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Identification of the *Arabidopsis thaliana* cell growth defect factor suppressing yeast cell proliferation

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Abstract

We identified cdf based on screening of the Arabidopsis cDNA library for functional suppressors of the AtBI-1 (a gene described to suppress the cell death induced by Bax gene expression in yeast). The cdf was located on Chr. V and was composed of 5 exons and 4 introns. It encodes a protein of 258 amino acid residues with a molecular weight of 28.8 kDa. The protein has 3 transmembrane domains in the C-terminal region. The cdf has one homologue, named cdf2, which was found in *Arabidopsis*. Like cdf, cdf2 also induced growth defect in yeast. The effect of the cell growth defect factor was somewhat lower than Bax. cdf could arrest the growth of yeast. Its localization to the nucleus was essential for the suppression of yeast cell proliferation. Morphological abnormality of intracellular network, which is a hallmark of AtBI-1, was attenuated by expression of cdf.

Key words : *Arabidopsis thaliana*, **AtBI-1, cdf, PCD, suppressing yeast**

Introduction

PCD, programmed cell death, plays a critical role in controlling the development and life of organisms by the removal of cells at the appropriate time. It is also an important biological process for the elimination of unwanted cells such as those with potentially harmful genomic mutations, auto reactive or virally infected cells (Fesik, 2000). In plants, the characteristics of PCD include the development of tracheary cells, root cap cells, aerenchyma formation (Kawai et al., 1998), tapetum cell degradation to sustain pollen development, stomium cell death for anther dehiscence, sexual organ formation, carpel senescence, leaf senescence, and responses to pathogens (Pennell and Casolo, 1997; Kawai and Uchimiya, 2000; Senda and Ogawa, 2004). For example, the hypersensitive response (HR) induced by certain plant pathogens involves PCD that restricts the spread of pathogens from the infection site (Lacomme and Crus, 1999; Greenberg, 1996). The mechanisms that control plant cell death, however, are not well understood. Several investigators have suggested parallels between PCD in plants and apoptosis in animals (Wang et al., 1996; Orzäez and Granell, 1997; Tanaka et al., 1997). Caspase-like proteolytic activity has been detected in tobacco tissue that was developing the HR following infection with a tobacco mosaic virus (TMV) (Pozo and Lam, 1998). The cell death associated with the HR in several plantpathogen systems has morphological similarities with animal apoptosis (Milttler and Lam, 1996). An over-expression of human Bcl-XL and caspase-specific peptide

inhibitors can abolish bacteria-induced plant PCD and confer stress tolerance (Mitsuhara et al., 1999). Coupled with this is the recent report of the abrogation of disease development in plants expressing animal anti-apoptic genes (Dickman, 2001). Lacomme and Cruz (1999) demonstrated by using a TMV vector that the expression of Bax triggered cell death in tobacco leaf cells, which closely resembled the HR to TMV in tobacco plants carrying the N gene. Recently, was reported that Bax-induced cell death can be biologically down-regulated by the ectopically expressed anti apoptotic protein AtBI-1 (Bax Inhibitor-1) in *planta* (Kawai-Yamada et al., 2001). Taken together, these reports suggest that cell death mechanisms in plants and animals may share common components that lead to similar cellular events.

The budding yeast *S. cerevisiae* has been employed extensively as a model for genetic analysis of a variety of complex pathways and processes (Matsuyama, 1999). A particular temperature sensitive mutant of cdc48 at the non-permissive temperature, exhibits nuclear fragmentation and membrane babbling reminiscent of apoptosis seen in animal cells (Madeo et al., 1997). Although yeast cells lack caspase and therefore do not possess the same apoptotic pathway found in animal species, these simple unicellular eukaryotes can be exploited in a wide variety of ways for apoptosis research. Utilizing the yeast functional screening system, the current study attempted to isolate cell death promoting factors that originated from plants and the activity of which could be suppressed by AtBI-1. After successive screening of the *Arabidopsis* cDNA library, one clone named cell growth defect factor (cdf) was obtained. In this paper, we describe cdf, a novel growth suppressor located in the mitochondria of yeast cells. The mammalian Bcl-2, a suppressor of Bax-induced lethality in both mammalian and yeast cells, also inhibited cdf-mediated growth defects. One homologue of cdf, named cdf2, induced a yeast growth defect in a manner similar to cdf. Furthermore, cdf did not show any growth inhibition in the Bax-resistant yeast mutant. It was found that the cellular localization of cdf in this mutant was also in mitochondria. These results suggest the functional similarity of cdf and Bax in arresting the growth of yeast cells.

Materials and Methods

Yeast strains

The yeast strain used in the current study was BF264-15*Dau* (MATa ade1 his2leu2-3, 112 trp1-laura3) (Lew et al., 1991). For genetic analysis, EGY48 (MAT_trp1 ura3 his3 leu2::plexAop6-LEU2) and BRM1 (MAT. trp1 ura3 his3 leu2::plexAop6-LEU2), Bax-resistant yeast mutant line, were also used (Matsuyama et al., 1998).

Functional screening in yeast

A yeast strain containing galactose-inducible AtBI-1 was obtained by transformation of pNMV4-AtBI, the TRP1-marked AtBI-1 into a plasmid NMV4, to wild type yeast (BF264-15 *Dau*). Yeast was maintained in a Synthetic Dropout medium lacking Triptophan (SD-T). mRNAs isolated from a 4-day old *Arabidopsis* cell suspension were used in synthesizing cDNAs which were then cloned into a pYX112 vector under the control of a TPI (triose-phosphate isomerase) promoter (Umeda et al., 1998). The obtained cDNA library was transformed into yeast cells and cultured on SD-galactose-T⁻-U⁻ plates, in which AtBI-1 was expressed. Colonies (about 40,000) appeared after being cultured for 2 days, which were then picked up and transferred to an SD-galactose-T⁻-U⁻ agarose medium. After being cultured for 3 days colonies grew on galactose medium, but not on the glucose medium that was used for further screening. Subsequently, these clones were again streaked on either glucose or galactose medium and cultured at 30℃ for 2 days. Finally, one clone named cdf was obtained.

Plasmid construction

The coding region of cdf and Bax were amplified

from pYX112-cdf and pYX112-Bax, respectively, by PCR using *Sph*I-tagged oligo-nucleotide primers: 5'-end (5'-GGCATGCGGATGGACGGGTCCGGGGAGCAG-3') and 3-end complementary strand (5'-GGCATG CTCAGCCCATCTTCTTCCAGAT-3') for cdf and 5'-end (5'-GGCATGCGGATGTCCTCGTC TCTTCTTC TC-3') and 3'-end complementary strand (5'-GGCATG CTTACTTGAGGAAAGTACAAGA-3') for Bax. PCR products were cloned into a pGEM T-easy vector (Promega) for sequencing. After digestion with *Sph*I, cdf and Bax fragments were cloned into TRP1-marked plasmid pTS909 that contains the yeast *GAL*1 promoter. Another plasmid pTS-cdf-GFP was constructed by inserting the GFP coding region between the *Bam*HI and *Xba*I sites of pTS909 that already contain cdf. The cdf homologue, cdf2, was isolated by the RT-PCR (reverse transcription polymerase chain reaction) method. To amplify the cdf2 gene from *Arabidopsis* total mRNA, the following primers were designed from the information of the EST sequence (accession number: AY035099): 5'-end (5'-GGCA TGCGGATGAATGC GTCCGGCTTAACT-3') and 3'-end complementary strand (5'-GGCATGCCTACCGGAGG TAACTCG AAGC-3'). The reaction was prepared from 10.0μg of total RNA samples isolated from 10 day old *Arabidopsis* plants. The conditions for RT-PCR were one cycle at 50℃ for 30 min and at 94℃ for 2 min; a 30 cycle at 94℃ for 30 sec, at 55℃ for 30sec, and at 72℃ for 1 min 30 sec using a One Step RT-PCR kit (TaKaRa, Tokyo, Japan) with a TaKaRa PCR Thermal Cycler. The expression plasmid, pTS-cdf2, was constructed in a way similar to that of cdf. The *Sph*I site of pTS909 was used for the fragment insertion.

Yeast growth assay

Yeast cells were grown for 2 days in YPD media and then transformed by the lithium acetate method with plasmids (pTS-cdf, pTS-cdf-GFP, pTS-cdf2, pTS-Bax, and pTS as control). The transformants were plated onto a SD glucose T medium and incubated at 30℃ for 2 days. The clones were then streaked onto both the SD-glucose-T$^-$ and SD-galactose-T$^-$ plates. To analyze the effect of AtBI-1, yeast lines containing pTS-cdf or pTS were co-transformed with pYX112-AtBI. The co-transformants were then streaked onto SD-glucose-T$^-$-U and SD-galactose-T$^-$-U$^-$ agar plates. The growth of the yeast cells was determined by measuring the OD of the yeast culture solution at 660nm. The yeast clones were inoculated and cultured for 2 days in proper selection media containing glucose, then washed, and transferred to galactose containing media to induce the expression of cdf and cdf-GFP. The OD660 of the cell culture used as the starting point was adjusted from 0.25 to 0.30. The value pf OD660 was measured again after culturing for 20 hrs.

Immunoblot analysis

Yeast cells cultured in glucose-containing medium to an OD600 of ~1.0 were washed three times and subjected to an additional 42 h culture in either fresh glucose- or galactose-containing medium. Yeast cells collected by a centrifugation were resuspended in a 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 vortex for 2 min and then boiled. Proteins separated by SDS-15% polyacrylamide gel electrophoresis were transferred onto a polyvinylidene fuoride membrane (Millipore, Bedford, MA, USA). After blocking with 5% skim milk in PBS at 4℃ 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 a polyclonal antibody for HA-tag (Upstate Biotechnology), followed by a 1:2000 dilution of horseradish HA-conjugated anti-rabbit IgG (Amersham) secondary antibody. Detection was accomplished by an enhanced chemiluminescence method (Amersham) with exposure to X-ray film (Fuji).

Microscopic Examination

Yeast cells harboring pTS-cdf-GFP and GFP were cultured for 2 days in SD-glucose-T$^-$ and were transferred to a view galactose-containing medium to induce gene expression. After 2 days of growth, GFP fluorescence was observed using a fluorescent microscope (DMRD, Leica, Germany) at a 488nm excitation wavelength. For electron microscopic study, sample preparation and treatment were essentially as described by Ueda et al (1996). After fixing of cells by freeze-substitution, frozen cells were treated with 4% osmium tetraoxide (OsO4) and embedded in Spurr's resin. Serial sections stained with uranyl acetate and lead citrate were observed using a Zmodel 2010 electron microscope (JEOL, Akishima-shi, Japan).

Results

Yeast cells containing pNMV4-AtBI were used to screen *Arabidopsis*-originated genes that can inhibit yeast growth. The *Arabidopsis* cDNA library was ligated into the *Eco*RI/XhoI site of the pYX112 vector. The pNMV4 possesses a galactose inducible promotor (*GAL*1) while pYX112 contains a constitutive promoter (*TP*I). The clones can survive on a galactose medium but die when the glucose medium is isolated. These clones are the genes that inhibit yeast growth and whose activity is suppressed by AtBI-1. In the first screening, yeasts co-transformed with pNMV4-AtBI and pYX112-*Arabidopsis* cDNA were plated onto SD-galactose-T$^-$-U$^-$ agar plates and the colonies were replicated to plates containing a glucose medium using a filter membrane. Out of 40,000 colonies 98 clones were isolated. In the second screening, each clone was streaked one by one to confirm the first screening. Thus, finally one cDNA that can suppress yeast growth was isolated and named cdf. A nucleotide sequence analysis of cdf indicated that this cDNA clone encoded a functionally unknown protein 958 nucleotides long and encodes a polypeptide of 258 amino acids (Fig. 1A, Fig. 1C). The cdf gene is located on Chr. V and is composed of 5 exons (Fig. 1B). A homology search indicated that one homologue of cdf, named cdf2 (accession no. AY035099), exists in the *Arabidopsis* genome (Fig. 1C). The cdf2 is 834 nucleotides long and encodes a polypeptide of 278 amino acids. The estimated molecular mass of cdf is 28.8kDa (Fig. 2C) and that of cdf2 is 31.6 kDa (data not shown). cdf and cdf2 have an overall homology of 18.7%. However in the middle part (61-98 aa of cdf) they are 71.2% homologous (Fig. 1C). Hydrophobicity analysis indicated that they have 3 transmembrane domains in their C-terminal region (Fig. 1D). cdf and cdf2 are novel genes and information about their function has not been previously reported.

To study the function of cdf, plasmid constructs of pTS-cdf, pTS-cdf2, pTS-cdf-GFP, and pTS-Bax were made. Since the pTS vector contains a galactose inductive promoter G*AL*1, the expression of the inserted gene can be induced onto a galactose containing medium. A wild type yeast strain (BF264-15*Dau*) was transformed with these vectors and was first cultured onto SD-glucose-T$^-$ plates. Colonies appeared after 2 days of culturing and were then streaked onto the galactose or glucose agar medium. While the yeast possessing an empty vector grew well on both of the media, the cells possessing pTS-cdf, pTS-cdf-GFP, or pTS-Bax hardly grew on the SD-galactose medium (Fig. 2A) caused by the gene expression. The Bax-resistant mutant of yeast (BMR1) was generated from wild type strain (EGY48) by Matsuyama et al. (1998). The expression of Bax in this mutant could not inhibit the growth of yeast cells. As shown in Figure 2A, when plated on the galactose containing medium, EGY48 cells transformed with pTS-cdf or pTS-Bax did not grow, whereas BRM1 cells transformed with the same plasmids survived and grew. This indicates that there may be a functional similarity between cdf and Bax protein. Although not as marked as Bax, a growth defect of cdf on the yeast cells was apparent. The fusion of GFP fluorescent tag to the C-terminal of cdf did not affect the function of cdf in the yeast. The effect of the cdf expression on yeast growth was also studied

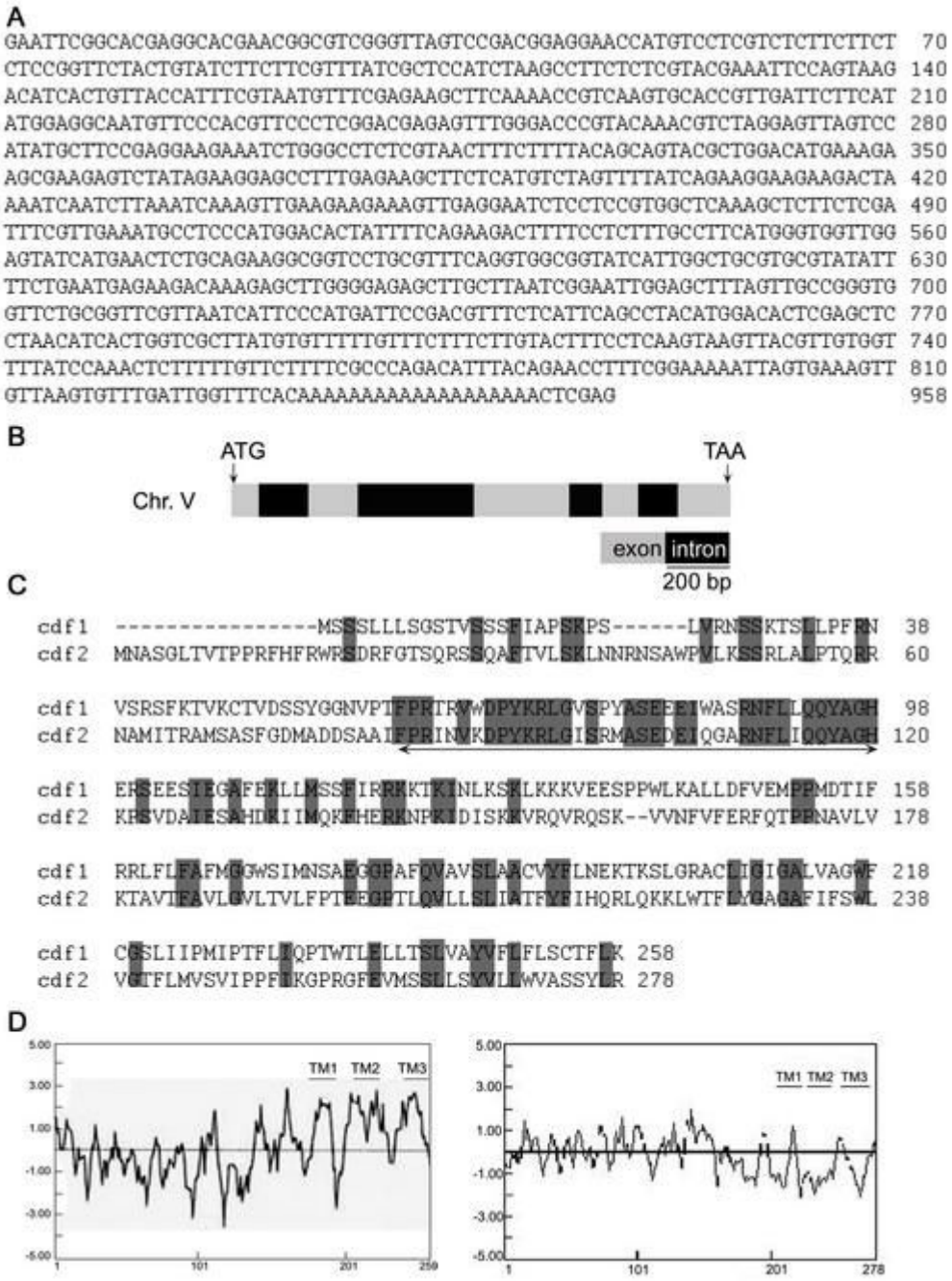

Fig. 1. Nucleotide sequence and genome structure of cdf.

A: The cdf cDNA is 958 nucleotides long. B: The gene is located on Chr. V in Arabidopsis genome and composed of 5 exons. C: Multiple sequence alignment of cdf and cdf2. The two proteins have an overall homology of 18.7 %; while in the middle part (61 98 aa if cdf), as indicated by an arrow, they are 71.2 % homologous. D: Hydrophobicity of cdf and cdf2 proteins and their putative protein structure. From the hydrophobicity analysis, the proteins were assumed to have 3 transmembrane domains in their C terminal.

by measuring the OD660 of the yeast culture solution. Yeast lines containing pTS-cdf, pTS-Bax and the empty vector (pTS) were cultured for two days in SD-glucose-T⁻ and then washed and cultured in a SD-galactose medium. The initial OD660 for these lines was adjusted to 0.25-0.30. The yeast cell growth was determined by measuring OD660 after 42 hrs of growth at 30℃. While the OD660 value of the yeast line possessing pTS reached 1.697, that of the yeast lines expressing cdf and Bax were 1.243 and 1.200 (Fig. 2B),

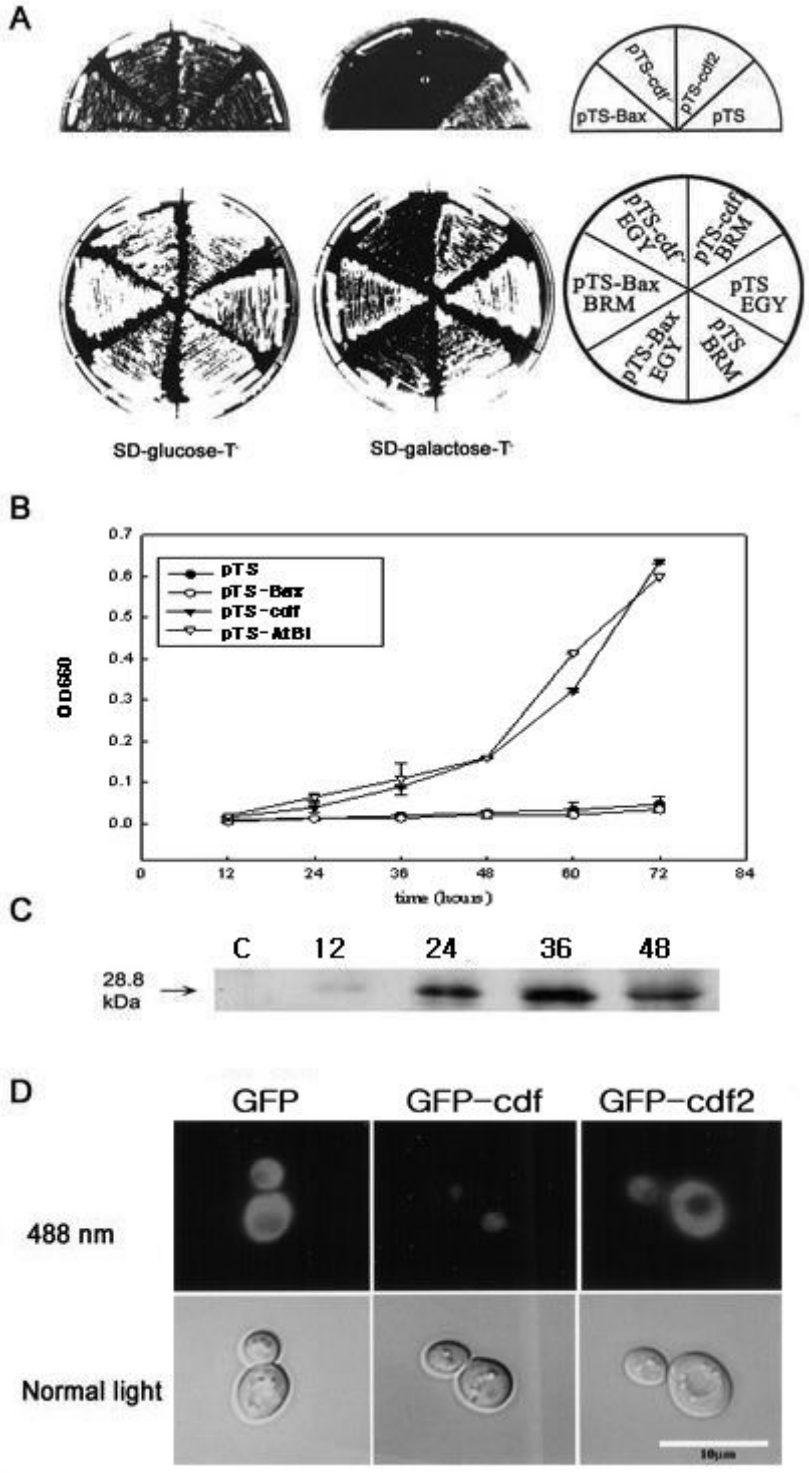

Fig. 2. The growth defect of yeast cells expressing cdf compared with that of cdf2 and Bax.

A: Yeast cells were transformation with pTS Bax, pTS cdf, pTS cdf2 and pTS empty vector and plated on SD glucose agar medium that lack of TRP. After 2 days of incubation, the colonies were streaked on both glucose and galactose containing SD medium. Expression of cdf could not inhibit growth of Bax resistant yeast mutant line. The result shows growth of yeast cells 2 days after streak.cdf on yeast. B: Growth defect of yeast expressing cdf shown by OD660 measurement. The 3 yeast lines were inoculated in SD glucose medium. After 42 hrs of shaking, yeast culture were washed and transferred to SD galactose liquid medium. The OD660 of the start point was adjusted to 0.25~0.30. Until 42 hrs every 6 hrs, the OD660 for each line was measured again. C: Immunological detection of cdf protein in yeast. D: The cellular localization of cdf in BF264 15Dau wild type yeast. The yeast lines possessing pTS cdf GFP, pTS GFP and pTS cdf were inoculated in glucose containing SD liquid medium, cultured for 2 days of culture and transferred to a galactose containing medium to induce the gene expression. The observation was performed after 8 hrs of inductive culture. The GFP fluorescence was examined at a 488nm excitation wavelength under a fluorescent microscope (DMFD, Leica, Wetzlar, Germany).

respectively. Moreover, the cdf protein was detected as a single peptide band of about 28.8 kDa in protein samples from yeast cells co transformed plasmid (pTS-cdf) and grown medium with SD-glucose-T (Fig. 2C). To investigate the cellular localization of the cdf protein in yeast, pTS-cdf-GFP and pTS-GFP were constructed. As previously demonstrated, the fusion of the C-terminal region of cdf to GFP did not affect its function. Yeast cells transformed with pTS-cdf-GFP were cultured first in a SD-glucose-T liquid medium for 2 days and then transferred to a galactose containing medium. After 8 hrs of being cultured, the fluorescence were observed after 2 days of growth on a galactose-containing agar medium. The EM analysis of yeast cells containing pTS-cdf and pYX112-AtBI showed a typical morphology of a normal S. cerevisiae with a round shape and normal size with a homogeneous vacuole and nuclear region and a number of mitochondria. In contrast, cells containing pTS-cdf and pYX112 developed a variety of morphological abnormalities, such as broken cytosolic vacuoles and an abnormally large number of mitochondria, which presumably reflected a continuum of severity (Fig. 3).

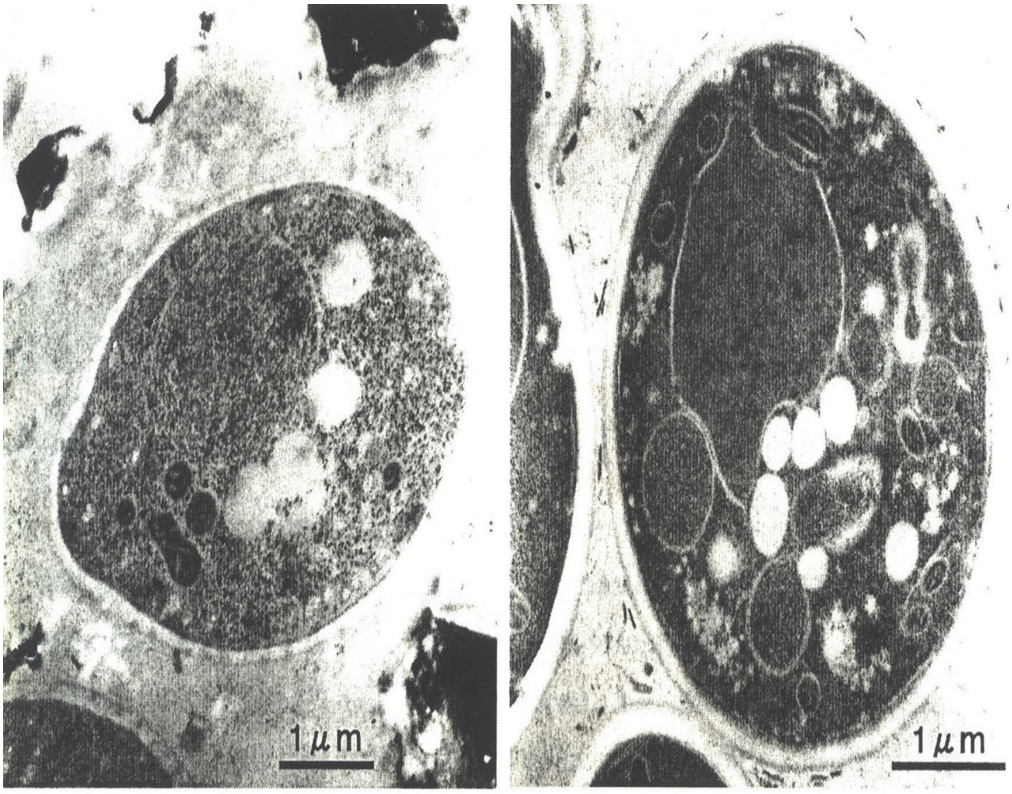

Fig. 3. An Electron Microscopic (EM) analysis of cdf mediated cell death in yeast cells.

BF264 15Dau cells containing pTS cdf and pYX112 AtBI (b) or pXY112 (a) were streaked onto a galactose agar medium to induce cdf expression. After 2 days culturing, cell morphology was analyzed by an EM.

2D). To further characterize the cell-death process induced by cdf in yeast cells, an electron microscopic of cdf-GFP and GFP (control) were observed under a microscope using a 488nm excitation wavelength (Fig. (EM) analysis was carried out. Cells harboring both pTS-cdf and pYX112 or pTS-cdf and pYX112-AtBI

Discussion

PCD is a major process in animal and plant development. In a database search of the *S. cerevisiae* genome, no obvious homologue of any crucial regulator of metazoan apoptosis [(members of the Bax/Bcl-2

family, caspases, apoptotic protease activating factor-1 (Apaf-1)] were. However, it has been noted that yeast cells, both *S. cerevisiae* and *Schizosaccharomyces pombe*, can be killed by the expression of a number of proapoptotic mammalian genes for example, Bax (Sato et al., 1994; Hanada et al., 1995; Greenhalf 1996) or p53 (Bischoff et al., 1992; Nigro et al., 1992). Recently, it was observed that the *S. cerevisiae cdc*48 mutant as well as cells over expressing Bax coordinately exhibit phenotype markers of apoptosis, chromatin condensation and fragmentation, DNA breakage, the exposition of phosphatidylserine, and the formation mini cells which approximates apoptotic bodies. This indicates the presence of a basic apoptotic mechanism in yeast (Matsuyama et al., 1999; Madeo et al., 1999). It is clear that Bax, Bak, or CED-4 do not simply act as cytotoxic substances in yeast, but seem to activate the same or a similar mechanism as in metazoan organisms. The observation of cell death accompanied by apoptosis like features in yeast suggests that apoptosis developed before the evolutionary separation between fungi and metazoans. Elements of the pathway conserved in yeast as well as animals should therefore belong to a basic but archaic evolutionary mechanism. Yeast should be useful in tracing the roots of apoptosis and solving some of the complications and apparent contradictions that are inherent in various models of apoptosis.

Many studies have demonstrated the commonality of the death machine existing in the whole mammalian, plant, and yeast systems. Though the function of cdf in the plant system has not been studied yet, and the mechanism of cdf in the resulting yeast growth defect should be conveyed further in detail, in the current study cdf caused an important phenotype on yeast and possibly could be considered to be one of the candidates for a plant PCD regulator. Since screening from the callus was used for screening cdf, it may be possible to get other positive clones for the death factor if the cDNA library was obtained from should this be other organs or another organ of *Arabidopsis*. Considering the similar function of cdf2 and cdf, the highly homologous middle part of the two genes may serve as a functional domain. There is estimated to be functional domain in the middle part of the two genes.

The BI-1 gene was identified by undertaking a functional screen for the Bax suppressors in yeast. The gene is highly conserved throughout evolution. It shares an identifiable similarity with the Bcl-2 family of proteins or any other proteins implicated in PCD. Thus, BI-1 represents a novel type of an apoptosis modulator. However, BI-1 does not interact with Bax directly, but acts on an element already present in yeast (Xu and Reed, 1998). Based on the apparent scarcity of BI-1 in the mitochondrial membranes, BI-1 might function as a dawn stream of Bax. AtBI-1 is the plant homologue of BI-1 and the expression of the protein could also suppress Bax-induced cell death in yeast (Kawai et al., 1999). Moreover, it has been reported that the plant anti apoptotic protein AtBI-1 is biologically active in suppressing the mammalian Bax in *planta* (Kawai-Yamada et al., 2001). The expression of AtBI-1 has also been found to be rapidly up-regulated in plants during wounding or pathogen challenge (Sanchez and Stephens, 2000).

The Bcl-2 family proteins are centrally involved in the control of PCD, with some inhibiting (Bcl-2 and Bcl-XL) and others promoting (Bax and Bak) apoptosis (Reed, 1994, Yang et al., 1997). The ability of the Bcl-2 family proteins to regulate cell life and death is conserved across evolution. The human Bcl-2 protein can block apoptotic cell death in insect cells (Alnemri et al., 1992), and can protect some mutant yeast strains from death induced by oxidative injury (Kane et al., 1993). The biochemical mechanism of the Bcl-2 action and its homologues are controversial. Since the anti-apoptotic Bcl-2 proteins can rescue yeast from Bax/Bak induced lethality, Bax-induced cell death is not a nonspecific toxic that is caused by an over-expression of a heterolog

ous protein. Moreover, mutants of Bcl-2 and Bcl-XL which fail to protect in mammalian cells are inactive in suppressing Bax-induced cell death in yeast.

During the process of identification of cdf from the

Arabidopsis cDNA library as a yeast growth defect factor, the function of cdf was suppressed by AtBI-1 and later it was also detected to have a functional interaction with Bcl-2. The result that the function of cdf can be suppressed by Bcl-2 indicates the commonality of the pathway for cdf and the Bcl-2 family. AtBI-1, one of the identified plant genes that has a high homology with BI-1, can play a vital role in studying plant PCD. The interaction of cdf and AtBI-1 in yeast has opened up the possibility of further research in identifying the responsible genes and the principle pathway of PCD in *planta*.

Mitochondria are implicated in yeast cell death that is induced by Bax overexpression. Bax targets mitochondria in both yeast and mammalian cells. The deletion of the trans membrane domain of Bax, which prevents Bax from being transported to mitochondria, renders it incapable of being lethal in yeast, suggesting that the mitochondrial targeting of Bax is both necessary and sufficient to induce cell death in yeast. The expression of Bax in yeast also induces cytochrome c release, a phenomenon observed in mammalian cells during apoptosis stimulation, suggesting that the primary an effect of Bax on mitochondria may be similar in both yeast and mammalian cells (Reed, 1997).

The existence of cdf in mitochondria suggests that the biological change and lethal influence which Bax causes on yeast, depending on its specific localization at mitochondria, is also involved in cdf induced yeast cell death. The cdf and Bax genes are currently supposed to follow a similar process during their inhibition of yeast growth. Although the real localization of cdf in plant cells has not been detected, its mitochondrial localization in the yeast system has made cdf a good candidate for the Bax-substituting plant element in yeast. Until now, there hasn't been any functional information about cdf in plant systems. The identification of the role of cdf in plants and the further investigation about its functional pathway needs to be clarified with more research.

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