The human dad-1 cDNA homolog was isolated from rice plants. The amino acid sequence of the predicted protein product is well conserved in both animals and plants. This rice dad-1 homolog can rescue the temperature-sensitive dad-1 mutants of hamster cells from apoptotic death, suggesting that the rice dad-1 homolog also functions as a suppressor for programmed cell death.

**Key words:** Apoptosis — cDNA — dad-1 — Rice (Oryza sativa).

Multicellular organisms eliminate harmful and/or unnecessary cells during differentiation and developmental process, called apoptosis. A typical phenomenon of apoptosis is the programmed cell death that enables multicellular organisms to maintain their homeostasis. These events are typified in vertebrates by plasma membrane blebbing, condensation of cytoplasm, compaction of chromatin, and degradation of nuclear DNA into oligonucleosome-size fragments (Sugimoto et al. 1995, see also reviews by Vaux et al. 1994, White and Evans 1995, Greenberg 1996). Plants may also carry out a mechanism of apoptosis similar to that found in animals, although the phenomenon has not yet been elucidated (Demura and Fukuda 1994, Greenberg 1996). In animals, apoptosis is activated during the course of several developmental pathways and in response to encountering certain viral pathogens and various environmental stimuli. Very recently, DNA fragmentation and formation of apoptotic bodies have been demonstrated in tomato cells treated with AAL toxin (toxin produced by a fungal pathogen, Alternaria alternata), fumonisins, or potassium cyanide (Wang et al. 1996). Apoptotic cell death was also shown in the events of the typical plant defense reaction, which is termed the hypersensitive cell death response (HR) (Ryerson and Heath 1996).

The dad-1 (defender against apoptotic death) gene is defective in a temperature-sensitive mutant cell line of hamster (tsBN7) that undergoes apoptosis at restrictive temperatures (Nakashima et al. 1993). The predicted product of this novel protein is well conserved among vertebrates (human, hamster, mouse and Xenopus) (Nakashima et al. 1993) and Caenorhabditis elegans (C. elegans) (Sugimoto et al. 1995). It is also found in two plant species, rice and Arabidopsis, on the sequence databases, although both plant sequences seemed incomplete. The dad-1 homolog of rice (Oryza sativa cv. Nipponbare) shows homology to C-terminal amino acid sequences deduced from animal dad-1. For this reason, we tried to clone nearly full length dad-1 cDNA and the corresponding genomic DNA to characterize the structure and organization of the dad-1 gene in rice.

In the present report, we describe the cloning of the cDNA and genomic DNA of dad-1 homolog in rice. Our experiments have demonstrated that the cloned rice cDNA complemented the temperature-sensitive dad-1 mutant of hamster BHK21 cell line, tsBN7.

Total RNA was extracted from a whole 3 months old seedling rice plant with ISOGEN (Nippongene, Toyama, Japan), and poly(A)+ RNA was purified with PolyATtract mRNA Isolation Systems™ (Promega, CA, U.S.A.). For the isolation of complete rice dad-1 cDNA, a modified RACE (Rapid Amplification of the cDNA End) method was performed using a Marathon cDNA Amplification Kit (CLONTECH; CA, U.S.A.) as described in the manuals from the supplier. To generate a nearly full-length cDNA, the following primers were designed; 5'-CGAAATCTGCTACGCCCCCTTTCC-3' (primer 2) for the 5'-RACE and 5'-TTTCCAACCTCTTCCCTCTGCG-3' (primer 1) for the 3'-RACE. These sequences were derived from the rice cDNA (R1413-1A) sequence in the EMBL database (accession number, D24136) that presumably lacked sequences corresponding to the N-terminus of the dad-1 homolog (Fig. 1). A thermal cycle consisting of 45 s at 94°C, 45 s at 60°C, and 3 min at 68°C was performed for 30 cycles with a mixture of Taq and Vent DNA polymerase for high fidelity. Both RACE products were annealed and amplified by PCR using AP1 (Amplification primer 1) supplied in the kit. The thermal cycle consisting of 45 s at 94°C, 1 min at 60°C, and 2 min at 68°C was performed for 30 cycles. The amplified cDNA fragment was purified from SeaPlaqueGTG agarose gel (FMC, Takara, Japan) after electrophoresis by digestion with β-agarase (Nippongene, Toyama, Japan).
Rice *dad-1* rescue tsBN7 cells from apoptotic death.

Japan) and subcloned either in pUC19 for sequencing or in pcDSRa296 for the complementation assay.

The nucleotide sequence of *dad-1* cDNA was determined by the dideoxy chain termination method (Sambrook et al. 1989) in both directions. The nucleotide sequences determined were compiled and analyzed with the GENETYX programs (SDC, Tokyo, Japan) (Fig. 1). The predicted translational initiation codon was located 24 nu-

![Diagram](image)

**Fig. 1** Schematic presentation of the structure of rice *dad-1* gene and the nucleotide and amino acid sequences. (A) Schematic presentation of the structure of rice *dad-1* gene. The *dad-1* homolog in rice is comprised of five exons (closed boxes). The restriction endonuclease recognition sites of EcoRI, HindIII and *PstI* are indicated at the top. The size of 100 bp is indicated with a solid line. (B) Nucleotide sequences of the *dad-1* gene and cDNA constructed and the deduced amino acid sequences of DAD1 protein. Nucleotide sequences of the exonic sequences and intronic sequences are indicated with capital letters and small letters, respectively. The deduced amino acid sequences are denoted by a single letter code below the nucleotide sequence. Adenine in the putative translational initiation codon (ATG) is arbitrarily set at +1 in the sequences. Numbers at the right side indicate the number of nucleotides from the translational initiation site.
Rice dad-1 rescue tsBN7 cells from apoptotic death

(A) Rice
1: PRATGKKTQKQQLGAAPFTPLNLFKIDILYVYFAGTVAFQALIQVYYGGSFPFFNSL560
Arabidopsis
1: S-TSTQKFTQGQLGAAPFTPLNLFKIDILYVYFAGTVAFQALIQVYYGGSFPFFNSL560
Human
1: SASVVSVQIS-FLEET-LSTQGKAGLNLKILVYLQAVLAVGTPFFNSL58
Xenopus
1: SVTVSVSIS-RLKDEY-VSSTQGKVLFVNLKILVYLQAVLAVGTPFFNSL58
c elegans
1: KTVTSTVSIS-LKIDPVKQAVGGLKVLFVNLKILVYLQAVLAVGTPFFNSL58

(B)
1.026

0.2027

0.0522

Human

0.1697

0.3220

0.1026

Rice

C elegans

Xenopus

Arabidopsis

Fig. 2 Comparison of the predicted amino acid sequences of rice DAD-1 and DAD-1 proteins from other organisms. (A) Residues that are identical in rice and one or more of the other proteins are printed as white-on-black letters. A hyphen indicates gaps introduced to maximize the alignment. Arabidopsis DAD-1 has a longer polypeptide at the N-terminal end (only 113 amino acid residues are shown). (B) Phylogenetic tree based on amino acid sequences of DAD-1 proteins from animals and plants. A phylogenetic tree was constructed with amino acid sequences of human and Xenopus DAD-1 (Kashima et al. 1993; accession number D15057 and D15059, respectively), C. elegans DAD-1 (Sugimoto et al. 1995) and D15057, partial sequence of Arabidopsis cDNA (127H23T7) (Newman et al. 1994; accession number T44943) by the UPGMA method (Kumar et al. 1994). Relative evolutionary distances between proteins are indicated.

cleotides downstream from the terminal of the 5′-cDNA end. It contained an open reading frame of 114 amino acid residues; other vertebrate dad-1 consisted of 113 amino acid residues. The deduced amino acid sequence is homologous, with 47% and 48% identical residues to dad-1 of human and Xenopus, respectively, and 77% identical residues to the Arabidopsis dad-1 homolog, although it has an extension sequence at the N-terminus unlike the other dad-1 sequences. Phylogenetic trees constructed by the UPGMA program (GENETYX: SDC, Tokyo, Japan) (Kumar et al. 1994) and based on these amino acid sequences demonstrated that the predicted protein products of the dad-1 genes from different kingdoms so far examined could be largely classified into three groups, vertebrate, invertebrate, and plant (Fig. 2).

To construct a genomic DNA library, genomic DNA was extracted from young seedlings of rice as described by Murray and Thompson (1980), and restriction endonuclease-digested DNAs were cloned into SuperCosI (STRATAGENE, La Jolla, CA, U.S.A.) according to the manufacturer's specification. Colonies of 1.5 × 10⁶ (0.1 μg of genomic DNA used in the construction of library) were transferred onto nylon membranes (HybondN+; Amer-

Fig. 3 Southern blot hybridization analysis of the presence of dad-1 in rice genome. Twenty micrograms of genomic DNA were digested with the restriction endonucleases depicted in the figure, electrophoresed, and transferred onto a nylon membrane. Hybridization was carried out using the 32P-labeled PCR product that contained nearly the entire rice dad-1 cDNA as a probe.
Rice dad-1 rescue tsBN7 cells from apoptotic death

sham, Tokyo, Japan) and screened with a 32P-labeled rice dad-1 cDNA fragment generated from PCR using the primers 3 and 4 (Fig. 1). Hybridization was performed overnight at 60°C in 6 x SSC, 5 x Denhardt's solution, 0.5% SDS, and 0.1 µg ml⁻¹ salmon sperm DNA. Hybridized DNA was washed 3 times with 0.1 x SSC and 0.5% SDS at 60°C. Three positive clones were obtained; one which appeared to cover the entire dad-1 genomic DNA was selected for further analysis. The nucleotide sequence of the dad-1 gene of rice revealed that it was comprised of five exons (Fig. 1), whereas animal dad-1 was comprised of three exons (Nakashima et al. 1993).

Genomic Southern blot hybridization analysis was performed as follows (Sambrook et al. 1989). Twenty micrograms of genomic DNA were digested with restriction enzymes, BamHI, EcoRI, HindIII, and PstI, fractionated by electrophoresis in a 0.7% agarose gel, and transferred onto a nylon membrane (HybondN+; Amersham, Tokyo, Japan). Hybridization and washing were performed as described above. Hybridization analysis demonstrated that a single copy of the rice dad-1 gene existed in rice genomic DNA; only a single major hybridized band appeared in BamHI-digested DNA fragments and two bands, in EcoRI-digested DNA fragments (Fig. 3). PstI- and HindIII-digestion did not give the expected number of the major hybridized bands, presumably because the band supposed to appear on this gel that contained a part of exon1 or mainly exon4 alone (PstI digestion) or exon1 and exon2 (HindIII digestion) was either too small to provide enough hybridization signal for this probe or so small that the hybridized band might have migrated away from the gel.

To check whether rice dad-1 is functionally interchangeable with mammalian dad-1, we examined the ability of the rice dad-1 homolog to complement the hamster tsBN7 mutant cells. pcDSRa296 carrying SRa promoter was used as an expression vector for rice dad-1 cDNAs, as described by Nakashima et al. (1993). Transformants of rice dad-1 cDNA were tested for temperature sensitive lethality in hamster tsBN7 cells. The rice dad-1 homolog rescued the temperature-sensitive mutant hamster cell

![A](image1.png)

![B](image2.png)

**Fig. 4** Complementation of tsBN7 cells by rice dad-1. Human- and rice-dad-1 cDNAs were transfected into tsBN7 cells and incubated at 33.5°C for two days followed by transfer at 39.5°C (non-permissive temperature for tsBN7) to examine the degree of complementation with human dad-1 (pcDSRa-hu-dad-1) and rice dad-1 (pcDSRa-rice-dad-1). As a control, the pcDSRa vector itself was introduced into tsBN7 cells. (A) Appearance of tsBN7 colonies at the non-permissive temperature due to complementation of human dad-1 and rice dad-1 cDNA. (B) The number of tsBN7 colonies which appeared at the non-permissive temperature (39.5°C). The number of colonies at 33.5°C supplemented with neomycin represents the relative transfection efficiencies of the recombinant plasmid, pcDSRa-hu-dad-1, pcDSRa-rice-dad-1, and pcDSRa vector alone.
Rice dad-1 rescue tsBN7 cells from apoptotic death

References


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