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Identification of Novel Mitochondrial Membrane Protein (*Cdf* 3) from *Arabidopsis thaliana* and its Functional Analysis in a Yeast System

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Abstract We screened the *Arabidopsis* cDNA library to identify functional suppressors of AtBI-1, a gene that suppresses cell death induced by Bax gene expression in yeast. *Cdf* 3 encodes a 118-amino-acid protein with a molecular mass of 25 kDa. This protein has two uncharacterized domains at amino acids residues 5-64 and 74-117. In the present study, CDF3 was found to induce growth defects in yeast and arrested yeast growth, although the cell-growth defect was somewhat less than that of Bax. Its localization in the inner mitochondria was essential for suppression of yeast-cell proliferation. The morphological abnormality of the intracellular network, which is a hallmark of AtBI-1, was attenuated by *Cdf* 3 expression.

Keywords: *Cdf* 3, PCD, suppressing yeast

Programmed cell death (PCD) plays a critical role in the development and vitality of organisms by removing cells at an appropriate time. PCD is also important in the elimination of unwanted cells such as those with potentially harmful genomic mutations or autoreactive or virally infected cells [2].

In plants, PCD is involved in the development of tracheary cells, root cap cells, aerenchyma formation [7], tapetum cell degradation to sustain pollen development, stomium cell death for anther dehiscence, sexual organ formation, carpel senescence, leaf senescence, and responses to pathogens [9, 20, 23]. For example, certain plant pathogens induce the hypersensitive response (HR) that involves programmed cell death to restrict the spread of pathogens from an infection site [4, 13]. However, the mechanisms that control plant-cell death are not well documented. Several investigators have suggested parallels between PCD in plants and apoptosis in animals [6, 26]. Caspase-like proteolytic activity has been detected in tobacco tissue that was developing HR following infection with tobacco mosaic virus (TMV) [19]. Furthermore, cell death associated with HR in several plant pathogen systems has morphological similarities to animal apoptosis [5]. Overexpression of human Bcl-XL and caspase-specific peptide inhibitors in plants can abolish bacteria-induced plant PCD and confer stress tolerance [18]. Recent reports outline the abrogation of disease development in plants expressing animal antiapoptotic genes [1, 27]. Using a TMV vector, Lacomme and Cruz [13] demonstrated that Bax expression triggered cell death in tobacco leaf cells that closely resembled the HR to TMV in tobacco plants that carry the N gene. Bax-induced cell death can reportedly be downregulated by ectopically expressed antiapoptotic protein AtBI-1 *in planta* [10]. Taken together, these studies 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 extensively employed as a model for the genetic analysis of a variety of complex pathways and processes [12, 14, 17]. At a nonpermissive temperature, a particular temperature-sensitive

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mutant of *cdc*48 exhibits nuclear fragmentation and membrane blebbing that are reminiscent of apoptosis in animal cells [16]. Although yeast cells lack caspase and, therefore, do not go through the same apoptotic pathway as animal species, these simple unicellular eukaryotes can be exploited in a variety of ways for apoptosis research. In the current study, we utilized the yeast functional screening system to isolate cell-death-promoting factors that were originated from plants, the activity of which could be suppressed by AtBI-1. We obtained three clones after successive screening of an *Arabidopsis* cDNA library and described one of these (Accession No. AB238795), a novel growth suppressor that was localized in yeast-cell mitochondria.

MATERIALS AND METHODS

Yeast Strains

The yeast strain BF264-15*Dau* (MATa ade1 his2leu2-3, 112 trp1-laura3) was used in this study [18].

Functional Screening in Yeast

A yeast strain containing galactose-inducible AtBI-1 was obtained by transformation of pNMV4-AtBI, the TRP1-marked AtBI-1 in an NMV4 plasmid, into wild-type yeast (BF264-15 *Dau*). Yeasts were maintained in Synthetic Dropout medium lacking tryptophan (SD-T). The mRNAs isolated from 4-day-old *Arabidopsis* cell suspensions were used to synthesize cDNAs, which were then cloned into a pYX112 vector under the control of the TPI (triose-phosphate isomerase) promoter [25]. The obtained cDNA library was transformed into yeast cells in which AtBI-1 was also expressed and cultured on SD-galactose-T^-U^- plates. Colonies (about 40,000) appeared after 2 days. The colonies were then transferred to SD-galactose-T^-U^- agarose medium. After three days, colonies had grown on galactose medium, but not on the SD-glucose medium that was used for further screening. Subsequently, these clones were streaked again on either SD-glucose [6.7 g/l Difco yeast nitrogen base+20 g/l glucose (Sigma)] or SD-galactose [6.7 g/l Difco yeast nitrogen base+50 g/l galactose (Sigma) +2 g/l sucrose (Sigma)] media and cultured at 30°C for two days.

Plasmid Construction

The coding region of *Cdf* 3 and Bax were amplified by PCR from pYX112-*Cdf* 3 and pYX112-Bax, respectively, using SphI-tagged oligonucleotide primers: 5'-end (5'-GGCATGCTATACCGACCTCACTACTTCAAC-3') and 3'-end complementary strand (5'-GGCATGCCATCACCT-CACGGTGTTGCAG-3') for *Cdf* 3, and 5'-end (5'-GGCA-TGCGGATGTCCTCGTCTCTTCTTCTC-3') and 3'-end complementary strand (5'-GGCATGCTTACTTGAGGA-AAGTACAAGA-3') for Bax. PCR products were cloned into pGEM T-Easy vector (Promega) for sequencing. After digestion with SphI, *Cdf* 3 and Bax fragments were cloned into TRP1-marked plasmid pTS909 [3] that contained the yeast *GAL*1 promoter. Another plasmid, pTS-*Cdf* 3-GFP, was constructed by inserting the GFP coding region between the BamHI and XbaI sites of pTS909 that contained *Cdf* 3.

Yeast Growth Assay

Yeast cells were grown for two days in YPD medium (20 g/l Difco peptone+10/l Yeast extracts+20/l Glucose) and then transformed with plasmids pTS-*Cdf* 3, pTS-Bax, or pTS (control) by the lithium acetate method. Transformants were plated on SD-glucose-T^- medium and incubated at 30°C for two days. The resulting clones were then streaked on SD-glucose-T^- and SD-galactose-T^- plates. To analyze the effects of AtBI-1, yeast lines containing pTS-*Cdf* 3 or pTS were cotransformed with pYX112-AtBI-1. The cotransformants were streaked on SD-glucose-T^-U^- and SD-galactose-T^-U^- agar plates. The yeast clones were inoculated and cultured for two days in proper selection media containing glucose. Yeast cell growth was determined by measuring the OD_{660} of the yeast culture suspension. The clones were washed and transferred to galactose-containing medium to induce expression of *Cdf* 3 and *Cdf* 3-GFP. The initial OD_{660} of the cell culture was adjusted to 0.25–0.30. The OD_{660} was measured again after culturing for 42 h.

Immunoblot Analysis

Yeast cells were cultured in glucose-containing medium to an OD_{600} of ~1.0, washed three times, and subjected to an additional 42 h of culture in either fresh glucose- or galactose-containing media. The yeast cells were then collected by centrifugation and resuspended in buffer containing 8 M urea (Sigma), 5% SDS (Sigma), 40 mM Tris-HCl (pH 6.8), 0.1 mM EDTA (Sigma), 0.4 mg/ml BPB (Sigma), and 10 μl/ml 2-mercaptoethanol (Sigma). An 80% volume of acid-washed glass beads (425–600 μm; Sigma) was added, and the tubes were vortexed for 2 min and then boiled. Proteins were separated by SDS-15% polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, U.S.A.). After blocking overnight with 5% skim milk in phosphate-buffered saline (PBS; 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 in 800 ml of distilled H_2O, pH 7.4) at 4°C, 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 (Sigma)] solution with a polyclonal antibody for the HA-tag (Upstate Biotechnology). This incubation was followed by washing and incubation with a 1:2,000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG (Amersham) secondary antibody. Detection was accomplished by the enhanced chemiluminescence method (Amersham) with exposure to X-ray film (Fuji).

Microscopy

Yeast cells harboring pTS-CDF3-GFP and pTS-GFP were cultured for two days in SD-glucose-T⁻ and then transferred to galactose-containing medium to induce gene expression. After two days, GFP fluorescence was observed using a fluorescence microscope (DMRD, Leica, Germany) with a 488-nm excitation wavelength. To visualize active mitochondria with transmembrane potential, yeast cells were stained with the fluorescent probe MitoTracker Red (650 nM; Invitrogen) for 5 min and examined under a fluorescence microscope at a wavelength of 568 nm. For electron microscopy, sample preparation and treatment were essentially as described by Ueda *et al.* [24]. After fixing the cells by freeze-substitution, frozen cells were treated with 4% osmium tetroxide (OsO_4, Sigma) and embedded in Spurr's resin. Serial sections were stained with uranyl acetate and lead citrate and observed using a Z-model 2010 electron microscope (JEOL, Akishima-shi, Japan).

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1   GAATTCGGCACGAGTGGCAAACCAACGTAAAACACTAACTAAGAGATCAATATGGCTGGA   60
1                                                         M  A  G    3
61  GTGATGAAGTTGGCATGCATGGTCTTGGCTTGCATGATTGTGGCCGGTCCAATCACAGCG  120
4   V  M  K  L  A  C  M  V  L  A  C  M  I  V  A  G  P  I  T  A     23
121 AACGCGCTTATGAGTTGTGGCACCGTCAACGGCAACCTGGCAGGGTGCATTGCCTACTTG  180
24  N  A  L  M  S  C  G  T  V  N  G  N  L  A  G  C  I  A  Y  L     43
181 ACCCGAGGTGCTCCACTTACCCAAGGGTGCTGCAACGGCGTTACTAACCTTAAAAACATG  240
44  T  R  G  A  P  L  T  Q  G  C  C  N  G  V  T  H  L  K  N  M     63
241 GCCAGTACAACCCCAGACTGTCAGCAAGCTTGCCGTTGCCTTCAATCTGCCTCTAAAGCC  300
64  A  S  T  P  D  R  Q  Q  A  C  R  C  L  Q  S  A  A  K  A     83
301 GTTGGTCCCGGTCTCAACACTGCCCGTGCAGCTGGACTTCCTAGCGCATGCAAAGTCAAT  360
84  V  G  P  G  L  N  T  A  R  A  A  G  L  P  S  A  C  K  V  N  103
361 ATTCCTTACAAAATCAGCGCCAGCACCAACTGCAACACCGTGAGGTGATGACGCAGGTC  420
104 I  P  Y  K  I  S  A  S  T  N  C  N  T  V  R  *                118
421 AAATGAAGCTACTAGCGGATGTTTCGAATATTATATAATAATCGATGAGATAATATTAAATAA  480
481 AGATGTTCGAATGGCTGTACTCTTTTCAATTTCCTGTCTTTTTTTATCGTGCGTTTCTA  540
541 TTATGTGATCGTCTGTACTATGTTCGCAAACAACGTTCTTTGAATTTCAGACTTGAAGTT  600
601 TAAGTTAAAAAAAAAAAAAAAAAAACTCGAG                               630
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Fig. 1. DNA and amino acid sequences of the *Cdf* 3 coding region.

RESULTS

Yeast cells containing pNMV4-AtBI-1 were used to screen *Arabidopsis* genes that can inhibit yeast growth. Thus, the *Arabidopsis* cDNA library was ligated into the EcoRI/XhoI site of the pYX112 vector. The pNMV4 possesses

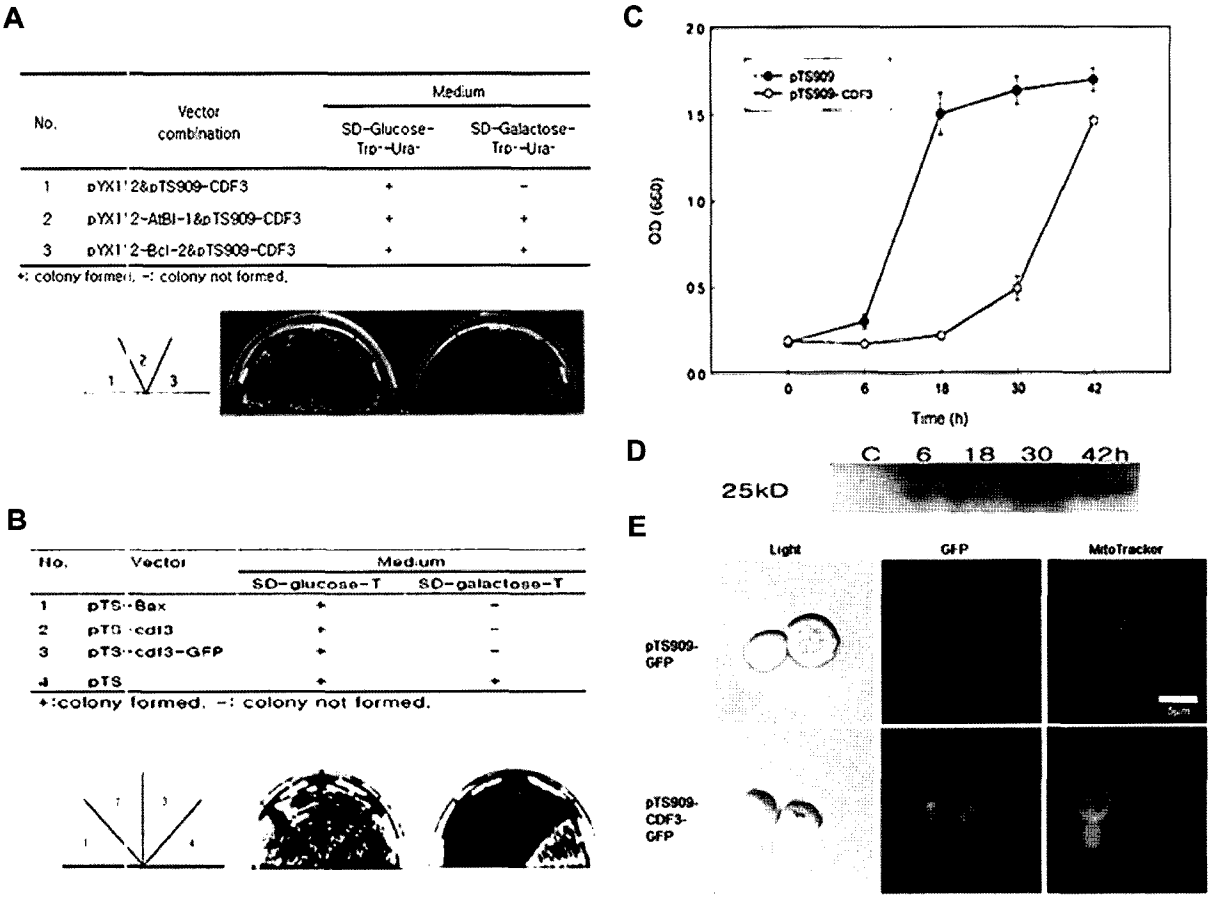

Fig. 2. Growth defects of yeast cells expressing *Cdf* 3.

A. *Yeast cells were transformed with the pTS-Cdf* 3 vector and plated on SD-glucose agar medium that lacked TRP and URA. **B.** Comparative growth of pTS-Bax, pTS-Cdf3, pTS-cdf3-GFP, and pTS vector on SD-glucose and galactose agar media that lacked TRP. **C.** Growth defect of yeast expressing *Cdf* 3 is shown by OD_{660} measurements. **D.** Immunological detection of CDF3 protein in yeast. **E.** The cellular localization of CDF3 in BF264-15*Dau* wild-type yeast. The GFP fluorescence was examined at 488-nm excitation wavelength with a fluorescence microscope (DMFD, Leica, Wetzlar, Germany). Active mitochondria were stained with the fluorescent probe MitoTracker Red.

a galactose-inducible promoter (*GAL*1), whereas pYX112 contains a constitutive promoter (*TPI*). Therefore, yeast cells carrying both plasmids can survive on galactose medium, but will die when transferred to glucose medium. These cells may have the genes that inhibit yeast growth and whose activity is suppressed by AtBI-1. In the first screening, yeasts that were cotransformed with pNMV4-AtBI and pYX112-*Arabidopsis* cDNA were plated on SD-galactose-T^--U^- agar plates, and the colonies were replicated to plates containing glucose medium using a filter membrane. Ninety-eight clones were isolated out of 40,000 colonies. In the second screening, each clone was individually streaked to confirm the first screening. One cDNA that suppressed yeast growth was isolated and named *Cdf* 3. Nucleotide sequence analysis of *Cdf* 3 indicated that this cDNA clone was 630 nucleotides long and encoded a functionally uncharacterized protein that had 118 amino acids (Fig. 1). Hydrophobicity analysis indicated two uncharacterized domains at amino acids residues 5-64 and 74-117 (Fig. 1).

To study *Cdf* 3 function, plasmid constructs of pTS-*Cdf* 3 and pTS-Bax were prepared. Since the pTS vector contains a galactose-induced promoter G*AL*1, expression of the inserted gene can be induced on medium that contains galactose. Thus, a wild-type yeast strain (BF264-15*Dau*) was transformed with these vectors and cultured on SD-glucose-T^- plates. Colonies appeared after two days and were streaked on galactose or glucose agar media. Whereas yeast possessing an empty vector grew well on both media, the cells that possessed pTS-*Cdf* 3, pTS- *Cdf* 3-GFP, or pTS-Bax showed little growth on SD-galactose medium (Fig. 2A), as a result of the gene expression. Bax expression did not inhibit yeast cell growth (Fig. 2B). As shown in Fig 2A, when plated on galactose-containing medium, yeast cells that were transformed with pTS-*Cdf* 3 or pTS-Bax (Fig. 2B) did not grow, whereas yeast cells transformed with the same plasmids survived and grew on SD-glucose-Trp^--Ura^- medium. This indicates that there may be functional similarities between *Cdf* 3 and Bax protein. Although not as marked as Bax, the growth defect of *Cdf* 3 in yeast cells was apparent (Fig. 2B). The effect of *Cdf* 3 expression on yeast growth was also studied by measuring the OD_{660} of yeast culture suspensions. Yeast lines containing pTS-*Cdf* 3, pTS-Bax, or the empty vector (pTS) were cultured for two days in SD-glucose-T^-. The cell lines were washed and then cultured in SD-galactose medium. The initial OD_{660} for these cell lines was adjusted to 0.25–0.30. Yeast-cell growth was determined by measuring the OD_{660} after 42 h at 30°C. Whereas the final OD_{660} of the yeast line with pTS reached 1.697, that of the yeast line expressing *Cdf* 3 was 1.420 (Fig. 2C). Moreover, CDF3 protein was detected as a single band of about 25 kDa in protein samples from yeast cells that were cotransformed with pTS-*Cdf* 3 and BF264-15*Dau* (yeast strain) and then

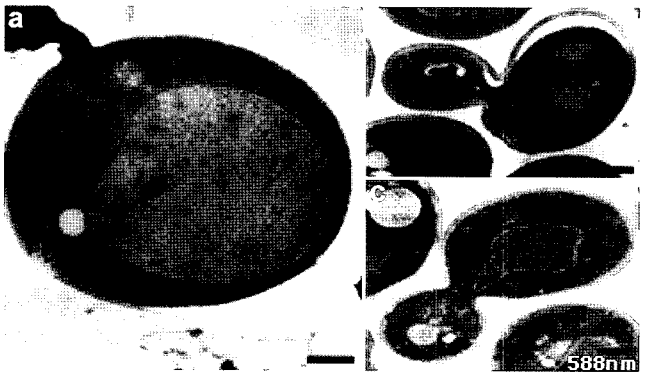

Fig. 3. Electron microscopic (EM) analysis of CDF3-mediated cell death in yeast cells. BF264-15*Dau* cells, containing pTS-*Cdf* 3 (b, c) and pXY112 (a) were streaked on galactose agar medium to induce *Cdf* 3 expression.
After two days, cell morphology was analyzed by EM.

grown on SD-galactose-T^- medium (Fig. 2D). To further investigate the cellular localization of CDF3 protein in yeast, pTS-*Cdf* 3-GFP and pTS-GFP were constructed. Yeast cells, transformed with pTS-*Cdf* 3-GFP, were cultured first in SD-glucose-T^- liquid medium for two days and then transferred to medium containing galactose. After 8 h, the fluorescence of *Cdf* 3-GFP and GFP (control) were observed with a fluorescence microscope at 488-nm excitation (Fig. 2E). The fusion of the GFP fluorescent tag to the C-terminus of *Cdf* 3 affected the function of *Cdf* 3 in yeast.

To further characterize the cell-death process induced in yeast cells by *Cdf* 3, an electron microscopic (EM) analysis was conducted. Cells harboring both pTS-*Cdf* 3 and pYX112 or pTS-*Cdf* 3 and pYX112-AtBI-1 were observed on galactose-containing agar medium after two days of growth. The EM analysis of yeast cells containing pTS-*Cdf* 3 and pYX112-AtBI-1 revealed typical morphology of normal *S. cerevisiae* (round shape and normal size) with the homogeneous vacuole, nuclear region, and a number of mitochondria. In contrast, cells containing pTS-*Cdf* 3 and pYX112 developed a variety of morphological abnormalities, such as broken cytosolic vacuoles and abnormally large number of mitochondria, presumably reflecting a continuum of severity (Fig. 3).

DISCUSSION

Many studies have demonstrated the commonality of the death machinery in mammalian [6], plant [8], and yeast [15] systems. Although the function of *Cdf* 3 in plants has not yet been fully studied and the mechanism of *Cdf* 3 in yeast growth defects still has to be further studied in detail, *Cdf* 3 expression resulted in an important phenotype in yeast and could possibly be considered as a candidate plant PCD regulator. Since the cDNA library used in the screen for

Cdf 3 was derived from the callus, it may be possible to get other positive clones for the death factor if the cDNA library were obtained from an *Arabidopsis* organ source. The estimated molecular mass of CDF3 was 25 kDa (Fig. 2D); a related protein, CDF1, is 31.6 kDa [11].

The BI-1 gene has been identified in a functional screen for Bax suppressors in yeast. The gene is highly conserved throughout evolution, and shares an identifiable similarity with the Bcl-2 protein family or other proteins that are implicated in PCD. Thus, BI-1 is a novel type of an apoptosis modulator. However, BI-1 does not directly interact with Bax, but rather it acts on an element already present in yeast [26]. Based on the apparent scarcity of BI-1 in mitochondrial membranes, BI-1 might function downstream of Bax. AtBI-1 is the plant homolog of BI-1, and expression of At-BI-1 protein also suppresses Bax-induced cell death in yeast [8]. Moreover, it has been reported that this plant homolog is biologically active in suppressing mammalian Bax *in planta* [10]. The expression of AtBI-1 has been shown to be rapidly upregulated in plants during wounding or pathogen challenge [22].

In the present study, during the process of identifying *Cdf* 3 from the *Arabidopsis* cDNA library as a yeast growth defect factor, AtBI-1 suppressed the function of *Cdf* 3. Since the function of *Cdf* 3 can also be suppressed by Bcl-2 [21], these observations indicate the commonality between the *Cdf* 3 pathway and that of the Bcl-2 family. AtBI-1, one of the identified plant genes that have high homology to BI-1, can play a vital role in the study of plant PCD. The presence of CDF3 in mitochondria suggests that Bax causes biological changes and lethal influence in yeast. The *Cdf* 3 and Bax genes are currently suggested to follow similar processes during their inhibition of yeast growth. Although CDF3 has not yet been localized and detected in plant cells, its mitochondrial localization in the yeast system makes it a good candidate for the plant Bax-substituting element. To date, there has been no functional information about *Cdf* 1 in plant systems, and therefore, the role of *Cdf* 3 in plants and its functional pathway require further investigation.

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