Journal of Plant Physiology 170 (2013) 1434–1441

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Molecular Biology

The ER luminal binding protein (BiP) alleviates Cd^{2+}-induced programmed cell death through endoplasmic reticulum stress–cell death signaling pathway in tobacco cells

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ARTICLE INFO

Article history:
Received 8 March 2013
Received in revised form 22 May 2013
Accepted 24 May 2013
Available online 16 July 2013

Keywords:
BiP protein
Bright yellow-2 cells
Cadmium
ER stress
Programmed cell death

ABSTRACT

Cadmium (Cd) is very toxic to plant cells and Cd^{2+} stress induces programmed cell death (PCD) in *Nicotiana tabacum* L. cv. bright yellow-2 (BY-2) cells. In plants, PCD can be regulated through the endoplasmic reticulum (ER) stress–cell death signaling pathway. However, the mechanism of Cd^{2+}-induced PCD remains unclear. In this study, we found that Cd^{2+} treatment induced ER stress in tobacco BY-2 cells. The expression of two ER stress markers NtBLP4 and NtPDI and an unfolded protein response related transcription factor NtbZIP60 were upregulated with Cd^{2+} stress. Meanwhile, the PCD triggered by prolonged Cd^{2+} stress could be relieved by two ER chemical chaperones, 4-phenylbutyric acid and tauroursodeoxycholic acid. These results demonstrate that the ER stress–cell death signaling pathway participates in the mediation of Cd^{2+}-induced PCD. Furthermore, the ER chaperone AtBiP2 protein alleviated Cd^{2+}-induced ER stress and PCD in BY-2 cells based on the fact that heterologous expression of *AtBiP2* in tobacco BY-2 cells reduced the expression of *NtBLP4* and a PCD-related gene *NtHsr203J* under Cd^{2+} stress conditions. In summary, these results suggest that the ER stress–cell death signaling pathway regulates Cd^{2+}-induced PCD in tobacco BY-2 cells, and that the AtBiP2 protein act as a negative regulator in this process.

Introduction

Cadmium (Cd), a toxic heavy metal, has been classified as a human carcinogen. Thus Cd is a tremendous danger to plants and animals (Bernard, 2008; DalCorso et al., 2010). The damage caused by Cd^{2+} in plant cells mainly includes reactive oxygen species (ROS) production, disturbances in photosynthesis, disorder of calcium (Ca) signaling, and induction of programmed cell death (PCD) (Chaffei et al., 2004; Ma et al., 2010; Rodriguez-Serrano et al., 2009; Zhang et al., 2005). Cd^{2+}-induced endoplasmic reticulum stress (ER stress) has been demonstrated in yeasts and mammals (Gardarin et al., 2010; Liu et al., 2006), but it has not been reported in plants yet.

The ER is a very important organelle for protein synthesis, signal transduction, and Ca homeostasis. ER stress is generally caused by an overload of unfolded proteins in the ER, which activates signal transduction of unfolded protein response (UPR) (Ron and Walter, 2007; Zhang and Kaufman, 2004). Inositol-requiring enzyme-1 (IRE1), activating transcription factor 6 (ATF6), and PKR-like ER kinase (PERK) act as transducers in the UPR signaling pathway in mammals (Calfon et al., 2002; Harding et al., 1999, 2000; Sidrauski and Walter, 1997; Yamamoto et al., 2007; Ye et al., 2000). Then, the ER luminal binding protein (BiP) and glucose-regulated protein 94 (GRP94) are induced by the UPR (Yoshida et al., 1998), and they enhance ER protein-folding capacity and maintain storage of ER Ca^{2+} (Lievremont et al., 1997). BiP is a central regulator of the UPR and a classical marker of UPR activation. When misfolded proteins accumulate in the ER, UPR is activated by BiP released from those three ER trans-membrane sensors IRE1, ATF6, and PERK (Bertolotti et al., 2000). Several UPR-related genes have been identified in plants in recent years. Two orthologs of IRE1 in *Arabidopsis* (AtIRE1a and AtIRE1b) are expressed in various organs and localized to the perinuclear ER membrane (Koizumi et al., 2001). Moreno and Hayashi found that AtIRE1b modulates activation of some basic-leucine zipper (bZIP) transcription factors (Hayashi et al., 2012; Moreno et al., 2012). Moreover, two groups of membrane-associated bZIP transcription factors play very important roles in

Abbreviations: ATF6, activating transcription factor 6; BiP, luminal binding protein; BY-2, bright yellow-2; bZIP, basic-leucine zipper; Ca, calcium; Cd, cadmium; ER, endoplasmic reticulum; GRP94, glucose-regulated protein 94; Hac1, ATF/CREB homolog 1; IRE1, inositol-requiring enzyme-1; NRP, N-rich protein; PCD, programmed cell death; PDI, protein disulfide isomerase; PERK, PKR-like ER kinase; UPR, unfolded protein response; Xbp1, X-box binding protein 1.

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0176-1617/$ – see front matter © 2013 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.jplph.2013.05.017

transducing ER stress signals in plant cells. One group, including AtbZIP60 and OsbZIP50, is activated by IRE1-mediated mRNA splicing, which is similar to activation of ATF/CREB homolog 1 (Hac1) in yeast and X-box binding protein 1 (Xbp1) in animals (Cox and Walter, 1996; Shen et al., 2001). The new proteins encoded by the spliced mRNA are trans-located to the nucleus where they activate transcription of some ER function-related genes (Deng et al., 2011; Hayashi et al., 2012; Iwata and Koizumi, 2005a; Nagashima et al., 2011). Activation of the other group of proteins such as AtbZIP28 and OsbZIP39 depends on regulated intra-membrane proteolysis and resembles the ATF6 process in animals in which site-1 and site-2 proteases are cleaved in response to ER stress (Liu et al., 2007; Tajima et al., 2008; Takahashi et al., 2012). As one of the most important ER chaperone proteins, BiP alleviates ER stress in tobacco (Alvim et al., 2001; Leborgne-Castel et al., 1999). However, PCD is the result of prolonged ER stress triggered by multiple stimuli, although the UPR alleviates ER stress damage.

The main apoptotic cell death signaling pathways demonstrated in mammalian cells are dependent on plasma membrane receptors, mitochondria, and the ER (Earnshaw et al., 1999; Nakagawa et al., 2000). ER-dependent apoptotic pathways have two different modes. The cleavage of procaspase 12 depends on calpain or caspase 7, which is released from ER membranes into the cytosol during prolonged ER stress and forms active caspase 12 to activate downstream apoptotic factors (Rao et al., 2002; Yoneda et al., 2001). BiP acts as an anti-apoptotic moderator and prevents activation of procaspase-7 and procaspase-12 by binding them at the ER membrane (Reddy et al., 2003). The mode of the other ER-dependent pathway is based on the lack of Ca^{2+} homeostasis. Whenever prolonged ER stress is triggered by depletion of Ca^{2+} in the ER, mitochondria overloaded with Ca^{2+} release cytochrome *c* into the cytosol, which causes activation of caspase-9 (Hacki et al., 2000). Similarly, PCD triggered by ER stress in plants has also been reported. In soybean cells, cyclopiazonic acid treatment induces ER stress, increases cytoplasmic Ca^{2+}, generates hydrogen peroxide, induces release of cytochrome *c* from mitochondria, and activates caspase-like proteases causing PCD (Zuppini et al., 2004). In addition, tobacco BY-2 cells, treated with a UPR inducer tunicamycin, may also lead to PCD along with up-regulation of *NtHsr203J*, a marker of PCD (Iwata and Koizumi, 2005b).

As studies on UPR and PCD become more in-depth, these signaling pathways have been revealed in plants as well as animals. BiP plays a very important role in stress-induced plant cell death, such as ER stress, osmotic stress, and water stress (Alvim et al., 2001; Leborgne-Castel et al., 1999; Reis et al., 2011). Moreover, BiP over-expression enhances tolerance to drought stress and delays leaf senescence induced by drought in soybean (Valente et al., 2009). The primary focus of the present study was on the pathway through which Cd^{2+} triggers PCD in plant cells and the mode of BiP action during this process.

Materials and methods

Plant expression vector construction

The reagent of TRIzol (15596026; Life Technologies Corp., Carlsbad, CA, USA) was used to extract *Arabidopsis* total RNA, and full length cDNA was produced using the SuperScript® II Reverse Transcriptase kit (18064-014; Life Technologies Corp.). The BiP coding the isoform *AtBiP2* (at5g42020) region was amplified by polymerase chain reaction (PCR) with the BiP2F1 and BiP2R1 primers (Table S1) to create two KpnI (E.C.3.1.23.26) sites located in front of the translation initiation codon and after the stop codon, respectively. After KpnI digestion, this fragment was inserted between the cauliflower mosaic virus (CaMV) 35S promoter and the 3′ un-translated end of the nopaline synthase gene present on pSN1301, resulting in pSN1301-BiP2. Using this same process, the AtBiP2 coding region was amplified by PCR with another two primer pairs (BiP2F2 and BiP2R2, BiP2F2 and BiP2R3), particularly the BiP2R3 primer containing the 6-His-tag codes (5′-GTGGTGGTGGTGGTGG-3′) before the termination codon (Table S1). Those fragments were separately inserted into pER8 (Zuo et al., 2000), which is an estrogen-dependent expression vector in plants, and were respectively named pER8-BiP2, and pER8-BiP2-6H. All plasmids obtained were identified by DNA sequencing before they were transformed into *Agrobacterium* C58.

Cell culture conditions

Tobacco cell (*Nicotiana tabacum* L. cv bright yellow BY-2) (Nagata et al., 1992) lines were grown in Murashige and Skoog liquid medium supplemented with 256 mg/L KH_2PO_4, 1 mg/L thiamine, 100 mg/L myo-inositol, 0.2 mg/L 2,4-dichlorophenylacetic acid, and 30 g/L sucrose. The cells were maintained on a gyratory shaker (120 rpm) in a dark, temperature-controlled room at 25 °C and sub-cultured weekly with 2% inoculum.

BY-2 cell transformations

Each expression vector was transformed into the C58 *Agrobacterium* rifampicin-resistant strain by electroporation. Two- or three-week-old tobacco BY-2 cell clusters were infused into a suspension of respective *Agrobacterium* stains with a BY-2 cell culture medium for 20 min. After the clusters grew on the normal medium for 2 days, they were washed three times, and then dropped into water containing 400 μg/mL cefotaxime for 45 min. Finally, the transformants were selected on tobacco BY-2 cell culture medium containing 20 μg/mL hygromycin B and 400 μg/mL cefotaxime.

Semi-quantitative reverse transcription (RT)-PCR

RNA isolation and the generation of cDNA were performed as described above. The primers for each target gene are shown in Table S2, which also includes the annealing temperature and PCR cycles. The amplified fragments of the target genes, such as *NtBLP4* (Accession No: X60057), *NtHsr203J* (Accession No: AF212184), and *NtEF1-α* (Accession No: D63396), were segregated by agarose gel electrophoresis under 6 V/cm pressure for 10 min. Gel Doc-It™ 300 (Ultra-Violet Products Ltd., Cambridge, UK) was used to scan the gels.

Real-time quantitative RT-PCR

RNA isolation and the generation of cDNA were performed as described above. For real-time quantitative RT-PCR, the final primer concentration was 0.2 μM in a total 20 μL reaction volume, and all primers were listed in Table S3. Real-time quantitative RT-PCR was performed with the StepOne™ Real-Time PCR System (Applied Biosystems, CA, USA) and prepared with the SYBR *Premix Ex Taq*™ GC Kit (DRR071A; TaKaRa Biotechnology Ltd, Dalian, China). The efficiencies of all cDNAs amplification were between 90 and 100%. Each RNA sample was assayed in triplicate. Expression levels of *NtBLP4*, *NtPDI* (Accession No: Y11209), and *NtbZIP60* (Accession No: AB281271) were calculated relative to the standard sample for calibration and then normalized to the *NtEF1-α* gene.

Protein gel blot hybridization

Each treated cell line was collected and quickly frozen in liquid nitrogen. Frozen samples were ground and transferred to tubes. Protein extraction buffer (100 mM HEPES, pH 7.5, 5 mM EDTA, pH