Available online at www.sciencedirect.com
ScienceDirect
Plant Science 172 (2007) 853–860
PLANT SCIENCE
www.elsevier.com/locate/plantsci

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

Emma Burbridge [a], Mark Diamond [b], Philip J. Dix [a], Paul F. McCabe [b,*]

[a] *Institute of Bioengineering and Agroecology, Department of Biology, National University of Ireland Maynooth, Maynooth, Co. Kildare, Ireland*
[b] *School of Biology and Environmental Science, University College Dublin, Dublin 4, Ireland*

Received 2 March 2006; accepted 6 March 2006
Available online 28 December 2006

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.

Keywords: Programmed cell death; Heat-shock; Peroxidase

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

DOIs of original articles: 10.1016/j.plantsci.2006.03.004., 10.1016/j.plantsci.2006.03.023.

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

* Corresponding author. Tel.: +353 1 716 2251; fax: +353 1 716 1153.
E-mail address: paul.mccabe@ucd.ie (P.F. McCabe).

0168-9452/$ – see front matter
doi:10.1016/j.plantsci.2006.03.024

H_2O_2 by cell wall peroxidases at the expense of NADPH 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 homeostatis 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 isothiocyanate (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× 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 [AACAGTCGTGGAAGTGCAGC] and 35S reverse [ACTGACGTAAGGGAT-