Ultraviolet-C Overexposure Induces Programmed Cell Death in Arabidopsis, Which Is Mediated by Caspase-like Activities and Which Can Be Suppressed by Caspase Inhibitors, p35 and Defender against Apoptotic Death*

Antoine Danon†, Vitalie I. Rotariš, Anna Gordon¶, Nathalie Mailhac, and Patrick Gallois‡

From the Laboratoire Gênome et Développement des Plantes, CNRS, Unité Mixte de Recherche (UMR) 5096, Université de Perpignan, 52 Avenue de Villeneuve, 66860 Perpignan Cedex, France

Plants, animals, and several branches of unicellular eukaryotes use programmed cell death (PCD) for defense or developmental mechanisms. This argues for a common ancestral apoptotic system in eukaryotes. However, at the molecular level, very few regulatory proteins or protein domains have been identified as conserved across all eukaryotic PCD forms. A very important goal is to determine which molecular components may be used in the execution of PCD in plants, which have been conserved during evolution, and which are plant-specific. Using Arabidopsis thaliana, we have shown that UV radiation can induce apoptosis-like changes at the cellular level and that a UV experimental system is relevant to the study of PCD in plants. We report here that UV induction of PCD required light and that a protease cleaving the caspase substrate Asp-Glu-Val-Asp (DEVDase activity) was induced within 30 min and peaked at 1 h. This DEVDase appears to be related to animal caspases at the biochemical level, being insensitive to broad-range cysteine protease inhibitors. In addition, caspase-1 and caspase-3 inhibitors and the pan-caspase inhibitor p35 were able to suppress DNA fragmentation and cell death. These results suggest that a YVADase activity and an inducible DEVDase activity possibly mediate DNA fragmentation during plant PCD induced by UV overexposure. We also report that AtDAD1 and AtDAD2, the two A. thaliana homologs of Defender against Apoptotic Death-1, 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.

Programmed cell death (PCD) is involved in some plant pathogen interactions (1) and in normal developmental processes during the plant life cycle. For example, it plays a role in the germination of seeds, the differentiation of the tracheary elements, reproduction, flower senescence (2), and senescence (e.g. Ref. 3). Building on the ancestral form of PCD, plants are expected to have evolved their own pathways to cope with plant-specific features such as the presence of cell walls that prevent dead cells from being engulfed by neighboring cells. Light dependence may be another example of a specific aspect of at least some forms of plant PCD. Induction of cell death requires light in a number of lesion-mimic mutants in Arabidopsis, lsd1 (4) and acd11 (5), and in maize, lls1 (6). Light is also required for PCD induced by the mycoxin fumonisin B1 (7). Whether and how light is required for activating cell death or for its execution are not yet clear.

Despite these specificities, some cellular aspects appear to be conserved in animals and plants, including DNA fragmentation (laddering), protoplast shrinkage, and chromatin condensation (8). In addition, caspase-like activities have been detected in plants (9). In animals, caspases are specifically activated during PCD. In particular, caspases initiate cell death by degrading several proteins essential for cell integrity (e.g. poly(ADP-ribose) polymerase), lamins, and gelsolin). Caspase activities can be measured using fluorogenic peptide substrates and can be blocked by the same peptide substrates coupled to an aldehyde. Caspase-like activities have been detected in tobacco showed that, during PCD induced by medamine in protoplasts, caspase inhibitors can block the induction of DNA fragmentation and of poly(ADP-ribose) polymerase cleavage (12). Caspase inhibitors (Ac-DEVD-CHO and Ac-YVAD-CHO) have also been shown to block PCD after pathogen induction (10). Expression of p35, a caspase inhibitor, has been reported to reduce the onset of apoptosis in embryonic callus in maize (13). This protein specifically inhibits caspases in insects, nematodes, and humans by blocking their active sites (14, 15). Key amino acid residues essential for inhibitor function have been identified using point mutations (15). More recently, it was...

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† Present address: Plant Genetics, Inst. of Plant Sciences, ETH-LFW D-31, Universitätstr. 2, CH-8092 Zürich, Switzerland.
‡ Present address: School of Biological Sciences, University of Manchester, Manchester M13 9PT, UK.
§ Present address should be addressed: School of Biological Sciences, University of Manchester, Manchester M13 9PT, UK. Tel.: 44-161-275-3922; Fax: 44-161-275-3938; E-mail: patrick.gallois@man.ac.uk.
shown that transgenic tomato plants bearing the p35 gene are protected against Alternaria alternata f. sp. lycopersici (Aal-toxin-induced death and pathogen infection, confirming that p35 can suppress PCD in plants (16).

The possible common origin of PCD in multicellular organisms and the conservation of some features of apoptosis might be expected to be partially reflected at a molecular level. However, despite the completion of the Arabidopsis genome sequence, only a few plant genes have been identified as orthologs of mammalian genes involved in apoptosis, e.g., At-BI1 (Arabidopsis thaliana BAX Inhibitor-1) (17, 18); At-DAD1 (A. thaliana Defender against Apoptotic Death-1) (19, 20); and cytochrome C, AIF, and PIG3 (reviewed in Ref. 8; see Ref. 21). The plant Bax inhibitor is localized in the endoplasmic reticulum (ER), and overexpression of the Bax inhibitor has recently been shown to suppress pathogenesis-induced plant PCD (22, 23). Finally, although there are no caspase orthologs, it has been proposed that plant metacaspases are homologous and functionally equivalent to animal caspases (24).

We have shown in a previous report that UVC stress induces apoptosis-like changes in Arabidopsis (25). These include detection of a DNA ladder, changes in nucleus morphology (crescent shape), and nucleus fragmentation. In protoplasts, DNA fragmentation was detected using the TUNEL reaction, and caspase inhibitors blocked the onset of DNA fragmentation. Using protoplast transfection, we show here that the DNA fragmentation induced by UVC can be suppressed by transient overexpression of p35 or At-DAD genes. These findings confirm that UV-induced cell death is a form of PCD and suggest a role for At-DAD or for ER stress in the PCD of plants.

EXPERIMENTAL PROCEDURES

Plant Material—Seeds from A. thaliana (Columbia 0) were sterilized, sown under aseptic conditions on agar plates containing Murashige and Skoog salts and vitamins supplemented with 0.4 M sucrose, 0.4 M glucose, and grown at 21 °C (17).

At-DAD1 cDNA (GenBank™/EBI accession number X95358) was excised from a library prepared in the λ-ZAP vector (Stratagene). At-DAD2 cDNA (GenBank™/EBI accession number AF030172) was cloned in the pMOSBlue vector (Amerham Bio-sciences). Plasmids p35S:At-DAD1 and p35S:At-DAD2 were constructed by inserting a BamHI/SphI fragment from the cDNA construction into the BamHI/SphI sites of the pDH51 plasmid (a gift of Dr. Paszkowski, Friedrich Miescher Institute, Basel, Switzerland). p35S:p35 was constructed by inserting an XbaI/ScaI fragment from the pPRM-35K-ORF mammalian expression vector containing p35 cDNA (a gift of Dr. P. Friesen, University of Wisconsin, Madison, WI) into the XbaI/ScaI sites of the pDH51 plasmid. p35S:p35-D78A was generated using p35S:p35 and primers according to the QuiikChange protocol (Stratagene). The pRTL2-GUS plasmid (a gift of Professor James Carrington, Texas A&M University, College Station, TX) was used as a transfection control. To obtain an At-DAD1-GFP fusion, At-DAD1 cDNA was amplified using PCR primers 5′-GFGF-CAGTCCAGGATGGCATGCTGTAATCCAGAGC-3′ and 5′-CTCATGACGGGTCGTTTGAATCCATGCAAGCTGGTGATTGATGATCA), which add a new NcoI site to either end of At-DAD1 cDNA. The At-DAD1 GFP construct was digested using NcoI (Roche Applied Science) and cloned into the NcoI site immediately 5′ to the EGFP coding sequence in the pK100 construct. The resulting p35S:At-DAD1-GFP cassette was then excised using HindIII and cloned into the HindIII site of the plant binary vector pZP111b (a gift of Dr. S. Michaels, University of Wisconsin, Madison, WI) and used in onion bombardment experiments. pK100 was created by excising the EGFP-1 gene (Clontech) with NcoI and cloned into the multiple cloning site of the pDH51 vector. pEGFP-Par was created by adding a basic chitinase secretion peptide and the ER retention signal KDEL to EGFP.

Protoplast Transfection—For transfection, protoplasts were resuspended at 10^6 protoplasts/ml in 0.4 M mannitol, 15 mM MgCl2, and 0.1% MES (pH 5.6) and kept for 30 min on ice. Protoplasts were then mixed with 50 μg of plasmid DNA in a 6-well Petri dish (Corning), and 1 ml of 40% polyethylene glycol (PEG) was carefully added. Protoplasts were incubated for 30 min at room temperature and washed sequentially three times for 5 min with 2, 4, and 8 ml of WS5 (25). Protoplasts were then pelleted at low speed centrifugation (60 × g, 5 min), resuspended in 1 ml of culture medium, and cultured for 24 h at room temperature. Transfection efficiency was between 40 and 60%. A transfection control using β-glucuronidase assays was routinely carried out. Semiquantitative RT-PCR and Transgene Expression—Briefly, RNA was extracted from aliquots of protoplasts transfected with various plasmids using the Invitek kit (Invirobi). 5 μg of total RNA was treated with 5 units of RNase-free DNase (Promega) in a 40 μl total volume. cDNAs were obtained using the PRO STAR™ first-strand RT-PCR kit (Stratagene) according to the manufacturer’s instructions with the following modifications. 1.2 μl of oligo(dT) primer was added to 2 μg of total RNA in a final volume of 15 μl in Milli-Q water. In the fourth step of the protocol, the reagent volumes were reduced by 2.5-fold. Absence of transfected plasmid or genomic DNA in the cDNA samples was verified by PCR amplification without a reverse transcription step. Semiquantification of target transcripts was carried out using PCR and gene-specific primers, selecting a number of cycles in the linear range of amplification. Actin-2 (GenBank™/EBI accession number U141998) was used as a reference gene to compare expression levels in different batches.

UV Irradiation of Plants and Protoplasts—Seeds were irradiated in open Petri dishes (9-cm diameter; Corning) using a UV Stratagene linker 2400 (Stratagene) fitted with 254-nm UVC light bulbs. Protoplasts were irradiated in an open 6-well Petri dish at 10^6 protoplasts/ml of culture medium. For caspase inhibition experiments, 10^5 protoplasts in 100 μl of culture medium were irradiated in an open 6-well Petri dish. Seedling and protoplasts were kept in white light: 400–700 nm, 150 μmol·m^-2·s^-1 (OSRAM) and 580–860 nm, 150 μmol·m^-2·s^-1 (Heliolux Cool White) after UV treatment unless stated otherwise. A UVC sensor fitted inside the Stratalinker irradiation chamber measured the UVC energy delivered in each experiment. The UVC doses used varied between 10 and 50 kJ/m². The UVC bulbs used emit a sharp wavelength band centered at 253.7 nm.

In Situ Detection of Cell Death Using Evans Blue—Protoplast samples were to be analyzed with 0.04% Evans blue for 5 min. Blue cells were scored as dead cells using a light microscope.

In Situ Detection of Nuclear DNA Fragmentation—The TUNEL reaction was carried out on fixed protoplasts according to Landon and Gallois (25). The protoplasts on slides were viewed with a fluorescence microscope (Zeiss Axioscop) using an FITC Blue 450 filter (Zeiss). For each sample, photographs of multiple microscopic fields were taken, and TUNEL-positive nuclei were scored on prints.

* R. Blanvillain, unpublished data.
Effect of Caspase Inhibitors in Vivo—Protoplasts were incubated for 1 h at room temperature with a caspase inhibitor (Ac-DEVD-CHO or Ac-YVAD-CHO (100 μM); Bachem Ltd.) and irradiated with 10 or 15 kJ/m² UVC. After various incubation times, protoplasts were harvested and incubated in the presence of Evans blue or fixed for the TUNEL reaction.

Protein Extraction and Caspase-like Activities—A. thaliana seedlings were grown for 3 weeks in Petri dishes (9-cm diameter) and then irradiated with 50 kJ/m² UVC and incubated under our standard culture conditions. At different time points, the plants were frozen in liquid nitrogen and ground to a powder with a mortar and pestle. The powder was collected in 1.5-ml tubes and kept at −80 °C until processing. The samples were then resuspended in assay buffer (20% glycerol, 0.1% Triton, 10 mM EDTA, 5 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride (PMSF), and 50 mM sodium acetate) and incubated with 200 μM Ac-DEVD-AMC or Ac-YVAD-AMC (Bachem Ltd.). AMC fluorescence was detected using a Labsystems Fluoroskan II every 5 min during a 1-h reaction. The enzymatic activity was calculated as the slope of the product concentration as a function of time. This activity was then standardized to the quantity of total proteins present in the sample (Bradford assay, Bio-Rad). For inhibitor assays, samples were resuspended in assay buffer and incubated for 1 h with a caspase inhibitor (DEVD-CHO or YVAD-CHO (200 μM); Bachem Ltd.) or with a protease inhibitor (pepstatin A (3 mM), leupeptin (4 mM), or E-64 (10 μM); all from Sigma). Extracts were then incubated with the substrate DEVD-AMC (200 μM).

Transfection of Onion Cells—Microprojectiles and DNA were prepared according to Hull et al. (30). Pieces of onion epidermis still attached to the ground tissue were placed on Petri dishes containing MS30 medium solidified with 10 g/liter Bacto-agar. Bombardments were carried out according to Hull et al. (30), and onion cells were incubated in the dark for 24 h at 22 °C. The epidermis was peeled off, and GFP-positive cells were observed with a Leica fluorescence microscope under blue wavelength excitation light using an FITC Blue 450–490 filter.

RESULTS

UV-induced PCD Is Light-dependent—To examine the effect of light on PCD induced by UVC, 5-day-old seedlings were subjected to increasing amounts of UVC and kept in the dark or in the light for 72 h. In the light, seedlings developed an obvious bleaching of their leaves with doses of 10 kJ/m² and above. In contrast, seedlings kept in the dark showed no bleaching (Fig. 1A). There was still no bleaching of seedlings kept in the dark for up to 7 days (data not shown). To confirm that bleaching was a measure of cell death, the same treatment was applied to protoplasts prepared from seedlings, and cell death was measured by Evans blue, a marker of plasma membrane integrity. Dead protoplasts accumulated the dye, whereas live protoplasts excluded the dye (Fig. 1A). When treated with UV and kept in the light, up to nearly 100% of the protoplast population was scored dead. The death rate in the dark was as little as 10% and comparable with untreated samples, although treated protoplasts appeared shrunken compared with untreated protoplasts (Fig. 1C).

DEVDase and YVADase Activities in UV-irradiated Seedlings—To assess whether cell death in UV-irradiated plants is partially homologous to animal PCD at the molecular level, we assayed for caspase-1-like activity, a protease that cleaves the substrate Tyr-Val-Ala-Asp (YVADase), and caspase-3-like activity, a protease that cleaves the substrate Asp-Glu-Val-Asp (DEVDase). Caspase-like activities were tested in extracts from irradiated and untreated seedlings using two caspase substrates: Ac-DEVD-AMC and Ac-YVAD-AMC (200 μM).

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radiation (data not shown). In contrast, induction of DEVDase activity was detected 30 min after irradiation and reached a peak at 1 h (Fig. 2A). Various protease inhibitors were tested to assign this DEVDase activity to a specific class of proteases. The protein extract was preincubated for 1 h with caspase inhibitors Ac-DEVD-CHO and Ac-YVAD-CHO (200 μM) before adding the substrate Ac-DEVD-AMC (200 μM). This final concentration is in the range used in animal studies. Inhibition with broad-spectrum cysteine and serine protease inhibitors that do not inhibit animal caspases was also tested: pepstatin A (3 mM), leupeptin (4 mM), or E-64 (10 μM). Inhibitor analysis (Fig. 2B) showed that the DEVDase activity was totally inhibited by Ac-DEVD-CHO and only partially by Ac-YVAD-CHO or by pepstatin A and leupeptin. E-64 had little effect on DEVDase activity, in agreement with inhibition data for animal caspases (31).

It has been proposed that cysteine proteases of non-caspase families (papain and legumain) are involved in PCD in plants (reviewed in Ref. 32). In addition, it has been shown that legumain can cleave Ac-YVAD-AMC, a substrate specific for caspase-1 (33). But in contrast to DEVDase activity, the activity of these two families of cysteine proteases was suppressed after UVC radiation (data not shown).

In the experiments presented here, the assay buffer used contained PMSF (serine protease inhibitor) and EDTA (metalloprotease inhibitor), two class-specific inhibitors that do not inhibit animal caspases. The inhibitors are used to distinguish possible plant caspase-like activities from other protease activities. In the absence of inhibitors in the assay buffer, there was a background activity in the non-induced samples that masked the activation of the DEVDase activity (data not shown). In the presence of the chosen inhibitors, the background activity was reduced, and activation results were clearer. The use of class-specific inhibitors to assay caspase-like activity in crude extract is consistent with protocols used in studies of animal caspases. (i) Stennicke and Salvesen (34) have reported that caspase substrates can be cleaved in crude extract by proteases other than caspases. (ii) PMSF (serine proteases) and EDTA (metalloproteases) do not inhibit animal caspases (31, 35). (iii) These inhibitors have been used in buffers to purify animal caspase-1 (35).

Further experiments showed that, in contrast to animal caspases, which are active at pH 7, DEVDase was active at pH 5 (data not shown). The activity was also relatively insensitive to a salt concentration of up to 200 mM (Fig. 2B), which has been shown to have an inhibitory effect on the detection of some caspase-like activity in plants (36).

In conclusion, UVC radiation induced DEVDase activity, which was specifically inhibited by Ac-DEVD-CHO and which was not significantly affected by other protease inhibitors. YVADase activity was detected in both induced and non-induced tissues.

DEVDase and YVADase Are Required for Induction of DNA Fragmentation and Cell Death—We have shown previously that, in plants, UVC radiation indirectly induces fragmentation of the genomic DNA, resulting in the formation of a DNA ladder (25). As in animal apoptosis, the rungs are multiples of 180 bp. This DNA fragmentation can also be detected in situ using the TUNEL reaction, which labels the free 3’-OH DNA extremities (25) and constitutes a marker of plant PCD. In animal cells, caspase-3 activates the DNase responsible for the appearance of the DNA ladder. Having detected DEVDase and YVADase activities in Arabidopsis extracts, we therefore investigated a possible link between these activities, cell death, and DNA fragmentation. Caspase inhibitors were analyzed for their potential ability to block DNA fragmentation and to rescue UV-irradiated protoplasts. Protoplasts were prepared using 3-week-old seedlings grown in vitro, incubated for 1 h at room temperature with a caspase inhibitor (Ac-DEVD-CHO or Ac-YVAD-CHO (100 μM)), and subsequently irradiated with UVC. After 4 h of culture in the light, protoplasts were harvested, fixed, and analyzed for DNA fragmentation using the TUNEL reaction. Non-irradiated protoplasts showed a background of 10% TUNEL-positive cells (Fig. 3A). This DNA fragmentation could be suppressed by YVAD-CHO and, to a lesser extent, by DEVD-CHO. Under the experimental conditions used, UV irradiation clearly induced DNA fragmentation, pushing it up to 35%. This induction could be totally prevented when protoplasts were preincubated with either of the two caspase inhibitors used.

The same experiment was repeated, and cell death was measured using Evans blue. 4 h after UV treatment, cell death reached 60% in the control sample without inhibitors. Preincubation in the presence of DEVD-CHO or YVAD-CHO reduced cell death by one-half. Suppression was less efficient than in the previous experiment, probably because Evans blue detected some necrosis (accidental death) that was not suppressed by the inhibitors. It remains that DEVD-CHO and surprisingly
YVAD-CHO were able to suppress both DNA fragmentation and cell death.

Transfection of p35 in Protoplasts Inhibits DNA Fragmentation and Cell Death—Transient expression analysis is a powerful tool to analyze the function of plant genes potentially involved in controlling PCD. Our UV and protoplast system is particularly suited for such analysis, as it allows protoplast transfection and cell death quantification. To assess the potential of this approach and to confirm the results obtained with the caspase inhibitors, we selected p35, an apoptosis inhibitor gene of viral origin that specifically suppresses caspase activity in animal cells to prevent the death of infected cells (14). Several mutations, including D87A, are known to prevent the inhibition of the caspase enzymes by p35 (15). To test whether the effect of p35 is conserved in plant cells, we made a construct with a p35 or a p35-D87A coding sequence under the control of the cauliflower mosaic virus 35S promoter. Preliminary experiments showed that the PEG treatment used or the transfection of plasmid DNA (50 μg of p35S::GUS) did not in itself protect protoplasts from the effect of UV and did not prevent the DNA fragmentation response (see Fig. 5) (data not shown). Protoplasts were then transfected with 50 μg of p35S::p35 plasmid. After 24 h of incubation, protoplasts were harvested or not (untreated) with UV and kept in continuous light. A, aliquots from the same transfection tube were harvested 0, 2, 4, and 8 h after irradiation to score cell death using Evans blue. Error bars are S.D. values for replicates.

Fig. 3. Caspase inhibitors suppress UVC-induced cell death and DNA fragmentation. A, protoplasts were incubated for 1 h at room temperature with no caspase inhibitor (Control), Ac-DEVD-CHO (100 μM), or Ac-YVAD-CHO (100 μM), and an aliquot was irradiated with 10 kJ/m² UVC. After 4 h of incubation in continuous light, protoplasts were harvested and fixed for the TUNEL reaction. The percentage of TUNEL-positive protoplasts was then calculated in the controls (no inhibitors) and in protoplasts incubated with caspase inhibitors with or without UV irradiation. Error bars are S.D. values for replicates. B, protoplasts were incubated for 1 h at room temperature with no caspase inhibitor (Control), Ac-DEVD-CHO (100 μM), or Ac-YVAD-CHO (100 μM), and an aliquot was irradiated with 15 kJ/m² UVC. After 4 h of incubation in continuous light, protoplasts were incubated with Evans blue. The percentage of dead protoplasts was then calculated in the controls (no inhibitors) and in protoplasts incubated with caspase inhibitors with or without UV irradiation. Error bars are S.D. values for replicates.

Fig. 4. Transfection of the p35 gene suppresses UVC-induced cell death and DNA fragmentation. Protoplasts were treated with PEG (control) or transfected with PEG and 50 μg of p35S::p35 plasmid (P35) or 50 μg of p35S::p35-D87A plasmid (P35-D87A). After 24 h of incubation, protoplasts were irradiated or not (untreated) with UV and kept in continuous light. A, aliquots from the same transfection tube were harvested 0, 2, 4, and 8 h after irradiation to carry out the TUNEL reaction. B, aliquots from the transfection tubes were harvested 4 h after irradiation to carry out the TUNEL reaction. C, aliquots from the transfection tubes were harvested 4 h after irradiation to score cell death using Evans blue. Error bars are S.D. values for replicates.
of p35S::p35 reduced the DNA fragmentation to a frequency of only 40% TUNEL-positive cells. In a second experiment, p35S::p35 or p35S::p35-D87A was transfected, and the effect of UV was analyzed at 4 h. RT-PCR experiments showed that the transgenes were expressed at a similar level. The point mutation D87A abolished the inhibitory effect of p35 (Fig. 4B). The same results were obtained using Evans blue to measure cell death, where the p35S::p35-D87A sample reached 70% dead cells, whereas the samples expressing functional p35 reached only 40% dead cells. As shown above, cell death measured using Evans blue correlated with the TUNEL results.

Cotransfection with another reporter construct such as a p35S::luciferase is clearly not necessary in our system, as the S.D. is already sufficiently small to allow meaningful comparisons. This experiment confirmed the results obtained using synthetic caspase inhibitors and indicates that the inhibition of DNA fragmentation is caspase-like specific. It also emphasizes the potential of protoplast transfection as a means to test the activity of putative suppressor genes.

Transfection of the At-DAD Genes in Protoplasts Inhibits DNA Fragmentation—DAD1 was originally discovered in hamster cells, where the cell line carrying the dad1 mutation dies via apoptosis (19). One A. thaliana homolog (At-DAD1) has been reported in the literature, and we demonstrated that the function of this protein is conserved between plants and animals by transformation of the original mutant hamster cell line with At-DAD1 (20). We identified a second homolog (At-DAD2) in the genome of A. thaliana (GenBankTM/EBI accession number AF030172). The two At-DAD genes share the same organization, with variations only in intron sizes (data not shown). At the amino acid level, the At-DAD1 and At-DAD2 sequences are 95.7% identical, with three of the five amino acid changes localized in the N-terminal cytoplasmic domain of the protein (Fig. 5A). The prediction of the membrane-spanning domains and the orientation of the protein N terminus relative to the cytoplasm have been confirmed experimentally for animal DAD1 (37).

To investigate the subcellular localization of DAD1 in plants, we constructed a C-terminal fusion of At-DAD1 with EGFP. Transfection of onion cells indicated that the At-DAD1-EGFP fusion has the same subcellular localization pattern as ER-targeted EGFP, indicating that the plant DAD1 protein is located in the ER (Fig. 5B). Using RT-PCR, we found that both genes were expressed at similar levels in all tissues and under all conditions tested (data not shown). Given that DAD1 may have an apoptosis suppressor role in animals, we wondered whether overexpression of At-DAD1 or At-DAD2 could protect cells from PCD in A. thaliana. To this end, we overexpressed both cDNAs in protoplasts before UVC irradiation. Protoplast samples were transfected with various plasmid constructions: p35S::GUS (negative control), p35S::At-DAD1, or p35S::At-DAD2. After 24 h of culture, RT-PCR experiments showed that the transgenes were expressed at a similar level. Protoplasts were irradiated with UVC, and aliquots were harvested after irradiation to carry out the TUNEL reaction. Fig. 5C shows that, in irradiated protoplast samples subjected to a mock transfection with no DNA or to transfection with p35S::GUS, the proportion of TUNEL-positive cells doubled, increasing from 25 to 50% 2 h after UVC irradiation. In contrast, in samples transfected with p35S::At-DAD1 or p35S::At-DAD2, a large proportion of the protoplasts were protected from UVC-induced PCD, as the percentage of TUNEL-positive cells reached only 30%. Overall, this suggests that the plant DAD proteins have the ability to suppress or significantly delay the induction of DNA fragmentation in transient expression assays. We obtained similar suppression of DNA fragmentation using At-BI1.3

DAD1 is localized in the ER membrane and is a possible anchorage protein for a structural unit within the oligosaccharyltransferase complex (37). This raises the possibility that DAD1 overexpression affects the induction of DNA fragmentation by limiting or preventing ER stress in UVC-treated cells. One possibility is that overexpression of DAD1/2 stabilizes or increases N-glycosylation. Therefore, we subjected the transfected protoplasts to ER stress downstream of glycosylation by pretreatment for 2.5 h with 20 μg/ml tunicamycin. This prevents glycosylation, promotes misfolding, and has been used to trigger the accumulation of unfolded proteins in protoplasts (38). Within the 6 h of our assay, At-DAD1 protected protoplasts from UVC-induced PCD as efficiently with or without tunicamycin in the culture medium (Fig. 5D).

DISCUSSION

UVC is a very convenient trigger to induce PCD in plants and protoplasts in a reproducible manner. UVC has been used in animal studies to study the triggering of apoptosis following DNA damage and the activation of p53. Whether a similar pathway is triggered or not in plants remains to be shown because no homolog of p53 has been identified to date. We have shown that UV-induced cell death displays apoptotic hallmarks (25), and we show here that it is a light-dependent process, possibly mediated by caspase-like activities. We take these two last results as additional evidence that UVC induces PCD in plant protoplasts. Using protoplasts is a reductionist approach that is a strength when studying intrinsic PCD pathways at the cellular level. Whole plants are complex systems to study cell death at the cellular level. Protoplasts are therefore an attractive alternative. It is possible that protoplasts are primed for cell death during isolation and do not recapitulate all aspects of PCD in plants, but our study showed that this is not detrimental to the analysis of the execution of PCD at the cellular level. At later stages, the findings made using protoplasts will be integrated at the whole plant level. As an example, our p35 results in protoplasts are validated by studies using stable transformants in other experimental systems where p35 expression has been shown to reduce cell death (13, 16).

We show here that this form of PCD shares a light requirement with other forms of reported PCD such as HR (4, 5) or the one induced by fumonisin B1 (7). One explanation for this light requirement might be that reactive oxygen species generated during photosynthesis (39) are implicated in the PCD process. The generation of reactive oxygen species has been shown to be involved in HR, a typical example of PCD in plants (40). It has been suggested that HR may need functional chloroplasts, although a mechanism for the involvement of chloroplast function in HR has not been established (39). Another possible explanation is based on the discovery that a salicylic acid synthesis pathway is localized in the chloroplast (41). The fact that salicylic acid is an important signal molecule for PCD in plants could indicate a role for chloroplasts in this process and explain light dependence. Interestingly, after exposure of Arabidopsis to UVC, salicylic acid has been reported to accumulate together with EDS5 mRNA, an essential component of salicylic acid-dependent signaling for disease resistance in Arabidopsis (42). Taken together, these results indicate that UVC induces salicylic acid biosynthesis in chloroplasts. It will be thus very interesting to investigate in the future further possible connections between UVC irradiation, salicylic acid production, light, and PCD.

We report here, using A. thaliana protoplasts, that caspase-1

3 N. Mailhac, manuscript in preparation.
and caspase-3 inhibitors can prevent DNA fragmentation and cell death induced by UVC irradiation. We used both YVAD and DEVD inhibitors and substrates to make our study comparable with other plant studies (e.g. Refs. 10 and 11). We also demonstrated that expression of the baculovirus pan-caspase inhibitor p35 was able to block PCD induced by UV in plants in a specific manner because the null mutant p35-D87A was unable to suppress DNA fragmentation. It has been shown that the D87A mutation prevents the cleavage of p35 by animal caspases and causes the protein to lose its caspase inhibitor activity (15).

The fact that caspase inhibitors are able to suppress cell death and DNA fragmentation suggests that proteases, which could be called caspase-like, are involved directly or indirectly with the execution of PCD induced by UVC. In support of this, we showed that, in our system, DEVDase activation took place within the first hour after induction of cell death. This is before the peak of TUNEL-positive cells measured at 4 h (25). This is different from other reports of late activation of caspase-like proteases (10, 11). This timing and the inhibition of DNA fragmentation by DEVD-CHO and p35 provide indirect evidence that the detected DEVDase possibly mediates the activation of DNA fragmentation. This DEVDase activity appears to be different from the one described in suspension cells by

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**Fig. 5. Transfection of the At-DAD genes suppresses UVC-induced DNA fragmentation.** A, At-DAD1 and At-DAD2 sequences are 95.7% identical. DAD proteins contain three membrane-spanning domains. Different color backgrounds map the predicted transmembrane domains. B, shown is the ER localization of an At-DAD1-EGFP fusion. Constructs were bombarded in onion epidermis cells, and EGFP fluorescence was detected after 24 h of incubation. The plasmids used were p35S::DAD1-EGFP, p35S::EGFPer (a positive control for ER localization), and p35S::EGFP (a cytosolic control). Images show a close-up of cytosolic strands inside onion cells. A reticulum pattern is visible with At-DAD1-EGFP and EGFPer. This pattern is absent with EGFP. C, protoplasts were treated with PEG (control) or transfected with 50 μg of various plasmid constructs: p35S::GUS (negative control), p35S::At-DAD1, or p35S::At-DAD2. After 24 h of incubation, protoplasts were irradiated with UVC and kept in continuous light, and aliquots from the same transfection tube were taken 0, 2, and 4 h after irradiation to carry out the TUNEL reaction. Error bars are S.D. values for replicates. D, shown is the effect of tunicamycin on the suppression of DNA fragmentation by At-DAD1. Protoplasts were treated with PEG or transfected with 50 μg of plasmid p35S::At-DAD1 (DAD1). After 24 h, an aliquote of PEG and DAD1 was preincubated in 20 μg/ml tunicamycin for 2.5 h (PEG + T, DAD1 + T). All samples were subsequently irradiated with UVC and kept in continuous light. Aliquots from the same transfection tube were taken 0, 2, and 4 h after irradiation to carry out the TUNEL reaction. Error bars are S.D. values for replicates.
Korthout et al. (36), as it was relatively insensitive to salt concentration and had a different pH optimum.

Although we observed a correlation between the activation of DEVDase and DNA fragmentation, the process involved may be different from that described during animal apoptosis. No caspase ortholog has yet been reported in plants, although it has been suggested that the distant metacaspase family contains functional homologs (24). However, purification experiments or expression in heterologous systems is required to tell us whether metacaspases possess caspase-like enzymatic activities. Caspase-1, which cleaves YVAD, is not involved in animal apoptosis, but in inflammation pathways instead (43). Therefore, inhibition of plant cell death by YVAD-CHO represents a difference between animal and plant cell death. In addition, we have measured a YVADase activity at various time points after irradiation, but no clear induction was detected, although the inhibitor data suggest that YVADase is required for the completion of cell death. We can speculate that a constitutive YVADase activity is necessary to prime the cell for cell death, but is not sufficient to induce cell death. For example, it is possible that YVADase activity is required to preactivate the DEVDase by cleavage. An alternative explanation is that the YVADase activity is repressed in vivo before PCD, but can be measured in cell extracts because its inhibition is relieved artifactually upon tissue homogenization.

In the absence of class-specific protease inhibitors (PMSF (serine proteases) and EDTA (metalloproteases)) and after UVC overexposure, there was a high background activity. In contrast, in the presence of these inhibitors, the background activity decreased, and the DEVDase was clearly up-regulated. This increase cannot be a result of an artifactual increase in the DEVDase activity due to the presence of inhibitors because we compared the activity before and after UVC treatment in the same extraction buffer. The most likely explanation is that, in the absence of class-specific inhibitors, several protease families may contribute non-specifically to the DEVDase activity. In the presence of inhibitors, this non-specific background is reduced.

The DEVDase activity detected could have been due to a non-specific cleavage of caspase substrates by other cysteine proteases such as papain and legumain. Cysteine proteases of the papain family are associated with PCD induced by H₂O₂ in soybean cells (44) and are also induced in tracheary element differentiation in Zinnia elegans (45). Legumains, another family of cysteine proteases, are expressed in senescent tissue of A. thaliana (46). In addition, we have shown that legumain can cleave the caspase substrate Ac-YVAD-AMC (33). In our experimental system, we have evidence that the DEVDase activity detected was not due to non-specific substrate cleavage by legumain or papain because the latter activities were down-regulated after UV treatment, and, in contrast, the DEVDase activity was up-regulated. Moreover, E-64 inhibited the papain activity, but not the DEVDase activity detected. Finally, leupeptin, pepstatin, and E-64 do not inhibit animal caspases and did not inhibit the DEVDase activity in our assay, which therefore behaves biochemically as an animal caspase. This suggests that DEVDase may be a true caspase-like protease, possibly a metacaspase. The proteases responsible for caspase-like activities in plants remain to be identified to establish their exact specificity and to establish whether plant PCD relies on a network of specific proteases. The results presented here form a sound basis for future purification schemes.

A transient assay using protoplasts has several advantages over using stable transformants to investigate the function of genes involved in PCD. (i) It circumvents the difficulties inherent to the study of lethal genes. (ii) It allows the easy quantification of a subtle induction or suppression effect that may be missed when scoring transgenic plants for altered cell death. In this context, we tested the suppression potential of the DAD1/2 genes. Our results point to a role of the DAD genes in suppressing PCD in plants. One conceptual difficulty for the role of DAD1 in PCD is its localization at the ER and its part in the N-glycosylation complex. In contrast, this protein was suggested to have a suppressor activity in PCD by studies in Caenorhabditis elegans, which its overexpression protects some of the cells destined to die by apoptosis during development (47). In addition, knockout mutants in mice show a reduced level of apoptosis in cultured embryos (48) or an altered interdigital cell death in heterozygotes (49), suggesting a role for DAD1 in developmental PCD. All these results favor a direct or indirect anti-apoptotic role for this gene in animal apoptosis. Moreover, a physical interaction with a member of the Bcl-2 protein family was reported (50), which may be correlated with DAD1 capacity to suppress apoptosis.

We found that At-DAD1 is localized in the ER and that its overexpression can rescue protoplasts from PCD independent of glycosylation. This suggests that the DAD proteins might be bifunctional proteins involved with the oligosaccharyltransferase complex and with PCD. It should be noted that the ER has been proposed to be a new gateway to PCD in animal cells, with the implication of ER calcium (51) and of a specific caspase (caspase-12) localized and activated in the ER (52). Interestingly, At-BBI is localized in the ER also and is able to suppress cell death induced in plants by BAX overexpression (53) or induced by a pathogen (22).

It remains to be shown whether PCD suppression by DAD1 is direct or indirect. An indirect involvement could be via an attenuation of an induced ER stress. A direct involvement could be via a physical association with a PCD regulator. In consequence, this work provides new exciting possibilities to investigate the molecular regulation of PCD in plants.

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