Use of cell morphology to evaluate the effect of a peroxidase gene on cell death induction thresholds in tobacco

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Abstract

Tobacco suspension cultures were subjected to a range of heat stresses and used to compare morphological aspects of programmed cell death (PCD) and necrosis. Cells undergoing PCD were found to display characteristic death morphology, caused by cytoplasmic retraction of the protoplast, and to have cleaved DNA. We evaluated if the morphological characteristics of PCD could be used to monitor changes in cell death induction thresholds in transgenic cell cultures with high levels of peroxidase activity. Again, using a heat shock assay, we show that tobacco cell cultures with elevated levels of peroxidase have higher cell death induction threshold levels than wild type tobacco cell cultures. Thus, assessing PCD associated morphological changes can report on the effect of altering peroxidase genes on cell death activation in tobacco. This study demonstrates that PCD morphology could routinely be used to monitor the effects of introduced genes on programmed cell death induction thresholds in plants.
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1. Introduction

Plant cells die in a number of ways. Two such death pathways include PCD and necrosis. Programmed cell death (PCD) is an active, genetically organized, process, which brings about the controlled disassembly of the cell. Necrosis is an uncontrolled type of cell death characterised by the cell losing its ability to osmoregulate leading to the cell swelling, bursting and releasing its cellular contents. Whether a biotic or abiotic stress initiates necrosis or PCD is generally dependent on the actual stress-dosage: low levels of stress induce cellular protective mechanisms, higher levels trigger PCD and higher levels again lead to necrosis. McCabe et al. [1] demonstrated this dose-dependence by killing carrot cells with increasing temperatures. They showed that plant cells that undergo PCD following moderate heat stresses die in such a way that the resultant corpse displays several hallmark features indicative of the PCD programme. Morphologically, the PCD protoplast condensed, leaving a visible gap between the cell wall and plasma membrane; additionally, nuclear DNA was cleaved, often into nucleosomal fragments [1,2].

PCD is essential for normal plant development and is an important component of a plants pathogen defence arsenal. However, the signalling mechanism that triggers PCD, or indeed the actual mechanism of cellular destruction, is poorly understood in plants. One set of molecules that have been implicated in both signalling and triggering the cell death cascade are reactive oxygen species (ROS). ROS levels in cells are influenced by peroxidases that are widely distributed among living organisms. Peroxidases have a multiplicity of physiological and biochemical roles in plants. Such roles include the cross-linking of molecules in the cell wall, auxin oxidation, lignin production and responses to biotic and abiotic stresses [3–5]. Peroxidase can regulate ROS within a cell by altering levels of hydrogen peroxide (H_2O_2). Peroxidase utilises H_2O_2 in the oxidation of many substrates such as lignin monomers [6–8] but paradoxically, it can also mediate the production of H_2O_2. This dual role of peroxidase means that it can play a part in H_2O_2 regulation. A reaction scheme for the production of H_2O_2 by cell wall peroxidases at the expense of NADPH

Abbreviations: PCD, programmed cell death; ROS, reactive oxygen species

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oxidase has been elucidated by Elstner and Heupel [9]. Plant homologues of two NADPH oxidase components, gp91[phox] and Rac have been cloned [10,11] and Kawasaki et al. [11] have shown that Rac protein might mediate the hypersensitive response (HR) in a ROS-dependent manner. A second proposed source for H_2O_2 involves an apoplastic peroxidase [12,13]. The O_2–heme complex of peroxidase is reduced to compound III and, under alkaline conditions, the complex is effectively catalysed to release H_2O_2.

Cells challenged by hypersensitive response elicitors will generate H_2O_2 which has threshold-specific effects on the plant cells: low doses induce antioxidant enzymes such as glutathione peroxidase preventing cellular damage, higher doses leads to programmed cell death (PCD) and very high levels lead to necrotic cell death [14]. H_2O_2 has also been shown to be a potent inducer of programmed cell death when added directly to plant cell cultures [1,15–17]. Houot et al. [17] have shown that H_2O_2 can induce PCD, in a process similar to animal apoptosis, but only in a dose-dependent manner. Catalase deficient cell lines have been used to show that an increase in H_2O_2 levels induces PCD suggesting that changes in H_2O_2 homeostasis can trigger PCD [18]. These findings demonstrate that H_2O_2 can play a role in cell death signalling. Since peroxidase can regulate the available levels of H_2O_2 within the cell, it may be able to influence the induction thresholds at which cell protection, PCD or necrosis are activated and may therefore be a key enzyme in manipulating these processes in plants.

We wished therefore to investigate the effect that over-expressing peroxidase genes may have on cell death rates in plants. We speculated that over-expressing peroxidase genes would have an effect on PCD rates but were unable to predict if this effect would be to lower or raise the threshold levels at which abiotic stress would trigger PCD. Tobacco has been shown to be an ideal model species to examine and identify the physiological effects of over and under expression of genes [19–21]. In this report, we established that a heat treatment could activate PCD or necrosis in tobacco cell cultures and, as has been shown in *Arabidopsis* and carrot [2], PCD in tobacco results in a condensed cytoplasm and PCD induced cleavage of DNA. We have utilised the cell morphology to report on cell death rates following heat treatment and were therefore able to evaluate, and report on, the effect of an introduced peroxidase gene on cell death rates in tobacco.

2. Materials and methods

2.1. Plant material

Nicotiana tabacum L. cv. Xanthi seeds were surface sterilised in 10% (w/v) hypochlorite and germinated on half strength MS medium [22]. Tobacco plants were maintained in a growth room at 25 °C under a 16-h light/8-h dark photoperiod regime. Tobacco leaf strips were placed on MS medium supplemented with 2 mg/l NAA and 0.25 mg/l kinetin to induce calli formation. Two hundred and fifty millilitre conical flasks with 50 ml of cell culture medium (MS medium supplemented with 100 mg/l, 2,4-D and 100 mg/l kinetin) were inoculated with friable callus to initiate cell suspensions and kept at 25 °C, under continuous light and on a rotary shaker at 110 rpm. Cell suspension cultures were subcultured at 10-day intervals by pipetting 10 ml of cell culture into 45 ml of fresh cell culture medium.

2.2. Induction and measurement of cell death

Seven-day-old cell cultures were centrifuged at 1500 rpm for 5 min to pellet cells. The medium was removed and replaced with fresh medium without growth regulators. The cells were then heat-shocked for 20 min at different temperatures, ranging from 25 to 85 °C, and then returned to 25 °C shaking at 100 rpm for 24 h. An equal volume of cells and fluorescein diacetate (FDA; a 0.1%, w/v, stock diluted 1:50 with culture medium), were placed on a microscope slide. Epifluorescence was observed through a fluorescein isothio-cyanate (FITC) filter. Only single cells and cells in groups of <6 were counted. Cells that did not stain with FDA were scored as dead. Cells whose cytoplasm had condensed and shrunken were scored as having undergone PCD. Cells that were FDA negative but showed no sign of cytoplasm condensation were scored as necrotic.

2.3. DNA extraction

DNA was isolated from the cells using a modified CTAB method. Cells were frozen and ground in liquid nitrogen. The powder was suspended in CTAB buffer (2% CTAB, 100 mM Tris, pH 8, 20 mM EDTA, 1.4 M NaCl, 1% PVP (M_W 40,000)) and incubated at 65 °C for 60 min. The supernatant was RNase A (10 mg/ml) treated at 37 °C for 30 min. An equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed well. The DNA was precipitated with $0.6\times$ isopropanol, washed in 70% ethanol, dried and resuspended in TE buffer. The DNA was then resolved on a 1.5% agarose gel.

2.4. Cloning of the barley peroxidase construct

A two-step cloning strategy was used to insert a barley peroxidase gene into a bar containing Ti-plasmid. A 1.2 kb *Pst* I/*Sph* I fragment of the cDNA clone, pBH6-301 [23], was cloned into the *Pst* I and *Sph* I sites a pPS48 vector derived from pBI121 (Clontech), which provided the enhanced cauliflower mosaic virus CaMV-35S promoter and terminator. A resulting 2.2 kb *Hind*III fragment was then isolated and cloned into the *Hind*III site of the Ti-plasmid CAMBIA 3301 and renamed HvHvPrx83201. The CAMBIA vector contained the Basta selection gene and the β-glucuronidase (GUS) reporter gene under the control of the CaMV-35S promoter. A positive clone was transformed into *Escherichia coli* (DH5α) using kanamycin as a selection pressure and positive clones were identified by PCR using the following primers: TDT15 [AACAGTCGTG-GAAGTGCAGC] and 35S reverse [ACTGACGTAAGGGAT-GACGC]. Clones were also checked by sequencing using a DNA sequencer model ABI 373, Perkin-Elmer.

2.5. Transformation

The construct was transformed into *Agrobacterium tumefaciens* (C58C1) using a freeze–thaw method as described previously [24] and into *N. tabacum* L. cv. Xanthi using a modified form of the leaf disc *Agrobacterium*-mediated transformation procedure [25]. Briefly, 1 cm^2 leaf discs were cut with a cork borer, placed into the bacterial suspension and left for 15–30 min with gently shaking. The discs were dried on sterile filter paper and placed onto co-cultivation medium (MS medium, coated with 150 µl, 10 mM acetosyringone (pH 5.1) and 15 µl, 1 M betaine, 1 day before use) for 2 days. The discs were then transferred to a shoot-inducing medium (MS medium supplemented with 0.1 mg/l NAA, 1 mg/l BA, 1 mg/l thiamine–HCl supplemented with 100 mg/l vancomycin, 100 mg/l timentin, 100 mg/l cefotaxime, to prevent the overgrowth of *Agrobacterium* and 10 mg/l Bialaphos (selective agent)). Regenerated shoots were excised after developing two leaves and placed onto MS medium to induce root formation, again supplemented with 100 mg/l vancomycin, 100 mg/l timentin, 100 mg/l cefotaxime and 10 mg/l Bialaphos. The reporter gene *uid*A encoding the enzyme β-glucuronidase [26] is used as a histochemical marker to identify putative transformants. A small amount of leaf tissue was immersed in the incubation solution (0.1 M Na$_2$PO$_4$, 10 mM EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 1.0 mM X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide), 0.1% Triton X-100) and left overnight at 37 °C [27]. The following day the tissue was incubated in ethanol to remove chlorophyll and to allow for the observation of the blue spots caused by the expression of β-glucuronidase. Rooted plants were screened for the presence of the transformed gene by PCR. Briefly, a master mix consisted of 1× PCR buffer, MgCl$_2$ to a final concentration of 0.05 mM, 300 µm HvPrx8 forward, 300 µm HvPrx8 reverse, dNTP mix to a final concentration of 200 µm and 1.5 U of *Taq* DNA polymerase. The PCR conditions were as follows: 96 °C for 1 min, followed by 30 cycles of 96 °C for 20 s, 55 °C for 20 s, 72 °C for 2 min and then finishing with 10 min at 72 °C. Tobacco plants that proved PCR positive were analysed for peroxidase activity. Once transformed plants were established, they were gradually weaned off the antibiotics by reducing the concentrations at periods of subculturing.

2.6. Protein extraction and peroxidase activity assay

Leaf material (100 mg/ml) was homogenized in 50 mM sodium phosphate buffer, pH 6.2 and centrifuged at 13,000 rpm for 10 min. The supernatant was used as total soluble protein. Total protein was quantified according to the standard Bio-Rad method, with BSA as the standard protein [28]. Activity of all samples was determined using the peroxidase substrate guaiacol. The reaction mixture consisted of 0.28% guaiacol and 0.30% H$_2$O$_2$ in 0.05 M Na$_2$HPO$_4$, pH 6.2. The sample extract (1–20 µl) was added to 1 ml of reaction mixture and the increase in absorbance was followed for 3 min at 30 s intervals at 420 nm. The rate of this reaction was used to express peroxidase activity.

3. Results

3.1. Heat shock induces programmed cell death morphology in N. tabacum cell cultures

Tobacco cell suspension cultures were chosen to investigate rates of PCD in wild type and transgenic plants. Cell cultures are amenable to this type of study as they are usually made up of rapidly dividing undifferentiated cells and are therefore relatively uniform. In addition, cells in culture are relatively assessable and a count of large numbers per treatment is straightforward. Fig. 1A demonstrates the morphology of a healthy living cell. Carrot and *Arabidopsis* cells, which have died by a 55 °C heat-induced PCD, have a very distinct morphology. Tobacco cells subjected to 55 °C for 20 min also display these PCD associated morphological characteristics (Fig. 1B). Following PCD inducing heat treatment, the protoplast condenses and the cell membrane retracts and moves away from the cell wall leaving a visible gap. As in carrot and *Arabidopsis* [1,2] cells, which were FDA negative but did not display the PCD morphology, were deemed to have been overwhelmed by stress and have died necrotically (Fig. 1C). In order to determine the levels of stress which result in either living, PCD or necrotic cells, cells from 7-day-old wild type tobacco cell cultures were harvested and subjected to a range of short heat treatments, as described in ref. [1]. The absence of FDA staining was plotted, as was the percentage of cells having died via PCD (FDA negative + cytoplasmic retraction). Fig. 1D shows that, from 25 to 55 °C, there is a steady increase in total cell death (FDA negative) in the culture. Practically, all these deaths are accompanied by the PCD morphology of retraction of the cytoplasm away from the cell wall. However, from 55 to 85 °C, the number of cells that die via PCD drops dramatically while conversely the number of cells dying via necrosis rises.

3.2. Heat shock triggers a PCD-induced cleavage in N. tabacum cell cultures

Confirmation of the activation of PCD was established by assaying for DNA cleavage. Cells from 7-day-old wild type tobacco cell cultures were heat-shocked at 55 °C for 20 min, returned to 25 °C and checked at regular intervals for PCD morphology. Even though PCD morphology was evident from 4 h after the treatment, we extracted total genomic DNA after 24 h as McCabe and Leaver [2] showed that initial PCD-induced cleavage generally begins 5–7 h after heat treatments with cleavage only becoming detectable after 12–24 h. Total genomic DNA extracted from cells subjected to 55 °C for 20 min was separated by electrophoresis on a 1.5% agarose gel along with genomic DNA from untreated cells. Evidence of DNA cleavage was detected 24 h after the PCD inducing heat treatment (Fig. 2).

3.3. PCR analysis and peroxidase activity of putative transformants

The transgenic nature of the resulting plant lines was established by β-glucuronidase (GUS) assay, PCR analysis and
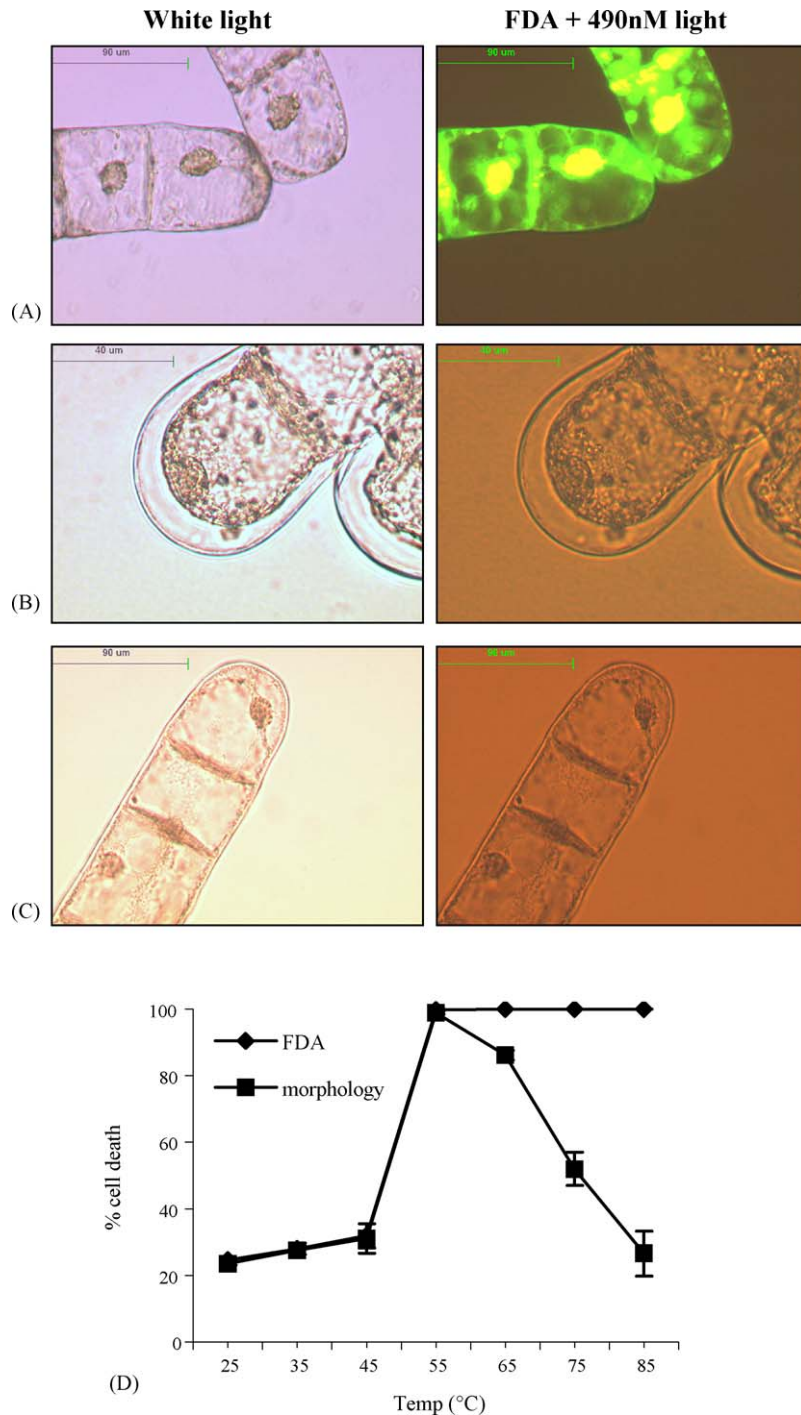
Fig. 1. Identification of live cells, necrotic cells and cells that have undergone PCD using FDA and cell morphology. (A) Cells that are alive have the ability to cleave FDA and fluoresce under light at a wavelength of 490 nM. (B) In cells that undergo PCD, the protoplast retracts from the cell wall. (C) Necrotic cells cannot cleave FDA so do not fluoresce and show no evidence of protoplast condensation. (D) Wild type cells were subjected to different heat treatments for 20 min and returned to 25 °C for 24 h. Cells were then scored under the light microscope for PCD morphology. The percentage of PCD cells was determined. The cells were also stained with FDA and examined under UV light to determine whether living or dead.

peroxidase activity assays. The first test carried out on these putative transformants was the GUS assay. A blue colour was observed when this reporter gene was expressed. The GUS assay is extremely useful as it is sensitive enough to detect expression within a single cell type and only a small amount of tissue is required. When a shoot began to develop on rooting medium a small amount of leaf tissue was removed and incubated in substrate solution. All rooting shoots were tested for GUS expression and all shoots that tested positive were maintained on rooting medium for further analysis. The presence of the gene was also confirmed by PCR. The primers, HvPrx8 forward [TGTTCAACAACGACACCACC] and
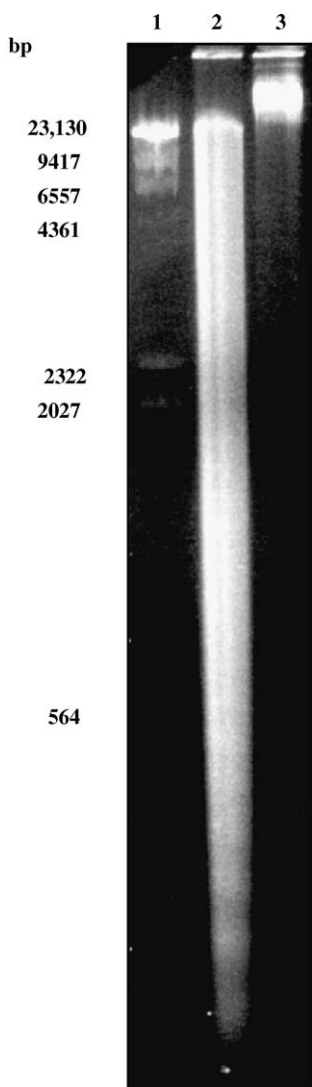
Fig. 2. Detection of DNA fragmentation in wild type tobacco cells induced to die by heat shock. Cells were subjected to 55 °C heat treatments for 20 min and 24 h later DNA was extracted and run on a 1.5% agarose gel. Lane 1 contains the λ-HindIII marker, lane 2 shows DNA from heat-treated cells and lane 3 shows DNA from cells not subjected to heat treatment.

HvPrx8 reverse [CATTCACGTGTCGTGCTAGC] amplified a 277 bp product which corresponded to the expected size of the introduced peroxidase gene (data not shown). Total soluble protein was isolated from leaf tissue and measured as described in Section 2. Peroxidase activity was determined using the peroxidase substrate guaiacol. Fig. 3A shows the peroxidase activity calculated for four representative tobacco plants transformed with the HvPrx83301 plasmid. Line HvPrx8.31 had the highest peroxidase activity.

3.4. Induction of cell death in tobacco cell cultures expressing a defence-related barley peroxidase

We used the heat shock assay to evaluate possible PCD activation differences in the sensitivity of wild type tobacco, and tobacco expressing barley peroxidase, following abiotic stress.
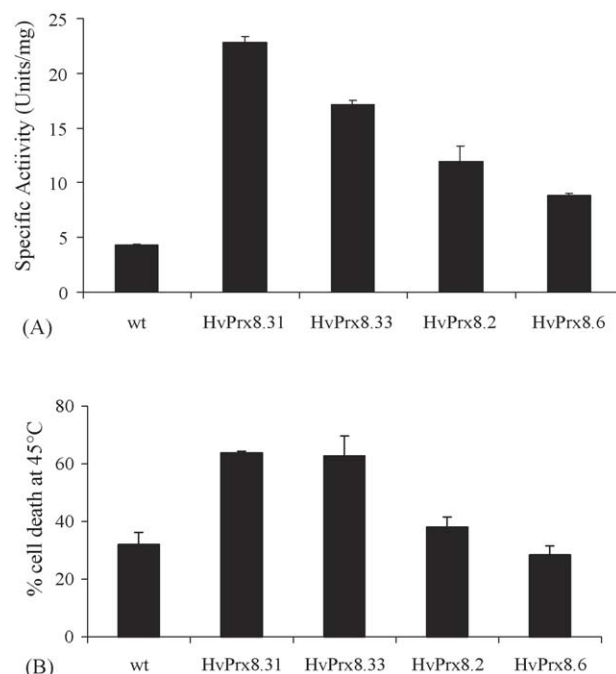

Fig. 3. Peroxidase activity and cell death rates in tobacco plants transformed with the HvPrx83201 plasmid. (A) Guaiacol peroxidase activity was determined in leaf material of primary transformants expressing the barley peroxidase gene, HvPrx8, under the control of the 35S-CaMV promoter. The results are the mean of three experiments. (B) Cells were subjected to a 45 °C heat shock treatment for 20 min and then returned to 25 °C for 24 h. Cells were then scored under the light microscope for PCD morphology. The percentage of PCD cells was determined. The cells were also stained with FDA and examined under UV light to determine whether living or dead.

Four independent transformants were first subjected to a 45 °C heat shock treatment. This temperature was chosen as it is on the boundary of the transition from cell staying alive to cells activating PCD (Fig. 1D). Any changes in PCD induction thresholds should be evident at this temperature. The percentage of cell death in four cell lines is shown in Fig. 3B. The cell line HvPrx8.6, which marginally over-expresses peroxidase shows no, or little, change in cell death rates at 45 °C. Cell line HvPrx8.2 has higher peroxidase levels and shows a small increase in cell death rates, while cell lines HvPrx8.31 and HvPrx8.33, with higher peroxidase activity again, have significant increases in cell death induction (Fig. 3A and B).

Using the HvPrx8.31 cell line, we subjected cells to a range of temperatures to investigate PCD activation at several temperatures including necrotically inducing levels of stress. Fig. 4A shows that the overall pattern of increased induction of PCD up to 55 °C and increased necrosis from 55 to 85 °C is very similar to the wild type response. Fig. 4B was created by combining the PCD morphology scores for wild type (Fig. 1D) and peroxidase over-expressing tobacco (Fig. 4A). In relation to the activation of PCD, there are two apparent trends that can be seen in Fig. 4B. Firstly, at temperatures below 55 °C, PCD is consistently higher in the peroxidase transgenic line. In fact, at 45 °C, the PCD rate is almost two-fold higher in the peroxidase transgenic than it is in the wild type cells. The second trend can be seen in temperatures of 55 °C and above where, this time, the
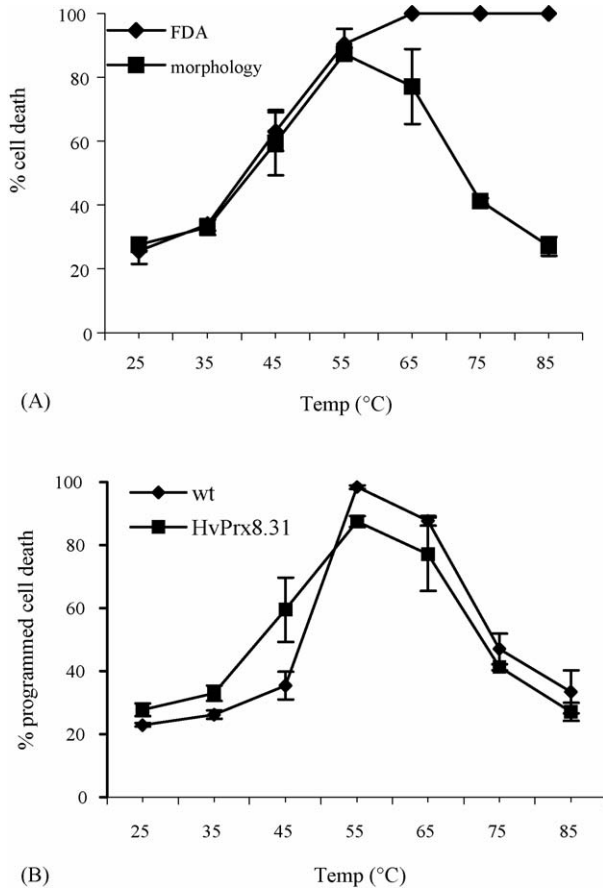
Fig. 4. Induction of programmed cell death in cell cultures expressing barley peroxidase, HvPrx8. (A) Cells, which were transformed with barley peroxidase, HvPrx8, were treated in the same way as wild type cells and scored as outlined above. Data shown are representative of three independent replicate experiments. Error bars represent standard error of the mean that is derived from numerous independent fields of at least 100 cells. (B) Morphology data from Figs. 1D and 4A were combined to compare PCD activation in wild type and transgenic tobacco expressing barley peroxidase.

peroxidase transgenic cells consistently undergo less PCD than the wild type cells.

4. Discussion

w?>McCabe et al. [1,2] have developed an inducible cell death assay with which they can distinguish living cells, those undergoing PCD and necrotic cells. This assay involves subjecting suspension culture cells to a range of temperatures over short periods. They could then analyse aspects of cell death using FDA staining, PCD-specific morphology and DNA cleavage. The ability to define exactly how many cells have died and to separate PCD from necrotic cell death allows one to score for subtle changes in cell death rates. This assay has been shown to work equally well with carrot and *Arabidopsis* cells [2].

In this paper, we have extended the assay to tobacco. We confirm that, as found in *Arabidopsis* and carrot [2] death induced by heat stress occurs via necrosis or PCD. At temperatures up to 55 °C, when cells die they activate PCD, however, at temperatures above 55 °C necrotic cell death predominates.

This biphasic pattern of cell death has been reported for other plant species [1,2] and human cell lines [29]. The pattern can also be repeated with a range of different noxious agents including H_2O_2 [30,15,1]. In the current study, PCD in tobacco cell cultures was accompanied by the characteristic heat-induced PCD morphology of cytoplasmic retraction, with practically all cells undergoing this form of PCD at 55 °C. PCD was confirmed by demonstrating that the dead cells at 55 °C were FDA negative and that their DNA had been cleaved.

As can be seen from our results, 55 °C is a pivotal temperature in the heat shock assay, the point at which necrosis replaces PCD as the predominant cellular death mechanism. At temperatures of up to 55 °C, cells are damaged by heat stress and will either, repair the damage and survive, or activate their programmed cell death mechanisms. Above 55 °C, the cellular damage is so severe that staying alive is no longer an option for cells. In this temperature range, cells will die and the challenge for the cell now is to activate a controlled programmed cell death and to avoid uncontrolled necrotic cell death.

The life or death choices a cell ultimately makes may be determined be a wide range of physiological considerations. Certainly, one such important consideration in determining whether a cell survives or mounts a PCD versus necrotic response may be determined by the redox state of the cell. Herein, we wished to see if we could utilise the heat stress assay to investigate the effect of over-expressing genes that may be involved in redox-associated signalling, or induction of cell death pathways, in tobacco. We chose to introduce a barley peroxidase gene into tobacco. There is documented evidence to suggest that peroxidase can generate H_2O_2 during the oxidative burst [12]. H_2O_2 is relatively stable and inert but it can produce the hydroxyl radical ($OH^•$), which is highly reactive and can initiate radical cascade reactions that lead to the rapid oxidation of lipid, proteins and nucleic acids. The HvPrx83201 plasmid was constructed incorporating a peroxidase gene, which was found to accumulate in response to the powdery mildew fungus attack [23]. This is possibly due to the induction of the hypersensitive response, which culminates in cell death following the rapid production of ROS. Since barley peroxidase in tobacco has been located to the apoplast, there is a strong conviction that this peroxidase could be a source of ROS [27].

Introduction of the peroxidase genes can lead to an increase in PCD in cells heat-shocked at 45 °C. It was seen that the transgenics with the highest peroxidase activity have the largest increase in cell death rates. In wild type cells, a 45 °C heat stress is on the border of the PCD induction threshold, cells while obviously stressed can cope with this stress and survive, certainly for at least 24 h. However, cell lines over-expressing high levels of peroxidase are much more likely to activate PCD following a 45 °C stress.

The balance of ROS within a cell has been shown to be critical to cell death decisions. For example, Levine et al. [15] demonstrated that H_2O_2 at low levels induce the protective gene glutathione S-transferase, at higher levels induces PCD and at higher levels again causes necrosis. The necrosis caused by extremely high levels of H_2O_2 is probably due to overwhelming lipid peroxidation and protein degradation. At PCD-inducing

levels, ROS will induce oxidative stress which is known to trigger the permeability transition (PT) pore opening in mitochondria leading to the release of cytochrome *c* and other PCD inducing mitochondrial factors [30,31]. Indeed, it has already been shown that a short 55 °C heat treatment of cucumber cotyledons results in cytochrome *c* being released from the mitochondria into the cytosol [32]. Additionally, mitochondrial release of nuclease activity has been shown to results in the degradation of nuclear DNA following PCD inducing stress [33]. Over-expression of a peroxidase gene would have a significant effect on the generation of ROS following stress treatments. Changing the balance of ROS within the cell would change the point at which cells activated PCD following heat shock. This could explain why we found higher PCD levels in peroxidase transgenics induced by heat stress at temperatures up to 55 °C. Above 55 °C, necrosis predominates in both wild type and transgenics. In this situation, as ROS levels may again be higher in the peroxidase transgenics than in wild type, this could lower the threshold for transition between PCD and necrosis in these cells. Therefore, increased levels of ROS and their dose-dependent effects on PCD versus necrotic cell death decisions can explain the apparently contradictory result of higher PCD levels in peroxidase transgenics induced by temperatures below 55 °C, but lower PCD levels at temperatures above 55 °C. Peroxidase, as with the majority of genes, will not act as a simple on/off switch for PCD. However, regulating the cellular levels of ROS will undoubtedly influence the threshold at which the cell mounts pro-survival versus PCD or necrotic responses. Since H_2O_2 is widely regarded as a cytotoxic agent, levels must be critically regulated by the action of antioxidant defence enzymes, which include peroxidases.

In the present study, we have shown the utility of a heat shock-based assay to monitor changes in PCD levels influenced by genes which affect cellular stress perception. The use of this assay to characterise and distinguish between PCD and necrotic modes of death provides an important tool to dissect the influence of introduced genes on cellular survival, in response to diverse stresses that have biological and commercial relevance.

Acknowledgement

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