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Rapid Report

dad-1, A Putative Programmed Cell Death Suppressor Gene in Rice

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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*), fumonisin, 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′-CGAAATCTGC AAACGCCCTTTC-3′ (primer 2) for the 5′-RACE and 5′-TTTCAACTCT TTCCTTTCTGGC-3′ (primer 1) for the 3′-RACE. These sequences were derived from the rice cDNA (R1413-1A) sequence in the EMBL databank (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,

The nucleotide sequences of rice *dad-1* cDNA and rice *dad-1* genomic DNA reported in this paper have been submitted to the DDBJ, EMBL and GenBank under the accession numbers, D89726 and D89727, respectively.

Japan) and subcloned either in pUC19 for sequencing or in pcDSRα296 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-

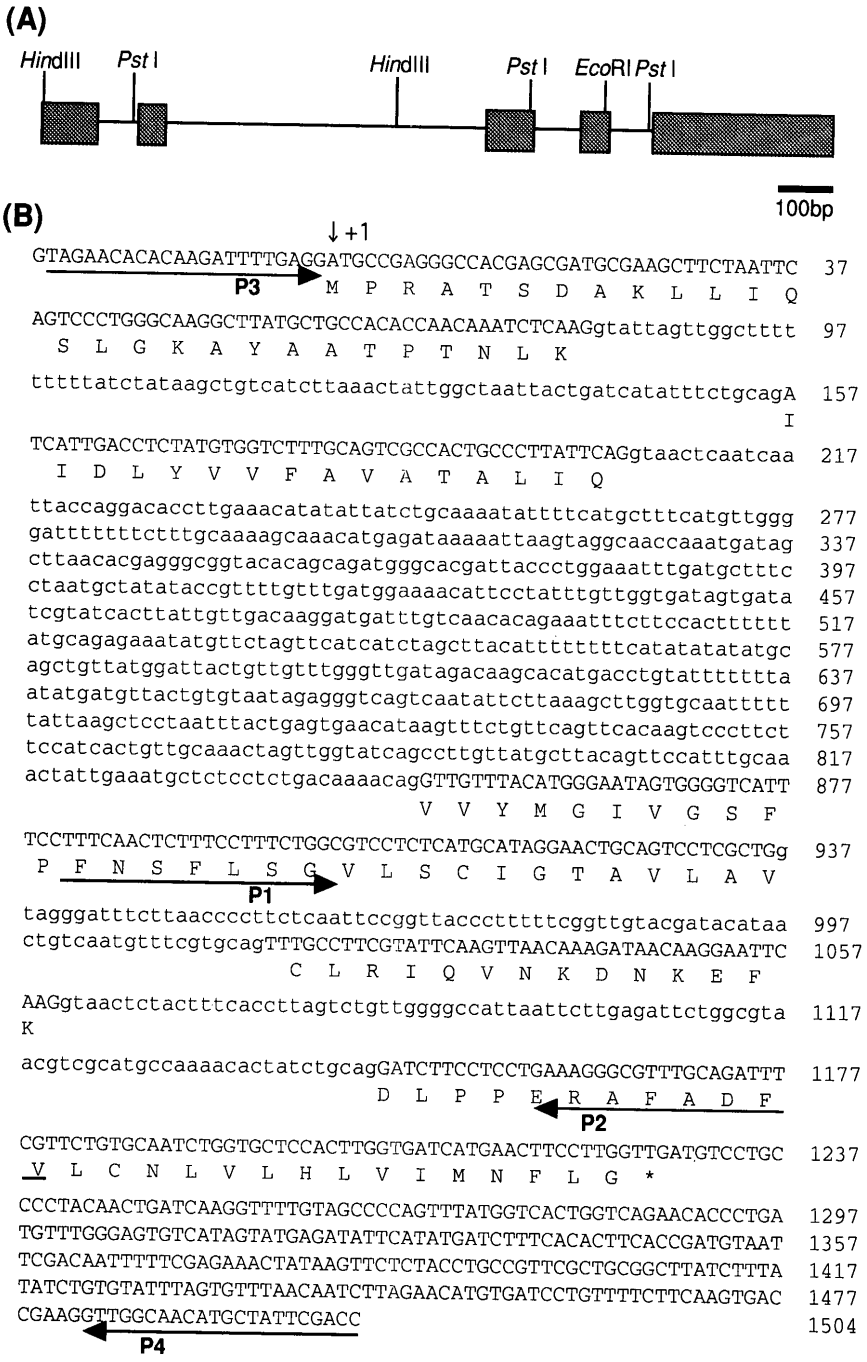


Fig. 1 Schematic presentation of the structure of rice *dad-1* gene and the nucleotide and amino acid sequences. (A) Schematic presentation of the structure of rice *dad-1* gene. The *dad-1* homolog in rice is comprised of five exons (closed boxes). The restriction endonuclease recognition sites of *Eco*RI, *Hin*dIII and *Pst*I 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 DAD-1 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.

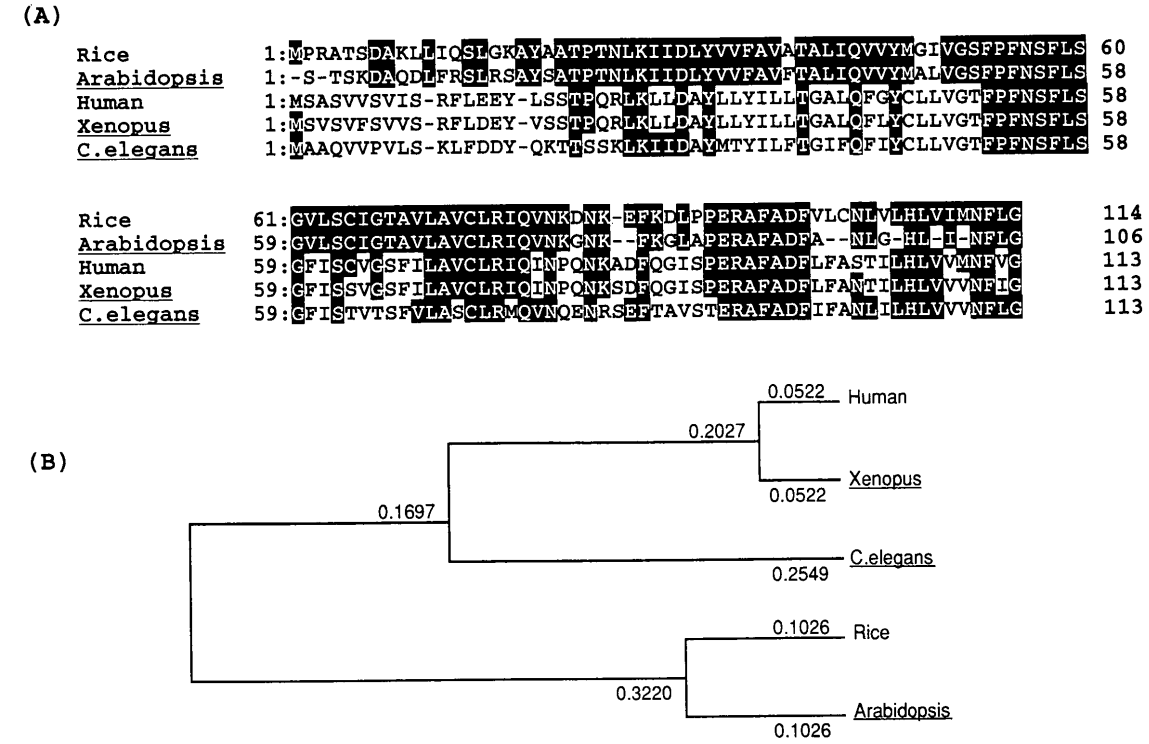


Fig. 2 Comparison of the predicted amino acid sequences of rice DAD-1 and DAD-1 proteins from other organisms. (A) Residues that are identical in rice and one or more of the other proteins are printed as white-on-black letters. A hyphen indicates gaps introduced to maximize the alignment. *Arabidopsis* DAD-1 has a longer polypeptide at the N-terminal end (only 113 amino acid residues are shown). (B) Phylogenetic trees 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 (Nakashima et al. 1993; accession number D15057 and D15059, respectively), *C. elegans* DAD-1 (Sugimoto et al. 1995; accession number X89080), 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 in SuperCosI (STRATAGENE, La Jolla, CA, U.S.A.) according to the manufacturer's specification. Colonies of 1.5×10^4 (0.1 μg of genomic DNA used in the construction of library) were transferred onto nylon membranes (HybondN+; Amer-

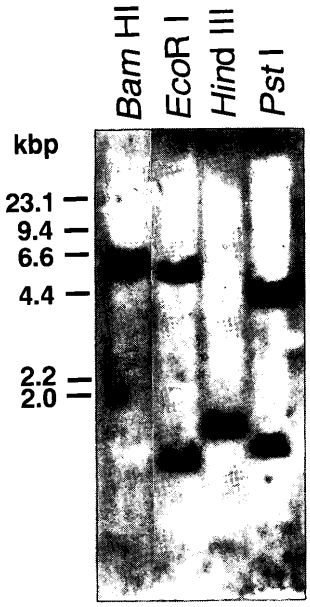


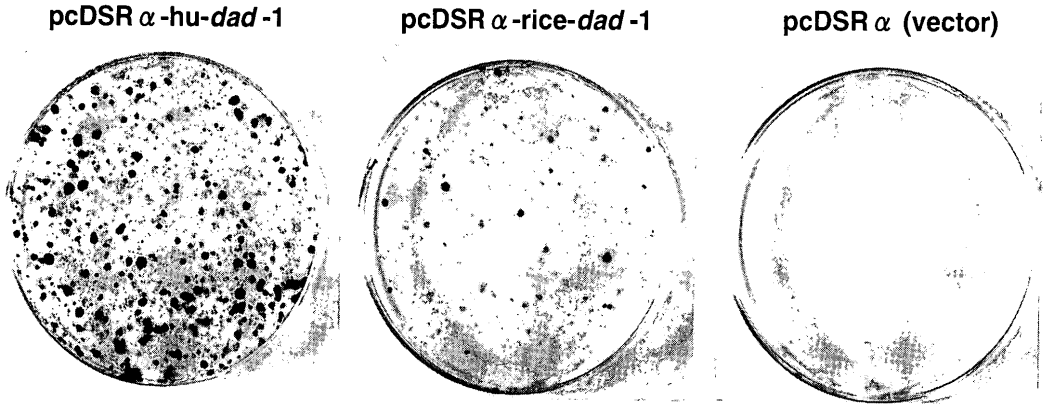
Fig. 3 Southern blot hybridization analysis of the presence of *dad-1* in rice genome. Twenty micrograms of genomic DNA were digested with the restriction endonucleases depicted in the figure, electrophoresed, and transferred onto a nylon membrane. Hybridization was carried out using the ^{32}P-labeled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

sham, Tokyo, Japan) and screened with a ^{32}P-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×SSC, 5×Denhardt's solution, 0.5% SDS, and 0.1 μg ml^{-1} salmon sperm DNA. Hybridized DNA was washed 3 times with 0.1×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, *Bam*HI, *Eco*RI, *Hin*dIII, and *Pst*I, 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 *Bam*HI-digested DNA fragments and two bands, in *Eco*RI-digested DNA fragments (Fig. 3). *Pst*I- and *Hin*dIII-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 (*Pst*I digestion) or exon1 and exon2 (*Hin*dIII 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. pcDSRα296 carrying SRα 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



B

Plasmid	No. of colonies (per 2 × 10^5 cells)	
	39.5°C	33.5°C + Neomycin
pcDSR α-hu-*dad*-1	215	51
pcDSR α-rice-*dad*-1	60	40
pcDSR α (vector)	0	45

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* (pcDSRα-hu-*dad-1*) and rice *dad-1* (pcDSRα-rice-*dad-1*). As a control, the pcDSRα 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, pcDSRα-hu-*dad-1*, pcDSRα-rice-*dad-1*, and pcDSRα vector alone.

lines; it was not as efficient as the human *dad-1* gene, but significant enough for the complementation of *dad-1*^{ts} of hamster cells when comparing to the transformants of the vector itself (Fig. 4). These results strongly support the hypothesis that the rice *dad-1* homolog may also function as a suppressor of programmed cell death in rice.

The *dad-1* gene, originally identified in a mutant hamster cell line (tsBN7) that undergoes apoptosis at restrictive temperature, has been shown to regulate programmed cell death in animals in a manner either independent of, or downstream from, *ced-9*, which was previously identified as a cell death suppressor gene in *C. elegans* (Sugimoto et al. 1995). A variety of positive and negative regulator genes involved in the events of apoptosis have been characterized in animal systems; however, very little is known about these genes in plants. Recent studies have demonstrated that higher plants also carry out programmed cell death events, including the appearance of apoptotic bodies and DNA laddering (Wang et al. 1996, Ryerson and Heath 1996) upon treatment with toxins or in the process of defense system typified by HR. Apoptosis-like cell death in barley roots due to high salt-stress has also been reported (Katsuhara and Kawasaki 1996). However, apoptosis-related genes other than *dad-1* have not been successfully isolated from higher plants.

As has been reported, the human *dad-1* and *C. elegans* *dad-1* can complement each other in each mutant cell line, and we have demonstrated here that the rice *dad-1* homolog can also complement mammalian *dad-1*, suggesting that cell death programs also exist in higher plants and that *dad-1* not only functions as a negative regulator of programmed cell death but also plays other vital roles in maintaining homeostasis in both the animal and plant kingdoms. Further elucidation of the role of this gene in many phyla is indispensable, since DAD-1 has been shown to be highly homologous in amino acid sequence with the subunit of oligosaccharyltransferase in yeast which is regarded as a house-keeping gene (Silberstein et al. 1995).

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References

Demura, T. and Fukuda, H. (1994) Novel vascular cell-specific genes whose expression is regulated temporally and spatially during vascular system development. *Plant Cell* 6: 967–981.

Greenberg, J.T. (1996) Programmed cell death: a way of life for plants. *Proc. Natl. Acad. Sci. USA* 93: 12094–12097.

Katsuhara, M. and Kawasaki, T. (1996) Salt stress induced nuclear and DNA degradation in meristematic cells of barley roots. *Plant Cell Physiol.* 37: 169–173.

Kumar, S., Tamura, K. and Nei, M. (1994) MEGA: molecular evolutionary genetics analysis software for microcomputers. *Computer Applications Biosci.* 10: 181–191.

Murray, M.G. and Thompson, W.F. (1980) Rapid isolation of high molecular weight plant DNA. *Nucl. Acids Res.* 8: 4321–4325.

Nakashima, T., Sekiguchi, T., Kuraoka, A., Fukushima, K., Shibata, Y., Komiyama, S. and Nishimoto, T. (1993) Molecular cloning of a human cDNA encoding a novel protein, DAD1, whose defect causes apoptotic cell death in hamster BHK21 cells. *Mol. Cell. Biol.* 13: 6367–6374.

Newman, T., de Bruijin, F.J., Green, P., Keegstra, K., Kende, H., McIntosh, L., Ohlrogge, J., Raikhel, N., Somerville, S., Thomashow, M., Retzel, E. and Somerville, C. (1994) Genes galore: a summary of methods for accessing results from large-scale partial sequencing of anonymous *Arabidopsis* cDNA clones. *Plant Physiol.* 106: 1241–1255.

Ryerson, D.E. and Heath, M.C. (1996) Cleavage of nuclear DNA into oligonucleosomal fragments during cell death induced by fungal infection or by abiotic treatments. *Plant Cell* 8: 393–402.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual.* 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Silberstein, S., Collins, P.G., Kellenher, D.J. and Gilmore, R. (1995) The essential OST2 gene encodes the 16-kD subunit of the yeast oligosaccharyltransferase, a highly conserved protein expressed in diverse eukaryotic organisms. *J. Cell Biol.* 131: 371–383.

Sugimoto, A., Hozak, R.R., Nakashima, T., Nishimoto, T. and Rothman, J.H. (1995) *dad-1*, an endogenous programmed cell death suppressor in *Caenorhabditis elegans* and vertebrates. *EMBO J.* 14: 4434–4441.

Vaux, D.L., Haecker, G. and Strasser, A. (1994) An evolutionary perspective on apoptosis. *Cell* 76: 777–779.

Wang, H., Li, J., Bostock, M.R. and Gilchrist, G.D. (1996) Apoptosis: a functional paradigm for programmed plant cell death induced by a host-selective phytotoxin and invoked during development. *Plant Cell* 8: 375–391.

White, M. and Evans, G. (1995) The last cut is the deepest. *Nature* 376: 17–18.

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