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Translocation of cytochrome *c* from the mitochondria to the cytosol occurs during heat-induced programmed cell death in cucumber plants

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Abstract In mammals mitochondria play a critical role in the activation of programmed cell death (PCD). One mechanism by which mitochondria can commit a cell to death is by translocating cytochrome *c* into the cytosol where it activates cell death caspases. However, release of cytochrome *c* does not appear to be a feature of caspase activation in nematodes or insects, similarly, there is no evidence for cytochrome *c* release during the caspase-independent PCD that can occur in *Dictyostelium* cells. In an attempt to understand the underlying regulation of PCD in plants we investigated if mitochondrial components were released into the cytosol when plant cells are induced to undergo PCD. PCD was triggered in cucumber cotyledons by subjecting them to a short 55°C heat treatment. This heat treatment has previously been shown to trigger PCD in other plant species and cell death was confirmed in cucumber using morphological (cellular condensation) and molecular (DNA 'laddering') markers of PCD. We present evidence that, unlike *Dictyostelium* and invertebrate PCDs, cytochrome *c* release is an early event in plant PCD. The mitochondrial release of cytochrome *c* following a PCD-inducing stimulus in both plants and mammals suggests the pathways have been conserved during evolution, having been derived from ancestral unicellular death programmes.

Key words: Programmed cell death; Cytochrome *c*; Mitochondrion; Cucumber

1. Introduction

Cell death occurs throughout the life cycle of higher plants, and developmental cell death has long been recognised as 'programmed' in a spatial and temporal sense. However, the biochemical and genetic programmes that regulate plant programmed cell death (PCD) are only now beginning to be revealed. It is possible to separate cell death programmes into three 'phases', *induction* – where the cell perceives the death-inducing stimuli, *effector* – where the signal activates the 'central executioner' and *cellular degradation* – where the cell is dismantled, often by caspases (a conserved family of cysteine proteases) [1]. Superficially, dying plant cells share many features with apoptotic animal cells, for example, the induction phase can be triggered by a range of diverse stimuli, such as abiotic stress [2], withdrawal of signalling molecules [3] or in response to pathogens [4]. Equally, the resulting degradation of the plant cell often resembles that of animal apoptosis with the activation of plant caspase-like molecules [4], cellular and nuclear condensation [3] and internucleosomal fragmentation of nuclear DNA [5]. However, despite the similarities of the induction and degradation phases, it is not known whether plant and animal cells share features of the execution phase of cell death. In elucidating the biochemical and genetic regulation of the plant cell death programme it would be useful to know the extent of homology the execution phase may share with the more extensively studied animal PCDs.

It is unclear if PCD evolved before the divergence of plants and animals and if the mechanism is conserved between the two kingdoms. If the programmes in both kingdoms were derived from a ancestral unicellular death process, then it is likely that the effector phase would utilise similar 'execution' molecules. In mammals mitochondria play a pivotal role in the commitment of a cell to death. Cell death inducing stimuli can result in the translocation of cytochrome *c* from the mitochondria to the cytosol. In the cytosol cytochrome *c* binds to the protein Apaf-1 and in turn this complex binds to, and activates, caspase-9, forming the 'apoptosome'. The apoptosome initiates a cascade of activated caspases which subsequently bring about the apoptotic destruction of the cell [6]. There are two main theories as to how cytochrome *c* release is achieved by the mitochondria. One possibility is a 'megachannel' opens allowing water and solutes to enter the mitochondria resulting in the rupture of the outer membrane and consequently cytochrome *c* leakage. Alternatively, there is evidence that cytochrome *c* may pass through the voltage-dependent anion channel (VDAC) which is located in the outer mitochondrial membrane. In mammals, the Bcl-2 family of proteins which positively and negatively regulate cell death have been shown to interact with the VDAC. Bcl-x_L, an anti-apoptotic member of the family, closes the VDAC by binding to it directly, while Bax and Bak, pro-apoptotic members, facilitate opening of the VDAC allowing cytochrome *c* to pass into the cytosol [7].

While plant caspase-like molecules have been shown to be present in tobacco cells it is not clear how they are activated [4]. Using a cell-free system, Zhao et al. [8] have shown that animal cytochrome *c* can activate caspase-like molecules in carrot cytoplasm which can then degrade rat liver nuclei in an apoptotic fashion. The activation of plant caspase-like molecules by animal cytochrome *c* suggests that the mitochondria are also important in regulating PCD in plant cells. To further elucidate the mechanism of action of PCD in plants we have investigated if the cellular perception of a cell death signal, and activation of the cell death programme, results in

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Abbreviations: PCD, programmed cell death; VDAC, voltage-dependent anion channel

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the release of cytochrome *c* from the mitochondria into the cytosol.

2. Materials and methods

2.1. Plant material

Cucumber seed (*Cucumis sativus* L. cv. Marketmore) was supplied by W. McNair, Portobello, Edinburgh, and grown in the dark as previously described [9]. Six-day-old cotyledons (55) were excised and incubated in a beaker with distilled water for 10 min at 25°C for controls, or 55°C to induce PCD [3]. Following heat treatment the cotyledons were incubated in a water bath at 25°C in the dark. Five cotyledons from the PCD-induced and control treatments were selected at successive time points over the following 12 h, frozen in liquid nitrogen and stored at −80°C until processing for DNA analysis. The remaining 50 cotyledons in each sample were used for subfractionation of mitochondria and cytosol.

2.2. DNA extraction and resolution on gels

At successive time points over a 12-h period following PCD induction, five cotyledons were ground to a fine powder in liquid nitrogen and the DNA was extracted as previously described [14]. DNA samples were digested with DNase-free RNase for 1 h at 37°C and the DNA content was estimated. To probe for oligonucleosomal fragments 500 ng of DNA was separated by electrophoresis on a 1.5% (w/v) agarose gel. The DNA was transferred to a nylon filter and hybridised to a radioactive probe prepared from *Sau*3AI-digested cucumber total genomic DNA. DNA probes were prepared using a random-primed DNA labeling method [15].

2.3. Isolation of proteins

PCD-induced or control cotyledons were homogenised in a pestle and mortar in 0.4 M mannitol, 25 mM MOPS pH 7.8, 1 mM EGTA, 8 mM cysteine and 0.1% (w/v) bovine serum albumin (BSA). Cell debris was pelleted by a quick centrifugation step where the rotor was stopped as soon as it reached $6000\times g$. The supernatant was removed and re-centrifuged at $12\,000\times g$ for 15 min to pellet mitochondria and the supernatant (cytosol) was stored for further analysis. The crude mitochondrial pellet was resuspended in 0.4 M mannitol, 10 mM Tricine pH 7.2, 1 mM EGTA (RB) and washed twice by recentrifugation prior to analysis in the oxygen electrode and quantification of cytochrome *c* by Western blot analysis (Fig. 3). The mitochondrial pellet was further purified on a 27 and 13.5% (v/v) Percoll (Pharmacia) step gradient in RB for Western blot analysis (Fig. 2). The buff-coloured fraction at the interface (purified mitochondria) was collected and washed by differential centrifugation in RB. Protein concentrations were determined using Coomassie Plus reagent (Pierce). The mitochondrial pellets were frozen and stored at −80°C prior to Western blot analysis.

2.4. Western blot analysis

Protein (50 μg) was separated on 15% SDS-PAGE (w/v) [10]. The proteins were transferred to nitrocellulose (Optitran BA-S 83, 0.2 μm, Schleicher and Schuell) and labeled with antibodies against cytochrome *c* (7H8.2C12, Pharmingen), as described by Bossy-Wetzel et al. [11]. The membrane was stripped, blocked in phosphate-buffered saline (PBS), 0.1% (w/v) Tween 20 (PBS-T)+5% (w/v) dried skimmed milk and reprobed with a polyclonal antibody against fumarase [12], 1:1000 in block buffer at 4°C overnight. After washing (3×5 min) in block buffer, the membrane was incubated in goat anti-rabbit HRP conjugate (Amersham), 1:10 000 in PBS-T for 1 h at room temperature and washed again. Labeling was detected by chemiluminescence (NEN), according to the supplier's manual. The signal was quantified by scanning densitometry of exposed film with the exposure time adjusted to non-saturating conditions.

2.5. Oxygen electrode assays

The integrity of the outer mitochondrial membrane, cytochrome *c* oxidase activity and respiratory activity (rate of O_2 consumption) were measured with a Clark-type oxygen electrode (Hansatech Ltd, UK). Mitochondria (50–100 μg) were assayed in 1 ml of 0.3 M mannitol, 10 mM TES–KOH (pH 7.5), 3 mM $MgSO_4$, 10 mM NaCl, 5 mM KH_2PO_4, 0.1% (w/v) BSA. Outer mitochondrial membrane integrity was measured as the ratio of O_2 consumption in the presence of cytochrome *c* (50 μg/ml) and ascorbate (5 mM) with or without the addition of 0.05% (w/v) Triton X-100 [13]. Maximal cytochrome *c* oxidase activity was considered to be the rate of O_2 consumption in the presence of Triton X-100 in the outer membrane integrity assay. In a separate assay, succinate (10 mM) and ADP (0.1 mM) were added to the mitochondria to measure the rate of O_2 consumption in state 3. All values were expressed as a percentage of the values obtained for non-treated control mitochondria.

3. Results

Plant cells subjected to 55°C for 10 min can activate a cell death programme that results in a characteristic series of morphological and biochemical changes [3]. We have used this induced PCD system on 6-day-old, dark-grown cucumber cotyledons to establish if cytochrome *c* release from the mitochondria occurs over the course of the cell death programme. Cotyledon cells die within 24 h of this heat treatment, and the dying and dead cells were seen to have condensed away from the cell wall, a morphological feature that is characteristic of heat-induced PCD (data not shown). Confirmation of the activation of PCD was established by assaying for the activation of cell death-specific endonucleases that cleave the DNA into oligonucleosomal units. Clear evidence of DNA laddering was detected within 12 h of the PCD-inducing heat treatment (Fig. 1).

PCD-induced and control cotyledons were homogenised

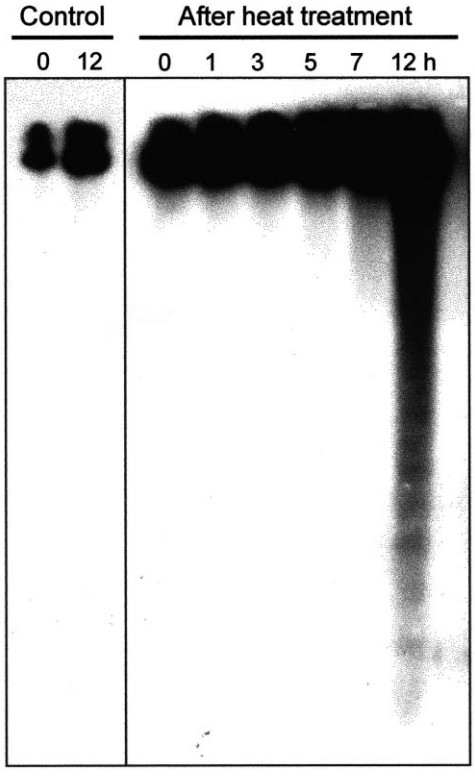

Fig. 1. Southern blot analysis of genomic cucumber DNA showing internucleosomal cleavage of DNA following heat treatment. Cucumber cotyledons were exposed to 55°C for 10 min and then incubated at 25°C. The cotyledons were frozen in liquid nitrogen at various time points over 12 h after heat treatment prior to extraction of genomic DNA. 500 ng of genomic DNA was separated on a 1.5% (w/v) agarose gel. After transfer to a nylon filter, the blot was hybridised with a cucumber genomic DNA probe obtained by radioactive, random-prime labeling with *Sau*3AI-digested DNA fragments and exposed to X-ray film.

and the mitochondria separated from the cytosol by centrifugation. The proteins in samples of each fraction were analysed by SDS-PAGE, followed by Western blot analysis with a monoclonal antibody raised against rat cytochrome *c*. The epitope recognised by this antibody has been mapped [16] and this region is identical in cucumber and rat cytochrome *c* amino acid sequences. The Western blot revealed that the mitochondria began to release cytochrome *c* to the cytosol during the 10-min heat treatment (Fig. 2), and that cytochrome *c* could no longer be detected in mitochondria after 3 h (Fig. 2). The Western blot analysis of the cytosolic samples demonstrated that cytochrome *c* was detectable in the cytosol immediately after the heat treatment and increased in the hour following heat treatment. To test the possibility that the heat treatment disrupts the mitochondria, we probed the membrane with an antibody against fumarase, a 50-kDa mitochondrial matrix protein. This protein remained associated with the mitochondria and was only detectable at a low level in the cytosol 3 h after heat treatment (Fig. 2).

To determine if the release of cytochrome *c* was due to the general disruption of the outer mitochondrial membrane, we assayed the outer membrane integrity using an assay based on the latency of cytochrome *c* oxidase activity [13]. The outer membrane showed a small decrease in integrity following heat treatment (statistically not significant, Student's *t*-test, $\alpha = 0.05$), and was still 75% of the control value after 2 h (Fig. 3). In contrast, the amount of cytochrome *c* in the same mitochondrial samples decreased to 20% of the control value upon heat treatment. Respiratory activity showed a decline to 70% of the control immediately after the 10-min heat treatment, and further decreased to about 30% of the control 30 min after the heat treatment. These results indicate that the decrease in respiratory activity is not a direct result of cytochrome *c* release. This was confirmed when addition of exogenous cytochrome *c* to the mitochondrial samples from the heat-treated cells did not restore oxygen consumption to control rates (data not shown). It is possible that the respiratory complexes are inactivated during the heat treatment, however our measurements show that the activity of cytochrome *c* oxidase itself is relatively unaffected by the heat treatment, provided that cytochrome *c* is added exogenously as a substrate (Fig. 3).

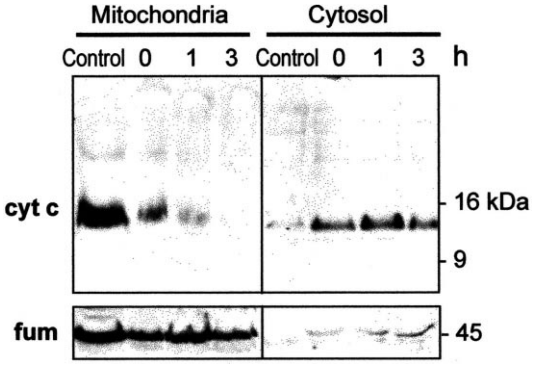

Fig. 2. Immunodetection of mitochondrial proteins in mitochondrial and cytosolic fractions of cucumber cotyledons following induction of PCD. Mitochondria were separated from the cytosol, at the indicated time points following 10 min at 55°C or 25°C (control) and subjected to Western blot analysis with antibodies against cytochrome *c* (cyt c) and fumarase (fum). Cytochrome *c* is shown to be selectively released from the mitochondria.

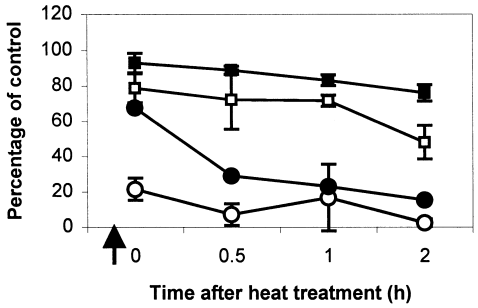

Fig. 3. Outer membrane integrity and respiration in mitochondria after PCD induction relative to non-induced controls. Standard oxygen electrode assays on mitochondria purified by differential centrifugation following 10 min at 55°C (arrow), at the time points as indicated. All values are duplicate measurements obtained from a typical experiment and are expressed as a percentage of the non-treated sample. The cytochrome *c* content was measured by quantitative Western blot analysis. Outer membrane integrity (■), cytochrome *c* oxidase activity (□), respiratory activity (oxygen consumption, ●) and cytochrome *c* (○).

4. Discussion

There are a number of similarities between plant PCD and animal apoptosis, especially during the initial and terminal stages [17]. Similarly, during the execution phase the degradative caspase molecules of PCD appear to be conserved among multicellular phyla such as mammals, nematodes, and *Drosophila*. In tobacco, hypersensitive-response cell death can be blocked by caspase inhibitors [4], however, it is not known if caspase-like molecules are widely involved in non-hypersensitive-response plant cell death programmes.

The effector molecules for plant PCD have not yet been identified. While caspase execution molecules appear to be conserved between phyla the effector molecules which trigger them vary [18]. In mammals mitochondrial proteins are important effectors of caspases and cytochrome *c* plays an important role as an initiator of the death machinery in cases where cellular damage is general (i.e. radiation, heat shock, cytotoxic drugs) [6]. Zhao et al. [9], using a cell-free system, showed that animal cytochrome *c* can activate molecules in carrot cytoplasm which can then degrade rat nuclei in an apoptotic fashion. This apoptotic destruction of nuclei could be blocked with caspase inhibitors suggesting cytochrome *c* was activating caspase-like molecules which were resident in the carrot cytoplasm. This demonstration that animal cytochrome *c* can activate carrot cytoplasmic factors suggests that plants may also use mitochondrial components as effectors of cell death initiation.

To investigate the activation mechanism of plant PCD we induced cell death in cucumber cotyledons to ascertain if mitochondrial components were released during the cell death programme. We induced cell death by exposing cucumber cotyledons to a short heat treatment. It has previously been shown that 55°C for 10 min causes carrot cells to undergo PCD with cells undergoing characteristic morphological changes, such as protoplast and nuclear condensation and associated DNA degradation in a time-dependent fashion [3]. A 55°C heat treatment also induces PCD in cucumber cotyledons with cells undergoing the same morphological changes as carrot cells, DNA is also degraded in a time-dependent fashion and Southern blot analysis using a ^{32}P-la-

beled total DNA probe demonstrated that the DNA was degraded into oligonucleosomal fragments ([3]; Delorme, McCabe, Kim and Leaver, unpublished results). We induced PCD by heat treatment in cucumber cotyledons to investigate if there was mitochondrial involvement in the cell death process and specifically to see if cytochrome *c* is released from the mitochondria to the cytosol. A comparison of the partitioning of cytochrome *c* between the mitochondria and the cytosol showed that cytochrome *c* release to the cytosol was an early event in cucumber cell death, occurring well before other PCD hallmark features such as DNA degradation. The retention of fumarase, a matrix protein, in the mitochondria and a minor decrease in outer membrane integrity during the time of maximal cytochrome *c* release suggest that the mitochondria were selectively releasing cytochrome *c* and were not simply damaged by the heat treatment. The assay for outer membrane integrity uses exogenous cytochrome *c* as a substrate. Only a little of this enters the mitochondria in the first hours after the heat treatment, and this is not sufficient to restore the drop in respiratory activity. This suggests that trafficking of cytochrome *c* is almost exclusively from mitochondria to cytosol, possibly through specific protein channels, as has been found in mammalian mitochondria-mediated cell death [7].

By triggering cell death with a known PCD-inducing abiotic stress we have shown that the release of cytochrome *c*, but not fumarase, from the mitochondria into the cytosol, is an early event in plant cell death. Cytochrome *c* release and activation of cell death is either an ancient effector of cell death, which evolved in an unicellular ancestor of multicellular organisms and has subsequently been lost from the programmes of several phyla, or has been a more recent evolutionary feature which arose independently in mammalian and plant cell death programmes. The similarities that are apparent in the induction, effector and degradation phases of plant and animal PCDs suggest a common single cell ancestor is more likely and therefore cytochrome *c* release is an ancient feature of PCD which has been secondarily lost from the regulation of PCD in many organisms. The full extent of the possible conservation of plant and animal cell death programmes remains to be established, but it may be fruitful to search for plant homologues of genes whose products are involved in the animal cell death process. Homologues of the mammalian family of Bcl-2 genes which regulate the release of cytochrome *c* from the mitochondria into the cytosol have not been found in plants, however, overexpression of a gene encoding Bcl-x_L inhibits PCD in tobacco plants [19]. If cytochrome *c* release does indeed trigger a plant caspase-like molecule then it is reasonable to assume there may exist in plants a functional homologue of molecules such as Apaf-1. Using BLAST searches van der Biezen and Jones [20] have already identified several plant resistance gene products which are involved in pathogen defence, that share sequence homology with Apaf-1. Whatever the full extent of conservation the fact that cytochrome *c* release is an early result of plant cell death will provide a basis for the further investigation of the mechanism and regulation of plant PCD.

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