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Evolutionally conserved plant homologue of the Bax Inhibitor-1 (BI-1) gene capable of suppressing Bax-induced cell death in yeast[1]

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Abstract **The plant homologue of Bax Inhibitor-1, a gene described to suppress the cell death induced by Bax gene expression in yeast, was isolated from *Oryza sativa* L. (rice) and *Arabidopsis*. The amino acid sequence of the predicted protein was well conserved in both animal and plant (45% in amino acids) and contained six or seven membrane-spanning segments. Northern blot analysis showed that OsBI-1 transcripts were present in all tissues examined. The OsBI-1 cDNA suppressed cell death induced by mammalian Bax in yeast, suggesting functional conservation of this BI-1 homologue in the plant kingdom.**

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Key words: Apoptosis; Bax; Bax inhibitor; Programmed cell death; *Oryza*

1. Introduction

Cell death is a highly regulated process involving intercellular signal transduction pathways. Studies in *Caenorhabditis elegans* have elucidated a cell suicide pathway which includes several participating genes that have homologues in vertebrates. Central among these death-regulating proteins is Ced-9, which suppresses programmed cell death in *C. elegans* [1]. Bcl-2, the vertebrate homologue of Ced-9, similarly inhibits cell death in vertebrate cells and functionally substitutes for Ced-9 in *C. elegans* [1,2]. Within the family of Bcl-2 like proteins, some inhibit apoptosis (e.g. Bcl-2 and Bcl-X_L), while others are involved in inducing apoptosis (e.g. Bax) [3,4]. Until now, proteins similar to Bcl-2 family have not been found in plants or in lower eukaryotes such as yeast.

Yeast provide a powerful tool for studying the molecular mechanisms of biological phenomena. It was recently found that expression of Bax is lethal in yeast [5–8]. This lethal effect is prevented by co-expression of anti-apoptotic genes such as Bcl-2 or Bcl-X_L [7,9]. Moreover, genetic analysis of the genes required for Bax-induced cell death in yeast has revealed commonalties in the mechanisms utilized by this death-inducing protein in both yeast and human cells [27]. These suggest that components of the cell death pathway controlled by Bax and other Bcl-2 family proteins may be conserved from simple unicellular eukaryotes (i.e. yeast) to multicellular organisms.

Xu and Reed [10] transformed yeast cells containing a galactose-inducible Bax plasmid with a human cDNA library in which cDNAs were fused to a constitutively active yeast promoter and isolated cDNAs that prevented Bax-induced lethality on galactose. This resulted in the identification of cDNAs encoding a protein, termed Bax Inhibitor-1 (BI-1), which is identical to a previously isolated human gene of unknown function called testis enhanced gene transcript (TEGT) [11,12]. The BI-1 was shown to have an ability to block cell death induced by Bax overexpression, growth factor deprivation, etoposide, and staurosporine treatment of cultured mammalian cells. Thus, BI-1 could be a new type of regulator of cell death pathways regulated by Bax/Bcl-2. Furthermore, similar DNA sequences were also detected in *C. elegans* and plants, suggesting evolutionary conservation of BI-1 genes.

In this work, we isolated the plant homologue cDNAs (*Oryza sativa*, OsBI-1; *Arabidopsis thaliana*, AtBI-1) of BI-1. The amino acid sequences of the predicted protein were well conserved in both animal and plant (41.5% in amino acids), and contained six or seven membrane-spanning segments. The OsBI-1 cDNA suppressed cell death induced by mammalian Bax in yeast, suggesting functional conservation of this BI-1 homologue in the plant kingdom.

2. Materials and methods

2.1. cDNA cloning and sequencing

A cDNA containing the full length open reading frame (ORF) of the rice BI-1 homologue was isolated using EST clone (97MJ0046, which was derived from National Institute of Science and Technology, Korea) as a probe. The rice root cDNA library in λZAP vector was prepared using a RNA isolation kit (Stratagene) and ZAP-cDNA synthesis kit (Stratagene) according to the manufacturer's instructions.

To generate a probe for screening of *Arabidopsis* cDNA libraries, the following primers were designed from the information of genomic sequence (accession number: Z97343); 5′-TCAAGCGTCCTGGTCACTGCATTTG-3′ and 5′-CGGTTATCATCTGTGTGTCCACCAC-3′. A partial gene fragment was amplified using the PCR method from *Arabidopsis* cDNAs obtained from suspension cultured cells. Using these partial cDNAs as hybridization probes, cDNA libraries were screened.

2.2. Southern hybridization

Two-week-old rice seedlings were harvested and genomic DNA was extracted by a CTAB method [13]. Ten micrograms of DNA were digested with restriction enzymes. The resulting DNAs were fractioned on 0.8% agarose gels, transferred to nylon membranes (Biodyne plus, Pall, NY, USA), and hybridized to the OsBI-1 cDNA

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[1] Accession numbers: OsBI-1, AB025926; AtBI-1, AB025927 for DDBJ/EMBL/GenBank.

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labeled with α-^{32}P in 10% dextran sulfate, 1 M NaCl, 1% SDS, and 100 μg/ml heat-denatured salmon testis DNA at 65°C. Washing was performed with low stringency (washed with 1×SSC and 0.1% SDS) or high stringency (0.1×SSC and 0.1% SDS).

2.3. Northern blot analysis of rice BI-1 cDNA

Total RNAs were isolated from different tissues using guanidine thiocyanate as described previously [14]. A ^{32}P-labeled cDNA representing the 3′-untranslated region (UTR) of rice BI-1 was used as a probe. Hybridization was performed as described in Southern blot hybridization, and washed with 0.1×SSC and 0.1% SDS at 65°C. The membrane was exposed overnight and analyzed using Imaging Plate scanner BAS 1500 (Fuji Film, Japan).

2.4. Yeast strains and expression

The *Saccharomyces cerevisiae* strain QX95001 is BF264-15Dau (*MATα ade1 his2 leu2-3, 112 trp1-1a ura3*) [15] containing the LEU2-marked mouse Bax-encoding plasmid YEp51-Bax [10], and was maintained in synthetic dropout medium lacking leucine (SD−Leu). Expression vectors, pTU1-OsBI-1 and pYX112-OsBI-1, were constructed using the *Eco*RI tagged coding sequence of OsBI-1 cDNA by ligation in the *Eco*RI site of Ura-marked vectors, pTU1 which includes a CEN replicon or pYX112 which includes a 2 μm replicon (Boehringer Mannheim). The inserts in the constructs were verified by sequencing on both DNA strands. Plasmid DNA transformations were performed by the LiCl method. Ura^{+} transformants that harbored pTU-OsBI-1 or pYX-OsBI-1 were then streaked on SD-glucose plates or SD-galactose plates. Plates were incubated at 30°C for 3 days.

3. Results

The mammalian BI-1 gene was isolated as a novel regulator of cell death using yeast [10]. During searching of EST clones, we identified a rice cDNA clone, 97MJ0046 (accession num-

A

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OsBI-1      1 MDAFYSTSSA YGAAASGWGY DSLKNFRQIS PAVQSHLKLV YLTLCVALAA SAVGAYLHVA   60
AtBI-1      1 MDAF-SSFFD SQPGSRSWSY DSLKNFRQIS PAVQNHLKRV YLTLCCALVA SAFGAYLHVL   59
human       1 M--------- -NIFDRKINF DALLKFSHIT PSTQQHLKKV YASFALCMFV AAAGAYVHMV   50
rat         1 M--------- -NIFDRKINF DALLKFSHIT PSTQQHLKKV YASFALCMFV AAAGAYVHVV   50

OsBI-1     61 LN--IGGMLT MLGCVGSIAW LFSVP--VFE ERKRFGILLA AALLEGASVG PLIKLAVDFD  116
AtBI-1     60 WN--IGGILT TIGCIGTMIW LLSCP--PYE HQKRLSLLFV SAVLEGASVG PLIKVAIDVD  115
human      51 THFIQAGLLS ALGSLILMIW LMATPHSHET EQKRLGLLAG FAFLTGVGLG PALEFCIAVN  110
rat        51 TRFIQAGLLS ALGALALMIC LMATPHSHET EQKRLGLLAV -AFLTGVGLG PALELCIAIN  109
(AtBI-2)                                         KRLYLLFL FALLKGASVG PMIMLVIDFD

OsBI-1    117 SSILVTAFVG TAIAFGCFTC AAIVAKRREY LYLGGLLSSG LSILLWLQFA ASIFGHSTGS  176
AtBI-1    116 PSILITAFVG TAIAFVCFSA AAMLARRREY LYLGRLLSSG LSMLMWLQFA SSIFGGSASI  175
human     111 PSILPTAFMG TAMIFTCFTL SALYARRRSY LFLGGILMSA LSLLLLSSLG NVFFG-SIWL  169
rat       110 PSILPTAFMG TAMIFTCFSL SALYARRRSY LFLGGILMSA MSLMFVSSLG NLFFG-SIWL  168
(AtBI-2)      SSVLVTAFVG TAVAFVCFSA AAMLATRREY LYHGASLACC MSILWWVQIA SSIFGGSTTV

OsBI-1    177 FMFEVYFGLL IFLGYMVYDT QEIIERAHHG DMDYIKHALT LFTDFVAVLV RILVIMLKNA  236
AtBI-1    176 FKFELYFGLL IFVGYMVVDT QEIIEKAHLG DMDYVKHSLT LFTDFVAVFV RILIIMLKNS  235
human     170 FQANLYVGLV VMCGFVLFDT QLIIEKAEHG DQDYIWHCID LFLDFITVFR KLMMILAMNE  219
rat       169 FQANLYMGLL VMCGFVLFDT QLIIEKAEHG DKDYIWHCID LFLDFVTLFR KLMLILAFNE  218
(AtBI-2)      VKFELYEGLL IFVGYIVVDT QMITEKAHHG DMDYVQHSFT FFTDFASLFV QIL

OsBI-1    237 SDKSEEKKRK KRS   249
AtBI-1    236 ADK-EEKKKK RRN   247
human     220 KDKKKEKK         227
rat       219 KDKKKEKK         226
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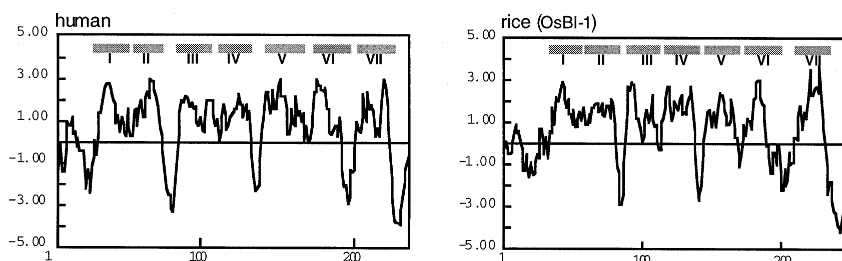
B



C

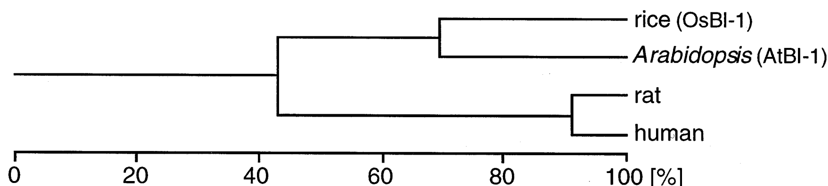


Fig. 1. A: Comparison of deduced amino acid sequences of BI-1 from rice (OsBI-1, accession number: AB025926), *Arabidopsis* (AtBI-1, accession number: AB025927) and other organisms: human, TEGT (BI-1) from Walter et al. [12]; rat, TEGT from Walter et al. [11]. Partial amino acid sequences estimated from *Arabidopsis* genomic clone (AtBI-2, accession number: Z97343) was also shown. Identical residues with OsBI-1 are shaded. A hyphen indicates gaps introduced to maximize the alignment. B: Hydrophobicity plot and transmembrane prediction of rice and human BI-1 proteins. Hydrophobicity was estimated by the method developed by Kyte and Doolottle [26]. Transmembrane domains were predicted using the SOSUI program [28]. C: Phylogenetic tree analysis of BI-1 proteins from animals and plants was constructed by the UPGMA method.

ber: AA754240), the predicted translation product of which showed similarity to the products of the mammalian BI-1 gene. This partial EST clone was used for a probe to screen libraries for full-length cDNAs containing the entire coding region. After successive screening, 12 positive clones were obtained from 2×10^5 plaques. The rice BI gene (OsBI-1, accession number: AB025926) encoded 248 amino acid residues. The resulting 12 clones were found to be derived from the same cDNA clone and showed the same nucleotide sequences. However, comparison of the 3′-ends indicated different polyadenylation sites.

We searched for *Arabidopsis* gene corresponding mammalian BI-1 homologue using the GenBank database. Consequently, we identified one genomic clone (accession number 97343), which was denoted to the AtBI-2 gene. To isolate the cDNA encoding full length AtBI-2, two primers described in Section 2 were prepared. By PCR method, we were able to amplify the partial DNA fragment from mRNA prepared from suspension cultured cells. Using the amplified fragment as a probe, we isolated one clone from the cDNA library of young *Arabidopsis* seedlings. This clone was not an identical one to the AtBI-2 (genomic clone), which showed 63% homology at nucleotide levels. Therefore, we denoted this newly isolated full length cDNA to AtBI-1 (accession number: AB025927). Further screening of the cDNA library did not result in the isolation of the AtBI-2 cDNA clone.

A comparison of the primary structure of OsBI-1 and AtBI-1 with other organisms is shown in Fig. 1A–C. The estimated molecular mass of OsBI-1 was 25.9 kDa, which is similar to the mammalian BI-1 protein. OsBI-1 showed homology with mammalian BI-1 (41.5% identical) and AtBI-1 (70.3%), respectively, at the amino acid level. As shown in Fig. 1B, the hydropathy plot and secondary structure prediction indicate that OsBI-1 is a membrane protein with six (by the transmembrane prediction program, TMpred) or seven (SOSUI, SPLIT) transmembrane domains. Similar transmembrane domains have been predicted at the same positions in the mammalian BI proteins (Fig. 1B, left) and AtBI-1 (data not shown). The predicted length of the BI-1 family proteins was conserved among the different species, with similarity spanning the entire length of the protein.

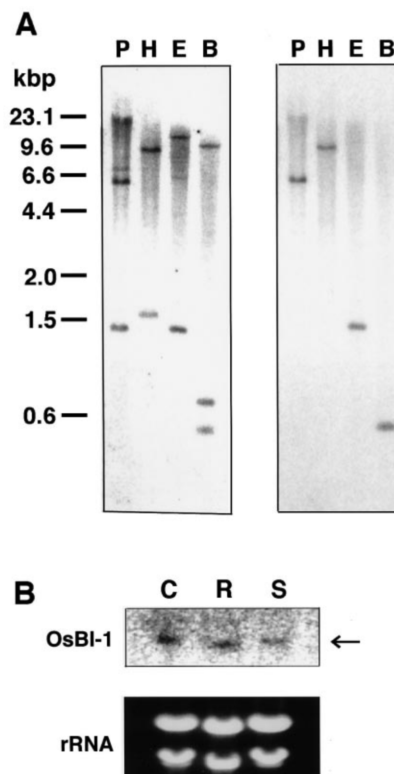


Fig. 2. A: Southern blot analysis of OsBI-1. Total genomic DNAs (10 μg/lane) digested with *Pst*I (P), *Hin*dIII (H), *Eco*RI (E) or *Bam*HI (B) were electrophoresed in a 0.8% agarose gel. Hybridization was performed using two different rice BI-1 cDNA probes: full length cDNA (left) or 3′-UTR (right). Washing was performed with low stringency (left, washed with 2×SSC and 0.1% SDS) or high stringency (right, 0.1×SSC and 0.1% SDS), respectively. B: Northern blot analysis of OsBI-1 cDNA. Total RNAs (10 μg/lane) were isolated from shoot (S) or root (R) tissues of 7-day-old seedlings and 4-day-old suspension cells (C). ^{32}P-labeled 3′-UTR of OsBI-1 was used as a probe (top). Washing was performed with 0.1×SSC and 0.1% SDS at 65°C. The ethidium bromide-stained gel confirmed the loading of approximately equal amounts of total RNA in each lane.

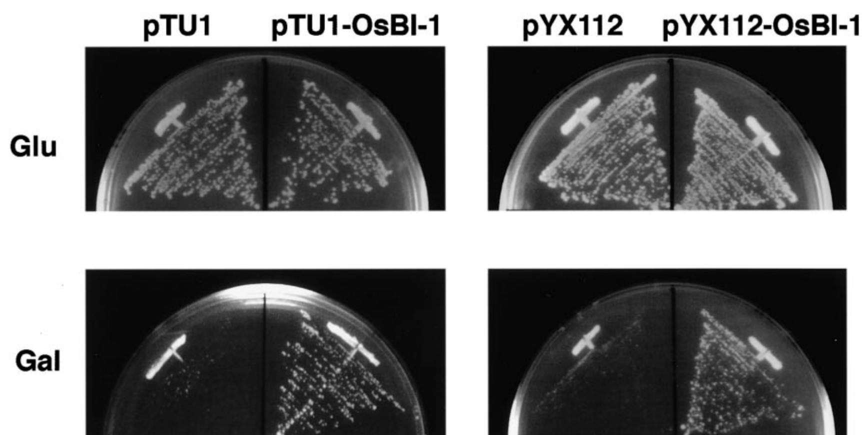


Fig. 3. Suppression of Bax-induced yeast cell death by plant BI-1. Either control vectors (pTU1 and pYX112) or OsBI-1 expression plasmids (pTU1-OsBI-1 and pYX112-OsBI-1) were transformed into cells of yeast strain QX95001 harboring YEp51-Bax. Transformants were streaked on glucose (Glu)- or galactose (Gal)-containing medium lacking uracil and leucine. Photograph was taken 3 days after incubation at 30°C.

To estimate the copy number of BI-1 genes in the rice genome, we performed genomic Southern blot analysis using a full length cDNA (Fig. 2A, left) and 3′-UTR region cDNA (Fig. 2A, right) as probes. OsBI-1 cDNA has an internal *Bam*HI, *Hin*dIII and *Pst*I site, therefore at least two bands with full length cDNA probe were predicted in these digests. Upon hybridization with a 3′-untranslated region probe in high stringency, a single band was observed in each digest. In contrast, the full length cDNA probe hybridized to several restriction fragments within the rice genome DNA at low stringency (Fig. 2A, left) or high stringency (data not shown), indicating the presence of supplementary sites in introns or the possible presence of additional rice genes with homology to OsBI-1. As mentioned above, all cDNAs screened (12 clones) from the rice root cDNA library were identical and derived from a single gene. However, other BI-1 homologues may not be contained in rice root cDNA libraries due to very low expression levels or because they may have a different tissue specific expression.

To study the expression of the OsBI-1 in rice tissues, Northern blot analysis was carried out using the 3′-untranslated region as a specific probe under the conditions of high stringency. As shown in Fig. 2B, the accumulation of the OsBI-1 mRNA was detected in all tissues (i.e. callus, root and shoot) examined.

The sequence and predicted structural similarity suggested that the rice protein is the plant homologue of mammalian BI-1 proteins. As a first step toward determining the role played by this protein in plants, we tested whether the plant protein has retained the same function as the animal BI-1 in terms of suppressing Bax-induced cell death in yeast. Expression of the proapoptotic protein Bax under the control of *GAL10* promoter in *S. cerevisiae* resulted in galactose-inducible cell death as demonstrated previously [10]. We subcloned the OsBI-1 cDNA into the yeast expression plasmids pTU1 and pYX112. These constructs were transformed into yeast strain, QX95001, which harbors a galactose-inducible Bax plasmid. As shown in Fig. 3, when plated on the galactose-medium, cells transformed with pTU-OsBI-1 or pYX-OsBI-1 survived and grew, whereas cells transformed with empty vector did not. As Xu and Reed [10] demonstrated in the mammalian BI-1 gene, the Bax protein was also expressed in yeast cells harboring either OsBI-1 or AtBI-1 (data not shown). Our unpublished results also showed that the OsBI-1 alone did not cause any growth defect in yeast lacking Bax expression.

4. Discussion

Programmed cell death (PCD) is a process of self-directed death of specific cells, representing an important component of growth and development of multicellular organisms [1,16]. In plants, PCD is characteristic of developing tracheary cells, root cap cells, aerenchyma formation [17,18], tapetum cell degradation to sustain pollen development, stomium cell death for anther dehiscence, sexual organ formation, carpel senescence, leaf senescence, responses to pathogens and other processes [19].

The mechanisms that control plant cell death are not well understood. Several investigators have suggested parallels between PCD in plants and apoptosis in animals [20–22]. Apoptotic DNA ladders have also been reported in the setting of plant PCD [21,23]. Expression level of the plant homologue of *dad*1 gene, known as a suppressor of programmed cell death in *C. elegans* and mammals [24], was down-regulated during petal senescence in peas [21]. Tanaka et al. [22] confirmed that the rice *dad* gene is a functional suppressor of cell death in animal cells. Recently, Lacomme and Santa Cruz [25] reported that Bax, a mammalian death-promoting member, triggered cell death when expressed in plants. These observations suggest that the common mechanism of animal PCD might be present in plants. However, no molecular relationship has been demonstrated between the mechanisms involving PCD in plants and apoptosis in animals. In particular, no functional homologues of the animal Bcl-2 family have been reported in plants.

A variety of positive and negative regulators of events involved in apoptosis have been characterized in animal systems. BI-1 represents a recently described suppressor of apoptosis which was identified based on its ability to inhibit Bax-induced cell death in yeast and mammalian cells [10]. Although the budding yeast *S. cerevisiae* and fission yeast *Saccharomyces pombe* do not contain identified homologues of Bcl-2 or caspases, it has been shown that Bax, Bak, and closely related death-promoting Bcl-2 family proteins induce cell death in these unicellular eukaryotes [5–9]. These cell deaths can be inhibited by co-expression of Bcl-2 or Bcl-X_L.

Homologues of BI-1 are found in rat, mouse, *C. elegans* as well as in plants (this work, *Oryza* and *Arabidopsis*), indicating that BI-1 is conserved throughout evolution. The OsBI-1 gene encodes a protein containing six or seven putative transmembrane domains similar to mammalian BI-1 proteins. Despite an identity of about 40% with the human protein, the plant BI-1 protein was able to suppress Bax-induced cell death in yeast. To our knowledge, this is the first report in which a plant protein has been shown to be able to functionally replace a Bcl-2/Bax-related cell death gene. The plant BI-1 protein is structurally and functionally similar to its mammalian counterpart, suggesting that at least some mechanisms of suppression of cell death have been conserved since the evolutionary separation of the plant and animal kingdoms. Interestingly, Xu and Reed [10] reported that the BI-1 protein associates with protein complexes that contain Bcl-2 and Bcl-X_L. Whether or not plant BI-1 homologues similarly form complexes with Bcl-2-like proteins or possess the ability to suppress cell death in plants will be the subject of further investigations.

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