethering or anchoring the ER to the plasma membrane was alluded to by optical tweezer experiments whereby a trapped Golgi body was used to stably remodel the ER around small islands of ER (Sparkes et al., 2009b). These experiments also highlighted that there is a physical connection between Golgi bodies and the ER bringing into question whether Golgi and ER dynamics are coordinated or are independent of one another? The common network upon which these interactions play out is the actin cytoskeleton.

Organelle movement in interphase plant cells is largely driven by actin and myosin, however the specificity of myosin-organelle interactions is poorly understood (Madison and Nebenführ, 2013 and references therein). In plants, organelle positioning and movement are correlated with responses to extracellular stresses such as pathogen ingress, wounding, and cadmium toxicity (Takemoto et al., 2003; Lipka et al., 2005; Hardham et al., 2008; Rodriguez-Serrano et al., 2009). At present, the precise role that organelle dynamics play in such processes is unclear. The best characterized study of the functional role of organelle movement is that of chloroplast repositioning upon high light, a response required to prevent photodamage (Wada et al., 2003). In addition there is a correlation between the morphology of the ER and the level of secretion with cisternal ER being more prevalent in cells producing more protein (Stephenson and Hawes, 1986; Ridge et al., 1999). Treating cells with latrunculin B to depolymerize

actin results in a cessation of movement of spheroid organelles such as Golgi (Boevink et al., 1998; Nebenführ et al., 1999), mitochondria (Van Gestel et al., 2002; Zheng et al., 2009), and peroxisomes (Jedd and Chua, 2002; Mano et al., 2002; Mathur et al., 2002), and produces a relatively static ER network (Quader et al., 1987; Liebe and Menzel, 1995; Sparkes et al., 2009a) with larger cisternae, indicating that an actively remodeling system affects the global morphology of the ER. The movement of Golgi, peroxisomes and mitochondria appear to be controlled by a subset of class XI myosins; XI-C, XI-E, XI-I, XI-K, XI-1, and XI-2 (Avisar et al., 2008, 2009, 2012; Peremyslov et al., 2008, 2010; Prokhnevsky et al., 2008; Sparkes et al., 2008, 2009a). Dominant negative tail domain expression of these different myosins shows some quantitative differences in the reduction of movement produced by each member of this subset, but the results do not preclude the simple interpretation that one myosin form may control the movement of several different organelles. Double, triple and quadruple *Arabidopsis* mutants in myosins XI-K, XI-1, XI-2, and XI-I have reduced organelle dynamics and display gross morphological defects (Prokhnevsky et al., 2008; Peremyslov et al., 2010; Ojangu et al., 2012). Although myosin XI-K has been shown to change ER form and dynamics (Sparkes et al., 2009a; Ueda et al., 2010), work with mutants of XI-1, and XI-2, shows that they have little effect on their own, but enhance the effect of XI-K when double or triple mutants are analyzed. Here, we further explore whether these different subclasses of myosin XI that reduce spheroid organelle mobility differentially affect the movement and remodeling of the ER network. Remodeling is assessed by quantifying the static elements in the network which should increase if motor activity is required to drive changes.

MATERIALS AND METHODS

PLANT MATERIAL AND CONSTRUCTS

Nicotiana tabacum plants were grown according to Sparkes et al. (2005). Fluorescent protein fusion constructs including the ER marker GFP-HDEL (Batoko et al., 2000), and mRFP-myosin XI-K, XI-I, XI-1, XI-2, XI-E, XI-C, XI-A tail domains (Sparkes et al., 2008; Avisar et al., 2009) were all infiltrated according to Sparkes et al. (2006) with an optical density of 0.1 except for GFP-HDEL, which required 0.04 optical density. Expression was analyzed 3 days following inoculation.

PROTEIN EXTRACTION AND WESTERN BLOTTING

Total proteins were extracted according to Gao et al. (2013). 0.5 g of tobacco leaf material 3 days post infiltration were ground in PEB (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% TritonX-100 plus protease inhibitors) and then centrifuged. Equal volumes of extract were separated by 10% SDS-PAGE and blotted onto PVDF membrane (Pall). mRFP fusions were detected using anti-mRFP primary antibody (Abcam) and HRP-conjugated goat anti-rabbit secondary antibody (Abcam). Chemiluminescence reaction was performed using ECL substrate (Pierce) followed by film exposure.

SAMPLE PREPARATION AND IMAGE ACQUISITION

The ER in the outermost cortical region of adaxial leaf epidermal pavement cells was imaged. Dual imaging of mRFP and GFP was done using multi-tracking in line switching mode on a Zeiss LSM510 Meta confocal microscope. GFP was excited with a 488 nm argon laser and mRFP with a 543 nm laser and their emissions detected using a 488/543 dichroic mirror and 505–530 and 560–615 nm band pass filters, respectively. All imaging was carried out using a 63 × 1.4 numerical aperture oil immersion objective.

PERSISTENCY MAPPING THE CORTICAL ER IN TOBACCO EPIDERMAL CELLS

For persistency mapping, time-lapse images of GFP-HDEL were captured using a 2–3 μm pinhole, 512 × 512 pixel resolution and 2.3× digital zoom. To reduce noise, 4× line averaging was used. The scan rate was increased by imaging a 955–960 μm^2 region of interest (ROI), so that 50 frames per 80 s were captured (0.63 frames/s). For samples where cells were coexpressing a fluorescent myosin tail domain and GFP-HDEL, coexpression was verified before time lapse imaging of the GFP-HDEL alone was performed.

Persistency maps were generated in Image J (version 1.45s, Wayne Rasband, National Institute of Health, Bethesda, MD) as described in Sparkes et al. (2009a) with the following modifications. As shown in the corrected tubule persistency maps in **Figures 2**, **3**, the persistent tubule subset was corrected by subtracting regions containing cisternae prior to making persistent tubule counts. This was done by directly subtracting the morphologically opened binary sum images from the morphologically closed skeletonized binary sum images. In the resulting image sets, only those with a projected area > 0.2 μm^2 were counted (excludes tubules less than 1 μm long, assuming a 200 nm projection of an individual tubule). This disconnected some of the tubules because punctae or small cisternae often occur at tubule junctions, producing shorter tubules of smaller percentage area than previously described. As shown in the corrected cisternal persistency maps of **Figures 5**, **6**, the persistent cisternal subset was corrected by subtracting regions that contained continuous flow (i.e., subtracting the sum of the 5-frame differences from the cisternal persistency map). Quantitation of the most persistent tubules, and cisternae was done, normalizing each movie to the total membrane area imaged in the percentage area and number/100 μm^2 values. Analysis of mesh size and mesh number per cytoplasmic volume was done with manual selection of the region containing in-focus signal for each movie, then measuring the number and area of selected mesh regions for each frame of every movie. The cytoplasmic volume was estimated as the area of the region containing in-focus signal times the z-dimension optical section acquired by the confocal microscope. Analysis of the size classes of persistent cisternae was done by making a binned histogram of the different sizes of persistent cisternae for the entire collection of movies acquired for each treatment. Note, highly persistent regions are defined as being present (persisting) for more than 23 s within the movie. Statistical comparison of the data was done using the 64 bit version of R, 3.0.2 (http://www.r-project.org/) using ANOVA and the Tukey HSD two-way comparison of means at 95% confidence interval. Each analysis is based on data generated from 15 movies in total taken from separate cells from three independent experiments.

Displaced frame difference images were made by subtracting every fifth frame, summing the differences between all frames, and dividing that value by an estimated cytoplasmic volume (area containing signal in a 3 μm optical slice) for each movie.

RESULTS

MYOSIN TAIL DOMAINS AFFECT ER MORPHOLOGY

As previously reported, transient expression of mRFP-myosin tail fusions in tobacco leaf epidermal cells are present in puncta or diffusely throughout the cytoplasm, with XI-I collating to the nucleus and motile puncta (**Figure 1**; Avisar et al., 2009). A similar perinuclear localization for both full length and tail fusions of XI-I in Arabidopsis and tobacco respectively were reported by Tamura et al. (2013). Here, we show how these fusions relate to, and affect the morphology and remodeling of the ER. **Figure 1** highlights that whilst these fusions do not solely collocate to the surface of the ER, they appear to differentially affect the level of ER cisternalization with XI-I tail resulting in the largest observable regions of cisternal ER (**Figures 1P–R**). This phenomenon is clearly visible when comparing the morphology of the ER in two adjacent cells where one has no detectable levels of mRFP-XI-I tail fusion (**Figures 1P–R**, white line defines cell boundary). These still images do not convey whether myosin tail expression affects the dynamic remodeling of the ER and so time lapse movies were taken and dynamics quantified.

MYOSIN TAIL DOMAINS AFFECT GLOBAL ER REMODELING

Movies were taken using consistent settings allowing comparisons between the effects of the myosin tail domains on ER dynamics to be drawn. In addition, the same settings were used to detect mRFP myosin tail fusions to determine whether any effects on ER dynamics were dependent on myosin tail expression level. Western blotting confirmed that the majority of the myosin tail fusions were stable under transient expression in tobacco leaf epidermal cells (**Figure S1**). Results indicated that whilst XI-A (**Movie S2**) tail domain didn't appear to affect global ER remodeling compared with control cells only expressing the ER marker (GFP-HDEL, **Movie S1**), the other tail domains perturbed remodeling to differing extents in a concentration independent manner; XI-2 slowed down movement (**Movie S3**), XI-I increased the level of cisternal ER which was still relatively mobile (**Movie S4**), whereas XI-C, XI-E, XI-K, and XI-1 all reduced active remodeling (**Movies S5–S9**).

QUANTIFICATION OF THE EFFECTS OF MYOSIN TAIL DOMAINS ON ER DYNAMICS

To quantify the effects of myosin tail domains on ER dynamics we implemented persistency mapping to tease out regions which had become more or less persistent compared with control cells. Persistency mapping can be used to measure the more static elements within the ER network over the given time frame. Morphological elements of the ER under study include tubules, cisternae (areas larger than 0.3 μm^2) and punctae (areas smaller than 0.3 μm^2). If the different myosins act as change agents, then their dominant-negative selective inhibition should decrease change, thereby increasing the number or size of the more persistent morphological elements. Therefore, we have chosen here and in prior work (Sparkes et al., 2009a) to measure the more persistent features (green to black in **Figures 2**, **3**, **5**, **6**) of the persistency map rather than less persistent features (yellow in **Figures 2**, **3**, **5**, **6**). The analysis here is further refined over prior work by

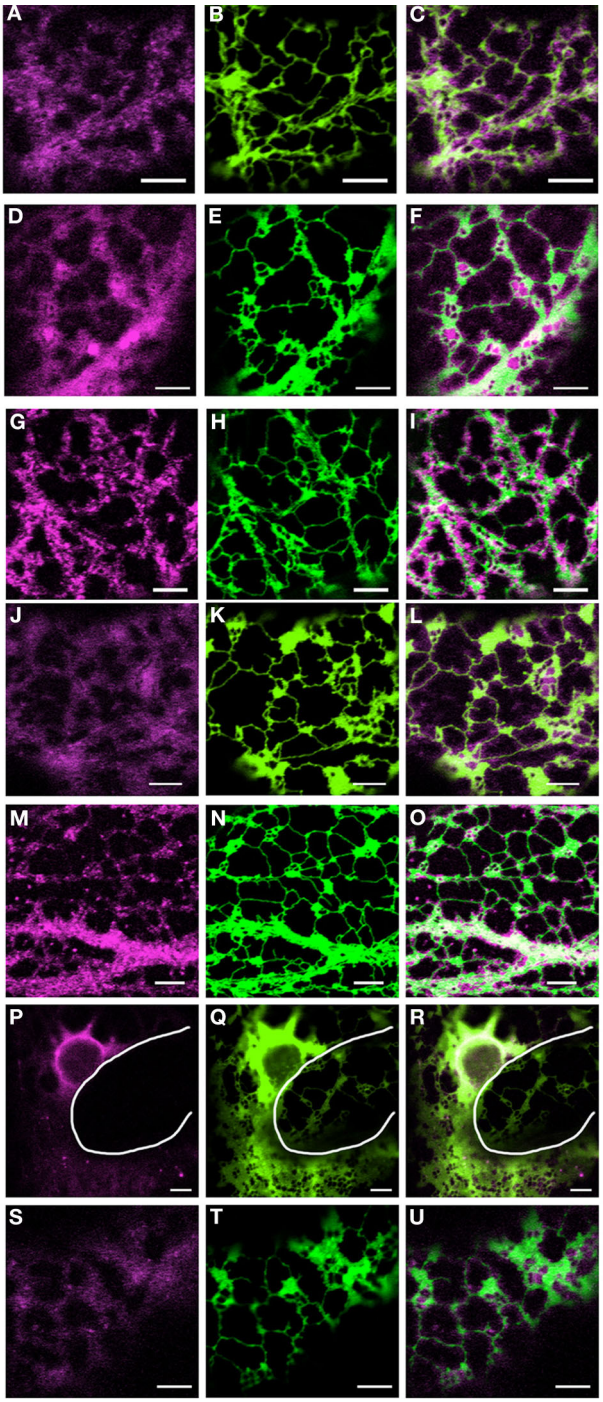

FIGURE 1 | Coexpression of myosin tail domains with an ER marker. Representative images of cells coexpressing GFP-HDEL (green) and mRFP myosin tail domain fusions [magenta; XI-A, **(A–C)**; XI-1, **(D–F)**; XI-2, **(G–I)**; XI-C, **(J–L)**; XI-E, **(M–O)**; XI-I, **(P–R)**; XI-K, **(S–U)**]. White line in panels **(P–R)** highlights the cell boundary between neighboring cells. Scale bar 5 μm.

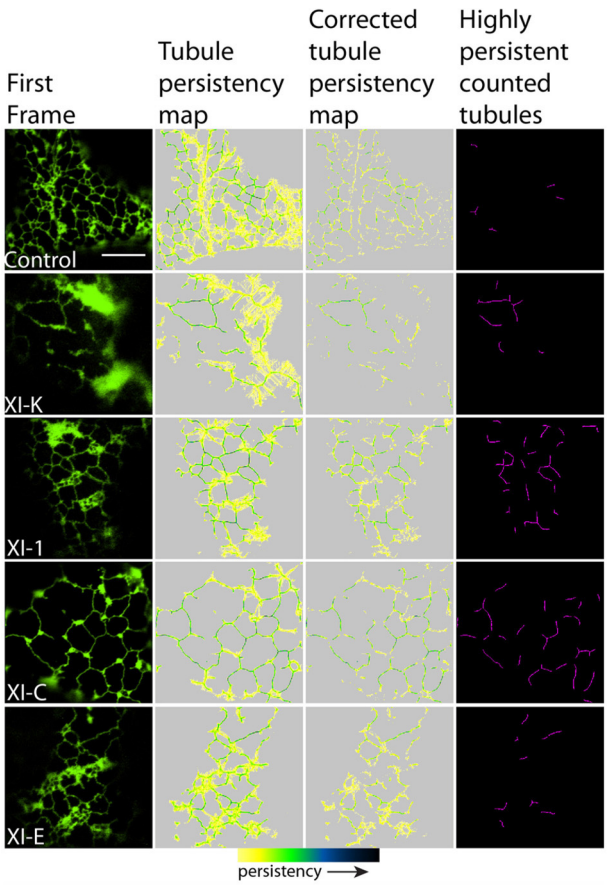

FIGURE 2 | Effects of XI-K, XI-C, XI-E, and XI-1 myosin tail domains on persistent ER tubules identified with co-expressed GFP-HDEL. Left-hand panel is the first frame of a 50 frame movie. Middle two panels are tubule persistency expressed as the sum of skeletonized tubules that persist for five frames (7.9 s).The corrected tubule persistency subtracts out contributing skeletons from cisternal regions. The yellow-green-blue-black persistency scale represents persistency from 7.9 to 80 s. Highly persistent tubules are those that are counted for **Figure 4**, being both greater than 0.2 μm^2 and persisting for at least a third of total movie time (black to green on the persistency scale with black representing persistency through the whole 80 s and green representing persistency for approximately 23 s). Note, control refers to cells only expressing the ER marker GFPHDEL (green, left hand panel).

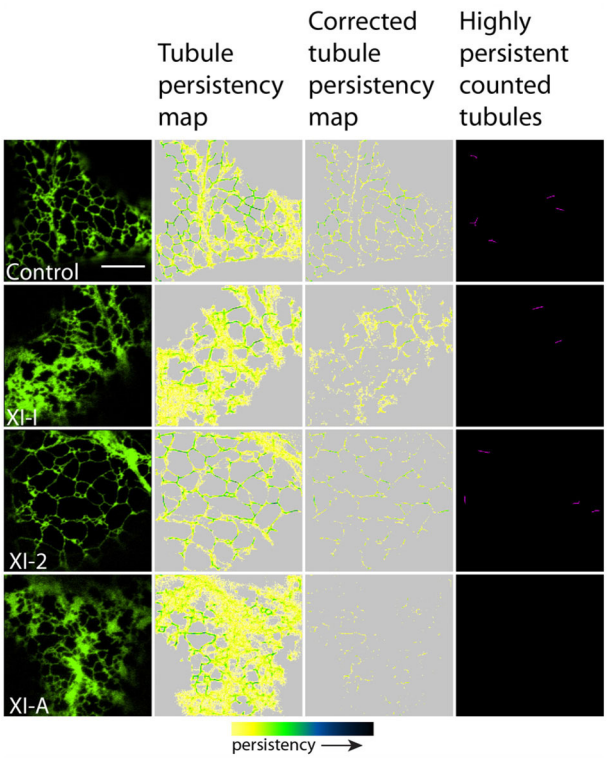

FIGURE 3 | Effects of XI-2, XI-A, and XI-I myosin tail domains on persistent ER tubules identified with co-expressed GFP-HDEL. Left-hand panel is the first frame of a 50 frame movie. Middle two panels are tubule persistency expressed as the sum of skeletonized tubules that persist for at least five frames (7.9 s). The corrected tubule persistency subtracts out contributing skeletons from cisternal regions. The yellow-green-blue-black persistency scale represents persistency from 7.9 to 80 s. Highly persistent tubules are those that are counted for **Figure 4**, being both greater than 0.2 μm^2 and persisting for at least a third of total movie time (black to green on the persistency scale with black representing persistency through the whole 80 s and green representing persistency for approximately 23 s). Note, control refers to cells only expressing the ER marker GFPHDEL (green, left hand panel).

excluding cisternal regions from tubular persistency analysis and by excluding large moving cisternal fields occurring throughout the analysis period (80 s) from the cisternal persistency analysis.

In general, persistency mapping shows an increase in persistent ER tubules (**Figures 2–4**), cisternae (**Figures 5–7**), and puncta (**Figures 5**, **6**, **8**) in cells expressing certain myosin tail fusions compared to control cells. In all of the persistency map figures (**Figures 2**, **3**, **5**, **6**) the image in the first column is the first frame from a representative movie and the additional columns highlight the process of isolating the highly persistent regions in the movie shown. These highly persistent regions (i.e., present for more than 23 s) are then quantified in terms of average number, size, and the percentage of imaged membrane represented by these morphological structures. Statistical analysis (HSD Tukeys test) of pair wise combinations of average values are shown in **Table 1** ($n = 15$). **Movies S1–S8** highlight the persistent ER elements in **Figures 2**, **3**, **5**, **6**.

Whilst there is a large variation within the sample population ($n = 15$), statistical analysis highlighted that average persistent tubule area and number were significantly higher in cells coexpressing the tails of XI-C, XI-E, XI-K, or XI-1 compared to the control (**Figures 2–4**, **Table 1**). This corresponded with an increase in mesh size of the tubular network (**Figure S2**). In addition, there appeared to be significant pair wise differences between several of these myosins except for XI-C, XI-E, and XI-1 which are presumably performing similar functions relating to ER tubule dynamics/formation.

There was also a significant increase in the percentage of total membrane imaged present in persistent cisternal regions for several myosin tails (XI-1, XI-C, and XI-K) compared with the control (**Figures 5–7**, **Table 1**, a histogram of persistent cisternal size classes for each combination is provided **Figure S3**).

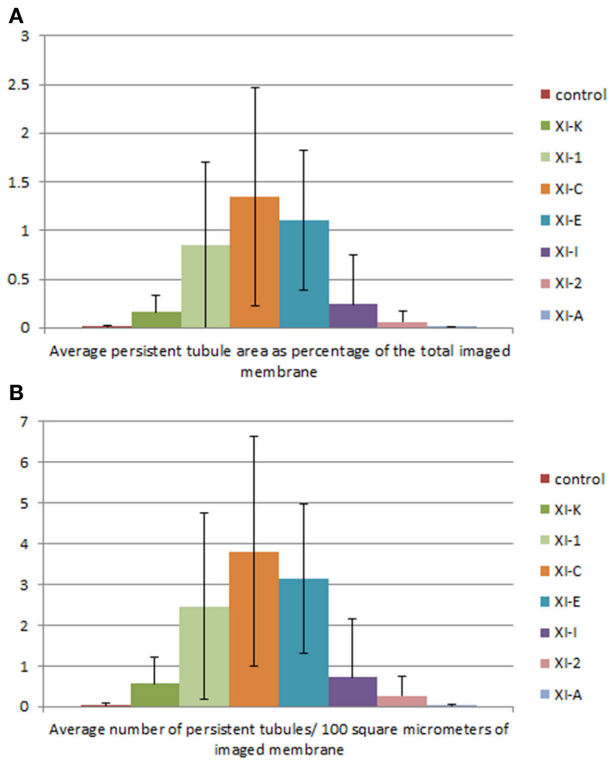

FIGURE 4 | Quantification of the effects of the myosin tail domains on persistent ER tubules. (A) Counts of the cumulative percentage area of highly persistent tubules (see **Figures 2**, **3** for examples). **(B)** Counts of the number of persistent tubules per hundred square micrometers of ER membrane analyzed for all of the myosin subclasses. Error bars represent standard deviation. The level of significance of the difference between the samples is described in **Table 1**.

These significant differences could relate to changes in either average size and/or number of persistent cisternae per given unit area. Both XI-1 and XI-K's effects were due to a combination of both increased number and average size of cisternal regions (**Figure 7**). XI-E and XI-I also resulted in significantly more cisternae. In addition the average area and percent area increased with XI-E and XI-I tail domain expression over control, but the data are so variable (note large standard deviations) that a firm statement of difference cannot be made. This high variability quite likely arises from the observations based on the movies, as described above, that cisternalization increases but a fairly large proportion remain motile. While the number of relatively immobile cisternae increases, there is enough general membrane cisternalization (hence larger amounts of membrane per field) that the area fraction of immobile cisternae does not reliably increase.

Pair wise statistical tests for significant differences between XI-1, XI-C, and XI-K persistent cisternal criteria indicate that XI-1 and XI-K are not different to one another. However, XI-C compared with either XI-1 or XI-K display differences in persistent cisternal number, but not persistent cisternal area. This could indicate that XI-1 and XI-K have conserved functions in generating the number and size of persistent ER cisternae, whereas XIC is more involved in maintaining cisternal number, but not size relative to the roles of XIK and XI-1.

Persistent regions of ER less than 0.3 μm^2, termed persistent punctae, are present in control cells and in all cells coexpressing the myosin tail domains. XIC tail domain is the only myosin to have a significant increase in the number of persistent punctae over control conditions, and when compared with other myosins (**Figures 5**, **6**, **8**, **Table 1**).

Finally we tried to monitor the active regions of the ER network using displaced frame differences (**Figure S4**). Here, the sum of the displaced frame differences summed over the imaged volume were plotted for all myosin combinations. As expected with increased persistency for XI-K, XI-C, XI-1, and XI-E we have a slower rate of movement in these samples compared to the control, XI-2 and XI-A. This provides an initial approximation to the dynamic elements of the network.

DISCUSSION

ER network structure (tubules/cisternae and three way junctions) is not completely dependent on cytoskeletal elements; depolymerization of actin results in an interconnected ER network which is more cisternal (Sparkes et al., 2009a, 2011). At present, additional molecular factors required to alter ER morphology in plants include reticulons and RHD3 (see references in reviews Sparkes et al., 2011, and Zhang and Hu, 2013). Here, we have begun to assess the relative role(s) of class XI myosins on ER geometry and remodeling. Several class XI myosins which appear to have a global effect on perturbing the movement of several organelle classes including peroxisomes, Golgi and mitochondria were investigated (XI-C, XI-E, XI-I, XI-K, XI-1, and XI-2; (Avisar et al., 2008, 2009; Peremyslov et al., 2008, 2010; Prokhnevsky et al., 2008; Sparkes et al., 2008, 2009a). XIA was also tested as the tail domain fusion did not affect global organelle dynamics in previous studies, and so was used here as a potential control for overexpressing a class XI myosin.

The main findings from this study indicate that whilst XI-A did not affect ER dynamics, XI-C, XI-E, XI-K, and XI-1 affected tubule persistency and the persistent nature of cisternae, and XI-C increased the number of persistent punctae. Whilst XI-I and XI-2 didn't affect the persistent regions within the network, visual inspection indicates that XI-I increases the level of cisternalization, while XI-2 reduces overall remodeling (**Movies S3**, **S4**). These differential affects could indicate specific roles for myosins in controlling tubulation (growth/shrinkage/lateral sliding to form polygons), cisternalization, and potential anchoring of the ER to the plasma membrane. For example, based on the differences these myosin tail domains exert on static regions within the ER network, it is interesting to speculate that XI-C, XI-E, XI-K, and XI-1 are required for most forms of tubule growth and shrinkage as overexpression of the tail domains increased the number of static tubules. This is assuming that the tail domains are acting in a dominant negative manner and the observed effect is due to a direct rather than indirect effect. It is likely that tubule growth draws upon, in part, a reservoir of membrane in the cisternal regions of the ER rather than being due to extension of completely newly synthesized membrane (Sparkes et al., 2009a). Mesh size of the tubular network increases in those treatments

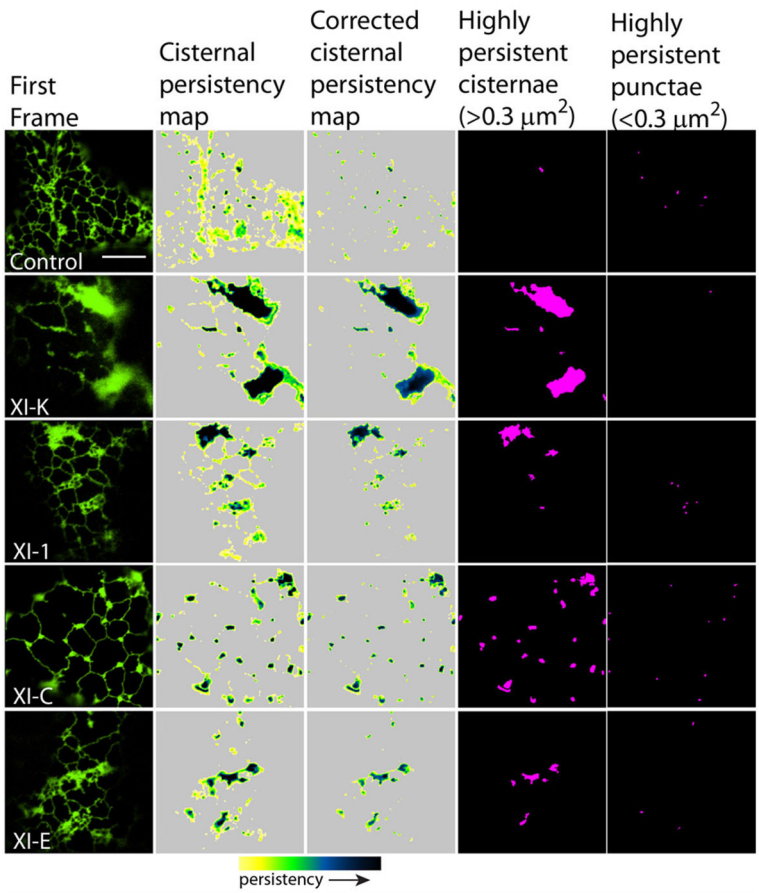

FIGURE 5 | Effects of XI-K, XI-C, XI-E, and XI-1 myosin tail domains on persistent ER cisternae and punctae identified with co-expressed GFP-HDEL. Left-hand panel is the first frame of a 50 frame movie. Middle two panels are cisternal persistency expressed as the sum of cisternae (morphometrically separated from tubules with a binary opening operation) that persist for at least five frames (7.9 s). The corrected cisternal persistency subtracts out regions of continuous flow (sum of five frame differences). The yellow-green-blue-black persistency scale represents persistency from 7.9 to 80 s. Highly persistent regions are those that are counted for **Figure 7**, cisternae being greater than 0.3 μm^2 and punctae less than 0.3 μm^2 and both persisting for at least a third of total movie time (black to green on the persistency scale with black representing persistency through the whole 80 s and green representing persistency for approximately 23 s). Note, control refers to cells only expressing the ER marker GFPHDEL (green, left hand panel).

that increase tubule persistency (**Figures 2**, **3** compared with **Figure S2**), indicating a lower amount of total tubulation. This explains why the myosin tail domains which increase the number of persistent tubules also have a concomitant increase in persistent cisternal size or number. The less obvious, but nonetheless significant effect, (**Figure 7**, **Table 1**) of XI-I increasing the number of persistent cisternae relative to control may relate to a shaping role in the ER of known cisternal structures, such as the nuclear envelope, where XI-I tends to accumulate (**Figure 1**). Hence, XI-I may have more shaping-related and less movement-related functions, while XI-C, XI-E, XI-K, and XI-1 may have more movement-related and less shaping-related functions. XI-2 tail domain expression doesn't affect the level of persistency in the ER, but appears to reduce overall modeling.

It is important that we comment upon the large variability seen within each sample population presented herein. Organelle dynamics are affected by growth and developmental cues, with larger, older cells showing faster ER and other organelle movement than smaller, younger cells (Stefano et al., 2014). The cell population analyzed here, the pavement epidermal cells between the minor veins of fully-expanded tobacco leaves, was chosen because it is amenable to rapid transient expression assays and provides easily-imaged, robust cells. However, this population varies somewhat in cell size and developmental stage. This could be one source of the variability. Other approaches, such as using myosin mutants, may provide analysis of a single developmental stage of a uniformly-sized cell population, but would suffer from the developmental defects caused by this mutation. Another source of variability in this work could arise from subcellular variations of movement within these puzzle-piece-shaped cells, which may have tip growth dynamics in the interdigitating lobes and non-tip growth dynamics along the plane of the outer wall. The pattern of cytoplasmic movement in cells undergoing polar tip vs. radial growth are radically different (Hepler et al., 2001). Unfortunately, technical limitations prevent acquiring data sets of adequate resolution

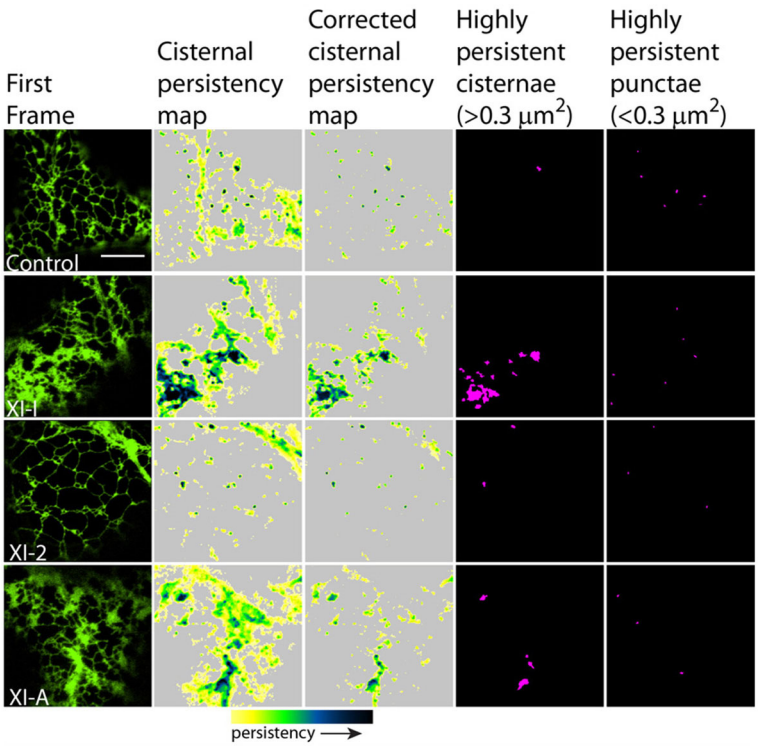

FIGURE 6 | Effects of XI-2, XI-A, and XI-I myosin tail domains on persistent ER cisternae and punctae identified with co-expressed GFP-HDEL. Left-hand panel is the first frame of a 50 frame movie. Middle two panels are cisternal persistency expressed as the sum of cisternae (morphometrically separated from tubules with a binary opening operation) that persist for at least five frames (7.9 s). The corrected cisternal persistency subtracts out regions of continuous flow (sum of five frame differences). The yellow-green-blue-black persistency scale represents persistency from 7.9 to 80 s. Highly persistent regions are those that are counted for **Figure 7**, cisternae being greater than 0.3 μm^2 and punctae less than 0.3 μm^2 and both persisting for at least a third of total movie time (black to green on the persistency scale with black representing persistency through the whole 80 s and green representing persistency for approximately 23 s). Note, control refers to cells only expressing the ER marker GFPHDEL (green, left hand panel).

to resolve the ER network in the entire cell within a fraction of a second. However, were that possible, different patterns of movement might be sorted and the ER dynamics within each pattern analyzed. Advances in imaging will hopefully overcome these limitations.

Previous studies using RNAi, expression of myosin truncations and observations in null mutant backgrounds have shown several myosins appear to have a global affect on the motility of all organelles tested (Avisar et al., 2008, 2009, 2012; Peremyslov et al., 2008, 2010; Prokhnevsky et al., 2008; Sparkes et al., 2008, 2009a). Interestingly, as detailed above, the same myosin truncations that globally arrest spheroid organelle movement have differential effects on ER dynamics and persistent regions. The effects of XI-I on spheroid dynamics could have been due to increased ER cisternalization reducing the effective void volume (cytoplasm) in which spheroid organelles could move thus reducing their motility. XI-2 reduces the movement of all organelles tested, including the ER to a certain extent, but doesn't affect the level of persistency. This could indicate that XI-2 affects a more global common element in organelle dynamics such as the actin cytoskeleton or perhaps cyclosis. Further examination of the nature of the change in the dynamics under control conditions, and in comparison with XI-2 tail domain expression (see **Movie S3**), awaits other forms of analysis. None-the-less, these results indicate that the myosin tail domains that globally affect the movement of spheroid organelles have differential effects on ER network dynamics; increased persistency (XI-1, XI-C, XI-K, XI-E) vs. reduced remodeling (XI-2) vs. drastic changes in ER cisternalization (XI-I).

The expression profiles of the myosins under study here indicate that XI-1, XI-2, XI-I, and XI-K are ubiquitously expressed throughout the plant, whereas XI-C and XI-E are mainly expressed in the stamen and pollen (Peremyslov et al., 2011; Sparkes et al., 2011). Unlike leaf epidermal cell, pollen tubes undergo polarized tip growth where organelle movement occurs via reverse fountain streaming (Hepler et al., 2001; Lovy-Wheeler et al., 2007). This type of growth and pattern of organelle movement could place additional demands on ER morphology; persistent punctae could possibly represent tethering/interaction with the plasma membrane to maintain geometric distribution throughout growth. Ridge et al. (1999) documented changes in ER morphology during developmental transitions in polarized root hair growth; upon completion of growth the ER is tubular whereas during the elongation phase a more cisternal form prevails some way behind the tubular ER network in the expansion zone at the tip.

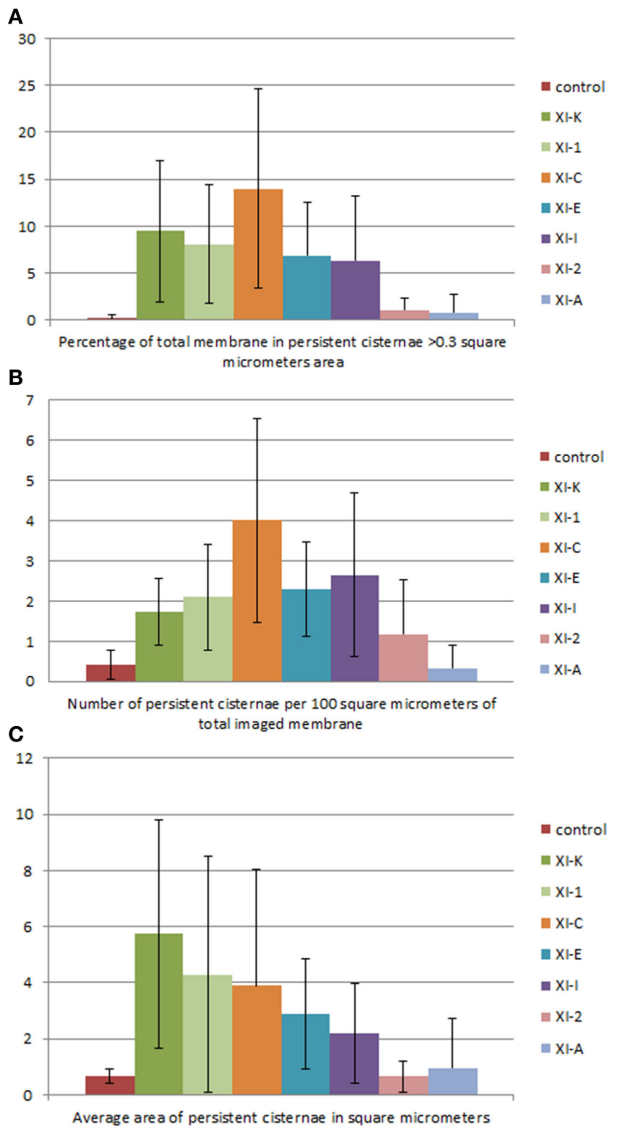

FIGURE 7 | Quantification of the effects of myosin tail domains on persistent ER cisternae. (A) Counts of the cumulative percentage area of highly persistent cisternae (see **Figures 5**, **6** for examples). **(B)** Counts of the number of persistent cisternae per hundred square micrometers of ER membrane analyzed for all of the myosin subclasses. **(C)** Average area of persistent cisternae in square micrometers. Error bars represent standard deviation. The level of significance of the difference between the samples is described in **Table 1**.

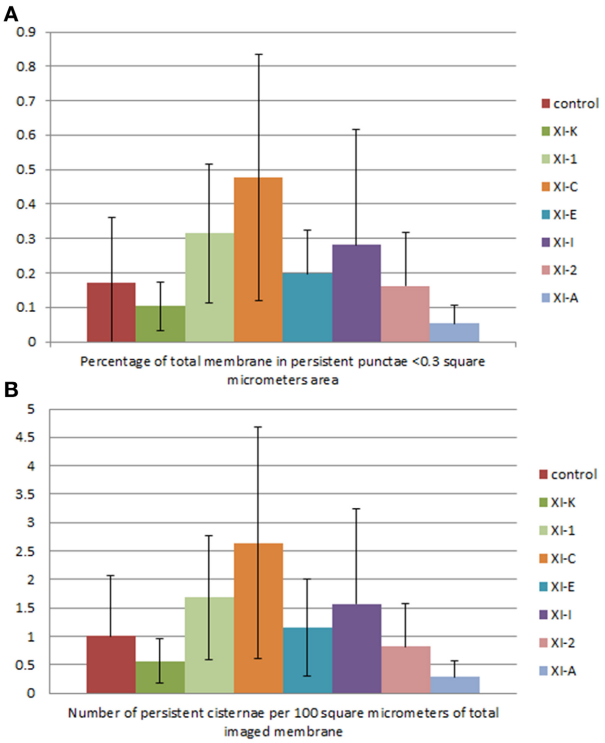

FIGURE 8 | Quantification of the effects of myosin tail domains on persistent ER punctae. (A) Counts of the cumulative percentage area of highly persistent punctae (see **Figures 5**, **6** for examples). **(B)** Counts of the number of persistent punctae per hundred square micrometers of ER membrane analyzed for all of the myosin subclasses. Error bars represent standard deviation. The level of significance of the difference between the samples is described in **Table 1**.

Interestingly, based on phylogenetic analysis of the myosin motor domains it appears that XI-C, XI-E, XI-K, and XI-1 from *Arabidopsis* are from closely related subfamilies XI(J) and XI(K). This could therefore indicate that they might perform similar/conserved functions in controlling ER dynamics (Peremyslov et al., 2011). Whereas XI-A, XI-I, and XI-2 are from more distantly related subfamilies XI(G), and XI(I).

Myosins have been implicated in ER formation and/or dynamics in several plant systems. Immunoblotting of subcellular fractions from Arabidopsis leaf material indicated that XI-K cosedimented with an ER fraction, and that the streaming of the ER is drastically reduced in *xi-k* mutants and to a lesser extent in *xi-1* and *xi-2* (Ueda et al., 2010). Independent studies place XI-K on motile vesicles, and appears to interact and collocate with members from a novel myosin receptor family (Peremyslov et al., 2012, 2013). Moreover, immunolocalization studies indicated that XI-2 partially localized to peroxisomes in *Arabidopsis* (Hashimoto et al., 2005), whereas a 175 kDa tobacco homolog with 75% identity to XI-2 was proposed to control ER movement in tobacco BY-2 cells (Yokota et al., 2009). *In vitro* reconstitution experiments from tobacco BY-2 cells further supported a role for the 175 kDa myosin in ER tubule formation (Yokota et al., 2011). More recently, expression of the tail domain of a maize XI-I homolog Opaque1 in *N. benthamiana* disrupted ER streaming, and subcellular fractionation indicated it is also associated with the ER in maize (Wang et al., 2012). Interestingly, Ueda et al. (2010) also highlighted that actin organization is perturbed in *xi-k xi-2* double mutants, but not single mutants, indicating that both myosins act in a cooperative/synergistic manner to control actin organization in elongating cells. Defects in actin organization were also seen in the midvein cells of triple (*xi-k/1/2*) and quadruple (*xi-k/1/2/i*) Arabidopsis mutants (Peremyslov et al., 2010). However, actin dynamics rather than organization were

Table 1 | Statistical analysis of persistency mapping.

Combinations	% Tubule area	Tubule number	% Cisternal area	Cisternal number	Cisternal area	% Punctae	Punctae number
XI1-control	**	**	*	*	*		
XI2- control							
XIA-control							
XIC-control	***	***	***	***		***	***
XIE-control	***	***		*			
XII-control				**			
XIK-control	**	**	**	***	***		
XI2-XI1	*	**	*		*		
XIA-XI1	**	**	*	*	*	*	*
XIC-XI1				*			
XIE-XI1							
XII-XI1		*					
XIK-XI1	*	*					
XIA-XI2							
XIC-XI2	***	***	***	***		***	***
XIE-XI2	***	***					
XII-XI2							
XIK-XI2			**		***		
XIC-XIA	***	***	***	***		***	***
XIE-XIA	***	***		*			
XII-XIA				***		*	*
XIK-XIA			**		**		
XIE-XIC				*		***	**
XII-XIC	***	***				***	*
XIK-XIC	***	***		**		***	***
XII-XIE	**	***					
XIK-XIE	***	***					
XIK-XII					*		

Tukeys HSD comparison of means test to compare persistent regions in the ER generated upon expression of myosin tail domains in tobacco leaf epidermal cells. Significant differences between pair wise combinations under test are shown ($p < 0.05$; **$p < 0.005$; ***$p < 0.0005$).*

affected in root hairs of an *xi-k* null line (Park and Nebenführ, 2013). In light of all of these studies, it is interesting to note that we found XI-2 may have a global affect on organelle dynamics which could be an indirect effect through perturbing actin organization. Therefore, organelle-myosin specificity, and the specific affect of myosins on actin dynamics, is still a matter of debate, and whether these differences relate to species or tissue specific effects is unclear. Determining the mechanism of myosin association and disassociation from targets is complex (Li and Nebenführ, 2007, 2008a,b). A recent publication from Peremyslov et al. (2013) highlighted a potential myosin receptor family in plants which will hopefully help resolve organelle-myosin specificity.

Persistency mapping is an excellent tool for quantifying the more static geometric elements within the dynamic ER network, which is continually undergoing rapid geometric rearrangements. It does not, however, monitor ER luminal or surface flow. Prior work (Sparkes et al., 2009a) has shown that non-directional surface flow as measured with FRAP and photoactivation still occurs when actin is depolymerized, a condition that drastically changes ER geometry. Optical flow analysis of fluorescent ER constituent proteins also shows that actin depolymerization does not completely inhibit movement (Stefano et al., 2014). In single mutant *xi-1*, there is only a small change in ER movement as analyzed with optical flow (Ueda et al., 2010), but XI-1 tail domain expression has a large effect on membrane geometry (**Figures 1**, **2**, **4**, **5**, **7**). Persistency mapping therefore provides the only tool currently available to quantify differences in ER network geometry. Further studies are required to determine whether the same myosins which affect ER network dynamics also affect surface flow. Better analytical tools are required to quantify the complex dynamic regions which consist of tubule growth, shrinkage, lateral sliding, changes from tubular to cisternal form, formation and movement of three way junctions, polygon formation, size, movement and potential filling to form cisternae. Whilst these dynamic elements can be broken down into various aforementioned categories, ER remodeling is a complex combination where each morphological element is interdependent requiring a more sophisticated cross correlative approach. Our initial displaced frame difference provides an initial observation of such dynamic elements. Furthermore, high persistency described here does not distinguish between elements which are static over sequential frames lasting more than 23 s vs. temporally episodic events over a similar discontinuous time frame. However, observation of the movies clearly shows that persistent elements are consistently static rather than being due to events which reoccur. In addition, it is worthwhile noting that whilst the studies presented here relate to real time events, they are limited by the spatial resolution of the microscope. In essence, the ER tubules studied are likely to be around the 50–70 nm diameter making the 200 nm resolution limit of the microscope prone to potentially imaging parallel tubules rather than reflecting single tubules. Only through the development of super resolution imaging systems, which allow imaging in real time, will these types of issues be resolved.

AUTHOR CONTRIBUTIONS

Imogen Sparkes designed the experiments, Lawrence R. Griffing carried out the persistency mapping and statistical analysis, Imogen Sparkes, Lawrence R. Griffing, and Hongbo T. Gao acquired the data. All authors were involved in writing and approving the manuscript.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2014.00218/abstract

Figure S1 | Western blot analysis of transiently expressed mRFP-myosin tail fusions in tobacco leaf epidermal cells. Arrow heads denote expected sizes of full length mRFP myosin tail fusions (∼100 kDa apart from XI-A which is ∼124 kDa). Asterisk shows detection of full length St-mRFP used as a control. Differences in relative myosin levels are due to differences in the level of transient expression. A large proportion of each myosin fusion is stable.

Figure S2 | Mesh number per estimated cytoplasmic volume and ER mesh size. The ER tubules generate open polygons that can be considered as elements of a mesh. Blue bars: The polygonal regions subtended by tubules were counted for each frame in each movie, averaged over the entire movie, divided by the estimated cytoplasmic volume, and shown as the average number of meshes per 300 μm^3 of cytoplasm for all of the movies taken for each treatment. XI-1, XI-C, XI-E, XI-I, and XI-K are all significantly different to the control and XI-A ($p < 0.005$; Tukeys HSD comparison of means). Red bars: The average area, in square micrometers, of each polygonal mesh structure for each frame of each movie was also calculated. The average mesh area over all of the movies of each treatment is shown. XI-C, XI-E, and XI-K are all significantly different to the control ($p < 0.005$; Tukeys HSD comparison of means). The mesh areas of XI-K and XI-E were also significantly different ($p < 0.05$) from XI-2, XI-A, and XI-I. Error bars are standard deviation.

Figure S3 | Histogram of the percent of each persistent cisternae in different size classes for each treatment. The distribution of the percentage that each different size class makes to the population of persistent cisternae produced by each treatment is shown. The value of each size class was chosen so that comparisons could be made between those that had predominantly small cisternae (e.g., control) and those that had very large cisternae (e.g., XI-1 and XI-K).

Figure S4 | Relative movement or flow as shown by average displaced frame differences summed over imaged volumes. The value of the intensity difference between every fifth frame, the displaced frame difference, is summed for each movie and divided by the approximate volume of the region imaged (area of signal in a 3 μm optical section). The average of these values is shown for all of the movies acquired for each treatment. Error bars are standard deviation. XI-1, XI-C, XI-E, XI-K, and XI-I are significantly different to the control ($p < 0.005$; Tukeys HSD comparison of means), and to XI-A ($p < 0.05$; Tukeys HSD comparison of means), and XI-1, XI-C, XI-E, and XI-K are significantly different to XI-2 ($p < 0.05$; Tukeys HSD comparison of means).

Movie S1 | Persistency mapping of ER dynamics in control tobacco leaf epidermal cells. Time lapse movie showing the movement of the ER, along with overlays of the persistent tubules, cisternae, and puncta (magenta). Scale bar 10 μm.

Movie S2 | Persistency mapping of ER dynamics in a tobacco leaf epidermal cell expressing the myosin tail domain XI-A. Time lapse movie of the ER in a cell coexpressing the tail domain of XI-A. Overlays of the persistent tubules, cisternae and puncta (magenta) are also shown for comparison. Scale bar 10 μm.

Movie S3 | Persistency mapping of ER dynamics in a tobacco leaf epidermal cell expressing the myosin tail domain XI-2. Time lapse movie of the ER in a cell coexpressing the tail domain of XI-2. Overlays of the persistent tubules, cisternae and puncta (magenta) are also shown for comparison. Scale bar 10 μm.

Movie S4 | Persistency mapping of ER dynamics in a tobacco leaf epidermal cell expressing the myosin tail domain XI-I. Time lapse movie of the ER in a cell coexpressing the tail domain of XI-I. Overlays of the persistent tubules, cisternae and puncta (magenta) are also shown for comparison. Scale bar 10 μm.

Movie S5 | Persistency mapping of ER dynamics in a tobacco leaf epidermal cell expressing the myosin tail domain XI-C. Time lapse movie of the ER in a cell coexpressing the tail domain of XI-C. Overlays of the persistent tubules, cisternae and puncta (magenta) are also shown for comparison. Scale bar 10 μm.

Movie S6 | Persistency mapping of ER dynamics in a tobacco leaf epidermal cell expressing the myosin tail domain XI-E. Time lapse movie of the ER in a cell coexpressing the tail domain of XI-E. Overlays of the persistent tubules, cisternae and puncta (magenta) are also shown for comparison. Scale bar 10 μm.

Movie S7 | Persistency mapping of ER dynamics in a tobacco leaf epidermal cell expressing the myosin tail domain XI-K. Time lapse movie of the ER in a cell coexpressing the tail domain of XI-K. Overlays of the persistent tubules, cisternae and puncta (magenta) are also shown for comparison. Scale bar 10 μm.

Movie S8 | Persistency mapping of ER dynamics in a tobacco leaf epidermal cell expressing the myosin tail domain XI-1. Time lapse movie of the ER in a cell coexpressing the tail domain of XI-1. Overlays of the persistent tubules, cisternae and puncta (magenta) are also shown for comparison. Scale bar 10 μm.

Movie S9 | Comparison of ER dynamics in two tobacco leaf epidermal cells expressing relatively different levels of mRFP-XI-1 tail domain fusion. Fluorescent image highlighting mRFP-XI-1 tail fusion (magenta) and the effects on ER dynamics in the same cell are shown (green). Two representative movies from two cells show similar dynamics in cells with relatively different levels of myosin tail expression.

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ER and vacuoles: never been closer

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The endoplasmic reticulum (ER) represents the gateway for intracellular trafficking of membrane proteins, soluble cargoes and lipids. In all eukaryotes, the best described mechanism of exiting the ER is via COPII-coated vesicles, which transport both membrane proteins and soluble cargoes to the *cis*-Golgi. The vacuole, together with the plasma membrane, is the most distal point of the secretory pathway, and many vacuolar proteins are transported from the ER through intermediate compartments. However, past results and recent findings demonstrate the presence of alternative transport routes from the ER towards the tonoplast, which are independent of Golgi- and post-Golgi trafficking. Moreover, the transport mechanism of the vacuolar proton pumps VHA-a3 and AVP1 challenges the current model of vacuole biogenesis, pointing to the endoplasmic reticulum for being the main membrane source for the biogenesis of the plant lytic compartment. This review gives an overview of the current knowledge on the transport routes towards the vacuole and discusses the possible mechanism of vacuole biogenesis in plants.

Keywords: endoplasmic reticulum, COPII vesicles, Golgi apparatus, trans-Golgi network, multivesicular body, vacuole

ENDOPLASMIC RETICULUM: ENTRANCE TO THE SECRETORY PATHWAY

The endoplasmic reticulum (ER) consists on a network of interconnected membrane tubules and cisternae ("reticulum") stretching across the entire cytoplasm ("endoplasmic"). First discovered in culture cells from chicken embryos (Porter et al., 1945), the ER is present in all eukaryotic cells, and is the intracellular compartment where membrane proteins, soluble cargoes and lipids are synthesized. From the ER, correctly folded membrane and soluble proteins are transported to other endomembrane compartments or to the extracellular space along the secretory pathway (Vitale and Denecke, 1999). For all eukaryotes, the best characterized mechanism of exiting the ER is the COPII-mediated transport. The coat protein complex II (COPII) assembles on specific locations of the ER membrane, called ER-exit sites (ERES), from which COPII-coated vesicles bud off. The assembly of COPII begins with the activation of the small guanosine triphosphatase (GTPase) SAR1 provided by the ER membrane-bound guanine nucleotide exchange factor (GEF) SEC12, which leads to the coordinated recruitment of the cytosolic heterodimers SEC23/SEC24 and SEC13/SEC31 to the ERES (Nakano et al., 1988; Barlowe and Schekman, 1993; Barlowe et al., 1994). Cargo recognition is provided by SEC24 and SAR1, whereas multiple adjacent SEC13/SEC31 subcomplexes drive the bending of the ER membrane using the energy of GTP hydrolysis (Brandizzi and Barlowe, 2013). Passive incorporation of soluble cargoes into COPII vesicles can occur (Wieland et al., 1987; Denecke et al., 1990; Matsuoka and Nakamura, 1991; Phillipson et al., 2001; Thor et al., 2009), instead membrane proteins and receptors require di-acidic or di-hydrophobic motifs in their cytosolic domains for efficient transport (Kappeler et al., 1997; Nishimura and Balch, 1997; Contreras et al., 2004; Hanton et al., 2005). In mammals, most COPII subunits have one or more paralogs, which generate a robust repertoire of COPII-coated vesicles with tissue specificities and selectivity for different cargo molecules (reviewed in Zanetti et al., 2011). In plants much less is known about specificities among different COPII-coated carriers, even though it has been recently shown that the concomitant function of all three SEC24 members of *Arabidopsis* is necessary for the development of the gametophytes (Conger et al., 2011; Tanaka et al., 2013). After a long debate whether COPII vesicles versus COPII-coated tubules existed in plant cells, ultrastructural analysis of high-pressure frozen samples and 3D tomography reconstructions have shown that COPII vesicles are present also in plants (Ritzenthaler et al., 2002; Donohoe et al., 2007; Robinson et al., 2007; Kang and Staehelin, 2008).

LYTIC VACUOLES

The plant lytic vacuole can occupy up to 90% of the total volume in mature vegetative cells. Its remarkable size allowed Antonie van Leeuwenhoek to notice the vacuole already in the 1670s, at the dawn of microscopy. The name "vacuole" was coined from "vacuum," because Felix Dujardin, in 1872, thought he was facing an empty space (Leigh and Sanders, 1997; De, 2000). On the contrary, the vacuolar content can generate a stationary turgor pressure of up to five bars (Zimmermann et al., 1980), which provides the driving force for plants' growth by pushing the cells to expand in oriented directions. Moreover, the lytic vacuole plays a crucial role in pH homeostasis, storage of ions, degradation of cellular waste, defense against pathogens, and in buffering abiotic stresses. The rapid release from or uptake to the vacuolar lumen of ions and water allow plants to efficiently cope with diversified environmental challenges. The multiple roles of plant lytic vacuoles are regulated by the activity of transporters that use the energy of the electrochemical gradient generated across the tonoplast by the vacuolar H^+-ATPase (V-ATPase) and vacuolar H^+-PPase (V-PPase). Despite good knowledge of the biochemistry and function of the

vacuolar proton pumps (Maeshima, 2001; Schumacher and Krebs, 2010), little is known about the mechanisms of their sorting and the intracellular routes they follow to reach the tonoplast. However, recent data has shown that both the V-ATPase and V-PPase of *Arabidopsis* are incorporated to the tonoplast via a novel mechanism that also challenges the current model for vacuole biogenesis (Viotti et al., 2013).

GOLGI- AND POST-GOLGI-MEDIATED TRANSPORTS TO THE LYTIC VACUOLE

Tonoplast-resident proteins and vacuolar soluble cargoes are synthesized in the ER, many of them are delivered to the *cis*-side of the Golgi apparatus via COPII vesicles, and from the Golgi they proceed further through the secretory pathway (**Figure 1**; Pedrazzini et al., 2013; Xiang et al., 2013). Vesicle transport between endomembrane compartments is mediated by different effector molecules, among which are the Rab GTPases, that are members of the ras superfamily of regulatory GTPases (Rutherford and Moore, 2002). The dissection of distinct steps of vacuolar transport using nucleotide-deficient mutants of different Rab GTPases in tobacco leaf epidermis cells has shown that tonoplast-resident proteins might follow at least three different routes (Bottanelli et al., 2011). In agreement with this finding, it has been shown that the sucrose transporter SUC4 and the *myo*-inositol transporter INT1 of *Arabidopsis* are delivered to the tonoplast in an adaptor protein complex 3 (AP3)-dependent and -independent manner respectively (Wolfenstetter et al., 2012). AP complexes sort cargo proteins into coated vesicles, and AP3 is involved in vacuolar trafficking. The exact localization of AP3 in plants is uncertain, because this adaptor seems to interact with clathrin (Lee et al., 2007; Zwiewka et al., 2011), which is present at the *trans*-Golgi network (TGN; Kang et al., 2011), whereas SUC4 accumulates at the Golgi apparatus in protoplasts isolated from *ap3* mutant seedlings, suggesting a Golgi-derived vesicle transport (Wolfenstetter et al., 2012).

From the TGN other two clathrin-mediated vacuolar transport carriers have been proposed to exist. Each of them has an EPSIN N-TERMINAL HOMOLOGY (ENTH) protein, EPSIN1 or MTV1 respectively, that acts as a monomeric adaptor for clathrin recruitment. Both EPSIN1 and MTV1 localize to the TGN, and both the respective knock-out mutants show defects in vacuolar transport (Song et al., 2006; Sauer et al., 2013).

The last and best known carrier in the vacuolar branch of the secretory pathway is the multivesicular body (MVB), which is an independent organelle (Tse et al., 2004) that arises from the TGN through a maturation process that involves the function of the TGN-located vacuolar proton pump VHA-a1, the calcium dependent phospholipid binding protein ANNEXIN 3, and the ESCRT-machinery (Scheuring et al., 2011). As in yeast and mammals, plant membrane proteins destined for degradation due to physiological turnover are incorporated to the MVB's intraluminal vesicles through the function of the ESCRT complexes, and then released inside the vacuole via MVB-to-vacuole fusion (Reichardt et al., 2007; Spitzer et al., 2009; Viotti et al., 2010; Scheuring et al., 2011). Soluble vacuolar cargoes dissociate from vacuolar sorting receptors (VSRs) in an acidic environment (Kirsch et al., 1996), therefore this event might occur in the lumen of the TGN, which is the most acidic organelle among the intermediate compartments

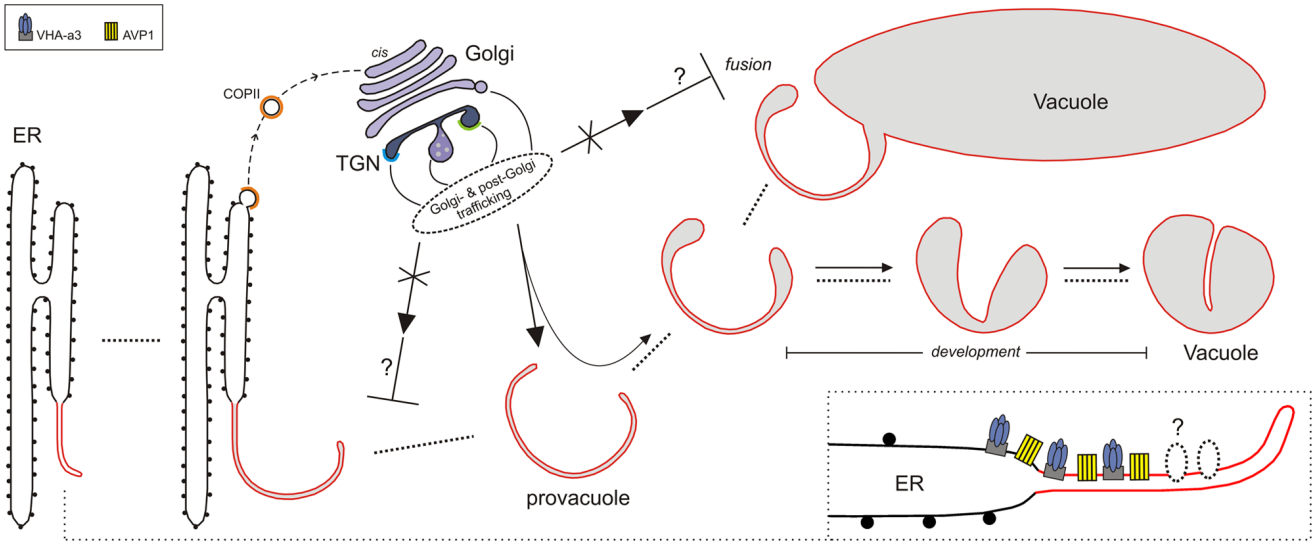

FIGURE 1 | Model for lytic vacuole biogenesis in *Arabidopsis*. The precursors of vacuoles, the provacuoles, arise from the endoplasmic reticulum (ER) via maturation. The vacuolar proton pumps VHA-a3 and AVP1 (and perhaps other tonoplast proteins) are incorporated to the nascent provacuole directly from the ER-membrane through an uncharacterized mechanism (lower right corner). Golgi- and post-Golgi trafficking (solid arrows) continuously contribute, during the different steps of vacuole formation (dotted lines), to the development of the vacuolar lumen via the delivery of soluble cargoes (e.g., acid proteases) and tonoplast-resident proteins that exit the ER with COPII-coated vesicles (dashed arrow). Provacuoles can also fuse with already developed vacuoles (*fusion*). Impairment of Golgi- and post-Golgi trafficking (crossed arrows) leads to aberrant multilayered provacuoles.This observation could be explained if impairment of Golgi- and post-Golgi trafficking would interfere (⊥) with the process of provacuoles-release from the ER, or with the fusion between provacuoles and vacuoles.

(Martinière et al., 2013; Shen et al., 2013). Soluble cargoes are then incorporated into the lumen of nascent MVBs, that arise from the TGN (Scheuring et al., 2011), for vacuolar transport. However, VSRs are localized both to the TGN and MVBs (Niemes et al., 2010; Stierhof and El Kasmi, 2010; Viotti et al., 2010), and the location from where they recycle is a matter of controversy (De Marcos Lousa et al., 2012; Robinson et al., 2012). An example of soluble cargo transported via MVBs is the cysteine protease aleurain (Miao et al., 2008), while regarding tonoplast resident proteins it has been recently shown that the auxin transporter WAT1 additionally co-localizes with the late endosomal marker RabG3f (Ranocha et al., 2013).

UNCONVENTIONAL ER-EXPORT OF PROTEINS TO THE VACUOLE

The conventional transport of proteins to the vacuole involves COPII-mediated ER-exit and the passage through several intermediate steps and compartments. Hence, the vacuole, together with the plasma membrane, may be seen as the most distal point of the secretory pathway.

However, it was shown by Gomez and Chrispeels (1993) that the tonoplast intrinsic protein α-TIP, unlike the soluble vacuolar protein phytohemagglutinin (PHA), can reach the lytic vacuole even after brefeldin A (BFA) or monensin treatment when transiently expressed in tobacco leaves, suggesting the presence of different vacuolar transport routes. Few years later, Jiang and Rogers (1998) showed that a chimera composed by the C-terminal domain of α-TIP fused to the transmembrane domain of the VSR BP80 reaches the protein storage vacuole (PSV) through a direct route from the ER. Evidence for an alternative mechanism of vacuolar trafficking was provided by the analysis of the calcineurin B-like (CBL) proteins, which are calcium sensors functioning in different locations within a cell (Batistič and Kudla, 2009). Among the ten members of *Arabidopsis*, CBL2, CBL3, CBL6 and CBL10 are targeted to the tonoplast in a COPII-independent manner, since overexpression of a dominant-negative mutant of SAR1did not interfere with their localization (Batistič et al., 2010). Moreover, it was shown that CBL6 is transported to the vacuole bypassing both the Golgi and post-Golgi compartments (Bottanelli et al., 2011). CBL proteins, however, seem not to enter the secretory pathway, but are rather synthesized in the cytosol and delivered to the tonoplast due to the presence of a tonoplast targeting signal (TTS) in their N-terminal domain (Bottanelli et al., 2011; Batistič et al., 2012; Tang et al., 2012). An example of soluble cargo transported to the plant vacuole through an unconventional route is the human α-mannosidase MAN2B1, which still reaches the vacuolar lumen even upon BFA treatment when transiently expressed in tobacco leaf mesophyll protoplasts (De Marchis et al., 2013).

The most abundant tonoplast resident protein, the vacuolar H^+-ATPase VHA-a3, is transported to the vacuole through a novel mechanism. By blocking COPII-mediated transport via BFA treatment of GNL1 BFA-sensitive *Arabidopsis* seedlings (Richter et al., 2007), VHA-a3 was not retained in the ER and was detected as normal at the tonoplast, whereas the TGN-located H^+-ATPase VHA-a1 was efficiently retained in the endoplasmic reticulum, indicating that VHA-a3 exits the ER in a COPII-independent manner (Viotti et al., 2013). Interestingly, while the N-terminal domain of the a1 subunit carries a typical di-acidic motif (EE--D) for COPII-mediated export, in those of the a2 and a3 isoforms there is none. Moreover, in a β-AP3 knock-out mutant (Feraru et al., 2010) VHA-a3 was detected as normal at the tonoplast (Viotti et al., 2013), and its transport was not stopped at the level of intermediate compartments by using the post-Golgi-transport inhibitor concanamycin A (ConcA). Similarly, the second *Arabidopsis* vacuolar proton pump, the H^+-PPase AVP1, did not accumulate to the Golgi/TGN interface upon ConcA treatment, and it did not localize to the limiting membrane of MVBs (Viotti et al., 2013). In other words, none of the known Golgi- and post-Golgi trafficking routes seemed to be involved in the delivery of the two vacuolar proton pumps.

AVP1 was not only detected to the limiting membrane of rounded vacuoles, but also uniformly present on the membranes of lytic vacuole precursors, the provacuoles. Provacuoles display a much finer (down to 30 nm thickness) tubular network in provascular cells of the root meristem, they are acidic, they carry VHA-a3 too, can fuse with already-developed vacuoles, and are distinct structures respect to autophagosomes (Viotti et al., 2013).

How is AVP1 transported from the ER to the provacuole and where does the latter originate from? A hypothesis is provided in the last section on this review.

MECHANISMS OF VACUOLE BIOGENESIS

Relatively little is known about the biogenesis of vacuoles in plants. Even the donor membrane from where newly formed vacuoles originate from is unclear. The model that boasts most of the credits in text books suggests that newly formed lytic vacuoles in root-tip cells originate from post-Golgi-derived vesicles (Marty, 1999; De, 2000; Robinson and Rogers, 2000). These vesicles would homotypically fuse to form tubular structures that represent the precursors of vacuoles, the provacuoles. The tubular provacuoles are supposed to fuse with one another, forming a complex network that finally will give raise to the central vacuole (Marty, 1999). This hypothesis is based on an early electron microscopy study that revealed tubular structures at the *trans*-side of the Golgi apparatus which were strongly electrondense after incubation with sodium β-glycerophosphate or cytidine 5′-monophosphate (Marty, 1978). These two compounds serve as substrates to detect acid phosphatase and thiolacetic acid esterase activity respectively, thus they were used as biochemical markers to highlight acidic compartments. The tubular-vesicular structure at the *trans*-side of the Golgi was named Golgi-associated endoplasmic reticulum (GERL; Marty, 1978), and later it was renamed as TGN (Griffiths and Simons, 1986). Due to its acidic intraluminal pH, the TGN was proposed to represent the donor membrane for the biogenesis of the lytic vacuole (Marty, 1999).

A few years ago it was shown in *Arabidopsis* that the vacuolar H^+-ATPase localizes also to the TGN, thus it is not a purely "vacuolar" enzyme. The V-ATPase (VHA) is a holoenzyme composed by two subcomplexes: the membrane-integral complex V_0, and the cytosolic complex V_1, both composed by multimeric subunits (Schumacher and Krebs, 2010). The subcellular localization depends on which isoform of the "a" subunit is incorporated in the V_0 subcomplex. Enzymes incorporating a1 are exclusively located to the TGN, instead enzymes incorporating either a2 or a3 localize

to the tonoplast (Dettmer et al., 2006). The presence of VHA-a1 at the TGN contributes to the acidification of tubules at the *trans*-side of the Golgi, and this might explain Marty's (1978) data. However, conclusive experimental evidence to unequivocally prove that the TGN represents the donor membrane for the biogenesis of the vacuole is lacking up to date.

THE ER IS THE MAIN MEMBRANE SOURCE FOR VACUOLE BIOGENESIS

Immunogold electron microscopy found AVP1-positive provacuoles directly connected to the ER, and immuno-fluorescent *in situ* visualization of sterols showed that ER-export of newly formed membranes can be COPII-independent in *Arabidopsis* roots (Viotti et al., 2013).These data point to the presence of an unknown mechanism in the endoplasmic reticulum that incorporates VHA-a3 and AVP1 to the provacuolar membrane that arises from the ER (**Figure 1**). It is important to mention that this mechanism could not occur anymore in fully mature cells where the growth of the central vacuole and the turnover of tonoplast resident proteins would involve only Golgi- and post-Golgi trafficking.

The hypothesis that the ER was the membrane source for the biogenesis of the vacuole was already proposed decades ago by Matile and Moor (1968) after ultrastructural analysis via freeze-etching of *Zea mays* seedlings, and one year later Mesquita (1969) published intriguing electron-micrographs showing connections between vacuoles and the rough ER in *Lupinus albus* roots. During the 1980s, other studies reproposed the ER to be the donor compartment for vacuolar biogenesis (Amelunxen and Heinze, 1984; Hilling and Amelunxen, 1985), nevertheless the absence of immunocytochemistry in these old works did not allow an univocal determination of structures' identity.

The *Arabidopsis* gene *VACUOLESS 1* (*VCL1*) is crucial for vacuole development, since embryo and suspensor cells in the *vcl1* knock-out mutant do not develop vacuoles and the mutant is embryo-lethal (Rojo et al., 2001). Interestingly in this study the authors reported the presence of a high number of auotophagosome-like structures in the embryo cells, which could have been, at least partially, provacuoles. From an ultrastructural point of view provacuoles appear indeed similar to autophagosomes. A recent study nicely depicted autophagosomes at the ultrastructural level for the first time in plants (Zhuang et al., 2013). Provacuoles and autophagosomes seem to be distinct entities, since the former were normally found in *atg2*, *atg5*, and *atg7* knock-out mutants, which lack these key players for the formation of the phagophore (Viotti et al., 2013). Since immunocytochemistry of AVP1 did not label several ring-like structures either in wild-type or in *pat2* seedlings (**Figure 2A**), this data point to the presence of distinct populations of circular double-bilayered membranes in the root meristem of *Arabidopsis*. It is likely that provacuoles and autophagosomes might at some point fuse, both contributing to the development of the vacuole (**Figure 2B**).

Several Golgi- and post-Golgi-trafficking mutants, such as *pat2*, *vps45*, and *amsh3*, display alterations in vacuole morphology (Zouhar et al., 2009; Feraru et al., 2010; Isono et al., 2010), and in all these mutants provacuoles were still present albeit often with aberrant profiles (Viotti et al., 2013). The Golgi and the TGN do

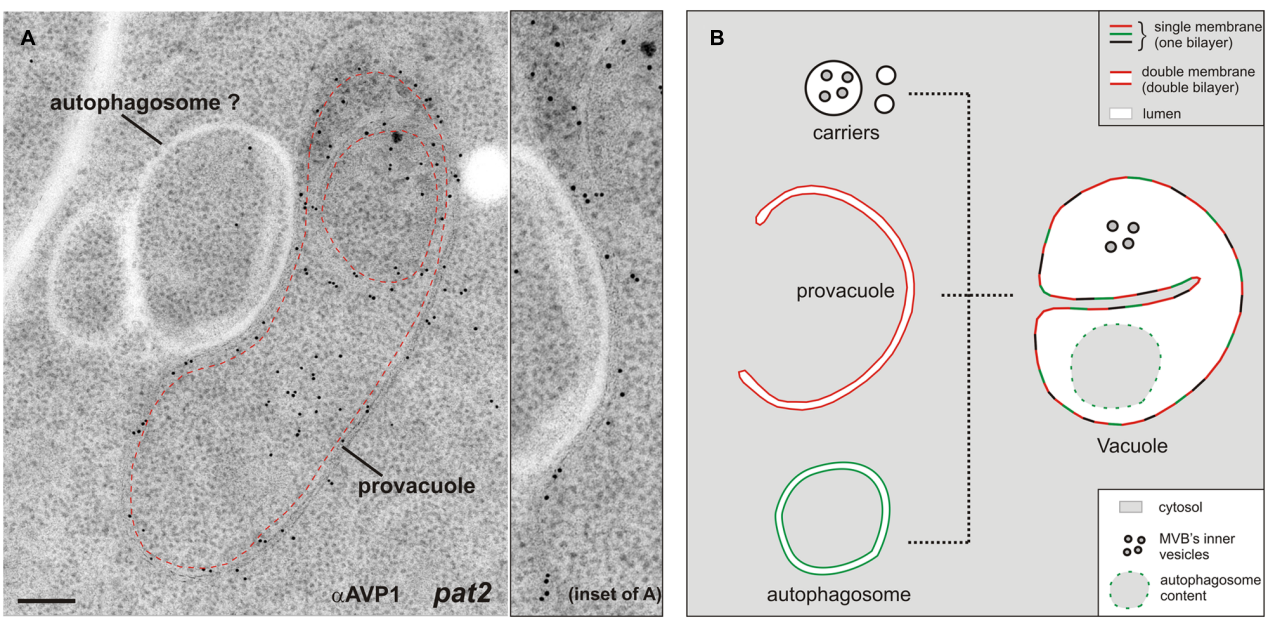

FIGURE 2 | (A) Immunogold labeling on ultra-thin sections of high-pressure frozen, freeze-substituted, and HM20-embedded *Arabidopsis pat2* root-tip cells shows a provacuole (dashed red line) carrying the V-PPase AVP1. Adjacent to the provacuole, a ring-like structure which is not labeled by the antibody (αAVP1) could be an autophagosome. This picture shows that at least two distinct populations of circular/semicircular double-bilayered structures existin *Arabidopsis* root-tip cells. Scale bar, 200 nm. **(B)** The development of mature vacuoles is probably the result of fusion events between provacuoles, autophagosomes and vacuolar transport carriers.

not seem to be the donor compartments for provacuole formation, but seem to be required for a rapid and efficient development of the vacuolar lumen, and for the delivery of some, although not all, tonoplast resident proteins. The presence of multilayered provacuoles in the *pat2* and *vps45* mutants suggests that an impaired Golgi- and post-Golgi-trafficking could affect the release of provacuoles from the ER, or the fusion between provacuoles and vacuoles, with the result of a proliferation of membranes that at some point start to curl concentrically forming multilayered compartments.

The incorporation of VHA-a3 and AVP1 already in nascent provacuoles at the ER could be explained through the immediate necessity of acidification of the lumen, which is a key feature of vacuolar activities. The molecular players involved in this putative mechanism of sorting and biogenesis are unknown. The functions of VHA-a2, VHA-a3 and AVP1 seem not to be required, since provacuoles and vacuoles are normally present in *vha-a2/vha-a3* and *avp1* mutants (Viotti and Schumacher, unpublished data). Since α-TIP transport in tobacco leaf cells was shown to be BFA-insensitive (Gomez and Chrispeels, 1993) and can be blocked by SEC12 overexpression (Bottanelli et al., 2011), it cannot be excluded that α-TIP follows the same route of VHA-a3 and AVP1, with SEC12 playing an additional role in this process. While we propose that newly formed lytic vacuoles arise from the ER, a subpopulation might originate from the conversion of protein storage vacuoles (PSVs) when seeds start to germinate (Zheng and Staehelin, 2011). Interestingly, direct transport from the ER to PSVs was reported in pumpkin cotyledons and seeds, where precursors-accumulating vesicles (PAC) arise from the endoplasmic reticulum and are delivered to PSVs (Hara-Nishimura et al., 1998). It is tempting to imagine PACs (diameter of 200–400 nm) being the precursors of PSVs, that slowly acquire their final size and identity via Golgi- and post-Golgi-mediated transport, as we have proposed to happen between provacuoles and lytic vacuoles. The idea that membraneous sheets (as provacuoles look like) arise from the ER (that also has similar structures, the cisternae) appears more reasonable in terms of geometry. One of the elements that could contribute to the formation of provacuoles is the different lipid composition of the nascent membrane compared to that of the ER. Theoretically, the clustering in discrete domains of one or more specific kind of lipids could drive the maturation of an organelle from another one. This could also be the case for the maturation of MVBs, which are enriched in phosphatidylinositol-3-phosphate (PI3P), while the Golgi and TGN mainly have PI4P (Vermeer et al., 2006; Vermeer et al., 2009).

More and more evidence is accumulating for direct ER-to-vacuole transport, and those that were supposed to be the "farest" intracellular compartments in plant cells could be, although briefly in time and space, even physically attached.

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Endoplasmic reticulum-mediated protein quality control in *Arabidopsis*

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A correct three-dimensional structure is crucial for the physiological functions of a protein, yet the folding of proteins to acquire native conformation is a fundamentally error-prone process. Eukaryotic organisms have evolved a highly conserved endoplasmic reticulum-mediated protein quality control (ERQC) mechanism to monitor folding processes of secretory and membrane proteins, allowing export of only correctly folded proteins to their physiological destinations, retaining incompletely/mis-folded ones in the ER for additional folding attempts, marking and removing terminally misfolded ones via a unique multiple-step degradation process known as ER-associated degradation (ERAD). Most of our current knowledge on ERQC and ERAD came from genetic and biochemical investigations in yeast and mammalian cells. Recent studies in the reference plant *Arabidopsis thaliana* uncovered homologous components and similar mechanisms in plants for monitoring protein folding and for retaining, repairing, and removing misfolded proteins. These studies also revealed critical roles of the plant ERQC/ERAD systems in regulating important biochemical/physiological processes, such as abiotic stress tolerance and plant defense. In this review, we discuss our current understanding about the molecular components and biochemical mechanisms of the plant ERQC/ERAD system in comparison to yeast and mammalian systems.

Keywords: endoplsamic reticulum-associated degradation, *Arabidopsis*, endoplasmic reticulum-mediated quality control, misfolded glycoproteins, receptor-like kinases

INTRODUCTION

It is well known that the proper function of a protein strictly depends on its native conformation, but protein folding is a fundamentally error-prone process. The endoplasmic reticulum (ER) is the cellular port of entry for secretory and membrane proteins to enter the secretory pathway and is a folding compartment for proteins to attain their native conformations through interactions with molecular chaperones, sugar-binding lectins, and folding enzymes (Gidalevitz et al., 2013). Misfolded proteins not only lead to functional deficiency but also induce dominant-negative and cellular toxicity effects, and it is thus essential that the ER should possess several highly stringent protein quality control mechanisms to closely monitor the folding process, allowing export of only correctly folded proteins to their final destinations but retaining incompletely/mis-folded proteins for additional rounds of chaperone-assisted folding. A high-efficient ER-mediated protein quality control (ERQC) system can also differentiate terminally misfolded proteins from folding intermediates and/or reparable misfolded proteins, stopping the futile folding cycles of the former proteins and eliminating them via a multistep degradation process widely known as ER-associated degradation (ERAD) that involves ubiquitination, retrotranslocation, and cytosolic proteasome (Smith et al., 2011). Our current understanding of the eukaryotic ERQC/ERAD system derived largely from studies in yeast and mammalian cells. However, recent genetic, biochemical, and cell biological studies in the reference plant *Arabidopsis thaliana* and other model plant species not only identified homologous ERQC/ERAD components but also revealed evolutionarily conserved features as well as unique aspects of the plant ERQC/ERAD mechanisms (Hong and Li, 2012; Huttner and Strasser, 2012; Howell, 2013), especially their connections with the stress tolerance and plant defense pathways.

N-GLYCAN-BASED ER RETENTION MECHANISM

Many secretory and membrane proteins are co-translationally glycosylated when entering the ER (Aebi, 2013). The so-called N-linked glycosylation occurs on the asparagine (Asn or N) residues within the Asn-X-Ser/Thr sequons (X indicating any amino acid except proline while Ser/Thr denoting serine/threonine residue) of a nascent polypeptide. This reaction is catalyzed by the enzyme oligosaccharyltransferase (OST), an integral membrane protein complex that transfers a preassembled oligosaccharide precursor $Glc_3Man_9GlcNac_2$ (Glc, Man and GlcNac denoting glucose, mannose and *N*-acetylglucosamine, respectively) from a membrane-anchored dolichylpyrophosphate (DolPP) carrier to the Asn residue (**Figure 1**; Mohorko et al., 2011). The assembly of $Glc_3Man_9GlcNac_2$ involves a series of highly specific asparagine-linked glycosylation (ALG) proteins that sequentially add a monosaccharide onto the DolPP linker or a DolPP-linked oligosaccharide precursor (Aebi, 2013; **Figure 1A**). The structure of a N-linked glycan plays an important role in the protein folding and quality control (Aebi et al., 2010). Immediately after transferring of $Glc_3Man_9GlcNac_2$ to an Asn residue,

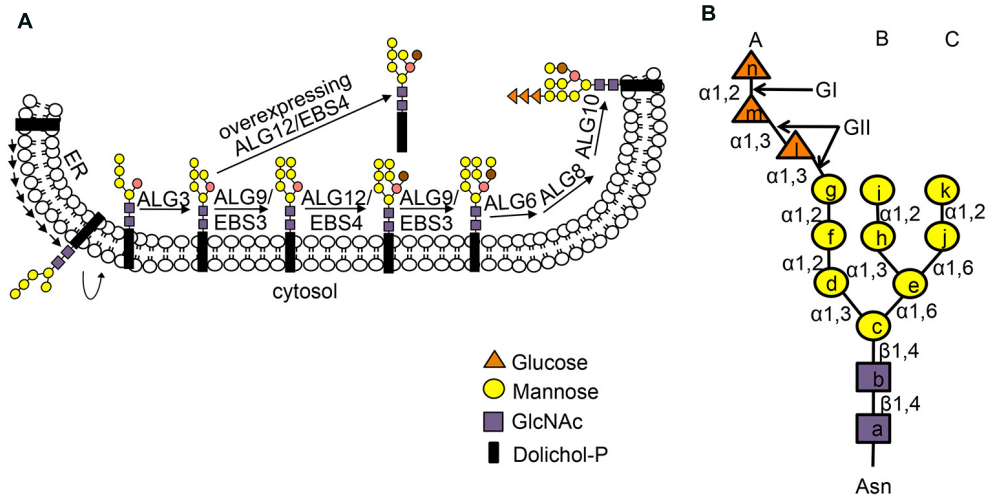

FIGURE 1 | (A) Stepwise assembly of *N*-glycan precursor on the ER membrane. The assembly of the *N*-glycan precursor starts at the cytosolic face of the ER membrane by adding two GlcNAc and five Man residues to the membrane-anchored Dol-PP linker. The resulting Dol-PP-$Man_5GlcNAc_2$ flips over into the ER lumen. Four Man residues are sequentially added to the flipped Dol-PP-$Man_5GlcNAc_2$ by three mannosyltransferases, ALG3, ALG9 (known as EBS3 in *Arabidopsis*) and ALG12 (known as EBS4 in *Arabidopsis*) with the ALG9 catalyzing two reactions of adding the terminal α1,2 Man residues on the middle and right branches. Three Glc residues are subsequently added to the right branch, generating the 14-sugar precursor, Dol-PP-$Glc_3Man_9GlcNAc_2$. Two α1,6 Man residues are marked by brown and salmon color. The Dol-PP linker and different sugar residues are indicated. **(B)** The structure of N-linked $Glc_3Man_9GlcNAc_2$ glycan with three dimannose branches (branch A, B and C). Lower case letters inside sugar residues represent the order of sugar addition. The sugar linkage bonds and enzymes (GI, GII) that remove the three Glc residues are indicated. Figure adapted from Hong et al. (2009).

the terminal and middle Glc residues are removed sequentially by glucosidase I (GI) and glucosidase II (GII), producing a monoglucosylated *N*-glycan, $GlcMan_9GlcNac_2$, which is recognized by the ER chaperone-like lectins, a membrane-anchored calnexin (CNX) and its ER luminal homolog calreticulin (CRT; Caramelo and Parodi, 2008; **Figure 2**). The high-specificity high-affinity binding between $GlcMan_9GlcNac_2$ and CNX/CRT is crucial for folding a nascent polypeptide as CNX/CRT can recruit other ER-chaperones and folding enzymes, including protein disulfide isomerases (PDIs) essential for generating inter/intra-molecular disulfide bonds. The removal of the remaining Glc residue by GII releases the nascent glycoprotein from CNX/CRT, thus terminating its folding process (Caramelo and Parodi, 2008). If the protein folds correctly, it will be transported out of the ER to reach its final destination. However, if the protein fails to attain its native conformation, it will be recognized by UDP-glucose:glycoprotein glucosyltransferase (UGGT), an ER-resident folding sensor consisting of a large non-conserved N-terminal domain presumably involved in recognizing non-native conformations and a smaller highly conserved C-terminal catalytic domain capable of catalyzing a glucosyltransferase reaction using uridyl diphosphate-glucose (UDP-Glc) as a substrate (D'Alessio et al., 2010). As a result, a single Glc is added back to deglucosylated *N*-glycans of the incompletely/mis-folded protein, permitting its reassociation with CNX/CRT and their associated proteins for another round of assisted folding. The alternate reactions of GII and UGGT drive many cycles of dissociation and reassociation of CNX/CRT with an incompletely/mis-folded glycoprotein [widely known as the CNX/CRT cycle (Hammond et al., 1994)], till the protein attains its native conformation (**Figure 2**). It is worthy to mention that the budding yeast (*Saccharomyces* cerevisiae), which is widely used for studying the ERAD process, lacks the CNX/CRT-UGGT system due to the presence of a catalytically inactive UGGT homolog (Meaden et al., 1990).

The *Arabidopsis* genome encodes only one UGGT homolog, and its physiological function was inadvertently found in a study for identifying additional signaling proteins of the plant steroid hormones, brassinosteroids (BR; Jin et al., 2007). A genetic screening for extragenic suppressors of an *Arabidopsis* dwarf mutant *brassinosteroid-insensitive 1-9* (*bri1-9*) led to the discovery of *Arabidopsis* UGGT (also known as EBS1 for EMS mutagenized bri1 suppressor 1; Jin et al., 2007). BRI1 is a cell surface-localized leucine-rich-repeat receptor-like-kinase that function as a BR receptor and contains a single transmembrane domain and 14 putative N-glycosylation sites in its N-terminal extracellular domain (Li and Chory, 1997). The mutant bri1-9, carrying a Ser662-Phe mutation in the BR-binding domain (Noguchi et al., 1999), was found to be retained in the ER by an EBS1/AtUGGT-dependent mechanism and subsequently degraded by a plant ERAD process (Jin et al., 2007; Hong et al., 2009). Loss-of-function mutations in EBS1/AtUGGT compromise such an ER-retention mechanism and allow some bri1-9 proteins to escape from the ER to reach the plasma membrane, resulting in phenotypic suppression of the dwarfism of the *bri1-9* mutant. The same genetic screen also identified CRT3 (Jin et al., 2009), a unique member of the *Arabidopsis* CNX/CRT family consisting of two CNXs and three CRTs, which actually retains bri1-9 via the CRT3-$GlcMan_9GlcNac_2$ binding. Both UGGT and CRT3 were also identified from two other independent genetic

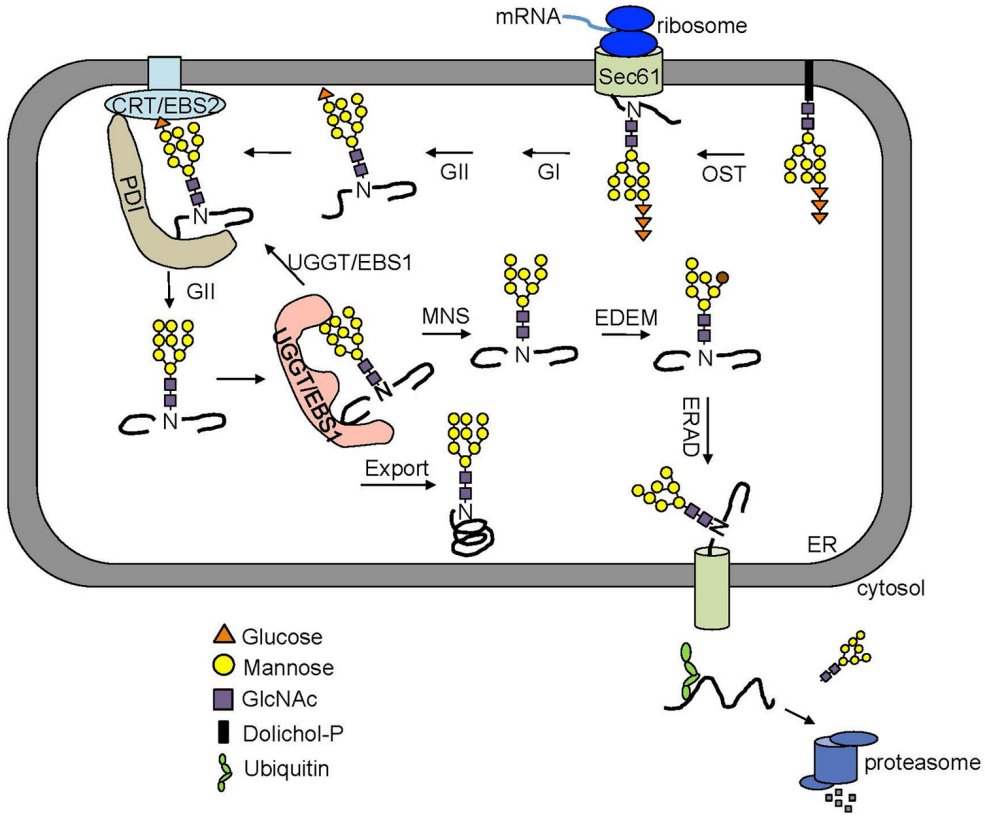

FIGURE 2 | An overview of the ERQC/ERAD system. Two Glc residues on the *N*-glycan of a nascent polypeptide are rapidly trimmed by GI and GII right after being transferred from the Dol-PP-linker. The resulting monoglucosylated *N*-glycans bind the two ER lectins CNX and CRT chaperone-assisted folding. The removal of this last Glc by GII releases a mature polypeptide from CNX/CRT. A correctly folded protein can leave the ER while an incompletely/mis-folded glycoprotein is recognized by UGGT (known as EBS1 in *Arabidopsis*) that adds back a Glc residue to the A branch, permitting its reassociation with CNX/CRT. A glycoprotein that fails to gain its native structure within a certain time window is removed from the folding cycle via sequential trimming of the two terminal α1,2 Man residues of the B and C branch by MNS1 (an ER-localized α1,2-Mannosidase, known as MNS3 in *Arabidopsis*) and Htm1/EDEM. A terminally misfolded glycoprotein with α1,6 Man-exposed glycan is selected to enter the ERAD pathway.

screens aiming to identify key regulators of the plant innate immune response to a bacterial translational elongation factor EF-Tu (Li et al., 2009; Saijo et al., 2009). Interestingly, while loss-of-function mutations in AtUGGT/CRT3 led to regaining partial sensitivity to BRs, *atuggt/crt3* mutants were insensitive to elf18, a biologically active epitope of EF-Tu. Further studies showed that both UGGT and CRT3 are absolutely required for the correct folding of EFR (EF-Tu Receptor; Saijo, 2010), a BRI1-like receptor-like kinase that binds elf18/EF-Tu to initiate a plant defense process (Zipfel et al., 2006). The importance of *N*-glycan-mediated folding control was further supported by discoveries that loss-of-function mutations in STT3A, a OST subunit, and GII resulted in significant reduction of the EFR protein abundance, presumably caused by incomplete folding and subsequent degradation (Lu et al., 2009; Haweker et al., 2010; von Numers et al., 2010).

In addition to the glycan-dependent ER retention system, the ER is equipped with additional retention systems to prevent export of misfolded proteins, especially those non-glycosylated ones. One system uses the family of ER-localized HSP70 proteins (known as BiPs), which have a N-terminal ATP-binding domain and a C-terminal substrate-binding domain that recognizes and binds to exposed hydrophobic patches of incompletely/mis-folded proteins in an ATP-dependent manner (Buck et al., 2007). The *Arabidopsis* has three BiP homologs, AtBiP1, AtBiP2 and AtBiP3, all of which were known to exhibit higher levels of gene expression under ER stresses (Sung et al., 2001). In *Arabidopsis*, BiPs were shown to bind both bri1-9 and bri1-5, another mutant variant of BRI1 carrying a Cys69Tyr mutation that destroys a disulfide bridge crucial for the structural integrity of the BR receptor, and were thought to contribute for the ER retention of the two mutant BR receptors (Jin et al., 2007; Hong et al., 2008). BiPs and their associated factors ERdj3B (an *Arabidopsis* ER-localized DNAJ homolog) and SDF2 (the *Arabidopsis* homolog of the murine stromal cell-derived factor 2) are also involved in the biogenesis/folding control of EFR (Nekrasov et al., 2009). BiPs were also known to interact with the orphan heavy chain of a murine IgG1 antibody or an assembly defective form of the trimeric vacuolar storage protein phaseolin in transgenic tobacco plants (Pedrazzini et al., 1997; Nuttall et al., 2002). Another glycan-independent ER retention mechanism relies on mixed disulfide bridges between incompletely/mis-folded

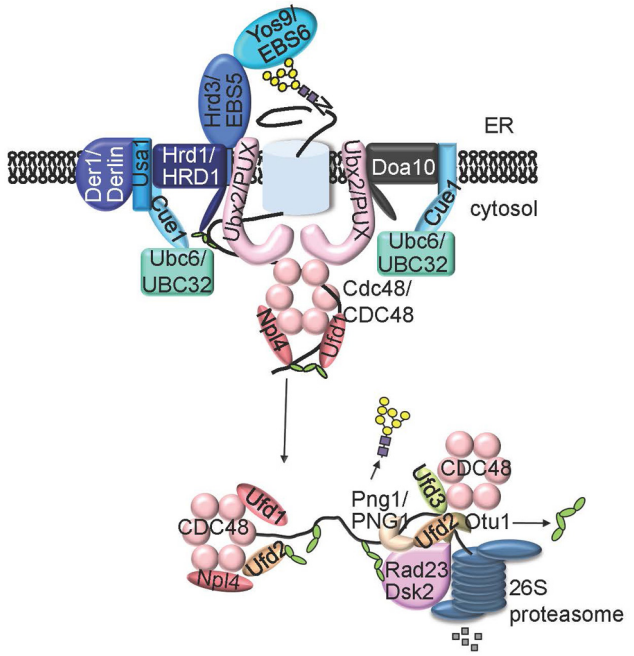

FIGURE 3 | A model of ERAD system. An ERAD client that has lesion in membrane-embedded segment ($ERAD_M$) or in ER lumen region ($ERAD_L$) is recruit by Hrd3 (EBS5 in *Arabidopsis*) and Yos9 (EBS6/OS9 in *Arabidopsis*) to the membrane-anchored E3 ligase Hrd1 (AtHrd1A and AtHrd1B in *Arabidopsis*) complex that also contains Cue1, Ubc6 (UBC32 in *Arabidopsis*), Usa1 and Der1. An ERAD substrate with a folding lesion in the cytosolic domain ($ERAD_C$) is recruited to a different ER membrane-anchored E3 ligase complex that contains Doa10 and Ubc6 (or Cue1/Ubc7). The two E3 ligase complexes share similar components on the cytosolic side of the ER membrane, including the substrate-extracting Cdc48-Ufd1-Npl4 trimeric complex and its membrane recruitment factor Ubx2 (one or more AtPUX proteins in *Arabidopsis*). An extracted polyubiquitinated ERAD substrate is processed by Ufd2, Ufd3, Otu1, and/or Png1 (AtPNG1 in *Arabidopsis*), delivered to the cytosolic proteasome with help of Dsk2 and Rad23, and eventually proteolyzed by the 26S proteasome.

proteins with PDIs and related ER-localized oxidoreductases (Reddy et al., 1996; Anelli et al., 2003, 2007). The *Arabidopsis* genome encodes 13 PDI-like proteins (Houston et al., 2005), none of which has been implicated in retaining misfolded proteins. However, a recent study on bri1-5 carrying an orphan cysteine residue (Cys62) suggested involvement of a thiol-mediated retention system in keeping the mutant BR receptor in the ER (Hong et al., 2008). Further biochemical studies are needed to verify this prediction and to identify one or more PDIs that form the predicted mixed disulfide bridge with the orphan Cys62 residue.

MARKING OF A TERMINALLY MISFOLDED GLYCOPROTEIN FOR ERAD

A protein that fails to attain its native conformation within a given time window is eliminated by ERAD (Vembar and Brodsky, 2008). One of the key events in ERQC is to terminate a futile folding cycle and to deliver an irreparable misfolded protein into the ERAD pathway. Although little is known about the marking mechanism for irreparable non-glycosylated ERAD clients, recent studies indicated that removal of the terminal α1,2-Man residue from the C-branch of *N*-glycan (**Figure 2**), catalyzed by homologous to mannosidase 1 (Htm1) in yeast and ER-degradation enhancing α-mannosidase-like proteins (EDEMs) in mammals, is required for generating the ERAD signal, an exposed α1,6 Man residue on an N-linked glycan (Quan et al., 2008; Clerc et al., 2009; **Figure 1B**). The *Arabidopsis* genome encodes at least two homologs of the yeast Htm1/mammalian EDEMs [known as MNS4 and MNS5 (Liebminger et al., 2009)], but their involvement in a plant ERAD process awaits functional investigation. Nevertheless, recent genetic screening for *Arabidopsis* mutants defective in ERAD of bri1-5/bri1-9 and subsequent molecular cloning and biochemical studies indicated that the glycan ERAD signal is well conserved in plants (Hong et al., 2009, 2012). Loss-of-function mutations in either EBS3 and EBS4 (homologs of the yeast/mammal ALG9 and ALG12, respectively) prevent complete assembly of the *N*-glycan precursor (**Figure 1**), resulting in glycosylation of the two ER-retained mutant BR receptor with truncated *N*-glycan lacking the α1,6-Man residue that would function as the ERAD signal and consequential inhibition of ERAD of bri1-5/bri1-9. In contrast, forcing the addition of the missing α1,6 Man residue to Dol-PP-$Man_6GlcNAc_2$ by over-expression of EBS4/ALG12 in an *Arabidopsis ebs3/alg9 bri1-9* mutant promoted the ERAD of bri1-9 (**Figure 1A**; Hong et al., 2012). Similarly, the ERAD of bri1-9 was presumably accelerated when its N-linked glycans carried a different exposed α1,6 Man residue (the inner α1,6 Man; Hong et al., 2012) caused by a loss-of-function mutation in ALG3 that adds an α1,3 Man to the inner α1,6-Man (Henquet et al., 2008; Kajiura et al., 2010; **Figure 1A**). The exposed inner α1,6 Man residue was shown to function as an alternative ERAD signal in both yeast and mammalian cells (Clerc et al., 2009; Hosokawa et al., 2009).

RECRUITMENT OF ERAD SUBSTRATES

The *N*-glycan ERAD signal is decoded by one or two ER luminal lectins, osteosarcoma 9 (OS9, also known as Yos9 in yeast) and XTP3-B (Yoshida and Tanaka, 2010; **Figure 3**). Yos9 and its mammalian homologs contain the mannose-6-phosphate receptor homology (MRH) domain that specifically recognizes and binds *N*-glycans with an exposed α1,6 Man residue (Hosokawa et al., 2010). In addition to OS-9/Yos9, selection of an ERAD client requires another ER resident protein, Hrd3 (HMG-CoA reductase degradation 3) in yeast and Sel1L (Suppressor of lin-12-Like) in mammals (Hirsch et al., 2009), a type I transmembrane protein with a large ER luminal domain consisting of multiple copies of the tetratricopeptide repeat motif. It was believed that Hrd3/Sel1L, exhibiting high affinity binding to exposed hydrophobic amino acid residues on misfolded proteins, makes the initial selection of a potential ERAD client, which is subsequently inspected by OS-9/Yos9 for the presence of an *N*-glycan ERAD signal (Denic et al., 2006; Gauss et al., 2006; **Figure 3**). Such a bipartite ERAD signal of a misfolded domain plus an α1,6-Man-exposed *N*-glycan ensures degradation of only terminally misfolded glycoproteins but not folding

intermediates carrying *N*-glycans with no exposed α1,6 Man residue.

The *Arabidopsis* genome has two *Hrd3/Sel1L* homologous genes, *AtSel1A* (also known as *EBS5 or HRD3A* or) and *AtSel1B* (also known as *HRD3B,* an apparent pseudogene) and just one *OS9/Yos9* homolog, *AtOS9* (also known as *EBS6*; Liu et al., 2011; Su et al., 2011, 2012; Huttner et al., 2012). AtSel1A/EBS5 complemented the ERAD-defect of the yeast Δ*hrd3* mutant assayed by ERAD of a mutant variant of carboxypeptidase Y (CPY*; Su et al., 2011), a commonly used ERAD substrate for many ERAD studies in yeast. By contrast, AtOS9 failed to rescue the defective ERAD of CPY* when expressed in a Δ*yos9* yeast strain (Huttner et al., 2012). Interestingly, a chimeric AtOS9-Yos9 protein consisting of the full-length AtOS9 and the Yos9's C-terminal region (amino acids of 277–542) promoted CPY* degradation in Δ*yos9* yeast cells (Huttner et al., 2012), suggesting that the MRH domain is interchangeable but the Yos9's C-terminal domain might be crucial for interacting with other components of the yeast ERAD machinery. Loss-of-function mutations in either AtSel1A/EBS5 or AtOS9/EBS6 inhibit ERAD of bri1-5, bri1-9, misfolded EFR (in an *ebs1/uggt* mutant background), and/or the transgenically expressed MLO-1 (Liu et al., 2011; Su et al., 2011, 2012; Huttner et al., 2012), a mutant variant of barley powdery resistance o (MLO) that carries a single amino acid change in the cytoplasmic region and was previously shown to be an ERAD substrate (Muller et al., 2005). As expected, AtSel1A/EBS5 and AtOS9/EBS6 physically interacted with bri1-9 or bri1-5 in a tobacco transient expression system or an *in vitro* pull-down assay (Huttner et al., 2012; Su et al., 2012). Consistent with what was known in yeast and mammalian cells, AtSel1A/EBS5 binds AtOS9/EBS and seems to be required for maintaining the stability of AtOS9/EBS6 (Huttner et al., 2012; Su et al., 2012). These results strongly suggested that the selection mechanism for a terminally misfolded glycoprotein for ERAD is conserved in *Arabidopsis.* It is important to point out that *Arabidopsis* mutants of AtSel1A/EBS6 or AtOS9/EBS6 are hypersensitive to NaCl-induced salt stress, suggesting a relationship between a cellular stress response and an environmental stress pathway (Liu et al., 2011; Huttner et al., 2012). It is quite possible that environmental stresses lead to decreased folding efficiency and increased accumulation of misfolded proteins in the ER, which require a highly efficient ERAD system for their removal to maintain ER homeostasis.

UBIQUITINATION OF CHOSEN ERAD CLIENTS

Hrd3/Sel1L and Yos9/OS9 not only select irreparable misfolded glycoproteins but also bring the chosen ERAD substrates to the membrane-anchored ERAD complexes responsible for ubiquitination and retrotranslocation. The central component of these ERAD complexes is a polytopic membrane protein with a RING finger-type ubiquitin ligase (E3) activity exposed to the cytosolic surface of the ER membrane, which not only ubiquitinates ERAD substrates but also connects to various ER luminal/cytosolic adapters (Hirsch et al., 2009). Yeast contains at least two distinct E3 ligases, 6 transmembrane-spanning Hrd1 (HMG-CoA reductase degradation) and 14-transmembrane-spanning Doa10 (Degradation of alpha2), that ubiquitinate three different types of ERAD substrates differing in the location of folding lesions: $ERAD_L$ (lesion in the ER luminal area), $ERAD_M$ (lesion in the transmembrane segment), and $ERAD_C$ (lesion in the cytosolic domain; Vashist and Ng, 2004; Carvalho et al., 2006). The Hrd1 complex ubiquitinates $ERAD_{L/M}$ substrates while the Doa10 complex deals with $ERAD_C$ clients. Mammals have at least 9 membrane-bound ERAD E3 ligases (Olzmann et al., 2013), including two Hrd1 homologs (HRD1 and gp78), one Doa10 homolog (TEB4), and several other RING-type E3 ligases such as RING membrane-anchor 1 (RMA1; Younger et al., 2006), whose founding member was initially discovered in *Arabidopsis* (Matsuda and Nakano, 1998).

The *Arabidopsis* genome encodes two Hrd1 homologs (AtHrd1A and AtHrd1B; Su et al., 2011; Huttner and Strasser, 2012), at least two Doa10 homologs (Doa10A/At4g34100 and Doa10B/At4g32670; Liu et al., 2011), and three homologs of RMA1, AtRMA1-AtRMA3 that were shown to be localized to the ER and exhibit *in vitro* E3 ubiquitin ligase activity (Son et al., 2009; **Table 1**), but it remains unclear if plants use distinct E3 ligases to removal different classes of ERAD substrates. Loss-of-function mutations in AtHrd1A or AtHrd1B had no detectable effect on bri1-5/bri1-9 degradation, but simultaneous elimination of the two Hrd1 homologs inhibited degradation of the two mutant BR receptors, indicating that AtHrd1A and AtHrd1B function redundantly in a plant ERAD pathway (Su et al., 2011). By contrast, the role of the two Doa10 homologs in the plant ERAD pathway remains unknown. Two recent genetic studies revealed important regulator roles of Doa10A (also known as SUD1 for SUPPRESSOR OF DRY2 DEFECTS1 or CER9 for ECERIFERUM9) in the cuticle lipid biosynthesis and in controlling the activity but not the protein level of an *Arabidopsis* HMG-CoA reductase (Lu et al., 2012; Doblas et al., 2013). Further studies are needed to determine if the *Arabidopsis* Doa10A is indeed involved in an ERAD pathway that regulates the protein abundance of key regulatory factors or metabolic enzymes involved in the cuticle lipid biosynthesis. Unlike yeast but similar to mammals, plants have additional membrane-anchored RING-type E3 ligases for ERAD. For example, the three *Arabidopsis* RMA1 homologs (Rma1H1) and a hot pepper (*Capsicum annuum*) Rma1H1 are involved in the degradation of a cell surface water channel to regulate its plasma membrane level (Lee et al., 2009). A recent study also suggested that a legume (*Medicago truncatula*) homolog of RMA1 seems to play a role in the regulation of biosynthesis of plant defense compounds, triterpene saponins that share the same biosynthetic precursors with sterols, through regulated degradation of HMG-CoA reductase (Pollier et al., 2013).

In a typical ubiquitination reaction, ubiquitin is attached to a substrate through a three-step process consisting of activation, conjugation, and ligation catalyzed by an ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and E3 (Pickart, 2004). In yeast, the Hrd1/Doa10 E3 ligases work together with a membrane-anchored E2 (Ubc6) and two cytosolic E2s (Ubc1 and Ubc7) that are recruited to the ER membrane by an ER anchor protein Cue1 (Hirsch et al., 2009), which also activates both E2 and E3 (Bagola et al., 2013; Metzger et al., 2013). *Arabidopsis* has a total of 37 E2 enzymes (Kraft et al., 2005), including

Table 1 | A list of known/predicted components of the *Arabidopsis* ERAD system.

Yeast/human gene name	*Arabidopsis* name	Accession number	Reference
Hrd1/HRD1	HRD1A	At3g16090	Su et al. (2011)
	HRD1B	At1g65040	
Hrd3/SEL1L	EBS5/HRD3A	At1g18260	Su et al. (2011)
	HRD3B	At1g73570	
Yos9/OS-9	EBS6/OS9	At5g35080	Huttner et al. (2012), Su et al. (2012)
Der1/DERLIN	DER1	At4g29330	Kirst et al. (2005)
	DER2.1	At4g04860	Wang et al. (2008), Kamauchi et al. (2005)
	DER2.2	At4g21810	
Ubc6/UBE2J1	UBC32	At3g17000	Kraft et al. (2005), Cui et al. (2012)
	UBC33	At5g50430	
	UBC34	At1g17280	
Htm1/EDEM	MNS4	At5g43710	Liebminger et al. (2009)
	MNS5	At1g27520	
Doa10/TEB4	SUD1/CER9/DOA10A	At4g34100	Liu et al. (2011), Doblas et al. (2013), Lu et al. (2012)
	DOA10B	At4g32670	
RMA1	RMA1	At4G03510	Matsuda and Nakano (1998), Son et al. (2009)
	RMA2	At4g28270	
	RMA3	At4g27470	
Ubx2/ERASIN	PUX1	At3g27310	Park et al. (2007), Rancour et al. (2004) Suzuki et al. (2001), Ueda et al. (2000)
	PUX2	At2g01650	
	PUX3	At4g22150	
	PUX4	At4g04210	
	PUX5	At4g15410	
	PUX6	At3g21660	
	PUX7	At1g14570	
	PUX8/SAY1	At4g11740	
	PUX9	At4g00752	
	PUX10	At4g10790	
	PUX11	At2g43210	
	PUX12	At3g23605	
	PUX13	At4g23040	
	PUX14	At4g14250	
	PUX15	At1g59550	
Cdc48/p97	CDC48A	At3g09840	Rancour et al. (2002)
	CDC48B	At2g03670	
	CDC48C	At3g01610	
Npl4/NPL4		At2g47970	
		At3g63000	
Ufd1/UFD1	UFD1	At2g21270	Galvao et al. (2008)
		At4g38930	
		At2g29070	
		At4g15420	
Ufd2/UFD2		At5g15400	Bachmair et al. (2001)
Png1/PNG1	PNG1	At5g49570	Diepold et al. (2007)
Rad23/RAD23	RAD23A	At1g16190	Farmer et al. (2010)
	RAD23B	At1g79650	
	RAD23C	At3g02540	
	RAD23D	At5g38470	

one potential Ubc1 homolog (UBC27), three putative homologs of Ubc7 known to be the cognate E2 for Hrd1 (UBC7 UBC13, and UBC14), and three likely homologs of Ubc6 associated mainly with Doa10 (UBC32, UBC33, and UBC34 each having a predicted transmembrane domain at their C-termini; Liu et al., 2011), but our understanding of the roles of these potential ERAD-participating E2s in the plant ERAD process is extremely limited. One of the *Arabidopsis* Ubc6-like E2 gene, *UBC32*, was recently found to be induced by salt, drought, and ER stress (Cui et al., 2012). Interestingly, the *Arabidopsis ubc32* mutant seedlings are more tolerant whereas *UBC32*-overexpressing transgenic *Arabidopsis* lines are more sensitive to salt and ER stress (Cui et al., 2012). The observed salt tolerance of the *ubc32* mutant is in contrast to the reduced salt tolerance of other known *Arabidopsis* ERAD mutants, including *ebs5/hrd3a*, *ebs6/os9*, and *hrd1ahrd1b* (Liu et al., 2011; Huttner and Strasser, 2012; Huttner et al., 2012). This discrepancy might be explained by different ERAD substrates being degraded by different E3 ligase complexes: $ERAD_{L/M}$ substrates by AtHrd1A/AtHrd1B in association with cytosolic E2s and $ERAD_C$ substrates by Doa10 using membrane-anchored E2s. Indeed, UBC32 was found to interact with *Arabidopsis* Doa10B and to stimulate the ubiquitination and degradation of a known ERAD substrate MLO-12, another variant of MLO carrying a single amino acid change in its cytosolic domain (Muller et al., 2005), in a tobacco leaf transient expression experiment (Cui et al., 2012). However, the tobacco result was quite different from the results obtained with the yeast MLO experiment showing that the ERAD of MLO-12 plus two other mutant MLOs (all carrying a cytosolic mutation) used the Ubc7-Hrd1 pathway but was unaffected by either *ubc6* or *doa10* deletion in yeast (Muller et al., 2005). Such inconsistency might be simply caused by heterologous expression of $ERAD_C$ substrates in two different eukaryotic systems. Nevertheless, UBC32 was implicated in the Hrd1-mediated degradation of bri1-9 (a presumed $ERAD_L$ substrate) as the *ubc32* mutation partially inhibited the degradation of the mutant BR receptor and weakly suppressed the corresponding dwarf phenotype (Cui et al., 2012). The partial inhibition could be attributed to a redundant role of UBC32 with its two close homologs or the potential *Arabidopsis* homologs of Ubc1/Ubc7. However, blast searches failed to find a single homolog of the yeast *Cue1* gene from published sequences of plant genomes and expressed sequence tags (our unpublished results), suggesting that plant ERAD processes might exclusively rely on ER-anchored membrane E2s. Alternatively, plants could recruit cytosolic E2s to the membrane-anchored E3 complexes via yet unknown recruiting factors that share no sequence homology but are functionally similar to Cue1.

The ubiquitination of ERAD substrates, especially those lacking *N*-glycan degradation signals, by the Hrd1 complex requires two additional adapters: U1-Snp1 associating-1 (Usa1; HERP in mammals), an ER membrane protein containing a ubiquitin-like (UBL) motif near it N-terminus and two predicted transmembrane domains in the middle, and Der1 (degradation in the ER; Derlins for Der1-like proteins in mammals), another integral ER membrane protein with four transmembrane segments (Kostova et al., 2007). Usa1 is thought to regulate the stability and/or oligomerization of Hrd1 and to recruit Der1 to the Hrd1 complex (Carvalho et al., 2006, 2010; Horn et al., 2009; Carroll and Hampton, 2010), while Der1 is believed to function either as a receptor for soluble non-glycosylated ERAD substrates or a potential retrotranslocation channel (Lilley and Ploegh, 2004; Ye et al., 2004; Kanehara et al., 2010). The *Arabidopsis* contains no homolog of Usa1/HERP1 but its genome encodes three Der1 homologs whose functional involvement in a plant ERAD pathway awaits detailed genetic and biochemical investigations (Kirst et al., 2005). An earlier study showed that at least two maize Der1 homologs could complement the yeast Δ*der1* mutant, suggesting a potential role for a plant Der1 homolog in an ERAD pathway; however, there is no genetic evidence for proving the hypothesis (Kirst et al., 2005).

RETROTRANSLOCATION OF ERAD SUBSTRATES

Because the catalytic domains of the ERAD-participating E2s and E3s are on the cytosolic surface of the ER membrane, ERAD substrates need to undergo retrotranslocation for ubiquitination and to access the cytosolic proteasome system for their degradation. However, the molecular nature of this retrotranslocon remains controversial (Hampton and Sommer, 2012). It was previously thought that the Sec61 translocon, which imports nascent polypeptides into the ER lumen during protein biosynthesis, is responsible for retrotranslocation ERAD substrates through the ER membrane (Pilon et al., 1997; Plemper et al., 1997). Other studies suggested that the yeast Der1 and its mammalian orthologs Derlins are the suspected retrotranslocon (Lilley and Ploegh, 2004; Ye et al., 2004). A recent study, however, showed that the E3 ligase Hrd1 itself could serve as the retrotranslocation channel for $ERAD_L$ substrates (Carvalho et al., 2010). It is quite possible that all three proteins are capable of retrotranslocation different ERAD substrates involving different adapter proteins.

Compared to the knowledge gained from the yeast and mammalian studies, we know almost nothing about the retrotranslocation step of a plant ERAD pathway. Several earlier studies did suggest the existence of a retrotranslocon in plant cells to move ERAD substrates into the cytosol. A confocal microscopic analysis of subcellular localization of a fusion protein between green fluorescent protein (GFP) with the P-domain of a maize CRT in tobacco leaf protoplasts suggested a retrotransport route from the ER to the cytosol (Brandizzi et al., 2003). In addition, a series of studies revealed that the A chain (known as RTA) of a ribosome-inactivating toxin, ricin that is normally produced as a dimeric protein of RTA covalently linked to a galactose-binding B chain via a single intramolecular disulfide bond and stored in the central vacuole of the endosperm cells of castor bean (*Ricinus communis*), was detected to be deglycosylated and eventual degraded in the cytosol when expressed alone in tobacco leaf protoplasts (Di Cola et al., 2001, 2005; Marshall et al., 2008). It is important to mention that ricin and a few other plant toxins were known to exploit the ERAD pathway to reach their cytosolic targets after being internalized by mammalian cells and retrograde-transported from the cell surface to the ER (Lord et al., 2003). In both yeast and mammalian systems, retrotranslocation of ERAD substrates was driven by ubiquitination (Bagola et al., 2011); however, a recent RTA study using plant protoplasts showed that retrotranslocation is

independent of ubiquitination as the lysine-lacking (hence non-ubiquitinated) variant of RTA could still be retrotranslocated from the ER into the cytosol (Di Cola et al., 2005), suggesting that the ubiquitination-retrotranslocation coupling might be substrate-dependent.

SUBSTRATE EXTRACTION, PROCESSING, AND DELIVERY TO THE PROTEASOME

Without regard to the identity of the actual retrotranslocons, ubiquitinated ERAD clients are extracted from the ER lumen ($ERAD_L$ substrates) or ER membrane ($ERAD_{M/C}$ substrates) by a trimeric complex consisting of a homohexameric Cdc48 (p97 or valosin-containing protein in mammals), an AAA-type ATPase and its two substrate-recruiting factors Ufd1 and Npl4 (each having a ubiquitin-binding domain; Wolf and Stolz, 2012). The $(CDC48)_6$-Ufd-Npl4 complex itself is recruited to the Hrd1/Doa10 E3 complexes by Ubx2 (VIMP for p97/VCP-interacting membrane protein in mammals), one of the 7 ubiquitin regulatory X (UBX) domain-containing proteins in yeast (13 UBX proteins in mammals; Neuber et al., 2005; Schuberth and Buchberger, 2005, 2008). The current working model posits that extracted ERAD substrates are further processed through antagonistic interactions between an U-box-containing E4 multiubiquitination enzyme Ufd2 and a WD40 repeat-containing protein Ufd3 with unknown enzyme activity plus a deubiquitylating enzyme Otu1, and/or through deglycosylation by the cytoplasmic peptide:*N*-glycanase (PNGase) Png1 (Raasi and Wolf, 2007). The processed ERAD substrates were subsequently delivered to the cytosolic proteasome by Cdc48 in association with two ubiquitin receptors Rad23 and Dsk2, each containing a UBL domain that interacts directly with the cytosolic proteasome and a polyubiquitin-interacting ubiquitin-associated (UBA) domain (Raasi and Wolf, 2007).

The *Arabidopsis* genome encodes three Cdc48 homologs, AtCDC48A, AtCDC48B, and AtCDC48C (Rancour et al., 2002). AtCDC48A was able to complement a yeast *cdc48* mutant (Feiler et al., 1995) and was shown to play a role in the ERAD of a mutant form of MLO and a mutant variant of the *Arabidopsis* vacuolar carboxypeptidase carrying the same Gly-Arg mutation as the yeast CPY* and in the retrotranslocation of RTA and the orphan subunit (RCA A) of another castor bean toxin agglutinin in plant cells (Muller et al., 2005; Marshall et al., 2008; Yamamoto et al., 2010). AtCDC48A is likely to be recruited to the ER membrane by UBX-containing proteins as the *Arabidopsis* genome encodes a total of 15 UBX-containing proteins (known as AtPUXs; **Table 1**), some of which were shown to interact with AtCDC48A (Rancour et al., 2004; Park et al., 2007). It remains to be determined which of the 15 AtPUX proteins are actually involved in recruiting an AtCDC48 to the ER membrane-anchored E3 ligase complexes and play a role in degrading known plant ERAD substrates. Our BLAST searches using the known ERAD components of yeast and mammals as query identified multiple homologs of the Ufd1, Ufd2, Ufd3, Npl4, Rad23, Dsk2 but only a single PNGase homolog in *Arabidopsis* (**Table 1**). The functional involvement of these potential ERAD components in an *Arabidopsis* ERAD process remains unknown except AtPNG1, which was recently shown to contain the suspected PNGase activity and could stimulate the degradation of two mutant variants of RTA in an *N*-glycan-dependent manner in yeast cells (Diepold et al., 2007; Masahara-Negishi et al., 2012).

CONCLUSION AND CHALLENGES

Despite rapid progress in recent years for identifying molecular components of plant ERQC/ERAD systems and studying their biochemical functions, our understanding of the plant ERQC/ERAD processes remains rather limited, especially about the later stages of the ERAD pathway, such as retrotranslocation, processing of polyubiquitin chains, and delivery (to cytosolic proteasome) of the known plant ERAD substrates. While forward genetic screens in *Arabidopsis* identified the GII-UGGT-mediated CNX/CRT cycle in retaining incomplete/mis-folded glycoproteins and ERAD components that function inside the ER lumen to promote the degradation of the two mutant BR receptors, reverse genetic approaches using T-DNA insertional mutants or RNAi-mediated knockdown of candidate ERAD genes listed in **Table 1** will certainly provide additional knowledge on the plant ERAD mechanisms. Transgenic *Arabidopsis* lines expressing carefully engineered substrates of glycosylated/non-glycosylated $ERAD_C$/$ERAD_M$ coupled with forward genetic screens and reverse genetic studies will reveal if *Arabidopsis* has several distinct ERAD subpathways using different E3 ligases and adapter proteins that recruits distinct ERAD clients. Similarly, genetic screens for enhancers/suppressors of the *Arabidopsis* wax mutant *cer9* [defective in Doa10A (Lu et al., 2012)] or *drought hypersensitive 2* mutant [that led to independent discovery of Doa10A (Doblas et al., 2013)] could uncover additional ERAD components, reveal unique features of the plant ERAD processes, and a better understanding of the regulatory function of the plant ERAD system in biosynthetic processes. Proteomic studies with the existing *Arabidopsis* mutants of the ERAD E3 ligases could lead to the discovery of additional biochemical pathways and/or physiological processes regulated by the plant ERAD machinery. However, the biggest challenges for the plant ERQC/ERAD research is whether the forward genetic approach in *Arabidopsis* could identify novel ERQC/ERAD components that haven't been discovered in other eukaryotic systems and if the combination of the *Arabidopsis* genetics with cutting-edge biochemical studies in *Arabidopsis* and transient expression systems could reveal novel biochemical functions of known or predicted ERAD components and provide satisfactory answers to some of the outstanding questions of the general ERQC/ERAD research field.

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Conserved and plant-unique strategies for overcoming endoplasmic reticulum stress

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Stress caused by environmental conditions or physiological growth can lead to an accumulation of unfolded proteins in the endoplasmic reticulum (ER) causing ER stress, which in turn triggers a cytoprotective mechanism termed the unfolded protein response (UPR). Under mild-short stress conditions the UPR can restore ER functioning and cell growth, such as reducing the load of unfolded proteins through the upregulation of genes involved in protein folding and in degrading mis-folded proteins, and through autophagy activation, but it can also lead to cell death under prolonged and severe stress conditions. A diversified suite of sensors has been evolved in the eukaryotic lineages to orchestrate the UPR most likely to suit the cell's necessity to respond to the different kinds of stress in a conserved as well as species-specific manner. In plants three UPR sensors cooperate with non-identical signaling pathways: the protein kinase inositol-requiring enzyme (IRE1), the ER-membrane-associated transcription factor bZIP28, and the GTP-binding protein β1 (AGB1). In this mini-review, we show how plants differ from the better characterized metazoans and fungi, providing an overview of the signaling pathways of the UPR, and highlighting the overlapping and the peculiar roles of the different UPR branches in light of evolutionary divergences in eukaryotic kingdoms.

Keywords: unfolded protein response, ER stress, UPR temporal activation, adaptation, autophagy, programed cell death, apoptosis, eukaryotes

INTRODUCTION

Environmental or physiological conditions that interfere with the proper protein folding in the endoplasmic reticulum (ER) lead to an accumulation of potentially toxic mis-folded proteins, a condition generally termed as "ER stress". To restore ER homeostasis, a network of inter-organelle signaling pathways mediates the "unfolded protein response" (UPR), leading to an increase of protein folding capacity in the ER (Walter and Ron, 2011). If these mechanisms of adaptation and survival to ER stress fail, the UPR signaling leads the cells toward cell death (Hetz, 2012). Even if several aspects of the UPR are conserved across eukaryotes, the mechanisms to counteract the ER stress can vary across plants, metazoans and yeast (Kimata and Kohno, 2011; Chen and Brandizzi, 2013a; Howell, 2013). This review focuses on the current understanding of how the UPR signaling pathways initiate and progress in response to the severity and duration of ER stress and addresses the overlapping and unique roles of the UPR response in eukaryotes with emphasis on multicellular eukaryotes.

UPR ARMS IN EUKARYOTES AND THEIR ER STRESS-SENSING MECHANISMS

In the yeast *Saccharomyces cerevisiae*, the UPR is mediated by inositol-requiring enzyme1 (IRE1; Ire1p; Cox et al., 1993; Mori et al., 1993), an ER-resident protein largely conserved in eukaryotes. IRE1 is a type I transmembrane protein, with an N-terminal ER luminal stress-sensing domain, and a Ser/Thr kinase domain and an endoribonuclease domain in the cytosol (**Figure 1A**). Metazoans and plants have expanded their UPR signaling pathways with additional ER stress sensors (**Figures 1B,C**). Indeed, in metazoans, at least three ER transmembrane sensors initiate the UPR: IRE1 (IRE1α and IRE1β isoforms), the activating transcription factor 6 proteins (ATF6α and ATF6β isoforms), and the protein kinase RNA-like ER kinase (PERK) (Hetz, 2012). ATF6 is a type II transmembrane protein, characterized by a C-terminal ER lumen domain and an N-terminal cytosolic domain containing a bZIP (basic leucine zipper) transcriptional factor domain, while PERK is a type I transmembrane protein, with an ER-luminal stress-sensing domain and a cytosolic Ser/Thr kinase domain (**Figure 1A**). In plants, the UPR regulators so far identified are two IRE1 homologs (IRE1A and IRE1B; Koizumi et al., 2001), a functional homolog of ATF6 (bZIP28; Liu et al., 2007a), and a component of the G protein complex (AGB1; Wang et al., 2007). Intriguingly, the involvement of G protein complex in UPR has not been observed in other eukaryotes possibly because of a redundancy of the multiple isoforms of the heterotrimeric GTP-binding proteins in metazoans. Moreover, a functional PERK ortholog has not been identified in plants.

The mechanisms of how ER stress is sensed have been partially defined: the UPR sensors may detect ER stress (1) through the dissociation of their ER-luminal stress-sensing domain from the ER chaperones, which would be induced by the binding of ER chaperones to unfolded proteins, as shown for IRE1α, PERK, and ATF6 in metazoans (Kimata and Kohno, 2011); (2) through the direct binding of the ER-luminal domain of the UPR sensors to the

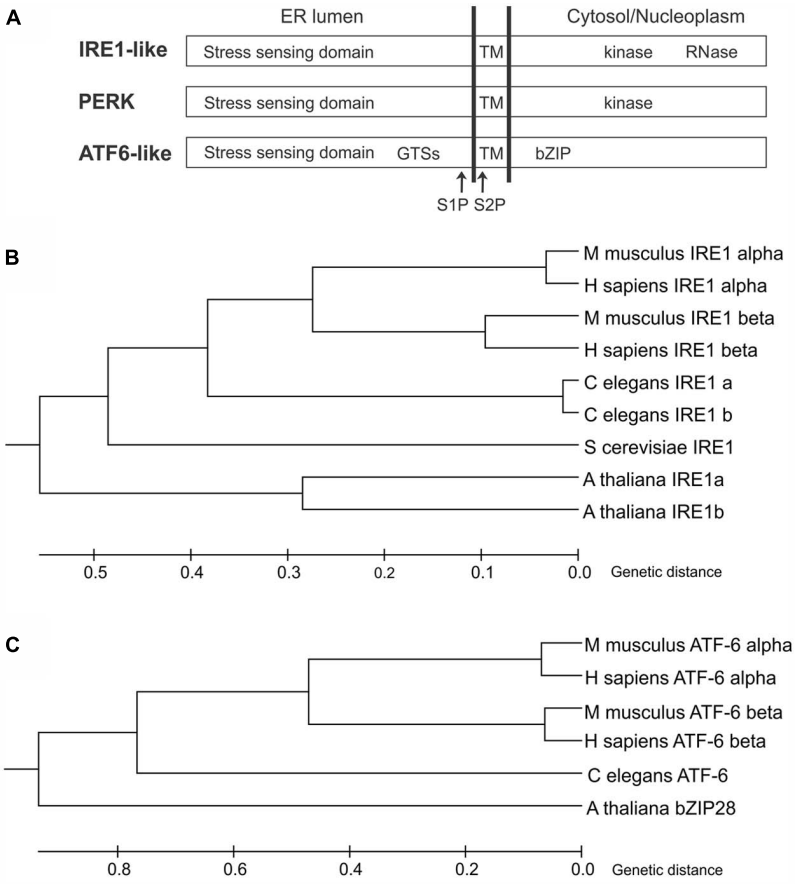

FIGURE 1 | ER stress-sensing proteins in eukaryotes. (A) Schematic diagram of IRE1-like, PERK and ATF6-like proteins (not to scale). GTSs, Golgi trafficking signals; TM, transmembrane domain; S1P, site 1 protease cleavage site; S2P, site 2 protease cleavage site; bZIP, basic leucine zipper domain. **(B)** Phylogenetic tree analysis of IRE1-like proteins from *Animalia* (*Homo sapiens*, *Mus musculus* and *Caenorhabditis elegans*), *Fungi* (*Saccharomyces cerevisiae*) and *Plantae* (*Arabidopsis thaliana*) was constructed by the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method using MEGA6 software. NCBI reference sequences: IRE1 alpha (*H.s*)-NP_001424, IRE1 beta (*H.s*)-NP_150296, IRE1 alpha (*M.m*)-NP_076402, IRE1 beta (*M.m*)-NP_036146, IRE1a (*C.e*)-NP_001254135, IRE1b (*C.e*)-NP_001254136, IRE1 (*S.c*)-NP_011946, IRE1a (*A.t*)-NP_565419, IRE1b (*A.t*)-NP_568444. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method. **(C)** Phylogenetic tree analysis of ATF6-like proteins from *Animalia* (*H. sapiens*, *M. musculus* and *C. elegans*) and *Plantae* (*A. thaliana*) was constructed by the UPGMA method using MEGA6 software. NCBI reference sequences: ATF6 alpha (*H.s*)-NP_031374, ATF6 beta (*H.s*)-NP_001129625, ATF6 alpha (*M.m*)-NP_001074773, ATF6 beta (*M.m*)-NP_059102, ATF6 (*C.e*)-NP_510094, bZIP28 (*A.t*)-NP_187691. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method.

unfolded proteins, as shown for IRE1 in yeast (Gardner and Walter, 2011); and (3) through post-translational modifications within the luminal domain, as observed in mammals for ATF6, where for example the hypoglycosylation is a monitoring mechanism for ER homeostasis to sense the glucose starvation or N-linked glycosylation impairment (Hong et al., 2004).

Upon ER stress induction, the UPR sensors are activated as follows: (1) the RNase domain of IRE1 is activated through its oligomerization and trans-autophosphorylation via its own kinase domain (Korennykh et al., 2009; Ali et al., 2011); (2) ATF6-orthologs are transported to the Golgi likely via specific trafficking signals (Shen et al., 2002; Srivastava et al., 2012) with the COPII vesicles (Schindler and Schekman, 2009; Srivastava et al., 2012), where the ATF6-orthologs are cleaved by the sequential action of the site 1 and site 2 proteases (S1P and S2P), thus releasing their N-terminal cytosolic transcription factor for translocation to the nucleus (Ye et al., 2000; Liu et al., 2007a); and (3) after PERK dimerization, one PERK homodimer likely inserts its flexible activation loop into the catalytic site of the adjacent homodimer, resulting in an interdimer trans-phosphorylation (Marciniak et al., 2006; Cui et al., 2011), that activates the kinase domain of PERKs. The mechanisms that lead to a role of AGB1 in the UPR are unknown (Wang et al., 2007; Chen and Brandizzi, 2012). In subcellular fractionation experiments, AGB1 has been found to be largely associated with the ER (Wang et al., 2007). Because AGB1 lacks a transmembrane domain, it is possible that post-translational modifications may modulate its UPR signaling function.

THE ACTIVATION OF THE UPR ARMS: THE TRANSLATIONAL ATTENUATION

The different arms of the UPR have evolved to activate overlapping but non-identical pathways in order to restore homeostasis, or, if the ER stress persists, to trigger programed cell death (PCD) in plants and yeasts, and apoptosis in metazoans (**Figure 2**). In the early phases of ER stress responses in metazoans, the overload of newly synthesized proteins into the protein-overloaded ER is reduced through the selective degradation of many mRNAs encoding ER-translocating proteins by the IRE1 endonuclease activity, a process termed "regulated IRE1 dependent decay" (RIDD), and through the transient attenuation of global protein translation via PERK. RIDD promotes the rapid mRNA decay of genes encoding secretory proteins (Hollien and Weissman, 2006; Han et al., 2009) and a similar mechanism most likely operates in plants (Mishiba et al., 2013). Interestingly, RIDD-mediated decrease in ER protein overload was also demonstrated in fission yeast *Schizosaccharomyces pombe*, where it functions as the exclusive UPR mechanism (Kimmig et al., 2012), but not in the budding yeast *Saccharomyces cerevisiae*, where, indeed, the UPR does not appear to attenuate protein translation (Spear and Ng, 2003).

To attenuate ER stress in metazoans in addition to RIDD, PERK phosphorylates the α subunit of the eukaryotic translation initiation factor 2 (eIF2α), inhibiting the 80s ribosome assembly and down-regulating protein synthesis (Harding et al., 1999). In mammals, the phosphorylation of eIF2α is a conserved mechanism to block general protein translation not solely restricted to ER stress, and it is carried out by different kinases activated by diverse cellular stresses. The only eIF2 α-kinase conserved among eukaryotes is the GCN2 protein: GCN2/eIF2 α pathway attenuates protein translation under nutrient limitation in yeasts and mammals (Berlanga et al., 1999; Harding et al., 2000), and under amino acid starvation, abiotic and biotic stresses and plants (Lageix et al., 2008; Zhang et al., 2008). Whether the plant GCN2 may function as the metazoan PERK in the UPR is yet unknown.

THE ADAPTIVE CELLULAR RESPONSE

Once the first layer of response to ER stress conditions is completed, in plants, metazoans and yeasts all the UPR stress sensors promote a coordinated adaptive response to protect the cell against oxidative stress, to augment protein-folding and secretory capacity in order to ensure that protein exit the ER productively, and to degrade potentially toxic unfolded proteins, by up-regulating the genes encoding oxido-reductases, ER chaperones, vesicle trafficking proteins, ER-associated degradation (ERAD) and ER-quality control (ERQC) components (Travers et al., 2000; Martínez and Chrispeels, 2003; Kamauchi et al., 2005).

IRE1 AND ITS UNCONVENTIONAL RNA-SPLICING ARM

The IRE1 endonuclease domain catalyzes the non-conventional cytoplasmic splicing of the mRNA encoding *bZIP60/XBP1/HAC1* (in *Arabidopsis*, metazoans and budding yeast respectively), leading to the translation of a transcription factor (bZIP60s/XBP1s/HAC1s) that mainly upregulates the expression of ERQC and ERAD-related genes (Yoshida et al., 2001; Iwata et al., 2008; Deng et al., 2011). Among the species, the amino acid sequences of these transcription factors are not highly conserved; however, a two stem–loop structure accompanied by a consensus sequence in each loop of the IRE1-splicing mRNA substrates is remarkably conserved and associated with IRE1-mediated cleavage (Oikawa et al., 2010; Deng et al., 2011; Nagashima et al., 2011). In yeast and metazoans, the spliced substrate becomes a potent transcriptional activator, since it gains a transcriptional activation domain in the new C-terminal tail (Mori et al., 2000; Yoshida et al., 2001). It is noteworthy that in the absence of induced ER stress in budding yeasts, the unspliced *HAC1* (*HAC1u*) mRNA is not translated, since an intron in the *HAC1u* mRNA blocks its translation (Chapman and Walter, 1997), while in mammalian cells the unspliced *XBP1* (*XBP1u*) mRNA is translated. XBP1u protein along with the *XBP1u* mRNA is associated peripherally with the ER membrane through an amphipathic region, where it facilitates the targeting of *XBP1u* mRNA to IRE1 presumably to increase the cytoplasmic splicing efficiency providing a rapid response to ER stress (Yanagitani et al., 2009). Upon prolonged ER stress, XBP1u forms a complex with XBP1s, leading it to be exported from the nucleus to the cytoplasm and rapidly degraded by the proteasome, presumably shutting down the transcription of the XBP1-target genes (Yoshida et al., 2006). Intriguingly, the IRE1-spliced *XBP1s* mRNA loses the ER membrane-anchor domain and it is released in the cytosol, indicating a different translational place for *XBP1u* and *XBP1s* mRNAs that presumably prevents the excess of degradation of the XBP1s by XBP1u protein during ER stress (Yanagitani et al., 2009). In plants, experiments based on the expression of in-frames fluorescent protein fusion with unspliced bZIP60u, have shown an association of bZIP60u with the ER through its putative C-terminal transmembrane domain (Deng et al., 2011). However, the biological roles of unspliced bZIP60 (bZIP60u) on the ER membrane are currently unknown. Unlike yeast and metazoans, spliced bZIP60 (bZIP60s) does not gain a transcriptional activation activity, since the transcriptional activation domain is located in the N-terminal tail along with a nuclear localization signal. The IRE1-splicing produces instead a new protein deprived of the transmembrane domain (Deng et al., 2011; Nagashima et al., 2011) and characterized by an improved fine regulatory modulation of its transcription activity as recently reported in rice (Lu et al., 2012). However, how this tuning is achieved is still not clear.

ATF6-LIKE TRANSCRIPTION FACTORS

Under ER stress, the transcription factor domain of bZIP28/ATF6 increases the expression of genes involved in protein folding and of other ER-stress related transcription factors such as bZIP60/XBP-1, providing a positive feedback for augmenting the UPR (Yoshida et al., 2001; Liu and Howell, 2010). Moreover, in metazoans, ATF6 enhances the expression of genes involved in ERAD, lipid biosynthesis and ER expansion, which are required to improve the capacity of the secretory pathway (Bommiasamy et al., 2009).

CONSERVED *cis*-ELEMENT IN THE UPR TARGET GENES

The UPR genes are induced through the recognition of *cis*-acting elements on their promoter regions by the UPR

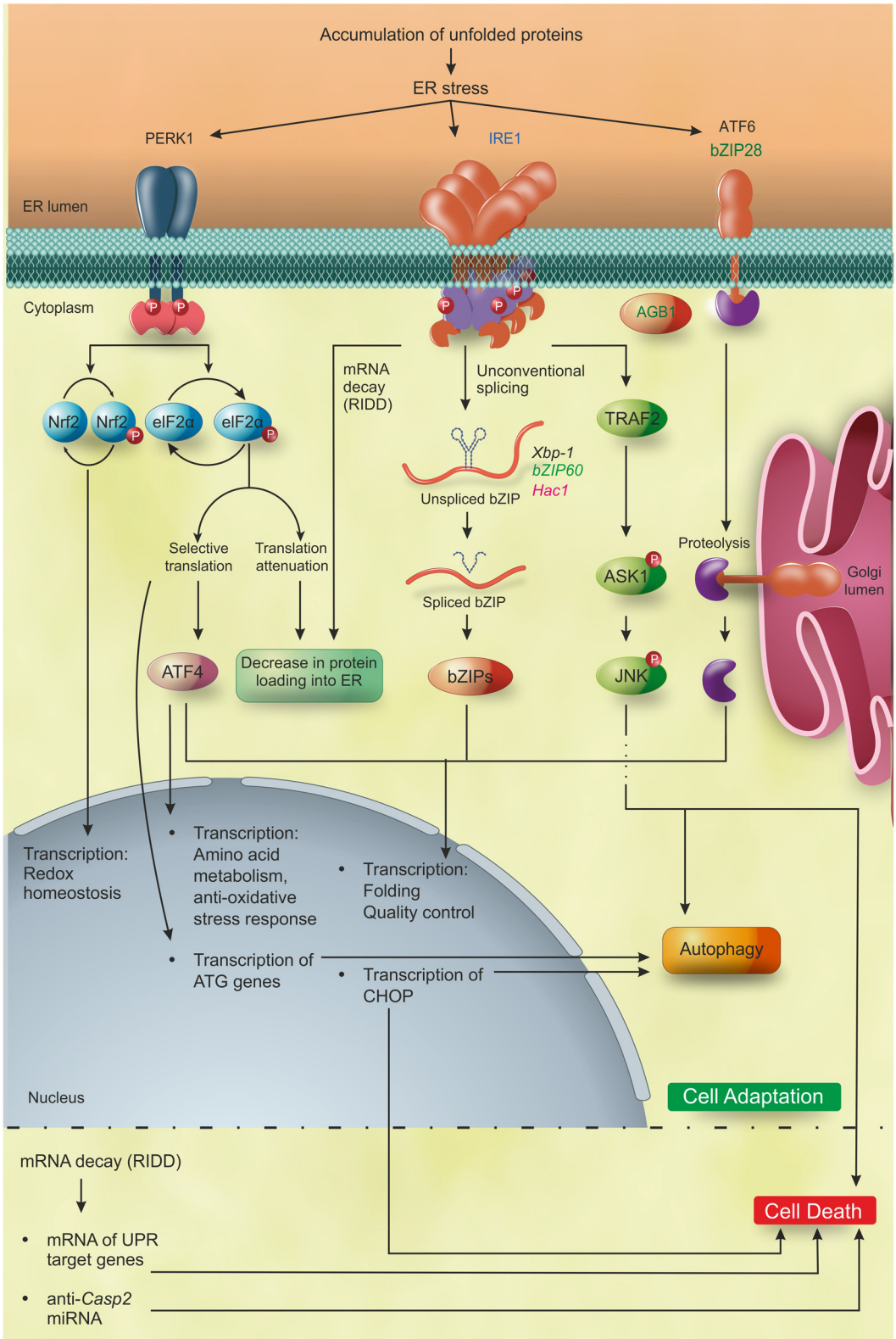

FIGURE 2 | Continued

FIGURE 2 | Continued
UPR pathways in eukaryotes. Accumulation of unfolded proteins inside the ER lumen triggers to UPR that, during the first layer of response, leads the cell to adaptation. IRE1 is the only identified ER stress sensor in yeast and it is widely conserved in metazoan and plants. Moreover, additional ER stress sensors so far identified are bZIP28 and AGB1 in plants, and ATF6 and PERK1 in metazoan. Activation of PERK leads to phosphorylation of two proteins: Nrf2 that activates the transcription of genes involved in redox homeostasis; and eIF2α that decreases the overload of newly synthetized proteins into the ER and increases the specific translation of the transcription factor ATF4. Subsequently, ATF4 induces the expression of genes involved in amino acid metabolism, anti-oxidative stress response, folding, quality control and autophagy. The activation of IRE1 leads (1) to the selective degradation of mRNAs (RIDD) encoding ER-translocating proteins, decreasing the protein loading into the ER, and (2) to the translation of the bZIP transcription factors Xbp1/bZIP60/Hac1 mRNA in metazoan, plants and yeast, respectively, that upregulate the UPR target genes mainly involved in folding, quality control and autophagy. ATF6 in metazoans and bZIP28 in *Arabidopsis* are translocated into the Golgi apparatus and cleaved by Site1 and Site2 proteases, releasing the ATF6/bZIP28 transcription factors domain, which then translocates to the nucleus where it increases the expression of genes involved in folding, quality control and autophagy. Upon excessive and prolonged ER stress, all the UPR pathways lead to cell death in yeast, metazoan, and plants. In plants, the cell death executioners are still largely unknown, while in metazoan they have been partially defined. In detail, IRE1 regulates the cell death through the mRNA decay of UPR target genes and of anti-apoptotic protein (anti-Casp2). In addition, IRE1 interacting with TRAF2 triggers the ASK1/JNK pathway promoting apoptosis. Moreover, PERK/eIF2α/ATF4, IRE1, and ATF6 induce the expression of the transcription factor CHOP, involved in the induction of apoptosis. Blue, eukaryotes; black, metazoan; green, plants; pink, yeast. Figure adapted from Chen and Brandizzi (2012).

transcription factors. In budding yeast, HAC1s binds and activates the UPR element-I (UPRE-I, consensus region CAGNGTG; Mori et al., 1996) and UPRE-II (TACGTG; Fordyce et al., 2012). In mammalians, XBP1 efficiently binds the UPRE-I (GA-TGACGT-G[G/A]; Wang et al., 2000; Yamamoto et al., 2004) and the ER stress responsive element-II (ERSE-II, ATTGG–N–CCACG; Kokame et al., 2001; Yamamoto et al., 2004), while ATF6 binds the ERSE-I (ERSE-I, CCAAT–N9–CCACG; Roy and Lee, 1999) and the ERSE-II (ATTGG–N–CCACG; Kokame et al., 2001) only in the presence of the nuclear transcription factor NF-Y (Yamamoto et al., 2004). Interestingly, the mammalian ERSE-I and UPRE-I elements are conserved in plants (Oh et al., 2003; Iwata et al., 2008; Liu and Howell, 2010), where other *cis*-elements have also been found, such as the pUPRE-II (GATGACGCGTAC; Hayashi et al., 2013) and the pUPRE-III (TCATCG; Sun et al., 2013). Similar to ATF6 in mammals, bZIP28 binds the ERSE-I element with assistance from the transcription factors NF-Y (Liu and Howell, 2010), while bZIP60s directly binds the pUPRE-III (Sun et al., 2013) and it regulates also promoters containing the *cis*-element ERSE-I and UPRE-I (Iwata and Koizumi, 2005). The multiple *cis*-elements involved in the ER stress response and their differential binding affinities for the UPR transcription factors presumably fine temporal modulate the UPR signaling and the ER stress response. Moreover, plants have evolved an additional layer of UPR regulation. The plant-specific nuclear transcription factor *NAC103* is indeed induced by ER stress presumably through bZIP60s, and the encoded protein NAC103 in turn regulates the UPR downstream genes (Sun et al., 2013). However, the precise mechanisms of the gene regulation networks are largely unknown.

PERK AS AN OXIDATIVE STRESS-ATTENUATOR

In metazoans, although PERK induces general protein translation attenuation, it also favors selective protein translation. Specifically, the PERK-phosphorylated eIF2α activates the translation of mRNAs with uORF (upstream open reading frame) within their 5' untranslated region (UTR), like the activating transcription factor 4 (ATF4; Harding et al., 2000). ATF4 protects cells against oxidative stress and ensures the supply of reducing substances (i.e., glutathione) by enhancing the metabolism of their precursors (i.e., sulfur-containing amino acids; Harding et al., 2003). Also PERK phosphorylates the transcription factor Nrf2 (nuclear factor erythroid2-releated factor 2), which translocates to the nucleus, heterodimerizes with the small Maf proteins and activates the transcription of genes involved in the redox homeostasis by binding to the antioxidant response elements on the target gene promoters (Cullinan et al., 2003).

AUTOPHAGY AS A PRO-SURVIVAL MECHANISM

Autophagy is an evolutionarily conserved process of bulk degradation, whereby large portions of cytoplasmic and organellar components are engulfed by double membrane vesicles (termed as "autophagosome") and delivered to the lysosome in metazoan, or to the vacuole in yeast and plants, for degradation and recycling of macromolecules (Liu and Bassham, 2012). During ER stress, autophagy is activated in yeast, mammals and plants, and it is involved in clearing unfolded protein from the ER by supplementing the ERAD pathway and, in turn, alleviating stress (Ding et al., 2007; Liu et al., 2012). In yeast, IRE1 regulates autophagy through the splicing of *HAC1*, which induces the production of Atg8p, an ubiquitin-like protein required for autophagosome formation (Yorimitsu et al., 2006). Unlike yeasts, in metazoans, autophagy is triggered by the kinase activity of IRE1 and PERK. IRE1, indeed, recruits the adaptor protein TNFR-associated factor2 (TRAF2) on the ER membranes, thus triggering the activation of the apoptosis signal regulating kinase1 (ASK1), which in turn regulates the activation of the c-Jun-N-terminal kinase (JNK), whose pathway induces the autophagosome formation (Pattingre et al., 2009). Moreover, PERK promotes autophagy through the phosphorylation of eIF2α, which induces the expression of ATF4, recently considered a key signal for autophagy activation (Matsumoto et al., 2013). ATF4 in turn activates the expression of autophagy-related (ATG) genes and of C/EBP-homologous protein (CHOP) transcription factor. CHOP and ATF4 together promote and modulate the induction of genes implicated in the formation, elongation and function of the autophagosome (B'chir et al., 2013). Similar to metazoans, in plants, it has been recently found that IRE1, specifically the IRE1b isoform, activates autophagy upon ER stress response independently from the IRE1-mediated *bZIP60* mRNA splicing (Liu et al., 2012). However, the mechanistic features of plant autophagy under ER stress are mainly unknown, in terms of the upstream regulator/s of IRE1b as well as its downstream targets.

UNRESOLVED ER STRESS LEADS TO CELL DEATH

UPR AND APOPTOSIS IN METAZOAN

Upon excessive and prolonged ER stress, in metazoans, all the three UPR signaling pathways lead to cell death through apoptosis

via the intrinsic mitochondrial pathway. Indeed, UPR regulates the activity of the pro-apoptotic members of the Bcl-2 family via transcriptional and post-transcriptional mechanisms, leading the BAX/BAK-mediated pore formation in the mitochondrial outer membrane, release of cytochrome c from the mitochondria, and subsequent activation of caspases, which are critical regulators of apoptosis via their role in propagating apoptotic signaling cascades (Rodriguez et al., 2011). However, it is not yet clear whether other types of cell death occur to eliminate terminally compromised cells under irreversible ER stress. Intriguingly in metazoans, under prolonged ER stress autophagy may switch from a pro-survival process to apoptosis. Several regulators of autophagy machinery are indeed involved in the apoptosis, such as JNK and CHOP (Rodriguez et al., 2011).

RIDD AS A PRO-APOPTOTIC EXECUTIONER

In mammals RIDD activity mediated by IRE1α enhances (1) ER stress intensity through the decay of mRNA encoding UPR target genes during the transition phase between the adaptive and apoptotic response (Han et al., 2009), and (2) expression of pro-apoptotic proteases, like the Caspase 2 (Upton et al., 2008), through the decay of selected antiapoptotic pre-miRNAs during the apoptotic response (Upton et al., 2012). In plants, the biological significance of RIDD activity in cell fate determination is still unknown.

ER STRESS INDUCED-CELL DEATH IN YEASTS AND PLANTS

In yeast, ER stress can induce PCD with apoptotic phenotypes (Hauptmann et al., 2006), as well as in a non-apoptotic process, where vacuole fragmentation and leaking of vacuolar materials are cell death features (Kim et al., 2012). Plant cell death executioners in the UPR are instead largely unknown. Unlike in metazoans, the plant IRE1 does not seem to have a pro-apoptotic role, given that the *Arabidopsis ire1* double mutants display compromised ER stress tolerance, instead of a greater survival rate (Nagashima et al., 2011; Chen and Brandizzi, 2012). Although neither homologs of Bcl-2 family proteins nor components of the PERK-CHOP pathways have been identified in plants yet, some regulators of ER-PCD seem to be conserved across kingdoms. These include the Bax inhibitor1 (BI-1)-like protein, an ER transmembrane protein that protects cells against ER-stress induced-cell death (Chae et al., 2003; Watanabe and Lam, 2008), and the chaperone BiP, that has a protective function against ER stress induced-cell death in both mammalian and plant cells (Kishi et al., 2010; Reis et al., 2011).

OTHER FACTORS THAT CONTROL THE UPR IN PLANTS

Plants have developed UPR roles for evolutionarily conserved gene family, like for the Bcl-2-associated athanogene7 (BAG7) protein, and AGB1, the Gβ subunit of heterotrimeric GTP-binding protein family. In detail, BAG7 a plant ER-localized protein involved in the UPR aids chaperones like BiP in the protection of cells via a co-chaperone activity, while in yeast and mammals BAGs have a nuclear/cytoplasmic localization and are not involved in the maintenance of the UPR, but rather in other processes ranging from proliferation to growth arrest and cell death (Williams et al., 2010). Furthermore, in plants, AGB1 and IRE1 have antagonistic roles in the UPR gene induction and they regulate essential and independent UPR signaling arms (Wang et al., 2007; Chen and Brandizzi, 2012, 2013b), but the underlying mechanisms are unclear.

CONCLUDING REMARKS

Environmental conditions, such as heat and salt stresses (Liu et al., 2007b; Deng et al., 2011), as well as physiological events, like growth and developmental processes (Chen and Brandizzi, 2012; Deng et al., 2013) evoked ER stress. Moreover, UPR has been recently linked to the phytohormone auxin, a master regulator of plant physiology, revealing a plant-specific strategy to maintain balance between stress adaptation and growth regulation (Chen et al., 2014). Additional studies are required to elucidate the plant UPR signaling and its molecular components, and how it is fine regulated during physiological events and environmental stresses. Also further work is needed to clarify the mechanisms leading the UPR to switch from cell survival to cell death and to identify the precise steps downstream of each UPR arm across different kingdoms.

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Stress sensing in plants by an ER stress sensor/transducer, bZIP28

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Two classes of ER stress sensors are known in plants, membrane-associated basic leucine zipper (bZIP) transcription factors and RNA splicing factors. ER stress occurs under adverse environmental conditions and results from the accumulation of misfolded or unfolded proteins in the ER lumen. One of the membrane-associated transcription factors activated by heat and ER stress agents is bZIP28. In its inactive form, bZIP28 is a type II membrane protein with a single pass transmembrane domain, residing in the ER. bZIP28's N-terminus, containing a transcriptional activation domain, is oriented towards the cytoplasm and its C-terminal tail is inserted into the ER lumen. In response to stress, bZIP28 exits the ER and moves to the Golgi where it is proteolytically processed, liberating its cytosolic component which relocates to the nucleus to upregulate stress-response genes. bZIP28 is thought to sense stress through its interaction with the major ER chaperone, binding immunoglobulin protein (BIP). Under unstressed conditions, BIP binds to intrinsically disordered regions in bZIP28's lumen-facing tail and retains it in the ER. A truncated form of bZIP28, without its C-terminal tail is not retained in the ER but migrates constitutively to the nucleus. Upon stress, BIP releases bZIP28 allowing it to exit the ER. One model to account for the release of bZIP28 by BIP is that BIP is competed away from bZIP28 by the accumulation of misfolded proteins in the ER. However, other forces such as changes in energy charge levels, redox conditions or interaction with DNAJ proteins may also promote release of bZIP28 from BIP. Movement of bZIP28 from the ER to the Golgi is assisted by the interaction of elements of the COPII machinery with the cytoplasmic domain of bZIP28. Thus, the mobilization of bZIP28 in response to stress involves the dissociation of factors that retain it in the ER and the association of factors that mediate its further organelle-to-organelle movement.

Keywords: endoplasmic reticulum stress, unfolded protein response (UPR), bZIP transcription factors, binding immunoglobulin protein (BIP), protein folding, Golgi apparatus, COPII vesicle transport system

INTRODUCTION

The endoplasmic reticulum (ER) engages in the folding and modification of proteins in the endomembrane system to ensure their correct sorting, secretion and function. Disturbances in the ER or overload in secreted protein production results in the accumulation of unfolded proteins, which has the potential to damage cells. This condition is sensed by specialized stress sensors/transducers in the ER membrane, which elicit the unfolded protein response (UPR). Plants have two kinds of sensor/transducers, ER membrane-associated basic leucine zipper (bZIP) transcription factors and RNA splicing factors. Upon stress, these sensors/transducers initiate several cellular responses that transduce signals to the nucleus to help restore ER homeostasis. One of the stress sensor/transducers in *Arabidopsis* is a membrane-associated transcription factor called bZIP28 that is activated by heat and ER stress agents. In this review, the mechanisms involved in stress sensing and mobilization of bZIP28 are discussed.

STRUCTURE OF bZIP28

bZIP28 is a type II membrane protein with a single pass transmembrane domain (TMD) that resides in the ER under unstressed conditions and in response to stress relocates to the nucleus where it upregulates stress response genes (**Figure 1**). bZIP28's N-terminus contains a bZIP domain and is oriented towards the cytoplasm. The C-terminus of the protein is inserted into the ER lumen and constitutes the lumenal domain (LD) which contains a Site 1 Protease (S1P) processing site (Liu et al., 2007) and a Site 2 Protease (S2P) recognition site, which is present within the TMD (**Figure 2**). Proximal to the TMD on the cytoplasmic side are present paired lysine residues that play an important role in the translocation of bZIP28 (Srivastava et al., 2012).

SENSING OF STRESS AND ACTIVATION OF bZIP28

Sensing of adverse environmental conditions is critical to bZIP28's function and in its ability to protect plants from stress. bZIP28 is a key player in UPR because it is activated by ER stress and directly targets typical UPR genes (Liu et al., 2007; Gao et al., 2008; Liu and Howell, 2010b; Iwata and Koizumi, 2012; Howell, 2013). Upon stress, bZIP28 exits from the ER and moves to the Golgi where it is proteolytically processed in a sequential manner by S1P and S2P (**Figure 1**) liberating its cytosolic component (Liu et al., 2007; Che et al., 2010). The cytosolic component containing the bZIP DNA

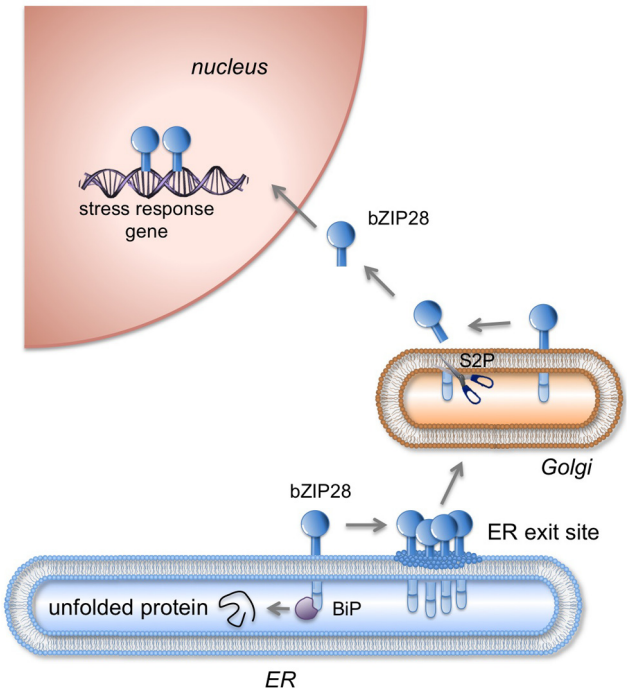
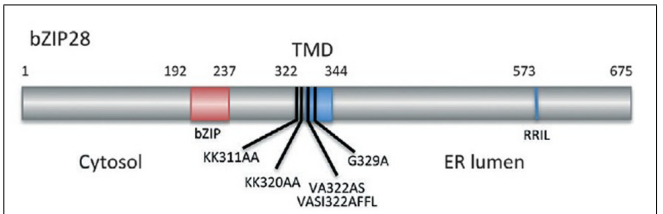


FIGURE 1 | Mobilization of bZIP28 in response to stress. Under unstressed conditions, bZIP28 resides in the ER membrane and is thought to be tethered there by the interaction of its lumenal tail with Binding Protein (BIP). In response to adverse environmental conditions or to an overload in the protein synthesis, unfolded proteins accumulate in the ER and BIP is competed away from its binding to bZIP28. Once freed, bZIP28 interacts with Sar1 GTPase a component of the COPII transport system and transported to the Golgi apparatus. In the Golgi, bZIP28 is processed by resident proteases including Site-2-Protease (S2P) liberating bZIP28's cytoplasmic domain, which relocates in the nucleus where it upregulates stress response genes. (Figure based on Srivastava et al., 2013).

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1   MTESTSVVAP PPEIPNLNPS MFSESDLFSI PPLDPLFLSD SDPISMDAPI
51  SDLDFLLDDE NGDFADFDFS FDNSDDFFDF DLSEPAVVIP EEIGNNRSNL
101 DSSENRSGDG GLEGRSESVH SQVSSQGSKT FVSDTVDASS SPESSNHQKS
151 SVSKRKKENG DSSGELRSCK YQKSDDKSVA TNNEGDDDDD KRKLIRQIRN
201 RESAQLSRLR KKQQTEELER KVKSMNATIA ELNGKIAYVM AENVALRQQM
251 AVASGAPPMN PYMAAPPLPY QWMPYPPYPV RGYGSQTPLV PIPKLNPKPV
301 SSCRPKKAES KKNEGKSKLK KVASISFTGI LFFVFLFGTL VPFMNVNFGG
351 ERGSFGGLSK YDGHRYYDEH KGRVLMVGDG SDVRRNSGIS EGNIHSSRIS
401 HGERDSCGGV DYNAHPKVEG RPSSLSNASD PLFASLYVPR NDGLVKIDGN
451 LIIHSVLASE KARGLGKKNI TETVKTKEPD LTIPGALSSA LAVPGVRGNA
501 AMLPHSTALS SEGKRLHQWF HEGGSGPLMD YSMCTEVFQF DIAPGAIVPS
551 SVSSISAEHL QNVTTHGKRM KNRRILEGLP VSLVASELNI TGTQPNKDAQ
601 NKTFNGNTNK PTSSSSMVVS VLLDPREVVD SETDRVVPPN PKSLSRIFVV
651 VLLDSVKYVT YSCVLPRSGL HLVAT
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FIGURE 2 | Map (above) of *Arabidopsis* bZIP28 showing its cytosolic, transmembrane (TMD) and lumenal domains. Also shown are the locations of the bZIP region in the cytosolic domain, the canonical S1P recognition site (RRIL) in the lumenal domain, several alanine substitution mutations and a deletion mutation (bZIP28Δ355) described in the text. Amino acid sequence of bZIP28 (below) highlights the bZIP domain and S1P recognition site (both underlined), the TMD in which the VASI sequence and helix-breaking G residue are boxed. Paired lysines involved in Sar1b binding and Golgi relocalization are indicated in bold. (Figure based on Srivastava et al., 2012).

binding and dimerization domain then relocates to the nucleus, and via recruitment of NF-Y subunits upregulates stress-response genes (Liu et al., 2007; Liu and Howell, 2010a)

ROLE OF BINDING IMMUNOGLOBULIN PROTEIN

As a sensor/transducer of UPR in plants, bZIP28 is thought to respond to ER stress in a manner similar to ATF6 in mammalian cells (Haze et al., 1999). ATF6 is also an ER membrane-bound bZIP transcription factor, with a sensor element located in the ER lumen (Chen et al., 2002).

bZIP28 senses stress through its LD (Srivastava et al., 2013; Sun et al., 2013) and through its interaction with the major ER chaperone, BIP, also known as the 78kDa glucose-regulated protein (GRP-78), which is located in the lumen of the ER (Ting and Lee, 1988; Hendershot et al., 1994; Hendershot, 2004). BIP binds to the newly synthesized proteins as they are translocated into the ER and assists in their proper folding and assembly.

BIP binds to bZIP28 under unstressed conditions preventing its mobilization in the absence of stress. It is not clear how the binding of BIP prevents bZIP28 mobilization. One idea derived from the mammalian literature is that BIP occludes the Golgi targeting signals on ATF6 (Shen et al., 2002). In *Arabidopsis*, BIP binds to the intrinsically disordered regions on bZIP28's lumen-facing tail (Srivastava et al., 2013), and it is not known whether that binding interferes with cargo recognition sites needed for bZIP28's transport from the ER to the Golgi. The crystal structure of the LD of bZIP28 has not been determined, but the predicted structure consists of a prominent β-barrel with two internal projections containing α-helix and random-coil regions (**Figure 3**). It is to the internal projections that BIP most avidly binds. In any case, BIP is released from bZIP28 in response to ER stress enabling it to exit from the ER.

The proposition that bZIP28's lumen-facing tail has Golgi localization signals (GLSs) is not in keeping with the observation that when its tail is eliminated, the protein is not retained in the ER but behaves as an activated form of bZIP28 (Srivastava et al., 2013; Sun et al., 2013). In the study by Srivastava et al. (2013), bZIP28Δ355 constitutively relocates to the nucleus where it upregulates stress response genes, such as *BiP3*. The movement of bZIP28 takes place via the Golgi and requires S2P processing. This was demonstrated by the fact that in an S2P mutant, bZIP28Δ355 does not move into the nucleus. It is interesting to note that bZIP28Δ355 lacks a S1P processing site. Cleavage at the S1P site is usually considered to be a prerequisite for S2P cleavage, which releases the transcriptional component of stress sensor/transducer from the Golgi for relocation to the nucleus (Espenshade et al., 1999; Shen and Prywes, 2004). This implies that S1P cleavage is not required for S2P proteolysis as long as the C-terminal tail of bZIP28 has been removed.

A model to account for the release of bZIP28 by BIP under stress is a dynamic competition model in which BIP is competed away

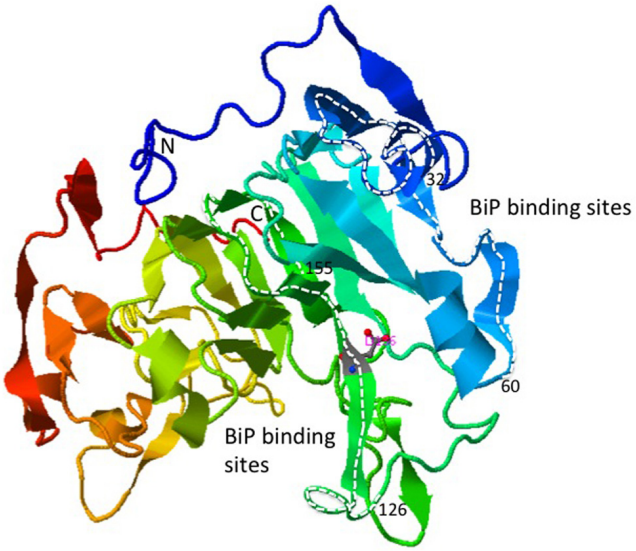

FIGURE 3 | Ribbon structure of the bZIP28 lumenal domain predicted by the template-based prediction program, I-TASSER (Zhang, 2008). The regions of the protein highlighted with the white dashed lines represent the peptides to which BIP preferentially binds in a phage display assay (Srivastava et al., 2013).

from bZIP28 by the accumulation of misfolded proteins in the ER. The model was developed in mammalian cells to explain the activation of ATF6 and IRE1 by the dissociation of BIP (Bertolotti et al., 2000; Shen et al., 2005). There are different ideas as to how BIP relinquishes its hold on ATF6 under stress conditions (Harding et al., 2002; Kaufman et al., 2002; Kimata et al., 2003; Parmar and Schroder, 2012). BIP bound to ATF6 is thought to be in equilibrium with free BIP and the BIP associated with the misfolded proteins. When unfolded proteins accumulate in the ER as a result of stress, the binding of BIP to ATF6 is competed away. Support for this model in mammalian cells has come from the overexpression of BIP, which was shown to attenuate UPR (Dorner et al., 1992; Kohno, 2010). The effects of BIP underexpression have been difficult to document in mammalian cells, which have only one BIP, and its knockout is lethal (Shen et al., 2002).

Arabidopsis has three BIP genes (*BIP1*, *-2*, and *-3*). BIP1 and 2 are almost identical proteins, and *BIP3* is expressed at elevated levels in response to abiotic stress or ER stress agents (Koizumi, 1996; Martinez and Chrispeels, 2003). The overexpression of either *BIP1* or *BIP3* by transgenesis delays the release of bZIP28 and does not allow the complete deployment of bZIP28 to combat stress. As for BIP underexpression, double homozygous mutant lines of BIP1 and 2 are lethal in *Arabidopsis*, but hemizygous knockout lines have been produced (Maruyama et al., 2010). In hemizygous BIP knockout lines, bZIP28 is released from the ER even under unstressed conditions (Srivastava et al., 2013). Thus, the results involving the overexpression and underexpression of BIP in *Arabidopsis* support the dynamic equilibrium model and demonstrate the critical role that BIP plays in the retention and activation of bZIP28 in *Arabidopsis*. The model in plants has been further supported by the observation that overexpression of BIP in tobacco helps to alleviate ER stress responses (Leborgne-Castel et al., 1999). It was found that overexpression of a BIP transgene downregulated endogenous BIP mRNA levels and reduced the UPR.

However, detractors of the dynamic equilibrium model argue that BIP is present in millimolar quantities in the ER and that slight fluctuations in folding state of ER proteins would not be able to shift the equilibrium and compete BIP away from its binding to ER stress sensor/transducers (Credle et al., 2005). Therefore, another model for the release of ATF6 from BIP in animal systems has been evoked that does not involve dynamic competition. Instead this model proposes that the association is stable but can be disrupted by a signal from misfolded proteins. Several arguments favor a stability model (Shen et al., 2005). BIP appears to recognize ATF6 as an unfolded client protein in that mutations in the substrate-binding domain of human BIP (such as P495L) inhibited the binding of BIP to ATF6. Also mutations in the ATPase domain (such as T37G) prevented the dissociation of BIP from ATF6 by ATP even when these complexes were purified. However such complexes were dissociated very efficiently when ER stress was induced by dithiothreitol (DTT), though not by detergents *in vitro*.

OTHER FACTORS THAT MAY REGULATE THE ACTIVATION OF bZIP28

The ER is a calcium-rich, oxidizing environment and imbalances in energy charge levels, redox conditions or interaction with DNAJ proteins could activate ER stress sensors. It has been observed in mammalian cells that the LD of ATF6 forms inter- and intramolecular disulfide bridges between its two conserved cysteine residues. In the absence of ER stress, ATF6 is found as monomer, dimer and oligomers in the ER (Nadanaka et al., 2007; Sato et al., 2011). Upon ER stress, due to the reduction of disulfide bridges, ATF6 is thought to depolymerize and to exit from the ER.

The ER has evolved specific posttranslational modifications and quality control mechanisms to prepare proteins for the extracellular environment. These modifications dramatically enhance the stability of secreted proteins. A major posttranslational modification of ER-synthesized proteins is disulfide bridge formation, which is catalyzed by the family of protein disulfide isomerases (PDIs; Ellgaard, 2004; Banhegyi et al., 2007; Andeme Ondzighi et al., 2008; Feige and Hendershot, 2011). PDI is the founding member of the ER PID family. Treatment of animal and plant cells with DTT results in ER stress due to the disturbance in the redox balance of the cells (Frand and Kaiser, 1998; Liu et al., 2007). The LD of ATF6 was found to associate physically with PDI under unstressed conditions implicating its role in imparting stability to ATF6 in the ER (Sato et al., 2011). Similar associations may also be expected with bZIP28 that might influence its function.

Hong et al. (2004) had shown that the glycosylation status of ATF6 is important in its interaction with the chaperone calreticulin. Under ER stress conditions, ATF6 is undergylcosylated, a condition which fails to promote its association with calreticulin and its retention in the ER. Liu et al. (2007) showed that the bZIP28 is glycosylated and, therefore, its glycosylation status may influence its interactions with ER chaperones and its retention in the ER.

DnaJ/Hsp40 (heat shock protein 40) proteins are important factors in chaperoning and protein folding primarily by stimulating the ATPase activity of Hsp70 proteins, which stabilizes the

interaction of these chaperones with their substrate proteins (Shen and Hendershot, 2005). Six ER localized DNAJ proteins that have been identified and are referred to as ERDdj1–6 (Jin et al., 2009). One of these, ERdj-3, is a soluble lumenal DNAJ family member. It is known to bind to BIP chaperone complexes in the ER and associates with a number of other unfolded proteins that are BIP substrates (Jin et al., 2008, 2009). An ERdj-3 mutant that does not bind to BIP still retains its ability to bind to unfolded proteins directly. BIP assists in the release of ERdj-3 from its substrate. The mutants of BIP that do not allow the release of ERdj-3 disrupt these association–dissociation processes (Awad et al., 2008; Jin et al., 2008). Erdj-3 is therefore a candidate for binding to the LD of ER membrane associated bZIP transcription factors such as bZIP28 and to contribute to their activation.

EXIT OF bZIP28 FROM THE ER AND FURTHER ORGANELLE TRANSLOCATION

The release of bZIP28 from BIP corresponds closely with its exit from the ER, but its release is not dependent on the ability of bZIP28 to traffick from organelle to organelle. As described above, in animal systems, it is thought that BIP binding retains ATF6 in the ER under unstressed conditions, putatively by blocking ATF6's GLSs, preventing its transport through the secretory pathway (Shen et al., 2002). BIP's release unmasks the two GLSs, GLS1, and GLS2 on ATF6. GLS1 binds to BIP while GLS2 is inactive. On dissociation of BIP, GLS2 directs ATF6 to the Golgi. Sequences similar to the GLSs in ATF6 have not been detected in bZIP28. Dissociation of BIP from bZIP28 only enables it to exit from the ER. Further organelle-to-organelle movement of bZIP28 is governed by other factors.

The mechanism of transport of proteins from the ER to the Golgi in plant cells is not completely resolved. It is unclear whether plants utilize intermediate compartments in the movement of ER cargo to the Golgi (Yang et al., 2005). The exit of cargo from the ER to the Golgi in yeast and animal cells involves COPII vesicles, but COPII vesicles have yet to be visualized convincingly in plant cells. However, mutations that affect the COPII system in plants disrupt protein transport from the ER to the Golgi (Marti et al., 2010). Some of the factors involved in the initiation of COPII vesicle assembly are a GTPase, Sar1, and Sec12, a guanine nucleotide exchange factor (Miller and Barlowe, 2010; Russell and Stagg, 2010). bZIP28 has been shown to interact with Sar1b and Sec12, and this association appears to play an important role in the translocation of bZIP28 from ER to the Golgi (Srivastava et al., 2012). Sar1b is one of the several plant Sar1 forms identified in *Arabidopsis* (Hanton et al., 2008). Sar1 can further recruit Sec23/24, the inner COPII vesicle components (Kuehn et al., 1998; Aridor et al., 2001; Bi et al., 2002).

A basal level of interaction between Sar1b and Sec12 with bZIP28 is seen even under unstressed conditions but stress treatment enhances this interaction several fold. This basal level of interaction between bZIP28 and COPII components is apparently not sufficient to initiate the transport of bZIP28 to the Golgi. Under stress conditions, a threshold level of interaction between bZIP28 and Sar1 is apparently met, and bZIP28 is mobilized (Srivastava et al., 2012). The interaction of bZIP28 with Sar1 requires the presence of dibasic residues on the cytosolic side of bZIP28 near the TMD (**Figure 2**). Two neighboring KK motifs at this location have a combined role in Sar1 binding to bZIP28, because substitution of charged residues to this pair of motifs results in a loss of Sar1b binding (Srivastava et al., 2012). It is believed that a charged pocket is created as a result of the paired lysines existing in close proximity to each other (Giraudo and Maccioni, 2003). ATF6 has also been shown to translocate to the Golgi with the aid of COPII vesicles (Schindler and Schekman, 2009).

Once bZIP28 exits the ER and moves into the Golgi, S2P proteolytically processes and releases the cytoplasmic-facing components of bZIP28 from the Golgi. S2P is an intramembrane metalloprotease (Feng et al., 2007) involved in regulated intramembrane proteolysis (RIP). The targets for intramembrane proteases are TMDs, and important residues in the TMDs of substrate proteins for a class of intramembrane proteases called rhomboids have been identified (Freeman, 2004). The substrates for S2P in mammalian systems are TMDs with a mid-domain helix-breaking residue and hydrophilic residues at the membrane boundary that are thought to provide a water channel for the intramembrane hydrolysis reaction (Urban and Freeman, 2003). The helix-breaking residues are thought to destabilize the TMD α-helix, causing a locally disordered conformation of the TMD and providing access of the protease to the substrate. Some of these residues are also found in the TMD of bZIP28, and a G329A mutation introduced into the middle of bZIP28's TMD demonstrated the importance of a helix-breaking G residue for proteolysis by S2P. The mutation resulted in a loss of bZIP28 processing, leading to its retention in the Golgi and a block in its translocation to the nucleus (Srivastava et al., 2012).

A hydrophilic VASI sequence at the cytosolic face of the TMD was hypothesized to be a channel permitting entry of water into the membrane interior for hydrolysis of the bond cleaved by rhomboid proteases (Urban and Freeman, 2003). To determine if the VASI sequence in bZIP28 was likewise required for proteolysis, it was substituted by a sequence to reduce the hydrophilicity of the region at the cytosolic boundary of bZIP28's TMD. These mutations had no effect on the proteolysis and movement of bZIP28 (Srivastava et al., 2012). Hence, *Arabidopsis* S2P may utilize a different mechanism for water entry to catalyze the intramembrane hydrolysis of bZIP28.

CONCLUSION AND FUTURE INSIGHTS

The sensing of ER stress involves the association or dissociation of the ER chaperone, BIP, from bZIP28. bZIP28 is retained in the ER during unstressed conditions by its interaction with BIP. Likewise, the mobilization of bZIP28 in response to stress involves its dissociation from the BIP and its association with COPII factors on its cytoplasmic face. Further organellar movement of bZIP28 is guided by residues in the TMD. Other unknown factors may also have a role in the activation and movement of bZIP28 and need to be explored.

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ER-mediated control for abundance, quality, and signaling of transmembrane immune receptors in plants

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Plants recognize a wide range of microbes with cell-surface and intracellular immune receptors. Transmembrane pattern recognition receptors (PRRs) initiate immune responses upon recognition of cognate ligands characteristic of microbes or aberrant cellular states, designated microbe-associated molecular patterns or danger-associated molecular patterns (DAMPs), respectively. Pattern-triggered immunity provides a first line of defense that restricts the invasion and propagation of both adapted and non-adapted pathogens. Receptor kinases (RKs) and receptor-like proteins (RLPs) with an extracellular leucine-rich repeat or lysine-motif (LysM) domain are extensively used as PRRs. The correct folding of the extracellular domain of these receptors is under quality control (QC) in the endoplasmic reticulum (ER), which thus provides a critical step in plant immunity. Genetic and structural insight suggests that ERQC regulates not only the abundance and quality of transmembrane receptors but also affects signal sorting between multi-branched pathways downstream of the receptor. However, ERQC dysfunction can also positively stimulate plant immunity, possibly through cell death and DAMP signaling pathways.

Keywords: ERQC, ER stress, MAMP/DAMP, immune receptor, LRR/LysM

INTRODUCTION

Plants sense their encounters to microbes through immune receptors that monitor extracellular or intracellular spaces for pathogen-associated ligands (Jones and Dangl, 2006; Boller and Felix, 2009; Dodds and Rathjen, 2010). Cell-surface receptors involve not only PRRs that recognize microbe-associated molecular patterns (MAMPs) or danger-associated molecular patterns (DAMPs) to confer pattern-triggered immunity (PTI), but also the resistance (R) proteins that recognize pathogen effectors to confer effector-triggered immunity (ETI). Among the former, FLS2 and EFR recognize the bacterial MAMPs flagellin (flg22 epitope) and the elongation factor EF-Tu (elf18 epitope), respectively (Gomez-Gomez and Boller, 2000; Zipfel et al., 2006). Among the latter, the tomato LRR-RLPs Cf proteins and Ve1 and the rice LRR-RK XA21 confer immunity against *Cladosporium fulvum*, *Verticillium dahliae*, and *Xanthomonas oryzae*, respectively (Song et al., 1995; Rivas and Thomas, 2005; de Jonge et al., 2012). Adapted pathogens can escape or overcome the defenses mounted by these extracellular receptors, with the aid of effectors. However, pathogen effectors acting within the host cells are directly or indirectly recognized by intracellular nucleotide-binding and LRR (NB-LRR) domain-containing R proteins, which leads to strong ETI activation that terminates pathogen growth.

The ER plays a central role in the biogenesis and intracellular distribution of transmembrane receptors according to their folding states and cellular demands. Hence, the ER homeostasis and prompt adaptation to ER stress are vital for plant life and health.

TRANSMEMBRANE IMMUNE RECEPTORS AND REGULATORS IN PLANTS

In plants, the extracellular leucine-rich repeat (LRR) and lysine-motif (LysM) domains define two major structural modules that recur in the extracellular domain of PRRs. PRRs include the LRR-RKs FLS2, EFR, and PEPR1/2 (for the endogenous Pep peptides; Krol et al., 2010; Yamaguchi et al., 2010), the LRR-RLPs LeEIX1/2 (for fungal xylanase; Ron and Avni, 2004), the LysM-RK CERK1 (for fungal chitin; Miya et al., 2007; Wan et al., 2008), the LysM-RLPs CEBiP (for fungal chitin; Kaku et al., 2006) and LYM1 and LYM3 (for bacterial peptidoglycan; Willmann et al., 2011). PRR-associated RKs and/or RLPs have been also described. The LRR-RK BAK1 (and/or its related SERK members) associates with numerous LRR-RKs, including the PRRs FLS2, EFR, and PEPR1/2, and also the defense regulators BIR1 and SOBIR1/EVR (Chinchilla et al., 2007; Ryan et al., 2007; Gao et al., 2009; Postel et al., 2010; Schulze et al., 2010). The tomato SOBIR1 ortholog interacts with Cf-4 and Ve-1 and is required for their ETI functions (Liebrand et al., 2013b). SOBIR1/EVR also acts together with the LRR-RLP RLP30, apparently in PTI against *Sclerotinia sclerotiorum* (Zhang et al., 2013). CERK1 also serves LYM1 and LYM3 in peptidoglycan perception (Willmann et al., 2011). Given the BAK1-independence of CERK1-mediated chitin signaling (Heese et al., 2007; Shan et al., 2008; Gimenez-Ibanez et al., 2009), the LRR-PRRs and LysM-PRRs might act in separate receptor complexes. In sum, extensive engagement of transmembrane receptors and regulators represents a key principle in plant immunity.

EVOLUTIONARILY CONSERVED ERQC PATHWAYS UNDERLIE PLANT-SPECIFIC SECRETORY PROCESSES

In eukaryotes, including plants, the biogenesis of transmembrane or secretory proteins occurs through the ER. Folding status of these proteins are monitored during their folding and maturation by a mechanism termed ERQC that ensures the delivery of properly folded proteins to their functional sites (Anelli and Sitia, 2008). This is essential in all eukaryotes tested (Kelleher and Gilmore, 2006). One pathway employs the Hsp70 family member BiP that acts in a multi-protein complex with the Hsp40 family members ERdJ and stromal cell-derived factor 2 (SDF2; Meunier et al., 2002). A second pathway relies on Asn (N)-glycosylation ($Glc_3Man_9GlcNAc_2$ conjugation) on the nascent client proteins catalyzed by the oligosaccharyltransferase (OST) complex. An N-glycosylation inhibitor, tunicamycin, is widely used as an inducer of ER stress in plants (Koizumi et al., 1999). Subsequent folding of N-glycosylated proteins occurs through a pathway involving glucosidases I and II (GI and GII), the folding cycle via the ER chaperones calreticulin (CRT) and calnexin (CNX), and UDP-glucose:glycoprotein glucosyltransferase (UGGT) that are highly conserved in eukaryotes (Kelleher and Gilmore, 2006). However, plants exhibit better tolerance to single gene disruptions in the N-glycosylation pathway downstream of the OST-mediated step (see below). *N*-glycoproteomics studies comparing seven model organisms including *Arabidopsis* also revealed the existence of lineage-specific N-glycosylated proteomes in a much larger portion than previously thought (Zielinska et al., 2012). Over-representation of extracellular functions in lineage-specific *N*-glycoproteomes implies a role for N-glycosylation in the divergence of extracellular and/or secretory functions unique to the plant lineage.

ERQC FOR PLANT IMMUNE RECEPTORS AND REGULATORS

Genetic tractability for ERQC components in plant models facilitates unraveling their roles in different branches of plant immunity. Genetic studies on *Arabidopsis* elf18-hyposensitive mutants led to the discovery for an ER N-glycosylation pathway involving the OST complex subunits STT3A and OST3/6, CRT3, UGGT, GII that is essential for EFR but not FLS2 biogenesis (Li et al., 2009; Lu et al., 2009; Nekrasov et al., 2009; Saijo et al., 2009; Haweker et al., 2010; Farid et al., 2013). Consistently, compared to FLS2, EFR is highly vulnerable toward chemical interference with the OST function or N-glycosylation site substitutions in the receptor LRR domain (Nekrasov et al., 2009; Saijo et al., 2009; Haweker et al., 2010; Sun et al., 2012). In addition, EFR biogenesis also specifically requires SDF2, ERdj3b, and BiP (Nekrasov et al., 2009). It remains elusive how these ERQC pathways are coordinated.

This N-glycosylation pathway is also required for SA-inducible but EFR-independent resistance (Saijo et al., 2009). Consistent with this, SA-induced resistance is reduced in the absence of BiPs or the OST subunit DAD1 (Wang et al., 2005). By contrast, DAD1 is dispensable for EFR accumulation and function (Haweker et al., 2010). In *Nicotiana benthamiana* that inherently lacks EFR, CRT3a is also required for PTI to the oomycete pathogen *Phytophthora infestans* (Matsukawa et al., 2013). These findings imply the existence of another ERQC client receptor(s) than EFR mediating these immune responses.

Genetic studies with misfolded but signaling-competent alleles of the LRR-RK brassinosteroid receptor BRI1, designated *bri1-5* and *bri1-9*, have also revealed a role for a common set of ERQC components, CRT3, UGGT, and BiP, in the ER retention of the BRI1 variants (Jin et al., 2007, 2009; Hong et al., 2008). However, OST3/6 seems to be dispensable for this ERQC (Farid et al., 2013). This again points to partial client-specific divergence in the usage of the OST complex subunits in the N-glycosylation pathway. The overall composition and precise mode of actions for the OST complex subunits remain to be elucidated.

Proteomics studies revealed BiPs and CRTs among Cf-4- and Ve1 interacting proteins *in vivo* (Liebrand et al., 2012, 2013a). Of four BiP members and three CRT members in tomato and *N. benthamiana*, only silencing of CRT3a (a plant-specific CRT; Christensen et al., 2010) compromises the proper glycosylation and biogenesis of functional Cf-4 protein. By contrast, silencing of single BiP members (except BiP4) and CRT members impaired Ve1-mediated resistance, without a significant decrease in the accumulation of complex glycan-conjugated Ve1. It is possible that these ER chaperones rather serve the LRR-RKs SOBIR1, SERK1, or BAK1 that is required for Ve1 function (Fradin et al., 2009, 2011; Liebrand et al., 2013b). Likewise, BiP3, SDF2, ERdj3B, CNX1, and CRT3 were recovered among XA21-associated proteins from rice plants (Park et al., 2010, 2013). BiP3 overexpression and SDF2 silencing both lower XA21-mediated resistance, pointing to a critical role for the ER homeostasis in this receptor pathway.

Compared to CRT3, CRT2 appears to have a minor role in PTI (Li et al., 2009; Christensen et al., 2010). True CRT2 function might be obscured by its dual role: CRT2 overexpression in *Arabidopsis* led to constitutive SA accumulation and *PR* gene activation in a manner dependent on its C-terminal Ca^{2+}-binding domain, but it rather lowered bacterial resistance (Qiu et al., 2011). However, the perturbation of the N-terminal chaperone domain allowed CRT2 to enhance bacterial resistance. It might be that CRT2 chaperone function serves a defense suppressor whilst Ca^{2+}-buffering function promotes SA-based immunity. In *N. benthamiana*, both CRT2 and CRT3 are required for the expression of the LRR-RK IRK1 that is essential for the NB-LRR R protein-mediated ETI to *Tobacco mosaic virus* (Caplan et al., 2009).

CRT-mediated defense suppression is exploited by the root-knot nematode *Meloidogyne incognita* for virulence promotion (Jaouannet et al., 2013). The nematode secretes CRT, which can suppress MAMP responses, into the apoplastic spaces during plant infection. In plants and animals, CRTs localize not only inside but also outside the ER (Baluska et al., 1999; Sharma et al., 2004; Krysko et al., 2013). Whether extracellular CRT pools modulate immunity in plants, like in mammals, requires future investigation.

In contrast to the aforementioned receptors that strictly require a subset of ERQC components for their biogenesis and/or function, there are also receptors that exhibit relative robustness to ERQC dysfunction, such as FLS2 and PEPR1/2 (Tintor et al., 2013). Future studies will be required to elucidate the molecular determinants for the differences in ERQC dependency.

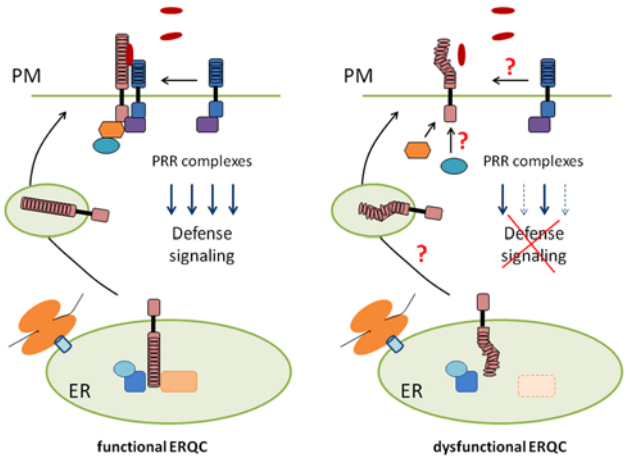

FIGURE 1 | A model for ER-mediated control of PRR biogenesis and signaling. In weakly dysfunctional alleles of ERQC components (right), folding defects of the extracellular domain of PRRs might affect stable accumulation at the plasma membrane (PM), subcellular trafficking, assembly of pre- and post-recognition complexes, or combinations thereof. Importantly, this can selectively impair a subset of diverse signaling outputs downstream of the receptor.

ROLE FOR THE LRR DOMAIN CONFORMATION IN IMMUNE RECEPTOR COMPLEX FORMATION AND SIGNALING

The question of how a single receptor governs diverse signaling outputs represents an important challenge in receptor biology. In weakly dysfunctional alleles of CRT3 and GII, the tested signaling outputs of EFR are differentially, but not uniformly, impaired without a significant decrease in the receptor steady-state levels (Lu et al., 2009; Saijo et al., 2009). In these alleles, the degree of signaling defects is correlated with that of decreases in EFR-ligand binding. It remains elusive how the lowered ligand binding differentially affects multi-branched signaling pathways emanating from the receptor. Nevertheless, these findings predict that signal separation for these diverse outputs occurs at the level of or in the proximity to the receptor, and can be influenced by the folding states of the LRR domain (**Figure 1**).

A crystal structure of flg22-bound FLS2 and BAK1 ectodomains revealed that the FLS2 ectodomain forms a superhelical structure with the flg22 binding site in the concave surface of the LRR 3–16 (Sun et al., 2013). This is in agreement with previous studies on the receptor-ligand binding (Dunning et al., 2007; Mueller et al., 2012). Importantly, flg22 binding occurs in the FLS2-BAK1 interface and seems to stabilize their interaction. Strikingly, perturbations of the FLS2-BAK1 interface differentially affect separate signaling outputs. BAK1 point substitutions in key residues of this interface almost abolished MPK4/MPK11 activation without substantial effects on MPK3/MPK6 activation. Flg22 variants incapable of BAK1 binding reduce FLS2-BAK1 interaction and a ROS burst without affecting MPK activation. These results suggest that changes in either FLS2 or BAK1 LRR conformation alter the receptor complex formation and function, and importantly, which selectively influences downstream signaling pathways. This model further predicts the existence of distinct signaling complexes defined by the LRR domain conformations of FLS2 and BAK1, which are specifically assigned to diverse signaling outputs. Such complexes might be separated spatiotemporally from each other and/or different in the composition of accessory proteins.

SUBCELLULAR PARTITIONING AND TURNOVER OF THE RECEPTOR IN PATTERN RECOGNITION RECEPTORS SIGNALING

The mechanisms underlying the subcellular partitioning of PRRs between their biogenesis and functional sites are still poorly understood in plants (Popescu, 2012). The reticulon-like proteins RTNLB1/RTNLB2 regulate the accumulation of functional FLS2 at the PM, possibly by controlling the ER exit of FLS2 (Lee et al., 2011). In rice, a chaperone complex consisting of Hsp90 and its co-chaperon Hop/Sti1 promote the delivery of CERK1 from the ER to the PM (Chen et al., 2010). This work also raises the possibility that PRRs associate with their signaling partners during their travel in the secretory pathway. Detailed cell biological studies are needed to precisely decipher the subcellular dynamics of these immune receptors.

PRR ubiquitination has emerged as a determinant for the receptor levels by targeting the receptors for degradation and/or modulating their membrane trafficking. Direct ubiquitination and subsequent degradation of the receptor contributes to signal attenuation for FLS2 (Lu et al., 2011). Upon flg22 binding, the FLS2 complex recruits two related U-box E3 ligases, PUB12 and PUB13, along with BAK1. A flg22-induced increase in BAK1 kinase activity on PUB12/PUB13 suggests a model in which BAK1-mediated phosphorylation promotes PUB12/13-mediated FLS2 ubiquitination. However, given that SOBIR1 is required to stabilize Cf-4 (Liebrand et al., 2013b), degradation of one of the receptor-interacting proteins might cause the receptor destabilization. Future investigation is required for how trans-phosphorylation between FLS2 and BAK1 influences the recruitment and phosphorylation of PUB12/PUB13 and how PUB phosphorylation influences the E3 ligase activity. Another E3 ligase triplet, PUB22/23/24 also acts as a negative regulator for FLS2, EFR, and PEPR signaling (Trujillo et al., 2008; Stegmann et al., 2012). Yeast two-hybrid screens for the E3 ligase interactors led to the identification of the exocyst subunit EXO70B2 as a target for PUB22-mediated ubiquitination (Stegmann et al., 2012). EXO70B2 contributes to different PTI-associated outputs, implying its influence on the receptor function, possibly through maintaining the PM receptor pool. The defects of *exo70b2* mutants in responses to different MAMPs and pathogens point to a role for EXO70B2 in a common step between different receptor pathways. Exocyst subunit degradation might lower the delivery of these receptors to the PM. However, compared to the *pub* triple mutants, the defects of *exo70b2* mutants in PTI-related outputs are much smaller, implying the existence of another ubquitination target(s) for these E3 ligases. Interestingly, the *Medicago truncatula* E3 ligase PUB1 has been also described to interact with and negatively regulate the LysM-RLK LYK3-mediated nodulation (Mbengue et al., 2010). This molecular logic might be widespread for transmembrane receptors in plant-microbe interactions.

Pattern recognition receptors undergo ligand-induced internalization from the PM, as described for FLS2 and LeEIX2 (Robatzek

et al., 2006; Bar and Avni, 2009). Although this process is closely correlated with immune signaling activation, it remains to be determined whether it serves signal activation or attenuation of the PRRs. Recent studies showed that flg22 perception transiently lowers the steady-state FLS2 levels and causes signal de-sensitization, which is followed by the replenishment of FLS2 accumulation and signal re-sensitization (Smith et al., 2013). It is of high interest to correlate this turnover event with subcellular dynamics of the receptor.

ENDOPLASMIC RETICULUM STRESS AND DEFENSE REGULATION

Overloading of misfolded proteins beyond the capacity of ERQC, termed ER stress, induces the so-called unfolded protein response (UPR) that is characterized by the induction of ER chaperones. Although close associations between UPR, disease, and immunity have been well documented in animals (Todd et al., 2008), much less information is available in plants (Vitale and Boston, 2008; Eichmann and Schafer, 2012). Two arms of UPR signaling have been described in *Arabidopsis* that involve the transmembrane transcription factors (TFs) bZIP17/bZIP28 and the protein kinase/ribonuclease IRE1 (Iwata and Koizumi, 2012; Howell, 2013). IRE1 serves to generate an active form of the TF bZIP60 via its mRNA processing. The two homologues IRE1a and IRE1b, as well as bZIP60, contribute to SA-mediated antibacterial immunity (Moreno et al., 2012). It seems likely that UPR-based increase of ERQC capacity ensures the supply of functional transmembrane regulators and alleviates cell death or damages that are caused by excessive ER stress (Howell, 2013). Indeed, *ire1a ire1b* plants show enhanced cell death upon ER stress, pointing to a role for the proper UPR in the ER homeostasis (Mishiba et al., 2013). ER stress-induced cell death is exploited by the mutualistic fungus *Piriformospora indica* for *Arabidopsis* root infection (Qiang et al., 2012). The fungal colonization is dependent on active suppression of the host UPR, ER disintegration and subsequent vacuolar processing enzyme-mediated vacuolar collapse leading to cell death in the colonized cells. Impaired ER integrity might disturb vesicular secretion of antimicrobial and/or defense signaling molecules, which could also facilitate fungal colonization.

Excessive ER stress also induces autophagy, another link to plant defense responses (see more details in Hayward and Dinesh-Kumar, 2011; Teh and Hofius, 2014). Both pro-survival and pro-death functions have been assigned to autophagy in the control of immune responses. Likewise, excessive ER stress can also positively influence plant immunity. In this respect, it is conceivable that ER stress-induced cell death emits DAMPs, as documented in animals (Krysko et al., 2013). This possibility remains understudied in plants. ER stress induces the TF OsWRKY45 that promotes SA-based defense in rice (Hayashi et al., 2012) and systemic immunity in cucumber (Sticher and Metraux, 2000). The mechanisms by which excessive ER stress is sensed and linked to defense activation represent an important future challenge.

CONCLUSION AND PERSPECTIVE

The ER regulates the abundance, quality and signaling function of transmembrane immune receptors. Genetic and proteomic studies led to the identification of ERQC components that are critical for PRR biogenesis in different plant species. Interestingly, emerging evidence points to a role for ERQC in receptor signal sorting, possibly through controlling the folding states and thus conformations of the extracellular domains. Future studies will be needed to reveal how ERQC modulates the turnover, subcellular dynamics, complex assembly, and post-recognition signaling of PRRs. It is also of importance to show how PRR biogenesis is rewired during pathogen challenges, according to the extent of ER stress arisen. Excessive ER stress might facilitate to engage DAMP-mediated immune systems that are tolerant to ERQC dysfunction, and thereby can ultimately enhance plant immunity.

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The ER quality control and ER associated degradation machineries are vital for viral pathogenesis

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The endoplasmic reticulum (ER) is central to protein production and membrane lipid synthesis. The unfolded protein response (UPR) supports cellular metabolism by ensuring protein quality control in the ER. Most positive strand RNA viruses cause extensive remodeling of membranes and require active membrane synthesis to promote infection. How viruses interact with the cellular machinery controlling membrane metabolism is largely unknown. Furthermore, there is mounting data pointing to the importance of the UPR and ER associated degradation (ERAD) machineries in viral pathogenesis in eukaryotes emerging topic. For many viruses, the UPR is an early event that is essential for persistent infection and benefits virus replication. In addition, many viruses are reported to commandeer ER resident chaperones to contribute to virus replication and intercellular movement. In particular, calreticulin, the ubiquitin machinery, and the 26S proteasome are most commonly identified components of the UPR and ERAD machinery that also regulate virus infection. In addition, researchers have noted a link between UPR and autophagy. It is well accepted that positive strand RNA viruses use autophagic membranes as scaffolds to support replication and assembly. However this topic has yet to be explored using plant viruses. The goal of research on this topic is to uncover how viruses interact with this ER-related machinery and to use this information for designing novel strategies to boost immune responses to virus infection.

Keywords: plant virus interactome, virus–host interactions, virus–membrane interactions, unfolded protein response, ubiquitin proteasome system, chaperones, ERAD

INTRODUCTION

The endoplasmic reticulum (ER) and Golgi apparatus comprise a fundamental endomembrane compartment for de novo protein synthesis. In the last 20 years, researchers have begun to uncover the protein quality control (QC) machineries that are housed in the ER and that tightly regulate protein production (Brandizzi et al., 2003; Zhao and Ackerman, 2006; Urade, 2009; Moreno and Orellana, 2011; Parmar and Schroder, 2012; Verchot, 2012). The ER QC machinery provides: (a) chaperone-assisted protein folding and assembly; (b) post-translational modification of proteins; and (c) protein transport out of the ER for maturation and secretion. The ER is also the major site for synthesis of membrane related phospholipids and membrane embedded proteins (Fagone and Jackowski, 2009). Cellular membranes are essential to compartmentalize functions, manage energy production, storage, and cell-to-cell communication. Cellular membranes function to segregate environments for protein synthesis, modification, secretion, and degradation.

Proteins that do not successfully progress through these mechanisms are categorized as malformed proteins and are subjected to ER associated degradation (ERAD). Typically, the toxic accumulation of malformed proteins as the result of biotic or abiotic stress, activates the unfolded protein response (UPR), which is a signaling network initiated at the ER. Protein sensors that reside in the ER (such as IRE1 and PERK) respond to ER stress by increasing transcription of a set of genes encoding ER resident chaperones (Parmar and Schroder, 2012) to enhance the protein folding capacity of the ER. Malformed proteins that cannot be refolded are sequestered, modified by ubiquitination, and degraded by the 26S proteasome (Meusser et al., 2005; Muller et al., 2005). The ubiquitin proteasome system (UPS) is a major regulatory system that contributes to all aspects of cell biology, not just ERAD. Both the ER QC and UPS machinery are widely conserved among eukaryotes. Researchers are just beginning to understand the role of the UPS in plant virus infection and immunity.

Serving as a model process in the field of stress biology, positive strand RNA viruses infecting mammals and plants pose an enormous biosynthetic burden on the ER. To aid cellular adaptation to infection, viruses trigger vigorous membrane and protein synthesis, and/or protein transfer to the Golgi apparatus (Netherton et al., 2007). Host gene expression is transiently enhanced to adapt to the immediate needs of virus gene expression, mitigate ER stress, and create a cellular environment that tolerates virus infection. Invading viral pathogens manipulate the ER QC machinery to: (1) support replication and protein production; (2) accommodate the translational needs of defense related transcripts; (3) subvert components of the system in a manner that promotes infection (Jelitto-Van Dooren et al., 1999). Researchers are working to determine when viruses highjack the intact ER QC machinery to promote viral protein production and when viruses isolate critical ER chaperones and divert them for other processes that are essential to infection (Nagy et al., 2011;

Verchot, 2012). One notion is researchers have considered is that membrane expansion to accommodate virus infection is somehow linked to UPR induction early in infection. However, recent data with *dengue virus* (DEN) indicates that early induction of UPR is correlated with membrane rearrangement and synthesis but is not directly responsible for changes in membrane composition (Pena and Harris, 2012). Thus, it is not clear if or how these two early events are directly linked.

In mammalian systems, researchers have described the relationship between the UPR, autophagy and oxidative stress (Ke and Chen, 2011b). Viruses that cause a huge burden to global health such *as hepatitis C virus* (HCV) and DEN exploit the UPR to modulate autophagy and oxidative stress pathways. This is essential to promote virus replication and evade host immunity (Ciccaglione et al., 2007; Urade, 2009; Costa et al., 2010; Jouan et al., 2012; Shinohara et al., 2013). The mechanism by which these and other viruses subvert the UPR is not resolved (Diehl et al., 2011; Estrabaud et al., 2011). In plants, UPR and autophagy are linked to plant immune responses (Moreno et al., 2012). The gene expression profiles of *potato virus X* (PVX) infected hosts have revealed enhanced transcription of ER QC machinery needed to expand the protein folding capacity of the ER (Ye and Verchot, 2011; Ye et al., 2013). Nonetheless, there have been fewer studies in plants examining the role of UPR and autophagy in immune evasion by plant viruses.

PLANT VIRAL INTERACTIONS WITH CELLULAR MEMBRANES ARE ESSENTIAL FOR REPLICATION AND EGRESS

In support of virus replication, positive strand RNA viruses cause extensive reorganization of cellular membranes and create subcellular compartments, called "viroplasms" (Castellano and Martelli, 1984; den Boon et al., 2010; Verchot, 2011). Such membrane bound compartments provide a protective environment and maintain the necessary viral and host proteins in proximity to the genomic template. Plant viruses belonging to the genera *bromovirus*, *comovirus*, *dianthovirus, nepovirus, pecluvirus*, *potexvirus*, recruit ER membranes to create viroplasms (Carette et al., 2000; Dunoyer et al., 2002; Ritzenthaler et al., 2002; Turner et al., 2004; Diaz et al., 2010; Verchot, 2011) while *potyviruses* use both ER and chloroplast membranes (Wei and Wang, 2008; Wei et al., 2010). Furthermore, many viruses induce massive membrane synthesis or alter the lipid composition of certain membranes needed for formation of these replication centers. In particular, *brome mosaic virus* (BMV), *cowpea mosaic virus* (CPMV), *grapevine fanleaf mosaic virus* (GFLV), and are among the well-studied examples (Carette et al., 2000; Ritzenthaler et al., 2002; Noueiry and Ahlquist, 2003). However, the signal transduction mechanism that controls ER membrane proliferation and their phospholipid profiles has not yet been elucidated.

Many plant RNA viruses encode movement proteins that interact with an active ER network to move from cell to cell across plasmodesmata (Boevink and Oparka, 2005; Tilsner et al., 2010, 2011; Genoves et al., 2011). Most known plant virus movement proteins are categorized into one of four recognized superfamilies (Melcher, 2000). The 30K superfamily encodes movement proteins related to the *tobacco mosaic virus* (TMV) 30K movement protein. Viruses belonging to the 30K superfamily are reported to encode movement proteins that carry viral genomic RNAs across the plasmodesmata. The TMV 30K protein, in particular, transports replication complexes across plasmodesmata in a manner that is dependent on both the ER and microfilament networks (Kawakami et al., 2004; Wright et al., 2007; Guenoune-Gelbart et al., 2008; Sambade et al., 2008; Niehl et al., 2013; Zavaliev et al., 2013). Two other key superfamilies are viruses that encode small hydrophobic movement proteins that insert into the ER. The double gene block (DGB) superfamily include viruses belonging to the genera *carmovirus*, *closterovirus*, *panicovirus*, and *sobemovirus*; and the triple gene block (TGB) superfamily includes viruses belonging to the genera *allexivirus, benyvirus, carlavirus, foveavirus, hordeivirus, pecluvirus*, and *potexvirus* (Vilar et al., 2002; Peremyslov et al., 2004; Sauri et al., 2005; Martinez-Gil et al., 2007, 2010; Verchot-Lubicz et al., 2010). In addition, the enveloped *tospovirus, tomato spotted wilt virus* encodes glycoproteins that localize to ER-export sites and Golgi complexes (Ribeiro et al., 2008).

EUKARYOTIC VIRUSES INTERACT WITH THE UPR MACHINERY TO PROMOTE PATHOGENESIS

As mentioned previously, positive strand RNA plant viruses pose an enormous biosynthetic burden on the ER, creating a higher than normal protein load. Therefore virus infection increases the potential for malformed proteins to accumulate thereby contributing to ER stress (Noueiry and Ahlquist, 2003). In this regard, the re-establishment of ER homeostasis by upregulating the ER protein folding and degradation machineries appears to be a coordinated adaptive response to virus invasion (Jelitto-Van Dooren et al., 1999; Urade, 2009; Liu et al., 2011; Wahyu Indra Duwi et al., 2013).

The best studied examples linking UPR to RNA virus infection are members of the family *flaviviridae*, such as DEN-2, HCV, *japanese encephalitis virus* (JEV), and *west nile virus* (WNV; Yu et al., 2006; Ambrose and Mackenzie, 2011; Paradkar et al., 2011). *Flaviviruses* depend upon the ER/Golgi network for replication and mature virions are released by budding through membranes of the ER/Golgi network. Three proteins prM, E, and NS1 enter the secretory system and are modified by glycosylation. Other non-structural proteins NS2A, NS2B, NS4A, and NS4B remain anchored to the ER. Each *flavivirus* has its own signature for activating the IRE1/XBP1-signaling pathways with unique benefits to virus infection (Iwata and Koizumi, 2012). For example, XPB1 activates genes involved in protein folding, ER biogenesis, and the ER degradation enhancing a-mannosidase-like protein 1 (EDEM-1; Mai and Breeden, 1997; Nekrutenko and He, 2006; Acosta-Alvear et al., 2007; Li et al., 2009). WNV NS4A and NS4B, as well as the HCV NS4B proteins activate XBP1 without altering EDEM-1 transcription (Zheng et al., 2005; Ambrose and Mackenzie, 2011). Researchers speculate that both HCV and WNV manipulate XBP1 signaling to promote the production of ER resident chaperones and membrane proliferation needed to support virus replication and protein production (Zheng et al., 2005; Ambrose and Mackenzie, 2011). The HCV E1 and E2 proteins are also reported to activate IRE1/XBP1 signaling events as well as PERK related oxidative stress pathways (Chan and Egan,

2005). For HCV, activation of these other pathways is linked to suppressing innate immunity while promoting virus replication (Ke and Chen, 2011a). The NS1 glycoprotein of JEV and DEN-2, as well as the NS2B/NS3 polyprotein of DEN-2 activate the XBP1-signaling pathway. Silencing XBP1 does not interfere with DEN-2 or JEV infection, but does exacerbate the cytopathic effects of these viruses. This suggests that the UPR is manipulated by these viruses to promote infection and counter host innate immunity (Estrabaud et al., 2011; Pena and Harris, 2011).

There are a few recent examples of plant viruses which interact with components of the UPR machinery to promote infection (**Table 1**). In plants IRE1 splices the bZIP60 transcription factor mRNA as a first step in UPR signaling (Deng et al., 2011; Iwata and Koizumi, 2012; Moreno et al., 2012). bZIP60, like XBP1, is reported to upregulate expression of the ER chaperone network that provides QC (Wahyu Indra Duwi et al., 2013) and likely benefits plant RNA virus infection. Importantly, it is not known whether the bZIP60 signaling pathway or other signaling pathways is responsible for the induced expression of membrane biosynthetic genes or changes in the host protein degradation patterns needed for virus infection. One virus example is PVX, which is a *potexvirus*. The PVX TGB3 movement protein is an 8 kDa movement protein that is tethered to the ER, induces expression of bZIP60 and ER resident chaperones as BiP, protein disulfide isomerase (PDI), and calreticulin (CRT; Garcia-Marcos et al., 2009; Ye and Verchot, 2011). Silencing bZIP60 gene expression in protoplasts greatly inhibited PVX replication. These data argue that although TGB3 is a movement protein, it nevertheless contributes to the regulation of virus replication by its impact on host gene expression (Ye and Verchot, 2011). Preliminary experiments indicated that BiP plays a role in preventing cytotoxic cell death during PVX infection which suggests that the UPR is an early event essential for persistent PVX infection and benefits virus replication (Jelitto-Van Dooren et al., 1999; Xu et al., 2005; Slepak et al., 2007; Urade, 2007). Two other virus examples include *papaya ringspot virus* (PRSV) and TMV, which require CRT to promote virus movement and possibly block calcium-dependent host defenses (**Table 1**). Since CRT is also regulated by bZIP60, it is worth further investigation to learn if PRSV interacts with this UPR signaling pathway. It is noteworthy that in our experiments, TMV did not appear to induce bZIP60 expression in a manner that is similar to PVX, which suggests that TMV could usurp CRT for its own processes without manipulating bZIP60 expression (Ye and Verchot, 2011; Ye et al., 2013). It is not known if silencing bZIP60 alters TMV or PRSV infection and such experiments are needed to better understand the role of UPR sensors in these virus infections.

In general, viruses have evolved to exploit the UPR machinery as a means to create environments that are favorable to infection. The UPR and ERAD mechanisms, by which plants and mammals respond to ER stress, have some significant similarities. While there is a greater body of research describing a role for XBP1-signaling pathways in mammalian virus infection, new evidence linking bZIP60 signaling pathways to plant virus infection suggest that RNA viruses infecting eukaryotes may generally manipulate the UPR to cope with ER stress, promote virus infection while reducing cytopathic effects, and possibly alter antiviral immunity.

There are also reports that viruses can perturb the cross talk between UPR signaling and other stress pathways including oxidative stress, autophagy, type I IFN antiviral response, and innate immune responses (Tardif et al., 2005; Sir et al., 2008; Garcia-Marcos et al., 2009; Ambrose and Mackenzie, 2011; Estrabaud et al., 2011; Evans et al., 2011; Yue et al., 2012). The exact mechanisms by which plant and mammalian RNA viruses manipulate UPR is not yet known, but given the universality of UPR regulation, the same machinery is likely to play an equally important role in plant virus replication and should be studied in more depth.

THE CONTRASTING ROLES OF CALRETICULIN IN STRESS RESPONSE AND VIRAL PATHOGENESIS

The expression of ER resident chaperones is transcriptionally coordinated in response to ER stress and bZIP60 is one of the identified transcription factors responsible for increased expression of a network of ER resident folding enzymes and chaperones. Among these are CRT and calnexin (CNX), which are highly conserved proteins critical to processing nascent glycoproteins and calcium homeostasis in the ER. In *Arabidopsis* there are three CRT isoforms (CRT1a, CRT1b, and CRT3) while in mammals there are only two CRT isoforms. CRT1a and CRT1b are similar isoforms that play general roles in maintaining protein folding and calcium levels in the ER (Persson et al., 2003; Thelin et al., 2011). The transcription of AtCRT1a and AtCRT1b is often coordinated, and is much more highly induced than AtCRT3 by such ER stress inducing compounds as tunicamycin (Jia et al., 2009; Christensen et al., 2010). Overproduction of CRT in response to pathogen attack or stress would increase the Ca^{2+} buffering capacity of the cell. Thus viruses could potentially target CRT gene expression to create an environment that is favorable to virus infection.

In contrast to mammalian systems, plant CRTs localize to several subcellular compartments. AtCRT1a/b also associate with plasmodesmata and research suggests that it plays a role in Ca^{2+} homeostasis in the plasmodesmata (Baluska et al., 1999; Christensen et al., 2010; Thelin et al., 2011). Localization of CRT to plasmodesmata requires the N-terminal signaling sequence for ER insertion, which implies that CRT moves through an ER-dependent route to reach the plasmodesmata (Wyatt et al., 2002). The AtCRT1a/1b co-localizes with the TMV movement protein in plasmodesmata (Chen et al., 2005; Ye et al., 2013). CRT1a was shown to bind TMV movement protein *in vitro* and *in vivo* by affinity chromatography, yeast two hybrid analysis, and fluorescence resonance energy transfer microscopy. Overexpression of CRT hinders TMV cell-to-cell movement and blocks movement protein accumulation in the plasmodesmata (Chen et al., 2005). GFLV also interacts with CRT to promote cell-to-cell movement. GFLV uses a tubule guided movement mechanism for intercellular movement (Laporte et al., 2003). Researchers proposed that CRT binds the viral movement protein and serves as a base for tubular assembly. The viral encoded movement protein moves through the secretory system to the destination where they form oligomers that build into tubules. Tubules extend across plasmodesmata and carry virion particles between neighboring cells (Laporte et al., 2003). Given that TMV and GFLV are not known to impact CRT expression, these data provide further support to the notion that TMV and GFLV are more likely to subvert CRT1a/1b from

Table 1 | Membrane related host proteins or post-transcriptional regulatory networks for compatible plant-virus interactions.

Virus genus	Virus species[a]	Cellular protein or machinery[b]	Type of association	Viral protein partner	Post-transcript regulatory network	References
potexvirus	PVX	bZIP60 SKP1	mRNA induction	TGB3	UPR and UPS turnover of TGB2	Ye and Verchot (2011), Ye et al. (2013)
tobamovirus	TMV	CRT	Direct protein interaction	30K	Plasmodesmata and virus movement	Chen et al. (2005)
	ToMV	UPS	Verified protein ubiquitination	30K	Protein modification and turnover, regulates virus movement.	Reichel and Beachy (2000)
nepovirus	GFLV	CRT	Co-localization	Movement protein	Receptor for viral movement protein at cell surface or in cytosol	Laporte et al. (2003)
potyvirus	PRSV	CRT	Direct protein interactions	HC-Pro	Interfere with plant Ca^{+2} signaling to block host defenses	Shen et al. (2010), Sahana et al. (2012)
	PRSV, LMV, PVY,	PPAA,alpha1 subunit of 2proteasome	Direct protein interactions	HC-Pro	Regulates protein turnover, HC-pro interaction	Ballut et al. (2005), Jin et al. (2007), Sahana et al. (2012)
	PSbMV	Poly ubiquitin	mRNA expression	Un-known	Unknown role in virus infection	Aranda et al. (1996)
polerovirus	BWYV	SCF E3 ubiquitin ligase	Potential interaction	P0 protein	P0 is an F box protein that inserts into the SCF complex and targets AGO1 for degradation	Pazhouhandeh et al. (2006), Baumberger et al. (2007), Bortolamiol et al. (2007)
enamovirus	PEMV	SCF E3 ubiquitin ligase	Potential Interaction	P0 protein	P0 is an F box protein that inserts into the SCF complex and targets AGO1 for degradation	Fusaro et al. (2012)
tombusvirus	TBSV	Nedd4-type Rsp5p ubiquitin ligase	Direct protein interactions	P92 replicase	Turnover of p92 replication protein	Barajas et al. (2009)
	TBSV	Cdc34p ubiquitin conjugating enzyme	Direct protein interactions	P33 replicase	Modify P33 necessary for interaction with host ESCRT1	Li et al. (2008), Barajas and Nagy (2010)
tymovirus	TYMV	UPS pathway	Protein interactions	66K replicase 69K move-ment protein	Modify and turnover of viral RdRp and movement protein	Hericourt et al. (2000), Drugeon and Jupin (2002), Camborde et al. (2010)

[a] *Virus names: PVX, potato virus X; TMV, tobacco mosaic virus; toMV, tomato mosaic virus; GFLV, grapevine fanleaf virus; PRSV, papaya ringspot virus; LMV, lettuc mosaic virus; PVY, potato virus Y; PSbMV, pea seedborne mosaic virus; BWYV, beet western yellow virus; PEMV, pea enation mosaic virus; TBSV, tomato bushy stunt virus, TYMV, turnip yellow mosaic virus.*

[b] *In this column UPS is identified when it is known that the virus protein is known to be ubiquitin modified and turned over. Protein interactions are verified by the ubiquitin modification, but the relationship with the proteasome is typically evidence by turnover not by direct protein interaction assays. However, there are times when the exact ubiquitin related enzyme is known and identified in this column. These are verified by either yeast two hybrid or affinity chromatography.*

their normal function to promote plant virus infection through interactions with the viral movement proteins.

All three CRT isoforms contain nuclear targeting signals in their central domain and C-terminal HDEL ER-retention signal. The wheat TaCRT3 was reported to translocate to the cytoplasm and nucleus (Jia et al., 2008; An et al., 2011). In *Nicotiana* ssp., CRT also localizes to the Golgi and plasma membrane (Jia et al., 2008; Matsukawa et al., 2013). Investigations of transcriptionally co-regulated gene networks in plants suggest that CRT1a and CRT1b are co-expressed with many ER chaperones while CRT3 is co-expressed with pathogen related signal transduction genes (An et al., 2011; Thelin et al., 2011). Given that transcriptionally coordinated genes typically provide related functions, the function of CRT3 is likely diverged from these ER resident isoforms.

As mentioned previously, PRSV and PVX represent another class of viruses that interacts with CRTs at the level of gene expression. Researchers showed that papaya CRT1a/b interacts with the PRSV HC-Pro protein using yeast two hybrid and BiFC assays in plant cells (Shen et al., 2010). HC-Pro is a well-studied viral protein that is involved in multiple functions including virus movement and suppression of post-transcriptional gene silencing (Shiboleth et al., 2007; Yap et al., 2009). As mentioned earlier, the PVX TGB3 movement protein increases the expression of bZIP60 and several ER resident chaperones including CRTs in *Arabidopsis* and *Nicotiana benthamiana* leaves. Silencing bZIP60 in protoplasts hampers PVX infection which led us to reflect on whether upregulation of ER chaperones, such as CRT1b is a necessary result of TGB3 activation of bZIP60. Considering that PRSV, like PVX, induces expression of CRT, it is reasonable to speculate that PRSV might interact with the co-expression gene network to enlist the ER QC machinery. However, the relationship of bZIP60 to a co-expression network involving CRT is not yet known. Further research is needed to better understand the roles of CRT in PRSV and PVX infection and determine if there are separate types of interactions involving plant viruses and such components of the ER QC machinery. Knowledge of whether bZIP60 is capable of activating CRT gene expression is also important for understanding how viruses interact with bZIP60 related gene networks.

To better understand the relationship of bZIP60 with ER QC machinery, we took advantage of the GeneCat co-expression analysis webtool (Mutwil et al., 2008; Usadel et al., 2009) to examine the whether the *Arabidopsis* bZIP60 (AT1G42990), CRT1b (AT1G09210), and CRT3 (AT1G08450) provide a chaperone framework for ER QC (**Figure 1**). Evidence that these genes are transcriptionally coordinated (using an r-cutoff value < 0.7) when induced during biotic stress, would support the hypothesis that they are related functionally and contribute to similar processes. Evidence of a weak relationship would suggest that bZIP60 expression is not solely tied to the expression of these genes. We also included SKP1 (AT1G75950) in the analysis query because it is induced alongside bZIP60 and CRT by PVX infection and is a co-factor in the UPS machinery that does not reside in the ER. This analysis identified genes that show strong rankings (when no average r-cutoff value was applied), appear to form a cluster that is connected within one or two nodes to the query genes, and appear to be mutually co-expressed. The GeneCat analysis output revealed that the expression of CRT1b, BIP1, PDI-like protein 2-1 and 2-3 (PDIL2-1, PDIL2-3), which are all components of the ER QC machinery, is strongly coordinated (**Figure 1**). The relatedness of these genes was also reported by Thelin et al. (2011) using the webtool PlaNet to explore the co-expression networks involving CRT. The comparative analysis using the query genes CRT1b, CRT3, and bZIP60, also demonstrates that CRT1b is co-expressed with ER chaperones and folding enzymes while CRT3 is either weakly linked or not linked to this network. Given reports linking CRT3 to a subnetwork of genes involved in pathogen-related signaling events, these data confirm that these CRTs have divergent functions (Thelin et al., 2011). Interestingly, there is no evidence that expression of bZIP60 is transcriptionally coordinated with the ER QC machinery when we use strict or relaxed cutoff ($r < 50$) to generate the network. CRT3 and SKP1 also lie outside the network and their expression is not coordinated with bZIP60 expression (**Figure 1**). It is possible that co-expression analysis does not reveal the regulatory relationships linking bZIP60 and CRTs and that these genes are linked in other ways. For example there might be other intermediate factors that connect to bZIP60 these ER resident chaperones.

THE PRO-VIRAL ROLE OF UPR AND AUTOPHAGY

Chaperone mediated autophagy is extensively studied in yeast and mammals and is recently identified to play an important role in infection and immunity in plants. In mammals, accumulation of malformed proteins in the ER activates UPR via PERK, IRE1, and ATF6 and all three sensors are required for induction of autophagy. Autophagy is a well-known degradation pathway for organelles and cytoplasmic components. Initially, the cellular autophagy protein LC3 associates with membranes forming crescent-shaped double membrane structures that sequester misfolded proteins and damaged organelles. These structures mature to form double membrane vesicles known as mature autophagosomes. These autophagosomes can fuse with endosomes to form amphisomes which then become acidified due to the presence of vacuolar ATPases and then fuse with the lysosomes to form autolysosomes that degrade their cargo.

Polivovirus (PV), HCV, DEN, and JEV are a few examples of the many positive strand RNA viruses that induce autophagic signaling and subvert autophagosomes or amphisomes to use as scaffolds supporting replication and assembly (Taylor and Jackson, 2009; Shi and Luo, 2012; Richards and Jackson, 2013). Since autophagy is also a pathway to degradation, viruses have developed strategies to block lysome fusion. Shi and Luo (2012) describe autophagic flux as the balance between the rate autophagosome formation and degradation. This concept is interesting with respect to virus infection, because viruses can act at different steps in the process to increase autophagosome formation, alter the rate of amphisome conversion, and reduce degradation, thereby triggering an incomplete autophagic response. DEN, for example localizes with immature autophagosomes. DEN and JEV are both enveloped viruses that rely on receptor-mediated endocytosis for cellular uptake. One possibility is that autophagosome-endosome fusion is a factor in virus entry and uncoating. HCV relies on autophagosome formation to support virion assembly and autolysosome for suppressing host immune responses. PV uses autophagosomes

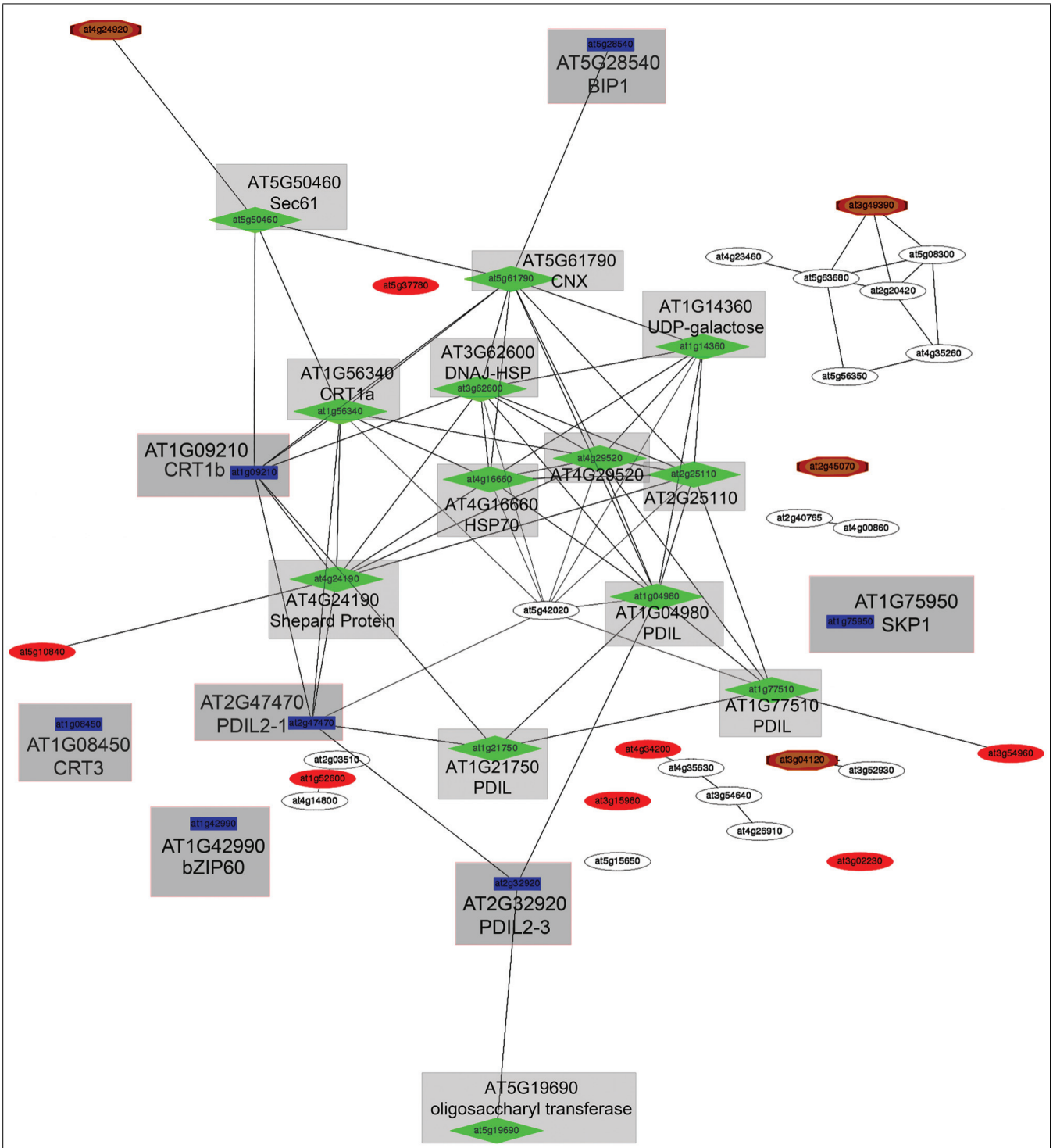

FIGURE 1 | Visualization of the genes associated with UPR in plants as determined by GeneCAT analysis. This is a simple subnetwork example (Mutwil et al., 2008). The nodes represent genes and the lines represent the relationships between the genes. The genes identified in blue are the query genes. The nearest co-expressed genes are highlighted in green and form a cluster of co-expressed genes based on an analysis of mutual co-expression ranks between the top 50 genes from the list. The bold lines, in this subnetwork, identify those genes that have low mutual ranks according to GeneCAT webtool analysis (Mutwil et al., 2008; Usadel et al., 2009). Query genes including bZIP60, SKP1, and CRT3 do have lines connecting them to the co-expressed gene network arguing that they are not connected in a manner that is statistically relevant. Thus the evidence that these genes are upregulated early in virus infection could suggest that the virus may be interacting with more than one signaling pathway. Each blue and green labeled node, has a gray box with the name of the gene and its accession highlighted in larger print.

as a scaffold for virus replication and the acidic amphisomes to promote virus assembly (Richards and Jackson, 2012, 2013).

With regard to plant viruses, there is no information yet that indicates a pro-viral role for autophagy in the infection process. Researchers have linked autophagy to plant innate immunity involving TMV infection. N-gene mediated immune response to TMV includes a form of programmed cell death known as a hypersensitive response (HR). In this regard, autophagy limits the extent of cell death to a local area on a leaf, preventing uncontrolled spread of HR throughout healthy tissues. This serves to contain the immune response to a localized region (Liu et al., 2005; Li et al., 2012).

MANIPULATING THE UBIQUITIN PROTEASOME SYSTEM FOR VIRUS INFECTION

The ubiquitin-26S proteasome system is the prevailing route for protein removal and is widely conserved among eukaryotes (Smalle and Vierstra, 2004; Vierstra, 2009). The ubiquitin conjugating pathway depends on the host E1, E2, and E3 ligases to link ubiquitin moieties to a protein substrate (Miura and Hasegawa, 2010). Certain types of ubiquitin modifications predestine a protein for degradation by the proteasome while others determine alternative subcellular locations or activations. Thus the ubiquitin ligase machinery, which includes the F-box protein that harnesses the substrate, modifies intact as well as malformed proteins. Ubiquitination can also be reversed by the action of de-ubiquitinating enzymes (DUBs) which can either trim a polyubiquitin chain or remove it from the substrate (Chenon et al., 2012).

There is a growing body of evidence that plant and mammalian viruses interact with both ubiquitin ligases and DUBs. There are many cases where eukaryotic viruses manipulate the UPS machinery to avoid immune clearance. For example the *human immunodeficiency virus* (HIV) Vpu protein is a low molecular mass protein with a single transmembrane domains that inserts into the ER. Vpu binds to the cellular CD4 protein in the ER and recruits the human F-box protein bTrCP targeting CD4 for degradation via the ubiquitin-proteasome pathway. CD4 is a cell surface receptor required for HIV uptake into cells, and the process of dislocation and degradation of CD4 in the ER reduces the number of available receptors at the cell surface and is important to free HIV gp160 in the ER for virus maturation and trafficking (Bour et al., 1995; Belaidouni et al., 2007; Malim and Emerman, 2008; Nomaguchi et al., 2008).

Among plant viruses, the UPS machinery can be manipulated to degrade components of the cell's gene silencing machinery, thereby promoting virus infection. *Poleroviruses* and *enamoviruses* encode the P0 protein which contains an F box protein motif (Baumberger et al., 2007; Fusaro et al., 2012). It is reported that the P0 inserts into the SCF complex and enables degradation of ARGONAUTE1 (AGO1) which is a core component of the RISC complexes and is an essential component of the RNA-silencing machinery. P0 acts as a silencing suppressor that enables AGO1 degradation, compromising RNA silencing, as well as the degradation of targeted viral RNAs. Furthermore, interactions between the *beet western yellow virus* (BWYV) P0 protein and SKP1 modulates programmed cell death during virus infection. Mutations that interrupt the ability of P0 to interact with SKP1 result in systemic necrosis, suggesting that the P0–SKP1 complex P0 is a silencing suppressor protein which might target components of the silencing machinery for proteasomal degradation through its interactions with SKP1 (Pazhouhandeh et al., 2006).

PVX is also known to interact with SKP1 but the role of this cofactor in virus infection is not yet clarified (Ye et al., 2013). As mentioned earlier the PVX TGB3 movement protein associates with the ER and upregulates expression of the bZIP60 transcription factor and several ER resident chaperones early in virus infection. TGB3 also induces SKP1 expression alongside several other genes suggesting that both the UPR and UPS systems are upregulated to handle the increased protein load in the cell. Another possibility is that TGB3 enhances the capacity of the UPS to degrade key host proteins that are either related to or independent of the RISC complex. The PVX TGB1 protein acts as a silencing suppressor protein that targets AGO1 for proteasomal degradation (Bayne et al., 2005; Chiu et al., 2010). It is worth to consider that TGB3 acts in concert with TGB1 to promote the degradation of AGO1.

Replication of *turnip yellow mosaic virus* (TYMV; a *tymovirus*) is broadly affected by the UPS and is a prime model for comparison with other positive strand RNA viruses. The TYMV genome encodes two non-structural proteins of 69 kDa and 206 kDa in size. The viral coat protein is expressed from a subgenomic RNA. The TYMV 69K protein is a viral movement protein that can be polyubiquitinated. Reports indicate that the 69K protein is turned over by the proteasome (Drugeon and Jupin, 2002). Protein turnover is suggested to either regulate virus movement or reduce cytotoxic accumulation of viral proteins. There are other viral movement proteins that are also regulated by proteasomal turnover including the TMV 30K movement protein and coat protein, the *polerovirus* 17 kDa movement protein, and the PV TGB3 (Mas and Beachy, 1999; Jockusch and Wiegand, 2003; Pazhouhandeh et al., 2006; Ju et al., 2008). These combined reports suggest that plant viruses may generally target the UPS to regulate the stability of virus movement proteins.

The TYMV 206 kDa polyprotein contains domains which provide methyltransferase, proteinase, helicase, and RNA-dependent RNA polymerase (RdRp) activities (Martelli et al., 2007). The proteinase domain autocatalytically cleaves the 206 kDa polyprotein to generate a 66K RdRp and a 140K protein which are both present in the active replicase complex. The 66K RdRp is modified by ubiquitin during infection and is a target for UPS turnover (Hericourt et al., 2000). Thus the UPS regulates TYMV replication (Camborde et al., 2010). The 140K protein is a precursor product that is further cleaved to produce the mature 98K proteinase and 42K helicase. The proteinase domain within the 140K or 98K protein also possesses deubiquitylating (DUB) enzyme activity (Chenon et al., 2012). Research suggests that 66K RdRp is the substrate for the deubiquitylating activity of these proteins. TYMV replication occurs along chloroplast membranes and the 66K protein is transported to this location by the driving interactions with the 140K protein. It is reported that the 140K or the 98K proteins monitor the ubiquitin moieties attached to the 66K RdRp to stabilize the replicase complex and promote virus replication early in infection. Then later in virus infection, ubiquitin modification of the 66K leads to proteasomal degradation and shuts

down replication. Thus, DUB activity also plays a role in directing viral RNA replication (Chenon et al., 2012).

Tomato bushy stunt virus (TBSV) is a member of the genus *tombusvirus* and provides another example whose replicase is impacted by the UPS machinery. Ubiquitination of the TBSV p33 controls interactions with the host ESCRT protein which is important for subcellular targeting of the viral replicase. The UPS system also monitors the accumulation of the TBSV p92 protein which is essential for virus replication (Li et al., 2008; Barajas et al., 2009; Barajas and Nagy, 2010). These combined examples suggest that there might be a common viral strategy to manipulate or alter the ubiquitin-mediated degradation machinery to promote plant virus infection.

CONCLUSION

Positive strand RNA viruses depend heavily on the ER for genome synthesis, protein production, and cell-to-cell movement. Given the biosynthetic burden that viruses pose on the cell, sustaining ER homeostasis and creating a membrane rich environment is an essential adaptation for infection to succeed. One question that remains to be answered is whether the extensive membrane expansion and remodeling is a means to compensate for the translational burden on the ER caused by virus infection, or is the direct outcome of UPR and/or autophagy. Given that virus translation causes a burden on the ER QC machinery, it is not known for certain whether this is the proximal cause of ER stress and activation of the UPR. It is arguable that the impact of translation on the ER causes cells to adapt and survive and that UPR activation is a component of cellular adaptation. However, there is a growing body of evidence to that indicates UPR is not just a cell survival response in the face of a toxic infection, but that many positive strand RNA viruses act in a targeted manner to upregulate UPR and benefits critical steps in virus infection. The examples provided here suggest that positive strand RNA viruses encode effector proteins that activate specific branches of the UPR in a targeted manner. For example, the HCV E1 and E2 trigger the IRE1/XBP1 pathway; the WSN NS4A also triggers the XBP1 pathway but does not activate EDEM-1; the JEV and DEN-2 NS1 proteins also activate XBP1 pathway; and in plants the PVX TGB3 activates the bZIP60 pathway.

One of the benefits of UPR activation is the increased availability of cellular chaperones which typically drive substrate protein folding and complex assembly in various cellular compartments. It seems obvious that viruses require the cell to have a greater protein folding capacity to accommodate the translational burden caused by infection, but viruses have an additional need to subvert certain chaperones from their normal function to help drive events during the infection process. As summarized in **Table 1**, we presented examples of *potyviruses, nepoviruses*, and *tobamoviruses* that pirate CRT to promote intercellular transport. However, the literature does not show whether many of these viruses activate signaling mechanisms to stimulate CRT expression. Furthermore, it is not known if other components of the ER QC machinery are diverted from their cellular roles to viral protein complexes. In general, virologists are currently working to uncover the parameters that determine which ER resident chaperones engage with viral proteins to promote viral pathogenesis and whether this benefits infection at the expense of cellular homeostasis or host immunity.

The relationship of the UPR to membrane biosynthesis or reorganization is not established but it is reasonable to predict that researchers are likely to be able to explain the mechanisms behind virus induced membrane synthesis as we explore the need for autophagic membranes. For example, membrane synthesis might be stimulated, not directly by the UPR machinery but by viruses interactions with the autophagic machinery. Perhaps viruses act on a parallel pathway to stimulate membrane synthesis needed for autophagosome and amphisome production. Given that viruses require changes in gene expression relating to both membrane synthesis and cellular chaperones, it would be intriguing to learn how the signal transduction events that relate the UPR and autophagy are connected.

The role of ubiquitin and DUBs in virus infection is an intriguing new topic with great potential to provide new insights into the host machinery involved in regulating virus infection. We presented examples of viruses belonging to a broad number of virus genera interact with the UPS machinery to either regulate its own replication cycle, modulate intercellular movement, or evade host defenses. As an aggregate, this work shows that viruses can manipulate the UPS machinery to suppress host defenses, modulate virus replication, and regulate viral protein turnover. The breadth of examples clearly shows that the UPS machinery plays a critical role in virus infection for a wide range of plant viruses.

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ER bodies in plants of the *Brassicales* order: biogenesis and association with innate immunity

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The endoplasmic reticulum (ER) forms highly organized network structures composed of tubules and cisternae. Many plant species develop additional ER-derived structures, most of which are specific for certain groups of species. In particular, a rod-shaped structure designated as the ER body is produced by plants of the *Brassicales* order, which includes *Arabidopsis thaliana*. Genetic analyses and characterization of *A. thaliana* mutants possessing a disorganized ER morphology or lacking ER bodies have provided insights into the highly organized mechanisms responsible for the formation of these unique ER structures. The accumulation of proteins specific for the ER body within the ER plays an important role in the formation of ER bodies. However, a mutant that exhibits morphological defects of both the ER and ER bodies has not been identified. This suggests that plants in the *Brassicales* order have evolved novel mechanisms for the development of this unique organelle, which are distinct from those used to maintain generic ER structures. In *A. thaliana*, ER bodies are ubiquitous in seedlings and roots, but rare in rosette leaves. Wounding of rosette leaves induces *de novo* formation of ER bodies, suggesting that these structures are associated with resistance against pathogens and/or herbivores. ER bodies accumulate a large amount of β-glucosidases, which can produce substances that potentially protect against invading pests. Biochemical studies have determined that the enzymatic activities of these β-glucosidases are enhanced during cell collapse. These results suggest that ER bodies are involved in plant immunity, although there is no direct evidence of this. In this review, we provide recent perspectives of ER and ER body formation in *A. thaliana*, and discuss clues for the functions of ER bodies. We highlight defense strategies against biotic stress that are unique for the *Brassicales* order, and discuss how ER structures could contribute to these strategies.

Keywords: endoplasmic reticulum, ER body, organelle biogenesis, β-glucosidase, plant defenses, secondary metabolites, glucosinolate

INTRODUCTION

The endoplasmic reticulum (ER) forms highly organized network structures composed of ER tubules and ER cisternae. In addition to this well-conserved ER network, different plant species develop unique ER-derived compartments that can be regarded as ER domains. Many of the ER-derived compartments accumulate specific types of proteins, such as the protein bodies (PBs) in maize and rice, which contain prolamin and zein, respectively (Herman and Larkins, 1999), the KDEL-tailed protease-accumulating vesicles (KVs) in mungbean (Toyooka et al., 2000), and the ricinosomes in castor bean that accumulate papain-type proteases (Schmid et al., 2001). These structures are thought to function as repositories of particular proteins until they are required.

In this review, we focus on what is called the ER body, also known as a fusiform body. This structure is an ER domain of unique shape and taxonomic distribution. In 1965, the ER body was first discovered in root epidermal and cortical cells of radish (Bonnett and Newcomb, 1965). It was described as dilated cisternae that had luminal continuity to the ER. During the following 15 years, researchers tried to characterize these structures and determine their functions (Iversen and Flood, 1969; Iversen, 1970a; Cresti et al., 1974; Hoefert, 1975; Endress and Sjolund, 1976; Jørgensen et al., 1977; Behnke and Eschlbeck, 1978; Gailhofer et al., 1979; Jørgensen, 1981). Three independent studies revealed that the dilated cisternae were primarily restricted to species of the order *Brassicales*, which were known to produce thioglucosides named glucosinolates (Iversen, 1970a; Behnke and Eschlbeck, 1978; Jørgensen, 1981). Given that glucosinolates required a specific enzyme called myrosinase to become active, this correlation implied that the dilated cisternae could act as a myrosinase repository. Indeed, activity-labeled transmission electron microscopy revealed that the dilated cisternae contained

potential myrosinase activity toward sinigrin (Iversen, 1970b), although this experimental system was subsequently questioned (Behnke and Eschlbeck, 1978). The question as to whether the dilated cisternae were involved in glucosinolate metabolism was left unsolved for decades, possibly due to a lack of appropriate molecular tools. In 1998, after several analyses using the model plant *A. thaliana*, it was suggested that the rod-shaped structures labeled with ER-localized green fluorescent protein (GFP) were equivalent to the dilated cisternae (Gunning, 1998). Hayashi et al. (2001) showed that these rod-shaped structures resembled the dilated cisternae described in past literature, and designated them as ER bodies. Matsushima et al. (2004) isolated the *nai1* mutant that lacked ER bodies ("nai" is a Japanese word for "absence"), which was the very first identification of genetic material in ER body research. Since then, genetic and biochemical studies have provided insights into the functions, importance, and biogenesis of this unique organelle, which will be summarized in this review.

Although most of ER-derived compartments form spherical structures, ER bodies have a rod shape. The constitutive presence of ER bodies in *A. thaliana* is strictly limited to roots in adult plants; they are absent in most cells in rosette leaves. Both wounding and jasmonic acid treatment induce *de novo* formation of ER bodies in rosette leaves. In combination with their unique shape, one can presume a tightly regulated and unique mechanism for ER body formation, which is discussed in the first section. The tissue specificity and the inducibility also suggest that ER bodies are involved in the response to wounding and some biotic stresses, as discussed in the second section. In the last section, we will discuss how ER bodies are conserved and evolved in the plant kingdom. Current research indicates that ER bodies are specific to the order *Brassicales*, especially to the families *Brassicaceae*, *Capparaceae*, and *Cleomaceae*. It is known that *Brassicales* plants have unique defense strategies against biotic stresses, which may lead to an interesting evolutionary story that includes ER bodies.

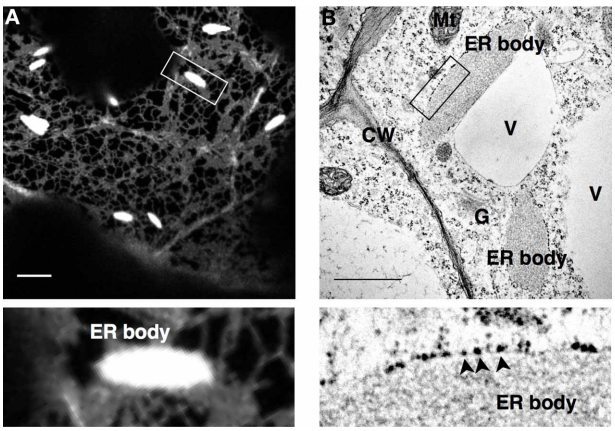

FIGURE 1 | ER bodies in *Arabidopsis thaliana*. A confocal micrograph **(A)** and an electron micrograph **(B)** of cotyledon and root epidermal cells, respectively, of *A. thaliana*. Arrowheads indicate ribosomes on the surface of the ER body membranes. ER-localized GFP (SP-GFP-HDEL) labels ER bodies as well as the typical ER network, and electron microscopy identifies ribosomes at the cytosolic surface of ER bodies, both of which indicate the luminal continuity between ER and ER bodies. Enlarged images of the squared regions are shown below. CW, cell wall; V, vacuole; Mt, mitochondrion; G, Gold body; Bars, 10 μm **(A)** and 1 μm **(B)**.

MOLECULAR MECHANISMS UNDERLYING ER BODY FORMATION

ER BODY IS A SUBDOMAIN OF ER THAT HAS SPECIFIC ORGANIZATION MECHANISM

The ER body in *A. thaliana* was first observed in a transgenic line (GFP-h) expressing ER-targeted GFP. In the cotyledons of GFP-h, bright rod-shaped structures of ER bodies were observed in addition to the generic ER network (**Figure 1A**; Ridge et al., 1999; Hawes et al., 2001; Hayashi et al., 2001). Electron microscopy analysis revealed that the ER body is covered by a single membrane surrounded by ribosomes, which is a characteristic of the ER (**Figure 1B**). The ER body is observed as a structure that is connected with ER tubules and/or ER cisternae in electron micrographs (Gunning, 1998; Hayashi et al., 2001). These results indicate that the ER body is continuous to the whole ER network; therefore, it is suggested to be a subdomain of the ER that has specific functions.

Plants develop several ER-derived structures that are thought to specifically function for protein storage in specific organs or during specific life stages. These include PBs and precursor-accumulating (PAC) vesicles in maturating seeds, KVs and ricinosomes in germinating seeds, and ER bodies in roots and seedlings. PBs accumulate seed-storage proteins in monocot plants such as rice (*Oryza sativa*) and maize (*Zea mays*) (Herman and Larkins, 1999). PAC vesicles accumulate precursors of seed-storage proteins in dicot plants such as pumpkin (*Cucubita maxima*) and castor bean (*Ricinus communis*), which undergo bulk transport to the protein-storage vacuole (Hara-Nishimura et al., 1998). KVs and ricinosomes accumulate papain-type proteases for the degradation of seed-storage materials in the cotyledon of mungbean (*Vigna mungo*) or endosperm of castor bean, respectively (Toyooka et al., 2000; Schmid et al., 2001). These vesicles are spherical with diameters of 0.5–1.0 μm and are surrounded by ribosomes, indicating that they are derived from the ER. Compared to these ER-derived vesicles, the ER body is longer and larger (~10 μm long and ~1 μm wide), and accumulates different kinds of proteins, namely the β-glucosidases. Therefore, the ER body is presumably different both in function and in biogenesis mechanisms from other ER-derived structures.

β-GLUCOSIDASE IS THE MAJOR COMPONENT OF ER BODY

The major protein component of ER body in *A. thaliana* is identified as a β-glucosidase called PYK10/BGLU23 (Matsushima et al., 2003). This β-glucosidase was identified by analysis of the *nai1* mutant in which ER bodies were absent. PYK10 is actively recruited from the ER network to ER bodies (Matsushima et al., 2003), in contrast to that for generic ER luminal proteins such as SP-GFP-HDEL. The level of PYK10 protein is high enough to be detected as one of the most abundant proteins in *A. thaliana* roots (Matsushima et al., 2003). Electron microscopy analysis revealed a relatively high electron density in the ER body lumen, suggesting that the ER body contains a large amount of proteins (Gunning, 1998; Hayashi et al., 2001). Consistent with this, ER bodies are enriched in a relatively heavier fraction after a centrifugation-based fractionation of subcellular compartments (Matsushima et al., 2003), which confirms a high protein density

within ER bodies. These studies of *A. thaliana* suggest that the ER body is a vessel for β-glucosidases to separate them from other proteins and substrates. Indeed, when cells are disrupted, PYK10 forms a huge protein complex that contains proteins originating from various subcellular compartments (Nagano et al., 2005, 2008). The *A. thaliana* genome encodes 47 β-glucosidases within 10 subfamilies (Xu et al., 2004), most of which (40 out of 47) possess signal peptides at their amino-termini, indicating localization in the endomembrane system or secretion to the apoplast (**Table 1**). All members of the subfamily that includes PYK10 and additional 7 β-glucosidases (BGLU18−25) also contain ER retention signals ([K/H/R][D/E]EL) at their carboxy-termini. Based on their sequence similarity, it can be assumed that these 8 β-glucosidases localize in ER bodies. Consistent with this, the subfamily member BGLU18 accumulates in ER bodies that are induced by wounding in rosette leaves (Ogasawara et al., 2009).

NAI2 AND PYK10: ER BODY COMPONENTS REQUIRED FOR ER BODY FORMATION

The protein factors involved in ER body formation in *A. thaliana* have been identified by analysis of the two mutants *nai2* and *long ER body* (*leb*). The recessive *nai2* mutant lacks ER bodies in seedlings and roots (Yamada et al., 2008). NAI2 accumulates in ER bodies but not in the ER network, indicating that NAI2 is an ER body component that determines ER body formation in *A. thaliana*. In the absence of NAI2, PYK10 is diffused throughout the ER network, and the protein levels are lower compared to those for wild type (WT). These results show that NAI2 enables the high accumulation and storage of PYK10, presumably by mediating production of the ER body. Based on sequence similarity, NAI2 homologs are only observed in *Brassicaceae* plants that produce ER bodies, further suggesting that NAI2 has a specific function in the generation of this organelle.

In *leb* mutant seedlings, there are fewer ER bodies and they have an elongated shape compared to those in WT seedlings (Nagano et al., 2009). A mutation in the *PYK10* gene that causes an amino acid substitution is responsible for the *leb* phenotype, revealing the importance of PYK10 localization in the ER body for ER body formation. PYK10 forms an oligomer linked by a disulfide bond (Nagano et al., 2005), presumably via a cysteine residue that is substituted in the *leb* mutant, because the mutation resulted in an altered oligomeric structure and reduced accumulation of PYK10 protein (Nagano et al., 2009). The *pyk10* single knockout mutant exhibited milder phenotype than that of the *leb* mutant, indicating that the *leb* mutation affects other ER body components that contribute to proper ER body organization. BGLU21 and BGLU22 are the two closest homologs of PYK10, which might contribute to ER body formation. A double knockout mutant *pyk10 bglu21* showed similar phenotype to the *leb* mutant, indicating that BGLU21 is involved in ER body formation. The *PYK10* mutation affects the nature of BGLU21 protein such as oligomeric states and/or protein conformation and induces the formation of aberrant ER bodies. These data suggest that PYK10, BGLU21, and perhaps BGLU22 are redundantly important for proper organization of this organelle in *A. thaliana* seedlings. The redundancy between BGLU21 and BGLU22 is also suggested by the fact that these two proteins are more similar to each other than to the closest homolog in *Arabidopsis lyrata*.

ER BODY MEMBRANE CONTAINS SPECIFIC PROTEINS

The membrane of ER bodies may have an important role in mediating the function and/or formation of ER bodies. Two integral membrane proteins, designated as MEMBRANE OF ER BODY (MEB) 1 and MEB2, have been identified to accumulate specifically at ER body membranes in *A. thaliana* (Yamada et al., 2013). These proteins were identified by coexpression analysis based on a public microarray database (ATTED-II; http://atted.jp) and transcriptomic analysis using the *nai1* mutant. MEB1 and MEB2 are homologous to each other with multiple membrane-spanning regions. They have weak similarity to Ccc1p and VIT1, an iron/manganese transporter in *S. cerevisiae* and *A. thaliana*, respectively (Li et al., 2001; Kim et al., 2006). MEB1 and MEB2 appear to possess metal transporter activity (discussed below), although their physiological role for plant fitness is still unknown (Yamada et al., 2013). MEB1 and MEB2 form a protein complex with NAI2, and are diffused throughout the ER network in the *nai2* mutant, suggesting the NAI2-dependent recruitment of MEB1 and MEB2 into ER body membranes. ER bodies in the seedlings of the *meb1 meb2* double mutant exhibited a comparable number and shape to those in WT seedlings, suggesting that these proteins are not necessary for ER body formation. However, these results indicate that the ER body membrane has a specific composition of proteins that differs from that of the ER network, and suggest that NAI2 is responsible for the organization of these ER body membrane proteins. Because NAI2 alone regulates ER body formation, the NAI2-dependent specification of the membrane by gathering specific proteins may have a crucial role for the biogenesis of ER bodies.

NAI1: THE TRANSCRIPTION FACTOR FOR ER BODY FORMATION

The basic helix-loop-helix- (bHLH) type transcription factor NAI1 (also known as AtbHLH20) solely regulates ER body formation, because disruption of this gene in *A. thaliana* completely disrupts ER body formation in seedlings and roots (Matsushima et al., 2004). A transcriptomic analysis of the *nai1* mutant revealed that ER body proteins, such as those encoded by *PYK10*, *NAI2*, *MEB1*, and *MEB2*, are expressed in a NAI1-dependent manner. This indicates that NAI1 is the master regulator for ER body formation, and it regulates the expression of these genes. NAI1 also regulates the expression of *JACALIN-RELATED LECTIN* genes (*JAL22*, *JAL23*, *JAL31*, and *PYK10 BINDING PROTEIN 1* (*PBP1*)/(*JAL30*), *GDSL LIPASE-LIKE PROTEIN* genes (*GLL23* and *GLL25*), and *MD2-RELATED LIPID RECOGNITION PROTEIN 3* (*ML3*) (Nagano et al., 2005, 2008; Hakenjos et al., 2013). JAL proteins lack signal peptides and are assumed to localize in the cytosol (Nagano et al., 2005), whereas GLL25 and ML3 have signal peptides and accumulate in vacuoles (Nakano et al., 2012; Hakenjos et al., 2013). JAL and GLL proteins form a large protein complex with PYK10 when cells are collapsed or disrupted (Nagano et al., 2005, 2008; Ahn et al., 2010), suggesting their functional link to PYK10 and ER bodies. Current research indicates that there are no morphological disorders of ER bodies when these proteins are depleted, indicating

Table 1 | A summary of 47 β-glucosidases encoded in the *A. thaliana* genome.

Subfamily§	Alias	Locus	Signal peptide†	ER retention	Proton donor‡	Proton acceptor‡	Aglycone binding‡	Expression¶	Notes
1	BGLU1	At1g45191	+	–	E	E	G	–	–
	BGLU2	At5g16580	–	–	E	E	G	–	
	BGLU3	At4g22100	+	–	E	E	G	Maturing seeds (late)	–
	BGLU4	At1g60090	+	–	E	E	G	Very low in all tissues	–
	BGLU5	At1g60260	+	–	E	E	G	–	–
	BGLU6	At1g60270	+	–	E	E	G	Aerial tissues except for reproductive tissues	–
	BGLU7	At3g62740	+	–	E	E	G	Seedlings and roots	–
	BGLU8	At3g62750	+	–	E	E	A	–	–
	BGLU9	At4g27820	+	–	E	E	G	Ubiquitous	–
	BGLU10	At4g27830	+	–	E	E	G	Ubiquitous	–
	BGLU11	At1g02850	+	–	E	E	G	Reproductive tissues and roots	–
2	BGLU12	At5g42260	+	–	E	E	Q	–	–
	BGLU13	At5g44640	+	–	E	E	Q	–	–
	BGLU14	At2g25630	+	–	E	–	Q	Pollen	–
	BGLU15	At2g44450	+	–	E	E	Q	Maturing seeds (early), flowers, and roots	–
	BGLU16	At3g60130	+	–	E	E	E	Ubiquitous	–
	BGLU17	At2g44480	–	–	E	E	L	Maturing seeds (late) and roots	–
3	BGLU18	At1g52400	+	+	E	E	A	Very high; aerial tissues	Induced by wounding; involved in ABA signaling
	BGLU19	At3g21370	+	+	E	E	S	Maturing seeds (late)	–
	BGLU20	At1g75940	+	+	E	E	A	Flowers	–
	BGLU21	At1g66270	+	+	E	E	A	–	–
	BGLU22	At1g66280	+	+	E	E	A	–	–
	BGLU23/PYK10	At3g09260	+	+	E	E	A	Seedlings and roots	ER body component
	BGLU24	At5g28510	+	+	E	E	A	Very low in all tissues	–
	BGLU25/GLUC	At3g03640	+	+	E	G	G	Ubiquitous	–
4	BGLU26/PEN2	At2g44490	–	–	E	E	A	Ubiquitous, except for maturing seeds	Atypical myrosinase; required for resistance against powdery mildew
	BGLU27	At3g60120	–	–	E	E	G	Very low in all tissues	–
5	BGLU28	At2g44460	+	–	E	E	A	Weakly in reproductive tissues and roots	–

(Continued)

Table 1 | Continued

Subfamily§	Alias	Locus	Signal peptide†	ER retention	Proton donor‡	Proton acceptor‡	Aglycone binding‡	Expression¶	Notes
	BGLU29	At2g44470	+	–	E	E	A	Maturing seeds	–
	BGLU30/DIN2/SRG2	At3g60140	+	–	E	E	A	Maturing seeds and roots	–
	BGLU31	At5g24540	+	–	E	E	S	Very low in all tissues	
	BGLU32	At5g24550	+	–	E	E	S	Very low in all tissues	
6	BGLU33	At2g32860	+	–	E	E	A	Ubiquitous in vegetative tissues	Involved in ABA signaling
7	BGLU34/TGG4	At1g47600	+	–	Q	E	K	–	Myrosinase in roots; resistance against insects?
	BGLU35/TGG5	At1g51470	+	–	Q	E	K	–	Myrosinase in roots; resistance against insects?
	BGLU36/TGG6	At1g51490	–	–	Q	P	K	Pollen	Myrosinase; pseudogene
	BGLU37/TGG2	At5g25980	+	–	Q	E	R	–	Myrosinase in leaves; resistance against insects?
	BGLU38/TGG1	At5g26000	+	–	Q	E	R	–	Myrosinase in leaves; resistance against insects?
	BGLU39/TGG3	At5g48375	+	–	Q	E	R	–	Myrosinase; pseudogene
8	BGLU40	At1g26560	+	–	E	E	Q	Ubiquitous except for roots	–
	BGLU41	At5g54570	+	–	E	E	Q	Reproductive tissues	–
	BGLU42	At5g36890	–	–	E	E	N	Ubiquitous except for maturing seeds	–
9	BGLU43	At3g18070	+	–	E	E	L	Very low in all tissues	–
	BGLU44	At3g18080	+	–	E	E	L	Ubiquitous	–
10	BGLU45	At1g61810	+	–	E	E	L	Stems and maturing seeds	–
	BGLU46	At1g61820	+	–	E	E	L	Maturing seeds and roots	–
	BGLU47	At4g21760	–	–	E	E	L	Leaves	–

§ *Xu et al., 2004.*

† *SignalP (http://www.cbs.dtu.dk/services/SignalP/).*

‡ *Burmeister et al., 1997.*

¶ *ATTED-II (http://atted.jp/).*

their lack of importance in ER body formation. **Figure 2** shows a proposed model of how the proteins are coordinated during ER body formation. Because a defect of either NAI2 or PYK10 causes disorganized ER body formation, these proteins are important for shaping ER bodies. It is possible that these proteins interact with each other to condense materials for ER bodies, followed by the NAI2-dependent recruitment of MEB1 and MEB2 to the pre-ER body membrane to form the ER bodies.

RELATIONSHIP OF ER NETWORK FORMATION AND ER BODY FORMATION

The ER body is derived from the ER network, and some ER proteins also localize in ER bodies, and thus it is possible that the organization of ER and ER bodies is coregulated by similar molecular mechanisms. Understanding how ER networks are formed and maintained provides insights into ER body organization. The molecular basis underlying ER morphology has been intensely studied for more than a decade using a variety of eukaryotic cells including mammals, yeasts, and plants such as *A. thaliana* and *Nicotiana benthamiana* (reviewed in Chen et al., 2013). It has been shown that ER network structures are determined by a series of membrane proteins [i.e., tubule-associated proteins such as reticulon family proteins (RTNs), Atlastin/ROOT HAIR DEFECTIVE 3 (RHD3) GTPases, and cisternae-associated proteins including CLIMP-63] (Nziengui et al., 2007; Sparkes et al., 2010; Tolley et al., 2010; Chen et al., 2011; Lee et al., 2011, 2013; Stefano et al., 2011; Zhang et al., 2013). Most studies of RTNs and Atlastin/RHD3 have been carried out using animal cells or *N. benthamiana*, which does not generate ER bodies. Therefore, it remains unclear how the plants developing ER bodies utilize these conserved mechanisms to generate these unique structures. Because reticulons and other similar proteins prefer localizing to the high curvature of ER membranes to retain a tubular structure, it is possible that these proteins are excluded from the ER bodies that have lower membrane curvature compared to that of the tubules. Alternatively, they might interact with ER body-specific proteins such as MEB1 and MEB2 on the ER body membranes, and contribute to its unique curvatures. In this context, the localization of RTNs and RHD3 in *A. thaliana* is of great interest.

In *A. thaliana*, a series of mutants designated as *endoplasmic reticulum morphology* (*ermo*) mutants have been isolated in a forward-genetic screen to identify the factors responsible for maintaining ER morphology (Nakano et al., 2009, 2012). In the *ermo1* and *ermo2* mutants, the cells develop a number of ER-derived spherical bodies, ∼1 μm in diameter, in addition to the typical ER network, whereas *ermo3* cells develop huge aberrant aggregate structures derived from the ER network and ER bodies. *ERMO1* and *ERMO2* encode *GNOM-LIKE 1* (*GNL1*) and *SEC24a*, respectively, which are involved in protein transport between ER and Golgi bodies. The unaffected morphology of ER bodies suggested that both *GNL1* and *SEC24a* were dispensable for the proper organization of ER bodies (Faso et al., 2009; Nakano et al., 2009). *ERMO3* encodes a GDSL lipase-like protein also known as MODIFIED VACUOLE PHENOTYPE1 (MVP1) or GOLGI DEFECTIVE36 (GOLD36), which localizes in vacuoles (Agee et al., 2010; Marti et al., 2010; Nakano et al., 2012). The ER aggregations in *ermo3* are absent in cells that do not accumulate ER bodies, and are suppressed in whole tissues by the introduction of the *nai1* mutation, suggesting a strong relationship between ERMO3 and ER bodies. The ER body structure is not significantly affected in *ermo3* cells (Marti et al., 2010; Nakano et al., 2012), and ERMO3 does not interact with NAI2 (Nakano et al., 2012), suggesting that the contribution of ERMO3 to ER body formation is not highly significant. ERMO3 was required for proper protein transport between ER and Golgi bodies, and ERMO3 formed a protein complex with PYK10, JAL, and MATH domain-containing proteins, which are regulated by NAI1 (Nakano et al., 2012). These results suggest that PYK10, JAL, and MATH domain-containing proteins form a large protein aggregate on ER, then alter the ER morphology to inhibit protein secretion in the absence of ERMO3. ERMO3 may be responsible for solving this aggregate in ER, because the expression of ERMO3 in ER rescues the phenotype in the *ermo3* mutant (Nakano et al., 2012). This tissue-specific requirement of ERMO3, in addition to the ubiquitous expression of ERMO3, clearly shows that each cell type requires its own regulatory systems to maintain their subcellular organization.

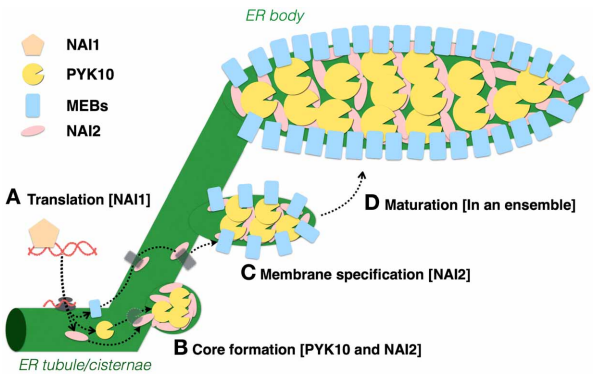

FIGURE 2 | Models of ER body formation in *A. thaliana* seedlings. (A) *NAI1* is expressed in epidermal cells to regulate ER body formation by inducing the expression of *PYK10*, *NAI2*, *MEB1*, and *MEB2*. **(B)** PYK10 and NAI2 may physically interact to form ER body structures from the ER. **(C,D)** NAI2 forms a complex with MEB1and MEB2, then recruits them to the ER body to form the ER body-specific membrane.

PHYSIOLOGICAL FUNCTIONS OF ER BODIES: PUTATIVE ASSOCIATION WITH INNATE IMMUNITY

TISSUE SPECIFICITY AND WOUNDING INDUCIBILITY INDICATE THE IMPORTANCE OF ER BODY IN PLANT DEFENSE

Constitutive ER bodies in *A. thaliana* are enriched in seedlings and roots of mature plants (Matsushima et al., 2002). In cotyledons, ER bodies develop only in epidermal cells but not in mesophyll cells. Similarly, no ER bodies are detected in the root vascular cylinder, in contrast to their presence in root epidermal, cortical, and endodermal layers (**Figure 3**). Shoot tissues including rosette and cauline leaves have fewer ER bodies compared to those of the underground tissues. They are absent in most of the rosette leaf cells, except for some epidermal cells along the primary and secondary veins or the edge of the leaves (Nakano et al., 2012). These specificities among plant tissues and cell types suggest that ER bodies are enriched at the interface between plants and surrounding organisms to protect plants from pathogens/herbivores that may enter or feed from the veins or leaf edge. This is supported by the fact that leaf wounding triggers local and systemic *de novo* formation of ER bodies in a jasmonic acid (JA)-dependent manner (Matsushima et al., 2002; Ogasawara et al., 2009). Atypical, elongated ER bodies are produced after wounding in the *nai1* mutant, suggesting that another factor in addition to NAI1 is involved in this response (Matsushima et al., 2003). Consistent with this, the induction

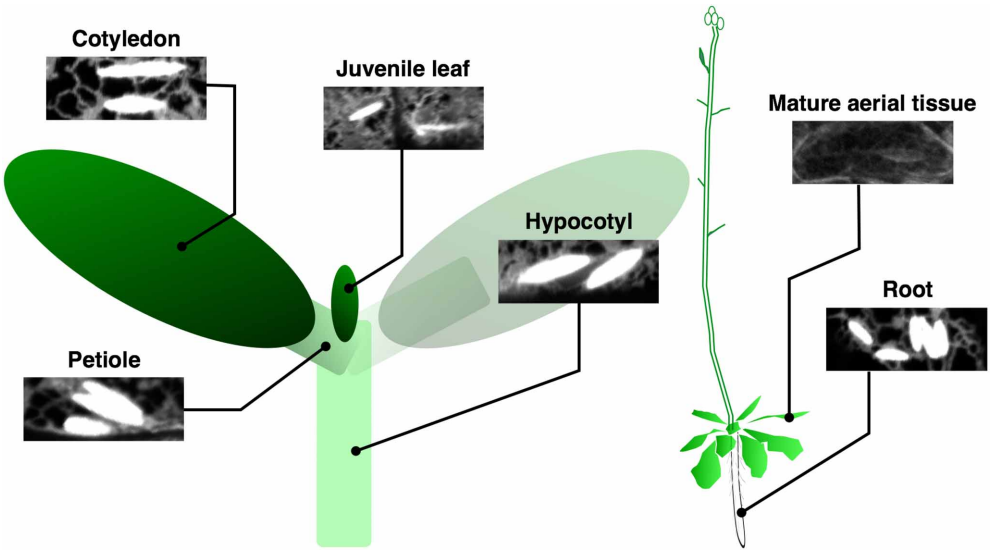

FIGURE 3 | Tissue localization of ER bodies. ER bodies are present in the epidermis of whole tissues in young seedlings, but are absent in aerial organs of mature plants. Root epidermal, cortical, and endodermal cells continue to develop ER bodies. Epidermal cells of a transgenic *A. thaliana* expressing ER-localized GFP from several tissues are shown.

of BGLU18, a major component of wound-triggered ER bodies in rosette leaves, is independent of NAI1 (Ogasawara et al., 2009). The observed high abundance of ER bodies in roots may contribute to the interaction between plants and surrounding (potential) pathogens and herbivores that inhabit soil (Yamada et al., 2011). For example, the soil environment contains 10^6–10^9 bacteria per gram, which is much greater than in the atmosphere (10^1–10^5 per cubic meter) (Bulgarelli et al., 2013). Many other organisms such as insects, worms, nematodes, and fungi also live in the soil and seek opportunities to exploit plant roots.

β-GLUCOSIDASE ACTIVITY OF PYK10 MAY CONTRIBUTE TO THE PLANT IMMUNE RESPONSE

The high abundance of PYK10 and other β-glucosidases in ER bodies implies that the enzymatic activities of these proteins play an important role in the function of this organelle. The reported *in vitro* substrates of PYK10 include 4-methylumbelliferyl (4MU)-glucoside, 4MU-fucoside (Matsushima et al., 2004), scopolin, and esculin (Ahn et al., 2010). Recombinant PYK10 protein expressed in insect cells hydrolysed with the highest efficiency scopolin (Ahn et al., 2010), a coumarin widely occurring in the plant kingdom, including *A. thaliana* (Bednarek et al., 2005; Shimizu et al., 2005; Kai et al., 2006; Bayoumi et al., 2008). Scopolin is a β-*O*-glucoside of scopoletin, which is regarded as a phytoalexin. It was shown that scopoletin and scopolin could inhibit germ tube growth of *Sclerotinia sclerotiorum* (Prats et al., 2006). Scopoletin was also found to possess toxic activity against *Fusarium oxysporum*, *Fusarium solani*, *Rhizopus stolonifer*, and *Lasiodiplodia theobromae* (Peterson et al., 2003). Growth inhibition of *F. oxysporum* was higher with scopoletin than with scopolin, suggesting that a β-glucosidase could have an important role in this activity.

Concerning the biological activity of scopoletin, it is possible that ER bodies are involved in plant defense by hydrolysing scopolin to this aglycone. As discussed above, JAL proteins, GLL proteins, and MATH domain-containing proteins formed a large protein complex with PYK10 after cell disruption (Nagano et al., 2005, 2008; Ahn et al., 2010). These proteins accumulate constitutively in separate cellular organelles: PYK10 in ER body, GLLs in vacuole, JALs in cytosol, and MATHs at plasma membrane (Nagano et al., 2005; Oelmuller et al., 2005; Marti et al., 2010; Nakano et al., 2012). The β-glucosidase activity of PYK10 is higher after complex formation, suggesting that JALs and GLLs serve as activators of PYK10 (Nagano et al., 2005, 2008). Stimulation of β-glucosidase activity can be solely carried out by PBP1, as revealed by an *in vitro* enzymatic assay (Ahn et al., 2010). This fact, combined with wound inducibility, suggests that these proteins are assembled with PYK10 when cells are damaged by feeding insects or pathogen infection, and that they produce substances that potentially target these invaders.

PYK10 MAY HAVE MYROSINASE ACTIVITY TOWARD INDOLE GLUCOSINOLATES

β-Glucosidases in the order *Brassicales* include a unique class of enzymes named myrosinases or β-thioglucoside glucohydrolases (TGGs), which are involved in the defense against insects, fungi, and bacteria (reviewed in Hopkins et al., 2009). Myrosinases are responsible for hydrolysing glucosinolates, thioglucosides that are specific to the order *Brassicales* (Halkier and Gershenzon, 2006). For a long time, myrosinases were considered to contain a unique amino acid signature that enables *in silico* prediction of their identity based on nucleotide sequence data (Burmeister et al., 1997). One of the unique features of this signature is a conserved basic residue (lysine or arginine) at the substrate pocket, which can form electrostatic interactions with the negatively charged sulfate group of glucosinolates (see **Figure 5**). It was found that the glutamate residue that serves as a proton

donor in *O*-glucosidases is substituted by glutamine in myrosinases, resulting in the strict reduction of *O*-glucosidase activity (Burmeister et al., 1997). In *A. thaliana*, six genes (*TGG1−6*) including two pseudogenes [*TGG3*, (Zhang et al., 2002); *TGG6*, (Andersson et al., 2009)] encode myrosinases. TGG1 and TGG2 are expressed primarily in leaves (Xue et al., 1995), whereas TGG4 and TGG5 are expressed primarily in roots (Barth and Jander, 2006; Andersson et al., 2009; Zhou et al., 2012). These four myrosinase have conserved lysine/arginine and glutamine residues at the substrate pocket, and have activity against glucosinolates. By contrast, the other β-glucosidases lacking these specific amino acids were thought to be β-glucosidases hydrolysing *O*-glucosides but not thioglucosides (Rask et al., 2000). However, PEN2, a β-glucosidase lacking these key amino acids, was recently shown to be an atypical myrosinase hydrolysing indol-3-ylmethyl glucosinolate (I3G) and 4-methoxy-I3G (4M-I3G) (Bednarek et al., 2009). PEN2 has a major role in *A. thaliana* immunity via its myrosinase activity (reviewed in Bednarek, 2012) against various microbes including fungal pathogens such as *Blumeria graminis* and *Plectosphaerella cucumerina* (Sanchez-Vallet et al., 2010), *Magnaporthe oryzae* (Maeda et al., 2009), *Leptosphaeria maculans* (Elliott et al., 2008), *Colletotrichum* species (Hiruma et al., 2010), oomycetes [e.g., *Phytophthora brassicae* (Schlaeppi et al., 2010) and *Pythium irregularum* (Adie et al., 2007)], and a growth-promoting endophytic fungus (*Piriformospora indica*; Jacobs et al., 2011).

PEN2 does not belong to the subfamily of PYK10, but both proteins have high sequence similarity (**Figure 4**), which suggests that PYK10 also has atypical myrosinase activity, whereas recombinant PYK10 was unable to hydrolyse sinigrin (Ahn et al., 2010). Sinigrin, which represents aliphatic glucosinolates, consists of the glucosinolate core structure with a short aliphatic side chain that differs significantly in its structure from the indolyl group present in I3G and 4M-I3G (indole glucosinolates) (**Figure 5**). It cannot be excluded that PYK10 may hydrolyse indole glucosinolates in addition to its inactivity toward aliphatic glucosinolates. The reported substrates for PYK10 have aglycones of coumarinyl moieties, suggesting that the substrate pocket of PYK10 is suitable for condensed-ring moieties. This also supports the possible activity of PYK10 to hydrolyse indole glucosinolates, which have condensed-ring moieties as aglycones (**Figure 5**). According to Brown et al. (2003), indole glucosinolates are the most abundant glucosinolates in *A. thaliana* roots (at least of Col-0 accession), where ER bodies and PYK10 are also highly abundant. Coexpression analysis based on a public microarray database (ATTED-II; http://atted.jp) revealed a strong correlation between *PYK10* and *CYP81F4* encoding an enzyme involved in the modification of I3G into 1-methoxy-I3G (Pfalz et al., 2011), further suggesting a functional link between PYK10 and indole glucosinolates.

PYK10 IN MUTUALISTIC RELATIONSHIP WITH *PIRIFORMOSPORA INDICA* AND DEFENSE AGAINST PARASITIC NEMATODES

It was reported that transgenic *A. thaliana*, which produced a novel type of glucosinolates, showed distinct bacterial and fungal communities in its rhizosphere compared to those of WT plants (Bressan et al., 2009). This result strongly suggests the

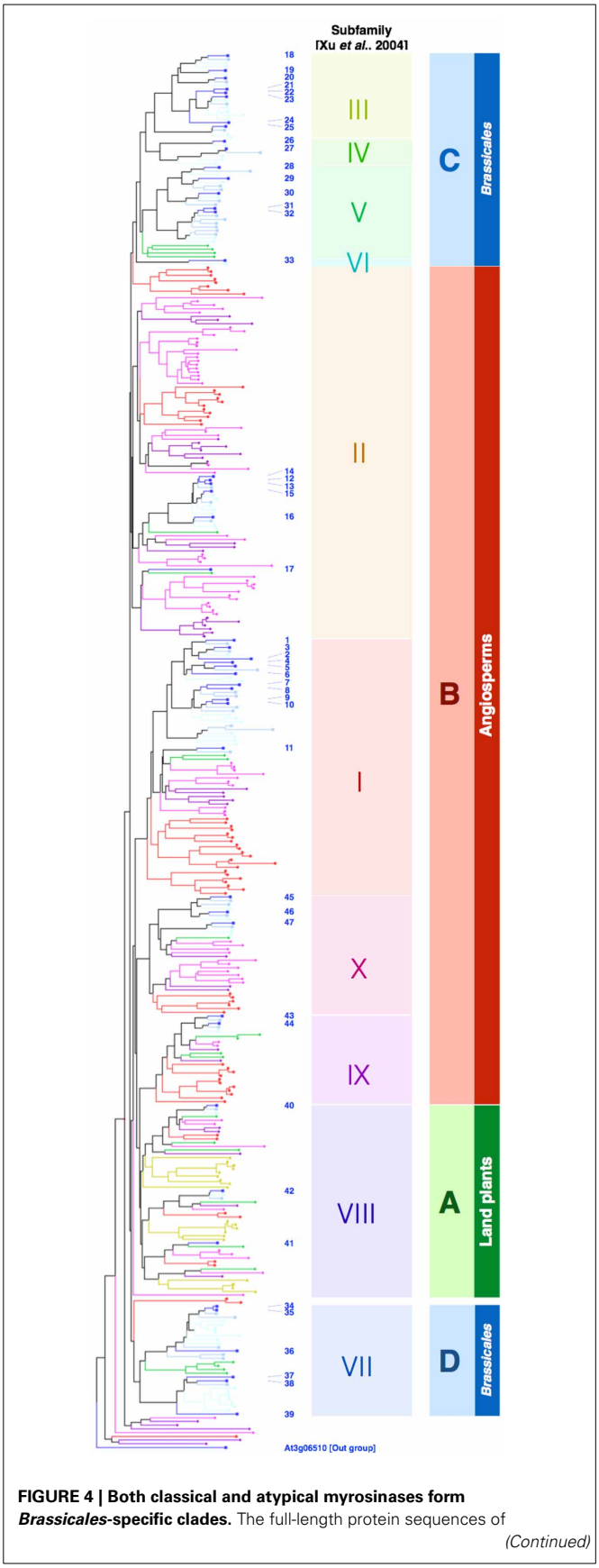

FIGURE 4 | Both classical and atypical myrosinases form *Brassicales*-specific clades. The full-length protein sequences of

(Continued)

FIGURE 4 | Continued
β-glucosidases in *A. thaliana* (blue squares), *Capusella rubella* (light-blue squares), *Thellungiela salsuginea* (cyan squares), *Carica papaya* (green diamonds), *Vitis vinifera* (dark purple diamonds), *Glycine max* (magenta diamonds), *Oryza sativa* (red circles), and *Physcomitrella patens* (dark yellow triangles) were retrieved from the Phytozome database (http://www.phytozome.net/) and aligned by ClustalW (http://clustalw.ddbj.nig.ac.jp/index.php). BGLU numbers of the β-glucosidases in *A. thaliana* are indicated by blue letters. At3g06510 was used as an out group. Subfamilies proposed by Xu et al. (2004) are indicated accordingly.

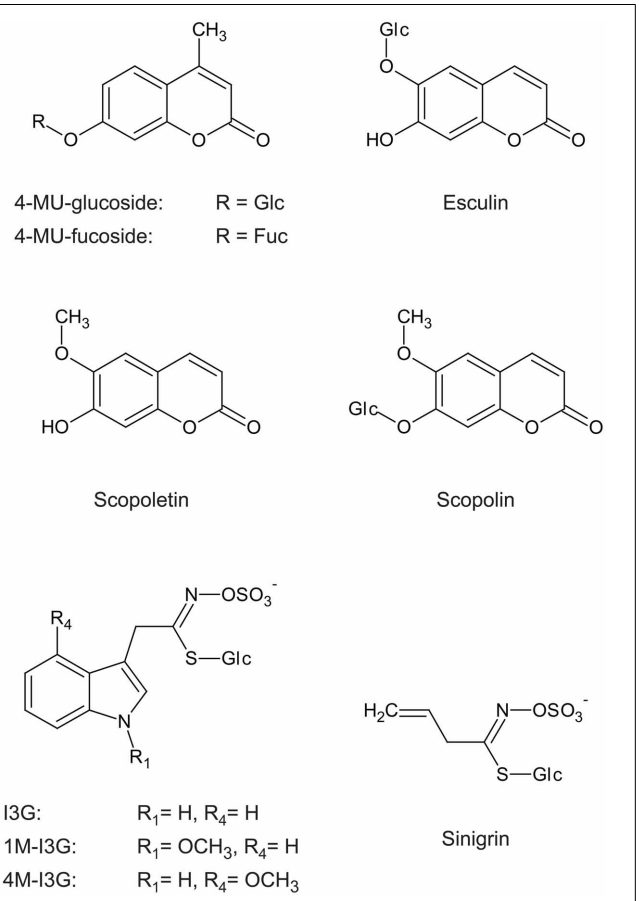

FIGURE 5 | Indole glucosinolate structure includes a side chain similar to that of scopolin and esculin, but not similar to that of sinigrin. I3G, indol-3-ylmethylglucosinolate; 1M-I3G, 1-methoxyindol-3-ylmethylglucosinolate; 4M-I3G, 4-methoxyindol-3-ylmethylglucosinolate; 4-MU-glucoside, 4-methylumbelliferyl-β-D-glucoside; 4-MU-fucoside, 4-methylumbelliferyl-β-D-fucoside.

involvement of glucosinolate in pathogenic and mutualistic plant microbe interactions in the soil environment. One of the most extensively studied root-colonizing microorganisms is an endophytic fungus *P. indica* that has a broad range of host species and a capacity to promote host-plant growth (Varma et al., 1999). Similar to the *pen2* mutant, *pyk10* and *nai1* mutant plants failed to suppress *P. indica* growth at a preferable level, resulting in fungal overgrowth that stimulated undesirable immune responses and loss of the beneficial interaction (Sherameti et al., 2008; Jacobs et al., 2011). In addition, depletion of two cytochrome P450 enzymes (CYP79B2 and CYP79B3) that are responsible for conversion of tryptophan into indole-3-acetaldoxime (IAOx), the first step of the biosynthesis of indole glucosinolates, also resulted in overgrowth of *P. indica* (Nongbri et al., 2012). By contrast, the phytoalexin camalexin, which is derived from IAOx and is absent in the *cyp79B2 cyp79B3* mutant, was dispensable for this suppression, suggesting that the proper control of this mutualistic fungus may involve indole glucosinolate metabolism. These results suggest that PYK10, PEN2, and indole glucosinolates have important roles in the establishment of the beneficial interaction with *P. indica*.

PYK10 was originally isolated as a root-specific gene with high expression level that became additionally elevated in nematode-infected tissue (Nitz et al., 2001). Because the degradation products of glucosinolates exhibited inhibitory activity against both a cyst nematode (*Globodera rostochiensis*; Buskov et al., 2002) and a root-knot nematode (*Meloidogyne incognita*; Lazzeri et al., 2004), it is possible that PYK10 protects roots from these parasites by its potential myrosinase activity. Cyst nematodes degrade the cell wall and infect *intracellularly*, whereas root-knot nematodes infect *intercellularly* (reviewed in Mitchum et al., 2012). In both cases, the cells surrounding infectious nematodes are disrupted and possibly collapse, which can passively trigger hydrolysis of glucosinolates. Alternatively, the plant may recognize the presence of these pests via nematode-associated molecular patterns or effectors injected into plant cells, and actively exert defensive glucosinolate metabolism.

Although both *pen2* and *pyk10* mutants showed greater *P. indica* colonization (Sherameti et al., 2008; Jacobs et al., 2011), there was no direct evidence that the control of fungal growth during this interaction was due to the myrosinase activity of both enzymes toward indole glucosinolates. Similarly, in addition to the inhibitory activity of glucosinolates against parasitic nematodes, it is not known if myrosinases are involved in these interactions. In addition to PEN2 and PYK10, *A. thaliana* roots express two classical myrosinases (TGG4 and TGG5), and TGG4 was shown to metabolize indole glucosinolates when overexpressed *in planta* (Bednarek et al., 2009). Future work will determine which of these four enzymes, and possibly other atypical myrosinases, are the key players during the interaction with endophytic fungi and parasitic nematodes.

ENHANCED SECRETION OF PROTEINS LOCALIZED IN ER BODIES (ESPER) IS A NOVEL IMMUNE RESPONSE AGAINST PATHOGENS

Recently, Watanabe et al. (2013b) reported a novel immune response designated as ESPER. They showed that two plant pathogenesis-related (PR) proteins, defensin (PDF1.2) and PR1, which contain antimicrobial activity, accumulated in ER bodies at a steady-state level and were secreted to the apoplast in response to the non-adapted pathogenic fungi *Colletotrichum gloeosporioides*. This suggests that ER bodies can serve as storage sites for various PR proteins until pathogenic attack, although these proteins have been reported to localize primarily in the vacuole or apoplast (Keefe et al., 1990; Dixon et al., 1991; Neuhaus et al., 1991; Van Loon et al., 2006; Sels et al., 2008). However, ER bodies

can be labeled with GFP fused to proteins in the secretory pathway, including both soluble and membrane proteins, which finally accumulate in other organelles (Teh and Moore, 2007), suggesting the non-selective entry of these proteins into ER bodies. The localization of PDF1.2 and other PR proteins was analyzed using GFP-fused protein constructs driven by the artificial cauliflower mosaic virus (CaMV) 35S promoter (Watanabe et al., 2013b). Therefore, it is possible that the observed protein accumulation in ER bodies could have been an artefact due to gene overexpression. Nevertheless, the drastic change in localization of these GFP-fused PR proteins under fungal attack indicates that the membrane trafficking system in plant cells can dynamically change in response to the surrounding environment. Although the ER body itself is not involved in ESPER, as suggested by the presence of ER bodies even after ESPER occurred, this, in turn, suggests that localization of PYK10 and other ER body components may also dynamically respond to abiotic/biotic conditions to become active.

POTENTIAL ER BODY FUNCTION IN METAL TOLERANCE OR UPTAKE

Recently, it was reported that iron starvation stimulated an ATP-binding cassette transporter ABCG37 (PDR9)-dependent secretion of scopoletin (Fourcroy et al., 2014). Roots of the *pdr9* mutants that lacked functional ABCG37 failed to secrete scopoletin and showed abnormal accumulation of its derivatives, including scopolin, inside the tissue. These mutants also exhibited decreased tolerance to iron deficiency, suggesting that secretion of scopoletin helped plants to take up iron. This is further supported by the results that some of the coumarin derivatives have the potential to chelate iron and serve as phytosiderophores (Mladenka et al., 2010). Therefore, scopoletin, produced by the action of PYK10 on scopolin, may contribute to iron uptake in *A. thaliana* roots. Notably, MEB1 and MEB2, the ER body-specific membrane proteins, were recently suggested to be iron transporters that can endow *S. cerevisiae* with iron tolerance when expressed heterologously (Yamada et al., 2013). These results suggest that ER bodies have a role in metal-stress response apart from plant microbe interactions. One possibility is that under iron-limiting conditions, ER bodies fuse with plasma membrane to release PYK10 into the apoplast, which consequently hydrolyses scopolin that is secreted to the apoplast via ABCG37 (**Figure 6**). Liberated scopoletin may enhance the availability of iron, which can be taken up via MEB putative transporters that are transferred on the plasma membrane. Mugineic acid, a phytosiderophore in barley, accumulates in ER-derived vesicles peripheral to the plasma membrane of root epidermal cells, and can be secreted to the soil to improve the iron availability (Negishi et al., 2002). To date, there is no report elucidating how ER bodies behave in iron-depleted conditions. In addition, it should be noted that the experiments reported by Fourcroy et al. (2014) were performed with roots of plants grown on agar plates and exposed to light. The accumulation of phenylpropanoids (the group of metabolites that includes scopolin and scopoletin) is strongly upregulated in *A. thaliana* roots artificially exposed to light, as compared to roots grown in soil (Hemm et al., 2004), suggesting that in soil the scopoletin-dependent iron-uptake mechanism may have reduced significance. Therefore, it is of great interest to test ER body behavior, together with scopoletin production, in *A. thaliana* roots grown in iron-depleted soil, which may validate the function of ER bodies in metal tolerance or uptake.

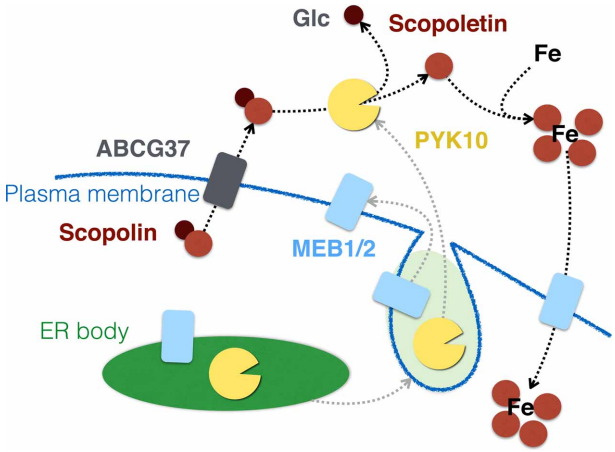

FIGURE 6 | PYK10 and ER bodies may have a role in iron uptake via hydrolysing scopolin. ER bodies may fuse with the plasma membrane under iron-deficient conditions, resulting in the relocation of MEB1 and MEB2 to the plasma membrane and secretion of PYK10 to the apoplast. Scopolin, secreted in an ABCG37-dependent manner, is then converted to scopoletin by PYK10, which in turn helps the cells to take up iron via the chelating activity of scopoletin. This uptake may be carried out by putative MEB transporters.

STRESS TRANSLATION VIA PHYTOHORMONE ACTIVATION

BGLU18/AtBG1 and BGLU33/AtBG2, which belong to the same clade with PYK10 and PEN2 (see **Figure 4**, discussed below), were reported to be involved in abscisic acid (ABA) signaling and drought resistance by hydrolysing ABA-*O*-glucoside, which is an inactive pool of ABA (Lee et al., 2006; Han et al., 2012; Xu et al., 2012; Watanabe et al., 2013a). BGLU18 is a member of the PYK10 subfamily. According to the ATTED-II database, BGLU18 is expressed primarily in aerial organs such as leaves and stems, but is limited in roots, which negatively correlates with the presence of constitutive ER bodies. However, BGLU18 is a component of ER bodies that are induced by wounding (Ogasawara et al., 2009). *BGLU18* is co-expressed with some homologs of genes related to constitutive ER bodies including *TSA1*, the closest homolog of *NAI2*, *JAL* genes (*JAL23* and *JAL35*), and a *GLL* gene, strongly suggesting that the mechanisms of BGLU18 accumulation and activation could be similar to those reported for PYK10.

Presuming that ABA is activated through sugar release from ABA-glucoside in drought-stressed leaves, it could be speculated that BGLU18 is engaged in ABA-activation in a manner independent from both constitutive root ER bodies and wound-inducible leaf ER bodies. Because the dynamics of ER bodies under drought stress are still unclear, it is possible that these organelles are induced under water-deficit conditions to mediate the conversion of ABA-*O*-glucoside to ABA, in response to the signal transduction of drought stresses. According to such a scenario, BGLU18 expression is induced by wounding and by

drought stress (ATTED-II), suggesting its dual functions in stress pathways. BGLU19, another member of the PYK10 subfamily, was suggested to be involved in cytokinin activation based on the highest sequence similarity with the β-glucosidase of *Brassica napus* that hydrolyses zeatin-*O*-glucoside (Falk et al., 1995; Xu et al., 2004). These results suggest that some β-glucosidases from this clade may have potential roles in phytohormone activation. The accumulation of various glucosylated phytohormones as precursors (Gachon et al., 2005; Poppenberger et al., 2005; Lee et al., 2006) suggests the involvement of glucosidases in the activation of these compounds, where the β-glucosidases may participate. Elucidating the involvement and behavior of ER bodies in phytohormone-mediated stress responses is required to further understand the functions of this organelle.

EVOLUTION OF ER BODIES AND β-GLUCOSIDASES IN THE ORDER *BRASSICALES*

ER BODIES ARE FORMED IN PLANTS BELONGING TO THE *BRASSICALES* ORDER

ER bodies were frequently observed by electron microscope using root tissues of plants belonging to the order *Brassicales* (Bonnett and Newcomb, 1965; Iversen and Flood, 1969; Iversen, 1970a; Cresti et al., 1974; Hoefert, 1975; Endress and Sjolund, 1976; Jørgensen et al., 1977; Behnke and Eschlbeck, 1978; Gailhofer et al., 1979; Jørgensen, 1981). This order includes *Brassicaceae* (mustard and cabbage family, including *A. thaliana*), *Capparaceae* (caper family), *Cleomaceae* (cleome family), *Resedaceae* (mignonette family) and *Tovariaceae*. The plants belonging to *Brassicaceae*, *Cleomaceae*, and *Capparaceae* are closely related to each other (Hall et al., 2004), and develop ER bodies or ER body-like structures in stems or roots. The ER bodies in these species occasionally contain internal filamentous structures (Hoefert, 1975; Jørgensen et al., 1977; Behnke and Eschlbeck, 1978; Gailhofer et al., 1979), suggesting their well-ordered formation. To date, no ER bodies have been observed in the *Resedaceae* (Iversen, 1970a). The *Tovariaceae* also do not develop ER bodies; however, filamentous aggregations of electron-dense materials similar to that of ER body were observed in the vacuoles of vascular-bundle cells (Behnke and Eschlbeck, 1978). Considering that *Tovariaceae* separated from *Brassicaceae* approximately 40−50 million years ago (Martin-Bravo et al., 2009), these filamentous structures might be the evolutionary intermediates of ER bodies. Collectively, ER bodies and related structures develop exclusively in a taxonomically limited group, raising the question of how this organelle evolved in these families.

The plant order *Brassicales* includes agriculturally important crops such as *Brassica rapa* and the model species *A. thaliana*, and plants in this order have been studied intensely. Many *Brassicales* plants are characterized with some unique features that evolved in this order, including the production of glucosinolates and other sulfur-containing secondary metabolites (Mithen et al., 2010; Bednarek et al., 2011), and the inability to accept arbuscular mycorrhiza as symbionts (reviewed in Delaux et al., 2013). Based on the public genome sequences, most of the proteins that localize in ER bodies and those that participate in the PYK10 protein complex belong to clades that occur in the family *Brassicaceae*. This suggests that *A. thaliana*, and most likely other species in *Brassicales*, have innovated a set of specific genes for developing these unique organelles.

β-GLUCOSIDASES OF THE PYK10 SUBFAMILY EVOLVED IN THE *BRASSICALES* ORDER

The *A. thaliana* genome encodes 47 β-glucosidases, which are grouped into ten subfamilies (Xu et al., 2004). **Figure 4** shows a neighbor-joining phylogenic tree generated for the *A. thaliana* β-glucosidases and related proteins retrieved from the genome sequences of *Capsela rubella*, *Tellungiella salsuginea* (formerly *T. halophila*), *Carica papaya*, *Vitis vinifera*, *Glycine max*, *Oryza sativa*, and *Physcomitrella patens*. The clade A includes BGLU40, 41, and 42, which corresponds to subfamily 8 (shaded with green); it also includes the β-glucosidases from *P. patens*, suggesting that these clades are ancestral among land plants. Clade B (shaded with red) corresponds to subfamilies 1, 2, 9, and 10; it does not include enzymes from *P. patens*, but does include enzymes from all other species, suggesting that this clade is specific to the angiosperms. By contrast, clades C and D correspond to the subfamilies 3–6, 7, and appear to be specific to *Brassicales*. Clade D consists of myrosinases, which are known to be specific for this order. The β-glucosidases from *C. papaya* were excluded from the subfamilies 3–5 that contain PYK10 and PEN2, showing the specificity of these three subfamilies to *Brassicaceae*, and possibly other species between *Brassicaceae* and *Caricaceae*. We identified a different lysine/arginine residue from the one conserved in classical myrosinases, which is located at the surface of the substrate pocket and completely conserved in all members of clade C (unpublished data). This positively charged residue might form electrostatic interactions with glucosinolates and explain the myrosinase activity of PEN2. Furthermore, it suggests that other β-glucosidases grouped into the clade are atypical myrosinases. As the glutamate proton donor residue is conserved within clade C, these enzymes appear to be intermediate forms between typical β-*O*-glucosidases and classical myrosinases. Twenty-two β-glucosidases in *A. thaliana* belong to clades C and D (hereafter called EE[K/R]-type and QE[K/R]-type, respectively), which is approximately half of the total β-glucosidases. Although myrosinases have been proposed to be special β-glucosidases, our phylogenetic analyses suggest that myrosinase activity could be a rather common feature of the β-glucosidases in *Brassicales*. The wide diversity within these β-glucosidases suggests that they have substrate specificity toward specific glucosides that are different in each plant tissue or under distinct conditions. This differential specificity would fit with glucosinolates that are highly diversified within *Brassicales* (reviewed in Agerbirk and Olsen, 2012).

It is unknown whether QE[K/R]-type β-glucosidases (classical myrosinases) evolved from EE[K/R]-type β-glucosidases (atypical myrosinases), or if they emerged independently. Two myrosinases that were recently isolated from *Carica papaya* possess the QE signature; however, they do not have any basic [K/R] residues that may mediate electrostatic interactions with glucosinolates. Consequently, both enzymes have a lower affinity for sinigrin compared to that of the classical myrosinases in *A. thaliana* (Nong et al., 2010). *C. papaya* is located almost at the edge of

the *Brassicales* phylogenic tree (Hall et al., 2004), and shared a common ancestor with *A. thaliana* approximately 72 million years ago (Ming et al., 2008), suggesting that myrosinases evolved from these ancient forms that lacked the glucosinolate-binding affinity. Because glucosinolates have been identified in *C. papaya* (Tang, 1973), evolution of this basic residue [K/R] might have been achieved after the innovation of glucosinolates. The specific positions of these conserved basic residues differ between classical and atypical myrosinases; thus, it is likely that these two groups emerged independently. According to the public genome database, the *C. papaya* genome does not encode a protein bearing significant similarity to NAI2 from *A. thaliana*, whereas other species in *Brassicaceae* do, suggesting that ER bodies have evolved specifically in plants belonging to the *Brassicaceae*, possibly due to the evolution of *NAI2*. The evolutionary process of producing ER bodies and atypical myrosinases will be elucidated by analysing species in between *C. papaya* and *Brassicaceae*.

PLANTS OF THE *BRASSICALES* ORDER DEVELOP DISTINCT GLUCOSIDASE-GLUCOSIDE DEFENSE SYSTEMS

To avoid constitutive hydrolysis of glucosinolates, which are proposed to accumulate in vacuoles, myrosinases have to be separated from their substrates into distinct cellular or subcellular compartments (**Figure 7**). In the case of classical myrosinases, the mustard-oil bomb system was proposed, in which the enzymes and substrates accumulate in distinct cells called myrosine cells and S cells, respectively, which allows suppression of the enzymatic reaction until the tissue is crushed. This intercellular partitioning does not require specific subcellular compartments for storage, and myrosinases accumulate in the vacuoles of myrosine cells possibly because they have the largest volume.

Table 1 shows that many β-glucosidases contain signal peptides, but some of them lost this motif. Differential localization might indicate that β-glucosidase activities are required for various cellular functions including those of endomembrane systems, apoplast, and cytosol. The evolutionary events of losing signal peptides appear to occur independently, suggesting that each of these events could reflect the acquisition of a novel molecular function. For example, PEN2 is known to localize at the periphery of peroxisomal membranes, which enables it to accumulate at the penetration sites in response to *Blumeria graminis* f. sp. *hordei* (*Bgh*) challenge (Lipka et al., 2005). This clearly shows the importance of protein localization for protein function. The mechanism of glucosinolate activation by PEN2 is totally different from that of classical myrosinases, because *Bgh* challenge does not inflict cell damage or cell disruption.

It is possible that *Brassicales* plants evolved ER bodies to store atypical myrosinases and separate them from their substrates on the subcellular level. A recent study addressing the cell-type specific metabolome in *A. thaliana* roots revealed the cellular distribution of glucosinolates (Moussaieff et al., 2013). According to this study, indole glucosinolates accumulate to relatively higher levels in columella and cortex, whereas lower levels accumulate in epidermis, endodermis, and stele, indicating that PYK10 and indole glucosinolates occur concurrently in the same cells in the cortex of *A. thaliana* roots, but in different organelles. ER bodies contain PYK10, whereas vacuoles presumably store indole glucosinolates, suggesting that plants in the *Brassicales* order evolved two distinct myrosinase-glucosinolate systems, one of which is the classical mustard-oil bomb system described above. The system proposed here considers that ER bodies separate both components into distinct organelles in a single cell (**Figure 7B**). This subcellular compartmentalization, which does not require tissue collapse to trigger the response, could be highly effective against fungal/bacterial pathogens that usually start the infection process by invading single plant cells. This system could be triggered by translocation of enzymes and/or substrates in living cells, such as the case of PEN2 (Lipka et al., 2005; Bednarek et al., 2009). It is also possible that chemical or protein factors such as pH or chaperones regulate the activity of ER body-accumulating β-glucosidases.

According to the literature, glucosinolates and myrosine cells are widely distributed throughout the *Brassicales* (Jørgensen, 1981). By contrast, ER bodies showed higher taxonomic limitation primarily in *Brassicaceae*, *Cleomaceae*, and *Cappraceae* (Iversen, 1970a; Behnke and Eschlbeck, 1978; Jørgensen, 1981), suggesting that ER bodies are a more recent innovation among

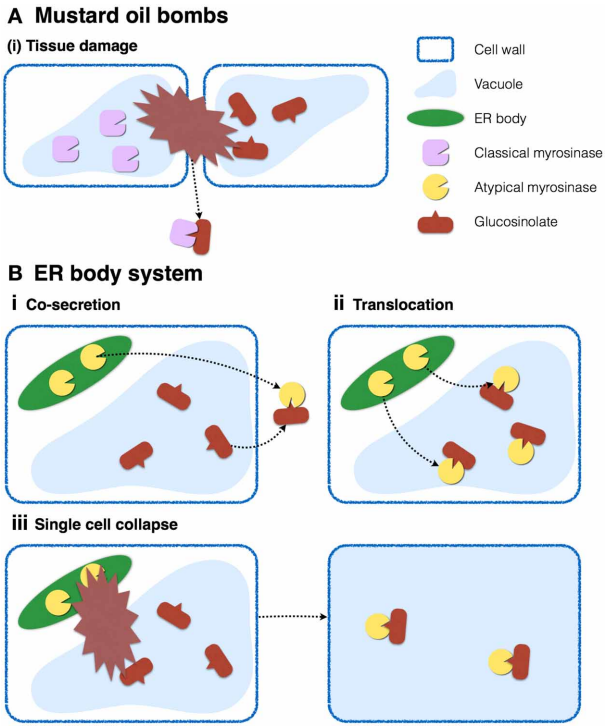

FIGURE 7 | Two distinct myrosinase-glucosinolate systems occurring in *Brassicales*. **(A)** In leaves, myrosinases and glucosinolates accumulate in vacuoles of distinct cells. Tissue collapse accompanied by destruction of multiple cells results in the mixing of enzymes and substrates, and the production of toxic products against herbivores. **(B)** In roots, myrosinases and glucosinolates accumulate in ER bodies and vacuoles, respectively, in the same cells. Enzymatic reactions may be activated by **(i)** cosecretion of enzymes and substrates, **(ii)** translocation of enzymes into vacuoles, or **(iii)** destruction of a single cell.

these families. Due to the lack of genetic/molecular tools in species belonging to other *Brassicales* families than *Brassicaceae*, the evolution of specific substrates, enzymes, and organelles remains unclear. Recent improvements in sequencing technologies have facilitated rapid progress in genome sequences for non-model plants, including many *Brassicaceae* species such as *T. salsuginea* (Wu et al., 2012; Yang et al., 2013), *B. rapa* (Wang et al., 2011), *C. rubella* (Slotte et al., 2013), and *A. lyrata* (Hu et al., 2011). Molecular and genetic tools are becoming available that will enable the inclusion of many other species in future research.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

We have shown how ER bodies are unique in their shape, biogenesis mechanism, functions, and evolution. The study of ER bodies provides novel insights into plant innate immunity systems, stress tolerance, and the subcellular organization of eukaryotic cells. Cell biological studies for understanding eukaryotic organelle organization are sometimes too focused on single cell types, including cultured cells. However, cultured cells are very different from endogenous cells in living individuals. For example, tobacco BY-2 cells are one of the most widely utilized plant cultured cell lines, but they have ER structures that are slightly different to those in *A. thaliana*, lacking ER bodies, and tubules and cisternae that appear different (Nakano et al., 2009). It is necessary to keep in mind what cell types are used for the experiments, and to clearly distinguish what is unique to the cell type and what is common to all eukaryotic cells, or at least to the species in use.

The ER body is a microdomain of the ER. Numerous studies have focused on how microdomains in the plasma membrane are organized and contribute to cell functions (reviewed in Lingwood and Simons, 2010). These membrane microdomains also play important roles in plant cells (reviewed in Malinsky et al., 2013). However, because these studies focused primarily on microdomains in plasma membranes, little is known about the organization of microdomains in organelle membranes including the ER. The organization of lipids and proteins should play an important role in the maintenance of organelle morphology and function. ER bodies may be a good model to understand microdomain organization in ER. The mechanisms underlying ER body formation will provide valuable insights into the general mechanisms of microdomain organization for organelle membranes. Combined with an understanding of physiological functions, ER bodies can be an interesting model case to connect subcellular microdomains to the overall fitness of individual plants.

Yet, we still have some unanswered key questions. The most important piece of the puzzle that is missing is the genetic evidence for ER body function in stress responses. As discussed in the second section, most results achieved in this decade suggest a role of ER bodies in interaction with microbes and in abiotic-stress responses via activation of phytohormones and/or phytosiderophores. However, there is still no direct evidence for the physiological functions of ER bodies. Identification of the native substrate(s) could help us in validating the function(s). Phenotypic analysis of knockout lines deficient either in ER-bodies, PYK10 or substrates of this β-glucosidase, as well as respective multiple mutants subjected to various conditions will be required toward this end. To confirm the involvement of PYK10 and its substrate in the response to a certain environmental stress it will be necessary to show that the mutants depleted in either the enzyme or the substrate show a similar defect at least in regard to that particular response. More importantly, the multiple mutant lacking both the enzyme and the substrate should exhibit comparable, but not additive or synergistic phenotype compared to the mutants lacking only the substrates or the enzymes. Performing such genetic analysis, particularly when both enzymes and substrates are involved in multiple pathways, will provide insights into which combination of enzymes and substrates is registered to the pathway of interest. What is also completely missing is the behavior of ER bodies in response to these stresses; thus understanding the dynamics of ER bodies and PYK10 is crucial. The proper response to stress requires activation of molecules that are involved in the resistance to stress, e.g., toxic compounds should be produced only when plants are subjected to undesirable visitors, because these compounds may also be toxic to the plant. This responsive activation may include dynamic translocation and/or secretion of enzymes, where the ER body β-glucosidases can become activated (**Figure 7**).

The advantage or disadvantage of developing specific storage organelles is not fully understood. Many plant species evolved specific organelles for protein storage (reviewed in Hara-Nishimura and Matsushima, 2003). The production of special compartments or compounds does require valuable resources, as shown for glucosinolates (Bekaert et al., 2012; Züst et al., 2012), which suggests that the possession of ER bodies should confer a beneficial advantage for plant fitness. To specifically address the significance of accumulating PYK10 in ER bodies, a population genetics approach using generations of *nai1*, *pyk10*, and *nai2* mutants under appropriate conditions will be required. Revealing the evolutionary processes during the achievement of ER bodies will also provide insight into this issue. Toward this end, a genome sequence of a species in between *Brassicaceae* and *Caricaceae* will be a great help.

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Endoplasmic reticulum KDEL-tailed cysteine endopeptidase 1 of *Arabidopsis* (AtCEP1) is involved in pathogen defense

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Programmed cell death (PCD) is a genetically determined process in all multicellular organisms. Plant PCD is effected by a unique group of papain-type cysteine endopeptidases (CysEP) with a C-terminal KDEL endoplasmic reticulum (ER) retention signal (KDEL CysEP). KDEL CysEPs can be stored as pro-enzymes in ER-derived endomembrane compartments and are released as mature CysEPs in the final stages of organelle disintegration. KDEL CysEPs accept a wide variety of amino acids at the active site, including the glycosylated hydroxyprolines of the extensins that form the basic scaffold of the cell wall. In *Arabidopsis,* three KDEL CysEPs (*AtCEP1*, *AtCEP2*, and *AtCEP3*) are expressed. Cell- and tissue-specific activities of these three genes suggest that KDEL CysEPs participate in the abscission of flower organs and in the collapse of tissues in the final stage of PCD as well as in developmental tissue remodeling. We observed that *AtCEP1* is expressed in response to biotic stress stimuli in the leaf. *atcep1* knockout mutants showed enhanced susceptibility to powdery mildew caused by the biotrophic ascomycete *Erysiphe cruciferarum*. A translational fusion protein of AtCEP1 with a three-fold hemaglutinin-tag and the green fluorescent protein under control of the endogenous AtCEP1 promoter (P_{CEP1}::pre-pro-3xHA-EGFP-AtCEP1-KDEL) rescued the pathogenesis phenotype demonstrating the function of AtCEP1 in restriction of powdery mildew. The spatiotemporal *AtCEP1*-reporter expression during fungal infection together with microscopic inspection of the interaction phenotype suggested a function of AtCEP1 in controlling late stages of compatible interaction including late epidermal cell death. Additionally, expression of stress response genes appeared to be deregulated in the interaction of *atcep1* mutants and *E. cruciferarum*. Possible functions of AtCEP1 in restricting parasitic success of the obligate biotrophic powdery mildew fungus are discussed.

Keywords: programmed cell death, plant immunity, sporulation, haustorium, cell wall

INTRODUCTION

Programmed cell death (PCD) is a genetically determined, highly regulated process in all multicellular organisms (Hadfield and Bennett, 1997). PCD causes the loss of unpollinated ovules and the collapse of nucellus cells; it eliminates tissues and cells serving temporary functions during development such as the tapetum cells in anthers, the elimination of suspensor cells connecting the embryo to the mother plant and dissolution of endosperm cells in germinating castor beans (Pennel and Lamb, 1997; Olsen et al., 1999; Young and Gallie, 2000). Plants furthermore limit the spread of fungal or bacterial pathogens by rapid cell death at the site of infection through a mechanism called the hypersensitive response (HR) (Dickman and Fluhr, 2013).

Diverse classes of proteases are involved in PCD, including cysteine proteases, serine proteases, aspartic proteases and metalloproteases (Beers, 1997; Beers et al., 2000, 2004; Schaller, 2004). In plant PCD, special functions are described for vacuolar proteases (Müntz, 2007; Hara-Nishimura and Hatsugai, 2011), metacaspases (Lam and del Pozo, 2000; Xu and Zhang, 2009; Tsiatsiani et al., 2011) or subtilisin-like proteases (Vartapetian et al., 2011). Specific for plant PCD is a unique group of papain-type cysteine endopeptidases (CysEPs) characterized by a C-terminal KDEL endoplasmic reticulum (ER) retention signal (KDEL CysEPs) with RcCysEP from castor bean (*Ricinus communis*) as the founding member (Schmid et al., 1998). KDEL CysEPs are not present in mammals or fungi, but are ubiquitous in plants (Hierl et al., 2012).

KDEL CysEPs exhibit a characteristic and unusually broad substrate specificity. The cleavage site ↓ within a substrate is denoted as P2-P1-↓-P1′-P2′. KDEL CysEPs have a clear preference for neutral amino acids with large aliphatic and

Abbreviations: CLSM, confocal laser scanning microscopy; EGFP, enhanced green fluorescent protein; 3xHA, three-fold hemagglutinin (HA) tag; HR, hypersensitive response.

non-polar (Leu, Val, Met) or aromatic (Phe, Tyr, Trp) side-chains in the P2 position and no clear preference in the P1 position, as it is typical for papain-type CysEPs. Unusually, they accept proline in the P1 and P1′ positions (Than et al., 2004; Hierl et al., 2013). Crystallization of the purified RcCysEP from castor bean as the founding member of KDEL CysEPs (Schmid et al., 1998) revealed that castor bean CysEP folds into two distinct domains of roughly equal size, as it is usual for papain-like CysEPs. The folding of RcCysEP is also very similar to the proline-specific cysteine peptidase from ginger (*Zingiber officinale*). The active site cleft of RcCysEP, however, is wider when compared to the ginger protease and papain (Than et al., 2004). RcCysEP can therefore digest extensins with its ability to accept glycosylated hydroxyproline near the cleavage site (Helm et al., 2008). The respective amino acids (Leu, Met, Ala, Leu, Asn, Gly), which are decisive for this generally more open appearance of the active site cleft, together with the amino acids defining the catalytic pocket (Cys, His, Gln, Asn), are highly conserved among all known KDEL CysEPs (Hierl et al., 2012). Possibly, all KDEL CysEPs share the same broad substrate specificity. Extensins build the basic scaffold of the plant cell wall (Cannon et al., 2008), and thus KDEL CysEPs might support final cell collapse. KDEL CysEPs seem to have a dual set of substrates: digesting cytoplasmic components in cells of dying tissues for recycling to the surviving parts of the plant or in cells of germinating seedlings for mobilization of storage proteins, respectively, and digesting cell wall extensins in the final stage of PCD in support of the general cell collapse for tissue break down. Furthermore, KDEL CysEPs are expressed during tissue remodeling, possibly for clearance of dead cells and for generating space for plant organ outgrowth (Helm et al., 2008; Hierl et al., 2013).

It is obvious that KDEL-CysEPs are found in tissues undergoing PCD, especially in cells that finally collapse, such as the hypogeous cotyledons of *Vicia sativa* (Becker et al., 1997), the maturing pods of *Phaseolus vulgaris* (Tanaka et al., 1991), the unpollinated ovaries of *Pisum sativum* (Cercos et al., 1999), the outer integument developing into the seed coat of *Phalaenopsis* (Nadeau et al., 1996), the senescing flower petals of *Hemerocallis* (Valpuesta et al., 1995) and *Sandersonia aurantiaca* (O'Donghue et al., 2002), the megagametophyte cells after germination of *Picea glauca* seeds (He and Kermode, 2003), and the epigeous cotyledons of *Vigna mungo* (Toyooka et al., 2000). KDEL CysEPs were found in the senescing endosperm of germinating castor bean seeds (Schmid et al., 1999, 2001) and in the nucellus in maturing castor bean seeds, where the endosperm expands at the expense of the nucellus cells (Greenwood et al., 2005). They are expressed in both developing and dehiscing tomato anthers (*Solanum lycopersicum*) (Senatore et al., 2009) and in endosperm cells of imbibed tomato seeds (Trobacher et al., 2013).

In *Arabidopsis*, three KDEL CysEPs: AtCEP1 (At5g50260), AtCEP2 (At3g48340), and AtCEP3 (At3g48350) have been identified that are expressed in tissues undergoing PCD. Determination of promoter activities using β-glucuronidase as reporter in *Arabidopsis* transformants elucidated a remarkable tissue- and organ-specificity: *AtCEP1* and *AtCEP3* promoter activities were found in generative tissues at several stages of seed and fruit development such as *AtCEP1* in the abscission zone and the nectaries of a silique or *AtCEP3* in the maturing carpels. *AtCEP1*, *AtCEP2*, and *AtCEP3* promoter activities were found in vegetative tissue such as *AtCEP1* in the course of lateral root formation, *AtCEP2* in roots within the root elongation zone and the beginning root cap, and *AtCEP3* at the hypocotyl-root transition zone or in trichomes of leaves (Helm et al., 2008; Hierl et al., 2013).

KDEL CysEP are synthesized as pre-pro-enzymes and are co-translationally transferred into the ER, where the pre-sequence is removed. KDEL CysEPs can be stored as enzymatically inactive pro-enzymes in ER-derived compartments.

A spherical organelle surrounded by a single ribosome-studded membrane with a diameter averaging 1 μm was found in senescing endosperm tissue from castor bean. This organelle was discovered in ultrastructural and cytochemical studies independently by two groups in 1970. It was called "dilated cisternae," since it seemed to develop from the ER (Vigil, 1970), or "ricinosome," since it was found only in castor bean at that time (Mollenhauer and Totten, 1970). The ricinosomes were "rediscovered" with the identification of their marker enzyme, the KDEL CysEP (Schmid et al., 1998). Ricinosomes with their KDEL CysEP have been identified by immuno-electron-microscopy in the endosperm of germinating castor bean seeds (Schmid et al., 1999, 2001), in the nucellus of maturing castor bean seeds (Greenwood et al., 2005), in flower petals of *Hemerocallis* (Schmid et al., 1999), in the cotyledons of *Vicia sativa* (Becker et al., 1997), the unpollinated ovaries of *Pisum sativum* (Cercos et al., 1999), in tomato anthers (Senatore et al., 2009) and in endosperm cells of tomato seeds (*Solanum lycopersicum*) (Trobacher et al., 2013). Hence, the accumulation of KDEL CysEPs and the appearance of ricinosomes may be used as an early predictor of PCD.

KDEL-tailed protease-accumulating vesicles (KDEL vesicles, KVs) in germinating mung bean (*Vigna mungo*) cotyledons are similar to ricinosomes in that they accumulate the KDEL-tailed cysteine protease SH-EP (Toyooka et al., 2000). In contrast to ricinosomes, immunocytochemistry identified KDEL vesicles to transport large amounts of SH-EP from the endoplasmic reticulum to protein storage vacuoles. The mass transport of the proteinase by KDEL vesicles is thus involved in the protein mobilization of plants (Toyooka et al., 2000; Okamoto et al., 2003).

Interestingly, two different types of ER-derived organelles were found in *Arabidopsis* seedlings for storage of KDEL CysEPs using the mCherry-AtCEP2 reporter fusion protein (Hierl et al., 2013). mCherry-AtCEP2 was detected in the epidermal layers of leaves, hypocotyls and roots; in the root, it was predominantly found in the elongation zone and root cap. Co-localization with an ER membrane marker showed that mCherry-AtCEP2 was stored in 10 μm long spindle shaped organelles as well as round vesicles with a diameter of approximately 1 μm. The long organelles appear to be ER bodies, which are found specifically in Brassicales. The round vesicles strongly resemble ricinosomes (Hierl et al., 2013).

In plant microbe-interaction PCD has to be tightly controlled. Biotrophic pathogens are restricted by PCD because they strictly depend on living host tissue to feed from. PCD is an integral part of the HR by which plants restrict biotrophs in particular if triggered by recognition of microbial effectors. By contrast, if host PCD is triggered by hemibiotrophic or necrotrophic pathogens,

it may foster disease by producing dead defenseless tissue that is easily accessible for the pathogen (Dickman and Fluhr, 2013). Papain-type cysteine proteases are involved in plant-microbe interactions. They are expressed in response to biotic stress and can be direct or indirect targets of microbial virulence effectors (Shindo and Van der Hoorn, 2008). Publicly available expression data (www.genevestigator.com; Zimmermann et al., 2004) suggested that *AtCEP1* (At5g50260, probe set ID 248545_at) is expressed in hormone response such as auxin in mutants of the constitutive photomorphogenic9 signalosome (*csn4 and csn3*; Dohmann et al., 2008) and in mutants that constitutively express defense responses such as *cpr5* (Bowling et al., 1997; Clarke et al., 2000). *AtCEP3* (At3g48350; probe set ID 252365_at) does not exhibit such a pronounced response, and no expression data are available for *AtCEP2* (At3g48340). We hence wanted to know, if AtCEP1 is involved in pathogen defense. Therefore, we chose the interaction with an obligate biotrophic powdery mildew fungus *Erysiphe cruciferarum* because it allows for observation of quantitative disease phenotypes. We further had observed that a certain degree of late epidermal cell death occurred in the interaction of *Arabidopsis* with *E. cruciferarum* and thus analyzed wild type *AtCEP1* and *atcep1* mutant phenotypes in this interaction. Data introduce a function for AtCEP1 in limiting susceptibility of *Arabidopsis* to *E. cruciferarum* and suggest a role in controlling late stages of the compatible interaction. Apparently, *AtCEP1*-dependent PCD at late stages of the compatible interaction fulfills a function in limiting parasitic growth of the fungus.

MATERIALS AND METHODS

GENERATION OF REPORTER LINES EXPRESSING PRE-PRO-3xHA-EGFP-AtCEP1-KDEL AND PRE-PRO-3xHA-EGFP-KDEL, RESPECTIVELY, UNDER CONTROL OF THE ENDOGENOUS AtCEP1 PROMOTER IN THE *atcep1* KNOCKOUT MUTANT

For the cloning strategy of the fusion gene coding for pre-pro-3xHA-EGFP-AtCEP1-KDEL under the control of the endogenous promoter of *AtCEP1* (P_{CEP1}::pre-pro-3xHA-EGFP-AtCEP1-KDEL) and the primers used see Supplemental Figure S1. The sequence approximately 2000 bp upstream of the start Met, that was previously shown to confer tissue specific expression (Helm et al., 2008), was used as the *AtCEP1* promoter region. We placed the first three amino acids Leu-Pro-Thr of the mature subunit C-terminal to the pro-sequence in front of the 3xHA tag in order to ensure processing of the propeptide during maturation of AtCEP1. The *AtCEP1* promoter with the adjacent 5'UTR and the coding region for the pre-pro-sequence were amplified from WT (Col0) genomic DNA isolated by cetyl-trimethyl-ammonium bromide (CTAB) extraction (Murray and Thompson, 1980). The 3xHA tag was amplified from pNIGEL18 (Geldner et al., 2009) and EGFP was amplified from pEZS-CL (Cutler et al., 2000). The mature AtCEP1 subunit with the 3'UTR was amplified from WT (Col0) genomic DNA. The resulting PCR products were cloned into pGREEN conferring kanamycin resistance (Hellens et al., 2000; www.ac.uk). The final plasmid construct was sequenced and transformed into *Agrobacterium tumefaciens* (pGV3101) by electroporation. The construct P_{CEP1}::pre-pro-3xHA-EGFP-KDEL as a non-functional reporter protein lacking the mature *AtCEP1* protease subunit was obtained in an analogous manner. It comprised the endogenous AtCEP1 promoter, the 5′UTR and the coding regions for the N-terminal pre-pro-peptide, for the 3xHA tag and EGFP and for the nine C-terminal amino acids of the mature AtCEP1 subunit, including the ER retention signal KDEL (for cloning strategy and primers used see Supplemental Figure S2). The resulting PCR products were cloned into pGREEN (Hellens et al., 2000). The final plasmid construct was sequenced and transformed into *Agrobacterium tumefaciens* (pGV3101) by electroporation.

Flowers from homozygous *atcep1* knock out mutant plants (SAIL_158_B06) were transformed by floral dipping (Clough and Bent, 1998) resulting in plants expressing the functional (P_{CEP1}::pre-pro-3xHA-EGFP-AtCEP1-KDEL) or non-functional (P_{CEP1}::pre-pro-3xHA-EGFP-KDEL) EGFP-reporter proteins, respectively. Eight different homozygous transformants for each construct were screened for high expression of the fusion protein by confocal laser scanning microscopy (CLSM) and three were chosen for further analysis.

LEAF INFECTION WITH POWDERY MILDEW AND SYMPTOMS RATING

Arabidopsis thaliana Col0 plants and the *AtCEP1* T-DNA insertion mutants (SAIL_158_B06 and SALK_013036) as well as *atcep1* knock out plants transformed with the functional reporter (P_{CEP1}::pre-pro-3xHA-EGFP-AtCEP1-KDEL in SAIL_158_B06) and *atcep1* knock out plants transformed with the non-functional reporter (P_{CEP1}::pre-pro-3xHA-EGFP-KDEL in SAIL_158_B06) were grown in a growth chamber at 22°C and a 10-h photoperiod with 120 µmol m^{-2} s^{-2} light and 65% relative humidity. The *Arabidopsis* compatible powdery mildew fungus *Erysiphe cruciferarum* was grown on Arabidopsis Col-0 plants and for increased conidia production on Arabidopsis pad4 mutant plants (Glazebrook et al., 1996) at the same conditions. Five-week-old *Arabidopsis* plants were inoculated with *E. cruciferarum* for macroscopy and microscopy evaluation of disease progression with a density of 5–7 conidia mm^{-2}. *Arabidopsis* susceptibility to *E. cruciferarum* was scored by visual examination of the whole plant 7, 9, 11, and 13 d after inoculation. Plants were distributed in three categories of susceptibility with 0–30%, 30–60%, and >60% diseased leaf area.

STAINING OF FUNGAL STRUCTURES WITH WHEAT GERM AGGLUTININE-TETRAMETHYLRHODAMINE

Wheat germ agglutinin tetramethylrhodamin conjugate (WGA-TMR, Invitrogen Molecular Probes, Germany) binds to extra- and intracellular chitin of fungi. To investigate the development of *E. cruciferarum* on *Arabidopsis*, inoculated leaves were harvested 5 days after inoculation and were discolored in ethanol-acetic acid glacial (EtOH-HAc; 6:1). Before WGA-TMR staining, the leaves were washed with H_2O_{dest} to remove the EtOH-HAc solution and were incubated for 6 min in PBS buffer (140 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.5 mM KH_2PO4, pH7.4). The leaves were placed into the staining solution [0.01 µg/µl WGA-TMR (Molecular Probes, Invitrogen), 0.01 µg/µl BSA, PBS buffer] and were vacuum infiltrated twice at −0,8 bar. After 24–48 h incubation in the dark at 4°C, three leaves from WT or *atcep1* plants each containing 54 colonies, respectively, were analyzed by fluorescence microscopy (Olympus BX61TRF, Japan). The results were

reproduced in a second independent inoculation with 48 colonies analyzed on three leaves of WT and *atcep1* plants, respectively.

CALLOSE STAINING WITH METHYL BLUE

Callose depositions in *Arabidopsis* cells were visualized by methyl blue (Sigma Aldrich Chemie GmbH, München, Germany) staining. The discolored and WGA-TMR stained leaves were rinsed with distilled water and transferred into 67 mM K_2HPO_4 buffer for 10 min followed by incubation for 3–4 h in the staining solution (0.05% methyl blue in 67 mM K_2HPO_4) in the dark and direct analysis by fluorescence microscope (Olympus BX61TRF, Japan).

qRT-PCR

Primers used for qRT-PCR are: ACT8 qRT fw: TGAGACCTTTA ATTCTCCAGCTATG; ACT8 qRT rv: CCAGAGTCCAACACA ATACCG; PR1 qRT fw: GATGTGCCAAAGTGAGGTGTAA; PR1 qRT rv: TTCACATAATTCCCACGAGGA; PDF1.2 qRT fw: GTT CTCTTTGCTGCTTTCGAC; PDF1.2 qRT rv: GCAAACCCC TGACCATGT. Total RNA was extracted from leaves before and 12 h (hpi) and 1, 2, 3, and 5 days (dpi) post mildew inoculation. Leaves were collected from at least five individual plants and total RNA was extracted with a NucleoSpin RNA plant kit (Machery-Nagel) and 2 µg of total RNA was reverse-transcribed with an oligo-dT primer and M-MulV Reverse Transcriptase (Fermentas) following the manufacturers' instructions. Quantitive real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad) in a CFX96 Real-Time System Cycler (Bio-Rad). A 50-cycle two-step amplification protocol (10 s at 95°C, 25 s at 60°C) was used for all measurements.

BIOCHEMICAL METHODS

Leaves were harvested 0–18 dpi days post inoculation with powdery mildew. A protein extract was prepared from one leaf (20–30 mg fresh weight). The plant material was ground with mortar and pestle under liquid N_2 followed by the addition of loading dye (60 mM Tris-HCl pH 6.6, 5% glycerine, 1.5% SDS, 1.5% β-ME, 0.1% bromophenol blue final concentration), incubation for 10 min/95°C and analysation by SDS-PAGE followed by western blot analysis with anti-HA antibodies (Roche).

CONFOCAL LASER SCANNING MICROSCOPY

CLSM (Fluoview FV 1000, Olympus, Japan) was performed using excitation at 488nm and emission detection between 503 and 550 nm for GFP. Single pictures or stacks of pictures with 0.5 µm increments at higher resolution and 2.5 µm increments at lower resolution were made.

RESULTS

HOMOZYGOUS *atcep1* KNOCK OUT MUTANTS EXHIBIT ENHANCED SUSCEPTIBILITY TO THE BIOTROPHIC FUNGUS *ERYSIPHE CRUCIFERARUM*

Two *AtCEP1* insertion lines, SAIL_158_B06 and SALK_013036, have the T-DNA insertion within the 3rd Exon. Both lines were homozygous, and RT-PCR using primers that amplified the coding region (spanning the T-DNA) confirmed the knock out of *AtCEP1* (**Figure 1**). Wild type Col0 plants and the two homozygous *atcep1* knock out mutants were inoculated with conidia of

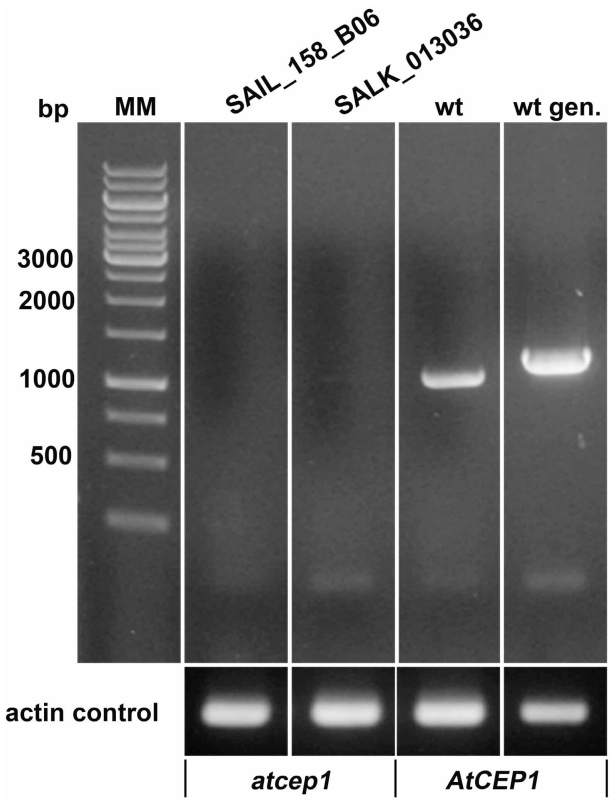

FIGURE 1 | Both AtCEP1 mutants SAIL_158_B06 and SALK_013036, harboring the T-DNA insertion in the 3. exon represents loss of function mutants. No corresponding transcript could be amplified by RT-PCR using primers that comprise the complete coding region from 7 days old seedlings, whereas the parent Col-0 wild type expressed the gene. wt, RT-PCR on wild type RNA; wt gen, PCR on wild type genomic DNA.

the powdery mildew fungus *E. cruciferarum*, an obligate biotroph that requires living cells for growth. Five week old plants were inoculated for evaluation of disease progression. *Arabidopsis* susceptibility to *E. cruciferarum* was scored by visual examination of the whole plant 7, 9, 11, and 13 days after inoculation. Both independent *atcep1* knock out mutants lines exhibited the same phenotype, that is enhanced susceptibility to *E. cruciferarum* as compared to the parental wild type, as can be seen by visual examination of the whole plant for scoring the leaf area covered by powdery mildew symptoms (**Figure 2**, Supplemental Figure S3, see also below).

A FUNCTIONAL REPORTER CONSTRUCT COMPLEMENTS THE PATHOGENESIS PHENOTYPE OF *atcep1* KNOCK OUT

We constructed the fusion genes under the control of the endogenous *AtCEP1* promoter for functional (P_{CEP1}::pre-pro-3xHA-EGFP-AtCEP1-KDEL) and non-functional (P_{CEP1}::pre-pro-3xHA-EGFP-KDEL) reporter constructs including EGFP with and without the mature AtCEP1 subunit (**Figure 3**, Supplemental Figures S1, S2, see Materials and Methods). The final plasmid constructs were sequenced and transformed into *Agrobacterium tumefaciens* for subsequent transformation into

atcep1 knock out plants SAIL_158_B06 in order to obtain plants expressing the functional or non-functional reporter proteins, respectively. Eight homozygous transformants for each construct were obtained. Three transformants for each construct exhibiting the highest fluorescence were chosen for further pathogen inoculation.

Five week old plants were inoculated with the powdery mildew fungus *E. cruciferarum* and were scored for disease progression by visual examination of the whole plant 9, 11, and 13 days post inoculation: Plants were distributed in the three categories of susceptibility with less than 30%, 30–60%, and more than 60% of infected leaf area (**Figure 4A**). Significantly more plants of the *atcep1* knock out line SAIL_158_B06 were classified in the category with >60% diseased leaf area compared to Col0 control plants. This was particularly obvious at later stages of the interaction, resulting in a super-susceptibility phenotype of the mutants. By contrast, *atcep1* knock out plants transformed with the functional reporter construct reporter P_{CEP1}::pre-pro-3xHA-EGFP-AtCEP1-KDEL exhibited a similar basal resistance to *E. cruciferarum* as compared to wild type Col0 plants thus proving the functionality of the reporter protein and complementation of the mutant phenotype. On the other hand, *atcep1* knock out plants transformed with the non-functional reporter construct reporter P_{CEP1}::pre-pro-3xHA-EGFP-KDEL behaved similar to the original *atcep1* knock out plants in showing super-susceptibility (**Figure 4B**).

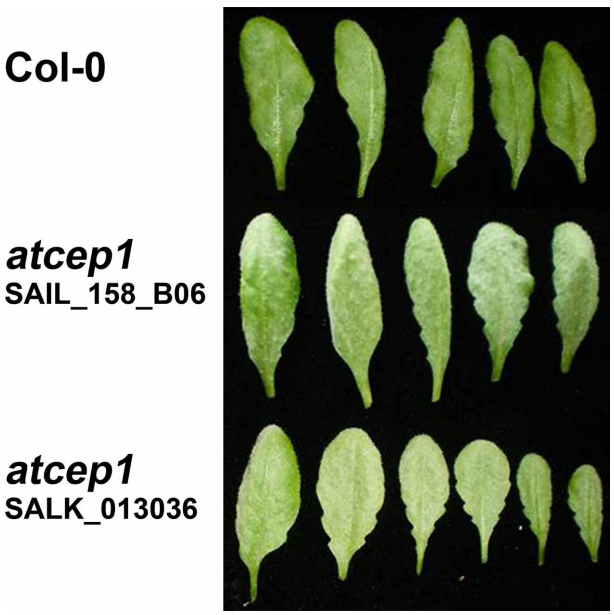

FIGURE 2 | Homozygous *atcep1* knock out mutants with a T-DNA insertion in the 3. Exon exhibit enhanced susceptibility upon leaf inoculation with the biotrophic fungus *Erysiphe cruciferarum*. Leaves are shown 11 days post low density inoculation.

EGFP-AtCEP1 EXPRESSION CORRELATES WITH THE APPEARANCE OF LEAF SYMPTOMS DURING INFECTION WITH POWDERY MILDEW

For detection of AtCEP1 protein expression, we scored the appearance of AtCEP1 on total protein extracts from infected leaves of *atcep1* knock out plants transformed with the functional reporter P_{CEP1}::pre-pro-3xHA-EGFP-AtCEP1-KDEL that are comparable to wild type Col0 plants and *atcep1* knock out plants transformed with the non-functional reporter P_{CEP1}::pre-pro-3xHA-EGFP-KDEL that are comparable to *atcep1* knock out plants by immunoblot analysis with anti-HA antibodies (**Figure 5**). Pro-3xHA-EGFP-AtCEP1-KDEL was detectable from day 9 on post inoculation and disappeared after day 15. Typically, two distinct protein bands are recognized by the anti-HA antibodies. Both proteins exhibit with molecular masses smaller than the 80 kDa marker the expected sizes based on their sequence with a calculated mass of 72.03 kDa and thus probably represent the intact pro-form of AtCEP1 with the C-terminal

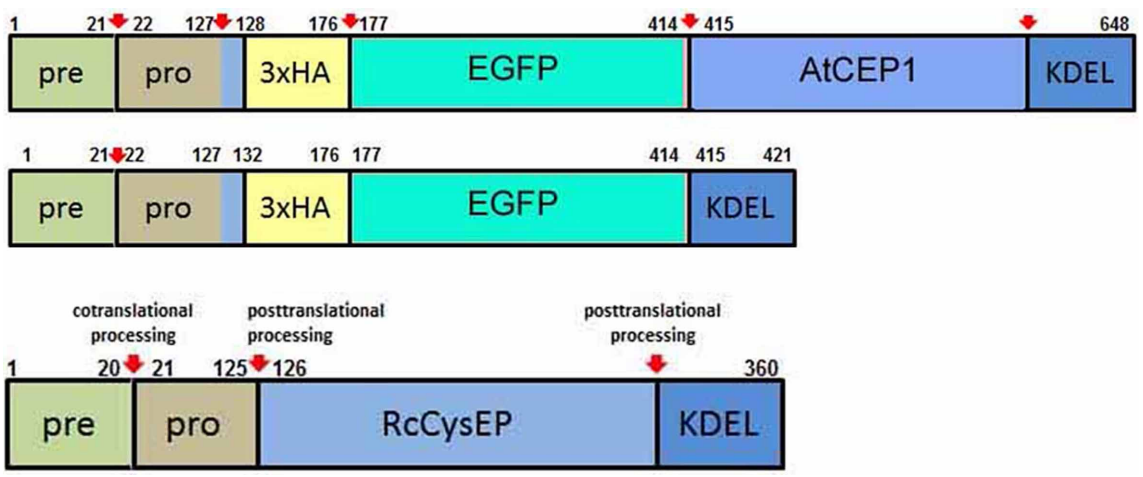

FIGURE 3 | Schematic representation of the functional reporter protein pre-pro-3xHA-EGFP-AtCEP1-KDEL and the non-functional reporter protein pre-pro-3xHA-EGFP-KDEL expressed under the control of the endogenous AtCEP1 promoter. Both constructs were transformed into *atcep1* knock out mutant line SAIL_158_B06. Pre-pro-RcCysEP is shown for comparison. Red arrows indicate proven or predicted protein cleavage sites.

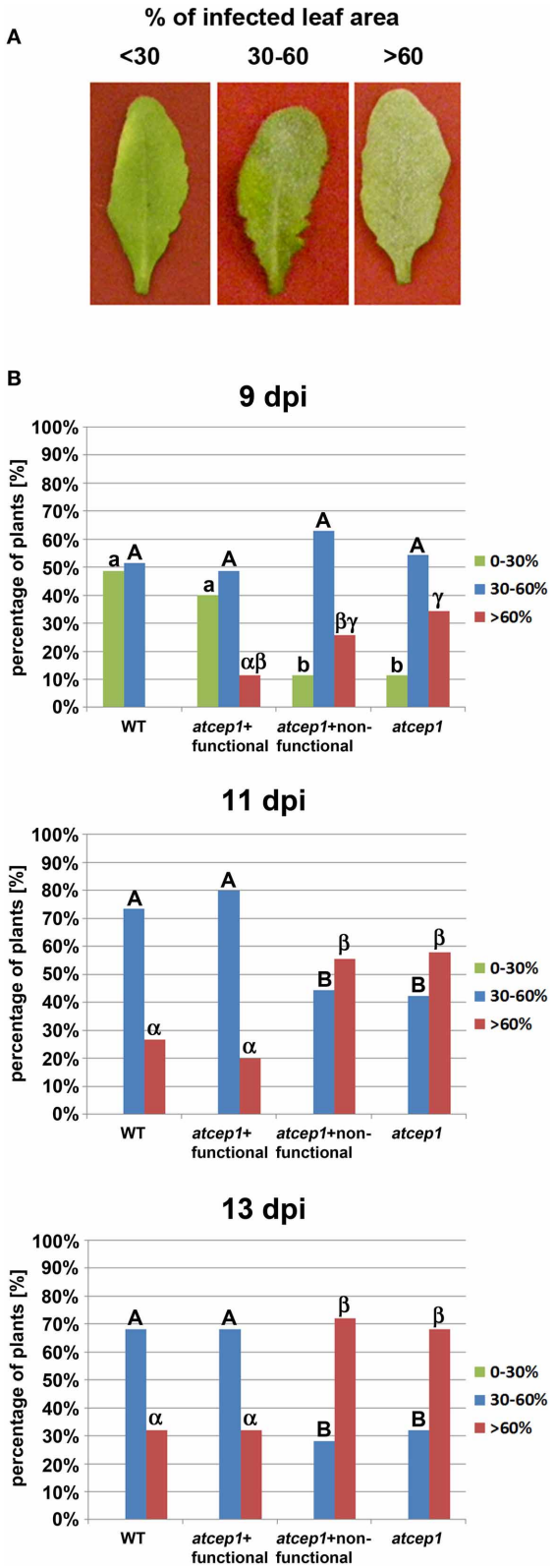

FIGURE 4 | Disease symptoms of wild type and *atcep1* mutant plants (SAIL_158_B06) upon leaf infection with *Erysiphe cruciferarum* spores. Wild type Col0 plants and *atcep1* (SAIL_158_B06) plants transformed with *(Continued)*

FIGURE 4 | Continued
the functional construct P_{CEP1}::pre-pro-3xHA-EGFP-AtCEP1-KDEL that are comparable to wild type plants, and *atcep1* mutant plants (SAIL_158_B06) transformed with the non-functional reporter P_{CEP1}::pre-pro-3xHA-EGFP-KDEL that are comparable to *atcep1* knock out plants were infected with *E. cruciferarum* and disease symptoms were scored after visual inspection of the whole plant 9, 11, and 13 days post inoculation (dpi). Infected leaf were distributed in the three categories <30%, 30–60%, and >60% diseased leaf area. **(A)** Representative leaves were excised and photographed 11 dpi. **(B)** Columns marked with different letters indicate statistically different groups according to the ANOVA- and Duncan test ($p < 0.05$) and represent the frequency of plants distributed in the three categories of susceptibility. Data represent the respective means of seven experiments from independent inoculation events of the mutants with the corresponding parent background control. Each experiment comprised 5 plants per line.

KDEL-motif still attached, however in different conformation. This double band was already found for the analogous pro-3xHA-mCherry-AtCEP2-KDEL reporter protein (Hierl et al., 2013). The non-functional reporter protein pro-3xHA-EGFP-KDEL was also detectable from day 7 post inoculation on and was found until day 18 post inoculation (**Figure 5**).

CELLS ATTACKED BY *ERYSIPHE CRUCIFERARUM* EXPRESS EGFP-AtCEP1 IN THE ENDOPLASMIC RETICULUM AND ACCUMULATE IT AROUND HAUSTORIA

To investigate the subcellular localization of AtCEP1 *in vivo*, we analyzed complemented mutant plants (*atcep1* knock out plants transformed with the functional reporter P_{CEP1}::pre-pro-3xHA-EGFP-AtCEP1-KDEL) during interaction with *E. cruciferarum* (12 dpi) by CLSM (**Figure 6**). Without inoculation, we never detected the functional EGFP fusion of AtCEP1. 12 dpi, the EGFP-AtCEP1 fusion protein displayed localization within the entire endoplasmatic reticulum of cells that were successfully penetrated by the fungus and accumulated especially around established haustoria (**Figures 6A–C**). A strong labeling around haustoria was also observed at the haustorial plane, where the network of the endoplasmic reticulum seemed to be very dense (**Figures 6D–F**).

WILD TYPE PLANTS EXHIBIT SIGNIFICANTLY MORE DEAD EPIDERMAL CELLS AND LESS ESTABLISHED HAUSTORIA AS COMPARED TO *atcep1* MUTANTS UPON INOCULATION WITH *ERYSIPHE CRUCIFERARUM*

Visual examination of wild type and *atcep1* mutant plants upon infection with *E. cruciferarum* suggested a role for AtCEP1 in restricting the development of a biotrophic fungus (**Figure 4**, Supplemental Figure S3). To assess the *atcep1* knock out phenotype on a microscopic level we used two different labels: red fluorescent wheat germ agglutinin that stains the chitin of fungal hyphae and methyl blue that stains the callose at papillae and encasements of established haustoria as well as whole cells that die in the course of epidermal HR-like cell death (**Figure 7**). At 5dpi, fungal colonies on the wild type were grown to a size that about 24 haustoria were established per single fungal colony. In an average colony, 17 fungal attempts to penetrate were associated with localized callose depositions (papillae) without a visible haustorium. More than 19 cells per fungal colony displayed whole-cell callose deposition indicative of cell death, which was associated with

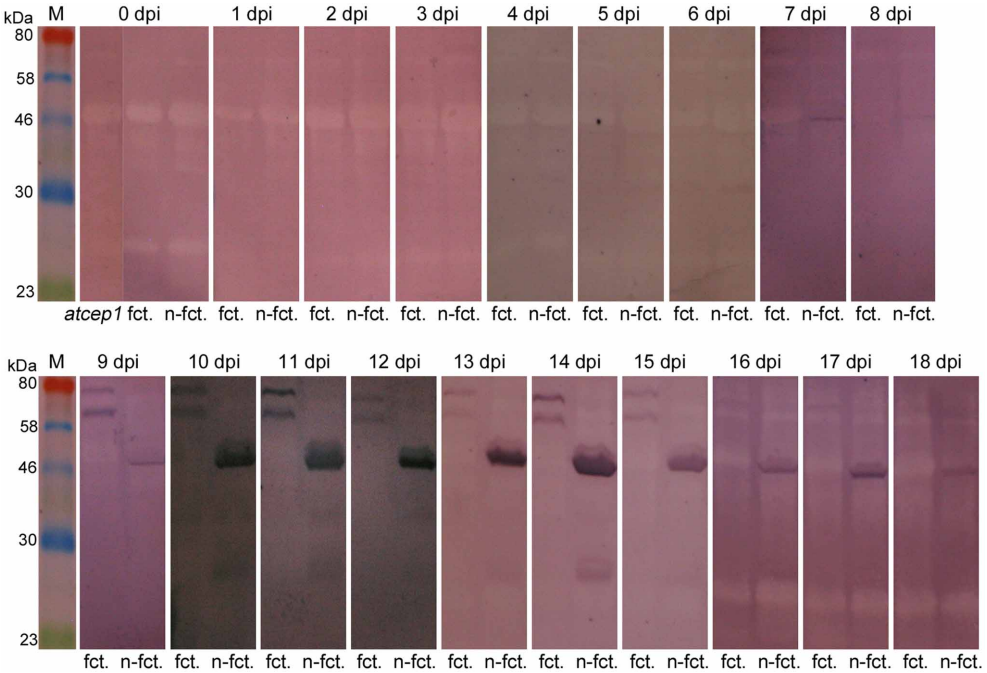

FIGURE 5 | Pro-3xHA-EGFP-AtCEP1-KDEL and pro-3xHA-EGFP-KDEL, respectively, are detectable from 9 days post inoculation (dpi) on upon leaf inoculation with *Erysiphe cruciferarum*. Immunoblots with anti-HA antibodies on total protein extracts from leaves of "wild type" plants (*atcep1* SAIL_158_B06 transformed with the functional construct P_{CEP1}::pre-pro-3xHA-EGFP-AtCEP1-KDEL) and *atcep1* knock out plants (SAIL_158_B06 and SAIL_158_B06 transformed with the non-functional construct P_{CEP1}::pre-pro-3xHA-EGFP-KDEL). *atcep1*, SAIL_158_B06; fct, *atcep1* plants transformed with the functional reporter pre-pro-3xHA-EGFP-AtCEP1-KDEL; n-fct, *atcep1* plants transformed with the non-functional reporter pre-pro-3xHA-EGFP-KDEL.

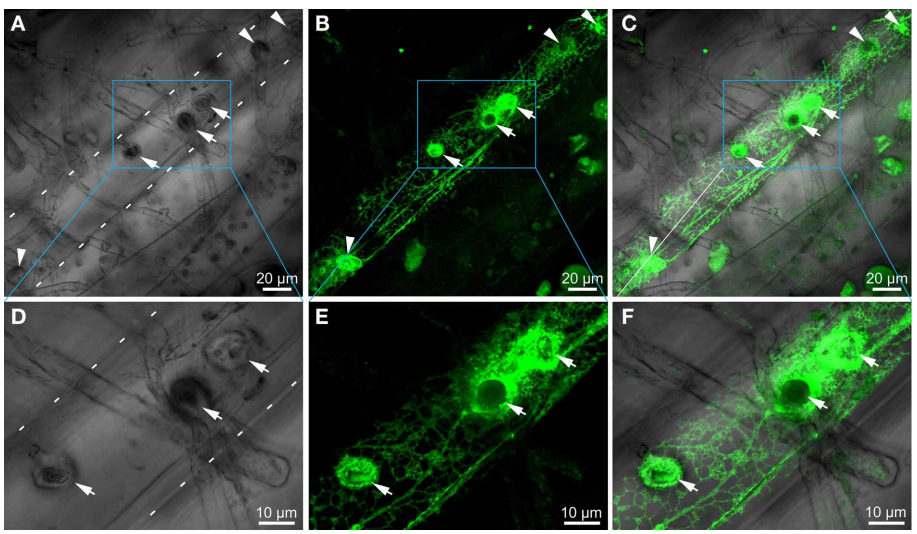

FIGURE 6 | Cells attacked by *Erysiphe cruciferarum* express EGFP-AtCEP1 in the endoplasmic reticulum and accumulate it around haustoria. Localization of EGFP tagged AtCEP1 in *Erysiphe cruciferarum* attacked cells of *Arabidopsis* WT cells (*atcep1* mutant plants transformed with the functional reporter P_{CEP1}::pre-pro-3xHA-EGFP-AtCEP1-KDEL) upon fungal leaf inoculation 12 dpi. Brick like epidermis cells close to the main vessel are shown; cell outlines are indicated by white dotted lines **(A,D)**. **(A)** Brightfield image of fungal structures on the leaf. Six bulbous haustoria are visible in the attacked cell (arrows and arrowheads). **(B)** EGFP-AtCEP1 shows localization in cortical net like structures and around haustoria. **(C)** Overlay of **(A,B)**. **(D–F)** Strong accumulation of EGFP-AtCEP1 can be observed around the haustoria. Pictures display a magnified optical section of the area marked by the blue box in the pictures **(A–C)** in the level of the intracellular haustoria. Pictures are maximum projections of 24 optical sections **(A–C)** or 12 optical sections **(D,E)**, respectively, with 0.5 µm increments.

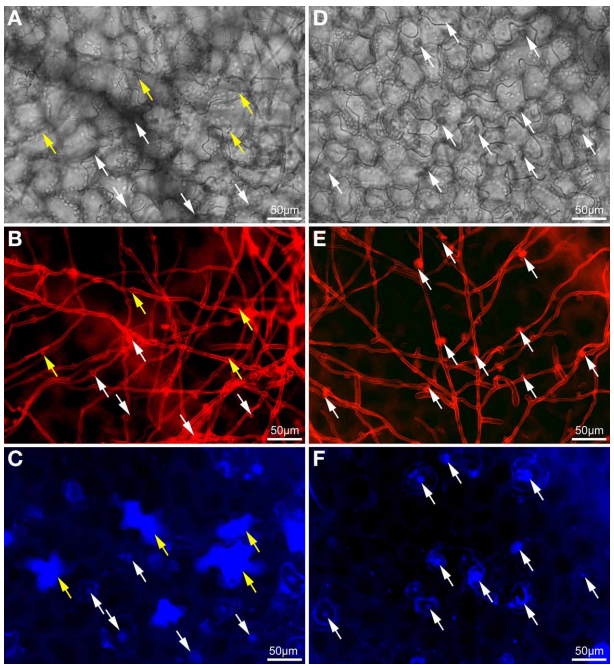

FIGURE 7 | Wild type plants exhibit significantly more dead epidermal cells and less established haustoria as compared to *atcep1* mutants upon inoculation with *Erysiphe cruciferarum*. Microscopic phenotype of powdery mildew attacked cells on *Arabidopsis* WT (Col0) and the *atcep1* knock out mutant (SAIL_158_B06) 5 dpi. WT cells show a frequent hypersensitive response (HR)-like reaction of penetrated cells **(A–C)** whereas no HR-like reaction is observed in the *atcep1* mutant **(D–F)**. **(A,D)** Brightfield image of the selected areas. **(B,E)** Fungal structures on the leaf surface and bulbous haustoria marked by white arrows were stained with wheat germ agglutinine-tetramethylrhodamine. **(C,F)** Callose was stained by methyl blue staining. Punctate methyl blue stained structures show papilla/cell wall appositions and encapsulated haustoria. Completely stained cells marked by yellow arrows indicate an HR-like cell death.

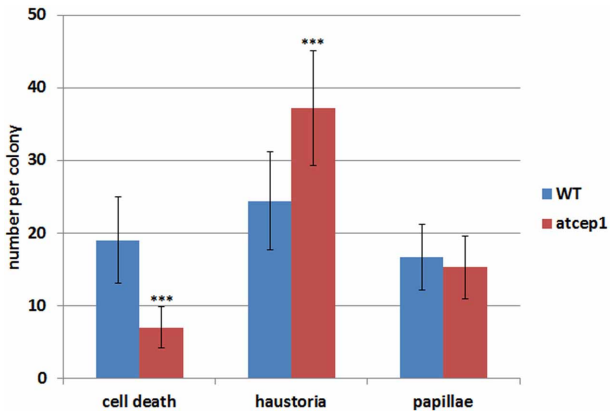

FIGURE 8 | The epidermal cell death is suppressed in cells attacked by *Erysiphe cruciferarum* on the *atcep1* mutant 5 dpi compared to the WT. Columns show the frequency per colony of epidermal cell death, haustoria and papilla in cells attacked by *E. cruciferarum*. Data present the mean of 54 colonies on three leaves per wild type and *atcep1* mutant line, respectively. Error bars are standard deviations. Differences between wild type and *atcep1* are highly significant after two sided student's *t*-test $p < 0.001$ (***). The results were similarly reproduced in a second independent inoculation with 48 colonies on three leaves per WT and *atcep1* mutant line, respectively.

fungal infection structures, in most cases collapsed haustoria. By contrast, the *atcep1* mutant displayed significantly less epidermal cell death (7 cells per colony) per fungal colony and at the same time supported the development of more haustoria (37 per colony) in living cells. Hence, the *atcep1* mutants displayed a failure to restrict establishment or maintenance of fungal haustoria under execution of an HR-like epidermal cell death (**Figure 8**).

PATHOGEN-RESPONSIVE GENES EXPRESSION IN *atcep1*

The difference in visual scoring leaf symptoms between wild type and *atcep1* mutant plants was most obvious at 9 to 13 dpi. However, differences were observed in fungal development and in plant cell death responses at the microscopic level already at 5 dpi. By contrast, no differences were observed in fungal development or host defense responses at 1 dpi (data not shown). We also tested wild type and *atcep1* mutants for differential expression of defense associated genes at 12 hpi, 1 dpi, 2 dpi, 3 dpi and 5 dpi. We measured relative expression of biotic stress markers *PR1* and *PDF1.2* (Reymond and Farmer, 1998) in wild type and *atcep1* plants after powdery mildew inoculation. Expression levels were normalized to the reference gene *ATC8*, and expression before inoculation in each genotype was set to 1. At distinct stages of the interaction, powdery mildew inoculation provoked very high induction of *PR1*. *PR1* gene expression was attenuated in super-susceptible *atcep1* mutants at 5 dpi, which was coinciding with lower levels of epidermal cell death at this time (**Figures 8**, **9**). By contrast, *PDF1.2* expression in *atcep1* transiently exceeded wild type level at 0.5 dpi and at 1 dpi (**Figure 9**).

DISCUSSION

PCD is part of the HR, which restricts growth of biotrophic and hemibiotrophic pathogens in both basal resistance and in effector-triggered immunity (Dickman and Fluhr, 2013). In plants like in animals, proteases often mediate the decisive or executing steps of PCD. Papain-type cysteine proteases are involved in plant-microbe interactions. They are expressed in response to biotic stress and can be direct or indirect targets of microbial virulence effectors (Shindo and Van der Hoorn, 2008). This supports a fundamental function of papain-type proteases in plant defense. However, the identity and regulation of the proteases that are functional in particular plant-microbe interactions or in specific stages of such interactions are largely unknown. We show here that an ER-resident papain-type protease, which has been implicated in developmental cell death and tissue remodeling, is co-opted for a late defense reaction in a compatible interaction with a biotrophic fungus.

We tested the ability of wild type and *atcep1* mutants to restrict growth of the biotrophic fungus *E. cruciferarum* to find possible evidence for a role of AtCEP1 in biotic stress responses and for a role of late epidermal cell death in restricting parasitic growth of an adapted powdery mildew fungus. This revealed super-susceptibility of two independent *AtCEP1* T-DNA insertion mutants to powdery mildew. Both mutants lacked expression of

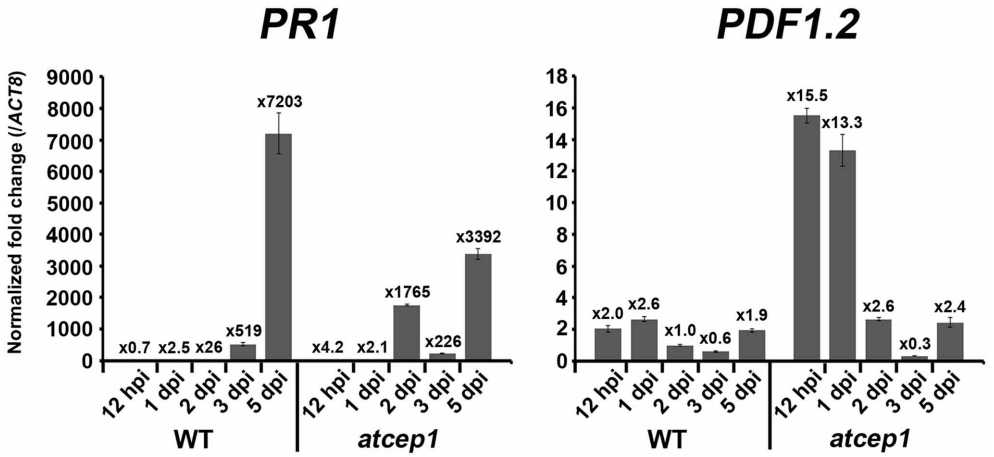

FIGURE 9 | Pathogen-responsive genes expression in *atcep1*. Relative gene expression of *PR1* and *PDF1.2* in wild type and *atcep1* plants 12 h post mildew inoculation (hpi) and 1, 2, 3, and 5 days post mildew inoculation (dpi). qRT-PCR was performed with *PR1* and *PDF1.2* gene-specific primers. Expression levels were normalized to the reference gene *ACT8* and expression before inoculation in each genotype was set to 1. The results were similarly reproduced by three independent inoculations. Error bars represent standard error of the mean (SE).

AtCEP1 and can thus be considered as loss-of-function mutants. Basal resistance of *Arabidopsis* was re-constituted by a functional but not by a non-functional complementation construct. A native promoter functional AtCEP1-reporter construct allowed for the detection of AtCEP1 protein accumulation during late stages of the interaction. Together, this suggests that AtCEP1 is involved in basal resistance of *Arabidopsis* to powdery mildew.

Little is known about the function and efficacy of plant defense responses in compatible plant-pathogen interactions. Late epidermal PCD, as observed here, could either be an indication of fungal failure to maintain compatibility at the single cell level for a long time or an effective basal defense response of the susceptible host. Our data suggest that this type of PCD is under genetic control of the host and partially restricts fungal development during late stages of the interaction and sporulation. It is generally believed that in a compatible interaction of a susceptible host with a virulent pathogen most plant immune responses are successfully suppressed by pathogen effector molecules. Indeed, host papain proteases, which function in defense against nematodes, fungi or oomycetes, are inhibited by host and pathogen protease inhibitors that are delivered during compatible interactions (Bozkurt et al., 2011; Lozano-Torres et al., 2012; van der Linde et al., 2012). Partial inhibition of AtCEP1 by a protease inhibitor from *E. cruciferarum* or the late accumulation of AtCEP1 protein during pathogen interaction would explain why the pathogenesis phenotype of *atcep1* was of quantitative nature. Furthermore, reporter expression of EGFP-AtCEP1 from P_{CEP1}::pre-pro-3xHA-EGFP-AtCEP1-KDEL was detectable but irregular in cells penetrated by *E. cruciferarum*. Basal expression of the *AtCEP1* gene is also low but detectable in leaves but higher in seeds, siliques, generative tissues and parts of the root (Helm et al., 2008 and unpublished results of the authors). Late defense-related expression points to possible hormone regulation of AtCEP1 during pathogenesis-induced physiological perturbations rather than expression in response to pathogen-associated molecular patterns, which trigger early immune responses. Alternatively, pathogenesis might have caused a loss of tissue identity.

Cells attacked by *E. cruciferarum* expressed EGFP-AtCEP1 in the ER and accumulated it around haustoria but not in ricinosome-like structures or ER-bodies, in which AtCEP2 can be found (Hierl et al., 2012, 2013). Interestingly, mutants lacking functional AtCEP1 did not show the same frequency of cell death in attacked epidermal cells, when compared to wild type. This suggests that AtCEP1 is required to undergo this type of PCD during interaction with *E. cruciferarum*. However, we cannot yet distinguish whether this is due to a function in regulating epidermal PCD or in affecting the functionality of fungal haustoria. The accumulation of ER and EGFP-AtCEP1 at the plant-fungus interface let us speculate about a possible leakiness of the plant ER to the apoplast or to the extrahaustorial matrix. There are examples of plant KDEL-motif containing ER proteins, including papain-type proteases, that have a second destination in the apoplast or in the vacuole (Jones and Herman, 1993; Okamoto et al., 2003). Late-endosomal multivesicular compartments, which are by default delivered to the vacuole, and the tonoplast can also fuse with the plasma membrane during plant-pathogen interactions. Additionally, it is still unclear whether the host extrahaustorial membrane is an extension of the plasma membrane or originates from an endomembrane (An et al., 2007; Hatsugai et al., 2009; Hückelhoven and Panstruga, 2011; Dickman and Fluhr, 2013; Drakakaki and Dandekar, 2013). Hence, AtCEP1 could function in inhibiting haustorial functions when the protein leaks into the apoplast. Since KDEL CysEPs contain a cleavable KDEL ER retention motif (**Figure 3**), which is processed during protein activation (Than et al., 2004; Hierl et al., 2013), a novel mechanism would need to be postulated for controlled release of proteins from the ER.

Haustoria are likely the fungal cells, from which effector proteins are released to suppress host immune responses including

the PCD. Therefore late expression of AtCEP1 could restrict haustorial functions in suppression of cell death. This is further supported because the reduction of cell death events in *atcep1* was accompanied by a complementary increase in haustoria per fungal colony. Indeed, most of the dead cells in the wild type were successfully penetrated and contained haustoria. Alternatively, AtCEP1 might directly function in epidermal PCD. KDEL-cysteine peptidases like AtCEP1 are considered as late-acting proteases that digest cell wall proteins during the final stages of PCD and tissue remodeling after cellular disintegration (Helm et al., 2008; Hierl et al., 2012). However, a sole post cell death digestive function of AtCEP1 would not explain restriction of fungal growth. Post-cell death tissue-clearance cannot determine the outcome of the interaction, because the fungus strictly depends on an intact host cell for biotrophy at the single-cell level. An alternative explanation for the phenotype would be a new function of AtCEP1 in the initiation of epidermal cell death.

Epidermal PCD occurred late in the interaction with the virulent powdery mildew fungus. This makes it different from a canonical HR, which is observed early in effector-triggered immunity and is based on specific nonself recognition. We do not yet know whether this type of late epidermal cell death mechanistically resembles HR or a novel type of PCD. Additionally, defense gene expression was altered in *atcep1* mutants before the pathogenesis-related phenotype became detectable. This might hind at a function of AtCEP1 in communication of the ER with the nucleus in stress-related gene expression.

CONCLUSION

Our data suggest a new function of AtCEP1 in late epidermal PCD in the interaction with the powdery mildew fungus. This PCD might possibly be a new type of PCD rather than classical HR. Future studies will shed light on the nature and regulation of this type of PCD and its role in interaction with other pathogens. Additionally, early differential expression of stress markers in *atcep1* points to a possible involvement of AtCEP1 in crosstalk between the ER and biotic stress responses, that takes place before cell death is observed. Apparently, the ER KDEL-peptidase AtCEP1, which otherwise may function in developmental PCD, has an additional and pivotal function in translation of pathogenesis-related stress into leaf epidermal cell death and pathogen defense.

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SUPPLEMENTARY MATERIAL

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When supply does not meet demand-ER stress and plant programmed cell death

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The endoplasmic reticulum (ER) is the central organelle in the eukaryotic secretory pathway. The ER functions in protein synthesis and maturation and is crucial for proper maintenance of cellular homeostasis and adaptation to adverse environments. Acting as a cellular sentinel, the ER is exquisitely sensitive to changing environments principally via the ER quality control machinery. When perturbed, ER-stress triggers a tightly regulated and highly conserved, signal transduction pathway known as the unfolded protein response (UPR) that prevents the dangerous accumulation of unfolded/misfolded proteins. In situations where excessive UPR activity surpasses threshold levels, cells deteriorate and eventually trigger programmed cell death (PCD) as a way for the organism to cope with dysfunctional or toxic signals. The programmed cell death that results from excessive ER stress in mammalian systems contributes to several important diseases including hypoxia, neurodegeneration, and diabetes. Importantly, hallmark features and markers of cell death that are associated with ER stress in mammals are also found in plants. In particular, there is a common, conserved set of chaperones that modulate ER cell death signaling. Here we review the elements of plant cell death responses to ER stress and note that an increasing number of plant-pathogen interactions are being identified in which the host ER is targeted by plant pathogens to establish compatibility.

Keywords: unfolded protein response, ER, autophagy, stress response and stress tolerance, PCD

INTRODUCTION

The endoplasmic reticulum (ER) contains the necessary machinery to ensure quality protein synthesis, maturation and secretion (Sparkes et al., 2009; Eichmann and Schafer, 2012). Adverse environmental stresses can impact this ER quality control (ER QC) machinery causing unfolded/misfolded proteins to accumulate in the ER. Perturbations in ER function are identified by transmembrane sensors which activate signal transduction that increases gene expression of ER resident chaperones and foldases. Collectively, this is known as the unfolded protein response (UPR). The UPR orchestrates restoration of ER homeostasis and protein production by: (i) inducing expression of chaperones and foldases to facilitate protein folding, (ii) translational repression to reduce the protein load on the ER and (iii) removal of unfolded proteins from the ER for degradation via the proteasome (Liu and Howell, 2010b).

In addition to protein synthesis and maturation, the ER also serves as the primary reservoir for intracellular Ca^{+2} storage. The Ca^{2+} that is concentrated in the ER facilitates the activity of ER resident chaperones and foldases. The mitochondria influences Ca^{2+} release from the ER as well as returning Ca^{2+} during a recovery phase (Berridge, 2002). Thus the ER and mitochondria cooperate to generate a continuous, tightly regulated Ca^{2+} signaling pathway. Moreover, ER stress is one stimulus that can trigger the release of Ca^{2+} from the ER into the mitochondria resulting in diminished protein folding capacity in the ER.

MAMMALIAN RESEARCH HAS PROVIDED A FRAMEWORK LINKING ER STRESS AND PROGRAMMED CELL DEATH

In mammals, chronic stress on the ER can promote oxidative stress, autophagy, and apoptotic cell death. In plants, the UPR is also linked to oxidative stress and PCD regimes that include autophagy. Cell death is seen as an adaptive and intended response to reinforce a system that is overwhelmed. Thus, in situations where the demand for protein and/or Ca^{+2} outweighs a given cells ability to cope, prolonged ER stress leads to harsher measures and activates a PCD that, in mammals, can be either autophagous and/or apoptotic (Hetz, 2012; Jager et al., 2012). In this review we focus on ER-directed cell death pathways in plants and discuss; (i) signal transduction mediating ER-stress induced cell autophagic/programmed cell death; (ii) the role of ER resident chaperones in suppressing PCD, (iii) the regulation/crosstalk of ER-induced PCD and autophagy pathways, and (iv) ER-stress regulated PCD during plant pathogen interactions.

ER SENSORS CONTROL STRESS RESPONSES AND CELL DEATH PATHWAYS

The detection of ER stress and mediation of UPR signaling occurs via sensors located at the ER membrane. In mammals there are three transmembrane embedded sensors: IRE1, ATF6, and PERK (Walter and Ron, 2011). Each of these sensors initiate signaling pathways that can restore ER homeostasis or under conditions of chronic stress and increasing damage, activate alternative

routes leading to cell death. The type 1 transmembrane protein, kinase/endoribonuclease inositol-requiring enzyme 1 (IRE1 a and b), is a dual functioning protein. IRE1 has ribonuclease activity and is responsible for splicing X-box binding protein-1 (XBP) mRNA, generating the transcription factor XBP1 (Yoshida et al., 2001). XBP1 translocates to the nucleus where it activates the expression of cytoprotective genes, including members of the ER QC pathway. IRE1 is also capable of activating the apoptotic-signaling kinase 1 (ASK1) and Jun-N-terminal kinase (JNK) that promote apoptosis (Koizumi et al., 2001; Nagashima et al., 2011; Humbert et al., 2012).

In plants, IRE1a and IRE1b localize to the perinuclear ER and the signaling pathways that emanate from these sensors include a completely separate set of intermediary factors from those identified in mammals (Koizumi et al., 2001). In fact recent data suggest a distinct and specialized role for IRE1b in ER stressed but not nutrient stressed induced autophagy and further suggests that an alternative pathway is in play during autophagy responses during nutrient deprivation in plants (Liu et al., 2012).

IRE1 activates mRNA splicing of the transcription factor, bZIP60 which recognizes promoters with a recently identified ER stress responsive *cis*-element UPRE-III in the NAC103 promoter. The NAC103 transcription factor activates several UPR related foldases including CRT1, CNX, PDI-5 (Sun et al., 2013a). Both IRE1 proteins have been reported to be expressed throughout the plant (Noh et al., 2002). While autophagy markers including the formation of autophagosomes were observed following nutrient starvation, wild type, IRE1a and IRE1b mutants resulted in similar phenotypes; (Liu et al., 2012). Treatment of *Arabidopsis* IRE1a and IRE1b knock out mutants with the well-known ER stress inducers, tunicamycin (TM) and dithiothreitol (DTT), resulted in contrasting phenotypes. Application of DTT and TM induced the formation of autophagosomes in IRE1a mutants and wild type plants. In contrast, autophagy was not induced in IRE1b mutants under the same conditions (Liu et al., 2012). ER stress-induced autophagy in plants occurs only via the IRE1b-mediated pathway (Koizumi et al., 2001; Liu et al., 2012).

In mammals, ATF6 is a type II transmembrane basic leucine-zipper (bZIP) domain-containing activating transcription factor (Yoshida et al., 2001). Upon ER stress ATF6 moves through the Golgi compartment and is cleaved by cellular proteases for maturation. The plant equivalents to the mammalian ATF6 pathway, are two key ER-localized, membrane tethered transcription factors, bZIP17 and bZIP28 (Liu et al., 2007a,b). Similar to ATF6, bZIP17, and bZIP28 are activated following detection of accumulating unfolded proteins in the ER and then translocate to the Golgi apparatus where they are cleaved by Golgi-localized proteases for maturation. In the nucleus bZIP17 and bZIP28 activate expression of cytoprotective chaperones and foldases, facilitating the formation of correct macromolecular structures and protein folding, respectively (Liu et al., 2007a, 2008; Liu and Howell, 2010a; Srivastava et al., 2012).

The third ER resident sensor identified in mammals is the type I transmembrane protein kinase RNA-like ER kinase (PERK). Upon detection of unfolded proteins and ER stress, PERK phosporylates and inactivates the translation initiation factor eIF2a to shut down protein synthesis (Harding et al., 2000). PERK also activates the transcription factor CHOP which induces gene expression that leads to apoptosis. Translational regulation may not be entirely conserved since no obvious PERK homologs have as yet been identified in plants (Urade, 2009; Eichmann and Schafer, 2012).

Calcium stores in the ER are critical for the functioning of certain ER resident foldases. Calcium imbalance in the ER can disrupt the functioning of this protein folding pathway causing malformed proteins to accumulate. Release of Ca^{+2} from the ER can interfere with protein folding and leads to increased Ca^{2+} levels in the mitochondria and can promote oxidative stress and ultimately cell death (Berridge, 2002). Given the critical role of calcium in protein folding, oxidative stress and programmed cell death, mammalian systems utilize several different regulators including Bcl-2 and family members (Bax, and Bak), which are cytoprotective calcium sensors that modulate the release of ER Ca^{+2} stores and regulate cell death. Given the importantance of calcium in protein folding in the plant ER, it is in some ways surprising that genome sequence comparisons between plants and mammals indicate that these Bcl-2 family members are not present in plants, at least at the level of primary DNA sequence. Remarkably, transgenically expressed cytoprotective Bcl-2 and others (e.g., nematode Ced-9, chicken Bcl-xl; insect IAP; viral p35) function in plants in a similar manner to what occurs in animals including inhibiting PCD in response to pathogen invasion and abiotic/environmental stresses, in accordance with transkingdom pathway conservation (Dickman et al., 2001; Lincoln et al., 2002; Williams and Dickman, 2008; Dickman and Fluhr, 2013). Thus mammalian anti-apopotic machinery functions in plants and points to a conserved apoptotic-like PCD mechanism for death in plants. A key issue that is not entirely reconciled, is that there is little evidence for conservation at the DNA/gene level. We have suggested that structural homologies independent of sequence account for functional conservation, and indeed we have shown this to be the case when we uncovered the *Arabidopsis* BAG gene family (Doukhanina et al., 2006; Kabbage and Dickman, 2008; Williams et al., 2010). Initial blast analyses of *Arabidopsis* nucleotide and amino acid sequences failed to identify homologs of the mammalian BAG family. Therefore more sensitive methods were used based on higher level conservation including Hidden Markov Modelling (HMM) and profile-profile alignment algorithims to identify seven BAG members in Arabidopsis (Doukhanina et al., 2006). Unlike animals, plant BAGs display unique sub-cellular localisation; the three predicted calmodulin-binding BAGs are localized in the ER, mitochondria and vacuole, all of which are known Ca^{2+} reservoirs and mediators of cell death pathways (Kabbage and Dickman, 2008; Williams et al., 2010; Dickman and Fluhr, 2013).

WHEN PROTEINS FAIL TO FOLD- PROTEIN DEGRADATION OR SELF-DESTRUCTION?

There are occasions when proteins fail to mature in the ER and as a result, they are exported from the ER for degradation by the ubiquitin-proteasome system as part of the endoplasmic reticulum assisted degradation (ERAD) pathway (Liu and Howell, 2010b; Huttner and Strasser, 2012). When plants are subjected to environmental stress, the levels of malformed proteins can overwhelm the ERQC and the ERAD associated systems. Continuing

ER stress requires harsher measures including oxidative stress that can lead to autophagy or apoptosis in mammals (Hetz, 2012; Jager et al., 2012; **Figure 1**). How these life/death decisions are made; where is the point-of no return from commitment to death; how these options are distinguished, identification of the key regulatory and the nature of PCD pathways promises to be of continuing interest.

Autophagy involves the sequestration of unwanted or damaged proteins and organelles in characteristic double membrane vesicles known as autophagosomes. There are more than 30 autophagy (ATG) related genes in yeast, many of which have been identified in mammals and plants. The mechanism of autophagosome formation has been described elsewhere but it is important here to recognize that the ER is active in the formation of these double membrane vesicles (Axe et al., 2008). Autophagosomes engulf cytosolic constituents and/or damaged organelles then deliver their "cargo" to either the vacuole (plants) or lysosome (mammals) where they are degraded. Autophagy is often viewed as a drastic measure taken by cells to eliminate damaged proteins or organelles with the goal of restoring cellular homeostasis, as

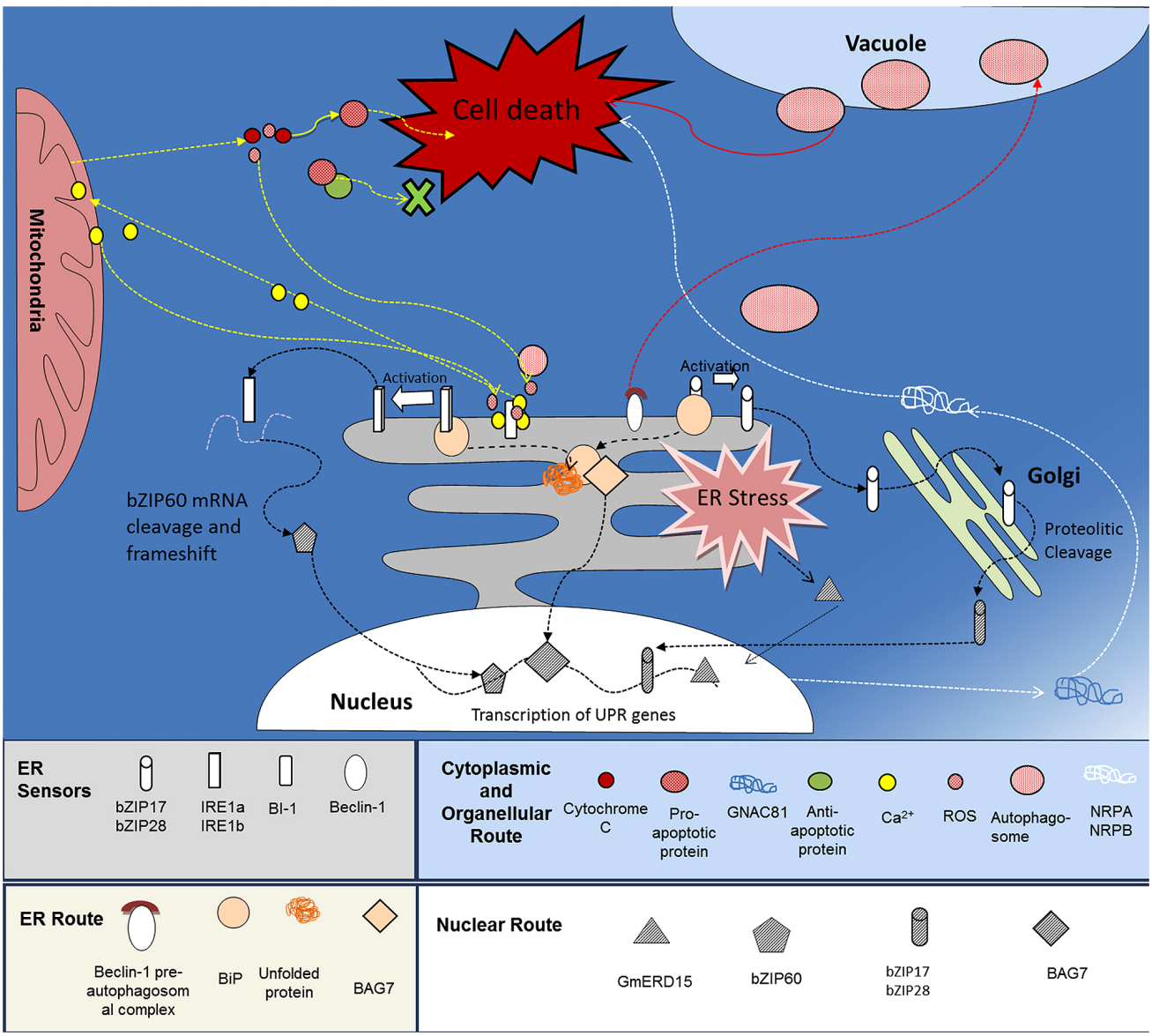

FIGURE 1 | ER-mediated cell death pathways in plants. The ER and ER stress pathways are key molecular switches of at least three routes leading to cell death, (i) intracellular signaling pathways - ROS triggers the mobilization of Ca^{2+} from the ER to the Mitochondria, resulting in a mitochondrial permeability transition pore (PTP), selective leakage of apoptotic factors, additional release of Ca^{2+} from the ER and cell death (Yellow arrows). (ii) ER-vacuole directed autophagy - ER-localized Beclin-1 initiates autophagosome formation, autophagy, and cell death, and (iii) NRP-directed cell death - detection of ER and osmotic stress activates the transcription factor GmERD15 which triggers expression of NRPA and -B. Once expressed, the NRPs activate the cell death effector GmNAC81 leading to DNA fragmentation and cell death (white arrows).

first noticed during conditions of yeast starvation (Takeshige et al., 1992).

Autophagy is involved with the UPR via the IRE1a pathway in mammals (Sano and Reed, 2013). In plants, autophagy is linked to IRE1b but splicing of bZIP60 mRNA is not required for autophagy. This dispensability of IRE1b splicing of bZIP60 suggests one of two possibilities; IRE1b may have an additional, as yet unknown splicing target that is linked to activation of autophagic machinery (Liu et al., 2012), or IRE1b may have additional functions (e.g., kinase activity) which activate an alternate signaling cascade.

Several environmental, nutritional, pathogenic and metabolic conditions including those that promote ER stress have been linked with autophagy. Thus a growing body of evidence suggests that autophagy can play opposing roles by promoting both cell survival and cell death (Liu et al., 2005; Hayward and Dinesh-Kumar, 2011). Moreover, as the ER is also crucial for maintenance of cellular homeostasis under stress, autophagy mediated trafficking of nutrients and removal of "damaged goods" can provide the cell necessary time for ER-stress adaptation. Thus, during times of excessive ER stress, autophagy may act as a cellular backup to ERAD bolstering UPR and ERAD capacity in addition to providing essential nutrients to penurious cells (Yorimitsu et al., 2006).

ER AND PLANT PCD

Programmed cell death is essential for normal growth and development (Kerr et al., 1972), however, disease occurs when PCD is inappropriately regulated (too much, too little). In mammals this can lead to a range of pathologies including cancer to Parkinson's disease. The UPR, autophagy and Ca^{2+} signaling between the ER and mitochondria are core components of the PCD machinery. These core components have recently been identified in plants thus linking the UPR to PCD and innate immunity (Ma and Berkowitz, 2007; Martinon and Glimcher, 2011; Stael et al., 2012).

In plants there are four key ER resident factors that link Ca^{+2} stores to UPR and cell death regulation. These are calreticulin, calmodulin, ER-type IIA Ca^{2+} pump, and type IIB Ca^{+2}-ATPase. Calreticulin is a Ca^{+2} binding protein that chaperones maturation of glycoproteins in the ER (Jia et al., 2009). Calmodulin is a Ca^{+2} sensor that recognizes changes in Ca^{+2} levels in the ER and activates immune related PCD (Ranty et al., 2006; Ma et al., 2008; Du et al., 2009). Application of the ER-type IIA Ca^{2+} pump inhibitor cyclopiazonic acid (CPA) leads to increased cytosolic Ca^{2+}, cross-talk with mitochondria and PCD in soybean (Zuppini et al., 2004). The formation of the so-called mitochondrial permeability transition pore (PTP), an established marker of PCD, allows the selective leakage of several stress signals and cell death regulators sensitizing ER channels to further release of Ca^{2+} in a positive feedback loop (Berridge, 2002). In addition, the ER localized type IIB Ca^{+2}-ATPase regulates N-mediated PCD in response to infection with Tobacco mosaic virus (TMV; Zhu et al., 2010). Perturbations in ER function, however, promote the release of Ca^{2+} from the ER into the mitochondria resulting in diminished protein folding capacity, ER stress, the formation of PTP and subsequent apoptosis (Berridge, 2002).

In addition to disruption of ER function and the accumulation of unfolded proteins, studies have shown that depletion/release of Ca^{2+} from the ER is promoted by pro-death members of the Bcl-2 family (Scorrano et al., 2003; Hammadi et al., 2013). Additionally, cytoprotective anti-PCD proteins such as Bcl-2 have been shown to prevent PCD by suppressing the release ER Ca^{2+} (Lam et al., 1994). One protein that may facilitate ER Ca^{2+} homeostasis by acting as a Ca^{2+} permeable channel is Bax inhibitor and is discussed below.

A plant-specific branch of the ER stress pathway was recently noted in soybean; N-rich protein (NRP)-mediated cell death. NRPs are asparagine rich proteins that represent the hallmarks of an integrative pathway linking ER and osmotic stress signaling (Reis and Fontes, 2012). The combination of ER-stress and osmotic stress activates the transcription factor GmERD15 which in turn binds to and triggers expression of N-rich protein genes including NRP-A and NRP-B (Reis and Fontes, 2012). Overexpression of these NRPs in soybean induces the cell death effector GmNAC81, activating a caspase-3-like activity promoting DNA fragmentation; features associated with PCD. The ER chaperone BiP limits spread of this death signal (Reis et al., 2011). In a yeast two-hybrid screen for GmNAC81 interactors, GmNAC30 was identified. Interestingly, a functionally relevant binding site for GmNAC81/ GmNAC30 was found in the promoter of vacuolar processing enzyme (VPE; Mendes et al., 2013). VPE is a plant protease the activity of which, is important for viral and bacterial-induced HR. VPEs have been reported on several occasions to induce PCD in plants following pathogen challenge. Although structurally unrelated to caspases, VPE has caspase-1 (an inflammatory caspase) activity (Hatsugai et al., 2004; Hara-Nishimura et al., 2005; Kuroyanagi et al., 2005). This system links ER stress, the UPR, and cell death regulation. Transgenic tobacco plants overexpressing BIP show enhanced water stress tolerance (Alvim et al., 2001; Valente et al., 2009), thus there is a potential biotechnological application by generating crop plants (e.g., soybean) overexpressing BiP that would mitigate ER stress and lead to enhanced water stress tolerance. Interestingly, NRP signaling is believed independent of the known ER resident stress sensor mechanisms; thus it will be of particular interest to identify key components and piece together these PCD pathway(s).

ROLE OF THE ER RESIDENT CHAPERONES *BAG-7, BIP* AND *BI-1* IN LIMITING CELL DEATH

BAG FAMILY OF PROTEINS

Members of the Bcl-2-associated athanogene (BAG) family are a conserved group of eukaryotic co-chaperones that assist in protein folding and diverse cellular processes including apoptosis and stress signaling. The *Arabidopsis* BAG family contains seven members and unlike its mammalian counterparts which are primarily cytosolic, different family members localize to different sub-cellular organelles (Doukhanina et al., 2006; Kabbage and Dickman, 2008; Williams et al., 2010). BAG proteins regulate Bcl-2, interact with Hsp70/Hsc70, and associate with the ubiquitin/proteasome systems in animals. BAG proteins function in processes that promote cell survival in both plants and animals (Kabbage and Dickman, 2008).

In the ER, AtBAG7 plays a key role in UPR pathways, particularly in response to heat and cold stress (Williams et al., 2010).

AtBAG7 directly binds to the ER-resident sensor and chaperone AtBiP2 (Williams et al., 2010) and likely provides essential co-chaperoning activity needed to prevent the build-up of unfolded proteins and limit cell death as a result. Interestingly, AtBAG7 mutants display hypersensitivity to the ER-stressor and autophagy inducer TM, suggesting that AtBAG7 plays a role in the regulation of autophagy pathways (Williams et al., 2010). Moreover such mutants were also sensitive to the ER stress stimuli heat and cold.

Subsequent staining of TM treated wild type and AtBAG7 mutant plants with the autophagosome detecting dye monodansylcadaverine (MDC) however, showed autophagosomes are formed in the presence or absence of AtBag7. Thus, AtBag7 does not appear to be necessary for autophagy. Our preliminary data indicates that AtBAG7 translocates from the ER to the nucleus in response to heat stress. Yeast two hybrid and BIFC studies show that following nuclear translocation, AtBAG7 binds to the transcription factor BTF3, a member of the NAC family of stress responsive transcription factors. It is reasonable to hypothesize that ER-localized AtBAG7 activates stress response pathways via translocation to the nucleus and transcriptional activation of genes associated with abiotic stress protection. In this respect AtBAG7 may not only play a role as an important ER co-chaperone but may be an important mediator of ER – nucleus signaling during ER stress responses. Studies are in progress to determine the contributions of AtBAG7 to the UPR and PCD.

BiP

The ER luminal binding protein (BiP/Grp78) is a member of the heat shock protein 70 (HSP70) family (Haas, 1994). It is the most abundant ER-chaperone, and a key player of the ERQC machinery. BiP binds to and suppresses the activity of the mammalian ER stress sensors, PERK, IRE1, and ATF6 and the plant ER stress sensors IRE1, bZIP17, and bZIP28 (Srivastava et al., 2013). Upon detection of unfolded proteins, however, BiP is released and binds to unfolded proteins thus leaving IRE1 free to oligomerise and splice bZIP60 mRNA, the result of which induces transcription of UPR associated genes.

Three BiP isoforms have been identified in *Arabidopsis* (Lin et al., 2001). Expression of BiP is induced by environmental stressors such as heat, salinity and pathogen invasion (Sung et al., 2001). Expression profiling has shown that BiP 1 and 2 are constitutively expressed while activation of BiP3 is triggered by bZIP60 as part of the UPR (Iwata et al., 2008; Deng et al., 2011). BiP3 expression is also induced in the absence of bZIP60, suggesting additional factors mediate transcription of BiP3 in the IRE/bZIP60 pathway. As mentioned BiP mediated inhibition of UPR is a factor in drought in transgenic tobacco (Alvim et al., 2001). BiP is also a negative regulator of ER stress related cell death during pathogen invasion (Jelitto-Van Dooren et al., 1999). Surface exposed leucine rich receptors are responsible for recognition of the bacterial pathogen-associated molecular patterns (PAMPs) and are critical in plant defense. Complex formation between BiP, and other ER resident proteins such as stromal-derived factor-2 (SDF-2) and calreticulin is essential for the glycosylation, activity and accumulation of the receptor kinases EFR and Cf-9, respectively. Similarly, loss of BiP complex formation causes ER retention and reduced levels of EFR and Cf-9 (Nekrasov et al., 2009; Liebrand et al., 2012). Taken together, in addition to reducing unfolded protein levels, BiP stabilizes immune receptors to facilitate host defense.

BAX INHIBITOR (BI-1)

Initially identified as a suppressor of BAX-induced (BI) cell death in yeast and mammalian cells, Bax-inhibitor proteins are highly conserved ER localized, multi-transmembrane proteins (Ishikawa et al., 2011). BI-1 genes are one of the few examples of a gene identified and characterized in both animals and plants that specifically regulate PCD. There is increasing evidence that BI-1 plays roles in both PCD and autophagy to regulate the switching of ER-mediated cell death pathways from autophagy to PCD (Castillo et al., 2011; Carvalho et al., 2013).

Over-expression of BI-1 reduces PCD as well as the cytosolic Ca^{2+} concentration, potentially by functioning as a pH sensitive regulator of ER calcium channel activity. Evidence for these functions is supported by topology studies which indicate that the C-terminus of BI-1 forms a Ca^{2+} pore that could act as the source for its Ca^{2+} -leaking properties (Bultynck et al., 2012). BI-1 also plays a role in autophagy responses via interaction with the ER stress sensor, IRE1; via interaction with BI-1, IRE-1 is activated leading to induction of UPR gene expression (Lisbona et al., 2009).

As in mammals *Arabidopsis*/plant Bax Inhibitor-1 (AtBI-1) also suppresses Bax when expressed in tobacco, as well as several abiotic and biotic stress-induced PCD (Watanabe and Lam, 2006, 2008). AtBI-1 mutants also display increased sensitivity to ER stress factors such as heat and TM. Conversely, *Arabidopsis* plants over expressing AtBI-1 are more tolerant to TM, heat and cold stress (Watanabe and Lam, 2008). Studies have indicated that BI-1 functions downstream of ROS generation; over-expression of BI-1 in *Arabidopsis* did not suppress BAX-induced ROS production but still prevented PCD (Kawai-Yamada et al., 2001, 2004). AtBI-1 prevents Bax-induced cell death at least in part, by maintaining ER Ca^{2+} homeostasis (Ihara-Ohori et al., 2007). AtBI-1 knockouts display accelerated methyl jasmonate-induced senescence pathways further linking the ER to autophagy (Yue et al., 2012).

A role for BI-1 in the regulation of plant autophagy responses is also suggested by starvation experiments. Carbon starvation is a known inducer of autophagy in plants and animals; down-regulation of the tobacco BI-1 homolog augments cell death upon starvation (Bolduc and Brisson, 2002). Importantly, *Arabidopsis* BI-1 knockouts did not display any developmental abnormalities suggesting a specific role for BI-1 during these stress conditions.

DEATH BY DESIGN? THE ROLE OF BECLIN-1 AND BCL-2 IN ER-INDUCED AUTOPHAGY AND PCD

Prolonged ER stress and/or excessive autophagy may induce PCD; paradoxically, autophagy mutants display increased PCD during nitrogen starvation. Thus either too much or too little autophagy can lead to PCD. The relationship and cellular decision process mediating autophagy vs. PCD is largely unknown. During the initial stages of stress, autophagy pathways may be triggered to reduce ER stress and prevent apoptosis. As a result homeostasis is maintained but whether the default state of autophagy is pro-survival or pro-death (or both) remains to be elucidated, as there

are examples of both situations (Hofius et al., 2009, 2011; Xu et al., 2013) As in the case of N-gene mediated resistance to TMV, pro-survival roles for autophagy in cytoprotective cell death, include the "controlled sacrifice" of select cells and organelles providing nutritional building blocks to the organism/cell, maintaining energy homeostasis during a potentially lethal situation. Importantly, for N-gene mediated resistance to tobacco mosaic virus, autophagy is needed to limit the extent of cell death surrounding the cells attacked by the virus (Liu et al., 2005). If autophagy is blocked, disease symptoms are no longer constrained. In other situations, sustained autophagy triggers apoptosis in mammals and in this case cell death may not be cytoprotective (Rikiishi, 2012). In this respect autophagy may be considered as a last ditch effort for cell survival, once the irreversible apoptotic cell decision is made, death is inevitable. The salient details as to how and when these life-death decisions are determined, also remain to be established. In mammals the interaction between Bcl-2 and Beclin-1 functions as a switch that drives the direction of signaling either toward autophagy or apoptosis (Oberstein et al., 2007).

The Bcl-2 family contains both pro-apoptotic proteins (e.g., BAX) and anti-apoptotic proteins including Bcl-2 (Reed, 1998). Family members are distinguished by 1-4 so-called BH (Bcl-2 homology domains). BH3 domains are of particular importance as all pro-apoptotic family members harbor this signature "death" domain required for apoptosis. Anti-apoptotic proteins inhibit cell death by binding to these domains. Beclin-1 (ATG6) is a conserved BH3 domain containing protein and primary component of the autophagy pathway that was initially identified as a Bcl-2 interacting protein. Bcl-2, if in sufficient concentration inhibits Beclin-1 mediated autophagy by binding to the BH3 domain in Beclin-1, thereby removing free Beclin-1. Thus Bcl-2 is not only anti-apoptotic, but also anti-autophagic. If Beclin-1 is in excess, Bcl-2 is tied up and autophagy ensues. Thus Beclin-1 serves as a checkpoint dictating the balance between apoptosis and autophagy pathways. The BH3 domain of Beclin-1 interacts with several anti-apoptotic Bcl-2 family members (Maiuri et al., 2010). Once bound, Beclin-1 is unable to assemble the pre-autophagosome complex thereby inhibiting autophagy while promoting apoptosis (Sinha and Levine, 2008). Plant Beclin-1 appears functionally and structurally similar to mammalian Beclin-1 (Sinha and Levine, 2008) with a notable difference; plant Beclin-1 lacks the mammalian BH3 death domain. Is there a functional or structural equivalent? What are the underlying mechanisms of Beclin-1 mediated autophagy and/or PCD?

ER-CELL DEATH PATHWAYS AND PLANT PATHOGEN INTERACTIONS

Recent research has begun to identify links between plant pathogens and ER stress related cell death. Studies with *Potato virus X* (PVX) show that the ER residing PVX TGBp3 movement protein activates the transcription factor bZIP60 to trigger the UPR as well as eliciting PCD (Ye et al., 2012, 2013). The mechanism appears to be conserved across host species, with expression of TGBp3 inducing the same sets of genes in *Arabidopsis thaliana* and *Nicotiana benthamiana. Tobacco rattle virus* mediated knockdown of the bZIP60 pathway significantly blocked accumulation of PVX in both protoplasts and whole plants; thus activation of bZIP60 is required for PVX replication. Additionally, infiltration of *Agrobacterium* carrying a TGBp3 expression system induced an HR in tobacco that could be abrogated by co-expression of BiP but not by anti-apoptosis genes Bcl-xl, Ced-9, Op-IAP. Such a hypersensitive response was not elicited upon infiltration of either TMV or PVX, thus indicating that TGBp3 elicits PCD but also the UPR for survival (Ye et al., 2013).

Reoviruses are double-stranded RNA viruses and infect a wide range of eukaryotes. Rice black-streak dwarf virus (RBSDV) P10 outer capsid protein induces ER stress and the UPR in *N. benthamiana.* Similar to the PVX TGBp3 protein, P10 associates with the ER and induces expression of bZIP60, BIP, PDI, and Calmodulin (CAM). Mammalian infecting reoviruses are also known to elicit the UPR to benefit virus replication and assembly. These viruses can sequester pro-death factors inside viroplasms to regulate antiviral defenses that could radiate from UPR initiation. These data suggest that virus interactions with the UPR machinery are conserved across eukaryotes and offer some unique perspectives on how viruses could control PCD via their interaction with the ER (Zambrano et al., 2011; Sun et al., 2013b).

Endoplasmic reticulum resident chaperones, BiP and calreticulin (CRT) have also been demonstrated to play a key role in resistance against the vascular wilt necrotrophic fungal pathogen *Verticillium dahliae.* Tomato Ve1, an LRR receptor-like protein confers resistance in tomato to *V. dahliae.* In a screen employing GFP fusions, immuno-screening and mass spectrometry, potential binding partners with Ve1 were identified including several ERQC chaperones including BiP and a lectin-like calreticulin (CRT) (Liebrand et al., 2014). Knockdown of tomato BiPs and CRT in the tomato plants carrying the Ve1 gene resulted in reduced resistance to *Verticillium* suggesting that both ERQC chaperones contribute to plant immunity. Interestingly, parallel experiments in tobacco and *N. benthamiana* failed to detect changes in glycosylation on Ve1 and unlike non-silenced tomato there was no suppression of HR, even though plants were more susceptible in the Ve1 CRT silenced tobacco lines. This suggests that a HR is not required and/or could indicate an uncoupling between cell death and resistance. An interesting example of ER targeting for compatibility was elegantly demonstrated in studies with *Piriformospora indica,* a fungal mutualist (Qiang et al., 2012). For successful root colonization *P. indica* initially colonizes living cells. During this period the fungus triggers ER stress but inhibits the UPR. Of note, VPE was also induced and mediated ER stress and cell death in large part by suppressing the host (*Arabidopsis*) UPR pathway. A similar scenario of colonizing living cells while preparing for their doom via ER stress regulation might be occurring in *Verticillium* as described above as well as the necrotroph *Sclerotinia sclerotiorum. Sclerotinia* is an aggressive, broad host range necrotroph that was recently shown to also colonize living cells prior to fungal induced host cell death. Sclerotinia oxalic acid mutants are non-pathogenic and elicit in the host a bona fide HR and autophagy (Kabbage et al., 2013). Treatment of this non-pathogenic mutant with DTT partially restored pathogenicity suggesting that the fungus may require ER stress control for successful infection (Williams et al., 2011).

Studies using differential lines of barley to the powdery mildew fungus *Blumeria graminis* f.sp. *hordei* (Bgh) suggest a functional

role for BI-1, the ER and UPR in response to fungal challenge (Huckelhoven et al., 2003). Several lines of evidence suggest an inverse relationship between BI-1 function and host penetration resistance against the powdery mildew fungus. Overexpression of barley BI-1 increased susceptibility to Bgh. Barley BI-1 expression is significantly suppressed following application of the salicylic acid analog, 2,6-dichloroisonicotinic acid (INA), an inducer of systemic resistance (Huckelhoven et al., 2003) Together the cell death suppressing activity of cytoprotective BI-1, compromises host defense mechanisms that generate a PCD –HR for resistance, providing a link between PCD host defense pathways and BI-1 expression.

CONCLUSION

We are just beginning to uncover the signaling pathways and regulatory circuits mediating ER stress and cell death in plants. Oxidative stress, Ca^{+2} influx to mitochondria, caspase-like activities, autophagy, and PCD related factors contribute to the ER stress response.

Cells appear to implement a hierarchal regime involving a series of checks and balances before succumbing to ER cell death. Prolonged ER-stress leads to oxidative stress and if sustained; autophagy can occur. If autophagy is not sufficient to right the ship, PCD removes the cell. Autophagy mutants display increased PCD during nitrogen starvation; thus too much or too little autophagy can result in PCD. Beclin-1 appears to serve as a key checkpoint illustrating the intricate balance between pro-survival and pro-death within autophagy pathways. By default, autophagy pathways appear to be pro-survival and can be considered a last ditch effort by the organism to cope with prolonged stress and prevent the "point of no return" that leads to the induction of apoptosis pathways. Intriguingly, although, Bcl-2 family members have not been found in plants, mammalian BCL-2 family members have been expressed in plants and demonstrate conserved function transkingdom manner, thus suggesting that structural /functional homologs of the Bcl-2 family exist in plants (Dickman et al., 2001; Doukhanina et al., 2006). The absence of a BH3 domain in plant Beclin-1 correlates with the failure to identify Bcl-2 family members and the question remains open as to whether there is a functionally operationally conserved mechanism or not in plants.

The ER and ER stress pathways are becoming increasingly more prominent as potential targets for the pathogenic success of microbial pathogens. We anticipate this to continue. For example, the fungus *P. indica* induces cell death by inhibiting the UPR related pro-survival machinery and then activating ER stress mediated cell death machinery (Qiang et al., 2012). Moreover, numerous plant viruses commandeer ER stress machinery to mitigate host defense and the HR further highlighting the ER as a master switch in biotic (and abiotic) environmental stresses.

Key future issues include: (i) Identifying the relevant players in plants and filling in the gaps in the in ER stress signaling pathways and (ii) Deciphering ER mediated cell decision processes. Although there are gaps in our knowledge surrounding ER-stress induced cell death pathways, it is apparent that the ER and UPR form a tight "cell death" regulatory network with several plant organelles that together facilitate homeostasis in mammals and plants in response to development and environmental cues.

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Endoplasmic reticulum stress-induced PCD and caspase-like activities involved

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Plant cells, like cells from other kingdoms, have the ability to self-destruct in a genetically controlled manner. This process is defined as Programmed cell death (PCD). PCD can be triggered by various stimuli in plants including by endoplasmic reticulum (ER) stress. Research in the past two decades discovered that disruption of protein homeostasis in the ER could cause ER stress, which when prolonged/unresolved leads cells into PCD. ER stress-induced PCD is part of several plant processes, for instance, drought and heat stress have been found to elicit ER stress-induced PCD. Despite the importance of ER stress-induced PCD in plants, its regulation remains largely unknown, when compared with its counterpart in animal cells. In mammalian cells, several pro-apoptotic proteases called caspases were found to play a crucial role in ER stress-induced PCD. Over the past decade, several key proteases with caspase-like enzymatic activity have been discovered in plants and implicated in PCD regulation. This review covers what is known about caspase-like enzymatic activities during plant ER stress-induced PCD and discusses possible regulation pathways leading to the activation of relevant proteases in plants.

Keywords: programmed cell death, VPE, proteasome, UPR, plant caspases

INTRODUCTION

Plant cells have the ability to self-destruct in a controlled manner. This process is called programmed cell death (PCD). Unlike death as a consequence of physical damage, which is not controlled by the cell itself, PCD is genetically regulated. PCD is a versatile tool for plants to cope with various situations or needs. In development, tracheary elements for example, undergo PCD to form the xylem (Groover et al., 1997; Bollhöner et al., 2012). PCD also is used in plant defense. When plant leaves are attacked by pathogens, PCD can be elicited locally where the tissue is invaded. In this case, PCD contributes to reducing the growth of the pathogen and possibly its spreading into other parts of the plant (Greenberg, 1996). Among the various stresses that induce plant PCD, endoplasmic reticulum (ER) stress is a new comer and is gaining more and more attention. The ER is an essential organelle. One of its functions is folding and *N*-glycosylation of secreted proteins. If for various reasons these processes do not function properly, misfolded proteins will accumulate inside the ER and cause ER stress. Cells experiencing ER stress will try to restore ER homeostasis by inducing the expression of unfolded-protein-response (UPR) genes. The products of these genes help cells escape ER stress by increasing the protein folding capacity and secretion ability within the ER. However, if the mounted rescue response is overwhelmed by ER stress, cells will eventually enter PCD. Such an ER stress-induced PCD has been observed in plants when *Arabidopsis* seedlings were treated with increasing concentration of PEG8000, which mimics drought. ER stress was induced and eventually resulted in PCD of root cells (Duan et al., 2010). In addition, when plants are challenged with biotic stress, for example a pathogen, ER stress is observed (Moreno et al., 2012) and this may contribute to the induction of PCD. In the past few years, there has been several reports on how ER stress regulators regulate UPR genes in plant cells (Liu and Howell, 2010; Howell, 2013). However, we still have a limited picture of the PCD pathway components activated when ER stress is unresolved. Among PCD regulators, caspases are central components that mediate animal PCD pathways in response to various stimuli, including ER stress. Caspases are cysteine-dependent aspartate specific proteases. As the name suggests, caspases cleave their substrates after an aspartate residue (in P1 position in the cleavage site). This led to the development of synthetic tetra-peptides conjugated with fluorescent reporters to determine caspase enzymatic activities. For example, Tyr(Y)-Val(V)-Ala(A)-Asp(D) is used to measure caspase-1 activity because it is the four amino acid pattern preferentially cleaved by caspase-1. Of note, YVAD can also be cleaved *in vitro* by other caspases or unrelated proteases such as legumains (Rotari et al., 2001; McStay et al., 2007). In plants, enzymatic assays using these synthetic caspase substrates have directly or indirectly implicated proteases with caspase-like activities in ER stress-induced PCD (Zuppini et al., 2004; Costa et al., 2008). The term caspase-like is used because there is no caspase ortholog in plant genomes and the plant proteases closest to animal caspases, the metacaspases, are unable to cleave synthetic caspase substrates and have instead Arg specificity. The plant caspase activities must therefore originate from proteases unrelated to animal caspases. Using various experimental systems, several research groups were able to identify plant proteases that possessed caspase-like enzymatic activities and regulated PCD. Plant proteases related to caspases and proteases with caspase-like activities have been extensively reviewed (Van der Hoorn, 2008; Xu and Zhang, 2009; Tsiatsiani et al., 2011;

Vartapetian et al., 2011). This review focuses on the caspase-like activities linked to ER-stress induced PCD and comments on the proteases involved.

COMPARISON OF THE REGULATION OF ER STRESS-INDUCED PCD BETWEEN PLANT AND OTHER MODEL ORGANISMS

As hinted above, PCD is an evolutionary conserved consequence of unresolved ER stress. PCD can be induced experimentally by a prolonged treatment using an ER stress-inducing chemical. Signal transduction from ER stress to apoptosis in mammalian cells has been established, although it is not fully complete. Three ER tethered proteins: protein kinase RNA-like ER kinase (PERK), activating transcription factor (ATF6) and inositol-requiring enzyme (IRE1α) are involved in the regulation of ER stress-induced apoptosis in mammalian cells. Inside the ER lumen, the protein chaperone binding immunoglobulin protein (BiP) contributes to both refolding of misfolded proteins and activation of PERK, ATF6, and IRE1α. During ER stress, PERK activation is due to its dissociation from BiP. Active PERK phosphorylates eukaryotic initiation factor 2 (eIF2) which results in translation inhibition (Harding et al., 1999; Harding et al., 2000). However, not all protein translation is blocked and a cAMP response element-binding transcription factor ATF4 escapes the inhibition of protein translation (Harding et al., 2003). Although many ATF4 target genes are pro-survival, one of ATF4 target genes, C/EBP homolog protein (CHOP), is pro-apoptotic (Zinszner et al., 1998). The mechanism of CHOP-induced apoptosis relies on (1) Restoration of translation via dephosphorylation of eIF2 by a protein phosphatase 1 (GADD34), which is a target of CHOP; (2) Inhibition of the pro-survival serine/threonine kinase Akt; (3) Repression of anti-apoptotic protein B-cell lymphoma 2 (BCL-2) expression level. In addition to PERK, ATF6 is involved in regulating CHOP expression as the CHOP gene is a direct target of ATF6 (Yoshida et al., 2000). However, not many evidences support that ATF6 is directly involved in the apoptosis signal pathway, apart from that ATF6 is able to increase CHOP transcript level. Finally, in the IRE1α branch, activation of IRE1α is also through dissociation from BIP (Szegezdi et al., 2006). Active IRE1α is able to recruit TNF-receptor-associate factor 2 (TRAF2), which in turn recruit apoptosis-signal-regulating kinase (ASK1; Nishitoh et al., 1998). The IRE1α-TRAF2-ASK1 complex activates c-Jun N-terminal kinase (JNK). Activation of JNK activates downstream pro-apoptotic signals. For instance, JNK represses BCL-2 via phosphorylation (Davis, 2000). Downstream of PERK, ATF6, and IRE1α mediating signals is the activation of caspases. Caspase-12 (in rodent) and caspase-4 (in human) were reported to mediate a caspase cascade in ER stress-induced PCD in mammalian cells (Nakagawa et al., 2000). A recent report implicated caspase-2 in ER stress-induced PCD of mouse embryonic fibroblasts (MEFs). Under severe ER stress, IRE1α, via its α RNase activity, degrades a selective set of miRNAs which repressed caspase-2 expression level (Upton et al., 2012). Supporting the importance of IRE1α in this process, no expression of *caspase-2* and no activation of caspase-3 were observed in IRE1α knock out MEFs, and knock out of the pro-apoptotic BAX/BAK did not affect caspase-2 expression level. The discovery that caspase-2 expression level was controlled by IRE1α and not by BAX/BAK suggested a direct signal pathway from IRE1α to caspase-2 and then to the mitochondrion apoptotic pathway involving BAX/BAK (Upton et al., 2012).

When looking into the ER stress-induced PCD signal pathway in plants, many of the regulators discovered in mammalian ER stress-induced apoptosis are missing from the genome sequences or at least functional equivalent are yet to be reported. For example, a PERK homolog has not been identified in plants and Kamauchi et al. (2005) reported that, unlike in animal cells, ER stress had no effect on protein translation in *Arabidopsis*. For this the authors used S^{35} labeled methionine and cysteine to measure nascent protein expression level under ER stress in *Arabidopsis*. In addition, the phosphorylation level of eIF2α showed a decrease rather than the increase described in animal cells (Kamauchi et al., 2005). All these experiments suggested that ER stress does not induce translation attenuation in plant cells. So it is possible that PERK and its downstream PCD regulator, CHOP, do not have functional homologs in *Arabidopsis*. Besides PERK and CHOP, there is no functional homolog of the BCL-2 family members reported in plants, in particular BAX/BAK, which control mitochondria membrane permeabilization during PCD. There are therefore clear differences in the regulation of ER stress-induced PCD between plant and mammalian cells. However, some ER-stress PCD regulators are conserved between plant cells and mammalian cells such as BAX-inhibitor and it is remarkable that despite the absence of caspase homologs in plants (Watanabe and Lam, 2008) caspase-like activities are induced during ER stress-induced PCD. In particular, caspase-3-like and caspase-9-like activities are triggered in soybean suspension cells during ER stress-induced PCD activated using cyclopiazonic acid (CPA), a chemical which inhibits ER Ca^{2+}-ATPase and causes Ca^{2+} efflux from the ER (Zuppini et al., 2004). Also in soybean, overexpression of N-rich proteins NRP-A and NRP-B or of a NAC transcription factor GmNAC81 (former GmNAC6), resulted in the induction of UPR and an increase of caspase-1-like and caspase-3-like activity (Costa et al., 2008; Faria et al., 2011). These few reports point out at caspases-like proteases being components of ER stress-induced PCD in plants. The next question is what are the proteases responsible for these caspase-like activities and do they regulate PCD?

PLANT CASPASE-LIKE ACTIVITIES AND ER STRESS-INDUCED PCD

Caspase-like activities has been observed in plant during PCD consistently since their first report (del Pozo and Lam, 1998). However, it took a long period of time to identify plant proteases responsible for the caspase-like activities. Initially, plant scientists pinned their hopes on metacaspases, as metacaspases were identified as distant relatives of animal caspases (Uren et al., 2000). Indeed metacaspases share some structural features with caspases. For example, metacaspases have a catalytic dyad based on cysteine and histidine (Uren et al., 2000). It is now clearly established, however, that metacaspases do not possess caspase-like enzymatic activity at all, as their cleavage site requires an Arg in P1 (R, e.g., RR) rather than Asp (D, e.g., YVAD, DEVD; Vercammen et al., 2004; He et al., 2008; Tsiatsiani et al.,

2011); Other plant proteases were found to exhibit caspase-like activities.

CASPASE-1-LIKE ACTIVITY: VACUOLAR PROCESSING ENZYME

Vacuolar Processing Enzyme (VPE) is a plant protease localized in the vacuole and responsible for processing vacuolar proteins. Hatsugai et al. (2004) reported that VPE, in addition to its activity against the tetrapeptide ESEN, exhibited caspase-1-like enzymatic activity as shown by activity labeling using the caspase-1 inhibitor YVAD-CHO. First, protein extracts from *Nicotiana benthamiana* were labeled with biotin-x-VAD-FMK. Two bands at 40kDa and 38kDa were detected and the labeling of these two bands was reduced with the addition of the caspase-1 inhibitor ac-YVAD-CHO or of a VPE antibody (Hatsugai et al., 2004). This indicated that VPE possessed caspase-1-like activity in plants. In addition, the recombinant γVPE recognized the VPE substrate ESEN with a Km of 30.3 μm and the caspase-1 substrate YVAD with a Km of 44.2 μm (Kuroyanagi et al., 2005) Further experiments suggested that VPE participate in plant PCD via controlling tonoplast rupture (Hatsugai et al., 2004). The fact that VPE has caspase-1-like activity had been documented for almost ten years, whereas a proof that VPE regulated ER stress induced PCD was only established recently using *Piriformospora indica* and *Arabidopsis* as an experimental system. In order for the mutualistic fungi *P. indica* to successfully colonize *Arabidopsis* roots, roots need to undergo a localized PCD (Qiang et al., 2012). A hallmark of PCD during the colonization of *P. indica* was the collapse of tonoplast. In addition, VPE null mutant reduced PCD as *P. indica* colonized roots (Qiang et al., 2012). Interestingly, Qiang et al. (2012) observed a swelling of ER, which was considered as an indication of ER stress, during *P. indica* colonization. To trigger ER stress-induced PCD, *P. indica* needs to overcome UPR, which tends to dampen the ER stress response. There is evidence that this is done by repressing UPR genes expression during colonization (Qiang et al., 2012). This set of experiments implied that VPE was downstream of UPR and part of the ER-PCD pathway. A recent report brings more details to this model. Two NAC transcription factors in soybean, GmNAC30 and GmNAC81, that induce PCD downstream of osmotic and ER stress, are able to interact with each other in a synergistic manner to activate directly *VPE* gene expression (Mendes et al., 2013). Both *GmNAC30* and *GmNAC81* transcript levels increased under ER stress induced using tunicamycin (Mendes et al., 2013).

CASPASE-3-LIKE ACTIVITY: PROTEASOME

26S proteasome is a proteases complex responsible for protein degradation in eukaryotic cells. The core unit of the 26S proteasome is the 20S proteasome. It consists of α and β subunits. Adding proteasome inhibitors, β-lactone and APnLD-CHO, to *Arabidopsis* leaf extract strongly reduced caspase-3-like activity as measured using the substrate DEVD (Hatsugai et al., 2009). In addition, the 20S proteasome β subunit 1 (PBA1) was detected by antibodies in protein-pull-down using the substrate analog inhibitor biotin-DEVD-FMK (Hatsugai et al., 2009). The activities of proteasome β subunit 2 (PBB) and 5 (PBE) were partially repressed by the caspase-3 inhibitor ac-DEVD-CHO at high concentration (>5 μM; Hatsugai et al., 2009). However, the authors suggested that neither PBB nor PBE possesses caspase-3-like activity, because the inhibition of their activities by ac-DEVD-CHO was not dose dependent (Hatsugai et al., 2009). Further to this, Han et al. (2012) purified a caspase-3-like activity from *Poplar* xylem tissues. In the elution fractions which showed highest caspase-3-like activity, the authors detected several proteins and mass spectrum analysis identified those proteins as proteasome subunit homologs in *Poplar* (Han et al., 2012). In other word, the caspase-3-like activity detected in plants is at least in part due to proteasome activity. In the context of ER stress-induced PCD, one major function of the proteasome is disposing of misfolded proteins in a process termed ER Associated Degradation (ERAD; Liu and Howell, 2010). Blocking proteasome activity is believed to reduce the degradation of misfolded proteins, which in turn causes more burdens to the ER. This implies that PBA1, as a proteasome subunit, may play a pro-survival role rather than a pro-cell death role in plant ER stress-induced PCD despite its caspase-3-like activity. So far, very few reports have depicted the role of the proteasome in PCD. Both pro-cell death and anti-cell death functions have been documented. Silencing *PBA1* blocked pathogen-induced PCD in *Arabidopsis* by blocking one of the first steps consisting of tonoplast and plasma membrane fusion (Hatsugai et al., 2009). On the other hand, silencing the 20S α6 subunit or the RPN9 subunit of 19S did elicit PCD in *N. benthamiana* (Kim et al., 2003). Similar contrasting observations have been made in animal cells. Proteasome inhibition using MG132 in human Jurkat cell increased the expression level of BIP, a marker of increased ER stress and resulted in more apoptosis (Park et al., 2011). Another report, based on vascular smooth muscle cells, also indicated that proteasome inhibition sensitized cells to ER stress-induced PCD (Amanso et al., 2011). This may be because applying both an ER stress inducer and a proteasome inhibitor may result not only in more misfolded protein aggregation, but also in a compromised UPR induction. In support of that, cells treated with the proteasome inhibitor MG132 exhibit a repressed UPR genes induction (Lee et al., 2003); how MG132 reduced UPR gene induction is not clear. On the other hand, a pro-apoptotic role of proteasome was also reported. Egger et al. (2007), reported that inhibition of the proteasome using lactacystin block ER stress-induced cell death in Rat 6 embryo fibroblasts (R6; Egger et al., 2007). The authors postulated that proteasome inhibition abolished the degradation of anti-apoptotic BCL-2 family members, so that apoptosis was blocked (Egger et al., 2007). It should be noted that a particular way of inducing ER stress was used in this research, as cells were co-incubated with brefeldin A (BFA) and cycloheximide (CHX). BFA blocks protein transport from ER to Golgi, causing ER stress. CHX represses bulk protein translation including pro-survival UPR proteins, such as proteins downstream of *ATF6* (Egger et al., 2007). With this combination of chemicals, protein input into ER was reduced and proteasome inhibition resulted only in a moderate unfolded protein burdens to ER, while BCL-2 degradation was blocked. Our lab is investigating whether the plant proteasome exhibits a pro- or an anti-cell death role in ER stress-induced PCD. Besides the proteasome, our unpublished results indicate that at least one other protease exhibit caspase-3-like activity in *Arabidopsis*.

Table 1 | Plant caspase-like activities and ER stress-induced PCD.

Protease	Preferred caspase substrate	Reference	Species	Comments
Vacuolar processing enzyme (VPE)	YVAD, caspase-1-like	Hatsugai et al. (2004), Qiang et al. (2012), Mendes et al. (2013)	*Arabidopsis*, *soybean*, *N. benthamiana*	Controls tonoplast rupture during PCD. Positively regulate ER stress-induced PCD.
Proteasome subunit β 1(PBA1)	DEVD, caspase-3-like	Hatsugai et al. (2009), Han et al. (2012)	*Arabidopsis*, *poplar*	Mediates PCD during HR. Activity detected during ER stress-induced PCD
Phytaspase	VEID, caspase-6-like	Chichkova et al. (2010)	*N. tabacum*, *rice*	Mediates HR and ROS-induced PCD. Not reported in ER stress-induced PCD.
Saspase	VEHD, caspase-6-like; IETD, caspase-8-like	Coffeen and Wolpert (2004)	*Oat*	Not reported in ER stress-induced PCD

OTHER CASPASE-LIKE ACTIVITIES

Besides the proteases responsible for caspase-1 and caspase-3-like activities involved in ER stress induced PCD, other proteases with caspase-like activities are known in plants. Phytaspase and saspase were found to exhibit caspase-like activities (Vartapetian et al., 2011). So far, however, no report has shown the involvement of these proteases in ER stress-induced PCD. Phytaspase, a subtilisin-like serine protease, takes its name from being a plant ASPartate-specific protease and was purified from tobacco and rice. It exhibits a preferential cleavage activity against caspase-6 substrate VEID although it can cleave other caspase substrates such as caspase 1 (YVAD) and 9 (LEHD) in particular (Chichkova et al., 2010). Phytaspase was shown to mediate HR-induced PCD and ROS-induced PCD (Chichkova et al., 2010). Saspase (Serine protease with an asp cleavage site) is a subtilisin-like serine protease, purified from *Avena sativa*, that exhibits an enzymatic activity against caspase-6 substrates (VEHD, VKMD, VNLD) and one caspase-8 substrate (IETD; Coffeen and Wolpert, 2004). A role for caspase-2 and caspase-4 have been described in mammalian ER stress, but caspase-2-like or caspase-4-like activity has not been reported in plants. As caspase-2 was reported to regulate ER stress-induced apoptosis in mammalian cells (Upton et al., 2012), caspase-2 activity could be tested in plant during ER stress-induced PCD although this activity in itself does not indicate a role in plant PCD. The caspase-like activities in plants and their correlations with ER stress-induced PCD are summarized in **Table 1**.

CONCLUSION

ER stress-induced PCD is gaining more and more attention in plant science research. So far, among plant proteases with caspase-like activity, only VPE, with a caspase-1-like activity, has been implicated in regulating ER stress-induced PCD. VPE is proposed to control indirectly tonoplast rupture during PCD. The detailed mechanism by which VPE controls tonoplast rupture is still unclear and could be addressed using ER-stress induced PCD as an experimental system. Another aspect that can be addressed is the role of caspase-3-like activity and of the proteasome. In particular, it is worth investigating whether in addition to being responsible for the degradation of misfolded proteins, the proteasome could also indirectly modulate UPR gene expression as suggested in some mammalian cell models (Lee et al., 2003; Amanso et al., 2011). Since three caspase-like activities have been directly or indirectly implicated in ER stress-induced PCD in plants, a complete profile of caspase-like activities during ER stress-induced PCD could be carried out using substrates and inhibitors. Following this, using relevant KO lines or KD lines would provide clearer experimental outcomes. Such an approach would give an insight into which additional proteases are involved in ER stress-induced PCD in plants. For example, whether phytaspase, a PCD regulator, has a function in ER stress-induced PCD is an interesting question that is still open. Finally, a complete understanding will not be achieved without knowledge of the *in vivo* protein substrates implicated.

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