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Review article

When is the unfolded protein response not the unfolded protein response?

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ABSTRACT

As sessile organisms, plants are subjected to variety of stresses for which they have evolved different protection mechanisms. One mechanism involves endoplasmic reticulum (ER) stress in which the process of protein folding is disturbed and misfolded proteins accumulate in the ER. ER stress elicits the unfolded protein response (UPR) whereby the stress conditions in the ER are communicated to the nucleus to regulate stress response genes. Since the UPR is one of a number of different mechanisms by which plants respond to stress, it is often difficult to distinguish the UPR from other stress responses. Many investigators have relied on the molecular signature of the UPR, the upregulation of UPR genes to implicate the UPR in response to various stresses. However, some of these genes are activated by other stresses making it problematic to know whether the UPR is truly activated in response to a given stress or is part of a complex response. Another challenge is to understand how plants actually perceive different stress conditions. Are all stress conditions that elicit the UPR response caused by an accumulation of misfolded proteins in the ER? Is this the case for salt stress, which induces the UPR? How about biotic stresses, such as bacterial or viral infections? Do they lead to the accumulation of misfolded proteins in the ER or are there other means by which they induce the UPR?

1. Introduction

*If it looks like a **duck**, swims like a **duck**, and quacks like a **duck**, then it probably is a **duck**. Anon*

Plants are sessile organisms and are subjected to many kinds of stresses – heat, drought, salt, herbivory, to name a few. To survive, they have evolved a variety of different mechanisms to deal with stress. Some of these are unique to plants; others are found in a variety of other organisms. Such is the case with endoplasmic reticulum stress (ER stress) and the unfolded protein response (UPR), which is found in eukaryotes, from yeast [1] to mammalian cells [2,3].

One of the major players in the UPR is Glucose Regulated Protein 78 (GRP78), an ER chaperone that was discovered by its upregulation following depletion of glucose from cultures of rapidly growing mammalian tumor cells [4]. It was subsequently found to bind to incompletely assembled immunoglobulin heavy chains in pre-β lymphocyte cells [5] and later observed to bind to other incompletely folded proteins preventing their export from the ER. Gething and Sambrook [6] proposed that agents, which induce GRP78, did so by altering the ER environment so as to interfere with protein folding. They went on to show that the overexpression of a chronically misfolded protein, a variant of the influenza hemagglutinin protein, induced the production of GRP78, which led them to coin the term the "unfolded protein response."

In plants, the UPR was first described in maize bearing a floury-2 mutation [7–9]. floury-2 is a mutation in an α-zein which results in the storage protein being abnormally folded or improperly assembled. The accumulation of the mutant protein led to the upregulation of Binding Protein (BIP) during endosperm development in maize. The induction of BIP expression and other ER stress related genes have since been used as indicators of the UPR and ER stress responses [10–13].

ER stress is defined as the accumulation of misfolded proteins in the ER. Protein folding is an important activity in the ER, because proteins are introduced in the ER largely as linear polypeptides. An incredible amount of experimental and computational work has been done over the years to describe protein folding *in vitro*. However, protein folding *in vivo* differs from that *in vitro* for several reasons as outlined by Hingorani and Gierasch [14]. 1) Secreted proteins folded *in vivo* may be largely folded cotranslationally, while proteins folded *de novo* are not. Cotranslational folding and not *de novo* folding may involve sequential folding of different protein domains as they emerge from the ribosome or the translocon. 2) Proteins folding *in vitro* involves a dynamic equilibrium between unfolded and folded states. It is not known whether proteins spend any time in an unfolded state *in vivo*. 3) *In vitro* protein-folding experiments are conducted in dilute protein solutions, while the concentrations of macromolecules *in vivo* are much higher. 4) *In vitro*, proteins fold on their own, while *in vivo* many proteins fold with the aid of chaperones. 5) Because proteins fold *in vivo* in a molecularly crowded environment, they are vulnerable to compet-

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ing intermolecular aggregation reactions.

2. The first responders

ER stress is defined as the accumulation of misfolded proteins in the ER, however, it is unusual for anyone to attempt measure the load of misfolded proteins in the ER in response to stress. One method that could be used for this purpose is to coimmunoprecipitate misfolded proteins with BiP [15–18]. BiP coimmunoprecipitates with many newly synthesized proteins in the process of folding, but this approach has not been used to measure the load of misfolded proteins in response to stress. The method has been used by Vitale et al. [18] to demonstrate that BiP coimmunopreciptates monomeric unassembled phaseolin, a misfolded form, but not the assembled trimeric protein.

However, other indirect methods have been developed to assess the relative changes in the load of unfolded proteins in the ER. The most innovative of these was reported by Eric Snapp [19,20] who developed a Fluorescence Recovery After Photobleaching (FRAP) method. The method assesses the binding of fluorescence tagged forms of Binding Protein (BiP) to misfolded proteins. Binding or molecular crowding alters the diffusion coefficient for BiP, which can be detected by FRAP analysis. Thus, the molecular movement of BIP can serve as a surrogate for changes in the burden of unfolded proteins in response to stress.

FRAP analysis is an *in vivo* method for assessing ER stress. The next best way to measure ER stress is to assess the activation levels of the ER stress transducers in cell extracts. The membrane-anchored transcription factors and the RNA splicing factor, inositol requiring enzyme 1 (IRE1) are the "first responders" to ER stress and are reliable indicators of UPR activity. bZIP28 is an example of a membrane-anchored ER stress transducer that is activated by the accumulation of misfolded proteins in the ER. Under unstressed conditions, bZIP28 is retained in the ER by its interaction with BiP (Fig. 1). In response to ER stress, BiP dissociates and bZIP28 is transported to the Golgi. This is similar in principle to the activation of cytosolic Heat Shock Factor1 (HSF1) in response to heat stress. Heat Shock Protein 90 (Hsp90) binds to HSF1, retaining it in the cytoplasm under nonstressed conditions [21,22]. In response to heat stress, the pool of free HSP90 is depleted by the binding of HSP90 to misfolded client proteins. As a result, HSP90 dissociates from HSF1, liberating it to enter the nucleus where it upregulates a constellation of heat shock protein genes.

Thus, in plants, the dissociation of BiP from lumen-facing domain of bZIP28 could be used as a marker of ER stress. The proteolytic processing of bZIP28 by the resident proteases in the Golgi could also be used as an indicator of ER stress. bZIP28 is first cleaved in by Site-1-protease (S1P) in its lumen-facing domain (Fig. 1). Cutting by S1P renders bZIP28 competent for cleavage in its transmembrane domain (TMD) by another Golgi resident protease, Site-2-protease (S2P). The cut within the membrane by S2P liberates the transcriptional, cytosolic domain, bZIP28p, which is then is transported into the nucleus. Cleavage by S2P happens in rapid succession after the cut by S1P. Thus, the S1P-cut intermediate is not found – only the S2P processed form can be detected by Western blotting.

The activation of IRE1 is similar to that for bZIP28, although IRE1's activation in yeast is thought to be a two-step process [23,24] in which the first step involves the dissociation of BiP from the lumen-facing domain of IRE1 (Fig. 1). BiP dissociation allows IRE1 to dimerize (or oligomerize) and become competent to bind misfolded proteins. It is argued that the binding of misfolded proteins to IRE1's lumenal domain is the actual switch, which activates IRE1's ribonuclease for mRNA splicing. Therefore, the dimerization of IRE1 could also be used as an indicator ER stress. Dimerization can be detected in living cells by fluorescence resonance energy transfer (FRET). By tagging IRE1 with different chromophores, one can measure the exchange of resonance energy between chromophores in response to stress. Fortunately, IRE1b in Arabidopsis can be tagged at its C-terminal cytosol-facing domain without sacrificing the ability of IRE1 to splice bZIP60 mRNA following

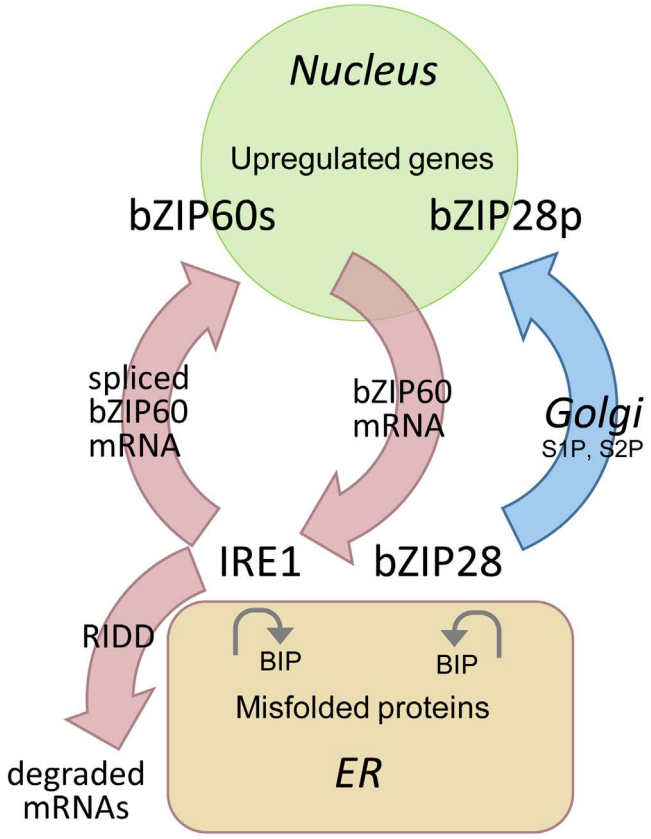

Fig. 1. Diagram of the UPR signaling pathway. There are two arms to the pathway in plants. One arm involves the membrane anchored transcriptional factor, bZIP28. In response to the accumulation of misfolded proteins in the ER, BiP dissociates from bZIP28, allowing it to exit the ER and move to the Golgi where it is processed by Golgi resident proteases, S1P and S2P. The processed transcription factor, bZIP28p, is liberated and enters the nucleus where it promotes the upregulation of stress response genes. The other arm of the pathway involves the RNA splicing factor IRE1 which splices bZIP60 mRNA. The splice occurs such that the mRNA encodes a transcription factor, bZIP60s, that is targeted to the nucleus where it can act in conjunction with bZIP28p to upregulate stress response genes. When activated, IRE1 also attacks and degrades other mRNAs in a process called Regulated IRE1-Dependent RNA Degradation (RIDD).

stress treatment. As an alternative to using FRET, one could monitor ER stress in living cells by detecting the clustering of fluorescent labeled IRE1. IRE1 clustering in response to stress has not yet been reported in plant cells, however, it has been observed in yeast [23,25] and mammalian cells [26].

3. Other UPR indicators

Moving yet another step downstream from primary UPR events, one can measure bZIP60 mRNA splicing by IRE1 as an indicator of ER stress (Fig. 1). An advantage in measuring splicing is that it can be monitored without gene tags or having to make transgenic plants. This is particularly useful in large scale assessments of the UPR, e.g. for crop plants in the field. bZIP60 splicing can be assessed by simple RT-PCR assays using splice-specific primers. RT-PCR assays are qualitative, but can be made quantitative by qRT-PCR. However, selection of primers and careful optimization of either assay is essential in order to discriminate between the spliced and unspliced forms of bZIP60 mRNA. Nonetheless, the RNA splicing assay is often the method of choice for monitoring ER stress.

Several years ago, it was found that IRE1 not only splices its principal target mRNA, but it also degrades other mRNAs encoding secreted proteins undergoing translation on the ER. This process was dubbed Regulated IRE1-dependent RNA degradation (RIDD) [27]. Because RIDD occurs when IRE1 is activated, it too can be used as an