The plant homologue of the *defender against apoptotic death* gene is down-regulated during senescence of flower petals

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Abstract Petal senescence is an example of a highly reproducible cell death programme. In this programme, DNA is fragmented internucleosomally and cells with condensed nuclei containing an increased number of 5′ ends can be detected with the TUNEL technique. The pea homologue of *defender against apoptotic death* (*dad*), a gene described to suppress endogenous programmed cell death in *Caenorhabditis elegans* and mammals was isolated. Expression studies show that *dad* declines dramatically upon flower anthesis disappearing in senescent petals, and is down-regulated by the plant hormone ethylene.

Key words: *Defender against apoptotic death* gene; *Pisum sativum*; Ethylene; Programmed cell death; Petal senescence; *dad* gene

1. Introduction

Programmed cell death (pcd) is an integral part of development in multicellular organisms, which is directed to eliminate unnecessary or harmful cells [1,2]. As many other developmental programmes, pcd has to be precisely coordinated both temporally and spatially [3]. To do so cells appear to have developed specific sets of genes to activate the death programme (*ced*3, ICE family, etc.) while another set (*bcl*2, *ced*9, *dad*, etc.) acts as suppressors. Both types of regulators ensure that the death programme is not activated in an untimely or uncoordinated manner [4,5].

Our knowledge of pcd in plant cells lags behind that of animal systems. Only recently some examples of pcd in plant systems have been reported [6–8]. These reports show that at least some characteristics – detection of apoptotic nuclei and DNA fragmentation — may be shared between animal and plant pcds. Inducer or suppressor genes of the plant cell death programme have been described in particular cases [9,10], but the expression of homologues of the animal genes controlling the pcd process has not been reported in plants.

Dad-1 appears to be an evolutionarily conserved inhibitor of programmed cell death [11]. *Dad*-1 was initially isolated as a mutation in a temperature-sensitive hamster cell line, tsBN7, that undergoes apoptosis when incubated at restrictive temperature [12]. A wild-type copy of *dad*-1 rescued tsBN7 cells from apoptotic death [12] and was sufficient to inhibit developmentally programmed cell death in *C. elegans* [13].

Senescence of the flower perianth (interestingly apoptosis, the name for a well established morphological type of pcd, means petal/leaf shedding) is a precise process of programmed cell demise [14]. To broaden the parallel between pcd in animal and plant systems we have isolated the pea homologue of *dad*, a repressor of pcd in animal systems and studied its pattern of expression during pea flower senescence. Its correlation with the occurrence of DNA fragmentation during petal senescence and the regulation of *dad* expression by the plant hormone ethylene is presented.

2. Materials and methods

2.1. Plant material

Pisum sativum cv. Alaska was grown in vermiculite and Hoagland's solution under controlled-room conditions as described previously [15]. Flowers were tagged at different stages of development corresponding to the number of days before and after anthesis. In the case of ethylene treatments plants were treated with this plant hormone at a concentration of 10 μl l^{-1} (0.4 μM) in an open flow system for different periods of time. Ethylene action inhibitors norbornadiene (1000 μl l^{-1}, 40 μM) and silver thiosulphate (20 μl of a 500 μM soln.) were applied 24 h before anthesis as described [6].

2.2. DNA fragmentation studies: DNA extraction/Southern analysis/TUNEL assay

Petals corresponding to different stages of senescence were harvested and immediately frozen in liquid nitrogen. For total DNA extraction samples were homogenized in liquid nitrogen and the DNA extracted as described previously [16]. Approximately 5 μg of DNA was loaded in a 2% agarose gel and electrophoresed at 60 V. DNA was stained with ethydium bromide. After visualization the DNA was blotted to Nylon membranes and hybridized to a ^{32}P-labelled total pea DNA probe (pea chromosomic DNA cut with Sau3A and labelled by the random hexamer priming method) following standard procedures. The final wash was in 0.1×SSC and 0.1% SDS at 65°C.

DNA fragmentation in situ was detected following a modification of the previously described TUNEL method [7,17]. Petal specimens (2×2 mm^2) were fixed in 4% formaldehyde, 100 mM phosphate buffer, pH 7.5, for 4 h. Washed throrougly with PBS the specimens were incubated at 37°C for 60 min with terminal deoxynucleotidyl transferase (TdT) from Calf thymus and fluorescein conjugated nucleotides (Boehringer Mannheim, GmbH). After rinsing 3 times in PBS, sections were incubated with a sheep anti-fluorescein antibody conjugated to alkaline phosphatase for 30 min at 37°C, according to the manufacturers instructions (Boehringer Mannheim). After rinsing with PBS color was developed by the addition of NBT/BCIP and incubating at RT for 10 min. Specimens were observed from the adaxial surface of the petal with a Nikon microscope.

2.3. cDNA cloning and sequencing

The pea *dad* homologue was isolated by PCR, using oligos (RD4: TCA ACC AAG GAA GTT CAT GA and RD5: GTG GGG TCA TTT CCT TTC AA) designed to hybridize to conserved regions of

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Abbreviations: pcd, programmed cell death; *dad*, defender against apoptotic death; TUNEL, terminal transferase UTP nick end labelling method

The nucleotide sequence reported in this paper will appear in the EMBL database under accession No. U79562.

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DAD sequences deposited in the database and vector specific primers. The cDNA template was a pea flower organ-specific cDNA library in lambda ZAP vector [18]. The sequence of cDNA inserts was determined on both strands by the dideoxy nucleotide chain termination method according to the Sequenase kit (USB, Amersham).

2.4. Northern hybridization

RNA was extracted and analysed in formamide gels as described previously [18]. RNA loading was equilized by OD and verified by ethydium bromide staining of the gels. *DAD* probe was random primed labelled with ^{32}P.

2.5. Southern analysis

DNA extracted from pea leaves as described [16] was digested with restriction enzimes and separated in a 0.8% agarose gel. DNA was blotted to Nylon membranes and hybridized to a ^{32}P-labelled *Eco*RI fragment of *dad* clone.

2.6. Computer analyses

Nucleotide and amino acid sequence searches and alignments were performed using the GCG (Genetics Computer Group, Madison, WI) package.

3. Results

3.1. Specific intranucleosomal DNA fragmentation can be observed in senescent petals and is blocked by inhibitors of the action of the plant hormone ethylene

DNA from petals at different stages of the senescent program was isolated and analyzed in agarose gels to see if it showed the same type of specific DNA degradation described in programmes of cell death for some animals and plants. To increase the sensitivity of the procedure, after transfer to Nylon membrane petal DNA was hybridized with a labelled total DNA probe.

In Fig. 1, chromatin fragmentation can be observed in DNA extracted from petals after the onset of senescence. The bands are approximately multiples of 180 bp, suggesting that the DNA has been digested at internucleosomal sites, and therefore before chromatin is non-specifically digested by nucleases and proteases. As can also be seen in Fig. 1A, the intensity of the DNA laddering increases with time to show the highest levels at day 3 after anthesis ($\approx$180 bp band in lane 6, can even be barely detected by ethydium bromide staining when loading 10 μg of total DNA).

As the intensity of DNA laddering continues to increase when many mesophyll cells have been emptied of their cellular content (Fig. 1A, and unpublished results), we investigated the morphology and the occurrence of nuclei showing DNA fragmentation in situ. To do this we used the TUNEL assay, in which nuclei with an increased number of DNA 5′ ends resulting from DNA fragmentation are highlighted. This technique is widely used in animal systems to identify cells undergoing programmed cell death and it has recently been shown to work also in plants [6–8,19]. As can be seen in Fig. 1B some cells in senescent petals are labelled by the TUNEL reaction and therefore must contain nuclei with fragmented DNA. The lower intensity of Acridine Orange staining observed in TUNEL-positive nuclei is due to the masking effect produced by the black precipitate from alkaline phosphatase reaction. Cells of STS-treated and of young (d-1) petals failed to exhibited TUNEL-labelled nuclei.

The relatively weak intensity of the DNA bands at early stages of senescence (Fig. 1A) probably reflects the small number of TUNEL-labelled cells observed on histological sections (Fig. 1B-b).

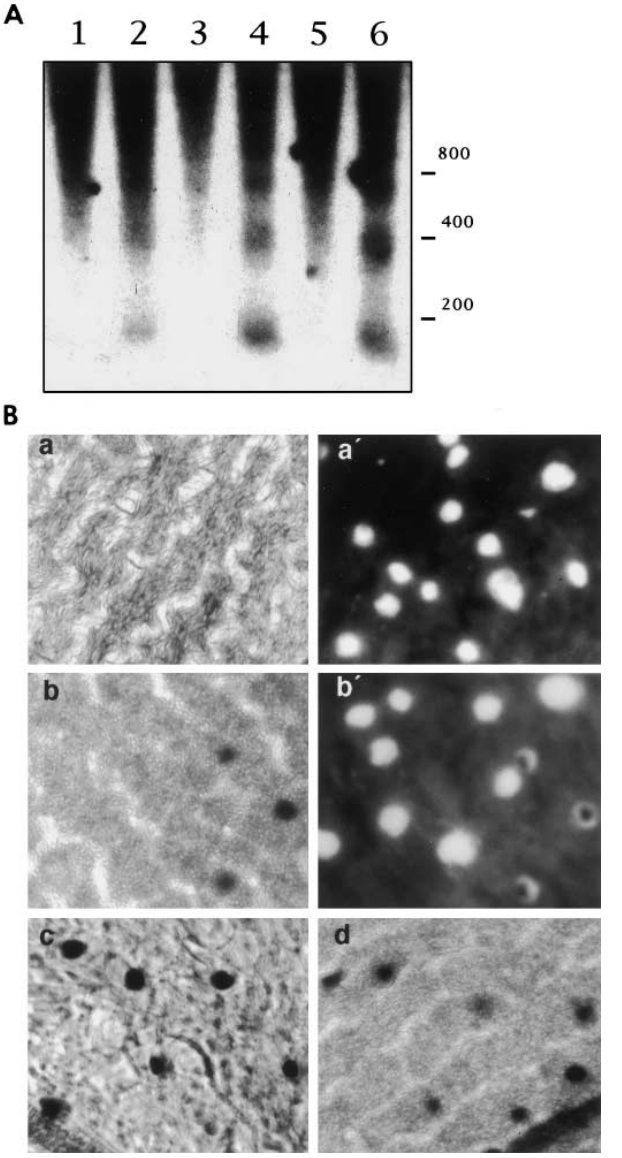

Fig. 1. DNA fragmentation during petal senescence. A: Southern analysis. DNA was isolated from petals at different stages, run on 2% agarose gels and transferred to nylon membranes. Filters were probed with random primed 32P-labelled pea DNA and autoradiographed. Lanes 1, 3 and 5: Petals treated with STS soln. on day 1 before anthesis (d-1) and collected 1, 2 and 3 days after anthesis, respectively. Lanes 2, 4 and 6: Untreated petals showing increasing senescence (collected 1, 2 and 3 days after anthesis respectively). B: TUNEL reaction. Whole mounts of petal pieces collected 2 days after anthesis were assayed with the TUNEL reaction. Increase in DNA ends was highlighted by addition of nucleotide analogues by using TdT, and the incorporated analogues decorated with the use of alkaline phosphatase linked to antibodies against the nucleotide analogues and NBT-BCIP substrates. a: Petals treated with STS at stage d-1. b–d: Non-treated petals. a′,b′: Same as (a) and (b) but after TUNEL staining petals were incubated with Acridine Orange to visualize the nuclei.

3.2. Isolation and expression of pea dad

The pea *dad*-1 homologue was isolated from a cDNA library of developing pea flowers, by using specific primers designed according to animal and Arabidopsis *dad* sequences present in the databanks and vector primers. A cDNA encompassing the complete coding sequence and 76 bp of untranslated leader region was obtained. Sequence alignment indi-

a

	Ps	At	Md	Ce	Mm	Ss	Hs	Xl	Sc	
Ps	---	86.9	81.2	50.9	50.0	50.0	50.0	49.1	37.7	
At	93.9	---	87.6	46.4	47.3	48.6	48.2	51.4	41.6	
Md	88.0	92.0	---	50.0	48.1	48.6	48.1	48.6	32.7	
Ce	70.9	66.6	70.9	---	60.1	61.0	61.9	63.7	42.6	
Mm	71.4	70.2	70.9	82.3	---	97.3	98.2	88.5	40.3	
Ss	71.4	70.2	69.3	82.3	100	---	99.1	90.2	41.2	
Hs	71.4	70.2	70.9	82.3	100	100	---	90.2	41.2	
Xl	72.3	76.9	71.1	83.1	95.5	96.4	95.5	---	39.4	
Sc	63.1	65.1	60.3	67.6	66.0	66.0	66.0	65.1	---	

% similarity

% identity

b

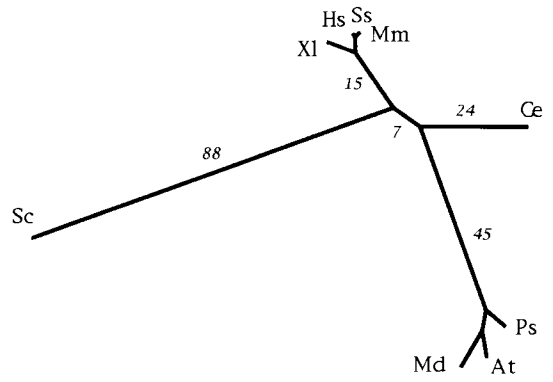

Fig. 2. Phylogenetic analyses of *dad*. a: Degree of similarity and identity of dad proteins from plant and animals calculated by using the Bestfit program from GCG. b: Phylogeny tree. The programmes DISTANCES and GROWTREE from GCG package with Kimura distances method were applied (University of Winsconsin). Sequences used for the alignments were: (*Pisum sativum*) (Ps), X95585(*Arabidopsis thaliana*) (At), U68560 (*Malus domesticus*) (Md), X98080 (*Caenorhabditis elegans*) (Ce), U22107 (*Mus musculus*) (Mm), D86562 (*Sus scrofa*) (Ss), D15057 (*Homo sapiens*) (Hs), D15059 (*Xenopus laevis*) (Xl) and U32307 (*Sacharomyces cerevisiae*) (Sc).

cates that *dad* is a highly conserved gene in both animal and plants (Fig. 2). Southern analysis indicates that *dad* gene is present in at least two copies in pea genome (Fig. 3).

Expression of pea *dad* homologue is constitutive in all tissues of the plant (data not shown). In the petal, however, expression of *dad* declines dramatically after anthesis stage and is below detection level in senescent petals (Fig. 4a). Petals of flowers where senescence has been blocked with ethylene action inhibitors, STS or NBD, maintain high levels of *dad* mRNA. Conversely petals whose senescence have been hastened by ethylene treatment show a faster decline in *dad* transcript levels than the untreated controls. These experiments were conducted in explants (Fig. 4b, lower panel) as well as in flowers attached to the plant (Fig. 4b, upper panel).

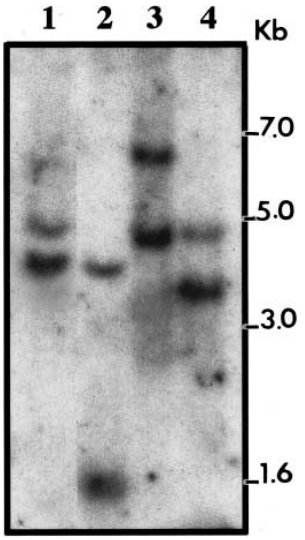

Fig. 3. Southern analysis of pea *dad*. Total pea DNA was digested with *Eco*RI (lane 1), *Eco*RI+*Ssp*I (lane 2), *Hin*dIII (lane 3) or *Hin*dIII+*Bam*HI (lane 4). Digested DNA was hybridized using a 365 bp *Eco*RI fragment of *dad* clone.

4. Discussion

Petal senescence is a precisely regulated cell demise process that occurs in flowering plants [14]. We are interested in the mechanism of cell death induction and in the transduction pathway leading to flower senescence as well as in learning

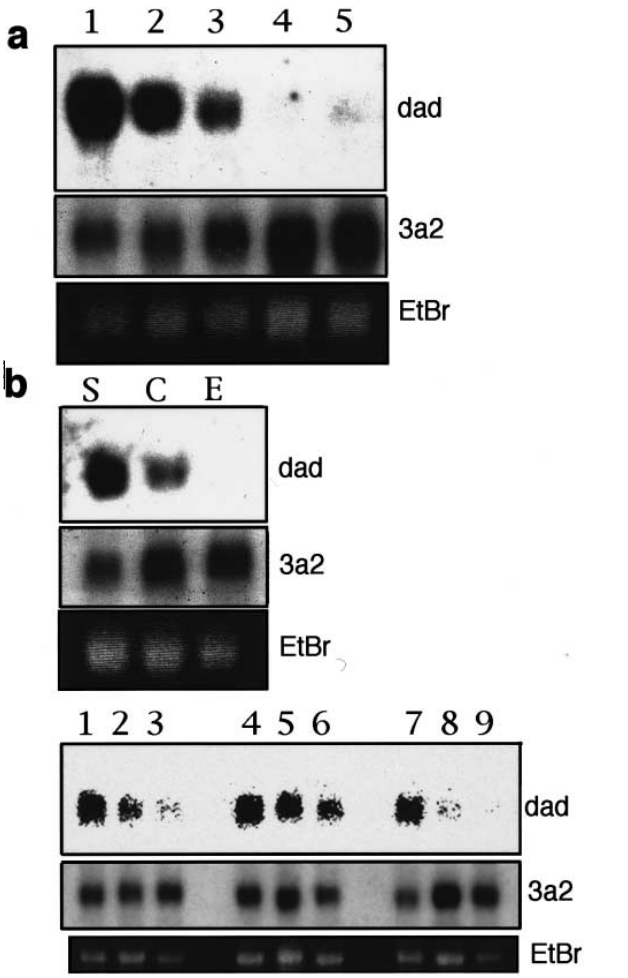

Fig. 4. (a) Expression of *dad* transcript during petal development and senescence. RNA samples were obtained from flower petals at different stages before senescence (lanes 1 and 2), at anthesis (lane 3) or at increased degrees of senescence (1 day after anthesis, lane 4; or 2 days after anthesis, lane 5). (b) Effect of ethylene and ethylene action inhibitor STS on *dad* expression in petals. RNA from flowers at the stage of anthesis which had been treated one day before with the senescence inducing plant hormone ethylene (Lane E), or with the inhibitor of ethylene action STS (lane S) were analyzed by Northern using the *dad*-1 cDNA probe. Control lane (C) corresponding to untreated flowers. In lower (b) panel, detached petals were collected after 0, 24, and 48h. of treatment with either ethylene action inhibitor NBD (lanes 4, 5 and 6 respectively), ethylene (lanes 7, 8 and 9) or left untreated (lanes 1 to 3). Total RNA samples (20 μg) were analyzed by Northern using the full-length *dad* cDNA (dad) and a constitutively expressed cDNA (3a2) as probes. rRNA stained with ethidium bromide (EtBr) is also shown.

to what extent the cell death programs of plant and animals share common mechanisms.

In this paper, senescence of the pea petals was characterised by the detection of specific DNA fragmentation. This fragmentation seems to occur at internucleosomal linker regions in some nuclei that showed condensed morphology and contained DNA with an increased number of 5′ ends. These characteristics are common to many apoptotic or programmed cell deaths described in animal systems [3] and to recent examples in plants [6–8].

Both DNA laddering and TUNEL-labelled nuclei are not produced if the flower is treated with STS or NBD which are known inhibitors of the action of the plant senescence hormone ethylene and therefore block flower senescence.

If *dad* is indeed a death supressor gene in plants, it makes sense that expression of this gene, which is high in metabolically active tissues declines abruptly once the organ is commited to die, and is finally absent in the senescent organ. Down-regulation of *dad* expression correlated with an increase in the fragmentation of DNA at internucleosomal linker regions in pea petals. In apoptotic tsBN7 hamster cells, loss of viability, degradation of DNA into oligonucleosomal and detection of cells containing nuclei with condensed chromatin, paralleled the disappearance of Dad protein. These results indicated that apoptosis of tsBN7 cells was induced by loss of the Dad-1 protein. These apoptotic caracteristics in hamster cells were reverted by introducing a wild-type copy of the gene encoding a stable Dad-1 protein. In the pea plant the involvement of *dad* in petal senescence is reinforced by the ability of the plant regulator ethylene, an efficient senescence-inducer in many flowers, to accelerate the drop-off in *dad* levels. Furthermore, petals whose senescence has been halted or slowed down by treating with ethylene action inhibitors do not show such a decrease in *dad* expression levels.

The availability of the pea *dad* cDNA clone will enable us to answer the question of whether constitutive or regulated expression of *dad* in transgenic plants will modify the cell death programme in plants as has been described in *C. elegans* and mammals [12,13].

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