UV-C radiation induces apoptotic-like changes in *Arabidopsis thaliana*

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Abstract With a view to studying programmed cell death in plants at the molecular level, we report here for the first time that apoptotic-like changes are induced by UV radiation in plant nuclei. In *Arabidopsis thaliana* seedlings a UV-C dose of 10–50 kJ/m^2 induces an oligonucleosomal DNA fragmentation which is reminiscent of the apoptotic DNA ladder described in animal cells. This DNA fragmentation was also detected in situ in protoplast nuclei as soon as 2 h after UV-C treatment. Moreover, UV-C induced a nuclear morphology characteristic of animal apoptotic nuclei. We propose that UV-C induction constitutes a powerful tool to compare the cellular response to irreversible UV damage in plants to that in animals and to study programmed cell death in *A. thaliana*.

Key words: Programmed cell death; Ultraviolet-C light; Plant; Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling; Protoplast; DNA ladder

1. Introduction

In multicellular organisms, cells self-destruct when they have been damaged or as part of normal development. Apoptosis is one process by which this programmed cell death (PCD) occurs and is well described in animal system. Apoptosis can be distinguished from accidental cell death on morphological criteria which include chromatin condensation, nucleus and cell fragmentation. Additionally a well accepted biochemical criterion is the detection of an oligosomal DNA ladder the rungs of which are multiples of 180 bp. This ladder is due to a caspase-activated DNase that degrades DNA during apoptosis [1]. The same apoptotic-like DNA ladder has been reported in plant PCD situations during development: in the monocot aleurone layer [2] and endosperm [3], in senescing petal [4] and carpel tissue [5]; and also after induction by different stresses such as cold [6], nutrient deprivation [7], salt stress [8], and during pathogen-induced death [9,10]. This suggests that plant PCD and animal apoptosis might share some common components. In animals a number of genes have been identified as being implicated in apoptosis; however, up to now only one plant homologue of such genes has been characterised: *AtDAD1* (defender against apoptotic death) [11] and the role of this gene in animal apoptosis suppression is unclear. This raises the question of to what degree the PCD process is conserved between plants and animals. It is thus important to set up systems in which this process could be easily induced and manipulated in plants and protoplasts.

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Abbreviations: PCD, programmed cell death; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling

UV radiation can damage many aspects of plant processes at the physiological and DNA level [12]. UV radiation is divided into UV-C (below 280 nm), UV-B (280–320 nm) and UV-A (320–390). UV-C is not physiologically relevant to plants since it is effectively blocked by the earth's stratosphere. However, because its DNA photoproducts are the same as those obtained with UV-B radiation, which reaches Earth's surface [13], UV-C radiation has often been used to study various physiologically relevant responses to DNA damage, and in particular has been shown to induce apoptosis in animal cells [14]. In the quest for a PCD inducer we have investigated the possibility of a similar UV-C response in plants. Plants require light for photosynthesis and thus had to develop strategies to protect themselves from UV damages, such as the inducible synthesis of UV-absorptive secondary metabolite sunscreen [15]. Therefore we used UV-C radiation doses higher than those which in animal cells are sufficient to induce apoptosis and we searched for apoptotic hallmarks in irradiated plants.

We report here for the first time that UV-C induces apoptotic-like changes in plant cells, i.e. nuclear fragmentation [16] and DNA laddering [17]. Protoplasts were used to quantify the effect and timing of cell death induced by UV-C. This constitutes a promising experimental model to study differences and similarities of UV response between plants and animal.

2. Material and methods

2.1. Plant material

Seeds from *Arabidopsis thaliana* (Columbia 0) were sterilised, sown under aseptic conditions on agar plates containing Murashige and Skoog salts and vitamins [18] (Sigma) supplemented with 20 g/l glucose and grown at 21°C, with a 14-h photoperiod.

2.2. Protoplast preparation

A. thaliana leaf protoplasts were isolated essentially as described in [19] from 3-week-old seedlings. Protoplasts were freed from cellular debris by filtration using first a 370-μm then a 74-μm mesh (Polylabo), followed by flotation on a 0.4 M sucrose solution, at $60\times g$ for 10 min. Protoplasts were then pelleted at low-speed centrifugation ($60\times g$, 5 min) and washed twice in a W5 medium [20] modified by reducing $CaCl_2$ from 125 mM to 1 mM and adding 0.1 M mannitol and 1 mM $MgCl_2$ (named W5A). Finally protoplasts were resuspended in culture medium (Murashige and Skoog salts and vitamins supplemented with 0.4 M sucrose, 0.4 M mannitol and 100 mg/l of cefotaxime (gift of Dr Chretien, Roussel Uclaf) at a density of 10^6 protoplasts per ml. For each treatment, 10^6 protoplasts were used and then incubated in one tube of 15 ml (Sarstedt), lying horizontally, in the dark.

The viability of the protoplasts was determined using fluorescein diacetate (FDA) stain (Sigma). Protoplast aliquots taken at different time points were incubated for 5 min in FDA at 10 μg/ml and mounted on glass slides. The slides were photographed using a fluorescence microscope (Zeiss axioplan), under a blue excitation wavelength for fluorescein detection in green (Zeiss, FITC filter BLUE-450–490). Protoplasts fluorescence was scored on colour prints.

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FDA-negative protoplasts (dead) were red because of chlorophyll fluorescence, FDA-positive protoplasts (live) were yellow (green+red).

2.3. UV-C irradiation of plants and protoplasts

Using a UV Stratalinker 2400 (Stratagene) fitted with 254-nm UV-C light bulbs, 3-week-old seedlings were irradiated in open petri dishes (9 cm diameter; Corning) and 10^6 protoplasts in 1 ml culture medium, were irradiated in open petri dishes (3 cm diameter; Corning). The UV-C energy delivered in each experiment was measured by a UV-C sensor fitted inside the Stratalinker irradiation chamber.

2.4. Isolation of nuclear DNA for fragmentation analysis

Total genomic DNA was extracted from seedlings following Dellaporta's procedure [21]. After RNase A treatment at 500 μg/ml (Eurogentec) for 1 h at 37°C, the DNA concentration in the samples was measured by addition of Hoechst 33342 (Sigma) [22] and the fluorescence read with a Fluoroskan (Labsystem, Fluoroskan II). Equal amounts of DNA were separated on a 2% agarose gel by electrophoresis in TBE, transferred onto a Hybond-N^+ membrane (Amersham), and hybridised with an α-^{32}P-radiolabelled *A. thaliana* genomic DNA probe at 65°C in phosphate buffer 250 mM, pH 7.2, SDS 7%, EDTA 1 mM, calf thymus DNA 10 μg/ml. The total genomic DNA used as probe was digested with *Sau*3A and radiolabelled using a random priming reaction (Rediprime kit; Amersham). Washes were done at 65°C in phosphate buffer 40 mM, SDS 1%, EDTA 1 mM.

After treatment, protoplasts were centrifuged at $3000\times g$ for 10 min, the pelleted protoplasts were resuspended in DNA extraction buffer (50 mM EDTA, 100 mM Tris, 1% SDS, pH 7.5), and frozen immediately in liquid N_2. Then the tubes were incubated for 10 min at 65°C, followed by two phenol/chloroform extractions and a final ethanol precipitation. After RNase digestion, the protocol for Southern blotting was the same as that used for seedling DNA.

2.5. In situ detection of nuclear DNA fragmentation

For in situ detection of DNA fragmentation, 100 μl of protoplasts were transferred onto a polylysine slide (Menzel-Glaser), fixed with an equal volume of 10% buffered formalin (Sigma) and dried for 1 h at 42°C. The fixed protoplasts were washed once with PBS buffer (pH 7.4), permeabilized with proteinase K 10 μg/ml (Bioprobe) for 10 min at room temperature, rinsed twice with PBS buffer (pH 7.4), dried at 42°C for 10 min and labelled for 1 h in the dark at 37°C with a commercially available TUNEL kit (fluorescein, in situ Cell Death Detection Kit, Boehringer Mannheim), at a dilution of 1:2 in reaction buffer. The TUNEL reaction incorporates fluorescein-dUTP at DNA strand breaks. After rinsing three times with PBS buffer (pH 7.4), the protoplasts were incubated with Hoechst 33342 (Sigma) at 10 μg/ml, for 1 h at room temperature in the dark, and rinsed three times with PBS buffer (pH 7.4). The slides were viewed with a fluorescence microscope (Zeiss Axioplan), under UV for Hoechst 33342 detection (Zeiss, UV-H365) and under a blue wavelength for fluorescein detection (Zeiss, FITC filter BLUE-450–490).

3. Results

3.1. UV-C radiation induces an apoptotic ladder in A. thaliana seedlings

Seedlings were grown for 3 weeks on agarose medium in sterile conditions and subjected to UV-C radiation. Total genomic DNA was extracted 24 h after treatment and separated by electrophoresis on a 2% agarose gel. The small genome of *A. thaliana* combined with the small number of seedlings we irradiated did not allow the purification of enough DNA to observe DNA fragmentation directly in the gel. So the DNA was transfer to a nylon membrane and hybridised to a radioactive probe prepared with total genomic DNA. This revealed that a DNA ladder is visible in the samples treated with a UV-C energy ranging from 10 kJ/m^2 to 50 kJ/m^2 (Fig. 1A). The intensity of the DNA ladder is faint in the 10 kJ/m^2 sample but becomes clearly present in the 50 kJ/m^2 sample. Using a DNA size marker, the sizes of the five smallest bands of the ladder were calculated to be around 180, 360, 540, 720 and 930 bp suggesting that they represent oligonucleosomal fragmentation. Lower energy treatments were inefficient in promoting DNA fragmentation (data not shown).

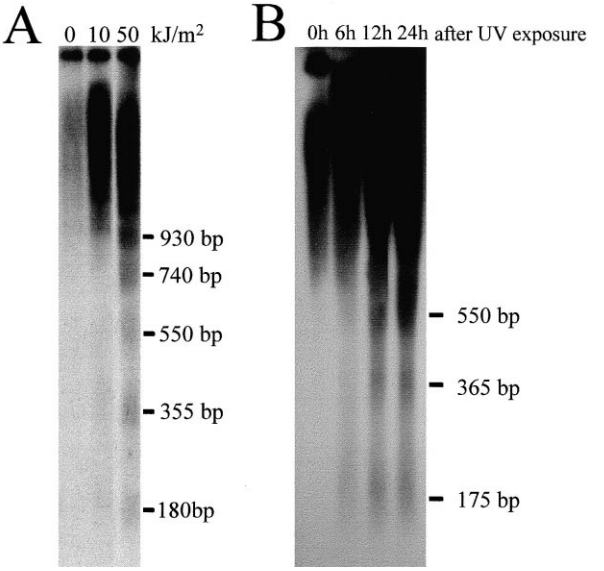

Fig. 1. Southern analysis of UV-C-induced DNA fragmentation in *A. thaliana*. A: UV-C energy range. 4 μg of total genomic DNA was loaded in each track. Radiolabelled genomic DNA was used as a probe. Lane 1, control DNA from 3-week-old plants; lanes 2 and 3, DNA from 3-week-old plants after 10 and 50 kJ/m^2 UV-C exposure respectively and harvested 24 h later. B: Kinetic analysis of DNA fragmentation. 2.5 μg of total genomic DNA was loaded in each track. Radiolabelled genomic DNA was used as a probe. Lane 1, control DNA from 3-week-old plants; lanes 2–4, DNA from 3-week-old plants after 50 kJ/m^2 UV-C exposure, harvested after 6, 12 and 24 h respectively.

A time course of DNA fragmentation was carried out using a 50 kJ/m^2 dose as the inducing treatment. Total genomic DNA was extracted at 6, 12 and 24 h after UV-C irradiation and separated on a gel along with genomic DNA from an untreated sample extracted 24 h after irradiation time. The Southern analysis (Fig. 1B) showed that the DNA ladder increased in intensity from 6 to 24 h. The three smallest bands visible were calculated to be around 180, 360 and 540 bp in size, thus confirming that they are multiples of the size of oligonucleosomal DNA. Background DNA degradation in the control is visible due to long exposure time of the autoradiograph to allow detection of the faint DNA ladder at 6 h after UV-C irradiation.

3.2. Quantification of UV-C-induced cell death using TUNEL reaction and FDA stain

The TUNEL reaction is used to visualise in situ the DNA fragmentation occurring during apoptotic cell death [23]. This reaction allows the measurement of the percentage of a cell population undergoing DNA fragmentation at a given time. To quantify easily the appearance of TUNEL-positive cells following UV-C irradiation we first of all set up a protoplast system. In vitro grown seedlings were harvested at 3 weeks and aerial parts used to prepare protoplasts. Samples of one million protoplasts in 1 ml liquid culture were transferred into 3 cm diameter petri dishes and irradiated with UV-C. To confirm that DNA fragmentation detected in seedlings is reproducible in protoplasts, a Southern analysis was carried out

on total genomic DNA extracted from protoplasts 6 and 24 h after UV-C treatment (5 kJ/m^2). Fig. 2 shows that a DNA ladder is visible 24 h after treatment although as in plants it is very faint at 6 h.

To quantify the UV-C effect on a plant cell population, protoplasts were harvested at 0, 2, 4 and 6 h after irradiation with UV-C doses ranging from 1 to 50 kJ/m^2, deposited on microscope slides, stained with FDA or fixed and treated with the TUNEL reaction. After the TUNEL assay, protoplasts were incubated with Hoechst 33342 to detect DNA, the samples were observed using a fluorescence microscope. Fig. 3 shows a typical result of the experiment; under UV light excitation a blue light emission revealed the position of the nucleus of each cell (Fig. 3A), under blue light excitation a green light emission indicated which proportion of nuclei were TUNEL-positive (Fig. 3B). Each sample was photographed and TUNEL-positive nuclei were counted and expressed as a percentage of total nuclei present in the sample. The use of photographic documents was necessary because FITC fluorescence faded too quickly to allow accurate counting on the slide.

In our culture conditions, *A. thaliana* protoplasts showed a low level of DNA fragmentation which reached 4% after 24 h of culture (data not shown). Fig. 4A shows that a 1 kJ/m^2 UV-C dose is not sufficient to induce DNA fragmentation in our system. With higher UV-C doses ranging from 10 to 50 kJ/m^2, the percentage of TUNEL-positive nuclei is proportional to UV-C dose, and increases with time. Protoplasts irradiated with a 50 kJ/m^2 exposure reach a maximum percentage of TUNEL-positive nuclei 4 h after UV-C exposure. The Southern result described above implies that nuclei positive for the TUNEL reaction could be attributed to oligonucleosomal fragmentation (PCD) and not to random DNA cleavage (accidental death: necrosis). The fact that the number of TUNEL-positive cells increases with time shows that

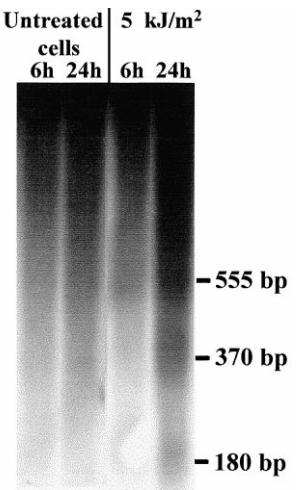

Fig. 2. Southern analysis of UV-C-induced DNA fragmentation in *A. thaliana* protoplasts. 0.5 μg of total genomic DNA was loaded in each track. Radiolabelled genomic DNA was used as a probe. Lane 1, control DNA extracted from 6-h protoplasts; lane 2, control DNA extracted from 24-h protoplasts; lane 3, DNA extracted from protoplasts 6 h after a 5 kJ/m^2 UV-C exposure; lane 4, DNA extracted from protoplasts 24 h after a 5 kJ/m^2 UV-C exposure.

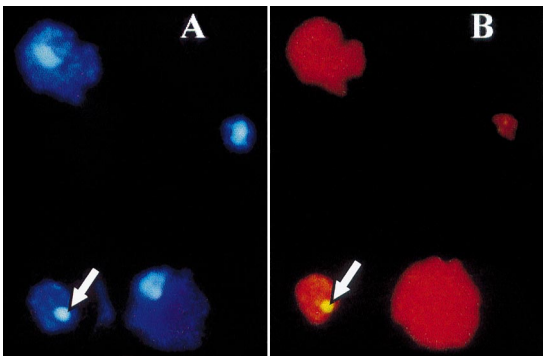

Fig. 3. Example of TUNEL-positive nuclei in UV-C-treated protoplasts (10 kJ/m^2). A: Protoplasts labelled with Hoechst 33342 (10 μg/μl) observed under UV light excitation. B: Protoplasts labelled with the TUNEL reaction observed under blue light excitation, ×105. White arrows indicate one TUNEL-positive nucleus.

the DNA fragmentation detected is not due to DNA breaks physically caused by UV-C but is a consequence of DNase activation.

We used FDA stain to link the DNA fragmentation detected using the TUNEL reaction and cell death. It should be noted that it has been shown using animal cells that FDA staining kinetics does not discriminate between apoptosis and necrosis [24]. Fig. 4B shows that as with TUNEL measurement, a UV-C dose of 1 kJ/m^2 does not affect the percentage of dead protoplasts. With a 10 kJ/m^2 UV-C exposure, the percentage of dead protoplasts increases with time and is correlated with the percentage of TUNEL-positive nuclei. However, with 50 kJ/m^2 the percentage of FDA-negative cells no longer correlates with the percentage of TUNEL-positive cells and immediately reaches 100%, showing a direct deleterious effect on the plasma membrane independent of time and DNA fragmentation.

3.3. Cell morphology of UV-C-treated protoplasts

In animals the nuclear changes associated with apoptosis are well defined and include nuclear fragmentation [16]. With a view to finding some common morphological changes, nuclei of several protoplast samples treated with 10 kJ/m^2 UV-C were analysed for their morphology. In Fig. 5 the protoplasts have lost their round shape due to fixation before the TUNEL reaction. The same unfixed protoplast population after irradiation is largely round and intact. TUNEL-positive cells could be assigned to three classes: (1) those containing normal-looking round nuclei (Fig. 5, 1), (2) those containing elongated, crescent-shaped nuclei (Fig. 5, 2,3), (3) those containing fragmented nuclei migrated to the cell periphery (Fig. 5, 4). It will be of interest to determine if these three classes constitute a morphological time course of UV-C-induced events as in animals, or if they reflect the population heterogeneity of protoplasts made of aerial parts and not of a defined tissue.

In addition in cells with fragmented nuclei, chlorophyll is not visible in white light, although some chlorophyll red fluorescence is visible under blue light excitation. The specific behaviour of chlorophyll and chloroplasts in relation to DNA fragmentation needs to be investigated since it is novel to the field.

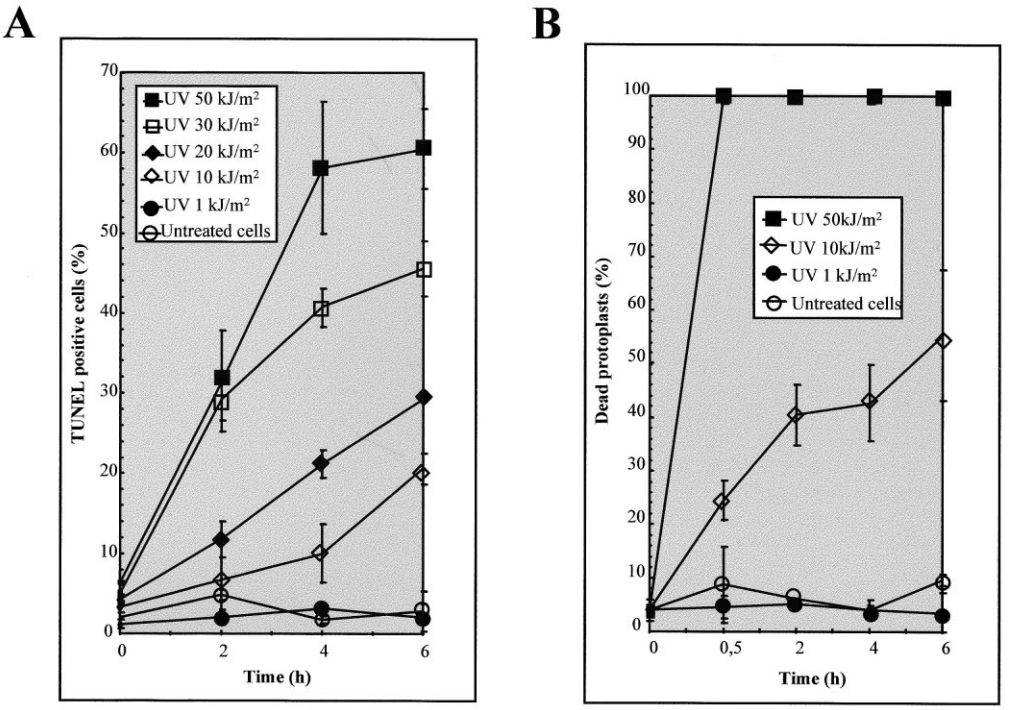

Fig. 4. Histochemical quantification of UV-C-induced cell death in *A. thaliana* protoplasts. Protoplasts were harvested 0, 2, 4 and 6 h after UV-C exposure ranging from 1 to 50 kJ/m^2. A: Percentage of protoplasts labelled using the TUNEL reaction. B: Percentage of unlabelled protoplasts (dead) using FDA stain (10 μg/μl). Each point corresponds to about 500 cells. Error bars give the S.D. for two experiments.

4. Discussion

This is the first report in plants that UV can induce a 180-bp DNA ladder which in animal cells is associated with apoptosis. Moreover, apoptosis in response to UV-C is well documented in animals [13,14]. Therefore molecular analysis of a UV-C response in plants is expected to give information on the evolutionary relationship between plant and animal PCD.

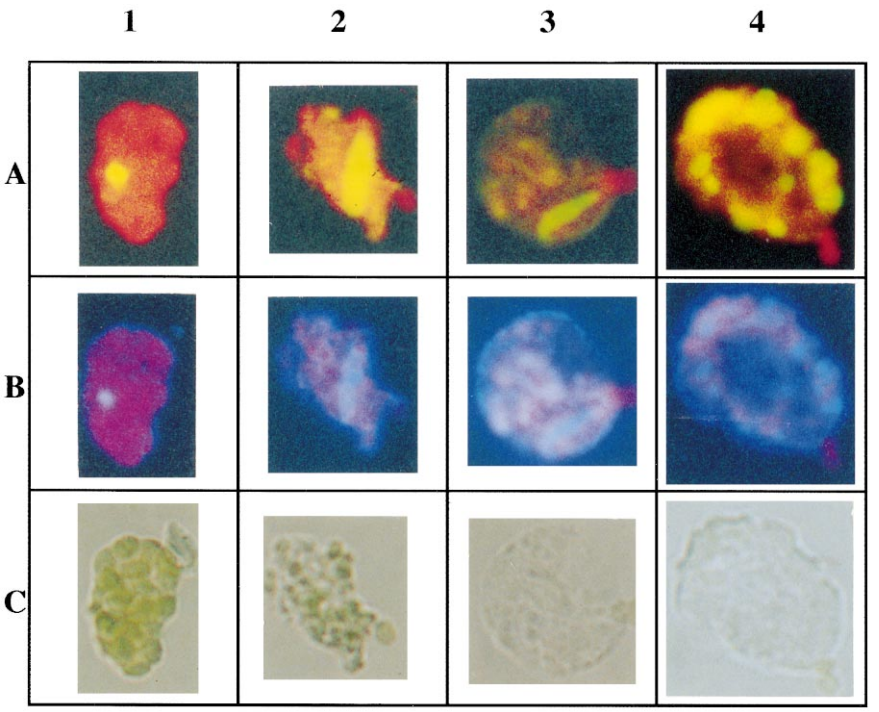

Fig. 5. In situ detection of nuclear changes associated with 10 kJ/m^2 UV-C-induced cell death in *A. thaliana*. 1–4 are protoplasts at different stages. A: Protoplasts labelled with the TUNEL reaction observed under blue light excitation. B: Protoplasts labelled with Hoechst 33342 (10 μg/μl) observed under UV light excitation. C: Protoplasts observed under white light. The red fluorescence corresponds to chlorophyll fluorescence. ×630.

In this report, DNA fragmentation was detected by two means: a qualitative one using Southern blotting and a quantitative one using the TUNEL reaction. This DNA fragmentation is not a direct physical effect of the UV-C irradiation used since for a given dose (a) the 180-bp DNA ladder increases in intensity with time and (b) the percentage of TUNEL-positive cells increases with time. We also showed that using a 10 kJ/m^2 UV-C dose, the appearance of TUNEL-positive nuclei induced by UV-C radiation is proportional to the percentage of dead protoplasts measured by FDA staining. These results confirm the direct link between cell death and TUNEL-positive nuclei. At the same UV-C dose, the detection of FDA-negative (dead) cells precedes the detection of the TUNEL-positive cells. This observation indicates that plasma membrane alteration is an early indicator of cells undergoing DNA fragmentation since it occurs prior to the detection of DNA strand breaks by the TUNEL reaction. This is consistent with what has been reported using animal cells and FDA [24]. It should be noted that in animal cells, FDA staining does not discriminate between apoptosis and necrosis [24]. As the number of FDA-negative cells is higher than those containing TUNEL labelling nuclei in our experiments, we cannot exclude that some of the FDA-negative cells are not apoptotic but necrotic.

At the highest dose used (50 kJ/m^2) all the protoplasts irradiated are FDA-negative immediately after UV-C exposure. It is therefore clearly a physical effect of the UV-C dose on the plasma membrane inducing the immediate release of fluorescein from the cells. It should be noted that it does not correlate with an immediate DNA fragmentation measured by TUNEL suggesting that the two phenomena are not linked. This reinforces the idea that in our system DNA fragmentation is a physiological response to UV-C radiation. If DNA fragmentation was a direct physical effect of UV-C we would expect the percentage of TUNEL-positive cells to reach a maximum level immediately after irradiation as observed using FDA and the highest UV-C dose.

Microscopic examination of TUNEL-positive nuclei revealed that, as in animal apoptosis [23], DNA fragmentation starts in morphologically normal nuclei. We have also observed crescent-shaped nuclei and fragmented nuclei. Crescent-shaped nuclei have been described during plant PCD induced by TMV [25]. Nuclear fragmentation occurs in the late stage of animal apoptosis [16], and has already been reported in one plant PCD study [9]. This suggests that the plant response to the UV-C doses used is a PCD response and this is the second report showing that nuclear fragmentation may occur in plant cells.

The UV-C doses used to induce apoptosis in animal studies with cultured cell lines are in the range of 10–50 J/m^2 [26,27] and are therefore about 500–1000 times lower than that used here with seedlings or protoplasts. The effect of UV-C on plant cells has already been tested in the context of DNA mutagenesis in *A. thaliana* with necessary UV-C doses reported to be at least equal to 1 kJ/m^2 [28]. However, in animal cells DNA mutations are induced by UV-C doses in the 5 J/m^2 range [29]. This corroborates our finding that plant cells are less sensitive to UV-C than animal cells with regard to a 180-bp DNA ladder response. In the case of seedlings, tolerance of high UV doses has been explained in the literature by several characteristics of plant cells: (i) the presence of provitamin D which has a UV absorption spectrum that overlaps with the one of DNA. This molecule has been shown to act as a natural sunscreen from high energy UV radiation [30]; (ii) the presence of flavonoids, since it has been shown in vitro that flavonoids protect DNA from UV-induced damage [31], and that *A. thaliana* mutants deficient in flavonoid content are hypersensitive to UV irradiation [15]; (iii) the presence of cell walls and cuticular waxes which reflect up to 99% of UV radiation [12]. With protoplasts some protection is expected to be provided by the liquid culture medium. In animal cell culture the liquid culture medium is removed before UV-C irradiation. A complementary but less favoured explanation would be that the threshold of cell death response to UV damage is higher in plants compared to animals or that repair mechanisms in plants are more efficient.

Although the induction of a 180-bp DNA ladder has been described in several plant species (reviewed in [32–34]), this is the first time that a system with a clear induction of apoptotic hallmarks is described using the model plant *A. thaliana*. This will facilitate the design of mutant screens for altered response phenotypes. Moreover, using UV-C, protoplasts and detection by TUNEL reaction we have set up a powerful and versatile induction system with which the effect of different molecules could be tested by addition to the protoplast culture medium. We propose that plant cells have a PCD response to UV-C probably triggered via DNA damage. Although the doses we used are not physiologically relevant they constitute an experimental set-up revealing the PCD response. We are now investigating whether some molecular components of the apoptosis regulation cascade known to be activated in animal cells by UV-C can be identified in our system (e.g. caspases).

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