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Review

Bax inhibitor-1: a highly conserved endoplasmic reticulum-resident cell death suppressor

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In spite of fundamental differences between plant and animal cells, it is remarkable that some cell death regulators that were identified to control cell death in metazoans can also function in plants. The fact that most of these proteins do not have structural homologs in plant genomes suggests that they may be targeting a highly conserved 'core' mechanism with conserved functions that is present in all eukaryotes. The ubiquitous Bax inhibitor-1 (BI-1) is a common cell death suppressor in eukaryotes that has provided a potential portal to this cell death core. In this review, we will update the current status of our understanding on the function and activities of this intriguing protein. Genetic, molecular and biochemical studies have so far suggested a consistent view that BI-1 is an endoplasmic reticulum (ER)-resident transmembrane protein that can interact with multiple partners to alter intracellular Ca^{2+} flux control and lipid dynamics. Functionally, the level of BI-1 protein has been hypothesized to have the role of a rheostat to regulate the threshold of ER-stress inducible cell death. Further, delineation of the cell death suppression mechanism by BI-1 should shed light on an ancient cell death core-control pathway in eukaryotes, as well as novel ways to improve stress tolerance.
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Programmed cell death (PCD) is a genetically controlled and conserved process in eukaryotes during development, as well as in response to pathogens and other stress signals.[1,2] This cell death has been well documented in animals at the genetic, biochemical and morphological levels, and many important regulators and effectors have been identified. In plants, understanding of PCD is relatively poor in terms of molecular mechanisms and our current view largely derived from analogies with animal systems.

One of the well-characterized mechanisms that can drive apoptosis in metazoans is that triggered by Bax protein, a pro-apoptotic member of the Bcl-2 family that interferes with mitochondrial function by forming pores at the outer mitochondria membrane.[3] Bax localization at the mitochondria results in cytochrome c release, followed by activation of death proteases called caspases, finally leading to cleavage of proteins essential for cell survival. This process is tightly controlled antagonistically by anti-apoptotic types of Bcl-2 protein family such as Bcl-2 and Bcl-X_L, which can inhibit Bax activation through their direct interaction. It has been reported that expressing animal and viral regulators of apoptosis such as Bax, Bcl-2, Bcl-X_L and p35 in transgenic plants resulted in

promotion or suppression of cell death phenotypes against infection of bacterial, fungal or viral pathogens (Figure 1).[4–7] As plant genomes lack the core PCD regulators such as caspases and Bcl-related proteins, the exact modes of action for these heterologous proteins in plants and fungi remain unclear. In the past decade, accumulating evidence support the idea that plants likely possess a similar set of core mechanisms that are utilized to orchestrate PCD events at the cytological and biochemical levels, such as accumulation of reactive oxygen species (ROS), cytochrome c release from mitochondria and activation of DNase and caspase-like proteases (Figure 1). However, it should be noted that the functional consequence of cytochrome c release from mitochondria in plant cell death remains controversial. Nevertheless, the ability of heterologous regulators of cell death to function across Kingdoms suggests that there should be a highly conserved cell death switching mechanism in eukaryotes that predates the divergence of plants and animals.

Bax inhibitor-1 (BI-1) is one of the most intensively characterized cell death suppressors conserved between plants and mammals.[8,9] In 1998, BI-1 was originally isolated from a human cDNA library, based on its ability to block cell
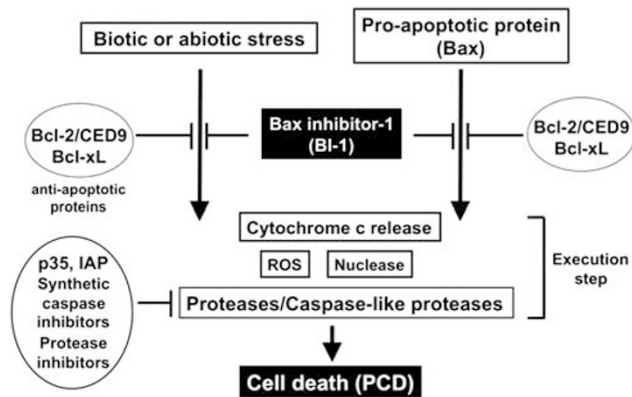
Figure 1 Schematic representation of the effects of heterologously expressed animal and viral PCD regulators and involvement of BI-1 in the control of plant PCD pathway. See text for explanation

death induced by ectopic expression of the mouse Bax gene in yeast.[10] Overexpression of human BI-1 can confer resistance to certain types of apoptotic stimuli that activate the intrinsic apoptotic pathway mediated by the mitochondria, whereas knockdown of BI-1 expression resulted in apoptosis in cancer cell lines.[10] BI-1 blocks Bax-induced cell death downstream of Bax action at the mitochondria, whereas Bcl-2 directly blocks Bax action by physical interactions,[10] suggesting that BI-1 is a cell death regulator in apoptosis. Subsequently, plant BI-1 genes from rice and *Arabidopsis* were isolated and shown to be an evolutionary conserved protein that, when overexpressed in yeast and plant cells, suppresses cell death induced by mammalian Bax.[11,12] This suggests the possibility that plants may have a conserved cell suicide mechanism that is present in animal and fungi, but could be activated by distinct cell death pathways that were elaborated later on in evolution. From this perspective, studying the mechanisms of cell death suppression by BI-1 may help us uncover the ancient 'core' program in eukaryotes that is used to determine cell suicide activation.

In this review, we will first summarize the recent progress on the role of plant BI-1 in anti-cell death pathways as revealed by molecular and genetic studies. Second, we will cover recent discoveries that lead to better understanding of the molecular and biochemical functions of plant BI-1 and its linkage to endoplasmic reticulum (ER) homeostasis, as well as its cytoprotective functions. Third, we will describe recent discoveries that identified interaction partners of plant BI-1, that is, calmodulin (CaM) and fatty acid hyroxylase (FAH), and their possible roles in the control of cell death. Finally, the possible scenario of how plant BI-1 may contribute to suppress a variety of stress-induced cell death in plants will be discussed.

BI-1 is a Broad-Spectrum Cell Death Suppressor in Plants

BI-1 proteins in eukaryotes are ER-resident trans-membrane proteins (25–27 kDa) that have a hydrophilic tail at their C-termini.[8,9] Like mammalian BI-1, plant BI-1 genes also express in diverse tissue types (leaf, root, stem, flower, fruit, etc.) and their expression levels are usually enhanced during

aging (senescence) and under stress conditions, suggesting that BI-1 function is physiologically associated with cell death control and/or stress management.[12–20] In fact, numerous studies by transgenic approaches have revealed that overexpression of plant BI-1 resulted in attenuation of cell death induced by biotic stresses (pathogens) and abiotic stresses such as heat, cold, drought, salt and chemical-induced oxidative stresses, which induce massive accumulation of ROS before cell death activation.[16–20] Interestingly, it was shown that elevated intracellular ROS levels by Bax expression was not abrogated in *AtBI-1* (*Arabidopsis BI-1*) overexpressing *Arabidopsis* plants, although plant cell death was strongly attenuated.[21] H_2O_2-induced cell death of tobacco BY-2 cells can also be protected by *AtBl-1* overexpression, whereas the relative ROS level was not significantly modulated.[22] ROS accumulation appears to be an important step to mediate Bax-activated cell death in plants, as several plant ROS scavenging enzymes such as ascorbate peroxidase, glutathione S-transferase and phospholipid hydroperoxide glutathione peroxidase were isolated as 'Bax inhibitors' from a cDNA library screen, using the yeast-Bax survival screen.[23–25] Together, these observations suggest that plant BI-1 functions downstream from the early steps of ROS-dependent cell death pathway.

Dynamic metabolic changes including enhanced capacity of several key amino acids metabolisms, glycolysis and components of redox and energy metabolism are known to be important contributors for acclimation to oxidative stress. In fact, BI-1 can apparently increase the capacity of cellular homeostasis, including primary metabolism, under oxidative stress conditions and thereby facilitate the repression of cell death, although *AtBI-I* overxpression does not trigger any obvious changes under normal growth conditions.[26] These metabolic changes under oxidative stress could be linked with alterations in Ca^{2+} signaling and sphingolipid metabolism (see below).

Involvement of plant BI-1 in plant defense response, as well as the hypersensitive response (HR), a well-characterized form of plant PCD, has been documented. In plant-fungal pathogen interaction systems, it was shown that overexpression of barley BI-1 resulted in hyper-susceptibility to the biotrophic fungal pathogen *Blumeria graminis,* which suppresses the cell death program of host plants for its successful infection and proliferation.[27] On the other hand, overexpression of barley BI-1 resulted in partial resistance to cell death induced by necrotrophic fungal pathogen *Fusarium graminearum*, which activates PCD of host plant cells.[27] Using stable transgenic RNAi lines that knocked down *BI-1* expression, Eichmann et al.[28] concluded that the anti-cell death function of BI-1 benefits biotrophic *B. graminis* to penetrate into host plant cells by modulating the capacity of cell-wall-associated defense responses in barley. Recent reverse genetic studies using two T-DNA insertion mutants of AtBI-1 (*atbi1-1* and *atbi1-2*) clearly demonstrated that these two loss-of-function mutants exhibited accelerated PCD phenotype upon treatment with a fungal toxin Fumonisin B1, when compared with wild-type plants.[16] In addition, involvement of BI-1 in HR-mediated disease resistance against a bacterial pathogen was also recently reported using the *Arabidopsis* and *Pseudomonas syringae* pathosystem.[29]

Collectively, the above reports clearly demonstrated that plant BI-1 acts as a negative regulator of PCD for two distinct types of stress-induced cell death by bacterial and fungal pathogens (biotic stress), and environmental stresses (extrinsic and intrinsic stress) such as heat shock[16] (Figure 1). Thus, further mechanistic analysis of AtBI1-dependent anti-PCD pathways should shed light on the regulation of disease resistance and stress response of plants.

Emerging Role of Plant BI-1 Linked with ER Homeostasis Control under PCD-Inducing Conditions

The fact that BI-1 apparently localizes to the ER membrane in both animal and plant cells led to our current working model of how BI-1 protein protects eukaryotic cells from a variety of cell death inducing signals. The ER is a highly dynamic and well-conserved organelle that has evolved specific mechanisms to ensure proper protein synthesis, folding and post-translational modifications, as well as the maintenance of its own home-ostasis. In metazoans, persistent or acute cellular stresses that cause disturbance of ER homeostasis, which is called 'ER stress', results in apoptosis.[30,31] As mammalian BI-1 was first shown to negatively regulate ER stress-mediated apoptosis,[32] plant BI-1 could also be involved in controlling cell death signaling from the ER as well.

Several studies have shown that treatment of plant cell cultures with ER stress-inducing agents such as tunicamycin (TM) and cyclopiazonic acid (CPA) results in growth arrest and/or cell death.[33,34] However, to what extent is this cell death mechanism evolutionarily conserved in plant cells remains to be elucidated. To obtain molecular and physiological insight into the process of ER stress in plants, the impact of drug-induced ER stress on the growth and survival of wild-type Arabidopsis (Col-0) plants was studied.[35] It was shown that TM perturbs root development, including elongation of primary and secondary roots and formation of lateral roots and root hair cells in a dose-dependent manner, concomitantly with the loss of cell viability and induction of PCD phenotypes.[35] It was also found that two other ER stress inducers, CPA (a calcium pump inhibitor) and the proline analog L-azetidine-2-carboxylic acid also induce cell death in Arabidopsis leaf in a similar manner to TM.[35] However, visible cell death phenotypes of Arabidopsis leaves as induced by a bacterial pathogen, FB1 and heat shock are very different from those by ER stress inducers (Figure 2). Notably, it was also found that such lethal effects of TM can be relieved by administration of two different chemical chaperones, 4-phenyl butyric acid and tauroursodeoxycholic acid (TUDCA), even in the presence of a lethal dose of TM.[35] These results suggest that TM induces root growth defect and PCD via defected protein folding that leads to ER stress. This idea is supported by a recent report that TUDCA can revert heat-sensitive phenotype of AtBAG7 and AtBip2 knockout mutants, which are defective in ER-resident heat shock protein 70 family members and exhibit increased sensitivity to TM.[36]

Furthermore, involvement of AtBI-1 in the ER stress response and its related cell death pathway in Arabidopsis root cells was shown genetically. It was shown that ER stress-mediated PCD can be manipulated by the disruption of AtBI-1 or overexpressing AtBI-1 proteins, resulting in accelerated or
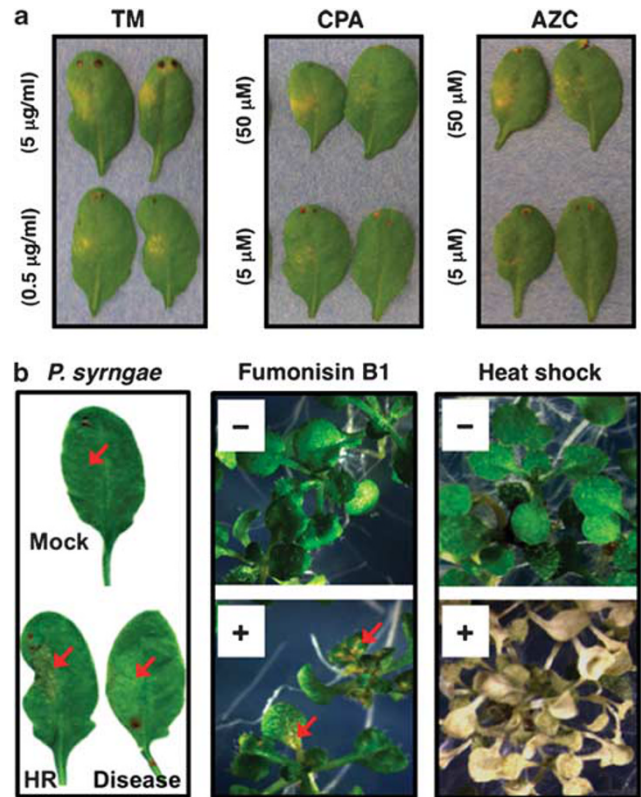

Figure 2 Visible cell death phenotypes of Arabidopsis leaves in response to biotic and abiotic stresses. (**a**) Infiltration of three ER stress-inducing agents into Arabidopsis leaves causes necrotic cell death. Yellowish cell death phenotype appeared at 3 days post infiltration. The concentrations used are shown at the left. TM, tunicamycin; CPA, cyclopiazonic acid; AZC, proline analog L-azetidine-2-carboxylic acid. (**b**) Bacterial pathogen-, Fumonisin B1- and heat shock-induced cell death. For bacterial pathogen challenge, compatible (disease-inducing strain, no avirulent gene) or incompatible (HR-inducing strain harboring an avirulent gene avrRpt2) Pseudomonas syringae (P. syringae) pv maculicola strain ES4326 (1×10^6 cfu/mL) suspended in 10 mM MgCl$_2$ solution was infiltrated into Arabidopsis leaves. For mock treatment, 10 mM MgCl$_2$ solution was infiltrated. Pictures were taken at 1 day (for HR) and 3 days (for disease and mock) post infiltration. HR developed in leaves after 12 h post infiltration. Necrotic disease symptoms developed by 3 days post infiltration. For Fumonisin B1 treatment, 5 day-old Arabidopsis seedlings grown in standard solid media were transferred to 1 μM Fumonsin B1-formulated solid media, grown for 7 days, and then picture was taken. For heat-shock treatment, 12 day-old Arabidopsis seedlings grown in standard solid media were incubated at 55°C for 20 min as described previously.[16] Pictures were taken at 1 day post treatment. $-$, control. $+$, stressed

attenuated PCD, respectively.[35] Expression of AtBI-1 can be induced transcriptionally through activation of the ER-resident bZIP transcription factors AtZIP60.[37] This up-regulation of BI-1 should increase the amount of BI-1 on the ER membrane and support its activity as a survival factor involved in an anti-PCD pathway. However, AtBI-1 appears to act in parallel to the unfolded protein response (UPR) that is induced during ER-stress and is not involved in the signaling of the UPR per se, as alterations in AtBI-1 levels did not significantly impact expression of UPR marker genes.[35] In addition, a recent study revealed that AtBI-1 has a role in root architecture development in response to water stress, which triggers the ER stress response and PCD in Arabidopsis root cells.[38] During water stress, Arabidopsis plants initiate PCD of meristematic cells in

the primary root, and eventually stimulates lateral root development for survival. These findings thus open the way for future studies to decipher the mechanisms of ER-mediated PCD and potential roles of this pathway in root development and stress response.

In mammalian cells, the tight regulation of ER stress response is a fundamental mechanism to maintain homeostasis and normal ER functions. This process is accompanied by conserved activation of transcriptional programs that can induce genes encoding enzymes that are capable of enhancing the protein folding capacity in the ER, as well as for ER-assisted degradation of unwanted proteins.[30] Lisbona *et al.*[39] recently demonstrated that mammalian BI-1 functions at the early stage of adaptive response against ER stress through its direct interaction with the ER stress sensor protein IRE1-α, which activates downstream transcriptional events that up-regulates ER-resident chaperones gene expressions mediated by XBP-1. The complex formation of BI-1 and IRE1-α is mediated through the C-terminal domain of BI-1 at the ER membrane. Importantly, this regulation is specific, which does not affect the other ER stress response pathways mediated by the ATF6- or PERK-dependent pathway. The IRE1-α/XBP-1 pathway was suppressed in BI-1 overexpressing animal cells, whereas their expression is hyperactivated in BI-1 knockout cells under ER stress-inducing drugs.[39] In the *Arabidopsis* root, alterations in AtBI-1 levels do not appear to significantly influence the expression levels of some of the key ER stress responsible genes, suggesting that AtBI-1 functions in parallel to this transcriptional upregulation.[37] However, it would be interesting if plant BI-1 function is associated with active transcriptional control in ER stress response through direct interaction with plant orthologue of IRE1[40] or other cryptic factors that may control ER stress-responsive genes in plants.

Another recent study identified NADPH-dependent cytochrome P450 oxidoreductase as an interaction partner of human BI-1, whose interaction is mediated through the C-terminal domain of BI-1.[41] Interestingly, this interaction decreases electron uncoupling between NADPH-dependent cytochrome P450 reductase and cytochrome P450 2E1, which is known to be a major source of ROS on the ER membrane. Thus, modulations of electron flow from NADPH-dependent cytochrome P450 oxidoreductase to P450 2E1 by BI-1 can result in a reduction of ROS production that potentially decrease unfolded and misfolded proteins accumulation in the ER. It was also found that overexpression of mice BI-1 reduces the level of ER stress-associated ROS accumulation in cultured mammalian cells through the modulation of heme oxygenase-1 expression.[42] The elevation of heme oxygenase-1 activity and modulation of electron flow from NADPH-dependent cytochrome P450 oxidoreductase to P450 2E1 following ER stress might be an adaptive response that allows the cells to reestablish ER homeostasis through limiting oxidative damage. Whether these mechanisms regulating ROS production in the ER occurs in plants as well remains to be determined.

Together, the recent discoveries on the role of plant and animal BI-1s described above strongly suggest that BI-1 has a conserved function at the convergence point of multiple stress signaling pathways that modulates the level of the 'pro-survival and pro-death' signals originating from the ER. These findings should facilitate future studies to decipher the mechanisms and pathways of ER-mediated PCD, as well as the role of this pathway in stress tolerance. Identification and characterization of the repertoire of plant BI-1-interacting factor(s) will be important to delineate the mechanism underlying the critical cell survival or suicide decision during ER stress.

Molecular Analysis of AtBI-1-Interacting Factors

CaM and Ca^{2+} signaling. Barley (*Hordeum vulgare* L.) Mlo protein is known as a negative regulator of cell death and of defense response to the biotrophic powdery mildew fungus *Blumeria graminis*.[43] Kim *et al.*[44,45] reported that binding of barley MLO to CaM (HvCaM3) is required for this Mlo-mediated defense response. Interestingly, Hückelhoven *et al.*[15] found that barley BI-1 overexpression resulted in breakdown of the penetration resistance to *B. graminis* in the barley *mlo* mutant, suggesting that BI-1 and Mlo may have similar functions in plant defense responses. The observation that Mlo binds to HvCaM3 led Ihara-Ohori *et al.*[46] to study CaM-binding ability of AtBI-1. Split-ubiquitin yeast two-hybrid (SuY2H) assays revealed that AtBI-1 could also interact with HvCaM3. The *Arabidopsis* genome contains seven CaMs (AtCaM1–7) and more than 50 CaM-related genes (CMLs). Among these proteins, the Ca^{2+}-dependent physical interaction between AtBI-1 and closely related *Arabidopsis* homolog of HvCaM3 (AtCaM7) was identified. *In vitro* overlay assays revealed the specificity of CaM binding with AtBI-1. In fact, AtBI-1 can interact with other AtCaMs (CaM3 and CaM6), but not with four CaM-like proteins (AtCML8, AtCML9, AtCML12 and AtCML23).[29] Thus, AtBI-1 activity may be mediated in part through its interaction wtih AtCaM7, AtCaM3 and/or AtCaM6 *in vivo*.

The C-terminus of AtBI-1 contains a cluster of charged amino acids (Lys and Arg for basic, and Glu and Asp for acidic residues) that are required for its anti-PCD activity under oxidative stress conditions.[22] Substitution of basic residues to non-charged amino acids was shown to result in loss of AtBI-1 function and CaM binding.[22,29,46] It should be noted that basic amino acid-rich amphiphilic clusters are present in other CaM binding proteins.[47] Thus, the C-terminal region of the AtBI-1 appears to be indispensable for its anti-cell death activity and correlates with its interaction with CaMs.

Arabidopsis microarray expression data analysis revealed that *AtCaM7* is expressed in mature leaves infected with an avirulent strain of *Pseudomonas syringae* carrying *avrRpt2* in a similar manner to the expression of *AtBI-1*, suggesting that AtCaM7 and AtBI-1 may work together in response to this pathogen.[29] In fact, the promoter activity of *AtBI-1* was upregulated in mature leaves in response to the avirulent *P. syringae,* especially in and around the area where pathogen was infiltrated, and this pathogen-induced HR cell death was enhanced in BI-1 knockout and knockdown mutants of *Arabidopsis*.[29] As transient influx of Ca^{2+} constitutes an early event in the signaling cascade that trigger plant defense and gene-for-gene HR cell death,[48] AtCaM7-AtBI-1 interaction might be important for modulating cell death activation

during *Rps2-avrRpt2* dependent HR to reduce the spread of the cell death signal and thus limit lesion size.

Although *AtCaM2* and *AtCaM3* were found as candidates co-expressed with *AtBI-1* under other biotic and abiotic stresses, *AtCaM1*, *AtCaM4* and *AtCaM5* show closer relationships with *AtBI-1* in tissue-specific expression patterns during normal plant development (Figure 3). The differential regulation of *AtCaMs* and *AtBI-1* expressions in various stresses and plant development suggests the possibility that plant CaM family proteins, which have diversified in plant species,[47] have distinct roles for the AtBI-1-mediated cell death regulatory mechanism in an isoform-specific fashion. Plant CaM proteins differentially regulate various cellular functions in stress conditions through binding to their target proteins, such as transcription factors, protein kinases, metabolic enzymes, ion channels, transporters and cytoskeleton proteins.[47,49] Further molecular, biochemical and genetic analyses of CaMs will be necessary to determine the precise functional roles of different CaMs and Ca^{2+} signaling as related to BI-1 activity.

An *Arabidopsis* mutant *atbi1-1* is predicted to express an aberrant form of the AtBI-1 proteins having a mutated C-terminal sequence that has lost its capability for forming coiled-coil structure.[16] *atbi1-1* was shown to exhibit enhanced sensitivity to various stresses including disturbed ion homeostasis during deficiency of Ca^{2+} or treatment with Mn^{2+} or CPA, an inhibitor of animal sarcoplasmic/ER Ca^{2+} ATPase (SERCA).[35,46] *AtBI-1* overexpression in tobacco BY-2 cells resulted in increased resistance to CPA-induced cell death. Interestingly, a transient increase of cytosolic free Ca^{2+} concentration after treatment with CPA was attenuated in *AtBI-1* overexpressing BY-2 cells. These results suggest the possibility that AtBI-1 directly or indirectly affects Ca^{2+} pumping activity at the ER, depending on AtBI-1-AtCaM7 interaction under oxidative stress or ER stress.

This possibility can be supported by the observation that inhibition of Bax-induced cell death by AtBI-1 was attenuated in yeast mutants in genes encoding homologs of animal SERCA, Pmr1p and Spf1p, which are involved in Ca^{2+} homeostasis through the ER.[46] Thus, Ca^{2+} homeostasis regulated by the inner membrane-localized Ca^{2+}-ATPases may have a vital role in cell death inhibitory activity of AtBI-1. Interestingly, CaM is known to bind directly to Ca^{2+}-ATPases localized at the ER,[50] suggesting a possibility that AtBI-1 may control cellular Ca^{2+} homeostasis mediated through interaction with CaM and Ca^{2+}-ATPase. Consistent with this hypothesis, an ER-resident Ca^{2+}-ATPase from tobacco (NbCA1) has recently been demonstrated to be an important modulator of PCD induced by pathogens and elicitors, with evidence that cellular Ca^{2+} fluxes are involved.[51]

Function of BI-1 in the regulation of cellular Ca^{2+} concentration has also been suggested by observations in animal BI-1 studies. Mammalian BI-1 was also reported to be involved in regulation of Ca^{2+} store in the ER. Chae *et al*.[32] found that the release of free Ca^{2+} from the ER store into the cytosol was reduced in BI-1-overexpressing cells, but enhanced in BI-1 knockout cells, when ER Ca^{2+}-pump activity was inhibited by thapsigargin that triggers ER stress and apoptosis. In the resting state, the Ca^{2+} pool level in the ER is controlled via a balance between active uptake by SERCA and passive efflux or basal leakage through other Ca^{2+} channels. Mammalian BI-1 may inhibit activity of Ca^{2+} release channels, or enhance/mediate Ca^{2+} uptake activity under ER stress. It should be noted that oxidative stress- or SERCA inhibitor-induced increase of cytosolic Ca^{2+} was reduced in *AtBI-1*-overexpressing BY-2 cells.[46] Thus, plant BI-1 may also be directly involved in Ca^{2+} transport through the ER membrane at the early stage of calcium signaling for cell death suppression.
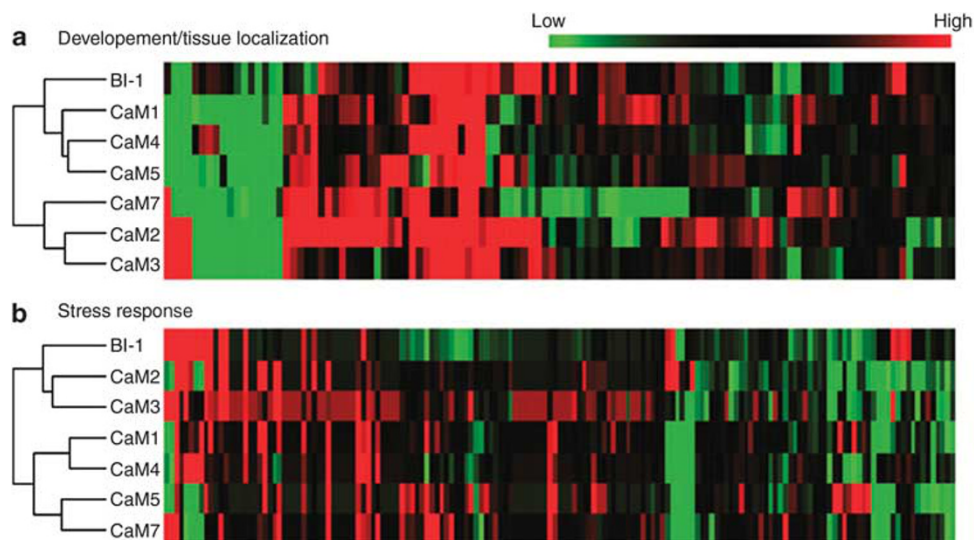

Figure 3 Classification of expression patterns of AtBI-1 and AtCaMs. Microarray data of *AtBI-1* and *AtCaMs* in various developmental stages/tissues (**a**) and responses to biotic and abiotic stresses (**b**) available in Arabidopsis eFP Browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi)[75] were used for hierarchical cluster analysis using R software. Heat maps indicate normalized expression values as scaled with color intensity, so that the highest and lowest expression corresponds to bright red and green, respectively. *AtCaM1/4/5* and *AtBI-1* show similar expression patterns in normal development/tissue localization (**a**), but *AtCaM2/3* are preferentially associated with *AtBI-1* in stress responses (**b**)

Kim *et al*.[52] recently revealed that the conserved C-terminal domain of BI-1 resembles the Lys-rich motif contained in the pH-sensing domain of ion channels. They also demonstrated that BI-1 acts as a pH-dependent Ca^{2+} transporter in BI-1 overexpressing cells and BI-1 reconstituted liposomes.[52] In acidic conditions, BI-1 formed homo-oligomers, and recently it was evidenced using the liposome reconstitution system that recombinant BI-1 possesses Ca^{2+}/H^+ antiporter-like activity.[53] These observations suggest that mammalian BI-1 itself acts as a Ca^{2+} pump in the ER under acidic pH condition. Very surprisingly, BI-1-overexpressing human cells in acidic conditions resulted in the activation of Bax-dependent apoptosis pathway.[52] These findings imply that human BI-1 can have death-promoting activity under low pH conditions. The pH sensing domain-like structure of mammalian BI-1 is also conserved in plant BI-1 proteins with some variations. Further studies should be undertaken to investigate whether or not plant BI-1 has a similar activity under low pH conditions, as one of the important events in plant PCD is manifested by rupture of the vacuole membrane that triggers a dramatic drop of intracellular pH from neutral ($\sim$7.3) to weakly acidic (5–6) levels.[54,55]

Cytochrome b_5 and sphingolipid metabolism. Nagano *et al*.[56] isolated cytochrome b_5 (Cb5) as another candidate partner with AtBI-1 by the SuY2H screening method, using an *Arabidopsis* cDNA expression library. In addition, a yeast mutant lacking *ScFAH1*, the ER-resident fatty acid hydroxylase containing a Cb5-like domain, failed to rescue Bax lethality of yeast by co-expression of *AtBI-1*. Interestingly, AtFAH1 and AtFAH2, the two *Arabidopsis* homologs of ScFAH1, lack the N-terminal Cb5-like domain and are thus distinct from their yeast and animal counterparts.[56,57] As the two AtFAHs can interact with AtCb5, the biochemical activities of AtBI-1 and AtCb5-AtFAH may be tightly coupled through physical interaction between these proteins.[56] Surprisingly, it was shown that *AtBI-1* overexpressing *Arabidopsis* plants accumulate 2-hydroxylated very long chain fatty acid (VLCFA) species, which are produced by FAHs in yeast and animals. The 2-hydroxylated VLCFAs are known components in the ceramide fatty acyl chains of sphingolipids,[58,59] whereas sphingolipids are believed to be involved in PCD signaling in both plants and animals. For example, pathogen-derived sphingolipids are known as an elicitor that induces PCD in plants.[60,61] A phytotoxin FB1 produced by *Fusarium* species have an inhibitory activity on ceramide synthesis leading to PCD, and AtBI-1 was shown to be involved in tolerance to this toxin.[16,62] Moreover, genetic analyses using sphingolipid metabolism-related *Arabidopsis* mutants have indicated that sphingolipids are involved in cell death regulation in plants as well.[63–67] This evidence suggest that sphingolipid metabolism is associated with PCD regulatory steps in plants, in which AtBI-1 may participate via modulation of fatty acid hydroxylation at the ER. Townley *et al*.[68] reported that cell death in *Arabidopsis* cells was induced by treatment with ceramide containing non-hydroxy short fatty acyl chain, but not by ceramide with 2-hydroxylation and longer acyl chains. This report supports the idea that 2-hydoxylated VLCFA synthesis catalyzed by

AtFAHs also can contribute to a sphingolipid-mediated PCD pathway, and AtBI-1 may be involved via physical interactions with some of the enzymes in this pathway.

In plants, the major portion of sphingolipids usually exist as complex glycosylated forms such as glucosylceramide and glycosyl inositolphosphoceramide. Recent advances in plant sphingolipidomics based on liquid column chromatography-mass spectrometry/mass analysis comprehensively revealed that the major sphingolipid pool within the complex glyco-sphingolipids contains mainly 2-hydroxylated fatty acyl chains, whereas non-hydroxylated forms are almost completely absent. In contrast, the smaller lipid pool of sugar-free ceramides contains non-hydroxylated forms to some extent.[69,70] This supports the possibility that activation of AtFAH by AtBI-1 contributes to enlargement of the glyco-sphingolipid pool.[56]

The AtBI-1/AtFAH complex may have a role in the formation of membrane microdomains such as 'lipid raft'. These domains are enriched in glycosphingolipids, sterols and certain membrane proteins to form highly ordered and tightly packed structures in lipid bilayers via lipid – lipid, protein – lipid and protein – protein interactions. In mammalian cells, these specialized domains can serve as platforms for various cellular processes, including receptor-mediated signal transduction, endocytosis, cell polarization, response to pathogens and ceramide-mediated apoptosis induction.[71] Recent studies have provided evidence that membrane microdomains consisting of typical lipids and proteins are also present in plant cells.[72] Minami *et al*.[73,74] revealed that detergent-resistant membrane fractions prepared from cold-acclimated *Arabidopsis* exhibit dynamic changes in microdomain-specific lipids, sterols and glucosylceramides, and in protein profiles. These evidences strongly suggest that plasma membrane microdomains have important roles for defense and acclimation in response to environmental stresses in plants. As sphingolipid metabolism and components of plasma membrane microdomains in plant cells can be affected by *AtBI-1* overexpression (Ishikawa *et al*., unpublished data), plasma membrane microdomain-localized cell death regulatory mechanisms might also be associated with BI-1 activity.

Concluding Remarks

The study of the mechanism of BI-1 action has revealed a number of fundamental roles that the ER may have in stress-induced cell death. These include controlling Ca^{2+} fluxes, lipid metabolism and ER stress response signaling (Figure 4). BI-1 can apparently influence these myriad activities through physical interactions with multiple proteins and enzymes on the ER-membrane. As a highly conserved subcellular compartment in eukaryotic cells, the ER is a prominent organelle that is highly sensitive to perturbations of cellular homeostasis. It is thus well suited to integrate multiple stress signals via the ER-stress response pathway and determine if PCD should be induced or not. The demonstration of the role for BI-1 in ER-stress tolerance, combined with the observation that BI-1 can suppress cell death activation by diverse types of stresses, thus supports the model that ER-stress is an ancestral integration point for stress-activated cell death.
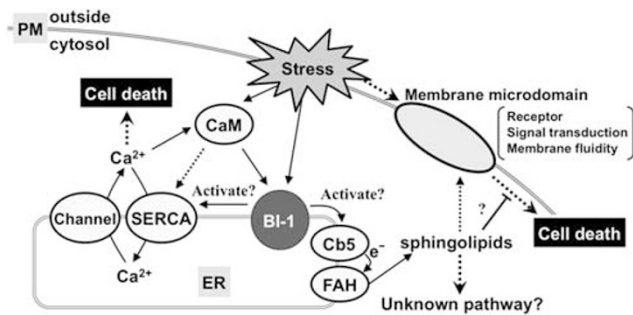
Figure 4 A schematic model for BI-1-mediated cell death regulation via modulation of Ca^{2+} signaling and sphingolipid metabolism in plant cells. Extrinsic and intrinsic stresses induce expression of *BI-1* and *CaMs*, resulting in increase of their protein levels, leading to accelerated interaction between them at the cytoplasmic face of the ER. This results in the modulation of cytosolic Ca^{2+} concentration via Ca^{2+} pumps such as the SERCA. This process could thus reduce the increase of cytosolic Ca^{2+} release from the ER through Ca^{2+} channels that is activated by stresses. Plant BI-1 can interact with the FAH via an electron donor Cb5 on the ER membrane, resulting in accumulation of 2-hydroxy VLCFA-containing sphingolipids. The resultant sphingolipids could affect the formation of plasma membrane microdomains, which would serve as a platform for cell death signal transduction in response to stresses. It remains to be determined how sphingolipids may regulate cell death induction downstream of BI-1 action at the ER

One of the current research foci is to determine how plant BI-1 and BI-1-regulated sphingolipid metabolism can affect the sphingolipid pool and functions of membrane microdomains. Another important area is to investigate if homologs to the binding partners of mammalian BI-1 that have been identified also have a role in BI-1-dependent anti-PCD pathway in plants. This may help to provide greater insights as to the extent beyond BI-1 that conserved mechanism(s) for PCD control may exist in diverse organisms. Thus, further study of BI-1 dependent pathways in the ER should reveal more details on this 'core' pathway of cell death regulation in eukaryotes.

Conflict of Interest

The authors declare no conflict of interest.

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