Chapter 7
ER Stress, UPR and Virus Infections in Plants

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Abstract The endoplasmic reticulum (ER) endomembrane is a central site for protein synthesis. Perturbation of ER homeostasis can result in an accumulation of unfolded proteins within the ER lumen, causing ER stress and the unfolded protein response (UPR). In humans, ER stress and UPR are closely associated with a vast number of diseases, including viral diseases. In plants, two arms that govern the UPR signaling network have been described: one that contains two ER membrane–associated transcription factors (bZIP17 and bZIP28) and the other that encompasses a dual protein kinase (RNA-splicing factor IRE1) and its target RNA (bZIP60). Although early studies mainly focus on the essential roles of the UPR in abiotic stresses, the significance of UPR in plant diseases caused by virus infections has recently drawn much attention. This chapter summarizes the latest scenario of ER stress and UPR in virus-infected plant cells, highlights the emerging roles of the IRE1 pathway in virus infections, and outlines exciting future directions to spark more research interest in the UPR field in plants.

7.1 Introduction

Protein folding guided by entropic and energetic forces has been one of the most intensely studied topics in biology in the past half century (Hartl and Hayer-Hartl 2009). Although the mechanism(s) by which the final folding is determined by the amino acid sequence still largely remains elusive, some important aspects in this field have become to be understood in the recent years. For instance, it has been known that a large fraction of proteins during synthesis (especially secreted and membrane proteins) are loaded in an unfolded state into the endoplasmic reticulum (ER) lumen (Hartl and Hayer-Hartl 2009). The newly synthesized polypeptides
trans-located in the ER undergo folding aided by ER-associated chaperons and organelle-specific posttranscriptional modifications to reach their higher-order three dimensional states efficiently on a biologically relevant timescale (Ellgaard and Helenius 2003; Hartl and Hayer-Hartl 2009; He and Klionsky 2009; Marcinak and Ron 2010). For those proteins that fail to fold and modify properly, a surveillance mechanism composed of the ER quality control (ERQC) system and the ER-associated degradation (ERAD) system is assigned to eradicate their deleterious effects in order to maintain cell health (Howell 2013).

However, the load of client proteins may exceed the assigned processing and eliminating capacities of the ER, leading to ER stress, which is a pervasive characteristic of eukaryotic cells (Ron and Walter 2007; Gao et al. 2008; Liu and Howell 2010; Marcinak and Ron 2010; Hetz et al. 2011; Iwata and Koizumi 2012). ER stress can be primed by developmental or physiological fluctuations and genetic mutations that erode the ER protein homeostasis networks (Brewer and Hendershot 2004; Schröder and Kaufman 2005; Balch et al. 2008; Kim et al. 2008; Marcinak and Ron 2010; Hetz et al. 2011). In eukaryotic cells, a substantial body of evidence has also shown that multiple types of environmental stimuli (abiotic and biotic stress), including pathogenic invaders, chemicals, and depletion of energy or nutrients, can exert stress on the ER by disruption of cellular redox equilibrium and calcium ($Ca^{2+}$) homeostasis, interference of post-translational modifications and assemblies, and demand for an increased protein synthesis capacity (Dimcheff et al. 2004; Gao et al. 2008; Liu and Howell 2010; Ye et al. 2011; Iwata and Koizumi 2012; Zhang and Wang 2012; Zhang et al. 2015). In general, perturbation of ER homeostasis associated with accumulation of unfolded proteins in the lumen of the ER triggers an evolutionarily conserved signaling pathway referred as the unfolded protein response (UPR) (Ron and Walter 2007; Kim et al. 2008).

The primary goal of the UPR is to reestablish cellular homeostasis, to relieve stress imposed on the ER, and to prevent the cytotoxic impact of malformed proteins via inhibition of mRNA translation and activation of adaptive mechanisms (Xu 2005; Kim et al. 2008; Preston et al. 2009; Ye et al. 2011). As a result of this adaptation, particular groups of genes are expressed to enhance the protein folding capacity of the ER and to promote the misfolded protein degradation capacity mediated by ERAD (Kim et al. 2008; Verchot 2014). The signal-transduction events that are commonly associated with innate immunity and host defense, including mitogen-activated protein kinases (MAPKs), c-Jun N-terminal kinase (JNK), reactive oxygen species (ROS) networks, $Ca^{2+}$ signaling and autophagy pathways, are also induced to produce a synthetic cellular response to ER stress (Fig. 7.1) (Kaneko et al. 2003; Tardif et al. 2005; Xu 2005; Chen et al. 2008; Kim et al. 2008; Ke and Chen 2011). However, if attempts to restore cellular native settings fail, a final mechanism called programmed cell death (PCD), also called apoptosis in eukaryotes, is triggered. Under this scenario, cell death is presumably useful to protect the organism from the expansion of potentially harmful substances produced by the damaged cells (Fig. 7.1) (Liu et al. 2005; Ron and Walter 2007; Kim et al. 2008).
Fig. 7.1 The UPR branches in eukaryotes. The PERK arm is identified only in animals. On ER stress induced by abiotic or biotic cues (such as virus infection), the PERK kinase oligomerizes in the ER membrane and is activated via trans-autophosphorylation. The activated PERK phosphorylates initiation factor 2 alpha (eIF2α), which inhibits the activities of eIF2B and the eIF2 complex. This accounts for all of the important consequences of PERK activity, such as translation inhibition of most mRNAs, which reduces protein synthesis and lowers ER loading. However, some mRNAs such as ATF4 gains a selective advantage for translation via phosphorylated eIF2α. ATF4 in turn contributes to the transcriptional activation of CHOP, XBP1, GADD34, and other genes involved in ROS signaling and apoptosis. The IRE1 arm is conserved in eukaryotes. IRE1 unconventionally splices the bZIP transcription factors XBP1, bZIP60 and HAC1 mRNA in mammals, plants, and yeast, respectively. The spliced bZIP transcription factors enter into the nucleus to regulate UPR, ERAD and autophagy target genes. In mammals, IRE1 arm also activates kinases such as JNK to initiate autophagy and apoptosis. ATF6 in animals and bZIP17/bZIP28 in plants reside in the ER membrane in unstressed cells. Upon ER stress, they are relocated from the ER to the Golgi apparatus, where they are subject to cleavage twice, first by the luminal S1P and then the intra-membrane S2P, to release their cytosolic transcription factor domains. Subsequently, the transcription factor domains then enter into the nucleus and activate a subset of UPR target genes.
In mammalian cells, the UPR is mediated by two types of ER transmembrane proteins (ER stress sensors). The type I ER stress sensor is composed of IRE1 (inositol-requiring transmembrane kinase/endonuclease) including two IRE1 isoforms IRE1α and IRE1β, and PERK (PKR-like ER kinase), whereas type II includes ATF6α and ATF6β (activating transcription factor 6) (Fig. 7.1) (Cox and Walter 1996; Sidrauski and Walter 1997; Oikawa et al. 2010; Hetz et al. 2011). In contrast to that in animals, the UPR in yeast (Saccharomyces cerevisiae) is controlled by only one signaling pathway, the type I transmembrane ER protein IRE1p (Fig. 7.1) (Cox and Walter 1996; Sidrauski and Walter 1997; Oikawa et al. 2010). Over the last 10 years, significant advances have been made in understanding the ER stress and UPR signaling pathways in plants (Urade 2007; Vitale and Boston 2008; Deng et al. 2011; Nagashima et al. 2011). Thus far, two UPR pathways have been identified in plants, one mediated by IRE1-bZIP60 (basic leucine zipper), and the other by site-1/site-2 proteases (S1P/S2P)-bZIP17/bZIP28 which is analogous to the animal ATF6 pathway (Fig. 7.1) (Koizumi et al. 2001; Liu et al. 2007a, b.; Urade 2007; Vitale and Boston 2008; Liu and Howell 2010; Deng et al. 2011; Nagashima et al. 2011). Most recently, the beta subunit of the heterotrimeric G protein complex AGB1 has also been found to be essential for the plant UPR, adding further complexity to the UPR pathways (Chen and Brandizzi 2012, 2013).

In humans, it has been known that ER stress is implicated in numerous diseases, including cancers, neurodegeneration, diabetes, inflammation, and viral diseases (He 2006; Hetz et al. 2011). Therefore, there is a significant biomedical interest in illustrating the UPR molecular mechanisms and developing procedures to manipulate this pathway (He 2006; Hetz et al. 2011; Zhang and Wang 2012). In plants, much of the work in this field has concentrated on ER stress induced by environmental cues (Irsigler et al. 2007; Liu et al. 2007b; Costa et al. 2008; Gao et al. 2008; Liu and Howell 2010; Deng et al. 2011; Iwata and Koizumi 2012). In contrast to mammalian systems, in which the virus-induced UPR has been extensively studied (He 2006; Zhang and Wang 2012), it has only been recently that the essential role of the UPR in plants in response to viral attack has drawn attention (Ye et al. 2011; Ye and Verchot 2011; Zhang and Wang 2012; Ye et al. 2013; Zhang et al. 2015). This book chapter presents the latest progress in and viewpoints on the research of virus-induced ER stress and UPR in plants, with a focus on a recent discovery that IRE1 and bZIP60 operate as a conserved pair to regulate virus infections. Through presenting evidence on how the ER transmembrane proteins sense the unfolded settings, we delineate the mechanisms of UPR activation under virus infections in mammals and plants, and discuss the functional implication of the UPR in virus infections and host responses. Finally, the physiological relevance of virus-induced ER stress with other signaling pathways and cellular processes is introduced and discussed, aiming to provide an integrated view of ER stress in multicellular eukaryotes and to suggest possible future directions of research on plant UPR.
7.2 The UPR Sensing Mechanisms during Virus Infections

Currently, there is general acceptance in the scientific community that the UPR signaling is initiated by UPR stress sensors, the ER resident transmembrane proteins (Fig. 7.1). Great efforts have been made in this area during the past decade (Bertolotti et al. 2000; Shen et al. 2002; Kimata et al. 2003, 2004; Credle et al. 2005; Gardner and Walter 2011), and several models have been proposed to depict the intricate mechanisms of how the protein misfolding is detected by UPR stress sensors (Hetz et al. 2011; Zhang and Wang 2012).

7.2.1 The Models of UPR Activation

Initially, the indirect recognition model proposes the binding immunoglobulin protein (BiP) as a repressor of UPR, which is dissociated from PERK, IRE1α or the yeast homolog IRE1p to bind unfolded proteins upon ER stress, leading to the activation of the ER transducers (Bertolotti et al. 2000; Kimata et al. 2003). This model is also suggested to operate in the control of type-II transmembrane sensor activation (Shen et al. 2002). However, two major observations challenge this model. First, genetic evidence shows that deletion of the BiP-binding site does not cause constitutive activation of IRE1p (Kimata et al. 2003, 2004). Second, the finding of central groove formed by α-helices in the crystal structure of IRE1p suggests that IRE1p itself has the intrinsic ability to sense ER stress (Credle et al. 2005). Thus, the unfolded protein is proposed to play a pivotal role in stabilizing the activated ER stress sensors after the releasing of BiP in order to trigger robust UPR, which is the so-called two-step activation model (also called semi-direct recognition model) (Kimata et al. 2003, 2004; Credle et al. 2005). Subsequently, time resolved analysis of IRE1p signaling and elegant biochemical assays reveal a new quantitative model (also called direct recognition model), in which IRE1p is in a dynamic equilibrium with BiP and unfolded proteins. This model mainly stresses that the unfolded protein binding to IRE1p is sufficient and the only prerequisite for activation of the UPR, ruling out BiP as the principal determinant that governs the state of the UPR, and regarding BiP as a buffer and a timer to fine-tune the sensitivity and dynamics of the UPR (Pincus et al. 2010; Gardner and Walter 2011). Nevertheless, the direct recognition model does not apply to human IRE1α, due to the facts that the groove in IRE1α is too narrow for peptide binding, and that the recombinant IRE1α does not interact with unfolded proteins in vitro (Zhou et al. 2006; Oikawa et al. 2009). Therefore, it is self-evident that the complexity of unfolded protein sensing is far beyond our initial expectation, and the task of identifying the fine molecular mechanisms in this field is far from complete. Interested readers are suggested to refer to the reviews for the details of the current recognition models (Hetz et al. 2011; Zhang and Wang 2012). In this section, we will mainly discuss how the UPR is activated upon virus infections.
7.2.2 The Strategies to Manipulate UPR by Human Viruses

During the course of millions of years of co-inhabitation with their hosts, viruses have evolved many sophisticated mechanisms of inducing and/or manipulating the UPR to assist in their own infections, which can be summarized into several categories as follows (Fig. 7.2). Viruses employ unfolded proteins encoded by their own genome to bind non-covalently the ER-resident protein BiP, thus leading to the activation of UPR (Fig. 7.2). These viral proteins include glycoprotein G of vesicular stomatitis virus, hemagglutinin-neuraminidase (HN) glycoprotein of paramyxovirus SV5, hemagglutinin of influenza virus, and E1 and E2 proteins of hepatitis C virus (HCV) (Kozutsumi et al. 1988; Hurtley et al. 1989; Ng et al. 1989; Machamer et al. 1990; Choukhi et al. 1998; Liberman et al. 1999). The second strategy is that viruses may exploit their own proteins(s) to directly and specifically modulate the ER stress sensors (Fig. 7.2). For instance, among seven proteins encoded by simian virus 5, only the HN glycoprotein that is inserted into the ER is capable of stimulating the UPR (Watowich et al. 1991). This also holds true for the ER-resident proteins encoded by flaviviruses and retroviruses (Tardif et al. 2004; Tardif et al. 2005). Recent studies with severe acute respiratory syndrome (SARS) coronavirus (SARS-CoV) have also revealed that one of the accessory proteins of SARS-CoV, the ER-resident protein 8ab protein, can bind directly to the luminal domain of ATF6, the type II ER stress sensor, to activate the UPR (Sung et al. 2009), whereas the ER-resident protein 3a selectively activates the PERK pathway (Minakshi et al. 2009). In addition, viruses may borrow the interaction of host factors and viral protein(s) in the ER site to induce the UPR (Fig. 7.2). This notion is supported by the finding that human cytomegalovirus protein US11 provokes the UPR in a manner depending on the interaction of US11 with Derlin-1 within the lipid bilayer of the ER (Tirosh et al. 2005). Lastly, but far from over, several other studies have also suggested a connection between the UPR and viral replication (Fig. 7.2). These include herpes simplex virus (HSV) 1, Japanese encephalitis virus (JEV), and HCV (Su et al. 2002; Cheng et al. 2005; Tardif et al. 2005).

7.2.3 The Possible Mechanisms of UPR Activation during Plant Virus Infections

In plants, several groups have independently shown through microarray analysis that the chaperon BiP is upregulated in *Arabidopsis* infected by *Turnip mosaic virus* (TuMV) and *Oilseed rape mosaic virus* (ORMV) (Whitham et al. 2003; Yang et al. 2007; García-Marcos et al. 2009). Consistently, other ER-resident chaperones have also been found to be induced in *Arabidopsis*, potato (*Solanum tuberosum*) and *N. benthamiana* (*Nicotiana benthamiana*) infected by *Potato virus X* (PVX) (Whitham et al. 2003; Yang et al. 2007; García-Marcos et al. 2009; Ye et al. 2011;
However, even though it is clear that the UPR downstream signaling is indeed activated during viral infections, we are only just beginning to understand the molecular mechanisms involved in the activation of virus-induced UPR in plants.

Studies with PVX identified the viral movement protein (MP) TGBp3, which resides in the ER, as an elicitor of the expression of ER-resident chaperones in Arabidopsis and N. benthamiana (Bamunusinghe et al. 2009; Ye et al. 2011, 2013). It seems that, similar to the ER-resident proteins encoded by human viruses, TGBp3 modulates the level of the ER chaperones as a means to cope with robust viral
protein synthesis (Tardif et al. 2004; Chan and Egan 2005; Sung et al. 2009). Nevertheless, the mechanism(s) by which TGBp3 activates the UPR pathway(s) in PVX infections still remain unknown. Recently, we have shown that, of the 11 viral proteins of TuMV, only 6K2 has the ability to induce the splicing of NtbZIP60 mRNA in N. benthamiana (Zhang et al. 2015). The potyviral 6K2 protein is an integral ER membrane protein and elicits the formation of ER-derived virus replication factories at ER exit sites (Laliberté and Sanfaçon 2010; Wei et al. 2010). Therefore, the finding of 6K2 as an inducer of the UPR pathway in plants is consistent with the well-documented conception in mammalian cells that virus-encoded ER targeting proteins induce the UPR (Fig. 7.2) (see discussion above). Based on all these observations (Bamunusinghe et al. 2009; Ye et al. 2011, 2013; Zhang et al. 2015), it is reasonable to conclude that the virus-encoded ER-targeting proteins may also be the potential inducers of the UPR in plants.

Although the mechanism of 6K2 triggering the UPR has yet not been fully understood, genetic and molecular analyses have revealed that the ER-resident chaperones are not only up-regulated, but the bZIP60 also undergoes unconventional splicing mediated by IRE1 in Arabidopsis and N. benthamiana under TuMV infections (Zhang et al. 2015). It was further been proven that the virus infections are suppressed in the ire1a-3 ire1b-4 double mutant, which can be rescued by complementing the mutant with not only IRE1A or IRE1B but also the spliced bZIP60 (bZIP60 S) (Zhang et al. 2015). Moreover, the virus infection suppression phenotype resulting from dysfunction of the IRE1-bZIP60 branch is independent of the S1P/S2P-bZIP17/bZIP28 arm (Zhang et al. 2015). These data directly show that the IRE1-bZIP60 branch, rather than the other arm, is responsible for the virus-induced UPR, and IRE1 and bZIP60 as a matched enzyme-substrate pair regulate virus infections, providing the first evidence that the IRE1-bZIP60 arm is preferably manipulated by plant viruses. Considering that no interactions between 6K2 and IRE1 in plants have been experimentally demonstrated (Zhang et al. 2015), it is unclear how the virus-encoded 6K2 manipulates the UPR pathway to benefit viral infection. It is possible that 6K2 induces the UPR through its physical interaction with the ER or subsequent ER remodelling (Fig. 7.2) (Laliberté and Sanfaçon 2010).

7.3 The Roles of UPR in Plant Virus Infections

Virus infections trigger an arm race between virus and the host. On one hand, the host mobilizes the UPR machinery in an attempt to hamper virus infections. On the other hand, viruses exploit or even manipulate the UPR branches for their own benefits (Chan 2014). In mammalian cells, the intimate and complicated relationship between virus and three UPR pathways has been well reviewed (He 2006; Zhang and Wang 2012; Jheng et al. 2014; Verchot 2014). Of them, the IRE1/bZIP60 branch is the most intensively studied (Jordan et al. 2002; Baltzis et al. 2004; Netherton et al. 2004; Sun et al. 2004; Tardif et al. 2005). Here, we will discuss the crosstalk between the UPR pathway mediated by IRE1 and virus
infections in plants as this is the only pathway found to be implicated in viral infections in plants.

Several lines of evidence suggest that the IRE1-mediated UPR branch promotes virus infections in plants. In the case of PVX, silencing \textit{NtbZIP60} in \textit{N. benthamiana} can suppress the expression of the UPR marker genes and reduce PVX accumulation (Ye et al. 2011, 2013). In virus-infected plants, membrane-associated virus replication or accumulation of large amounts of viral proteins can disrupt the fine equilibrium within cells (Ye et al. 2011, 2013; Smith 2014); therefore, the activation of the UPR mediated by \textit{bZIP60} may serve as a compensatory mechanism required for the host to alleviate cytotoxicity and to restore cellular normal state and functions. This notion is supported by several earlier reports that the infections caused by a set of viruses, including \textit{Cucumber mosaic virus}, \textit{Oil seed rape mosaic virus}, \textit{Turnip vein-clearing virus}, \textit{Potato virus Y} and TuMV, can up-regulate the expression of \textit{bZIP60} and ER marker genes in plants, suggesting a general role of this UPR pathway and ER chaperones in virus infections (Whitham et al. 2003; Yang et al. 2007; García-Marcos et al. 2009).

From the angle of the virus, the increased expression of ER-resident chaperones may facilitate virus infection through assisting in the assembly of replication complexes and the synthesis and folding of viral proteins as well as the assembly of viral particles (Hafrén et al. 2010; Howell 2013). In single-celled yeast, the host Ssa1/2p molecular chaperone (yeast homologue of HSP70) is required for the assembly of the tombusvirus replicase and to enhance viral RNA replication (Serva and Nagy 2006; Pogany et al. 2008). In plants, up-regulation of \textit{HSP70} by potyvirus infection depends on the cytoplasmic UPR pathway (Aparicio et al. 2005; Sugio et al. 2009). Moreover, HSP70 is a component of the membrane-associated viral ribonucleoprotein complex, playing a critical part in viral genome expression and replication (Hafrén et al. 2010; Jungkunz et al. 2011). In agreement with these findings, we have recently shown that treatment of \textit{N. benthamiana} with pharmacological small molecular chaperones can promote TuMV infection (Zhang et al. 2015).

The IRE1-\textit{bZIP60} pathway is also crucial for TuMV infection. First, in response to TuMV infection, the IRE1-\textit{bZIP60} arm of the UPR is activated in both locally and systemically infected leaves (Zhang et al. 2015). Second, the mutant \textit{bzip60-2} without detectable \textit{bZIP60} S significantly inhibits viral accumulation and remarkably suppresses the development of disease symptoms, which can be rescued by genetic transformation of \textit{bZIP60} S into the mutant (Zhang et al. 2015). Third, two different double mutants of \textit{IRE1A} and \textit{IRE1B}, in which the \textit{bZIP60} splicing is blocked, also display the reduced levels of viral RNA accumulation and suppress viral symptom development. The absence of \textit{bZIP60} S and suppression of virus infection in the double mutants can be rescued by complementation with either \textit{IRE1} or \textit{bZIP60} S (Zhang et al. 2015). Collectively, these data demonstrate that IRE1 and its processed \textit{bZIP60} S function as a projected cognate system to promote viral infection in plants. This result is in accordance with several recent studies in mammalian cells that the UPR can be hijacked by viruses, such as influenza A virus, to favor viral infection, and inhibition of IRE1 activity compromises viral replication (Hassan et al. 2012).
The UPR has emerged to be more than an independent cellular response to virus infections. It is intimately linked to a variety of signaling networks and cellular responses either by modulating signaling pathways or as part of the cellular responses (Fig. 7.1) (Chan 2014). Here, we outline the physiological relevance of virus-induced ER stress with the ROS signaling network, autophagy and the ER quality control system mediated by ERAD.

7.4.1 Virus-Induced ER Stress and ROS Signaling Network

It has been known that ROS plays a pivotal role in various clinical diseases, including those associated with atherosclerosis and viral infection (Tardif et al. 2005). Many studies in human cell lines have proved that there exists a crosstalk between ER stress and the ROS pathway during virus infection (Tardif et al. 2005). For instance, infection by HCV in liver cells leads to the leakage of Ca^{2+} from ER stores, which is then taken up by mitochondria, inducing a sustainable accumulation of ROS (Fig. 7.3) (Ivanov et al. 2013; Paracha et al. 2013). ROS in turn activates cellular tyrosine and serine/threonine kinases to cause the translocation of NF-κB and STAT-3 transcription factors into the nucleus in favor of its genome translation and replication (Fig. 7.3) (Gong et al. 2001; Tardif et al. 2005; Ivanov et al. 2013; Paracha et al. 2013).

In plants, ROS function as signaling molecules to control a variety of physiological and pathological processes (Apel and Hirt 2004; Zhang and Xing 2008; Zhang et al. 2009). Early studies with Tobacco mosaic virus (TMV) showed that the activity of the gp91phox NADPH oxidase homologs is enhanced in virus-infected leaves, and ROS is induced within seconds after challenging the tobacco epidermal cells with virus (Allan et al. 2001; Sagi and Fluhr 2001). These studies also demonstrate that the rapid induction of ROS bursting can be prevented by specific inhibitors of NADPH oxidase, pointing to the plasma membrane NADPH oxidase, also known as the respiratory burst oxidase (RBO), as a biochemical source of ROS production in virus-infected plants (Allan et al. 2001; Sagi and Fluhr 2001). This notion is further confirmed by the observation that the rapid systemic generation of ROS in response to Cauliflower mosaic virus (CaMV) infection is abolished by the double mutation of AtrobhD and AtrobhF (two Arabidopsis genes homologous to gp91phox, Arabidopsis RBO homolog) in Arabidopsis (Love et al. 2005).

Although virus-induced ROS production is linked to NADPH oxidase, the mechanisms underlying the activation and regulation of plant NADPH oxidase homologs in response to virus infections have yet to be understood. Studies with Pseudomonas syringae pv. tomato have indicated that calcium is one of the fastest responses upon pathogen infection, and is required for ROS production in plant
Fig. 7.3 Virus-induced ER stress and ROS signaling. In mammals, upon sensing virus-induced increase in Ca\(^{2+}\), mitochondria initiate ROS signaling. ROS in turn activates tyrosine/serine/threonine kinases-NF-κB pathways, to assist in viral genome translation and replication. In plants, ER stress also play a role in maintaining persistent virus infection and promoting virus spread through manipulating ROS and Ca\(^{2+}\) signaling networks. Here, we propose a ROS signaling cascade in virus infection in plants. Virus-induced ER stress generates a calcium signature during the early stage, which primes the downstream effectors such as NADPH oxidases to initial ROS production. The increased ROS further activate plasma membrane Ca\(^{2+}\) channels for eliciting a Ca\(^{2+}\) influx. The ROS and Ca\(^{2+}\) signaling networks contribute in a synergetic relation to virus infection through alerting host defense systems.
defense response (Blume et al. 2000; Grant et al. 2000). Interestingly, in the case of *Pseudomonas syringae pv. glycinea*, ROS is also required to prime Ca\(^{2+}\) influx to activate a physiological cell death program (Levine et al. 1996), indicating that calcium functions not just upstream but also downstream of ROS production in response to bacterial pathogens (Nürnberger and Scheel 2001; Apel and Hirt 2004). Therefore, all these data indicate a complex and common spatiotemporal connection of ROS and Ca\(^{2+}\) signaling networks in plant biotic stress responses (Fig. 7.3). Along with the fact that all plant Rboh proteins contain two EF-hand motifs in their N-terminal region that are regulated by Ca\(^{2+}\) (Torres et al. 2006), it is logical to propose a possible signaling cassette for virus infection as follows. Virus-induced ER stress may produce a calcium signature during the early stage, which is sensed by the downstream effectors such as NADPH oxidases to initial ROS production. The elevated ROS may further activate plasma membrane Ca\(^{2+}\) channels for eliciting a Ca\(^{2+}\) influx to virus’ own advantages or to alert host defense system(s) (Fig. 7.3).

It should be noted that the links of ER stress to ROS signaling during plant virus infection may not be limited to just turning on the ROS signaling cascade. During the long-term evolution, ER stress and the ROS signaling network might have developed a sophisticated and coordinated relationship to cope with environmental cues. Recently, it has been shown that the expression of PVX movement protein TGBp3 in *N. benthamiana* leads to ROS accumulation and cell death, which can both be prevented by the co-expression of ER molecular chaperon BiP (Ye et al. 2011, 2013; Ye and Verchot 2011). These data indicate that ER stress may play a contributing role towards maintaining persistent virus infection and/or promoting virus spread through manipulating the ROS signaling pathway. In addition, in *Plum pox virus* (PPV)-infected cells, accompanying with ROS production, the levels of apoplastic antioxidant enzymes are also increased in susceptible peach cultivar to maintain sustainable oxidative stress conditions, creating a co-existence environment for the host and the virus (Díaz-Vivancos et al. 2006; Hernández et al. 2006). In this sense, the role of ER stress-induced manipulation of ROS pathways is associated with the host antioxidant systems and is required for the establishment of persistent virus infection. Further studies are needed to elucidate the molecular link and functional relevance of the UPR branches and the ROS signaling network upon virus infections.

### 7.4.2 Virus-Induced ER Stress and Autophagy

Autophagy is a double-membrane vesicular process that results in the degradation of the sequestered components (Blázquez et al. 2014; Jheng et al. 2014). In mammalian cells, autophagy executes its cell-context specific functions in a four-step program: (1) autophagy induction mediated by activation of the Unc51-like kinase (ULK1) complex (Inoki et al. 2003; Mizushima 2010; Egan et al. 2011; Kim et al. 2011; Markus et al. 2011; Randhawa et al. 2015); (2) vesicle nucleation regulated by the Beclin1-PI3KC3 complex through the recruitment of PI3P
effectors and lipids required for auto-phagosome construction (Proikas-Cezanne et al. 2004; Axe et al. 2008; Hayashi-Nishino et al. 2009; Matsunaga et al. 2009); (3) vesicle expansion followed by the conjugation of microtubule-associated protein light chain 3 (LC3) with phosphatidylethanolamine (PE) and vesicle completion, represented by the enclosure of cytosolic cargos into double membrane vesicles, leading to the formation of auto-phagosomes (Geng and Klionsky 2008; He and Klionsky 2009; Shpilka et al. 2011); (4) auto-phagosome maturation into autolysosome by sequential fusion with endosomes and lysosomes, which is related to the expression of lysosomal-associated membrane protein 2 (Lamp-2), leading to the degradation of the loaded contents by internal hydrolases (Liang et al. 2008).

In mammalian cells, although the activation of the UPR and the induction of autophagy have been described during infections by a wide variety of viruses, the relationships between these two cellular processes remain controversial. In the case of HCV infection, the down-regulation of a variety of UPR modulators by siRNA has been found to result in a suppression of HCV-induced LC3-PE conjugation and a decrease of HCV RNA replication (Chen et al. 2008; Ke and Chen 2011). It has also been reported that HCV-induced eIF2α phosphorylation through PERK activates autophagy (Dreux and Chisari 2011), and CHOP activated by the PERK-ATF4 and ATF6 pathways plays a leading role in promoting ATG12 and LC3 protein expression (Ke and Chen 2011; Wang et al. 2014). Moreover, knockdown of IRE1 is found to inhibit the formation of auto-phagosomes as well as the conversion of LC3-I to LC3-II (Joubert et al. 2012). All these data suggest an epistasis role of the UPR in autophagy activation. However, the independence of autophagy induction on the UPR has also been observed. The expression of a sub-genomic replicon of a pegivirus results in an increased LC3-II level, but does not induce the UPR (Howell 2013). Furthermore, the cause-effect relationship between UPR and autophagy is also far from fully understood (Mohl et al. 2012). For instance, WNV triggers the UPR but does not always up-regulate the autophagy pathway (Vandergaast and Fredericksen 2012). All these mixed observations show that further studies are still needed to unravel the connection between the UPR and autophagy pathway during virus infection.

It is well known that the basic process and the essential components of autophagy are highly conserved among eukaryotes from yeast to animals and plants (Liu and Bassham 2012). Unlike animal autophagy, however, the cargo of autophagy is destined to the vacuole in plants (or, in yeast, the analogous vacuole) for degradation (Chen and Klionsky 2011; Liu and Bassham 2012). Like the implication of autophagy in health and disease processes such as cancer, neurodegeneration, aging, and longevity in animals (Yang and Klionsky 2010), autophagy in plants are associated with a variety of stresses, pathogen infections, and senescence (Bassham 2007; Hayward and Dinesh-Kumar 2011). Consistent with the findings from studies using mammalian cells (Chen et al. 2008; Ke and Chen 2011), autophagy genes play a role in host defense against virus infection in plants. For example, when tobacco plants are infected with TMV, autophagy is induced in both the infected and the uninfected area (Liu et al. 2005). Similar to mammalian BECLIN1, the plant orthologs of three autophagy genes, BECLIN1, ATG3 and ATG7, restrict viral replication (Liu et al. 2005). In contrast to
mammalian \textit{BECLIN1}, which prevents cell death in virus-infected tissues \citep{Orvedahl2010}, plant autophagy genes play a role in preventing cell death in uninfected tissues \citep{Liu2005}. Similar results have also been observed in \textit{Arabidopsis} \textit{ATG6} knockdown plants and \textit{ATG5} knockout mutants \citep{Yoshimoto2009} challenged with an avirulent bacterial pathogen, \textit{Pseudomonas syringae pv. tomato (Pst)} DC3000 \textit{(avrRPM1)} \citep{Patel2008}. These results suggest that autophagy functions to prevent runaway cell death in plants.

In plants, autophagy is known to function during ER stress. When ER stress is induced by tunicamycin (TM) or dithiothreitol (DTT), auto-phagosomes accumulate in \textit{Arabidopsis} root cells; therefore autophagy is activated by ER stress in plants \citep{Liu2012}. Moreover, the activation of autophagy by TM and DTT is mediated by the ER transducer IRE1B, rather than IRE1A \citep{Liu2012}. Although IRE1B is identified as an upstream regulator of autophagy during ER stress in plants, the detailed regulatory mechanism is still unclear \citep{Liu2013}. In yeast, ER stress-triggered autophagy relies on the endoribonuclease splicing activity of IRE1 toward its mRNA substrate. In animal cells, ER stress-triggered autophagy is mediated by the kinase activity of IRE1 through the JNK pathway (Fig. 7.1), rather than its splicing activity \citep{Liu2013}. In \textit{Arabidopsis}, ER stress-induced autophagy also does not depend on the splicing activity of IRE1B toward \textit{bZIP60} \citep{Liu2012}. Considering that the JNK pathway does not appear to exist in plants, it is possible that either IRE1B has other splicing targets besides \textit{bZIP60}, or IRE1B has unidentified functions in addition to its splicing activity related to induction of autophagy \citep{Liu2013}.

### 7.4.3 Virus-Induced ER Stress and ERAD

During virus infection, viral activities, such as viral genome replication and protein translation, pose an enormous biosynthetic burden on the ER, leading to ER stress \citep{Noueiry2003}. In this regard, the re-establishment of ER homeostasis by activating UPR pathways to slow down protein synthesis and to upregulate the capacity of the ER to fold client proteins is an adaptive cellular strategy that is beneficial to invaders and hosts \citep{Verchot2014}. To prevent congestion of the ER folding machinery with terminally misfolded proteins, eukaryotic cells have evolved an ERQC surveillance mechanism and an ERAD degradation system for removal of proteins unable to re-fold properly or unable to fold within a reasonable time (Fig. 7.1) \citep{Meusser2005, Byun2014, Verchot2014}. It has been shown that ERAD is vital to the maintenance of healthy cells, and its failure to destroy misfolded proteins is associated with a growing number of illnesses, such as Parkinson’s, Alzheimer’s and Huntington’s diseases \citep{Howell2013, Byun2014}.

Basically, the ERAD system recognizes misfolded proteins to be eliminated, and then extracts them through membrane channels in an energy-dependent manner for poly-ubiquitination, and subsequently degradation in proteasomes \citep{Howell2013, Byun2014}. The identification of terminally misfolded proteins from the
nascent glycoproteins in the process of being folded is a key step for the ERQC system. This step involves the bipartite recognition of the terminal α-1, 6-linked mannose on the C chain and the misfolded protein moiety, which are the two major features for terminally misfolded proteins (Denic 2011). In yeast, this recognition is performed by the Hrd1 complex, which is composed of E3 ubiquitin ligases Hrd1 and Hrd3 as well as Yos9 lectin. Yos9 recognizes in collaboration with Hrd3 the terminal α-1,6-mannose linkage on the C chain of the N-glycan of a glycoprotein, which is exposed by the action of the mannosidase Htm1 (Gauss et al. 2011). The ERAD substrates recognized by Yos9 and Hrd3 are recruited as a client protein for cytosolic ubiquitination by the E3 ligase Hrd1. Therefore, the Hrd1 complex spares nascent glycoproteins from early degradation, and misfolded proteins bearing modified glycans are consigned to ERAD (Gauss et al. 2011). The misfolded protein recognized and labelled by the Hrd1 complex is extracted from the ER lumen by CELL-DIVISIONCYCLE protein 48 (CDC48, an AAA-ATPase motor) and then targeted to the 26S proteasome for degradation.

In mammalian cells, some viruses, including hepatitis B (HBV) or HCV, exploit the ERAD to reduce the amounts of glycoproteins and particles produced to avoid the innate and adaptive immune systems, leading to chronic infections (Byun et al. 2014). Interestingly, both viruses can induce the UPR, which in turn increases the levels of certain ERAD components (Byun et al. 2014). Therefore, virus-induced UPR and ERAD benefit virus infections. The 69K MP of the plant virus *Turnip yellow mosaic virus* (TYMV) is poly-ubiquitinated for subsequent rapid and selective proteolysis by the proteasome in the *in vitro* reticulocyte lysate translation system (Druegon and Jupin 2002). In plants, *Potato leaf roll virus* (PLRV) MP and the PVX protein TGBp3 are the targets of the proteasome (Vogel et al. 2007; Ju et al. 2008). Trans-locating TGBp3 from the ER to the cytoplasm for degradation depends on the ERAD pathway (Ju et al. 2008).

As a conserved chaperone controlling protein fate in yeast and animal cells by extracting protein substrates from membranes or complexes (Meyer et al. 2012), CDC48 has been recently identified as a cellular factor regulating viral MP accumulation patterns in plant cells. *Arabidopsis CDC48* is upregulated upon TMV infection, and the encoded protein interacts with viral MP in ER-associated inclusions, the viral factories (Niehl et al. 2012). Later in the infection cycle, more misfolded MPs further accumulate in the ER-inclusions, and then are extracted by the CDC48 complex to the cytosol for poly-ubiquitination and subsequent degradation in an ERAD-dependent pathway (Reichel and Beachy 2000; Niehl et al. 2012). As a result, the 26S proteasome becomes saturated, and increased amounts of MP stabilize microtubules to hinder the transport of viral complexes along the ER into the neighboring cells (Niehl et al. 2012). In this regard, CDC48 function may represent a host defense mechanism by which the viral protein is removed to ensure membrane maintenance and to control viral movement (Niehl et al. 2012). However, this system may be exploited by the virus to increase replication efficiency since the extraction of MP from ER-inclusions by CDC48 may render the RNA translatable and consequently assist further replication of the virus. It will be interesting to test the importance of CDC48 in regulation of the cell-to-cell transport and replication of the virus during viral infection (Niehl et al. 2013).
7.5 Conclusions

The UPR was originally thought to maintain and re-establish cellular homeostasis upon ER stress. Now it is clear that the signaling of the ER stress-induced UPR pathways has broad associations with other signaling networks and cellular events, bringing important consequences for diverse physiological and pathological processes. Moreover, the elements of the UPR systems can be exploited or/and manipulated by pathogenic viruses in favor of genome translation and replication, viral particle assembly and persistent infections.

Despite the recent advances made in plants towards understanding the UPR implicated in abiotic and biotic stress, including viral infection, we are still far away from fully dissecting the UPR. The molecular and structural basis for recognition of the unfolding settings by the ER stress sensors are still missing in plants. The understanding of virus-induced UPR will be very rudimentary without a thorough elucidation of the structural basis of ER stress sensors. As discussed above, in mammalian cells, a variety of approaches can be used by viruses to activate and/or to block specific UPR pathway(s). However, in plants, viral proteins are the only known inducer of the UPR, and just one pathway is identified in virus infections thus far. Therefore, extensive studies are needed in the future to disclose the relationship between virus infections and the host UPR.

In addition, although ER stress and UPR are closely associated with other signaling networks and cellular processes, how those processes are coordinated to function is still unclear. A comprehensive study on these questions will certainly shed new light in the UPR pathways, lead to a better understanding of host-virus interactions, unlock novel antiviral mechanisms and targets and, in the long run, assist in developing novel effective antiviral strategies.

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