Cell Death Suppressor Arabidopsis Bax Inhibitor-1 Is Associated with Calmodulin Binding and Ion Homeostasis

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Cell death suppressor Bax inhibitor-1 (BI-1), an endoplasmic reticulum membrane protein, exists in a wide range of organisms. The split-ubiquitin system, overlay assay, and bimolecular fluorescence complementation analysis demonstrated that Arabidopsis (Arabidopsis thaliana) BI-1 (AtBI-1) interacted with calmodulin in yeast (Saccharomyces cerevisiae) and in plant cells. Furthermore, AtBI-1 failed to rescue yeast mutants lacking Ca\(^{2+}\) ATPase (Pmr1 or Spf1) from Bax-induced cell death. Pmr1 and Spf1, p-type ATPases localized at the inner membrane, are believed to be involved in transmembrane movement of calcium ions in yeast. Thus, the presence of intact Ca\(^{2+}\) ATPases was essential for AtBI-1-mediated cell death suppression in yeast. To investigate the effect of AtBI-1 on calcium homeostasis, we evaluated sensitivity against cyclopiazonic acid (CPA), an inhibitor of sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\) ATPase in AtBI-1-overexpressing or knock-down transgenic Arabidopsis plants. These plants demonstrated altered CPA or ion stress sensitivity. Furthermore, AtBI-1-overexpressing cells demonstrated an attenuated rise in cytosolic calcium following CPA or H\(_2\)O\(_2\) treatment, suggesting that AtBI-1 affects ion homeostasis in plant cell death regulation.

In higher plants, programmed cell death (PCD) plays a vital role in proper biogenesis and morphogenesis similar to apoptosis in animal cells, where some biotic or abiotic stimuli trigger the death pathway, resulting in mitochondrial dysfunction, generation of reactive oxygen species (ROS), and activation of specific proteases. In apoptosis, Bax, a proapoptotic member of the Bcl-2 family proteins, is activated by certain apoptotic stimuli and then translocates into the mitochondrial membrane, causing mitochondrial dysfunction (Wei et al., 2001). Some features of plant PCD, including hypersensitive reaction (HR), resemble the apoptotic process. Thus, in plant PCD: (1) caspase-like protease activity is observed during Tobacco mosaic virus (TMV)-induced HR (Chichkova et al., 2004); (2) vacuolar processing enzyme has caspase-1 activity and mediates TMV-induced HR in tobacco (Nicotiana benthamiana; Hatsuagai et al., 2004; Hara-Nishimura et al., 2005). In addition, mammalian Bax is capable of inducing cell death in yeast (Saccharomyces cerevisiae; Zha et al., 1996) and also plant cells (Lacomme and Cruz, 1999). Such evidence suggests the existence of a common mechanism for animal and plant cell death pathways.

In animal cells, intracellular calcium level plays an important role in death pathways triggered by apoptotic stimuli. Several studies suggested that the amount of calcium ion stored in the endoplasmic reticulum (ER) determines the sensitivity of the cells to apoptotic stress (Pinton et al., 2001; Scorrano et al., 2003). It was also suggested that translocation of calcium ions from the ER to the mitochondrion may be required to induce cell death by some apoptotic signals; alteration of calcium level in the ER could signal apoptotic cell death (Scorrano et al., 2003). On the other hand, overexpression of Bcl-2 reduces the ER calcium level (Foyouzi-Youssefi et al., 2000), suggesting that the effect of antiapoptotic proteins on apoptosis may be mediated by regulation of calcium flux.

Bax inhibitor-1 (BI-1) was first identified as a suppressor of Bax-induced cell death on screening of a human cDNA library in yeast cells (Xu and Reed, 1998). Although no homologs of the Bcl-2 family proteins have been identified in plants and yeast, BI-1 is...
widely conserved in organisms, including Caenorhabditis elegans and Xenopus laevis (Hückelhoven, 2004). Overexpression of Arabidopsis (Arabidopsis thaliana) BI-1 (AtBI-1) suppresses Bax-induced cell death in Arabidopsis and yeast (Kawai et al., 1999; Sanchez et al., 2000; Kawai-Yamada et al., 2001, 2004). Bax expression in plant cells causes ROS generation, organelle disruption, and ion leakage from cells (Baek et al., 2004; Yoshinaga et al., 2005). AtBI-1 prevents ion leakage, but not ROS generation, when overexpressed together with Bax in Arabidopsis (Kawai-Yamada et al., 2004). In addition, H2O2-, salicylic acid (SA)-, elicitor-, heat-, and cold-induced cell death were suppressed in plant cells overexpressing AtBI-1 (Chae et al., 2003; Matsumura et al., 2003; Kawai-Yamada et al., 2004). Moreover, tobacco Bright Yellow-2 (BY-2) cells became sensitive to Suc starvation after overexpression of antisense BI-1 (Bolduc and Brisson, 2002; Bolduc et al., 2003). It was recently reported that cells from BI-1-deficient mice showed hypersensitivity toward agents that induce ER stress, such as tunicamycin or thapsigargin (TG; Chae et al., 2004). However, the mechanism of BI-1-mediated suppression of cell death is still unclear.

The barley (Hordeum vulgare) Mlo protein, a plantspecific plasma membrane (PM) protein, is known as a modulator of cellular defense and cell death (Piffanelli et al., 2002). Recently, it was shown that overexpression of barley BI-1 in mlo5-resistant cultivars restored Mlo function when penetrated by Blumeria graminis (Hückelhoven et al., 2003), suggesting that AtBI-1 and Mlo may possess similar functions in cellular defense and cell death modulation. Moreover, it was demonstrated that the barley Mlo protein binds to calmodulin (CaM; HvCaM3) and the interaction is related to the control of its function (Kim et al., 2002). CaMs facilitate Ca2+-dependent responses through binding to their target proteins and modulating their activities. We were inspired by these facts to study the interaction of BI-1 and CaM. Here, we show that AtBI-1 can also interact with CaM and the cell death suppression activities of AtBI-1 in plant cells are mediated, at least in part, by modulation of ion homeostasis.

RESULTS
AtBI-1 Interacts with CaM

Overexpression of barley BI-1 in resistant mlo5 barley plants results in almost complete restoration of susceptibility to penetration by B. graminis (Hückelhoven et al., 2003), suggesting that BI-1 and Mlo, CaM-binding proteins, possess similar functions in the plant defense system. Thus, we tried to evaluate the interaction between CaM and BI-1 proteins. The plasmid possessing HvCaM3 protein was kindly provided by Dr. Ralph Panstruga (Max-Planck Institute). The yeast split-ubiquitin system demonstrated that AtBI-1 interacted with HvCaM3 (Fig. 1). As reported previously, AtBI-29, AtBI-30, and AtBI-32 are C-terminal mutants of AtBI-1 (Kawai-Yamada et al., 2004). Although the AtBI-29 and AtBI-32 mutants maintained inhibitory function toward Bax-induced cell death in yeast, the AtBI-30 mutant lacked the C-terminal coiled-coil structure and function (Kawai-Yamada et al., 2004; Fig. 1A). As shown in Figure 1B, AtBI-29 and AtBI-32 maintained the interaction with HvCaM3, whereas AtBI-30 did not. These results suggest the possibility that such interaction may be necessary for the suppressive action of AtBI-1 on Bax-induced cell death.

To further investigate the interaction between AtBI-1 and Arabidopsis CaM, an in vitro overlay assay was performed. HvCaM3 is almost identical to Arabidopsis CaM7 (AtCaM7; Zielsinski, 2002), with only one amino acid substitution (A111→S111). S- and His-tagged AtCaM7 expressed in Escherichia coli was purified by nickel-nitrilotriacetic acid resin. Maltose-binding protein (MBP)-tagged, C-terminal 14 amino acids of AtBI-1 (BI-C) or β-galactosidase (Gal; control) purified by amylose resin were used for the overlay assay. As shown in Figure 2, purified BI-C and Gal proteins were

Figure 1. AtBI-1 interacts with CaM in yeast. A, Bait and prey vector constructs used for the yeast split-ubiquitin two-hybrid system. Nubi and Cubi represent the N and the C terminus of ubiquitin protein, respectively (Stagljar et al., 1998; Kim et al., 2002). The full length of AtBI-1 was used as a bait protein, and the C-terminal 14 amino acids were replaced in mutants (AtBI-29, 32, 30) as presented. Replaced amino acids are underlined. Amino acids with positive charges are indicated in red and those with negative charges in blue. Calculated scores for coiled-coil structure and cell death suppression are indicated (Kawai-Yamada et al., 2004). pCup, pCup promoter; CYC1, CYC1 terminator; pMet, pMet promoter; URA3, reporter protein. B, Yeast split-ubiquitin system was used to analyze the interaction between AtBIs (AtBI-1, AtBI-29, AtBI-30, and AtBI-32) and HvCaM3. Yeast cells containing different combinations of bait (AtBI-1, AtBI-29, AtBI-30, and AtBI-32) and prey (Nul-HvCaM3) constructs were tested for their growth phenotype on minimal medium containing 5-FOA. For the negative control, yeast cells possessing NuA-HvCaM3 and AtBI-1 (BI-1 + NuA) were also plated. The results show the growth of each line after 2-d (without 5-FOA) and 6-d (with 5-FOA) incubation at 30°C.
immunoblotted with anti-MBP antibody. The membrane was reprobed with recombinant AtCaM7 protein in the presence of 1 mM CaCl2 or 5 mM EGTA. AtCaM7 did bind to BI-C, although this protein did not bind to the Gal protein (control). EGTA inhibited such binding, confirming the importance of calcium in CaM-mediated binding.

To verify AtBI-1/CaM binding in planta, a bimolecular fluorescence complementation (BiFC) assay was performed in Agrobacterium-infiltrated tobacco leaves. As previously reported, the green fluorescent protein (GFP)-tagged AtBI-1 protein (AtBI-GFP) localized to the perinuclear region, which is indicative of ER localization, in tobacco epidermal cells (Fig. 3F). When the AtBI-1 fused to the N-terminal yellow fluorescent protein (YFP) fragment and AtCaM7 fused with the C-terminal YFP were coexpressed in tobacco leaves, a strong BiFC signal was detected in the epidermal cells (Fig. 3, A, C, and E), confirming the interaction of AtBI-1 and AtCaM7 in planta. Control experiments in which pSPYNE-AtBI-1 and unfused pSPYCE were coexpressed did not show any fluorescence (Fig. 3, B and D).

Involvement of the Calcium Pump in BI-1 Function

An increase in cytosolic Ca2+ has been implicated in the signal transduction pathway that mediates apoptosis (Pinton et al., 2001). It is possible that members of the Bcl-2 family affect the apoptotic process by modulating ion fluxes in the cell (Lam et al., 1994). Based on the above background, we then investigated the role of proteins involved in calcium transport on the inhibitory function of AtBI-1 against Bax-induced cell death in yeast. As shown in Table I, we employed several mutants deficient in calcium homeostasis-related proteins. Mid1, Yvc1, and Cch1 are Ca2+ channels located in the ER and PM, vacuole, and PM, respectively (Bertl and Slayman, 1990; Locke et al., 2000; Yoshimura et al., 2004). Spf1, Pmc1, and Pmr1 are Ca2+ ATPases localized in endomembranes (Antebi and Fink, 1992; Cunningham and Fink, 1994; Cronin et al., 2002). Vcx1 is a vacuolar H+ /Ca2+ antiporter, and Ccc1 localized to the vacuole is involved in iron, manganese, and Ca2+ homeostasis in yeast (Cunningham and Fink, 1994; Lapinskas et al., 1996). To evaluate the antiapoptotic function of AtBI-1, the wild-type yeast line and mutants possessing Yep51-Bax plasmids were transformed with pYX112-AtBI, which were then subjected to spot assay. The growth curve of these yeast strains expressing AtBI-1 was almost the same, and sensitivity of these yeast strains against Bax-induced cell death was also similar (data not shown). As shown in Figure 4, A and B, wild type and most mutants expressing both Bax and AtBI-1 grew on Gal-containing medium. However, Δpmr1 and Δspf1 cells failed to grow by the expression of Bax even when AtBI-1 was overexpressed. Reverse transcription (RT)-PCR demonstrated that deficiency in Pmr1 and Spf1 did not

Figure 2. Overlay assay reveals that Arabidopsis CaM protein interacts with the C-terminal region of AtBI-1. To detect the interaction between AtCaM7 and the C-terminal region of AtBI-1, an overlay assay was performed. The purified MBP-tagged, C-terminal 14 amino acids of AtBI-1 (BI-C) and Gal proteins were run by gel electrophoresis and blotted on a membrane as described in “Materials and Methods.” Coomassie Brilliant Blue staining and western blotting with anti-MBP antibody were performed as controls of protein loading. The overlay assay was performed by an S-tagged AtCaM7 protein with 1 mM CaCl2 or 5 mM EGTA-containing solution.

Figure 3. BiFC analysis of AtBI-1/AtCaM7 interaction in plant cells. A, Schematic representation of BiFC plasmids. The coding region of AtBI-1 and AtCaM genes was fused with the N-terminal (YFP-N; 1–155 amino acids) and C-terminal (YFP-C; 156–239 amino acids) region of YFP, respectively. B to E, BiFC visualization in Agrobacterium-infiltrated tobacco leaves with pSPYNE-AtBI-1/pSPYCE (empty vector; B and D) and pSPYNE-AtBI-1/pSPYCE-AtCaM7 (C and E). F, Visualization of AtBI-GFP. Arrows indicate nuclei. Bars in B and C = 100 μm; bars in D to F = 10 μm.
firmed perinuclear localization of AtBI-1 in microscopic analysis using GFP-tagged AtBI-1 protein.

To Cyclopiazonic Acid Enhanced Resistance of AtBI-1-Overexpressing Plants

Yeast strains used in this study

<table>
<thead>
<tr>
<th>Disrupted Gene</th>
<th>Name, Putative Intracellular Localization, and Estimated Function of the Mutated Protein</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>YNL291c</td>
<td>Midl, ER and PM, stretch-activated Ca(^{2+}) channel</td>
<td>Locke et al. (2000); Yoshimura et al. (2004)</td>
</tr>
<tr>
<td>YLR220w</td>
<td>Ccc1, vacuole, involved in iron, Mn(^{2+}) and Ca(^{2+}) transport</td>
<td>Lapinskas et al. (1996)</td>
</tr>
<tr>
<td>YEL031w</td>
<td>Spf1, ER, P-type ATPase involved in Ca(^{2+}) homeostasis</td>
<td>Cronin et al. (2002)</td>
</tr>
<tr>
<td>YOR087w</td>
<td>Yvc1, vacuole, Ca(^{2+}) channel</td>
<td>Bertl and Slayman (1990)</td>
</tr>
<tr>
<td>YGR217w</td>
<td>Cch1, PM, voltage-gated Ca(^{2+}) channel</td>
<td>Locke et al. (2000)</td>
</tr>
<tr>
<td>YGL006w</td>
<td>Pmc1, vacuole, Ca(^{2+}) ATPase</td>
<td>Cunningham and Fink (1994)</td>
</tr>
<tr>
<td>YDL128w</td>
<td>Vcx1, vacuole, H(^{+}/Ca^{2+}) antiporter</td>
<td>Cunningham and Fink (1996)</td>
</tr>
<tr>
<td>YGL167c</td>
<td>Pmr1, Golgi, Ca(^{2+}) ATPase</td>
<td>Antebi and Fink (1992)</td>
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</tbody>
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Enhanced Resistance of AtBI-1-Overexpressing Plants to Cyclopiazonic Acid

Pmr1 and Spf1 are members of the SERCA family and plant homologs have also been identified (Geisler et al., 2000). To understand the relationship between intracellular calcium homeostasis and BI-1 in plant cells, we evaluated the effect of cyclopiazonic acid (CPA), a specific inhibitor of SERCA, using transgenic Arabidopsis plants with AtBI-1 overexpression (OX) or knock-down (KD) lines (Fig. 4A). RT-PCR analysis demonstrated that *AtBI-1* was overexpressed in lines OX1 and OX2 and reduced in lines KD1 and KD2 (Fig. 5B). To evaluate CPA sensitivity, each plant line was grown on 0.5× Murashige and Skoog medium with or without 5 μM CPA. Although these transgenic plants showed no obvious phenotype under general growing conditions, slight growth retardation was observed in control and KD lines on CPA-containing medium (data not shown). To examine the effect of CPA on leaves, we measured chlorophyll content using threelfifths of leaves of 3-week-old plants (Fig. 5C). Although the chlorophyll content measured in untreated plants was similar in all lines, KD and control lines (wild type and GFP) demonstrated reduced chlorophyll content on CPA-containing medium. In contrast, OX lines showed elevated chlorophyll content with unknown mechanisms, suggesting that overexpression of AtBI-1 overcame the effect of SERCA inhibition by CPA. We also investigated the effects of TG, which is also a well-known SERCA inhibitor in animals. However, no obvious phenotype was evident in TG-treated plants (20 μM; data not shown). In fact, ECA1, an Arabidopsis homolog of SERCA, is CPA sensitive, but TG insensitive (Li and Ste, 1998).

To gain more insight into the function of AtBI-1, we investigated ion stress sensitivity of AtBI-1 transgenic plants. It is reported that Arabidopsis ECA1, an ER-localized SERCA-type Ca\(^{2+}\) pump, plays a role in both Ca\(^{2+}\) and Mn\(^{2+}\) homeostasis and is required to support plant growth under conditions of Ca\(^{2+}\) deficiency or Mn\(^{2+}\) toxicity (Wu et al., 2002). To evaluate ion stress sensitivity in plants with altered AtBI-1 expression, AtBI-1 transgenic plants were grown on a Ca\(^{2+}\)-deficient or Mn\(^{2+}\)-rich (0.5 mM) plate. When plants were grown on such plates for 3 weeks, KD lines showed severe growth defects and less chlorophyll content (Fig. 6). In contrast, OX plants had elevated chlorophyll content and less growth retardation in both ion stress conditions, suggesting AtBI-1 affects ion homeostasis in plant cells.

Measurement of [Ca\(^{2+}\)]\(_{cyt}\) in AtBI-1-Overexpressing Plant Cells

Alterations in cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{cyt}\)) have been implicated in apoptosis control mechanisms. The anti-apoptotic protein Bcl-2 reduces Ca\(^{2+}\) efflux through the ER membrane (Lam et al., 1994). A rapid increase in [Ca\(^{2+}\)]\(_{cyt}\) is a common response to pathogen challenge in plant cells (Blume et al., 2000) and oxidative stress (Rentel and Knight, 2004). We therefore explored the effects of BI-1 overexpression on Ca\(^{2+}\) homeostasis. To investigate the role of AtBI-1 on calcium flux in plant cells, transgenic tobacco BY-2 cells were examined using the calcium-sensitive luminescent protein aequorin. In our previous work, BY-2 cells overexpressing AtBI-1 showed resistance to H\(_2\)O\(_2\)- or SA-induced cell death (Kawai-Yamada et al., 2004). The vectors pBIG-AtBI-MBP (BI) and pBIG-MBP (VC) were introduced into tobacco BY-2 cells expressing proaequorin protein (Takahashi et al., 1997). Transformed cells were treated with H\(_2\)O\(_2\) or CPA and their viability was measured. It is known that H\(_2\)O\(_2\) and CPA induce cell death (Kawai-Yamada et al., 2004). The vectors pBIG-AtBI-MBP (BI) and pBIG-MBP (VC) were introduced into tobacco BY-2 cells expressing proaequorin protein (Takahashi et al., 1997). Transformed cells were treated with H\(_2\)O\(_2\) or CPA and their viability was measured. It is known that H\(_2\)O\(_2\) and CPA induce cell death (Kawai-Yamada et al., 2004). The vectors pBIG-AtBI-MBP (BI) and pBIG-MBP (VC) were introduced into tobacco BY-2 cells expressing proaequorin protein (Takahashi et al., 1997). Transformed cells were treated with H\(_2\)O\(_2\) or CPA and their viability was measured. It is known that H\(_2\)O\(_2\) and CPA induce cell death (Kawai-Yamada et al., 2004).
rapid \([\text{Ca}^{2+}]_{\text{cyt}}\) elevation and cell death (Berridge, 1993; Akaishi et al., 2004). Although CPA or \(\text{H}_2\text{O}_2\) treatment caused massive cell death in the control BY-2 cell line (VC), the AtBI-1 overexpressed line (BI) repressed such effects (Fig. 7A). Using the same cell lines, we monitored \([\text{Ca}^{2+}]_{\text{cyt}}\) after \(\text{H}_2\text{O}_2\) or CPA treatment. As shown in Figure 7, B and C, rapid elevation in \([\text{Ca}^{2+}]_{\text{cyt}}\) appeared as a peak at 1 to 2 min after \(\text{H}_2\text{O}_2\) or CPA treatment. The luminescence traces showed individual properties between CPA and \(\text{H}_2\text{O}_2\) treatment. The duration and peak time was similar between cells expressing vector control and AtBI-1 (data not shown). Treatment of AtBI-1-expressing cells with \(\text{H}_2\text{O}_2\) resulted in a slight decrease of \([\text{Ca}^{2+}]_{\text{cyt}}\) at peak point compared with control cells. The CPA-released calcium concentration in AtBI-1-overexpressing cells was reduced to 75% to 80% of the control at peak point. The resting value of \([\text{Ca}^{2+}]_{\text{cyt}}\) before treatment was around 100 nM in all transformed strains (data not shown).

**DISCUSSION**

**BI-1 Interacts with CaM**

Rapid elevation in \([\text{Ca}^{2+}]_{\text{cyt}}\) is a common response of organisms against various external stresses. Evidence from previous studies suggests that such \(\text{Ca}^{2+}\) fluxes trigger various cellular responses, such as microtubule depolymerization, defense gene activation, and cell death (Lecourieux et al., 2002). Using the yeast split-ubiquitin system, we demonstrated the interaction of AtBI-1 with CaM protein. Furthermore, mutants of AtBI-1 lacking the function of cell death inhibition lost the ability to interact with CaM, suggesting that interaction with CaM is essential in yeast for suppressing cell death by AtBI-1. Furthermore, overlay assay indicated that the C-terminal 14 amino acids of AtBI-1 interacted with the AtCaM7 protein. Our previous study demonstrated that the C-terminal 14 amino acid-deleted mutant protein failed to inhibit plant cell death caused by \(\text{H}_2\text{O}_2\) or SA (Kawai-Yamada et al., 2004), suggesting that the C-terminal region is essential for the inhibition of cell death by AtBI-1.

Recent studies have demonstrated close interactions between CaM and several enzymes involved in oxidative cell death responses. For instance, CaM interacts with and activates some plant catalases (Yang and Poovaiah, 2002) and NAD kinase, which generates NAD(P)H for NAD(P)H oxidase (Turner et al., 2004). In addition, the barley Mlo protein, a plant-specific modulator of cellular defense and cell death, also binds to CaM and the interaction was related to the control of...
pathogen defense (Kim et al., 2002). In this study, we showed that AtBI-1 also interacted with CaM, suggesting that CaM may be one of the key factors regulating cell death or survival. The biochemical function of CaM-binding BI-1 remains to be elucidated.

**Anti-Cell Death Function of AtBI-1 Is Associated with Calcium Flux**

Several investigators point to a direct role of calcium in controlling life and death of plant cells as well as mammalian cells (Sanders et al., 2002). This study indicated that AtBI-1 did not suppress Bax-induced cell death in yeast mutants lacking Pmr1 and Spf1. The two proteins are homologs of animal SERCA pumps localized at the Golgi and ER, respectively (Antebi and Fink, 1992; Cronin et al., 2002) and are known to be involved in ER calcium homeostasis (Strayle et al., 1999; Vashist et al., 2002). The inability of AtBI-1 to suppress cell death in Δpmr1 and Δspf1 suggests that an accurate calcium flux at the Golgi or ER may be needed for the cell death inhibitory function of AtBI-1 in yeast. Mutants deficient in other calcium transporters located at the area, such as the PM or the vacuole, did not influence the antiapoptotic function of AtBI-1, supporting the conclusion that cell death suppression by AtBI-1 involves ER or Golgi calcium translocation.

To investigate the effect of AtBI-1 on calcium response to plant cell death stimuli, we measured changes in \([\text{Ca}^{2+}]_{\text{cyt}}\) in response to \(\text{H}_2\text{O}_2\) and CPA using proaequorin protein-expressing tobacco BY-2 cells. The finding that \(\text{H}_2\text{O}_2\) treatment on tobacco plants induced a transient increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) was previously reported.

**Figure 5.** CPA resistance in Arabidopsis plants overexpressing AtBI-1. A, Schematic diagrams of the vectors used for production of OX and KD transgenic lines. P35S, CaMV 35S promoter; LB, left T-DNA border; RB, right T-DNA border; NosT, nopaline synthetase terminator; KanR, kanamycin resistance gene. B, RT-PCR analysis of AtBI-1 expression in wild type (WT), OX, KD, and GFP transgenic plants. Total RNA was prepared from 2-week-old seedlings. *Actin8* (*ACT8*) was used as an internal control. Ethidium bromide staining of RNA was served as a loading control. C, Chlorophyll content in three-fifths of leaves obtained from transgenic plants grown on 0.5× Murashige and Skoog medium (control) or 0.5× Murashige and Skoog medium with CPA for 3 weeks. Data are mean ± se; \(n = 4\) to 6.
In this study, a rise in \([\text{Ca}^{2+}]_{\text{cyt}}\) was found at 1 to 2 min after H\(_2\)O\(_2\) or CPA treatment, similar to the result described by Price et al. (1994). In contrast, Lecourieux et al. (2002) showed biphasic elevation caused by cryptogein of *Phytophthora cryptogea* and H\(_2\)O\(_2\) in *Nicotiana plumbaginifolia* cells. The \([\text{Ca}^{2+}]_{\text{cyt}}\) elevation caused by various treatments might be different in magnitude, duration, and frequency, leading to diverse cellular responses, such as adaptation to various stresses and cell death (Lecourieux et al., 2002).

Recently, Kadota et al. (2005) demonstrated that the Ca\(^{2+}\)-permeable channel NtTPC1A/B located at the PM plays a role in a H\(_2\)O\(_2\)-induced rise in \([\text{Ca}^{2+}]_{\text{cyt}}\) in tobacco BY-2 cells. Inhibition of SERCA by CPA treatment also leads to a rise in \([\text{Ca}^{2+}]_{\text{cyt}}\) (Berridge, 1993). Because of leaking of Ca\(^{2+}\) through an unidentified channel, inhibition of the Ca\(^{2+}\) pump by CPA induces an efflux of Ca\(^{2+}\) from the ER into the cytosol, producing

![Figure 6](image-url)  
*Figure 6.* Phenotypes of transgenic plants associated with ion stress conditions. A. Phenotypes of transgenic plants grown on 0.5× Murashige and Skoog medium with 0.5 mM MnCl\(_2\) (+Mn) or without Ca\(^{2+}\) (−Ca) for 3 weeks. B. The third leaves obtained from transgenic plants grown on the 0.5× Murashige and Skoog medium with 0.5 mM MnCl\(_2\) or without Ca\(^{2+}\) for 3 weeks. C. Chlorophyll content in transgenic plants grown on 0.5× Murashige and Skoog medium with 0.5 mM MnCl\(_2\) or without Ca\(^{2+}\) for 3 weeks. Data are mean ± se; n = 4 to 6.

![Figure 7](image-url)  
*Figure 7.* Measurement of \([\text{Ca}^{2+}]_{\text{cyt}}\) following H\(_2\)O\(_2\) or CPA treatment in AtBI-1-overexpressing BY-2 cells. A. Inhibition of H\(_2\)O\(_2\)- or CPA-induced cell death by AtBI-1. Viability of BY-2 cells expressing empty vector (VC) or AtBI-MBP (BI) after 3 mM H\(_2\)O\(_2\), treatment for 16 h, or after treatment with 1, 5, or 10 μM CPA for 16 h. Evans blue negative (living) and positive (dead) cells were counted under a microscope. Each bar represents the mean ± se values of three independent experiments (n = 10–18). B. Typical aequorin luminescence traces of cells expressing AtBI-1 treated with 3 mM H\(_2\)O\(_2\), or 10 μM CPA. Arrows indicate the time of CPA and H\(_2\)O\(_2\) administration. AL, Aequorin luminescence. C. Measurement of a peak \([\text{Ca}^{2+}]_{\text{cyt}}\) after treatment with 3 mM H\(_2\)O\(_2\) or 10 μM CPA. Each bar represents the mean ± se values of three independent experiments. n = 12 to 14; **, P < 0.01; *, P < 0.05.
an increase in cytosol-free Ca$^{2+}$ concentration (Zuppini et al., 2004; Sivaguru et al., 2005). In this study, CPA- and H$_2$O$_2$-induced calcium elevation was reduced by AtBI-1 overexpression in tobacco cells. This result is in agreement with those of previous studies demonstrating that human BI-1 overexpression led to decreased ER-releasable calcium concentration in human fibrosarcoma cells as well as Bcl-2 overexpression in various animal cells (Foyouzi-Youssefi et al., 2000; Chae et al., 2004). As mentioned above, inhibition of the SERCA pump causes the passive release of calcium from the ER and an increase in cytosolic calcium, which induces cell death (Zhou et al., 1998). However, overexpression of SERCA can also result in induction of cell death (Ma et al., 1999). These results indicate that accurate management of intracellular calcium homeostasis is necessary for cell living/death regulation.

We found that Arabidopsis plants with overexpressed or knocked-down AtBI-1 demonstrated altered CPA or ion stress tolerance, as reported in ECA1 transgenic plants (Wu et al., 2002), suggesting that AtBI-1 also plays a role in regulation of calcium homeostasis. Whether AtBI-1 acts solely in modulating ER-related [Ca$^{2+}$]$_{cyt}$ flux or also regulates other steps remains to be determined.

Ca$^{2+}$ pumps are widely distributed on membranes, including the PM, vacuole, Golgi, and ER. The role of Ca$^{2+}$ in the cellular signaling pathway is well recognized in mammalian cells, whereas regulation of Ca$^{2+}$ homeostasis in plants is still poorly understood. CPA and TG are well-known inhibitors of the SERCA, causing an increase in [Ca$^{2+}$]$_{cyt}$ (Wuytack et al., 2003).

In our experimental system, TG treatment did not cause effective Ca$^{2+}$ efflux to the cytosol for unknown reasons. Conflicts of such experimental results need to be solved by isolation of target pumps.

Sze et al. (2000) classified plant Ca$^{2+}$ pumps in two different classes based on similarity to animal proteins: type IIA, homologous to the SERCA class of Ca$^{2+}$ ATPases located in the ER, and type IIB, homologous to the PM-type Ca$^{2+}$ ATPases. Interestingly, Arabidopsis type IIA Ca$^{2+}$ ATPase ECA1 can complement the yeast mutant defective in both Golgi and vacuole Ca$^{2+}$ pumps (pmr1 and pmc1, cnb1; Liang et al., 1997; Sze et al., 2000). Furthermore, ECA1 is inhibited by CPA, but not by TG (Liang and Sze, 1998). In contrast, ACA2 of Arabidopsis encoding a type IIB Ca$^{2+}$ pump is unaffected by both CPA and TG (Hwang et al., 2000). A pump showing mixed characteristics of both ECA and ACA pumps was also identified in maize (Zea mays; Subbaiah and Sachs, 2000).

There is no experimental evidence for the regulation of plant type IIA Ca$^{2+}$ pump activity. The most closely related ER-type calcium pump in animals, SERCA, is highly regulated by phosphorylation (East, 2000). Because the plant type IIA pump is not directly regulated by CaM (Liang and Sze, 1998), AtBI-1 may act as the mediator between CaM signaling and Ca$^{2+}$ pumps; or it is possible to speculate that the AtBI-1 may recruit or remove CaM to or from the pump. Our data suggesting the involvement of Ca$^{2+}$ ATPases localized at the ER or Golgi for BI-1 function in the yeast system are in good agreement with this model. To elucidate the real function of AtBI-1 in plant cells, further analysis in AtBI-1/AtCaM binding, calcium flux measurement, and target pumps in Arabidopsis is needed.

It is demonstrated that overexpression of BI-1 overcomes various stress-induced defects. For example, H$_2$O$_2$, SA-, elicitor-, heat-, and cold-induced cell death were suppressed in plant cells overexpressing AtBI-1 (Chae et al., 2003; Matsumura et al., 2003; Kawai-Yamada et al., 2004). Expression of plant BI-1 genes is rapidly up-regulated during wounding, pathogen attack (Sanchez et al., 2000), and senescence (Coupe et al., 2004), but down-regulated by elicitor treatment (Matsumura et al., 2003). These results suggest the involvement of BI-1 in the stress response and the HR in plants. Furthermore, AtBI-1 OX and KD plants grew normally under general growth conditions. This suggests that AtBI-1 is not essential for normal development and growth in Arabidopsis. Recently, two Arabidopsis mutants with a T-DNA insertion in the AtBI-1 gene (atbi1-1 and atbi1-2) were characterized by Watanabe and Lam (2006). Both mutants also showed no obvious phenotype under general growth conditions. However, these mutants exhibited accelerated cell death upon exposure to the fungal toxin fumonisin B1. BI-1-deficient mice also showed normal growth; however, increased susceptibility to ER damage and oxidative stresses was reported (Chae et al., 2004). In conclusion, BI-1 is presumably not required for physiological regulation of development of plant PCD. Rather, BI-1 possesses evolutionarily conserved functions that allow it to provide tolerance against stress-mediated cell death.

**MATERIALS AND METHODS**

**Yeast Split-Ubiquitin Two-Hybrid System**

Protein interaction with AtBI-1 was analyzed using the yeast (Saccharomyces cerevisiae) split-ubiquitin system (Stagljar et al., 1998; Kim et al., 2002). Plasmid vector (Hy-CaM3) and yeast cells were kindly provided by Dr. Ralph Panstruga and Dr. IE Somisich (Max-Planck Institute). The C-terminal mutants of AtBI-1 were constructed using PCR, as described previously (Kawai-Yamada et al., 2004). The coding region of AtBI-1 and the C-terminal mutants (AtBI-29, 30, and 32) were cloned into pMet-Cub-Ura3 vector to construct the bait plasmids. Yeast cells (ID53 strain) were transformed with different combinations of bait (AtBI-1 and the mutants) and prey constructs, and the His ‘Trp’ Ura’ transformants were streaked on minimal medium containing 5-fluoroorotic acid (5-FOA; 0.1%) and were incubated at 30°C for 4 d. Replacement of Ile-13 of wild-type Nubi by Ala (NuA) decreases the affinity to Cubi (Stagljar et al., 1998; Kim et al., 2002). The low Cubi-affinity NuA prey variant served as a negative control.

**Plant Materials**

Transgenic plants expressing AtBI-1 and GFP (Arabidopsis [Arabidopsis thaliana] ecotype Col-0) were described previously in Kawai-Yamada et al. (2001, 2004). To generate KD plants, an RNAi plasmid, pKGW1WG2(CII)-AtBI, was constructed. Briefly, the 3’-terminal fragment of AtBI-1 cDNA (+291/+744; translation initiation is +1) was introduced into the RNAi plasmid pKGW1WG2(CII) (Karimi et al., 2002) by the Gateway system. The resulting
plasmid was transferred to Agrobacterium tumefaciens strain EHA105 (Hood et al., 1993) using the freeze-thaw method (An et al., 1998). Transformation of Arabidopsis plants was carried out by the floral-dip method (Bechtold andPelletier, 1998). Obtained plants were grown at 23°C under continuous light conditions (60 μmol m⁻² s⁻¹).

**CaM Overlay Assay**

We used an overlay assay to examine the ability of the C-terminal region of AtBI-1 to bind with Arabidopsis CaM 7 (AtCaM7) protein in vitro. The AtCaM7 cDNA was inserted into pET32- vector (Novagen) to obtain His- and S-tagged AtCaM7 protein. The resultant vector pET32-AtCaM7 was transformed into Escherichia coli BL21 strain and the recombinant His- and S-tagged CaM7 protein was purified according to the manufacturer’s instructions (Invitrogen).

The 14-amino-acid C terminus of AtBI-1 was ligated into pMAL vector (NEB) to express MBP-tagged protein. The resultant vector was transformed into E. coli BL21 strain and MBP-tagged Bi-C protein was purified according to the instructions provided by the manufacturer (NEB). The empty pMAL vector possessing in-frame Gal was used as a control (MAL-Gal). The purified Bi-C and MAL-Gal proteins were applied to SDS-PAGE, then blotted onto polyvinylidene difluoride membranes. The membranes were immunoblotted with anti-MBP antibody (NEB) or overlay assayed with recombinant AtCaM7 protein in the presence of 1 mM CaCl₂ or 5 mM EGTA as described by Kim et al. (2002). To detect CaM7 binding, horseradish peroxidase-conjugated S-protein (Novagen) and an ECL kit (Amersham Biosciences) were used because CaM7 protein was expressed as S-tagged protein.

**BiFC Analysis**

Plasmids for BiFC analysis were kindly provided from Dr. Klaus Harter (University of Köln) and Dr. Hironori Kaminaka (University of Tottori; Walter et al., 2004). PCR-amplified coding regions of AtBI-1 and AtCaM7 were introduced into the Xhol/ BamHI site. The resultant plasmid, pSPYNE-AtBI-1, was fused with the N-terminal (1–155 amino acids) region of YFP, and pSPYCE-AtCaM7 was fused with the C-terminal (156–239 amino acids) part of YFP. BiFC was performed in 6-week-old Nicotiana benthamiana plants after Agrobacterium-mediated transient transformation according to Walter et al. (2004). For the BiFC signal, a fluorescence microscope (DMRd; Leica) was applied. For the control experiment, plBiN-AtBI-GFP (Kawai-Yamada et al., 2001) was infiltrated to N. benthamiana leaves and GFP fluorescence was examined.

**Yeast Strain and Cell Death Suppression Assay**

Yeast disruption mutants were purchased from Euroscarl. Plasmids used here were described previously (Kawai et al., 1999; Kawai-Yamada et al., 2001). Yeast disruption mutants and wild-type (BY4741) cells were transformed with pMAL vector harboring MBP (pYX112) and was incubated at 23°C for 2 d (Glc) or 4 d (Gal).

**Cytological Analysis**

GFP-tagged AtBI-1 (pYX112-AtBI-GFP) was transformed into yeast cells (Kawai-Yamada et al., 2001). GFP fluorescence was examined with a fluorescence microscope (DMRd; Leica). For measurement of cell viability, Nicotiana tabacum BY-2 cells were treated with the indicated reagents for 16 h and stained with 0.03% Evans blue. The numbers of stained (dead) and unstained (live) cells were counted under microscopy.

**RT-PCR**

Arabidopsis and yeast total RNA was extracted using the RNasy ministart kit (Qiagen) and 0.2 μg were used as a template for RT-PCR with specific primers for AtBI-1 (5'-GGATATGAGGCTTGATGCTGATC'- and 5'-GTGTAGTCTGTTTTCTCC-3' and Actin8 (5'-GCCAGACTACCCATCGTCGACAG'- and 5'-TCGCTTCTGCTGTATGACAC'-3'). The conditions for direct amplification with Ready-to-Go RT-PCR Beads (Amersham-Pharmacia) were 1 cycle at 42°C for 20 min and at 95°C for 5 min; 28 cycles at 95°C for 30 s, at 58°C for 30 s, and at 72°C for 1 min.

**Ion Stress Assay**

Ion stress treatment was performed as described by Wu et al. (2002). Briefly, surface-sterilized seeds were germinated on an 0.8% agar plate with 0.5X Murashige and Skoog medium with 0.5 mM MnCl₂ or calcium-free medium under constant illumination (60 μmol m⁻² s⁻¹). To measure chlorophyll content, leaves obtained from each seedling were homogenized with 80% acetone. The supernatant, collected after centrifugation, was examined at 663 and 645 nm, and the chlorophyll content was calculated as described by Kawai-Yamada et al. (2001).

**Cytosolic Calcium Concentration**

N. tabacum BY-2 suspension culture cells expressing proaequorin in the cytosol (Takahashi et al., 1997) were used for the measurement of [Ca²⁺]cyt. The coding sequence of MBP was amplified from pMAL vector by PCR and was cloned into pBVG vector containing the 35S promoter of the Cauliflower mosaic virus (CaMV) and the nopaline synthase terminator to generate pBIG-MBP. To express AtBI-MBP in BY-2 cells, PCR-amplified fragments of AtBI-1 were cloned into pBVG-MBP. The resultant vectors, pBVG-AtIB-MBP and pBVG-MBP (control), were used for the Agrobacterium-mediated transformation of N. tabacum BY-2 cells expressing aequorin protein (Takahashi et al., 1997).

[Ca²⁺]cyt was measured by a method as described previously (Takahashi et al., 1997; Kawanuo et al., 1998). Transformed BY-2 cells (3 d old) were incubated with 1 μM coelenterazine in the culture medium in darkness for 8 h to reconstitute the calcium-sensitive luminescent protein aequorin. Cells were washed with and resuspended in fresh medium and used after 30 min of resting incubation. H₂O₂ or CPA was added to the cells and [Ca²⁺]cyt- depend- ent aequorin luminescence was monitored with a CHEM-GLow photom- eter (American Instrument Co.) equipped with a pen recorder (Rikadenki Co.), and expressed as relative peak height of aequorin luminescence. After each experiment, all remaining aequorin was discharged with 1 μM CaCl₂ and 10% ethanol, and the resultant luminescence was measured to estimate the amount of remaining aequorin. [Ca²⁺]cyt was calculated using the calibration equation: pCa = 0.332988(–log k) + 3.5993, where k represents luminescence counts per second divided by total counts (Knight et al., 1996).

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**LITERATURE CITED**


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Bax Inhibitor-1 Associated with Ion Homeostasis
