The *Arabidopsis thaliana* ethylene-responsive element binding protein (AtEBP) can function as a dominant suppressor of Bax-induced cell death of yeast

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Abstract We identified genes based on screening of an *Arabidopsis* cDNA library for functional suppressors of mouse Bax-induced cell death of yeast cells. Interestingly, the cDNA encoding AtEBP, known as *Arabidopsis thaliana* ethylene-responsive element binding protein, was isolated numerous times in the functional screen (82% of all suppressors). Full-length AtEBP and its localization to the nucleus were essential for the suppression of Bax-induced cell death. Morphological abnormality of intracellular network that is a hallmark of Bax-induced cell death was attenuated by expression of AtEBP. **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: *Arabidopsis thaliana* ethylene-responsive element binding protein; Bax; Cell death; Yeast; *Arabidopsis*

1. Introduction

The mammalian Bax is a pro-apoptotic protein of the Bcl-2 family that can induce cell death in the budding yeast, *Saccharomyces cerevisiae* [1–3]. In fact, a mammalian cell death inhibitor was identified as a suppressor of Bax-induced cell death of yeast [4]. These results indicate that a cell death process for which Bcl-2 family proteins are pivotal positive or negative effectors must function in yeast; although the molecular entities are not structurally similar since bioinformatics analysis does not reveal their identity from the yeast genome database. Recently, we established that transgenic *Arabidopsis* plants overexpressing Bax protein exhibit marked cell death and characteristics of apoptosis such as cell shrinkage, membranous destruction and other apoptotic phenotypes [5]. Plants expressing the plant homolog of mammalian Bax inhibitor-1 (AtBI-1 [6]), together with Bax, were able to sustain growth (or better rather than those expressing Bax), indicating that AtBI-1 can function as an anti-apoptotic protein in planta [5]. Since AtBI-1 was first demonstrated as a Bax inhibitor in yeast, we were prompted to identify other plant genes that could suppress Bax-induced lethality using the yeast system.

In this study we employed an *Arabidopsis* cDNA library to screen suppressors against lethal effects of mouse Bax in yeast. Consequently, AtBI-1 and genes involved in the detoxification of ROS (reactive oxygen species) such as Fe-SOD (Fe-superoxide dismutase), peroxidase and GST (glutathione *S*-transferase) were isolated. The most abundant gene was found to be AtEBP, known as *Arabidopsis thaliana* ethylene-responsive element binding protein [7].

We report here that a full-length of AtEBP and its nuclear location are essential for the suppression of Bax-induced cell death in yeast. Intracellular defects induced by Bax were restored by the co-presence of AtEBP in yeast.

2. Materials and methods

2.1. Yeast strains and transformation

The *S. cerevisiae* strain QX95001 is BF264-15Dau (*MATα ade1 his2 leu2-3, 112 trp1-1a ura3*) [8] containing the LEU2-marked mouse Bax-encoding plasmid YEp51-Bax [4], and was maintained in synthetic dropout medium lacking leucine. Yeast transformations were performed by the lithium acetate method [9]. Transformants were grown on glucose-containing synthetic dropout medium lacking leucine and uracil at 30°C.

2.2. Preparation of expression plasmids for full-length and truncation series of AtEBPs

The full-length or deletion mutants of AtEBP sequences, tagged with *Eco*RI and *Hin*dIII sites by PCR, were subcloned into *Eco*RI–*Hin*dIII-digested pYX112 (Ura-marked), which includes a 2 μm replicon (Boehringer Mannheim).

2.3. Immunoblot analysis

Yeast cells cultured in glucose-containing medium to an OD_{600} of ~1.0 were washed three times, and subjected to additional 16 h culture in either fresh glucose- or galactose-containing medium. Yeast cells collected by a centrifugation were re-suspended in the buffer containing 8 M urea, 5% SDS, 40 mM Tris–HCl (pH 6.8), 0.1 mM EDTA, 0.4 mg/ml BPB and 10 μl/ml 2-mercaptoethanol. 80% vol. of acid-washed glass beads (425–600 μm) (Sigma) was added and the tubes were vortexed for 2 min and boiled. Proteins separated by SDS–15% polyacrylamide gel electrophoresis were transferred onto polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). After blocking with 5% skim milk in PBS at 4°C for overnight, membranes were incubated in 5% skim milk in TBST (50 mM Tris–HCl [pH 7.5], 150 mM NaCl and 0.05% Triton X-100) solution with polyclonal antibody for Bax (Upstate Biotechnology), followed by a 1:2000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG (Amersham) secondary antibody. Detection was accomplished by an enhanced chemiluminescence method (Amersham) with exposure to X-ray film (Fuji).

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2.4. Cellular localization of green fluorescent protein (GFP)-tagged AtEBP

Chimeric genes possessing GFP (pBINm-gfp5-ER, ER signal removed) [10–13] at either the N- or C-terminal end of AtEBP were constructed, and cloned into pYX112 for transformation of QX95001. For the observation of GFP fluorescence and DAPI staining, yeast cells were fixed in 70% ethanol and subjected to fluorescence microscope (Leica, HC).

Oligonucleotide-directed mutagenesis was used for the introduction of mutations or deletion on the putative nuclear localization signal (NLS) for AtEBP. The proper construction of all plasmids was confirmed by DNA sequencing.

2.5. Electron microscopic study

Sample preparation and treatments were essentially the same as described by Ueda et al. [14]. After the fixation of cells by the freeze-substitution, frozen cells were treated with 4% osmium tetraoxide (OsO_4) and embedded in Spurr's resin. Serial sections stained with uranyl acetate and lead citrate were observed using the Zmodel 2010 electron microscope (JEOL, Akishima-shi, Japan).

3. Results and discussion

A cDNA library derived from *Arabidopsis* suspension cultured cells was constructed into the yeast expression vector [15], which was then screened for suppressors of Bax-induced cell death of the *S. cerevisiae* strain QX95001. This strain was obtained by transforming BF264-15 Dau (*MATα ade1 his2 leu2-3, 112 trp1-1a ura3*) [8] with the plasmid YEp51-Bax from which the full-length mouse Bax protein is expressed under the control of the galactose-inducible yeast *GAL10* promoter [3]. Bax-induced cell death of this strain is triggered by transferring cells from glucose- to galactose-containing medium [3]. Accordingly, an *Arabidopsis* cDNA library was introduced into the QX95001 strain and screened for Bax-resistant transformants. A primary screen of 10^6 colonies identified about 600 that survived after induction of Bax expression on galactose-containing medium. The cDNAs were

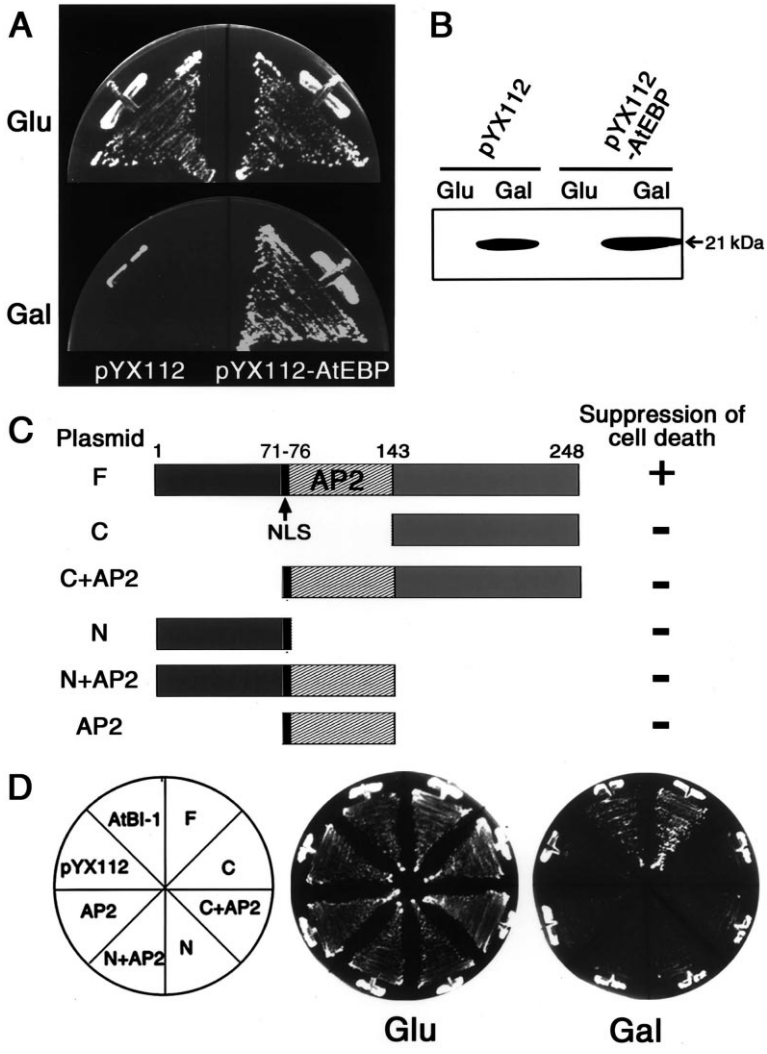

Fig. 1. Suppression of Bax-induced cell death by AtEBP in yeast. A: Either control vector (pYX112) or AtEBP expressing plasmid (pYX112-AtEBP) were transformed into yeast QX95001 strain possessing YEp51-Bax. Transformants were streaked on either glucose- (Glu) or galactose- (Gal) containing medium, and incubated at 30°C for 3 days. B: Immunological detection of Bax protein in yeast expressing AtEBP. Yeast strain possessing Bax was transformed with pYX112 or pYX112-AtEBP, cultured in glucose medium until an OD_{600} of ~1.0, then transferred to glucose (Glu) or galactose (Gal) medium for additional culture (16 h). C,D: Evaluation of cell death inhibition activity in truncation series of AtEBPs. The control plasmids (pYX112 and AtBI-1), full-length AtEBP (F) and constructed AtEBP mutants (C, C+AP2, N, N+AP2 and AP2) were transformed to QX95001 and streaked on glucose (Glu) or galactose (Gal) medium. The photographs were taken after 3 days of incubation at 30°C. The results of cell death suppression are given on the right in C.

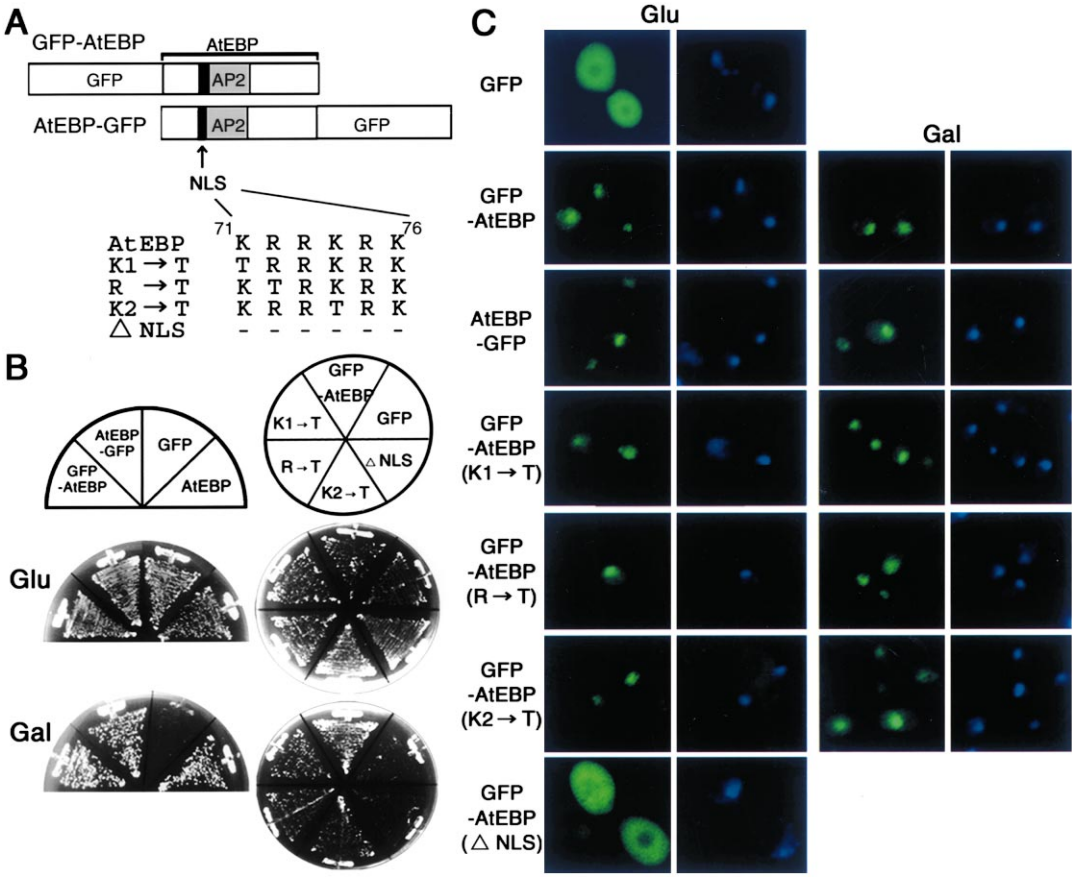

Fig. 2. Intracellular distribution and cell death suppression of GFP-tagged AtEBP and NLS-mutations in yeast. A: A schematic figure showing AtEBP tagged with GFP (GFP–AtEBP or AtEBP–GFP) and mutations (K1→T, R→T, K2→T and ΔNLS) in NLS region (71–76). GFP (m-gfp-ER, ER signal removed) was fused to either the N-terminal (GFP–AtEBP) or C-terminal (AtEBP–GFP) of AtEBP, respectively. Using GFP–AtEBP, NLS mutated plasmids were constructed. B: Yeast cells possessing Bax gene were transformed with each plasmid and streaked on the glucose (Glu) and galactose (Gal) medium. As a control, GFP or AtEBP alone in the presence of Bax in yeast was also presented. C: Fluorescence distribution of GFP and GFP-tagged AtEBP (WT and NLS-mutations) in yeast possessing Bax. GFP fluorescence (left) and DAPI staining (right) are presented. Yeasts were cultured in glucose or galactose medium until an OD_{600} of ~0.5 before observation.

isolated from the colonies and retransformed into QX95001 and 34 colonies were recovered that exhibited substantial suppression of Bax-induced cell death on galactose-containing medium.

Nucleotide sequence analysis of DNA inserts indicated that two of 34 cDNA clones encoded AtBI-1 [6]. The predicted products of these clones; Fe-SOD [16], peroxidase [17] and GST [18], could be involved in oxidative metabolism. In this respect, Madeo et al. [19] suggested an accumulation of ROS in Bax-induced apoptosis in yeast. Kampranis et al. [20] isolated a novel tomato GST/peroxidase that suppressed Bax lethality in yeast. Expression of GST/GPX significantly enhanced resistance to H_2O_2-induced stress in yeast, underlining the relationship between oxidative stress and Bax-induced death in yeast cells. One cDNA clone encoded a GTP-binding protein highly similar to a member of the YPT/rab subfamily in pea [21]. Interestingly, 82% of the clones (28 of 34) encoded AtEBP which was isolated as an ethylene-inducible, GCC box DNA-binding protein that interacts with the *Arabidopsis* OBF4 protein [7]. AtEBP has one AP2/ERF domain [22], which binds specifically to the GCC box, as established by electrophoretic mobility-shift assay and DNase I footprint analyses [7]. *AtEBP* expression is inducible by exogenous ethylene in wild-type plants and *AtEBP* transcript abundance is

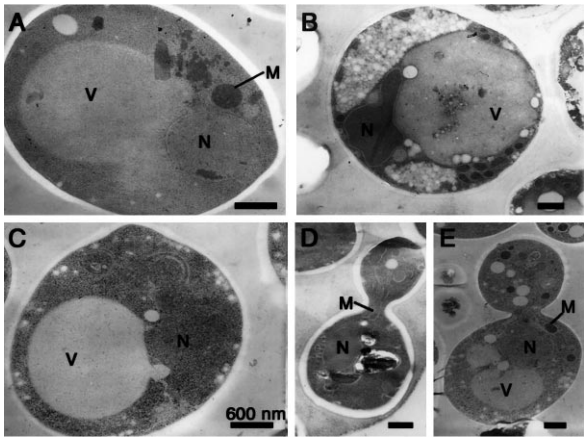

Fig. 3. Electron microscopic observation of yeast cells. Yeast strain QX95001 containing YEp51-Bax was transformed with either pYX112 (A,B,D) or pYX112-AtEBP (C,E). Cells were cultured on agar medium containing either glucose (A) or galactose (B–E) for 3 days, which were then provided for electron microscopic observation. N, nucleus; M, mitochondrion; V, vacuole. Bar: 600 nm.

greater in the *ctr1-1* mutant, in which ethylene-regulated signal pathways are constitutively activated [7].

As shown in Fig. 1A, QX95001 transformed with AtEBP could grow on SD-galactose medium, showing a strong inhibition of AtEBP against Bax-induced cell death. Moreover, the Bax protein was detected as a single peptide band of about 21 kDa in protein samples from yeast cells co-transformed with either control vector (pYX112) or AtEBP expressing plasmid (pYX112-AtEBP) and grown on medium with galactose (Fig. 1B), confirming that AtEBP did not down-regulate the Bax production in yeast. To determine the essential regions in AtEBP necessary for suppressing Bax-induced cell death in yeast, we generated the following deletions: C-terminal region (amino acids 143–248), NLS signal peptide, AP2 domain and C-terminal region (amino acids 71–248), N-terminus and NLS (amino acids 1–76); N-terminus, NLS and AP2 domain (amino acids 1–143) and NLS and AP2 domain (amino acids 71–143). After transformation of QX95001, only cells expressing the full-length AtEBP survived on galactose-containing medium, while those expressing deletion variants did not (Fig. 1C,D). Thus, it remains to be seen whether or not any specific domain is essential for the cell death suppression activity of this protein, since such deleted mutant protein may potentiate in the destruction of protein structure and other ambiguities.

To explore the intracellular localization of AtEBP in yeast, AtEBP proteins tagged with GFP on the N- (GFP–AtEBP) or C-terminus (AtEBP–GFP) were expressed in QX95001. Both proteins had the capacity to suppress Bax-induced cell death on galactose-containing medium, while GFP alone did not (Fig. 2A,B). This indicated that fusion of GFP protein to either the N- or C-terminus of AtEBP did not alter its capacity to suppress Bax-induced lethality in yeast. Fluorescence microscopy established that GFP–AtEBP and AtEBP–GFP were localized to the yeast cell nucleus as indicated by DAPI staining (Fig. 2C). In contrast, GFP as a control was distributed throughout the cell. NLS-deleted AtEBP did not translocate to the nucleus, indicating that nuclear targeting [23] AtEBP was essential for its suppression of Bax-induced lethality.

Electron microscopic observation was carried out to investigate intracellular morphology in yeast cells possessing Bax and AtEBP. As seen in Fig. 3B,D, Bax caused an abnormal morphology of nucleus, large accumulation of autophagic bodies in vacuole and aberrant cell division. Such intracellular abnormalities caused by Bax were restored by the co-presence of AtEBP in yeast (Fig. 3C,E).

Vergani et al. [24] isolated *CKC1*, an identical gene to *Arabidopsis ANT* belonging to AP2-related gene family. They also demonstrated that LexA–ANT fusion protein activated transcription of a reporter gene from promoters containing *lexA* operators in yeast. Therefore, it may be needed to analyze any transcriptional activity of AtEBP in yeast.

At present, we were unable to identify any deposited DNA sequences similar to AtEBP in yeast or metazoan. The possible existence of unknown proteins conferring functional similarity can be anticipated in evolutionally distinctive species. In addition, studies on the biological significance of AtEBP in plant cell death pathway will be a subject of future investigation.

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References

[1] Sato, T., Hanada, M., Bodrug, S., Irie, S., Iwama, N., Boise, L.H., Thompson, C.B., Golemis, E., Fong, L., Wang, H.-G. and Reed, J.C. (1994) Proc. Natl. Acad. Sci. USA 91, 9238–9242.
[2] Greenhalf, W., Stephan, C. and Chaudhuri, B. (1996) FEBS Lett. 380, 169–175.
[3] Zha, H., Fisk, H.A., Yaffe, M.P., Mahajan, N., Herman, B. and Reed, J.C. (1996) Mol. Cell. Biol. 16, 6494–6508.
[4] Xu, Q. and Reed, J.C. (1998) Mol. Cell 1, 337–346.
[5] Kawai-Yamada, M., Jin, L., Yoshinaga, K., Hirata, A. and Uchimiya, H. (2001) Proc. Natl. Acad. Sci. USA, in press.
[6] Kawai, M., Pan, L., Reed, J.C. and Uchimiya, H. (1999) FEBS Lett. 464, 143–147.
[7] Büttner, M. and Singh, K.B. (1997) Proc. Natl. Acad. Sci. USA 94, 5961–5966.
[8] Lew, D.J., Dulic, V. and Reed, S.I. (1991) Cell 66, 1197–1206.
[9] Gietz, R.D. and Woods, R.A. (1994) in: Molecular Genetics of Yeast: Practical Approaches (Johnston, J.A., Ed.), pp. 121–134, Oxford University Press, Oxford.
[10] Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W. and Prasher, D.C. (1994) Science 263, 802–805.
[11] Heim, R., Prasher, D.C. and Tsien, R.Y. (1994) Proc. Natl. Acad. Sci. USA 91, 12501–12504.
[12] Siemering, K.R., Golbik, R., Sever, R. and Haseloff, J. (1996) Curr. Biol. 6, 1653–1663.
[13] Haseloff, J., Siemering, K.R., Prasher, D.C. and Hodge, S. (1997) Proc. Natl. Acad. Sci. USA 94, 2122–2127.
[14] Ueda, T., Matsuda, N., Anai, T., Tsukaya, H., Uchimiya, H. and Nakano, A. (1996) Plant Cell 8, 2079–2091.
[15] Umeda, M., Bhalerao, R.P., Schell, J., Uchimiya, H. and Koncz, C. (1998) Proc. Natl. Acad. Sci. USA 95, 5021–5026.
[16] Camp, W.V., Bowler, C., Villarroel, R., Tsang, E.W.T., Montagu, M.V. and Inzé, D. (1990) Proc. Natl. Acad. Sci. USA 87, 9903–9907.
[17] Capelli, N., Tognolli, M., Flach, J., Overney, S., Penel, C., Greppin, H. and Simon, P. (1996) Plant Physiol. 112, 446.
[18] Zhou, J. and Goldsbrough, P.B. (1993) Plant Mol. Biol. 22, 517–523.
[19] Madeo, F., Fröhlich, E., Ligr, M., Grey, M., Sigrist, S.J., Wolf, D.H. and Fröhlich, K.U. (1999) J. Cell Biol. 145, 757–767.
[20] Kampranis, S.C., Damianova, R., Atallah, M., Toby, G., Kondi, G., Tsichlis, P.N. and Makris, A.M. (2000) J. Biol. Chem. 275, 29207–29216.
[21] Nagano, Y., Murai, N., Matsuno, R. and Sasaki, Y. (1993) Plant Cell Physiol. 34, 447–455.
[22] Fujimoto, S.Y., Ohta, M., Usui, A., Shinshi, H. and Ohme-Takagi, M. (2000) TPlant Cell 12, 393–404.
[23] Kalderon, D., Richardson, W.D., Markham, A.F. and Smith, A.E. (1984) Nature 311, 33–38.
[24] Vergani, P., Morandini, P. and Soave, C. (1997) FEBS Lett. 400, 243–246.