A homologue of the defender against the apoptotic death gene (*dad1*) in UV-exposed *Chlamydomonas* cells is downregulated with the onset of programmed cell death

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We report here the isolation of a homologue of the potential anti-apoptotic gene, defender against apoptotic death (*dad1*) from *Chlamydomonas reinhardtii* cells. Using polymerase chain reaction (PCR), we investigated its expression in the execution process of programmed cell death (PCD) in UV-C exposed dying *C. reinhardtii* cells. Reverse-transcriptase (RT)-PCR showed that *C. reinhardtii dad1* amplification was drastically reduced in UV-C exposed dying *C. reinhardtii* cells. We connect the downregulation of *dad1* with the upregulation of apoptosis protease activating factor-1 (APAF-1) and the physiological changes that occur in *C. reinhardtii* cells upon exposure to 12 J/m^2 UV-C in order to show a reciprocal relationship between proapoptotic and inhibitor of apoptosis factors. The temporal changes indicate a correlation between the onset of cell death and *dad1* downregulation. The sequence of the PCR product of the cDNA encoding the *dad1* homologue was aligned with the annotated *dad1* (C_20215) from the *Chlamydomonas* database (http://genome.jgi-psf.org:8080/annotator/servlet/jgi.annotation.Annotation?pDb=chlre2); *Annotation?pDb=chlre2*); this sequence was found to show 100% identity, both at the nucleotide and amino acid level. The 327 bp transcript showed an open reading frame of 87 amino acid residues. The deduced amino acid sequence of the putative *C. reinhardtii* DAD1 homologue showed 54% identity with *Oryza sativa*, 56% identity with *Drosophila melanogaster*, 66% identity with *Xenopus laevis*, and 64% identity with *Homo sapiens*, *Sus scrofa*, *Gallus gallus*, *Rattus norvegicus* and *Mus musculus*.

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1. Introduction

Programmed cell death (PCD) or apoptosis is a process in which unwanted cells are removed during the growth and development of multicellular organisms. In response to an array of internal and external stimuli, cells exhibit the capacity to forfeit themselves by means of a complex machinery of interconnected signal transduction pathways (Raff 1998). Recent studies have convincingly shown the existence of PCD in unicellular organisms such as animals (Debrabant *et al* 2003 and references therein), plants and algae such as *Dunaliella teritolecta* (Segovia *et al* 2003; Bidle and Falkowski 2004), *Chlamydomonas reinhardtii* (Moharikar *et al* 2006) and yeast (Madeo *et al* 2002; Del Carratore *et al* 2002; Herker *et al* 2004).

It is now known that animals, plants and unicellular eukaryotes use PCD for defence or developmental mechanisms (Danon *et al* 2004). This argues for the presence of a common ancestral apoptotic machinery among eukaryotes. However, at the molecular level, very few

Keywords. *Chlamydomonas reinhardtii*; defender against apoptotic death (*dad1*); UV-C.

Abbreviations used: APAF-1, apoptosis protease activating factor-1; *dad1,* defender against apoptotic cell death 1; IEP, isoelectric point; OST, oligosaccharyltransferase; PCD, programmed cell death; PCR, polymerase chain reaction

regulatory proteins or protein domains have been identified as conserved across all eukaryotic PCD forms. An important goal is to determine the molecular components used in the execution of PCD in plants, which have been conserved during evolution.

Studies on the genetic control of apoptosis in both animal and plant cells presently focus on specific proteins that have been shown to either inhibit the process, i.e. bcl-2 (Vaux *et al* 1992; Antonsson and Martinou 2000), inhibitor of apoptosis protein (Zaffaroni *et al* 2005; Nachmias *et al* 2004), defender against apoptotic cell death 1 (*dad1*; Yamada *et al* 2004; Nakashima *et al* 1993), or to promote it, i.e. apoptotic protease-activating factor 1 (APAF-1; Lindholm and Arumae 2004), bax and bak (Cory and Adams 2005), caspases (Kim *et al* 2005), metacaspases (Uren *et al* 2000; van der Hoorn and Jones 2004), apoptosis-inducing factor (Hong *et al* 2004).

Among all these components of apoptosis, it must be noted that the DAD1 protein is unique, since it is a part of the oligo saccharyltransferase (OST) complex and has been suggested to play an important role in *N*-linked glycosylation, with a role connecting it to the apoptotic machinery (Makishima *et al* 2000). *dad1* is a putative anti-apoptosis gene identified in several distantly related organisms. In the evolutionary perspective it has been shown that DAD1 proteins are conserved among human (Nakashima *et al* 1993), mouse (Apte *et al* 1995), *C. elegans* (Sugimoto *et al* 1995), *Arabidopsis thaliana* (Gallois *et al* 1997), pea (Orzaez and Granell 1997) and many other species. Dysfunction and downregulation of *dad1* has been linked to PCD in both animals and plants. This gene was originally isolated from a temperature-sensitive mutant hamster cell line that undergoes apoptotic cell death when incubated at a non-permissive temperature, and encodes a protein that has been described to inhibit developmental PCD in *C. elegans* (Nakashima *et al* 1993). A range of studies have demonstrated considerable evolutionary and functional conservation and, therefore, it was postulated that DAD1 is a universal negative regulator of PCD. The plant homologue was shown to complement DAD1 mutation in animal cells (Tanaka *et al* 1997), and may therefore function as a gene in plant cell death. Interestingly, the DAD1 protein is a subunit of the OST complex, which catalyses *N*-linked glycosylation. It interacts with Mcl-1, an anti-apoptotic protein of the bcl-2 family (Makishima *et al* 2000). DAD1 seems to have a role to play in preventing apoptotic cell death and in regulating the levels of *N*-linked glycosylation in *Saccharomyces cerevisiae* and the BHK hamster cell line (Makishima *et al* 2000).

However, *dad1* expression has not been reported from unicellular plants. In this study, we report the isolation of a *dad1* homologue from a unicellular plant *C. reinhardtii* and follow its expression during UV-C induced apoptotic-like cell death. Interestingly, we also show that *dad1* homologue expression is significantly downregulated in *C. reinhardtii* cells exposed to UV, thereby rendering them prone to apoptosis. Our findings demonstrate a direct regulation at the level of transcription with a stress signal such as UV-C. The *in silico* work shows a high level of conservancy of this particular molecule, tempting us to conclude that the apoptotic machinery is conserved in this unicellular species too. We connect the downregulation of *dad1* with the upregulation of APAF-1 and show a reciprocal relationship between proapoptotic factors and those that are inhibitors of apoptosis. A summary of the morphological, biochemical and physiological changes that occur in *C. reinhardtii* cells post exposure to 12 J/m^2 UV-C is given in table 1 (unpublished data and Moharikar *et al* 2006). The temporal changes indicate a correlation between the onset of cell death and *dad1* downregulation. We believe that our analyses provide a functional insight into the process of apoptosis, bringing us closer to dissecting this pathway further.

2. Materials and methods

2.1 *Cell culture conditions*

C. reinhardtii (strain CC-125) cells were obtained as a gift from E. Harris, Genetic Centre, Duke University, USA. Cell growth conditions, exposure of cells to UV-C and calculation of UV-C doses in J/m^2 were done as mentioned in Moharikar *et al* (2006). Briefly, asynchronous mid-log phase cultures with initial cell concentrations of 1 X 10^5 cells/ml were used. 1 X 10^5 cells/ml from the logarithmic growth phase were washed and re-suspended in fresh Tris–acetate–phosphate (TAP) medium to obtain a cell concentration of 1 X 10^8 cells/ml and then exposed to UV-C irradiation (254 nm) using Bio-Rad GS Gene linker™ at doses of 1–100 J/m^2. After exposure, the cells were kept at 22–23^{o}C on a gyratory shaker in the dark for various time periods (1–18 h) to allow expression of morphological and biochemical alterations. All chemicals were of molecular biology or analytical grades and were purchased from GE healthcare, UK Limited Buckinghamshire, England; Sigma, St Louis, Mo, USA; Qualigens Fine Chemicals, Mumbai; and Molecular Probes, Eugene, USA.

2.2 *RNA extraction and cDNA synthesis from* Chlamydomonas

For RNA extraction, 2 ml of the 10^6 cells/ml were harvested by a quick spin (2′/8000 rpm/4^{o}C) to which 400 μl of GTC-RNA extraction buffer was added (4 M GTC, 25 mM sodium citrate, 0.1% SLS 0.1 M β-mercaptoethanol). To this lysed suspension, 40 μl of 2 M sodium acetate (pH 4.0) was added followed by the addition of 400 μl of phenol and mixed by inversion. This was followed by the addition of 700 μl

of chloroform after which the suspension was thoroughly mixed and centrifuged at 12,000 *g*/15′/4°C. The aqueous phase was removed and RNA was precipitated by the addition of an equal volume of isopropanol at 4°C/30 min. The RNA pellet was recovered by centrifugation at 12,000 *g*/15′/4°C, followed by a 70% ethanol wash, after which the pellet was air-dried and resuspended in 50 μl RNase-free TE buffer, and quantified by UV absorbance. The consistency of RNA quality was assured by the visualization of 18 S and 28 S RNA bands on a 1.2% formamide agarose gel. cDNA synthesis was carried out using SuperScript™ Reverse Transcriptase purchased from Invitrogen™ Life Technologies, using buffers and instructions provided by the manufacturer.

2.3 *RT-PCR and sequencing of the PCR product*

In order to semi-quantify the transcript, *Chlamydomonas* actin gene was used as an internal reference. The primers were designed for the *dad1* sequence annotated in the Chlamy database. Complementary DNA was amplified by following two primer mixes for the *dad1* gene; FW Primer 5′ CGC GGA TCC ATG CTC GCC GAG ATC 3′-24; RW Primer 5′ CCC CTC GAG TCA GCC CAT GTA GTT CC 3′-26; with the following PCR conditions: initial denaturation at 94°C for 2 min followed by 30 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 2 min. The last extension was done at 72°C for 10 min. The PCR product thus obtained was purified using the Qiagen PCR purification kit and was then sequenced from MWG¸ Biotech Private Limited, Bangalore, India.

2.4 *Detection of APAF-1 protein from* C. reinhardtii *cells*

Two ml of 1 X 10^8 cells/ml *C. reinhardtii* cells, both exposed and unexposed to UV-C, were collected at various doses (12–100 J/m^2) and time-points, as indicated. Protein was extracted according to the method of D'Souza and Johri (2002), with some modifications. Briefly, cells were harvested by centrifuging at 4,000 rpm for 10 min at 4° C. The mixture was then boiled in homogenization buffer for 10 min, followed by centrifugation at 14,000 rpm for 10 min at 4° C and the supernatant containing the proteins was used for western blot analysis. The protein was estimated with Bradford reagent (Bradford 1976) and 150 μg was loaded per treatment per lane. Western blots were probed with a commercial antibody against the mammalian APAF-1 (Sigma). Detection was done using the biotin–avidin method as per the Vectastain kit.

2.5 In silico *analyses*

Using nineteen randomly chosen GenBank-registered amino acid sequences of DAD1 (for accession no., *see* legends to figure 5) a multiple alignment was carried out using the ClustalW multialignment tool. The conserved sequences were simultaneously confirmed using the Multalin program (*www.multalin.com*).

3. Results

When *C. reinhardtii* cells were exposed to increasing doses of UV-C (6–50 J/m^2), there was a sizeable decrease in the cell number as compared with the control (Moharikar *et al* 2006). The cells exhibited hallmarks typical of apoptosis and our experiments (as described in Moharikar *et al* 2006) confirmed a UV-C induced cell death-like process in *C. reinhardtii*.

3.1 *Isolation and expression of dad1 from* C. reinhardtii

To understand the components involved in the molecular mechanism of UV-C induced apoptosis in *C. reinhardtii*, an *in silico* search for the apoptosis molecules was adapted using the *Chlamydomonas* database (www.chlamy.org/chlamydb.html). The annotated JGI Chlamy database matched with only one putative gene for *dad1* (C_20215). Primers (both forward and backward, one set) were designed using the mRNA transcript for this gene. Expression profiles of the putative *C. reinhardtii dad1* gene were then observed

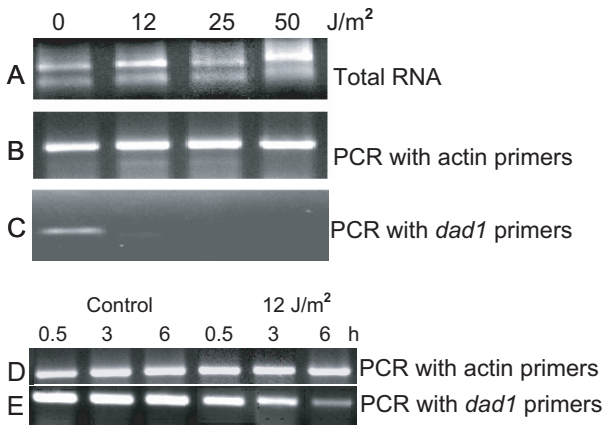

Figure 1. Expression of *dad1* gene using RT-PCR. **(A)** Total RNA was isolated from cells unexposed (Lane 1) and exposed to various doses of UV (Lanes 2–4) and separated by 1% formaldehyde agarose gel electrophoresis. **(B)** Levels of *C. reinhardtii* actin cDNA in control and UV-exposed cells. **(C)** Levels of *C. reinhardtii dad1* cDNA in control and UV-exposed cells. The *dad1* transcripts were semi-quantified with RT-PCR with *C. reinhardtii* actin gene as a control. **(D)** The *dad1* transcripts were semi-quantified with RT-PCR with *C. reinhardtii* actin gene as a control. **(E)** Levels of *C. reinhardtii dad1* cDNA in control and UV-exposed cells at increasing time-points and a constant UV-C dose of 6 J/m^2.

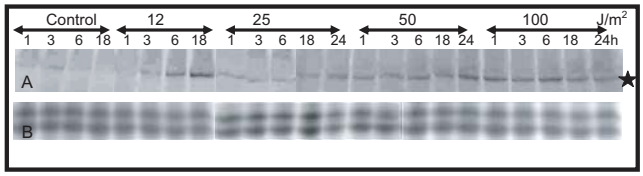

Figure 2. Western blot analyses **(A)** of total protein extract from control and UV-induced *C reinhardtii* cells with commercial antibodies raised against mammalian APAF-1. Cells were exposed to increasing doses of UV-C and collected at varying time-points post-UV exposure. The star indicates the position of the protein (130 kDa) that shares epitopes with the antibody. **(B)** The Coomassie-blue stained gels as loading controls.

in UV-C exposed and unexposed cells at the transcriptional level by using a semi-quantitative RT-PCR technique (figure 1). Total mRNA was isolated from control and UV-C exposed cells (12–50 J/m^2 at various time-points from 0.5 to 6 h post UV exposure, dark incubation). cDNA synthesis was carried out using SuperScript™ Reverse Transcriptase. The PCR amplified products were respectively electrophoresed on an agarose gel and the results showed abundant amplification of a ~300 bp product in the control cells, however, there was, no such product seen in any of the UV-C exposed cells (figure 1). As a gel loading and experimental control, total RNA (figure 1A) was also electrophoresed. To semi-quantify the transcript, the *C. reinhardtii* actin gene was used as an internal reference (figure 1B). Further, with increasing time at a lower dose of UV-C, a semi-quantitative analysis of the *Dad1* transcript was carried out. As compared with the control, we observed a reduction in the transcript from 0.5 to 6 h at 6 J/m^2 (figure 1E). There was no difference in the levels of the actin transcript (figure 1D).

3.2 *Detection of APAF-1 protein from C. reinhardtii cells*

To connect the downregulation of *dad1* with another apoptotic marker protein, we checked the pattern of regulation of a proapoptotic protein, APAF-1. The APAF-1 protein level was checked in the UV-C unexposed and exposed *C. reinhardtii* cells. Electrophoretically resolved total protein extracts from such cells were probed with a commercial antibody raised against mammalian APAF-1. The APAF-1 antibody cross-reacted to a protein of 130 kDa (figure 2A), indicating the presence of APAF-1 in *C. reinhardtii*. The intensity of the 130 kDa protein was low in control cells (1–18 h). On the other hand, in all the doses (12–100 J/m^2) of UV-C-treated cells (figure 2A) the intensity of this protein showed an increase from 1 h, with the maximum intensity being reached at 6 h (post UV exposure). In the light of the loading control (figure 2B), there was an apparent gradual increase in comparative levels from 12 to 100 J/m^2.

3.2 In silico *studies using the putative* dad1 *(C_20215)*

The PCR product obtained with the *dad1* specific primers was isolated and sequenced. The isolated cDNA (figure 3), which was designated as *C. reinhardtii dad1*, was found to be of 327 bp and showed an open reading frame encoding 108 amino acids with methionine at the start position encoding a protein of 12.0 kDa. The *C. reinhardtii dad1* and C_20215 (from the Chlamy database *http://genome.jgi-psf.org:8080/annotator/servlet/jgi.annotation Annotation?pDb=chlre 2*) sequences when aligned showed 100% identity, both at the nucleotide and amino acid levels (figures 4 A, B). Therefore, it is believed that the sequence isolated by us is indeed a homologue of the potential anti-apoptotic gene *dad1* from *C. reinhardtii* cells. When the DAD1 protein was checked *in silico* for domain searches, as in other DAD1 proteins, we found that *C. reinhardtii* DAD1 showed the presence of three transmembrane domains (figure 4C). Further, several (nineteen in number, *see* figure 5 legends for accession numbers) DAD1 protein sequences were then used to make an *in silico* comparison with *C. reinhardtii* DAD1 (data not shown). The molecular weight predicted for all these proteins ranged between 12 and 13.5 kDa and the DAD domain extended over the entire length of these proteins. Interestingly, the predicted isoelectric points (IEPs) showed three groups, those proteins with an IEP of approximately 7.38–7.53 were from mouse, human, pig, bovine, *Xenopus*, apple, rat and *Chlamydomonas*; the second group that showed an IEP of 8.81–9.07 were from slime mold, mosquito, *Arabidopsis*, rice, tomato, chicken, petunia, barley and tobacco; the exception to all these was the DAD1 protein from pea which showed an IEP of 6.32 (data not shown). The significance of the differences in IEPs may reflect the difference in composition of amino acids for all these proteins. A multiple sequence alignment of the deduced protein sequence of *C. reinhardtii dad1* gene with other *dad1* sequences is shown in figure 5. Alignment of the *C. reinhardtii* DAD1 protein sequence with those for DAD1 from several other species indicates the extent of the identity. While 28 exact matches were seen, there were stretches of

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  1 atgctcgccgagatcgtgaaggcgttctcggacgagtacaagaag
    M  L  A  E  I  V  K  A  F  S  D  E  Y  K  K
 46 actccagtccgtgtgaaggttctggatgcgttcctagtctacgca
    T  P  V  R  V  K  V  L  D  A  F  L  V  Y  A
 91 ctggcgactgcggcggttcagttcgcgtacatgctgctggtgggg
    L  A  T  A  A  V  Q  F  A  Y  M  L  L  V  G
136 acgttccccttcaacgcgttcctggctggcttcctttcgtgcgtg
    T  F  P  F  N  A  F  L  A  G  F  L  S  C  V
181 ggcttcttcgccctgactgtgtgcctgcgcatgcaagtggaccca
    G  F  F  A  L  T  V  C  L  R  M  Q  V  D  P
226 gccaacaaggagttctcggggatatcgcccgagcgc 261
    A  N  K  E  F  S  G  I  S  P  E  R
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Figure 3. Nucleotide sequence and putative ORF of *dad1* from *C. reinhardtii*. The nucleotide and deduced amino acid sequence of *C. reinhardtii dad1* gene isolated from the control cells.

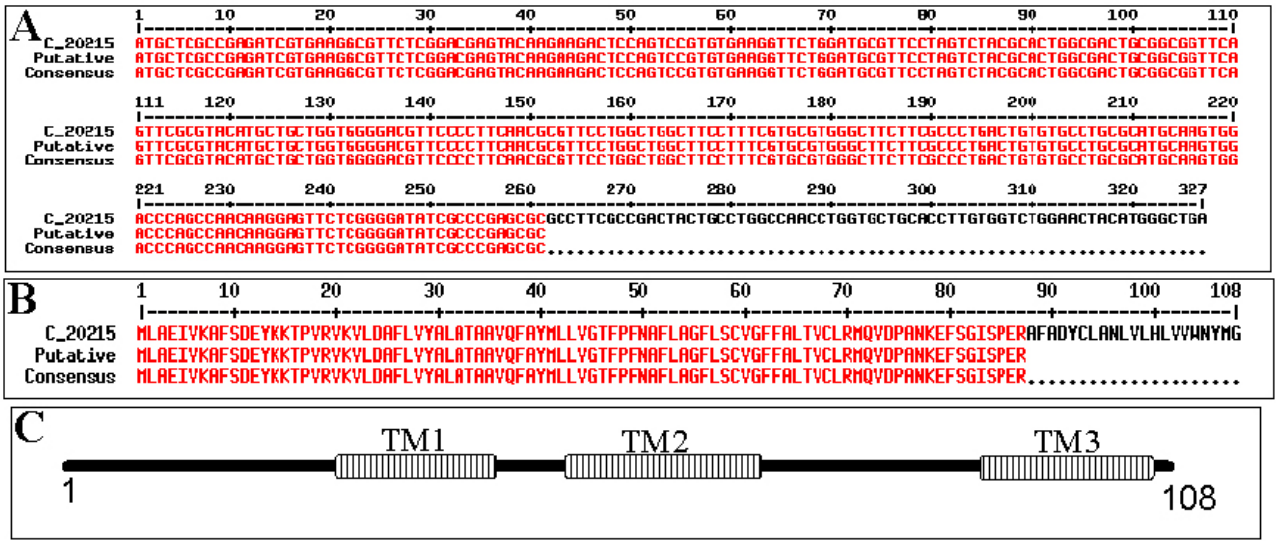

Figure 4. Pair-wise alignment of the nucleotide **(A)** and amino acid **(B)** sequences of *dad1* from the *Chlamydomonas* database (C_20215) and the putative partial sequence of the *dad1* isolated from cells grown at the logarithmic phase. **(C)** The domain organization of *dad1* from *C. reinhardtii*. Note the three stretches of transmembrane domains (TM1–3).

amino acid sequences that were highly conserved among all the species; these seem to encompass the transmembrane domains. The deduced protein sequence of *C. reinhardtii* DAD1 had a high homology with that of DAD1 from other species (figure 5). This alignment shows that *C. reinhardtii* DAD1 is 54% identical to *Oryza sativa*, 56% identical to *Drosophila melanogaster*, 66% identical to *Xenopus laevis*, and 64% identical to *Homo sapiens*, *Sus scrofa*, *Gallus gallus*, *Rattus norvegicus* and *Mus musculus*.

4. Discussion

Earlier laboratory studies demonstrated the presence of an apoptotic-like cell death process in *C. reinhardtii* cells exposed to UV-C irradiation (Moharikar *et al* 2006; and table 1). We observed typical hallmarks of apoptosis including cell shrinkage, associated nuclear morphological changes, flipping of phosphatidylserine and DNA fragmentation detected by the TUNEL assay and oligonucleosomal DNA laddering assay. In our parallel pursuit of understanding the molecular mechanisms of the UV-induced cell death process in *C. reinhardtii*, we developed two approaches. In the "functional" approach, we observed that there are certain "factors" in the spent medium that protect the UV-exposed bystander cells from death (Moharikar *et al* 2006). Another approach is based on identification of the molecules involved in the apoptotic process, assuming that the mechanism is by and large conserved in animals and plants across evolution. We have either used antibodies or designed primers to first identify the molecules that are universally common to the apoptosis process and isolated these molecules in *C. reinhardtii*. To initiate this strategy, we used antibodies against a mammalian caspase-3 and showed that it shared epitopes with a protein of 28 kDa, whose pattern of expression correlated with the onset of cell death (Moharikar *et al* 2006). In the present study, we have used primers designed against the putative *dad1* (as annotated from the *Chlamydomonas* database) to isolate it from *C. reinhardtii* and followed its expression during UV-C induced apoptotic-like cell death.

The *dad1* gene was first isolated from humans; the defect in this gene caused apoptotic cell death in hamster BHK21 cells (Nakashima *et al* 1993). The gene mapped to human chromosome 14q11-q12 and mouse chromosome 14, and was shown to have plant and nematode homologues (Apte *et al* 1995). The mouse DAD1 protein showed an expected high homology with previously cloned human- and *Xenopus* DAD1-encoding cDNAs. Also, this sequence had remarkable homology with partial cDNA sequences reported from *O. sativa* (rice) and *C. elegans* (nematode), suggesting the existence of plant and invertebrate homologues of this highly conserved gene. A direct role as an inhibitor of apoptosis was revealed when *dad1* was deleted using gene targeting (Brewster *et al* 2000). Analysis of embryos

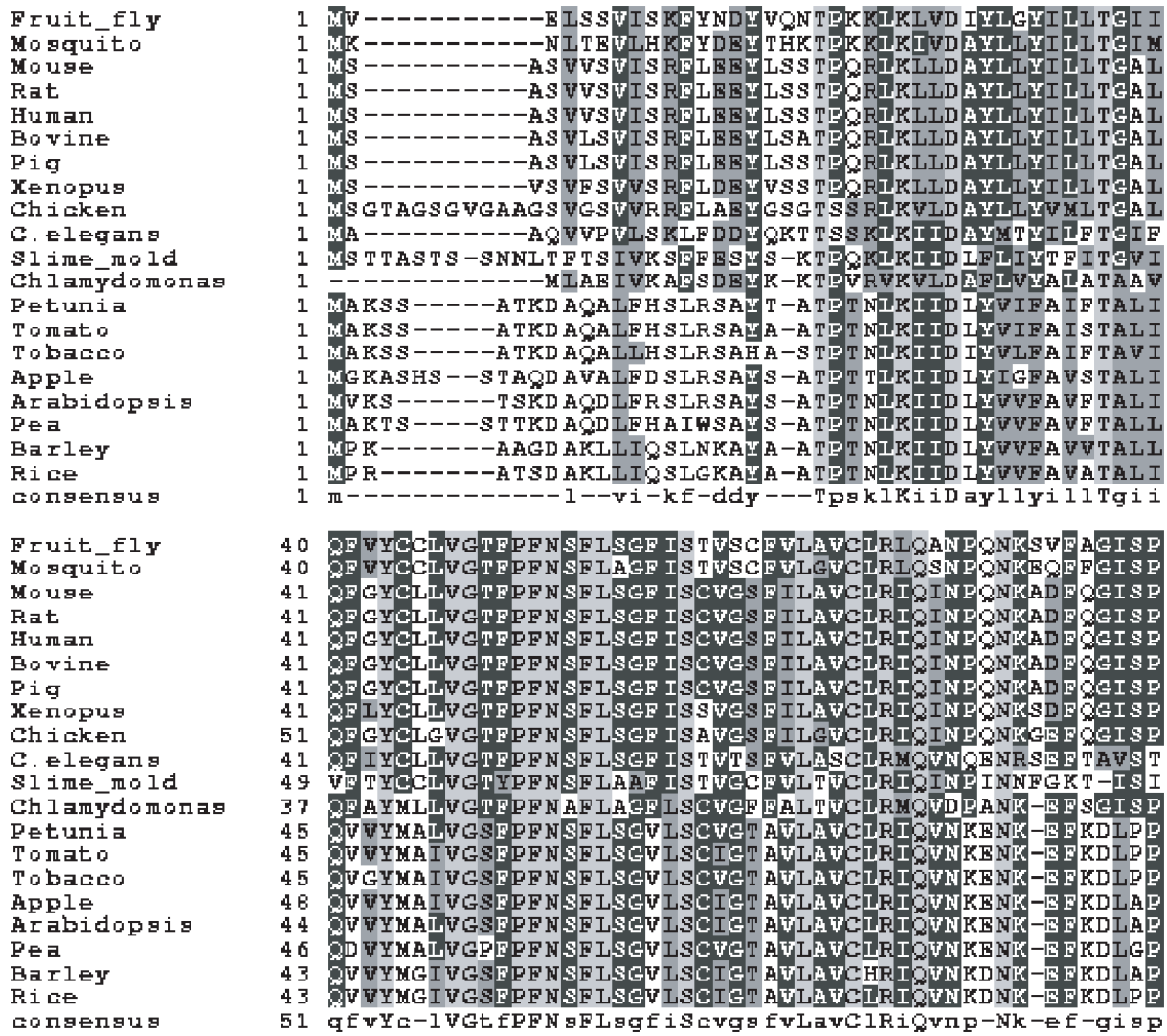

Figure 5. Multiple alignment of the deduced amino acid sequences of the *C. reinhardtii dad1* homologue gene with other DAD1 protein sequences using the ClustalW program. The DAD1 protein sequences aligned were: *C. reinhardtii* (C_20215, this study), *H. sapiens* (P61803, Nakashima *et al* 1993), *M. musculus* (P61804, Apte *et al* 1995), *Sus scrofa* (Q29036, Suzuki *et al* 1996, direct submission to NCBI), *B. taurus* (NP_001029933, Sonstegard *et al* 2002), *X. laevis* (P46967, Nakashima *et al* 1993), *D. discoideum* (Q54FB6, unpublished but available in GenBank), *A. gambiae* (AAQ94040, unpublished but available in GenBank), *A. thaliana* (Q39080, Gallois *et al* 1997), *C. elegans* (AAB96727, *C. elegans* sequencing consortium), *Oryza sativa* (O50070, Tanaka *et al* 1997), *Lycopersicon esculentum* (Q9SMC4, Hoeberichts and Woltering 2001), *G. gallus* (O13113, Wang *et al* 1997), *P. hybrida* (AAO73434 unpublished but available in GenBank), *Malus domestica* (O24060, Dong *et al* 1998), *Pisum sativum* (Q9ZRA3, Orzaez and Granell 1997), *Hordeum vulgare* (Q9SME8, Lindholm *et al* 2000), *N. tabacum* (BAB40808, Yamada 2001, direct submission to NCBI), *D. melanogaster* (Q9VLM5, Misra *et al* 2002), *R. norvegicus* (P61805, unpublished but available in GenBank). Completely conserved residues, light grey; identical residues, dark grey; similar residues, gray 2; different residues, white.

from heterozygous matings of adult mice (+/-) detected *dad1* null (-/-) embryos at E3.5 but no later, suggesting that *dad1* is required for development beyond the late blastocyst stage. Also, increased levels of apoptosis were observed in cultured embryos lacking a functional copy of the gene, consistent with an anti-apoptotic role for *dad1* (Brewster *et al* 2000). These embryos showed delayed development by embryonic day 7.5, exhibiting aberrant morphology,

Table 1. Summary of the morphological, biochemical and physiological changes that occur when *C. reinhardtii* cells are exposed to 12 J/m^2 of UV-C

No.	Time	Physiological changes	Reference
1	2 min	Activation of an MBP kinase and a Jun kinase	Unpublished data
2	10 min	De-flagellation; cells become immotile	Dharmadhikari *et al* 2006
3	20 min	Recruitment of RecA-like molecule to the nucleus	Unpublished data
4	30 min	Increase in the total nuclear protein quantity	Unpublished data
5	1 h	Formation of nuclear DNA nicks	Moharikar *et al* 2006
6	1.5 h	APAF-1 upregulation	Present study
7	2 h	Caspase-like activation	Moharikar *et al* 2006
8	~3 h	*dad1* downregulation	Present study
9	3 h	Chloroplast DNA degradation	Moharikar *et al* 2006
10	15 h	Genomic DNA laddering	Moharikar *et al* 2006
11	18 h	50 % cell death	Moharikar *et al* 2006

impaired mesodermal development and increased levels of apoptosis in specific tissues. These defects culminate in homozygous embryos failing to turn on the posterior axis and subsequent lethality by embryonic day 10.5 (Hong *et al* 2000). In *C. elegans*, overexpression of *dad1* defends against developmentally regulated PCD (Sugimoto *et al* 1995). In mammalian cells, mutated *dad1* can be complemented with cDNA clones encoding DAD both from *Arabidopsis* and rice (Gallois *et al* 1997; Tanaka *et al* 1997) and downregulation of *dad* has been correlated with onset of DNA fragmentation during petal senescence in pea (Orzaez and Granell 1997).

Gallois and co-workers provided the first experimental proof of the existence of a homologue of an animal gene for *dad1* in plants (Gallois *et al* 1997). They isolated the first plant *dad1* clone from an *Arabidopsis thaliana* cDNA library whose predicted translation product showed marked similarity to the mammalian defender against DAD1 protein (Gallois *et al* 1997). The *A. thaliana* protein was found to be 49% identical to the hamster protein and could be substituted for its animal counterpart and suppress apoptosis in a mutant cell line tsBN7 (Nakashima *et al* 1993). This demonstrates that the plant protein is as efficient as human DAD1 in rescuing these hamster cells from apoptosis. This was also the first demonstration of complementation of a vertebrate mutant by a plant cDNA. These results suggested that the process of apoptosis may be conserved in animals and plants (Gallois *et al* 1997). In a similar manner, the human *dad-1* cDNA homologue isolated from rice plants could also rescue the temperature-sensitive *dad1* mutants of hamster cells from apoptotic death, suggesting that the rice *dad1* homologue also functions as a suppressor of programmed cell death (Tanaka *et al* 1997). From deductive analogy, one can then conclude that the *C. reinhardtii* gene which is 50–60% identical to its animal counterparts must be able to fulfil the criteria exhibited by the *A. thaliana* and rice proteins.

The expression pattern of the *dad1* gene has been shown in several different organisms, including carcinoma cell lines. A mixed pattern emerges, although the common trend is a general downregulation with apoptotic stimuli. Among the differentially expressed genes following stimuli such as neuronal differentiation (Satoh and Kuroda 2000), *dad1* was significantly upregulated, but there was no change in the levels of *dad1* in a gamma-irradiated human lymphoblastoid cell line (Bishay *et al* 2000). Endothelin-regulated gene expression in human mesangial cells also showed downregulation of the *dad1* gene (Mishra *et al* 2003). A human hepatocellular carcinoma (Tanaka *et al* 2001) and a mantle cell lymphoma (de Vos *et al* 2003) showed a raised transcript level, while a malignant cell line showed a decline in the levels of *dad1* transcripts (Verneris *et al* 2000). The *dad1* homologue cloned from a cDNA library of the spider *A. ventricosus* revealed a 339 bp cDNA with an open reading frame of 113 amino acid residues (Sik *et al* 2003). Northern blot analysis showed that the transcript of the *dad1* homologue gene was present in all tissues examined, but interestingly, the transcript levels were particularly high when exposed at low (4^{o}C) and high (37 ^{o}C) temperatures, suggesting that the gene is induced with temperature stress (Sik *et al* 2003). In suspension-cultured tomato cells, cell death can be triggered by treatment with camptothecin, an inhibitor of topoisomerase 1. A differential display of the camptothecin-induced suspension-cultured tomato cells showed almost 50% downregulation in the *dad1* transcript (Hoeberichts *et al* 2001). Yamada and co-workers (2004) isolated a homologue of the *dad1* gene from *Gladiolus* petals as full-length cDNA (*g1dad1*), and showed that *g1dad1* expression in petals was drastically reduced before the first visible symptom of senescence (petal wilting). A few days after downregulation of *g1dad1* expression, processes specific for the execution phase of PCD such as DNA and

nuclear fragmentation were observed (Yamada *et al* 2004). Also, a tomato lipase gene homologous to *Arabidopsis dad1* (lipase homologous to *dad1*; LeLID1) increased rapidly during germination of seeds and reached a maximum level at four days after germination, after which it decreased rapidly. Little expression could be found in flowers or fruits (Matsui *et al* 2004). UV-C overexposure induces PCD in *Arabidopsis* (Danon *et al* 2004). The process required light and a protease cleaving the caspase substrate Asp–Glu–Val–Asp (DEVDase activity) was induced within 30 min and peaked at 1 h. In addition, At-DAD1 and At-DAD2, the two *A. thaliana* homologues of DAD1, could suppress the onset of DNA fragmentation in *A. thaliana*, supporting an involvement of the endoplasmic reticulum in this form of the plant PCD pathway. Another cDNA homologue for *dad-1* (*citdad-1-1*) was isolated from the *Citrus unshiu* fruit (Moriguchi *et al* 2000). It was found to be 345 bp long, with a deduced protein sequence of 115 amino acids. The expression of *citdad-1-1* was progressively downregulated in leaves as they matured (Moriguchi *et al* 2000). Northern blot analysis of germinating barley scutella show that the expression of only *dad1* declined before the onset of DNA fragmentation. In contrast to this, the expression of another *dad* transcript *dad2* and *ost1* (a cDNA encoding another subunit of the same OST complex) increases before the onset of DNA fragmentation (Lindholm *et al* 2000). On the other hand, the plant homologue of the *dad* gene is downregulated during senescence of flower petals in pea (Orzaez and Granell 1997). Its expression pattern indicates that *dad1* declines dramatically on flower anthesis disappearing in senescent petals, this downregulation is by the plant hormone ethylene. In summary, it appears that downregulation of *dad1* seems to occur with senescence or apoptosis and PCD, while its expression is upregulated when the signal to a given cell is developmental. In a similar manner, our *in silico* findings have shown that *dad1* isolated from *C. reinhardtii* is a gene that exists in a single copy and encodes a small protein of 12.5 kDa. There was a significant downregulation of the PCR product upon UV exposure of the cells. The downregulation of *dad1* coincides with the upregulation of a proapoptotic protein APAF-1, leading us to conclude that the *dad1* expression pattern is indicative of PCD. It also shows homology to DAD1 proteins from several species, indicating that the machinery of apoptosis that seems to be conserved in evolution may include a unicellular organism such as *C. reinhardtii*.

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