Programmed Cell Death in *Arabidopsis thaliana*

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Abstract

Programmed Cell Death in *Arabidopsis thaliana*

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Programmed Cell Death (PCD) describes an orderly cellular breakdown that occurs in both plants and animals throughout development and in response to biotic and abiotic stresses. The molecular machinery that functions in the induction and execution of animal PCD has been characterised in great detail. Conversely, few genes and proteins involved in plant PCD have been identified. While certain features of animal PCD may be conserved, the induction and execution of plant PCD is also likely to involve novel proteins and mechanisms.

The aim of the work presented in this thesis was to investigate experimental approaches for studying plant PCD and to gain an understanding of the molecular mechanisms involved. To this end, an *Arabidopsis thaliana* cell suspension system was developed in which PCD could be induced by both a heat treatment (55°C, 10 min) and senescence (13 to 14 days-old). This system allowed for the molecular responses related to programmed cell death to be distinguished from those that were a specific response to the inducing stimulus.

The *Arabidopsis* cell suspension system was utilised for an analysis of transcriptomic and proteomic changes that occur following the induction of PCD. A custom cDNA microarray analysis of ~100 putative cell death-related genes was used to measure the abundance of transcripts of these genes during PCD, and this work was extended to a whole-genome transcriptomic analysis of PCD. A number of candidate genes that may play a role in plant PCD were identified. These included those encoding antioxidant enzymes, cytosolic heat shock proteins, the mitochondrial adenine nucleotide translocase, ion transporters, a two-component response regulator (ARR4), several pathogenesis-related proteins, phospholipases and proteases, extracellular glycoproteins and enzymes (including a subtilisin-like protease, chitinases, and glucanases), and transcriptional regulators such as a homeobox leucine zipper and NAC-domain proteins.

The induction and execution of plant PCD is also likely to involve mechanisms that are not transcriptionally regulated. A proteomic analysis of changes in the total cellular protein profile during heat- and senescence-induced PCD was therefore used to identify 12 proteins that are modulated in both systems and may play a PCD-specific role. These included the mitochondrial voltage-dependent anion channel (Athsr2), catalase, mitochondrial superoxide dismutase, an extracellular glycoprotein, and aconitase.

Selected genes and proteins identified in the transcriptomic and proteomic analyses were further investigated in an attempt to define their role in plant PCD. Since PCD is difficult to quantitatively analyse at the whole-plant level, initially a strategy of transient expression of genes of interest in *Arabidopsis* protoplasts was adopted. However, it proved to be technically difficult to accurately quantify the number of dead cells in this system. As an alternative, *Arabidopsis* T-DNA insertional mutants within genes of

interest were investigated for PCD-related phenotypes. Mutants in *Senescence-Related Gene 3*, the mitochondrial voltage-dependent anion channel (*Athsr2*), and cytosolic *Heat shock protein 70-3* were isolated. The mutant lines were not visibly affected in their development, formation of xylem, onset and progression of senescence, or responses to abiotic and biotic stresses. This indicated that these genes are either not involved in the PCD pathway or that their functional role can be fulfilled by other gene products.

Abbreviations

All symbols used for units and their decimal prefixes are in SI units. The chemical formulae used are based on standard symbols for the elements. Both are as described in the Handbook of Chemistry and Physics (67^{th} edition, 1986-1987). Abbreviations that are less common are listed below.

$\Delta\psi$	electrochemical gradient
2-D	2-dimensional
ADP	adenosine disphosphate
AGI	*Arabidopsis* Genome Initiative
AIF	Apoptosis Inducing Factor
ANT	adenine nucleotide translocase
AOX	alternative oxidase
Apaf-1	apoptotic protease-activating factor-1
At3	*Arabidopsis thaliana* cell suspension culture media (see section 2.1.1)
ATP	adenosine triphosphate
Avr	avirulence
Basta	glufosinate ammonium
BLAST	basic local alignment search tool
bp	basepair
BSA	bovine serum albumin
C. elegans	*Caenorhabditis elegans*
CAD	caspase-activated DNase
cDNA	complementary DNA
cfu	colony-forming units
CMS	cytoplasmic male sterility
CsA	cyclosporin A
CyP-D	cyclophilin D
D	dilution
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
DEPC	diethylenepyrocarbonate
ddH_2O	double distilled water
dGTP	2'-deoxyguanosine 5'-triphosphate
DNA	deoxyribonucleic acid

ds	double stranded
DTT	dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
dUTP	2'-doxyuridine 5'-triphosphate
DW	dry weight
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetra-acetic acid
EGTA	ethyleneglycol-bis(β-amino-ethylether) N, N, N', N'-tetraacetic acid
EndoG	Endonuclease G
EST	Expressed Sequence Tag
FDA	fluorescein diacetate
FW	fresh weight
GTP	guanosine triphosphate
GUS	β-glucuronidase
HR	hypersensitive response
Hsp	heat shock protein
IAP	inhibitor of apoptosis
ICAD	inhibitor of caspase-activated DNase
IEF	isoelectric focusing
IMM	inner mitochondrial membrane
IMS	intermembrane space
IPG	immobilised pH gradient
IRE	iron regulatory element
IRP	iron responsive protein
kb	kilobase
kDa	kiloDalton
LB	Luria-Bertani
LSD	lesion simulating disease
M2	mutagenised line, generation 2
M3	mutagenised line, generation 3
MALDI	matrix-assisted laser desorption/ionisation
menadione	2-methyl-1,4-naphthoquinone
MES	4-morpholineethanesulfonic acid monohydrate 2-(N-morpholino)ethanesulfonic acid
MMP	mitochondrial membrane permeability

MnSOD	manganese superoxide dismutase (mitochondrial)
MPT	mitochondrial permeability transition
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MUG	4-methylumbelliferyl-β-D-glucuronide
MW	molecular weight
NADH	nicotinamide adenine dinucleotide
NCBI	National Center for Biotechnology Information
OGDC	oxoglutarate dehydrogenase complex
OMM	outer mitochondrial membrane
p	probability
PAGE	polyacrylamide gel electrophoresis
paraquat	methyl viologen or 1,1'-dimethyl-4,4'-bipyridinium dichloride
PARP	poly(ADP-ribose) polymerase
PBS-T	phosphate-buffered saline-Tween-20
PCD	programmed cell death
PCR	polymerase chain reaction
PDC	pyruvate dehydrogenase complex
PEG	polyethylene glycol
pI	isoelectric point
PMSF	phenylmethanesulfonyl fluoride
PMT	photo-multiplier tube
PPT	D-phosphinothricin
PS	phosphatidylserine
Pst	*Pseudomonas syringae* pathovar *tomato*
PTP	permeability transition pore
R	resistant
RING	really interesting new gene
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcriptase-polymerase chain reaction
S	susceptible
SAIL	Syngenta *Arabidopsis* Insertion Library
SDS	sodium dodecyl sulphate
SOD	superoxide dismutase

TAIR	The *Arabidopsis* Information Resource (http://www.arabidopsis.org)
TCA	trichloroacetic acid
TCA cycle	tricarboxylic acid cycle
T-DNA	transferred-DNA
TE	buffer of Tris and EDTA
TE	tracheary element
ToF	time-of-flight
tRNA	transfer RNA
TUNEL	TdT-mediated dUTP nick-end labelling
UTR	untranslated region
UV	ultraviolet
v/v	volume to volume
VDAC	voltage-dependent anion channel
w/v	weight to volume
X-gluc	5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, cyclohexylammonium salt

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CHAPTER ONE

1. Introduction

1.1. Programmed Cell Death

1.1.1. Defining and identifying programmed cell death

A simple search of the PubMed journal index for papers published in 2001 and containing the words 'programmed cell death' (PCD) retrieves over 8,000 articles matching these criteria. The phenomenal interest in PCD attests to the fundamental importance of this event in the development of almost all multicellular organisms. PCD is generally defined as an active cellular suicide employed for the purpose of removing unwanted, aged, or damaged cells (Pennell and Lamb, 1997; Zamzami *et al.*, 1997). Although the term PCD is often used interchangeably with the term 'apoptosis', the latter describes a specific morphological phenomenon commonly associated with cell death in animals. Apoptotic cell death is characterised by chromatin condensation and nuclear fragmentation (pyknosis), plasma membrane blebbing and, ultimately, the breakage of the cell into small, membrane-enclosed 'apoptotic bodies' which are engulfed by phagocytic cells (Figure 1.1A) (Reed, 2000). The presence of a cell wall in plants precludes the possibility for apoptotic cell death (Jones, 2001) and, instead, cell death in plants (associated with cellular condensation and nuclear fragmentation) is described by the term 'programmed cell death' (Figure 1.1B) (Pennell and Lamb, 1997). This term takes into account different morphologies and biochemical pathways associated with cell death, while emphasising that these deaths are 'programmed' and therefore a normal part of the organism's development (Lockshin and Zakeri, 2001).

The morphological features of PCD arise from the fact that this type of cell death is controlled by a genetic programme and, in turn, involves molecular and biochemical

A

B

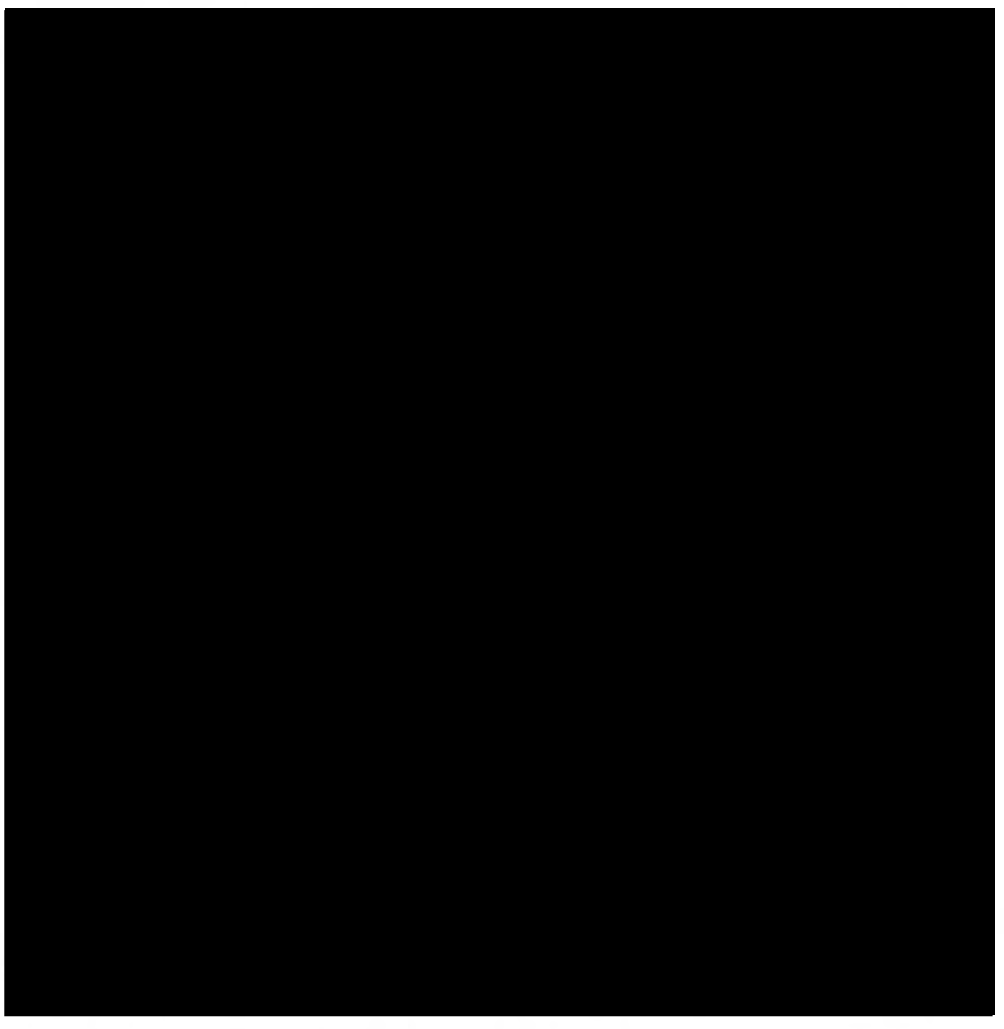


Figure 1.1. Changes in cellular morphology during PCD.
A. The cellular morphology of apoptosis in mammalian cells (arrow A) involves plasma membrane blebbing and cytoplasmic condensation. Normal, viable cells (arrow N) do not show this morphology. From Afford and Randhawa, 2000. **B.** Electron micrograph of the cellular morphology of plant PCD, which is associated with a condensed cytoplasm and plasma membrane blebbing. CC, condensed cytoplasm; PM, plasma membrane; CW, cell wall. From McCabe *et al.*, 1997.

cellular pathways that include protease and nuclease activation, altered cellular redox states, and changes in ion fluxes (especially Ca^{2+}) (Pennell and Lamb, 1997; Zamzami *et al.*, 1997). The induction of this programme, which can occur in response to a variety of developmental factors, often does not occur in response to severe cellular insults such as toxins or physical or chemical damage (Lennon *et al.*, 1991). In these cases, cell swelling and plasma membrane lysis occur before the activation of the cell death programme and, in animal cells, lead to the leakage of cellular contents into surrounding tissues, thereby provoking an inflammatory response. Such unregulated, accidental cell death is defined by the term 'necrosis' and is not accompanied by the cellular signalling pathways that control PCD.

1.1.2. An historical perspective of PCD

The recognition of cell death as a normal part of animal development can be traced back to the 19th century, during which time anatomists studying processes such as notochord formation and the metamorphosis of tadpoles and insects recognised the cellular features associated with apoptotic death (Afford and Randhawa, 2000; Lockshin and Zakeri, 2001). In some instances morphological characteristics of both apoptosis and necrosis were identified and therefore the existence of more than one type of cell death was realised. In 1964, the term 'programmed cell death' was coined (Lockshin and William, 1964), but it was not until 1972 that the term 'apoptosis' was employed to describe the specific ultrastructural morphology of this cell death (Kerr *et al.*, 1972).

Over the last two decades, knowledge about the molecular basis of PCD has increased dramatically as many of the inducer and effector molecules have been identified. This understanding of PCD began most significantly with studies of cell death lineages in *C. elegans* development that uncovered the underlying genetic basis (Ellis and Horvitz, 1986; Metzstein *et al.*, 1998). The recognition of the importance of

this work and its significant impact on medical research was highlighted recently with the award of the 2002 Nobel Prize in Physiology or Medicine to H. Robert Horvitz, Sydney Brenner, and John E. Sulston for their discoveries concerning the genetic regulation of organ development and programmed cell death (http://www.nobel.se). The continued interest in and enthusiasm for the topic has been fuelled by the recognition of the importance of PCD in normal animal development.

1.1.3. The significance of PCD in animal development

It is sobering to realise that, over the span of a human life, over 99.9% of the body's cells undergo cell death — one hundred thousand cells die on a daily basis (Vaux and Korsmeyer, 1999). The occurrence of cell death permeates all aspects of normal development. For example, cellular suicide is an effective mechanism used to ward off viruses and prevent their spread throughout the body (though viruses have unsurprisingly evolved mechanisms to inhibit host cell death). Cell death occurs as part of the programme of ageing, limiting the maximum lifespan of an organism to that which is determined by their genetic make-up. Perhaps most importantly, however, cell death is an integral mechanism in the construction and maintenance of the multicellular organism (Meier *et al.*, 2000). The over-production of cells that can later be ablated in a flexible manner to meet developmental demands is commonly witnessed in animals. This occurs in the deletion of female sexual organs during male embryonic development (and vice versa) and that of other embryonic tissues. During amphibian and insect metamorphosis, muscle cells and neurons no longer required for the new life phase are eliminated by apoptosis. The importance of cell death in controlling animal shape and size has been seen in countless instances; it is generally accepted that, in the absence of the appropriate survival signals or cues from surrounding cells, cell death becomes a

default mechanism to ensure that each organ contains the right number of cells and that cells survive only in the appropriate time and place (Raff, 1996).

Cell death is also invoked when cells are injured or experience some developmental or genetic error, serving to sacrifice these damaged or mutated cells for the sake of viability at the whole-organism level (Duke *et al.*, 1996). The failure of cell death to occur thus constitutes the underlying basis for cancer development, in which cells grow and proliferate in an unregulated manner. Conversely, inappropriate cell death contributes to the development of several diseases, including PCD of helper T cells in AIDS, heart cells and neurons in strokes, and various brain degeneracies such as amyotrophic lateral sclerosis and Alzheimer's, Huntington's, and Parkinson's diseases. Examples such as these highlight the fundamental importance of PCD in normal development, defense, and maintenance, and justify the enormous amount of interest that has been generated in the scientific community concerning the understanding of this cellular event.

1.1.4. The significance of PCD in plant development

It is rather satisfying for the plant biologist to consider that the first description of a 'cell' was made by the English scientist, Robert Hooke, whom, in 1665, examined sections of cork tissue under the microscope (Vaux and Korsmeyer, 1999). In fact, the 'cells' that Hooke observed were actually remnants of plant cells that had undergone PCD, forming a protective layer around the stem. PCD is required throughout plant development, serving to eliminate unwanted cells and to rid the plant of those cells which have served their developmental role. For example, a number of specialised cell types undergo PCD during gamete formation. These processes include the deletion of floral organs in the formation of unisexual flowers and megaspore abortion to yield the remaining mature megagametophyte (Gray and Johal, 1998). Additionally, cell death in

the tapetal layer occurs during anther dehiscence, and various mutants that fail to undergo cell death result in male sterility. Recent molecular and biochemical evidence has suggested that cell death in the tapetal layer is programmed in nature (Balk and Leaver, 2001).

PCD is involved in the degradation of tissues that are no longer required. The removal of suspensor cells during embryo development is by PCD (Gray and Johal, 1998), as is post-germination cell death of seed aleurone and endosperm tissues (Kuo *et al.*, 1996; Fath *et al.*, 1999; Bethke and Jones, 2001; Fath *et al.*, 2002). In addition to the removal of such specialised cells, PCD is also used to form specific structures by cell removal. Examples include the formation of oxygen carrying conduits known as aerenchyma and the sculpting of leaves (Gray and Johal, 1998). PCD is also recognised to be the endpoint of senescence, a process in which cellular material and nutrients are recycled in an orderly fashion (Gan and Amasino, 1997; Quirino *et al.*, 2000). This may be useful in containing the dying and dead cells and therefore preventing infection or the spread of disease in the plant (Pennell and Lamb, 1997). Finally, the generation of root cap cells to protect the root apical meristem involves cell death that exhibits biochemical features of PCD (Wang *et al.*, 1996a).

PCD is also required in plants in response to abiotic stresses for the removal of damaged cells (Jones, 2001). With respect to biotic stress, PCD in plants is associated with the defensive hypersensitive response (HR) in plant-pathogen interactions, serving to limit the spread of the pathogen throughout the plant and its acquisition of nutrients (Greenberg, 1997; Heath, 2000a). Indeed, in many instances that mirror those of animal cells described above, PCD is an essential mechanism in development to construct and maintain plant tissues, and also to protect them throughout the plant life cycle. In these regards, and particularly in light of the important applications of PCD research in

agriculture (for example, in maximising disease resistance or delaying post-harvest senescence) (Lam *et al.*, 1999a), it is not surprising that research into the molecular mechanisms underlying plant PCD has become one of the hottest topics to emerge in plant biology in recent years.

1.2. Molecular mechanisms of mammalian PCD

The process of cell death can generally be divided into three phases (Green and Kroemer, 1998). During the first induction phase, a signal, either extrinsic or intrinsic, is perceived and the cell is triggered to die. In the effector phase, the molecular machinery that executes PCD is activated and the cell becomes committed to die. Finally, it is during the degradation phase of PCD that the associated morphological and biochemical features are acquired. To understand the molecular machinery involved in mammalian PCD (the most widely studied form of cell death), it is useful to consider the main molecular players in each phase and how they contribute to the cell death programme (Figure 1.2).

1.2.1. Induction phase of PCD

1.2.1.1. Mammalian cell death is mediated by caspases

A discussion of the molecular machinery of mammalian PCD cannot begin without first defining caspases. Caspases are cysteine aspartyl-specific proteases, meaning they contain a cysteine residue at their active site and cleave substrates after an aspartate residue (Hengartner, 2000). Caspases are evolutionarily well-conserved throughout the metazoa; one of the first cell death genes discovered in *C. elegans* was found to encode a caspase homologue (Yuan *et al.*, 1993). In humans, over 12 caspases have been identified (Hengartner, 2000). While it must be noted that not all cell deaths are caspase-dependent and that certain cells are even able to withstand the activation of caspases (Leist and Jaattela, 2001), at least 8 of the identified caspases in humans are implicated in cell death (Hengartner, 2000).

Caspases are synthesised as inactive zymogens and are activated by cleavage at aspartate residues to yield large (~20 kDa) and small (~10 kDa) subunits that associate

Figure 1.2. Molecular mechanisms of mammalian PCD.
Mammalian PCD, whether triggered developmentally or as a consequence of stress or other simuli, can involve extrinsic or intrinsic induction. Extrinsic, receptor-mediated PCD may signal through the mitochondrion. Intrinsic, mitochondrial-mediated pathways of PCD typically begin with the release of cytochrome *c* from the intermembrane space and may involve the action of the Permeability Transition Pore (PTP). PCD can involve both caspase-dependent and caspase-independent mechanisms, the latter of which includes Apoptosis Inducing Factor (AIF) and Endonuclease G action. Other proteins, including Bcl-2 family members and Inhibitors of Apoptosis (IAPs), serve to regulate the cell death process. (Figure courtesy of J. Balk and L. Sweetlove).

as heterotetramers to form the active enzyme (Cryns and Yuan, 1999). The fact that caspases are activated by cleavage at aspartate residues combined with their known substrate specificity at this site means that one caspase can activate another, resulting in an auto-amplification cascade. Caspases are thus arranged in a proteolytic cascade that relays and amplifies cell death signals. Specific family members can be classified as either upstream (initiator) or downstream (effector) caspases (Cryns and Yuan, 1999; Slee *et al.*, 1999; Reed, 2000). Large N-terminal prodomains in the former are important for interaction with other proteins that trigger the cell death cascade. Conversely, the effector caspases serve to cleave specific target proteins, either degrading them or activating them (for example, by cleavage of a negative regulatory domain), to bring about cell death and the associated morphological features.

1.2.1.2. Extrinsic induction of caspase-dependent cell death

Two pathways initiating caspase-dependent cell death have been identified (Figure 1.3) (Budihardjo *et al.*, 1999). The Type I, or extrinsic pathway of cell death, involves the perception of an extracellular ligand that binds to specific death receptors (DR) on the target cell. Such receptors include Fas/CD95, tumour necrosis factor-α (TNF-α) receptor 1, and both DR4 and DR5 that bind to the TNF-α-related apoptosis-inducing ligand (TRAIL) (Ashkenazi and Dixit, 1999). These receptors are characterised by possessing a cytoplasmic 'death domain' (DD) that serves to mediate interactions between other DD-containing proteins. The interaction of DD proteins eventually leads to their recruitment of and binding to the initiator procaspase-8, which is then cleaved and activated by induced proximity to other procaspase-8 molecules via a weak autocatalytic protease activity present in the zymogen form (Reed, 2000; Kaufmann and Hengartner, 2001). In some human lymphoid cells, caspase-8 cleaves and directly activates the effector caspase-3 (Budihardjo *et al.*, 1999). In most other cells, however,

Figure 1.3. Extrinsic and intrinsic pathways for induction of cell death.
Two major pathways leading to caspase activation exist in mammalian cells, the extrinsic (left) and intrinsic (right). The extrinsic pathway is mediated by death receptors, such as TNFR and Fas, which then recruit signalling proteins with Death Domains (DD). This eventually leads to the activation of caspase-8. The intrinsic pathway can be triggered by a variety of stimuli. This pathway involves the translocation of Bax from the cytosol to the mitochondrion (see section 1.2.2.3) and the translocation of cytochrome *c* from the mitochondrial intermembrane space to the cytosol. This leads to the activation of the apoptosome, which includes caspase-9. Active caspase-8 and caspase-9 then cleave and activate the effector protease, caspase-3, leading to a downstream caspase cascade. From Reed, 2000.

caspase-8 activity cleaves the cytoplasmic protein Bid (a pro-apoptotic member of the Bcl-2 family), liberating a fragment that then acts upon the mitochondria to induce cell death (see below) (Li *et al.*, 1998; Luo *et al.*, 1998).

1.2.1.3. Intrinsic mitochondrial induction of caspase-dependent cell death

In addition to the Type I, extrinsic induction of death, cells also possess a Type II, intrinsic mechanism for caspase activation and death induction (Song and Stellar, 1999; Kaufmann and Hengartner, 2001). The activation of this pathway requires the involvement of mitochondrial proteins, most notably the electron transport chain protein cytochrome *c*, that is normally localised to the mitochondrial intermembrane space. Upon perception of the appropriate death-inducing signal, cytochrome *c* is translocated to the cytosol (the mechanism by which this translocation occurs is still a source of debate; see below). Released cytochrome *c* then binds a cytoplasmic protein, Apaf-1 (apoptotic protease-activating factor-1) (Li *et al.*, 1997; Zou *et al.*, 1997), which is homologous to the *C. elegans* cell death protein, CED-4. Apaf-1 undergoes an ATP- or dATP-dependent conformational change that allows it to bind the prodomain of procaspase-9. Together, these 3 proteins (cytochrome *c*, Apaf-1 and procaspase-9) constitute what is commonly referred to as the 'apoptosome'. By an as yet ill-defined mechanism, procaspase-9 is then activated to caspase-9, and this initiator caspase is responsible for proteolytically activating the effector caspase-3 (Rodriguez and Lazebnik, 1999).

In summary, two mechanisms of caspase activation exist in mammalian cells: The extrinsic pathway involves death receptors and the activation of the upstream caspase-8, while the intrinsic, mitochondrial pathway involves the release of cytochrome *c* and the activation of the upstream casapse-9 (Hengartner, 2000). Generally, upstream caspases are activated by protein-protein interactions, while the downstream effector

caspases, including caspases-3, -6, and -7, are proteolytically activated by the former. The two pathways of caspase induction may not be mutually exclusive. For example, activation of procaspase-8 can eventually lead to activation of the mitochondrial pathway as well (Kaufmann and Hengartner, 2001).

1.2.2. Effector phase of PCD

An examination of the effector phase of PCD, in which the executioners of cell death become activated and the cell is committed to die (Green and Kroemer, 1998), requires an understanding of changes in mitochondrial structure and function during this stage. Specifically, mitochondrial membrane permeabilisation (MMP), which is induced during the initiation of PCD, may be responsible for taking the cell beyond the 'point of no return'. It is useful to first understand what MMP entails at a biochemical and physiological level, and to then examine some of the key proteins, such as the Permeability Transition Pore (PTP) components and Bcl-2 family members, that are thought to regulate this process.

1.2.2.1. Mitochondrial changes during cell death

Mitochondria in healthy cells can be divided into two sub-compartments (Kroemer and Reed, 2000). The internal matrix is surrounded by the cristae of the highly-folded inner mitochondrial membrane (IMM), while the intermembrane space (IMS) is defined as that region between the IMM and outer mitochondrial membrane (OMM). Under normal physiological conditions, the electrochemical gradient ($\Delta\psi$), which involves the accumulation of protons in the IMS, is established. $\Delta\psi$ is generated by the activity of the electron transport chain and is maintained by the impermeability of the IMM to protons. Ultimately, $\Delta\psi$ allows for the production of ATP in the matrix. The adenine nucleotide translocase (ANT), which is localised to the IMM, functions to

exchange this mitochondrially-produced ATP for cytosolic ADP (Belzacq *et al.*, 2002). The cytosolic-mitochondrial exchange of ADP/ATP also requires the function of an OMM protein, the voltage-dependent anion channel (VDAC or porin) (Tsujimoto and Shimizu, 2002). VDAC, the most abundant OMM protein, is a non-specific pore permeable to solutes of up to ~5,000 Da, and thereby allows for equilibrium of solutes to be achieved between the cytosol and the IMS. During the MMP, the IMM becomes permeable in a stepwise manner to molecules of up to 1,500 Da, thus dissipating the proton gradient and $\Delta\psi$ completely (Waterhouse *et al.*, 2002). This not only results in a loss of ATP synthesis, but is also associated with the release of pro-apoptotic proteins from the IMS, including cytochrome *c*, the Apoptosis Inducing Factor (AIF) (Lorenzo *et al.*, 1999), and Smac/DIABLO (Du *et al.*, 2000; Verhagen *et al.*, 2000) (see discussion below).

The underlying mechanism for the release of IMS proteins during cell death is still widely debated. The MMP may be caused by the association of existing mitochondrial proteins to form the PTP, a non-specific pore that allows free exchange of matrix solutes with the cytosol and loss of mitochondrial volume homeostasis (Figure 1.4A) (Desagher and Martinou, 2000). Swelling of the mitochondrion and an expansion of the IMM sufficient to rupture the OMM would lead to the non-specific release of all contents of the IMS, including proteins, into the cytosol (Desagher and Martinou, 2000). Alternatively, specific proteins such as Bcl-2 family members and VDAC may form channels in the OMM sufficiently large to allow for the regulated passage of soluble proteins (Figure 1.4B) (Desagher and Martinou, 2000). It must be stressed that evidence for both models is often based on *in vitro* studies or indirect observations, and no single model has been conclusively shown to function in all cell deaths (Martinou and Green, 2001; Halestrap *et al.*, 2002). Nevertheless, a consideration of the various models

A

B

Figure 1.4. Possible mechanisms for the release of intermembrane space proteins during PCD.
A. Permeability Transition Pore (PTP) opening may increase mitochondrial inner membrane permeability, eventually causing matrix swelling and a rupture of the outer membrane. **B.** A channel may form in the mitochondrial outer membrane, composed of Bax alone, or Bax in complex with other proteins, such as VDAC. Adapted from Desagher and Martinou, 2000.

presents an opportunity to introduce some of the major protein players in mammalian cell death.

1.2.2.2. Permeability transition pore formation in cell death

Dissipation of the $\Delta\psi$ during cell death may be due to the formation of a complex between existing mitochondrial proteins in a PTP. The basic structure of the PTP is thought to include outer membrane VDAC, inner membrane ANT, and the matrix cyclophilin D (CyP-D) (Figure 1.5) (Desagher and Martinou, 2000). The elucidation of PTP components and regulatory mechanisms (reviewed by Crompton, 1999) began most notably with the discovery of inhibitory compounds of PTP opening, as studied by methods including matrix swelling, the loss of mitochondrial membrane potential, the release of Ca^{2+}, and the measurement of [^{14}C]-sucrose permeation (Halestrap *et al.*, 2002). It was initially found that PTP activity was inhibited by the drug cyclosporin A (CsA) (Crompton *et al.*, 1988a), a compound that binds specifically to CyP-D (Connern and Halestrap, 1992), thus providing the impetus for the elucidation of other PTP components.

The ability of CyP-D to catalyse the conversion of *cis* and *trans* peptide bonds adjacent to proline residues suggested that it may be involved in causing conformational changes in a membrane protein that was part of the PTP. It was indeed shown that, in the presence of Ca^{2+}, binding of CyP-D to ANT allowed for the formation of a non-specific membrane channel, and that this process was enhanced under conditions of oxidative stress (Halestrap and Davidson, 1990). Other studies demonstrated that ANT also forms complexes with VDAC during normal mitochondrial function (McEnery *et al.*, 1992) and it was later shown that these complexes could be purified using CyP-D (Crompton *et al.*, 1998b). Furthermore, PTP activity was reconstituted in complexes containing all three proteins and involving contact between the two membranes. Since other groups

Figure 1.5. Possible components of the mitochondrial permeability transition pore (PTP).
In the open state, water and solutes (<1500 Da) enter the matrix, causing swelling of the matrix and eventual outer membrane rupture. Putative PTP components include cyclophilin D (Cph. D), the adenine nucelotide translocase (ANT), and the voltage-dependent anion channel (VDAC). Opening of the PTP is also affected by a number of pharmacological agents and cellular conditions as indicated.
ROS, reactive oxygen species. Adapted from Desagher and Martinou, 2000.

have demonstrated that only ANT and CyP-D are required to reconstitute PTP activity (Halestrap *et al.*, 2000), it remains for future studies to determine whether VDAC is a component of the pore *in vivo* (Crompton *et al.*, 2002). Finally, mechanisms of MMP requiring only a change of VDAC to the closed state have also been proposed (Vander Heiden *et al.*, 1997; Vander Heiden *et al.*, 2000), as well as other studies which have demonstrated that only the overexpression of a specific isoform of human ANT (ANT-1) is required to dominantly induce apoptosis (Bauer *et al.*, 1999).

1.2.2.3. Bcl-2 family members as regulators of mitochondrial permeability

The release of intermembrane space proteins to the cytosol during cell death is known to be regulated by members of the Bcl-2 family of proteins (Vander Heiden and Thompson, 1999). These proteins are either anti-apoptotic (Bcl-2-like proteins, such as Bcl-2 or Bcl-X_L), or pro-apoptotic (such as Bax and Bak) in their effects. Beginning with the discovery that Bcl-2 was capable of inhibiting mitochondrial-dependent apoptosis in a cell-free system (Newmeyer *et al.*, 1994), studies of the various family members have revealed unique biochemical properties that are suggestive of their function in regulating mitochondrial homeostasis and cell death. For example, Bcl-2 proteins possess a highly conserved carboxy-terminal transmembrane tail that is capable of targeting the proteins to membranes, including the OMM (Nguyen *et al.*, 1993). Indeed, the translocation of cytosolic Bax to the mitochondria is required for its cell death-inducing effects (Hsu *et al.*, 1997; Wolter *et al.*, 1997; Gross *et al.*, 1998). Additionally, Bcl-2 proteins are known to undergo a variety of protein-protein interactions, particularly with other members of the family, resulting in the formation of homo- and heterodimers that determine the overall balance of available pro- or anti-apoptotic proteins (Reed, 1997). In *C. elegans*, the Bcl-2 homologue, CED-9, interacts directly with, and inhibits the activity of, the Apaf-1 homologue, CED-4. Evidence for a

similar regulatory action between Bcl-2 and Apaf-1 in mammalian cells has not been definitively shown (Metzstein *et al.*, 1998; Hengartner, 2000).

The biochemical features of Bcl-2 proteins, combined with the elucidation of Bcl-X_L structure that revealed its similarity to the pore-forming unit of diptheria toxin (Muchmore *et al.*, 1996), suggest that these proteins may exert their effects by forming channels in the mitochondrial membrane (Reed, 1997; Vander Heiden and Thompson, 1999; Hengartner, 2000). These pores may be responsible for generating the MMP by destabilising the planar phospholipid bilayer or may, perhaps, be large enough for IMS proteins to pass through directly (Desagher and Martinou, 2000). In either case, targeting of Bcl-2 proteins to the outer mitochondrial membrane is key to their function in cell death. While some family members may be constitutively localised to the OMM, others can require cleavage by caspases (Bid), phosphorylation or dephosphorylation (Bad), or other undefined mechanisms (Bax) to translocate from the cytosol to the OMM following cell death induction (Reed, 2000). Various lines of evidence also suggest that the interaction of Bcl-2 proteins with other mitochondrial membrane proteins, particularly VDAC (Shimizu and Tusjimoto, 1999) and ANT (Marzo *et al.*, 1998) (in reconstituted liposomes), may be important in regulating mitochondrial membrane permeability. However, Bax-induced cytochrome *c* release can occur independently of the PTP (Eskes *et al.*, 1998). Thus, the precise biochemical mechanisms and protein interactions involved in Bcl-2 regulation of apoptosis remain to be fully elucidated. Nevertheless, the central role for these proteins in regulating mitochondrial homeostasis and the translocation of IMS proteins to the cytosol during cell death is firmly established.

1.2.2.4. Other regulators of cell death

As alluded to above, several other proteins in addition to cytochrome *c* are released from the mitochondrial IMS during cell death execution. Some of these proteins add a further layer of regulation to caspase-dependent cell death, particularly after cytochrome *c* release, while others induce cell death in a caspase-independent manner. For example, additional regulation of caspase activity is provided by a protein simultaneously discovered by two groups and named Smac/DIABLO (for second-mitochondria-derived activator of caspases or direct IAP-binding protein with low pI) (Du *et al.*, 2000; Verhagen *et al.*, 2000). The release of this pro-apoptotic protein from the mitochondrial IMS allows it to bind and inhibit another class of cell death regulators, Inhibitors of Apoptosis (IAPs).

IAPs comprise a family of evolutionarily conserved cell death suppressors present in mammals, insects, and some animal viruses (Deveraux and Reed, 1999; Miller, 1999). These proteins are characterised by the presence of a BIR domain (baculovirus IAP repeat) and a zinc-binding fold, and may also contain RING zinc-fingers that are important in their interactions with other proteins. IAPs exert their function by binding to and inhibiting activated caspases. This interaction is selective for specific caspases, and IAP inhibition of cell death extends to both the extrinsic and intrinsic induction pathways downstream of Bcl-2 protein function and cytochrome *c* release. It is suggested that IAPs may serve to ensure that spontaneous caspase activation does not lead to cell death, and therefore only the release of mitochondrial Smac/DIABLO will lead to a full execution of the cell death programme (Hengartner, 2000). Finally, it should be noted that, in addition to caspase inhibition by IAPs, the activity of the apoptosome is negatively regulated by an interaction of cytosolic Heat shock protein 70 (Hsp70) with Apaf-1 (Saleh *et al.*, 2000). In accordance with this

inhibitory role of Hsp70 on cell death, a downregulation of Hsp70 expression is necessary to allow cell death to proceed.

In addition to the various checks and balances that exist to ensure caspase-dependent cell death occurs in only the appropriate time and place, caspase-independent mechanisms of cell death have also been identified (Leist and Jaattela, 2001). (In these cases, other proteases such as cathepsins, calpains, serine proteases, or proteosomes may be responsible for producing the distinctive apoptotic morphology). The regulation of caspase-independent death has been most extensively documented with respect to two mitochondrial proteins, Apoptosis Inducing Factor (AIF) and Endonuclease G (EndoG). AIF, a protein normally resident in the mitochondrial intermembrane space, is translocated directly to the nucleus upon apoptosis induction to induce nuclear condensation and nuclear DNA fragmentation (albeit differing from that of caspase-dependent cell death; see below) (Lorenzo *et al.*, 1999; Cande *et al.*, 2002). The AIF protein is evolutionarily conserved and resembles bacterial NADH ferredoxin reductases and eukaryotic ascorbate oxidoreductases. Interestingly, AIF pro-apoptotic activity is also negatively regulated by the cytosolic Hsp70 (Ravagnan *et al.*, 2001). Additionally, the mitochondrial nuclease EndoG has been demonstrated to be involved in cell death in both *C. elegans* (Parrish *et al.*, 2001) and mammals (Li *et al.*, 2001). Upon release from the mitochondria (at least in mammals), EndoG is translocated to the nucleus where it induces DNA fragmentation in a caspase-independent fashion. Though the precise localisation of EndoG within the mitochondrion is currently unclear — but is suspected to include at least a sub-fraction in the IMS (Hengartner, 2001) — the fact that both AIF and EndoG are normally resident in this organelle again highlights its central role in cell death regulation in both caspase-dependent and –independent manners.

1.2.3. Degradation phase of PCD

As mentioned earlier (see section 1.1.2), apoptosis was first recognised and described by the striking cellular morphology exhibited by apoptotic cells (Kerr *et al.*, 1972), though programmed cell deaths that do not necessarily exhibit the characteristic morphology of apoptosis also exist (Hengartner, 2000). In any case, the process of programmed cell death, apoptotic or not, is hallmarked by certain morphological and biochemical changes that are generated during the degradation phase of PCD (Green and Kroemer, 1998). These include changes to cellular and nuclear morphologies, fragmentation of nuclear DNA, hallmark cleavage of certain cellular proteins, and changes in membrane topology. In many cases, cellular changes during the degradation phase are the result of caspase action, though hallmark features of cell death can also be recognised independently of caspase action (Leist and Jaattela, 2001).

1.2.3.1. Changes in cellular morphology

The degradation phase of PCD is most notably recognised by the distinct cellular morphology which accompanies it (Gorman *et al.*, 1994). Generally, cells undergoing death are characterised by a shrinkage of the cell that may be as great as 30% of the cellular volume due to a net movement of fluid out of the cell (Wilcock and Hickman, 1988; Gorman *et al.*, 1994). The shrinkage of the cell may also be due to increased activity of transglutaminases, enzymes that serve to catalyse Ca^{2+}-dependent crosslinking of cytoplasmic proteins (Fesus *et al.*, 1989). At the subcellular level, electron microscopy has indicated that the mitochondrion, endoplasmic reticulum, and Golgi apparatus remain intact (Kerr *et al.*, 1972; Wyllie *et al.*, 1980), though other studies have documented swelling of the mitochondrion during cell death (Vander Heiden *et al.*, 1997). Additionally, mammalian cells undergoing death tend to lose contact with and detach from neighbouring cells (Gorman *et al.*, 1994). The loss of

membrane integrity that is associated with changes in cellular morphology provides the basis for many vital staining techniques used to detect cell death, including Trypan Blue exclusion and Acridine Orange/ethidium bromide uptake.

Changes in plasma membrane topology are commonly associated with mammalian cell death and are thought to be a means by which dying cells may be recognised and engulfed by macrophages and other phagocytes (Martin *et al.*, 1994). One of the hallmark features of cell death is the exposure of phosphatidylserine (PS) on the external face of the plasma membrane (Fadok *et al.*, 2001). In healthy cells, this particular phospholipid is present almost entirely on the inner side of the plasma membrane. During apoptosis, however, exposure of PS on the outer leaflet provides a requisite signal for macrophage engulfment of the dying cell, thereby protecting surrounding tissues from inflammation. This feature of cell death is commonly detected using a specific PS probe, Annexin V, that can then be visualised by fluorescence microscopy or detected and quantified using flow cytometry (Martin *et al.*, 1994).

1.2.3.2. Nuclear changes

Mammalian programmed cell death is accompanied by widespread nuclear breakdown and the fragmentation of nuclear DNA (Gorman *et al.*, 1994). In fact, the distinctive nuclear morphology acquired during PCD was one of the features originally used to distinguish it from necrotic cell death (Kerr *et al.*, 1972). During PCD, the chromatin collapses against the nuclear periphery — often to a 'half-moon' shape — and progressively condenses (reviewed by Earnshaw, 1995; Martelli *et al.*, 2001). In a process that defines apoptosis, balls of chromatin surrounded by the nuclear envelope sometimes bud out, forming the characteristic 'apoptotic bodies' (Halicka *et al.*, 2000). Key nuclear proteins, such as lamin and PARP (Poly(ADP-ribose) polymerase), are cleaved in a signature manner during cell death (Robertson *et al.*, 2000). With respect to

the former, the cleavage of lamin proteins at the nuclear envelope is controlled by specific caspases and is thought to aid in the detachment of the chromatin from the nuclear envelope (Rao *et al.*, 1996; Buendia *et al.*, 1999). PARP is an enzyme common to eukaryotes that is activated by DNA breakage and catalyses the poly(ADP-ribosyl)ation of chromosomal proteins (Lindahl *et al.*, 1995; Wood, 1996). During cell death, caspase-3-dependent cleavage of nuclear PARP produces a characteristic 85 kDa fragment (Nicholson *et al.*, 1995; Tewari *et al.*, 1995), and this cleavage may ensure that DNA repair processes are not active during a time of general DNA breakdown (Robertson *et al.*, 2000).

Several other nuclear proteins are specifically cleaved during cell death (reviewed by Robertson *et al.*, 2000), most of which are involved in the processes of chromatin condensation and nuclear dismantling. However, the activation of endonucleases also occurs at this time (Wyllie, 1980), giving rise to the production of hallmark DNA oligonucleosome fragments as first observed in irradiated lymphocytes (Skalka *et al.*, 1976). Cleavage of the DNA between nucleoesomes is thought to be specified primarily by chromatin structure (rather than DNA sequence) (Robertson *et al.*, 2000). The endonuclease responsible for this 'laddering' pattern has been identified as a caspase-activated DNase (CAD), which is activated upon caspase-3 cleavage of its inhibitor (ICAD) (Enari *et al.*, 1998; Sakahira *et al.*, 1998). The elucidation of the CAD-ICAD endonuclease activation has allowed for an explanation of the characteristic DNA laddering pattern that is so frequently used to identify PCD. Notably, however, DNA cleavage associated with AIF induction of cell death results in the production of large, 50 kb fragments that can be visualised by pulsed-field gel electrophoresis, indicating differences in the mechanisms of DNA cleavage in caspase-dependent and –independent cell death.

1.3. Molecular mechanisms of plant PCD

The significance of PCD as a part of normal plant development has long been acknowledged. Examples include the terminal differentiation of a variety of cell types, including tracheary elements, PCD as the endpoint of senescence, and as a component of disease resistance mechanisms (Jones, 2001). Importantly, the finding that these cell deaths are programmed in nature, i.e. they involve *active* genetic and metabolic components that respond to appropriate signals, has led to obvious interest in elucidating the molecular machinery controlling cell death in plants. Studies of plant PCD morphology and active mechanisms have revealed striking similarities with mammalian PCD. Additionally, molecular investigations have highlighted the possible conservation of some animal cell death genes and proteins in plants and a central role for the mitochondrion in plant PCD (Lam *et al.*, 2001). Taken together, these studies suggest that some aspects of mammalian PCD may be conserved in plants, but also that plants have evolved unique mechanisms for controlling cell death.

1.3.1. Morphology of plant PCD

Just as the fate of the dying cell is known to vary in mammalian systems, with 'apoptosis' describing only one morphology, so does the morphology of a dying plant cell differ depending upon the type of cell death (Groover and Jones, 1999). In general, two types of plant cell deaths can be recognised: Vacuolar cell death that typifies tracheary element differentiation, and apoptotic-like cell death that shares many morphological characteristics with mammalian PCD (Fukuda, 2000). In addition, a third type of cell death that occurs specifically during leaf senescence has been defined (Fukuda, 2000). The latter classification is purely on the basis of this cell death occurring 'slowly' and involving a high recovery of cellular contents. However, it is the

opinion of this author that senescence-induced PCD bears sufficient morphological and biochemical similarities to apoptotic-like cell death to be classified as apoptotic-like. This view is shared by several other researchers (Callard *et al.*, 1996; Yen and Yang, 1998; Delorme *et al.*, 2000). The considerable amount of prolonged, cellular recycling associated with this event can be considered as part of the senescence, rather than the cell death, process.

1.3.1.1. Vacuolar cell death in plants

The key feature of vacuolar cell death is that of active vacuole collapse preceding other degradative events (Fukuda, 2000). While this type of death has been seen in endocarp cells during ovary senescence in pea (Vercher *et al.*, 1987) and aleurone cells in wheat (Kuo *et al.*, 1996), it is most commonly associated with the differentiation of tracheary elements (TE) during xylogenesis (Fukuda, 2000). Pioneering work by Fukuda in the study of TE cell death and the molecular processes involved was achieved using *Zinnia* cell cultures in which TE can transdifferentiate from mesophyll cells *in vitro* (Fukuda and Komamine, 1980a; Fukuda and Komamine, 1980b). Three stages of this differentiation are recognised: During Stage I, dedifferentiation unique to the *in vitro* system occurs. In Stage II, immature xylem cells differentiate and, during Stage III, secondary wall thickening and, ultimately, cell death occurs. It is the transition from Stage II to III that is considered to be the critical step in cell death. Increased cellular Ca^{2+} and brassinosteroid signalling are both necessary events for the transition to Stage III, and the accumulation of specific mRNAs, proteases, and nucleases are associated with the cell death process.

The morphology of vacuolar cell death is unique in many respects (Fukuda, 2000). The most obvious difference between this and other forms of plant cell death is the degeneration of organelles, notably beginning with the vacuole within approximately

six hours of secondary wall thickening. Plant cells are distinguished from animal cells in having a large central vacuole, and the mechanisms surrounding the disruption of this organelle and its contribution to cell death have been studied in detail (Groover *et al.*, 1997). The process is marked by the cessation of cytoplasmic streaming and the implosion of the vacuole, at which time sequestered hydrolytic enzymes are released and proceed to degrade the cellular contents. Single-membrane organelles, such as the endoplasmic reticulum and the Golgi bodies, are seen to swell and then disappear, followed by that of double membranous organelles such as the chloroplast and mitochondrion. Eventually, this degradation leaves behind a hollow corpse that functions as a conducting element with reinforced secondary cell walls.

The degradation of the nucleus, which is triggered by vacuole rupture, is not accompanied by hallmark chromatin condensation or nuclear fragmentation (Obara *et al.*, 2001). Rather, nucleases released from the vacuole serve to digest nuclear DNA and, while these nucleases may be specific to TE cell death (Thelen and Northcote, 1989; Aoyagi *et al.*, 1998), they do not produce a laddering pattern of DNA characteristic of apoptosis (Fukuda, 2000). Proteolytic activity during TE cell death is also due to the activation of specific proteases, both of the cysteine and serine protease families, many of which are also released from the vacuole. It is of interest that many of the proteases and nucleases responsible for executing TE cell death are often not induced during other types of plant cell death, such as that following senescence or abiotic stress, suggesting that the molecular mechanisms and morphologies associated with vacuolar cell death may be unique to this process. (It should be noted that the terms 'proteinase' and 'protease' are both used in animal and plant PCD literature. As noted by Beers *et al.* (2000), 'proteinase' refers to those enzymes that act internally on polypeptide chains, while 'protease' refers to those that act near the terminus of the polypeptide chain. For

the sake of consistency and because the target cleavage site is not always known, the more commonly-used 'protease' is adopted hereinafter).

1.3.1.2. Apoptotic-like cell death in plants

In contrast to vacuolar cell death described above, there are an increasing number of examples of plant PCD that bear considerable morphological similarity to apoptotic cell death in mammals. These include similarities at the whole cell level, changes in membrane topology, and the degradation of nuclear proteins and DNA (Danon *et al.*, 2000). A striking cell death morphology is evident in plant PCD due to the unique presence of a cell wall (see Figure 1.1B). Condensation of the cytoplasm and shrinkage of the plasma membrane away from the cell wall bears remarkable similarity to cytoplasmic condensation in mammalian cell death. This morphology has been observed in carrot cell suspension cultures when grown at low cell densities or subjected to heat treatment (McCabe *et al.*, 1997), *Arabidopsis* cell suspension cultures in response to Yariv reagent (Gao and Showalter, 1999), in tomato root cap cells or protoplasts treated with AAL toxin (Wang *et al.*, 1996a), and following senescence in cucumber cotyledons (Delorme *et al.*, 2000). The presence of the cell wall prevents engulfment by neighbouring cells (such as by macrophages in mammals). Instead, the cell is most likely disposed of by autolysis (Danon *et al.*, 2000).

Changes in nuclear morphology and DNA degradation patterns reminiscent of mammalian apoptosis are frequently evident in plant PCD (Danon *et al.*, 2000). Condensation of the nucleus and chromatin has been observed repeatedly in a wide variety of plant cell deaths, suggesting that this is a common feature (Callard *et al.*, 1996; McCabe *et al.*, 1997; O'Brien *et al.*, 1998a; Gao and Showalter, 1999; Wang *et al.*, 1999; Clarke *et al.*, 2000; Balk and Leaver, 2001; Yao *et al.*, 2001; Saviani *et al.*, 2002). Moreover, the cleavage of DNA into hallmark 'ladders' that can be visualised by

gel electrophoresis has been reported in a number of instances. While smeared DNA is indicative of a passive mechanism most commonly associated with necrosis, DNA fragmentation, particularly when combined with morphological observations (Renvoize *et al.*, 1998), provide strong support for an apoptotic-like death in plants.

Specifically, DNA laddering has been reported in a number of developmentally-associated occurrences of PCD. Examples include the death of monocot aleurone (Wang *et al.*, 1996b), nucellus (Dominguez *et al.*, 2001), and endosperm cells (Young and Gallie, 2000), senescence of leaves, petals, and cotyledons from a variety of species (Yen and Yang, 1998; Delorme *et al.*, 2000; Xu and Hanson, 2000), and in anther development of sunflower and barley (Wang *et al.*, 1999; Balk and Leaver, 2001). Additionally, DNA laddering into oligonucleosome fragments has been detected in experimentally-induced plant PCD, including that following low density culture and heat treatment (carrot) (McCabe *et al.*, 1997), cold stress (tobacco) (Koukalova *et al.*, 1997), ageing/nutrient deprivation (*Arabidopsis*) (Callard *et al.*, 1996), UV radiation (*Arabidopsis*) (Danon and Gallois, 1998), D-mannose stress (*Arabidopsis* and maize) (Stein and Hansen, 1999), and in response to pathogen infection or elicitation (oat, cowpea, and barley) (Ryerson and Heath, 1996; Wang *et al.*, 1996a; Navarre and Wolpert, 1999). DNA degradation as detected by the TUNEL (TdT-mediated dUTP nick-end labelling) method that labels free 3'-OH ends has also been used in additional studies (Danon *et al.*, 2000). However, this method is considered somewhat inferior to the detection of oligonucleosome fragments by gel electrophoresis, since cells that die necrotically will stain TUNEL-positive (McCabe *et al.*, 1997) and the procedure can often give false-positive results (Wolvekamp *et al.*, 1998).

A variety of endonucleases responsible for DNA fragmentation in plant cell death have been described (Goldman *et al.*, 1994; Mittler and Lam, 1995; Wang *et al.*,

1996a; Fath *et al.*, 1999; Stein and Hansen, 1999; Young and Gallie, 2000; Yao *et al.*, 2001). Both Ca^{2+}-dependent and Zn^{2+}-dependent endonucleases that cleave dsDNA have been isolated and characterised, with the former generally catalysing limited DNA fragmentation and the latter being involved in DNA hydrolysis. The mechanisms of induction of these enzymes is relatively unclear (reviewed by Sugiyama *et al.*, 2000), and any particular instance of plant PCD can involve nucleases of both classes. However, it is important to note that no homologues of the mammalian CAD/ICAD complex (section 1.2.3.2), either at the genetic or biochemical level, appear to exist in plants. (Sugiyama *et al.*, 2000). Moreover, Zn^{2+}-dependent nuclease activity is absent in mammalian cells, suggesting divergence of the endonucleolytic pathway of mammals and plants that is responsible for DNA degradation during PCD.

Two additional features of mammalian cell death have been identified in plant cells: Phosphatidylserine (PS) exposure on the outer leaflet of the plasma membrane and PARP cleavage (reviewed by Danon *et al.*, 2000). With respect to the former, the detection of PS can be achieved by working with protoplasts in which the cell wall has been digested, thus exposing the plasma membrane to the Annexin V probe (O'Brien *et al.*, 1997). Using camptothecin as an inducer of cell death and flow cytometry to detect Annexin V-labelled cells, O'Brien *et al.* (1997) were able to demonstrate that tobacco protoplasts exhibited PS exposure prior to DNA breakage in a manner analogous to mammalian cells. The purpose of PS exposure during PCD in plants remains unclear, though the authors suggest it may play a role in cellular signalling. Finally, the characteristic cleavage of PARP has been reported in heat-shocked tobacco suspension cells (Tian *et al.*, 2000). PARP inhibitors are capable of reducing the degree of cell death in soybean cell cultures, while overexpression of PARP increases the percentage of cell death in this system (Amor *et al.*, 1998). These studies suggest that a role for

PARP in DNA breakage and cell death in plants may exist, and future studies may confirm that cleavage of PARP is a characteristic feature of plant PCD.

1.3.2. Conservation of mammalian cell death-related genes in plants

Given the similarities between mammalian and plant PCD at a morphological and biochemical level, the post-genomics era has presented the opportunity for systematic genome screens to identify homologues of mammalian cell death-related genes in plants (Aravind *et al.*, 1999). The results have shown that, while many of the key mammalian cell death genes appear to be absent in plants, the conservation of other genes and functional domains exists. For example, the complete sequencing of the *Arabidopsis* genome has revealed that IAPs and Bcl-2 family proteins, such as Bcl-2 or Bax, are not encoded in the plant genome (The *Arabidopsis* Genome Initiative, 2000). This may seem to contradict a previous report that detected a Bcl-2 homologue in tobacco cells (Dion *et al.*, 1997). This study used an antibody against human Bcl-2 with Western blot analysis and showed immunolocalisation of the reacting protein to internal membranes, including the mitochondrion. In the absence of identifying the plant protein that the anti-Bcl-2 antibody reacts with, however, this study does not demonstrate the existence of Bcl-2-like proteins in plants. Interestingly, it has recently been shown that the pro-apoptotic Bid protein bears both sequence and functional homology to plant lipid transfer proteins (Degli Esposti, 2002). While the author uses this data to suggest a possible functional mechanism for Bid action, approaching this finding from the plant perspective indicates that some seemingly innocuous plant proteins may, in the future, prove to play a cell death-related role.

A recent study examining transcripts upregulated in response to wounding and pathogen challenge in *Arabidopsis* identified *AtBI-1,* a homologue to mammalian *Bax Inhibitor-1* (Sanchez *et al.*, 2000). A subsequent study isolated a rice homologue, *OsBI-*

1, that was capable of suppressing Bax-induced lethality in yeast (Kawai *et al.*, 1999). Human *BI-1* was originally identified in a screen for suppressors of Bax-induced cell death in yeast, and BI-1 functions to inhibit Bax activity. It is noteworthy that BI-1 proteins do not bear sequence similarity to those of the Bcl-2 family (Xu and Reed, 1998). Given that plants do not contain an obvious homologue to Bax, the function of the plant Bax-Inhibitor protein remains obscure, although the amino acid sequence suggests it may encode an ion channel protein.

It is of interest that plants do not seem to encode typical caspase homologues (Lam and del Pozo, 2000), since these are the key effectors of many forms of mammalian cell death (see section 1.2.1.1). However, a recent report has identified caspase-like proteins in plants, termed 'metacaspases', which are also found in fungi and protozoa (Uren *et al.*, 2000). Interestingly, one class of metacaspases contains a proline-rich prodomain and Zn finger motif that are characteristic of HR-related proteins, such as LSD1 (Dietrich *et al.*, 1997; Uren *et al.*, 2000). To date, no analysis of metacaspase activity has been undertaken and a role for these proteins in plant PCD has not been established.

Two other genes can be cited as examples in which the plant genome encodes definite homologues of mammalian cell death-related genes. The first of these is Apoptosis Inducing Factor (AIF). As originally noted in the isolation of mammalian AIF, the gene is most similar to plant oxidoreductases (Lorenzo *et al.*, 1999; Cande *et al.*, 2002). However, while the effect of mammalian AIF has been well-characterised and a recent study has implicated the homologue of this protein in *Dictyostelium* cell death (Arnoult *et al.*, 2001), no role for the plant AIF homologues has been demonstrated. However, several analyses of plant PCD have demonstrated the formation of large, 50 kb DNA fragments (alone or in conjunction with oligonucleosome

cleavage), such as in the somatic embryogenesis of gymnosperms (Filonova *et al.*, 2000) and HR-related cell death of tobacco (Mittler and Lam, 1997; Mittler *et al.*, 1997) and soybean (Levine *et al.*, 1996). This breakdown of DNA is reminiscent of that which occurs during AIF-dependent cell death in mammals (Lorenzo *et al.*, 1999). While one of the two AIF homologues in plants possesses a mitochondrial targeting sequence, proteomic analysis of *Arabidopsis* mitochondria have localised this enzyme to the matrix rather than the IMS (Millar *et al.*, 2001). Therefore, what role, if any, the plant AIF homologue plays in cell death remains to be seen, as does the mechanism responsible for large-scale DNA fragmentation.

Secondly, a plant homologue to the mammalian *Defender Against Apoptotic Death 1* (*DAD1*) gene has been described in plants. *DAD1* encodes a subunit of mammalian oligosaccharyltransferase complexes (Hong *et al.*, 1999), and mutant cell lines die by apoptosis (Nakashima *et al.*, 1993). Conversely, overexpression of the gene protects against cell death in *C. elegans* (Sugimoto *et al.*, 1995). The *Arabidopsis* genome encodes at least two homologues of this gene, one of which can complement the cell death phenotype of mutant mammalian cell lines (Gallois *et al.*, 1997). Thus, the function of the protein appears to be evolutionarily conserved between plants and animals, though its precise role in cell death is still under investigation (Hong *et al.*, 1999).

In addition to whole gene conservation, there are several examples of conservation of functional domains of mammalian cell death-related proteins in plants (Aravind *et al.*, 1999). For example, the cysteine-rich repeats that are conserved in the extracellular domain of Tumour Necrosis Factor Receptor (TNFR) proteins bear homology to those of maize Crinkly receptor kinases involved in epidermal differentiation (Becraft *et al.*, 1996; Aravind *et al.*, 1999). Even more striking, however,

is the homology of Apaf-1 and CED-4 to the plant Ap-ATPase domain (van der Biezen and Jones, 1998; Aravind *et al.*, 1999). This domain, which includes a nucleotide-binding (NB) adaptor, is typically found in plant resistance (*R*) gene products that are involved in the HR. The carboxy-terminus of *R* gene products encodes Leucine Rich Repeats that are often involved in protein-protein interactions. Therefore, has been suggested that *R* gene products may function as adaptors to activate a cell death-inducing complex (similar to the apoptosome) in a pathogen recognition-dependent manner, in which ATP/GTP hydrolysis at the NB site is responsible for activating downstream effectors.

1.3.3. A role for the mitochondrion in plant PCD

A central role for the mitochondrion in regulating mammalian cell death has repeatedly been demonstrated, both in its sequestration of pro-apoptotic proteins that are released upon the appropriate stimulus and the signalling pathways involving key cell death proteins that impinge upon this organelle (Jones, 2000; Lam *et al.*, 2001). Some of these proteins (i.e. caspases or Bcl-2 family proteins) appear to be lacking in plants, while for other homologues of mammalian cell death proteins (i.e. AIF, cytochrome *c*, or putative PTP components), a role in plant PCD has not been conclusively demonstrated. Nevertheless, there are several lines of evidence to indicate that the plant mitochondrion, like its mammalian counterpart, may serve as a regulator of the plant cell death programme (Jones, 2000).

There are an increasing number of studies which demonstrate that plant PCD is associated with a translocation of cytochrome *c* from the mitochondria to the cytosol. The mitochondria-to-cytosol translocation of this protein has been evidenced during cell death in heat-treated cucumber cotyledons (Balk *et al.*, 1999), premature cell death of anthers in cytoplasmic male sterile sunflower (Balk and Leaver, 2001), following

oxidative stress-induced cell death of *Arabidopsis* cell cultures (Tiwari *et al.*, 2002) and tobacco (Robson and Vanlerberghe, 2002), in D-mannose-induced cell death of maize cell cultures (Stein and Hansen, 1999), and in menadione-treated tobacco protoplasts (Sun *et al.*, 1999). Additionally, cytochrome *c* addition to carrot cytosol was found to cause apoptosis of mouse nuclei in a cell-free system (Sun *et al.*, 1999). These observations are in contrast to a single study that found cell death following petal senescence in petunia to be independent of cytochrome *c* release (Xu and Hanson, 2000). A causal role for released cytochrome *c* in plant PCD, however, has yet to be demonstrated. Interestingly, the addition of plant mitochondria to a *Xenopus* cell-free system was found to increase the activation of caspase-3 (Korthout *et al.*, 2000).

The association of mitochondrial cytochrome *c* release with plant PCD prompts the question of what might be the underlying mechanism(s). To this end, evidence has accumulated that a mitochondrial permeability transition also occurs during plant PCD in a manner analogous to mammalian systems. Ca^{2+}- and phosphate-dependent swelling of purified potato mitochondria can lead to outer membrane rupture and cytochrome *c* release (Arpagaus *et al.*, 2002). As with the mammalian MPT, this effect is inhibited by cyclosporin A (an inhibitor of cyclophilin D) and enhanced by anoxic conditions. Additionally, nitric oxide-induced cell death of *Citrus* cell suspension cultures is preceded by a loss in $\Delta\psi$, while oxidative stress-induced cell death of *Arabidopsis* cell suspension cultures causes mitochondrial swelling (Tiwari *et al.*, 2002). In both of these instances, the MPT and cell death are inhibited by cyclosporin A. Finally, the addition of bongkrekic acid — an inhibitor of the mammalian PTP component, ANT — is sufficient to prevent the induction of pathogen- and stress-related genes following antimycin A treatment of tobacco cell suspension cultures (Maxwell *et al.*, 2002). Taken together, these studies suggest that an MPT is a general feature of plant PCD, particularly that

induced by oxidative stress, although the existence of a PTP or the mechanisms behind the MPT remain to be elucidated.

Finally, a role for the mitochondrion in plant PCD can also be indirectly inferred from studies demonstrating that mitochondrial dysfunction can lead to cell death. For example, a mitochondrial DNA mutation that produces a novel open reading frame is the causative basis for cytoplasmic male sterility in sunflower and is associated with premature PCD of anther tissue (Balk and Leaver, 2001). A recent study has shown that a mutation in the mitochondrial chaperone, GFA2, results specifically in defective synergid cell death during *Arabidopsis* female gametophyte development (Christensen *et al.*, 2002). The authors suggest that this is most likely due a defect in normal mitochondrial function, perhaps involving a compromise in the folding of proteins involved in cell death, but that GFA2 is unlikely to be directly involved in the cell death process. Additionally, evidence that the mitochondrial protein alternative oxidase (AOX) plays a role in attenuating H_2O_2- or salicylic acid-induced cell death in tobacco suggests that mitochondrial control of ROS production may be an important mechanism in regulating cell death (Robson and Vanlerberghe, 2002), including that related to the HR (Ordog *et al.*, 2002). Robson and Vanlerberghe (2002) have also provided intriguing evidence that mitochondria-dependent and –independent pathways of plant PCD — as assayed by mitochondrial electron transport chain function and cytochrome *c* release — exist, depending upon the method of PCD induction.

In summary, current support for a role of the mitochondrion in plant PCD is drawn from correlative data for cytochrome *c* release, permeability transitions, mitochondrial dysfunction, and ROS production. Further investigations are needed to determine what causative role plant mitochondria play in PCD, and to elucidate the molecular mechanisms involved.

1.4. Plant PCD: Methodologies, systems, and insights

The agronomic importance of plant PCD and the relative novelty of the subject have prompted a flood of investigations aiming to unravel the molecular mechanisms involved. To this end, both mutant analyses and studies that attempt to demonstrate mammalian cell death mechanisms in plants have been undertaken. Additionally, genetic suppressor screens and transcript expression analyses have been successful in identifying novel genes that may be related to plant PCD. The following section offers a brief survey of methodologies and systems employed to investigate plant PCD and the associated advantages and limitations of the various approaches.

1.4.1. Mutant isolation

Forward genetics approaches have had some success in identifying genes that function in plant PCD, particularly that related to the HR. Two types of mutant screens may be undertaken in this regard: (1) For mutants that fail to undergo the appropriate HR cell death; or conversely, (2) for so-called 'lesion mimic mutants' that exhibit the HR or cell death in the absence of pathogen infection (Greenberg and Ausubel, 1993; Dietrich *et al.*, 1994). A successful example of the first screen is that of the *defense, no death* (*dnd1*) mutant which was isolated as an *Arabidopsis* line that failed to induce HR cell death in response to pathogen attack (Clough *et al.*, 2000). Cloning of *DND1* revealed that it encoded a cyclic nucleotide-gated ion channel, though several other *dnd* mutants which have recently been isolated have not yet been cloned (Yu *et al.*, 2000).

Conversely, there are many examples of lesion mimic mutants, of which one of the first identified was *Arabidopsis* LSD1. LSD1 encodes a zinc finger protein that functions as a negative regulator of cell death and was originally identified as a mutant that failed to limit the spread of HR-induced cell death (Dietrich *et al.*, 1997). Other

lesion mimic mutants that have been cloned in *Arabidopsis* include that of the recessive accelerated cell death (*ACD*) mutants, *acd2* and *acd11*, and the *constitutive pathogen response5* (*cpr5*). *ACD2* encodes a red chlorophyll catabolite reductase (Mach *et al.*, 2001) and *ACD11* encodes a sphingosine transfer protein (Brodersen *et al.*, 2002). Both *acd* mutants exhibit constitutive activation of defense responses and spontaneous cell death. *cpr5* mutant plants exhibit spontaneous cell death, misregulation of cell division, and develop smaller leaves than wildtype. Cloning of *cpr5* revealed that it encodes a novel putative transmembrane domain protein with a nuclear-targeting sequence in the cytoplasmic domain (Kirik *et al.*, 2001). Finally, an *Arabidopsis* mutant recently isolated which exhibits humidity-sensitive cell death and constitutive disease resistance was found to be caused by a defective copine protein, CPN1. Copines are Ca^{2+}-dependent, phospholipid-binding proteins that have only recently been described in animals (Jambunathan *et al.*, 2001).

Thus, there are a growing number of mutants that exhibit abnormal HR-related cell death phenotypes. However, there do not seem to be any examples in which HR mutants were studied and found to be defective in other types of cell death, for example that following abiotic stress or as the endpoint of senescence. Therefore, a role for genes identified in these studies as general plant cell death regulators cannot yet be ascribed. Importantly, while screens for mutants that undergo spontaneous cell death seem to be successful in identifying mutant lines, very few of the corresponding genes encode regulatory proteins (i.e. transcription factors) or signal transduction components. In many instances, these genes encode proteins that are generally involved in cellular function and viability. This is, perhaps, the primary limitation of a genetic cell death screen and the improbability of identifying genes that are directly related to PCD by this approach: Any protein that is vital to cellular function, when mutated or underexpressed,

may result in cell death, even though the gene product does not function in the cell death programme *per se*.

1.4.2. Pharmacological studies

In order to investigate biochemical features of PCD and, often, to draw parallels to mammalian cell death, several studies involving the use of pharmacological agents in plant PCD have been undertaken. These are most often related to assays for caspase-like activity in plants or studies on the role of Ca^{2+} signalling. With respect to the former, the lack of identifiable caspases in the plant genome has not discouraged the use of caspase inhibitors to attempt to experimentally prevent plant cell death (Lam and del Pozo, 2000). Peptide inhibitors for both caspase-1 and caspase-3 were found to be effective in preventing HR-related cell death in tobacco (del Pozo and Lam, 1998) and the induction of apoptosis in mouse liver nuclei with carrot cytosol in a cell-free system (Zhao *et al.*, 1999). The use of a synthetic caspase-3 substrate in heat-treated tobacco cell cultures revealed caspase-3-like activity (Tian *et al.*, 2000). Caspase-3 inhibitors were found to prevent PARP cleavage in menadione-treated tobacco protoplasts but not to affect cytochrome *c* release (Sun *et al.*, 1999). Finally, caspase-3-like activity was detected in the cytosol of *Chara* cells microinjected with a synthetic substrate (Korthout *et al.*, 2000). However, in the absence of identified caspases (or, in fact, any plant proteases that function in a regulatory cascade analogous to that of mammalian systems), it is impossible to conclude how these inhibitors may be affecting plant cell death.

Evidence acquired in recent years has demonstrated that, in mammals, prolonged increases in Ca^{2+} ion concentration will induce cell death and that Ca^{2+} signalling is involved in PCD (Berridge *et al.*, 1998). Very high Ca^{2+} concentrations often lead to unorganised or necrotic cell death via the activation of Ca^{2+}-sensitive proteases. While the exact nature and function of the Ca^{2+} signal in mammalian PCD is unclear,

mitochondrial and endoplasmic reticulum Ca^{2+} fluxes, in addition to cytosolic Ca^{2+} transients, are thought to be important. Ca^{2+} fluxes may be altered by Bcl-2 family proteins and are known to affect the PTP function (Crompton, 1999). In light of these findings, several groups have attempted to demonstrate a similar involvement of Ca^{2+} in plant PCD.

Studies of Ca^{2+} and plant PCD generally involve the use of Ca^{2+} channel blockers or Ca^{2+} ionophores in the HR (tobacco and oat) (Mittler and Lam, 1997; Navarre and Wolpert, 1999). A detailed analysis of soybean HR demonstrated Ca^{2+} fluxes in response to H_2O_2 and that cell death, including the production of 50 kb DNA fragments, could be blocked by incubation of cells in a Ca^{2+}-free medium (Levine *et al.*, 1996). Conversely, treatment of cells with a Ca^{2+} ionophore was capable of inducing cell death and the production of 50 kb DNA fragments. However, in cowpea HR, increased Ca^{2+} signalling was present only during fungal penetration of the plant cell and not during fungal growth in the cell lumen, thereby implying that Ca^{2+} may not be directly able to trigger HR cell death (Xu and Heath, 1998). It was also suggested that perhaps a *decrease* in cytosolic Ca^{2+} levels may be important in mediating the HR in tobacco under certain conditions (Mittler *et al.*, 1999a).

Collectively, the above studies indicate that Ca^{2+} signalling is associated with plant PCD. However, the Ca^{2+} studies must be interpreted with some caution, since the methodology employed generally involves the use of Ca^{2+} channel blockers or Ca^{2+} ionophores. These chemicals are not well characterised and therefore complicate the ability to draw definitive conclusions about downstream effects (Price *et al.*, 1994). Moreover, without identifying the downstream molecular events of Ca^{2+} signal transduction, the application of pharmacological agents does little to advance our understanding of the regulatory mechanisms that function during plant PCD.

1.4.3. Transgenic expression of animal cell death genes in plants

Attempts to determine whether mechanisms of PCD are evolutionarily conserved have largely focused on identifying plant homologues of animal cell death proteins. It may have initially been assumed, before the availability of the complete *Arabidopsis* genome sequence (The *Arabidopsis* Genome Initiative, 2000), that Bcl-2 family proteins also existed in plants. This prompted investigations into the activity of these proteins when expressed heterologously in plants (Lam *et al.*, 1999b). Indeed, while yeast also do not encode Bcl-2 family proteins, expression of pro-apoptotic Bax is sufficient to cause cell death in yeast (Zha *et al.*, 1996). Similarly, it was found that transgenic tobacco plants expressing murine Bax exhibited cell death and the expression of defense-related transcripts (Lacomme and Santa Cruz, 1999). The mechanism of Bax-induced cell death in transgenic plants has not been determined. In the absence of plant Bax homologues, these experiments merely demonstrate that animal Bax can function in a heterologous plant system, possibly by forming pores in mitochondrial membranes (Danon *et al.*, 2000).

It was recently demonstrated that the extent of Bax-induced cell death in both yeast and *Arabidopsis* could be decreased by simultaneous overexpression of *Bax Inhibitor-1* from either rice (*OsBI-1*) or *Arabidopsis* (*AtBI-1*), suggesting that OsBI-1 and AtBI-1 actively suppress Bax action (Kawai *et al.*, 1999; Kawai-Yamada *et al.*, 2001). Surprisingly, the transgenic expression of AtBI-1 in mammalian cells was observed to induce cell death in a manner similar to Bax, possibly due to a dominant inhibition of the endogenous BI-1 protein (Yu *et al.*, 2002). Conversely, the expression of the cell-death suppressors, Bcl-X_L and CED-9, were sufficient to suppress UV-, oxidative stress-, and HR-induced cell death in tobacco (Mitsuhar *et al.*, 1999). Further studies demonstrated the ability of human Bcl-2 and Bcl-X_L, *C. elegans* CED-9, and

baculovirus IAP transgenes to negatively regulate pathogen-induced cell death in tobacco (Dickman *et al.*, 2001).

Since plants, like yeast, do not encode Bcl-2 family proteins or IAPs, there are at least two possible explanations for the effect of their transgenic expression on plant cell death (Fraser and James, 1998). One possibility is that the endogenous death programme in mammals and plants is conserved, and ectopically expressed Bax, Bcl-2, etc., are acting directly upon these cellular components (for e.g. the permeability transition pore (Jones, 2000)). An alternative explanation is that these proteins are affecting an ancestral cell death pathway that is shared between mammals and plants (Fraser and James, 1998). In this regard, the generation of reactive oxygen species (ROS) has been suggested to constitute an evolutionarily ancient cell death programme. Bax and Bcl-2, etc., may exert their pro- and anti-apoptotic effects, respectively, through ROS generation (Madeo *et al.*, 1999; Fleury *et al.*, 2002).

1.4.4. Genetic suppressor screens and transcript expression analyses

Two of the more successful methods employed to isolate novel genes and proteins that are activated during, and can affect, plant PCD are genetic suppressor screens for plant inhibitors of Bax-induced cell death in yeast, and analyses of transcript expression patterns during plant PCD. With respect to the former, the ability of mammalian Bax expression to induce cell death in yeast allows for a systematic screen of plant cDNA libraries for suppressors of this effect. This type of screen was originally employed to isolate mammalian *Bax Inhibitor-1* from a mammalian cDNA library (Xu and Reed, 1998). Similar screens using plant cDNA libraries have identified a novel tomato glutathione-*S*-transferase/peroxidase (Kampranis *et al.*, 2000), an *Arabidopsis* ethylene-responsive element binding protein (AtEBP), Fe-superoxide dismutase, peroxidase, and glutathione-*S*-transferase (Pan *et al.*, 2001), an *Arabidopsis* vesicle-

associated membrane protein (Levine *et al.*, 2001), and a soybean ascorbate peroxidase (Moon *et al.*, 2002). In all cases, the antioxidant activities of the proteins encoded by these genes suggests that the primary cause of Bax-induced cell death in yeast is the formation of ROS. In fact, Bax-expressing yeast do contain higher levels of ROS (Kampranis *et al.*, 2000; Levine *et al.*, 2001; Moon *et al.*, 2002). In light of these findings, it would be logical to return to the effects caused by ectopic Bcl-2-like protein expression in plants. Measurements of ROS in these transgenic plants may indicate increased or reduced levels of ROS associated with the expression of pro- or anti-apoptotic proteins, respectively.

Transcript expression analyses during plant PCD have also been successful in identifying novel genes involved in plant PCD and, in some cases, demonstrating that altered expression of the identified genes can affect plant PCD. For example, the identification of *AtBI-1* was originally achieved in a differential cDNA-AFLP display of transcripts upregulated during wounding and pathogen challenge in *Arabidopsis* (Sanchez *et al.*, 2000). Additionally, an analysis of changes in transcript expression during cell death in tomato cell cultures identified several novel genes with altered expression patterns (Hoeberichts *et al.*, 2001). Similarly, the identification of a matrix metalloproteinase whose transcripts are upregulated at the onset of PCD in cucumber cotyledons suggested that the encoded protein may be involved in plant PCD (Delorme *et al.*, 2000). This was supported by a recent study demonstrating that reduced expression of an *Arabidopsis* matrix metalloproteinase affects the onset and progression of senescence and/or PCD (Golldack *et al.*, 2002). Finally, a screen to identify new molecular markers of the *Arabidopsis* HR identified seven genes (several of which encode unknown proteins) that exhibited increased transcript expression during the HR (Daniel *et al.*, 1999; Lacomme and Roby, 1999). A subsequent study demonstrated that

misexpression of one of these genes, which encodes a MYB transcription factor, affects HR cell death in *Arabidopsis* (Vailleau *et al.*, 2002). Therefore, genetic suppressor screens and transcript expression analyses to identify genes that may be involved in plant PCD have identified many genes that may not otherwise have been predicted. These studies are also advantageous in helping to understand the underlying mechanisms and biochemical features of the plant cell death programme (i.e. as identification of plant antioxidant genes as suppressors of Bax-induced lethality in yeast would indicate).

1.5. Aims of this project

In spite of the fundamental importance of plant PCD in many aspects of plant growth, development, and defense, our current understanding of the molecular mechanisms involved in this process is limited (Pennell and Lamb, 1997). Attempts to identify analogous molecular mechanisms in plants to those that occur during mammalian PCD have been met with limited success. Indeed, the anticipated conservation of key cell death proteins, such as caspases and Bcl-2 family members, have failed to materialise. Nevertheless, there exist several similarities in cell death morphology and in the likely role of the mitochondrion that suggest that at least some aspects of mammalian PCD may be conserved in plants (Danon *et al.*, 2000). Therefore, while it is reasonable to draw some parallels between mammalian and plant PCD, advancement in our understanding of the latter will additionally require an expectation of uncovering novel mechanisms.

The methodologies and systems traditionally employed to study plant PCD at the molecular level include mutant isolation (particularly related to the HR), pharmacological assays, transgenic expression of animal genes in plants, genetic suppressor screens, and transcript expression analyses. The problems associated with each approach have been outlined above (see section 1.4). Continued progress in our understanding of plant PCD requires that the problem is studied in a wider context and is not limited to one specific developmental event or process, and that the experimental strategy allows the discovery of novel mechanisms.

The current project aims to meet both of the above objectives by studying plant PCD induced by more than one stimulus and by undertaking a non-hypothesis-driven characterisation of transcript and protein expression during plant PCD. Specifically, an *Arabidopsis thaliana* cell suspension culture will be utilised as the experimental system,

since it is amenable to a variety of treatments and cell death-inducing stimuli (McCabe and Leaver, 2000). In addition, the availability of the complete sequence of the *Arabidopsis* genome will facilitate a wide-scale transcriptomic and proteomic analysis of plant PCD and should aid in the discovery of novel genes associated with PCD (The *Arabidopsis* Genome Initiative, 2000). Methods of inducing and examining PCD in the cell cultures will be established, such that further investigations can utilise more than one PCD-inducing stimulus. It is anticipated that this system will allow the genetic response associated with PCD to be dissected away from the specific response to the death-inducing stimulus. In this manner, it will be possible to identify genes and proteins that are generally involved in the core cell death programme and function similarly irrespective of the method of induction.

The *Arabidopsis* cell suspension system will be utilised in analyses to identify transcripts and proteins that are altered in expression levels during plant PCD. Not only have analyses of altered transcript expression patterns been previously used to successfully identify plant cell death genes (see section 1.4.4) but, in the current bioinformatics era, transcriptomics and proteomics are innovative and powerful tools with which to study large numbers of transcripts and proteins simultaneously and to uncover novel molecular targets (Brown and Botstein, 1999; Pandey and Mann, 2000). Importantly, employing both techniques provides a complementary approach since the molecular mechanisms of plant PCD may be regulated at a transcriptional, translational, and/or post-translational level. Following the identification of putative plant PCD genes and proteins, a reverse genetics approach will be utilised to assay the effect of over- or underexpression of genes of interest on plant cell death. These studies should allow for the identification of novel genes and proteins that may be involved in plant PCD — an

approach that has only recently been made possible by exploiting revolutionary genetic resources in the post-genomics era.

CHAPTER TWO

2. Materials and Methods

2.1. *Arabidopsis* cell cultures

2.1.1. Cell culture maintenance

Arabidopsis thaliana var Landsberg Erecta suspension cultures (May and Leaver, 1993) were maintained in Murashige and Skoog medium (Murashige and Skoog, 1962) containing 0.5 mg/L naphthaleneacetic acid, 0.05 mg/l kinetin, and 3% (*w/v*) sucrose, and adjusted to pH 5.75 with NaOH (At3 medium). Cell suspensions were subcultured every 7 days by pipetting 10 ml of the suspension into 100 ml of fresh At3 medium in a 250 ml conical flask. Suspension cultures used as stocks for weekly subculturing were maintained in a controlled environment chamber: 24°C constant temperature, light intensity of ~110 $\mu mol \cdot m^{-2} sec^{-1}$ supplied by 2-foot fluorescent tubes during a 16 h photoperiod, and incubated on a rotary shaker at 150 rpm. Suspension cultures used for experimental purposes were subcultured from cultures in the above conditions and grown in a controlled environment chamber: 24°C constant temperature, 24 h darkness, and incubated on a rotary shaker at 150 rpm.

2.1.2. Measuring packed cell volume

Packed cell volume was measured by transferring cells into 2×50 ml sterile conical tubes. Cultures were centrifuged at 500 *g* for 3 min and the total packed cell volume measured. Cultures were then transferred back to the conical flask under sterile conditions and returned to the rotary shaker.

2.2. Chemicals

Chemicals, including Murashige and Skoog medium, were purchased from Sigma-Aldrich or Merck, unless otherwise specified.

2.3. Analysis and induction of PCD

2.3.1. Scoring of PCD morphology

To score the viability of cell suspension cultures, 20 µl of suspension culture was removed with a cut pipette tip and mixed with an equal volume of fluorescein diacetate (FDA; Molecular Probes) diluted in At3 media. FDA was used at a final concentration of 0.002% (*w/v*) according to the manufacturer's protocol, with an excitation wavelength of 490 nm and an emission wavelength measured at 515 nm. Condensed and shrunken cells were scored using phase-contrast microscopy and cell viability confirmed by the presence of FDA staining.

2.3.2. Detection of DNA laddering

Oligonucleosome-sized DNA fragments were visualized by fractionating 10 µg of genomic DNA (see section 2.4.1) by electrophoresis on 1.5% (*w/v*) agarose gels stained with ethidium bromide following standard procedures (Sambrook and Russell, 2001).

2.3.3. Heat treatment

Six day-old cell cultures were placed in a shaking water bath (150 rpm) at the appropriate temperature for the specified time period, and then returned to the rotary shaker at 150 rpm (24°C).

2.3.4. Menadione treatment

Menadione (2-methyl-1,4-naphthoquinone) dissolved in 95% (*v/v*) ethanol was added to 6 day-old cell cultures at a final concentration of 400 µM. Control cultures were treated with an equal volume of 95% (*v/v*) ethanol.

2.3.5. Measurement of medium carbohydrate concentration by Anthrone Reagent

One ml of culture medium was removed and centrifuged at 1,500 *g* at room temperature for 1 min to pellet cells. An aliquot of the supernatant was incubated in 1 ml of Anthrone Reagent (9(10H)-Anthracenone) (1.4 g/L in 85% (*v/v*) H_2SO_4) at 40°C for 20 min. The sample was centrifuged at 12,000 *g* at room temperature for 5 min. 200 µl aliquots of the sample were transferred to a microtitre plate and the absorbance read at 620 nm. The samples were compared against a calibration curve of 0 to 100 nmol sucrose.

2.3.6. Induced starvation and culture rescue

Six day-old cell cultures were filtered through sterile 177 µM nylon meshes (Plastok, Wirral, UK), washed with 50 ml of the respective medium into which they were to be transferred, and then scooped with a sterile spatula into either fresh At3 medium, At3 medium lacking sucrose, or At3 medium lacking sucrose but containing 88 mM sorbitol. Cultures 'rescued' on various days were either transferred by a similar method into fresh At3 medium, or sucrose was added directly to a final concentration of 88 mM.

2.4. Nucleic Acids

2.4.1. Isolation of genomic DNA

Total DNA used for the detection of oligonucleosome-sized cleavage of DNA was isolated as follows: Cells were harvested, the medium removed by filtration and snap-frozen in liquid nitrogen before being stored at -80°C. 0.5 to 1.0 g of tissue was ground to a fine powder under liquid nitrogen. The frozen powder was added to 15 ml extraction buffer (100 mM Tris-HCl, pH 8.0; 50 mM EDTA, pH 8.0; 500 mM NaCl; 10 mM β-mercaptoethanol), followed by the addition of 1 ml 20% (*w/v*) sodium dodecyl sulphate. After incubation at 65°C for 10 min, 5 ml 5 M potassium acetate was added and the sample was incubated on ice for 20 min. The proteins were pelleted by centrifugation at 17,400 *g* at 4°C for 20 min. The supernatant was filtered through Miracloth (Calbiochem, Nottingham, UK) into a tube containing 10 ml cold isopropanol and incubated on ice for 30 min. The nucleic acids were pelleted by centrifugation at 17,400 *g* at 4°C for 15 min. The pellet was air-dried, resuspended in 0.7 ml 50 mM Tris-HCl (pH 8.0) and 10 mM EDTA (pH 8.0), transferred to a microfuge tube, and centrifuged at 12,000 *g* at 4°C for 5 min to remove non-dissolved debris. 1/10 volumes 3 M sodium acetate (pH 5.2) and 2/3 volumes cold isopropanol were added to the supernatant, and the samples were incubated on ice for 15 min. The nucleic acids were pelleted at 12,000 *g* at 4°C for 10 min, dried, and resuspended in TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0). RNA was digested in the presence of 0.2 mg/ml RNase A at 37°C for 30 min. DNA concentration and quality was determined spectrophotometrically by scanning the absorbance of the sample from 220 nm to 300 nm.

2.4.2. Isolation of genomic DNA for PCR analysis

For high-throughput isolation of plant genomic DNA to be used in PCR analysis only, the following procedure was used: One rosette leaf (1 to 2 cm in length) was ground for 10 sec with a mini pestle in a 1.5 ml microfuge tube. 200 µl of extraction buffer (200 mM Tris-HCl, pH 7.5; 250 mM NaCl; 25 mM EDTA, pH 8.0; 0.5% (*w/v*) SDS) was added and the sample ground for a further 10 sec, followed by vortexing for 5 sec. At this point, samples were left at room temperature for up to one hour while other samples were extracted. When all samples had been extracted, cellular debris was pelleted by centrifugation at 12,000 g at room temperature for 3 min. 150 µl of the supernatant was transferred to a fresh microfuge tube containing 150 µl isopropanol, mixed briefly, and incubated at room temperature for 2 min. Nucleic acids were then pelleted by centrifugation at 12,000 g at room temperature for 5 min. The pellet was air-dried and resuspended in 100 µl TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0), and the samples then centrifuged at 12,000 g at room temperature for 1 min to remove non-dissolved debris. In a standard 50 µl PCR reaction, 2.5 to 5 µl of the sample was used as a template. For the attempted PCR-based analysis of SAIL T-DNA insertional mutants (see section 8.2.2), the T-DNA-specific primers used were as described by McElver *et al.* (2001):

LB1, 5'-GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC-3'

QRB1, 5'-CAAACTAGGATAAATTATCGCGCGGTGTCA-3'

RB1, 5'-ATTAGGCACCCCAGGCTTTACACTTTATG-3'.

2.4.3. RNA isolation

Total RNA was isolated from cell cultures using a TRIzol method obtained on the *Arabidopsis* Functional Genomics Consortium website (http://afgc.stanford.edu).

Cells were harvested, the medium removed by filtration and snap-frozen in liquid nitrogen before being stored at -80°C. 1.0 g of tissue was ground to a fine powder under liquid nitrogen. The frozen powder was added to a 30 ml plastic screw-cap centrifuge tube containing 15 ml TRIzol reagent (38% (*v/v*) phenol; 0.8 M guanidine thiocyanate; 0.4 M ammonium thiocyanate; 0.1 M sodium acetate, pH 5.2; 5% (*v/v*) glycerol). Samples were incubated at room temperature for 5 min, and then homogenised for 15 sec with a polytron. Cell debris was pelleted by centrifugation at 12,000 *g* at 4°C for 10 min. The supernatant was decanted into a new screw-cap tube and the pellet discarded. To achieve phase separation, 3 ml of chloroform was added, the tubes were vortexed for 15 sec, incubated at room temperature for 3 min, and then centrifuged at 10,000 *g* at 4°C for 15 min. Following centrifugation, the upper aqueous phase was carefully pipetted into a clean screw-cap centrifuge tube and the interphase and lower phase discarded. RNA was precipitated by adding 0.5 volumes of the aqueous phase each of isopropanol and 0.8 M sodium citrate/1.2 M sodium chloride. Tubes were covered, mixed by gentle inversion, and incubated at room temperature for 10 min.

Precipitated RNA was pelleted by centrifugation at 10,000 *g* at 4°C for 10 min. The RNA pellet was then washed with 20 ml of 75% (*v/v*) ethanol, vortexed briefly, and centrifuged at 10,000 *g* at 4°C for 10 min. The supernatant was decanted and the pellet air-dried for 5-10 min. The pellet was resuspended in 250 µl DEPC-ddH_20 by pipetting up and down and incubating at 55°C for 5 min. The sample was transferred to a microfuge tube and centrifuged at 12,000 *g* at 4°C for 5 min to remove non-dissolved debris. The supernatant was transferred to a new microfuge tube and the RNA concentration and quality was determined spectrophotometrically by scanning the absorbance of the sample from 220 nm to 300 nm. RNA integrity was further assayed by

separating 3 to 5 µg of RNA on a 1.0% (*w/v*) agarose gel stained with ethidium bromide following standard procedures (Sambrook and Russell, 2001).

For RNA isolation from protoplast suspensions, 3 volumes of mannitol/W5 (0.4 M mannitol; 1 mM glucose; 30 mM NaCl; 25 mM $CaCl_2$; 1 mM KCl; 0.3 mM MES; adjusted to pH 5.7 with KOH) was added to the protoplast suspension culture and protoplasts pelleted by centrifugation at 500 *g* at room temperature for 10 min. The supernatant was aspirated and the protoplasts snap-frozen in liquid nitrogen. TRIzol reagent (Life Technologies) was used for small-scale RNA isolation according to the manufacturer's protocol, and the final RNA pellet was resuspended in 50 µl ddH_2O.

2.5. Cloning and molecular biology

2.5.1. Standard cloning techniques

Molecular techniques such as subcloning, agarose and polyacrylamide electrophoresis, and blotting of nucleic acids and proteins onto nylon or nitrocellulose membranes were carried out according to the methods described by Sambrook and Russell (2001). All DNA used designed in these studies were synthesised by and purchased from MWG Biotech AG (Ebersburg, Germany).

2.5.2. Expressed Sequence Tags

All *Arabidopsis thaliana* Expressed Sequence Tags (ESTs) were obtained from the *Arabidopsis* Biological Resource Centre (ABRC) at Ohio State University, USA (http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.htm). ESTs were received as stab cultures and handled according to ABRC directions. The sequence of each EST received was independently verified by sequencing the plasmid from the appropriate primer (i.e. T7, T3, SP6, etc.) (see section 2.5.8).

2.5.3. Preparation of cDNA

Unless otherwise specified, all RNA used for cDNA cloning was obtained from healthy, 6 day-old cell cultures. RNA was first DNase-I treated to digest any contaminating genomic DNA. 1x React 4 Buffer (Life Technologies), 1 mM DTT, 10 u RNasin (Promega), 50 µg total RNA, and 50 u RNase-free DNAse-I (Boehringer) were mixed to a final volume of 100 µl and incubated at 37°C for 30 min. RNA was then recovered by phenol-chloroform extraction and isopropanol precipitation overnight at -20°C. RNA was pelleted by centrifugation at 12,000 *g* at 4°C for 20 min, washed in 500 µl 75% (*v/v*) ethanol, dried briefly at room temperature, and resuspended in 15 µl

DEPC-ddH_2O. RNA concentration was determined spectrophotometrically by absorbance at 260 nm, and 10 µg of DNase-treated RNA was then used for cDNA synthesis. 0.5 µg oligo$(dT)_{12-18}$ (Amersham) and 10 µg RNA were mixed with 10 mM each of dATP, dCTP, dTTP, and dGTP (Amersham) and incubated at 65°C for 5 min to denature the RNA. After a brief chilling on ice, 1x First Strand Buffer (Life Technologies), 10 mM DTT, and 5 u RNasin (Promega) were added to a final volume of 19 µl and incubated at 42°C for 2 min, and then 1 µl (200 u) Superscript II (Life Technologies) was added. The reaction was incubated at 42°C for 60 min, and subsequently heat inactivated by incubation at 70°C for 10 min.

2.5.4. Amplification of cDNAs using RT-PCR

cDNAs were amplified using RT-PCR and, unless otherwise specified, the cDNA source was that isolated from 6 day-old cell cultures, and then diluted 10 times from the original synthesis reaction. 1 µl cDNA was used as a template, and primers were used at a concentration of 0.1 µM to 0.4 µM. Specific cDNAs were typically amplified in 30 rounds of PCR (94°C, 3 min; [94°C, 30 sec; 50-55°C, 30 sec; 72°C, 1 min] x 30; 72°C, 3 min).

2.5.5. Competitive RT-PCR

Ten µg of DNase-treated RNA was used for Tag 10 and Tag 50 (Ambion) cDNA synthesis to compare the abundance of transcript levels in RNA isolated from either control or PCD-induced (heat or senescence) cell cultures. 1 µl Tag 10 (control RNA) or Tag50 (heat and senescence RNA) oligo(dT) primer and 10 µg RNA were mixed with 10 mM each of dATP, dCTP, dTTP, and dGTP and denatured at 65°C for 5 min. Tag10/Tag50 cDNA synthesis was carried out in a final volume of 20 µl (1x First Strand

Buffer (Life Technologies); 10 mM DTT; 5 u RNasin (Promega); 1 µl (200 u) Superscript II (Life Technologies)) at 42°C for 60 min. This generated cDNA populations which differed by 40 bp. RT-PCR was carried out simultaneously with 1 µl control Tag10 cDNA and 1 µl Tag50 heat or Tag50 senescence cDNA ('competitive RT-PCR') in a 25 µl volume using standard PCR techniques with 35 rounds (94°C, 3 min; [94°C, 30 sec; 55°C, 30 sec; 72°C, 1 min] x 35; 72°C, 3 min). Primers used for each RT-PCR reaction consisted of a Tag-sequence-specific primer (Ambion) and a gene-specific primer to generate a PCR product between 250 and 500 bp. Gene-specific primer sequences were:

β-tubulin, 5'-AGTTCACTGAAGCAGAGAGTA-3'

Athsr5, 5'-GCCCGATTTGCTTGACGAAT-3'

KNAT2, 5'-GGGCATCATCGCGAATGAAT-3'

SRG3, 5'-ATGAAGCCATCCAACAGAGAT-3'

Athsr2/VDAC, 5'-GGGATTGCTCTCGCTCTCAA-3'.

15 µl of the RT-PCR reaction was separated on a 2.0% agarose gel (*w/v*) stained with ethidium bromide following standard procedures (Sambrook and Russell, 2001). The intensity of each Tag10 and Tag50 RT-PCR product was measured using a Fluor-S Imager and Multi-Analyst Software (Bio-Rad). The Tag50/Tag10 ratio for each transcript was calculated and normalised to that of a *β-tubulin* RT-PCR reaction carried out in parallel.

2.5.6. Electro-transformation of *E. coli*

Electro-transformation of *E. coli* XL1Blu competent cells was carried out using a Gene Pulser (Bio-Rad) according to the manufacturer's protocols. Voltage was set at 2.5 kV and the pulse controller set to a resistance of 200 Ω. Briefly, 1 to 10 ng of purified

plasmid or 3 to 4 µl of ligated, dialysed plasmid was mixed with 40 µl competent cells and incubated on ice for 30 sec. The mixture was then transferred to a cold electroporation cuvette and tapped gently. The cuvette was inserted into the cuvette slide chamber and placed in contact with the chamber electrodes, and a pulse delivered. After ensuring that the time constant was 4 to 5 msec, 1 ml SOC medium (Sambrook and Russell, 2001) was added to the cuvette and mixed briefly by pipetting. The cell suspension was then transferred to a 15 ml conical tube and incubated at 37°C for 1 h while shaking. 10 to 100% of the suspension (depending on the expected efficiency of the ligation reaction) was plated on selective Luria-Bertani (LB) medium (Sambrook and Russell, 2001) at 37°C for 16 h.

2.5.7. Plasmid purification and glycerol stocks

In all cases where plasmid preparation was required, single bacterial colonies on selective LB medium were used. 5 ml selective LB medium was inoculated with a single colony using a toothpick under sterile conditions. The cultures were grown at 37°C for 16 h (overnight) with shaking, and 3 ml of the culture was used for plasmid purification using the Plasmid Mini-Prep kit (Qiagen) according to the manufacturer's protocols. For large-scale plasmid preparations to be used in protoplast transformations, 600 µl of overnight culture was used to inoculate 300 ml selective LB medium and the culture grown for 16 h at 37°C with shaking. A Plasmid Maxi-Prep kit (Qiagen) was used to purify plasmid DNA according to the manufacturer's protocols. For the production of glycerol stocks, 800 µl of bacterial culture was added to 200 µl of glycerol and snap-frozen in liquid nitrogen before being stored at -80°C.

2.5.8. DNA sequencing

All sequencing reactions for plasmids were carried out as follows: 0.3 to 0.5 µg of purified plasmid, 5 pmol of the appropriate primer, 4 µl of Big Dye (ABI), and 4 µl Big Dye Dilution Buffer (200 mM Tris-HCl, pH 9.0; 5 mM $MgCl_2$) were mixed to a final volume of 20 µl. PCR cycle sequencing was achieved in 25 rounds (96°C, 1 min; [96°C, 20 sec; 50°C, 10 sec; 60°C, 4 min] x 25). Post-sequencing precipitation of the DNA was carried out by adding 2 µl 3 M sodium acetate (pH 5.2) and 50 µl 95% (*v/v*) ethanol to the sequencing reaction. The sample was incubated at room temperature for a maximum of 15 min, and DNA pelleted by centrifugation at 12,000 *g* at room temperature for 20 min. The supernatant was immediately removed and the pellet washed with 200 µl of 70% (*v/v*) ethanol, followed by centrifugation at 12,000 g at room temperature for 3 min. The supernatant was discarded and the sample dried by speed-vacuum for approximately 5 min. The pellet was then resuspended in 1 µl ddH_20 and stored at 4°C until sequencing. Sequencing reactions were performed in an ABI 310 sequencer according to the manufacturer's protocols as a service run by the Department of Plant Sciences (University of Oxford, UK).

2.6. Construction of a custom cDNA microarray

2.6.1. Preparation of target DNA

2.6.1.1. PCR of target DNA

Target DNAs for spotting on the microarray were amplified using standard PCR techniques. Template plasmids were either Expressed Sequence Tags (ESTs) clones obtained from the *Arabidopsis* Biological Resource Centre (Ohio State, USA), cloned cDNAs obtained as gifts from colleagues, or independently-cloned cDNAs. For ABRC plasmids and cDNAs received from colleagues that were already cloned into standard plasmid vectors (e.g. pBluescript), target DNAs were amplified using universal plasmid primers (e.g. T3, T7). For cDNAs that were cloned from *Arabidopsis* cDNA (see section 2.5.3) the primer combinations are listed in Table 2.1. These cDNAs were cloned directly into the pGEM-T Easy Vector System (Promega). For cDNAs that were received as gifts from colleagues, the cDNA and donating researcher is listed in Table 2.2.

Briefly, 100 µl PCR reactions were set-up containing 1x PCR Buffer (Sigma), 10 mM each dNTP (Amersham), 0.1 µM each primer, 2 ng template plasmid DNA, and 5 u Taq DNA polymerase (Sigma). Target DNA was amplified in 40 rounds of PCR (94°C, 3 min; [94°C, 30 sec; 40°C, 30 sec; 72°C, 1 min] x 40; 72°C, 3 min). 3 µl of each PCR reaction was then run on a 1.0% (*w/v*) agarose gel stained with ethidium bromide following standard procedures (Sambrook and Russell, 2001) to verify successful amplification of target DNA.

2.6.1.2. PCR product purification

PCR products were purified using a PCR purification kit (Qiagen) according to the manufacturer's protocols, with the exception that products were eluted by adding 30 µl ddH_20 to the column, incubated at room temperature for 5 min, centrifuged at 12,000 *g* at room temperature for 5 min, and the eluate was then reapplied to the column and centrifuged again to ensure maximum elution. 1 µl of the eluate was run on a 1.0% (*w/v*) agarose gel stained with ethidium bromide following standard procedures (Sambrook and Russell, 2001) alongside 1 kb and 100 kb molecular weight markers (New England Biolabs) to ensure that the final concentration of each PCR product, as estimated by visual comparison with the DNA marker, was at least 100 ng/µl.

2.6.2. Printing of arrays

Microarrays were printed on a BioRobotics MicroGrid arrayer according to the manufacturer's instructions. Arrays were printed on poly-lysine coated microscope slides (BDH) and each target DNA was spotted in replicate 4 times. In total, there were 16 blocks per slide, with each block containing 10 rows, and each row containing one replicated DNA or control. Each spot, or 'feature' on the array, was approximately 200 µm in diameter, and the spacing between spots (from centre to centre) was approximately 400 µm. The purified PCR products were prepared in 1x spotting buffer (150 mM sodium phosphate, pH 8.5; 0.01% (*w/v*) SDS) on a 384-well microtitre plate.

2.6.3. Post-processing of arrays

Once printed, DNA was fixed to the slides by crosslinking with UV-C light in a crosslinker (Stratagene) set to 650 (x100) J. After fixation, the slides were chemically treated to block the remaining binding sites not covered by the DNA array and to denature the DNA array on the slide surface. The position of the array was first marked

on the slide using a diamond pen (since the array is no longer visible after treatment). 6 g of succinic anhydride was dissolved in 325 ml of 1-methyl-2-pyrolidinone, and 15 ml of 0.1 M sodium borate (pH 8.0) was added. The slides were immediately plunged into this solution using a slide plunger and shaken for 15 min on a rotary shaker. The slides were then immersed in a 95°C water bath for 2 min and then in 95% (*v/v*) ethanol for 1 min. The slides were then air-dried and stored in a dark slide box.

Table 2.1. Primer combinations for cloning cDNAs used in custom microarray.

Gene	Genbank Accession ID	5' Primer (5' → 3')	3' Primer (5' → 3')
cytochrome c	AF000657	GTCGTTTGATGAAGCACCAC	TCCTTCAAGTAGGCGATGAT
ANT1 (*adenine nucleotide translocase*)	X65549	TCAAGTTCAGCACCCCACTA	CAGAAAGTCAAGGGCAAAGT
CSD1 (*Cu/Zn superoxide dismutase*) (cytosolic)	X60935	GGGTGTTACGGGGACTATCT	GACAACAACAGCCCTACCAA
CSD3 (peroxisomal)	AF061520	ACATTTCCGGAACTACTCAT	TACTGCCCACTAAGCGGTAT
MSD1 (*Mn superoxide dismutase*) (mitochondrial)	AF061518	GTAGTGCCATTGACGCTCAC	TTCTCATAAACCTCGCTTGC
AtBI-1 (*At Bax-Inhibitor 1*)	AF208124	TTAGCAAGACGCAGGGAGTA	GAGAATCCGAACAAACACAG
SRG1 (*Senescence-Related Gene 1*)	X79052	GTTCCTGTTAAACCCCTCCC	AAGCTTTTCCGTCGAGAGTA
SRG2C (*Senescence-Related Gene 2* C-term)	X82624	ATATTACGCATGGTCATTGA	TTAAACCATTTGACCGAATC
SRG2N (*Senescence-Related Gene 2* N-term)	X82623	GATCGGCATAGTTTTCCTGA	CGATGATAAAAATCAATTGC
SRG3 (*Senescence-Related Gene* 3)	X98376	TGAATCATGATAGCAGCACC	CATACGGAAAGCTAAACAAT
unknown	AF069442	TACTGCAGGAAGAGCTCAAGAG	TTACCCGCGAGCAAAACGAG
unknown	AC005278	AAGTGCATCAGAATCGCTCACG	AAGAACTCGCTCCAAAGACACG

Primers were used to amplify specific cDNA fragments from total cDNA synthesised from 6 day-old *Arabidopsis* cell suspension cultures, typically using 30 rounds of PCR and standard PCR techniques ((see section 2.5.4).

Table 2.2. Souce of cloned cDNAs used in custom microarray that were obtained as gifts from colleagues.

Gene	Genbank Accession ID	Researcher
At1-MMP (*matrix metalloproteinase*)	Z97341	Ian Clark (University of East Anglia, UK)
At2-MMP	AC002062	Ian Clark
At3-MMP	AC002396	Ian Clark
At4-MMP	AF062640	Ian Clark
At5-MMP	AC005966	Ian Clark
AtPUMP/UCP1 (*uncoupling protein*)	AJ223983	Akio Watanabe (Aktia Prefectural University, Japan)
UCP2	AB021706	Akio Watanabe
ANT2 (*adenine nucleotide translocase*)	X68592	Wolfgang Schuster (Freie University Berlin, Germany)
VDAC/Athsr2	AJ131391	Dominique Roby (INRA Toulouse, France)
AOX/Athsr3 (*alternative oxidase*)	AJ131392	Dominique Roby
Atp1 (*ATP synthase complex, subunit α*) (mitochondrial)	Y08502	Philippe Giegé (University of Ulm, Germany)
gsh2 (*glutathione synthetase*)	Z50153	Chris Leaver (University of Oxford, UK)
Athsr5	AJ243378	Dominique Roby
Athsr6	AJ243379	Dominique Roby
Athsr7	AJ243380	Dominique Roby
malate synthase	T04620	Steve Smith (University of Edinburgh, UK)
thiolase	AB008854	Steve Smith
isocitrate lyase	Z18772	Steve Smith
C4H (*cinnimate-4-hydroxylase*)	U71081	Clint Chapple (Purdue University, USA)

2.7. Probing of cDNA microarrays

2.7.1. Probe preparation

50 µg of total RNA was reverse transcribed to synthesise either Cy3- or Cy5-dCTP-labeled cDNA probe. Briefly, 0.5 µg oligo $d(T)_{12-18}$ (Amersham) and 50 µg RNA were mixed to a final volume of 29 µl with ddH_20. The mixture was heated at 65°C for 5 min to denature the RNA and then immediately cooled on ice. After brief centrifugation to collect the sample, a master mix containing 9 µl 5x First Strand Buffer (Life Technologies), 2 µl low-C dNTP mix (25 mmol each of dGTP, dATP, and dTTP and 10 mmol dCTP) (Promega), 1 nmol Cy3 or Cy5 dCTP (Amersham), 2 µl 0.1 M DTT, and 15 u RNasin (Promega) were added. The sample was heated at 42°C for 2 min and then 2 µl (400 u) Superscript II (Life Technologies) was added. The reaction was incubated at 42°C for 2 h, and stopped by the addition of 5 µl 100 mM EDTA. Template RNA was hydrolysed by adding 10 µl 1 M sodium hydroxide and incubating at 70°C for 10 min. The reaction was neutralized by adding 10 µl 1 M Tris-HCl (pH 8.0) and 3 µl 2 M HCl. The labelled cDNA was then precipitated by the addition of 5 µl 3 M sodium acetate (pH 5.2), 5 µl yeast tRNA (10 mg/ml) (Life Technologies), and 90 µl isopropanol. RNA was pelleted by centrifugation at 20,000 *g* at room temperature for 30 min. The pellet was resuspended in 9 µl ddH_20, and the Cy3- and Cy5-labeled probes were then combined to a final total volume of 18 µl.

2.7.2. Pre-hybridisation of arrays

Arrays were pre-hybridised by adding 30 µl pre-hybridisation solution (3x SSC; 2.5x Denhardt's Reagent; 1% (*w/v*) BSA; 0.1% (*w/v*) SDS) onto the microarray surface. A clean coverslip was placed on top and the microarray slide was placed into a

hybridisation cassette (Telechem International, USA). 30 µl 3x SSC was aliquoted into each of the sunken channels in the cassette to provide humidity during incubation. The cassette was assembled and the array incubated for 40 min in a 42°C oven. Following incubation, the cassette was dismantled and the slide quickly transferred into a rack immersed in ddH_2O for 1 min. The slide was then removed and allowed to air-dry.

2.7.3. Hybridisation

3.75 µl 20x SSC and 1.25 µl 50x Denhardt's Reagent were added to the purified, combined Cy3- and Cy5-labelled probes. The probe was denatured at 98°C for 2 min. 2 µl of 1% (*w/v*) SDS was added, mixed carefully, and then the entire mixture (24 µl final volume) was aliquoted onto the array and a coverslip quickly placed on top. The slide was placed in the microarray cassette, 30 µl 3x SSC aliquoted into each of the sunken channels, and the cassette assembled. Arrays were hybridised for 18 to 24 h in a 65°C oven.

2.7.4. Slide washing

All washes were performed at room temperature. Following hybridisation, the cassettes were removed from the oven, allowed to cool for a few minutes, and then dismantled. The slide was transferred into a rack immersed in 0.5x SSC/0.01% (*w/v*) SDS for 5 min, and then transferred into a rack immersed in 0.06x SSC for 5 min. The slide was removed, allowed to air-dry, and stored in a light-proof box until ready for scanning.

2.7.5. Microarray scanning and data analysis

2.7.5.1. Capture of microarray data

An Axon GenePix 4000A Microarray scanner and GenePix 3.0 data acquisition software (Axon Instruments, Union City, USA) were used according to the manufacturer's instructions to capture the data. Briefly, the arrays were scanned at a final resolution of 10 μm using laser excitation simultaneously with individual 635 nm and 532 nm lasers at 100% power. These are the optimum wavelengths to excite the fluorophores Cy5 and Cy3, respectively. After the initial scan, the pixel intensity distribution for the Cy3 and Cy5 channels were examined by a histogram analysis. PMT (photo-multiplier tube) settings were evaluated to ensure that neither channel was saturating (above 65535) and that that signal levels in both channels overlapped to give a final ratio of 1.0. If the ratio was not 1.0, PMTs were adjusted accordingly for each wavelength independently and slides were re-scanned until a ratio of 1.0 was achieved. After scanning and capture of the data using the GenePix 3.0 software, the raw data was imported into Microsoft Excel for further analysis.

2.7.5.2. Quality control of microarray data

Several quality control measures were taken for each array independently to determine whether the array was of sufficient quality to continue with further analysis. These measures included determining that the mean and median intensity values at each wavelength for a given feature were nearly identical (giving rise to a line of best fit with slope=1 when the mean intensities of all of the features were plotted against the respective median intensities), since high quality microarrays will yield similar, nearly identical median and mean values. It was checked that no pixels in the array were saturating (i.e. at the maximum 16-bit intensity value of 65535) since such signals

exceed the dynamic range of the detection system and thus cannot be measured accurately.

Unreliable data was also sorted by ensuring that the fluorescence signal at each feature was significantly above background levels, since values close to background were highly variable and the error associated with these values could give rise artificially high or low ratios. To this end, data points where expression was not greater than two times above the overall background for at least one channel were discarded. Background fluorescence values at each wavelength were also calculated for the buffers and blanks in each block independently and checked to be equal to or less than the local background, thus ensuring that no fluorescence was being obtained from the buffer solution itself. Genes which exceeded the threshold of only one wavelength were further examined to determine if the data could be considered reliable, particularly noting if the coefficient of variation of fluorescence for the 3 replicates of each gene did not vary by more than 10%. At all stages, the data was manually checked and the array visually inspected to identify any unreliable features.

Generally, it is noted that the feature ratio can be calculated by three different methods: Ratio of Medians (the ratio of the background subtracted median pixel intensity in one channel, to the background subtracted median pixel intensity in the second channel), Median of Ratios (the median of the pixel-by-pixel ratios of pixel intensities that have had the median background intensity subtracted), and the Regression Ratio (the relationship between wavelength 1 and wavelength 2 determined by computing a linear regression between these two populations of pixels and taking the slope of the line of best fit to be the regression ratio). The latter ratio does not use background subtraction, and is therefore an independent ratio calculation compared with the former two ratios which do subtract background intensities. It was noted in the

GenePix instructions that, if a feature has similar values using all three ratio calculations, then the value for that feature may be considered reliable. Thus, any genes of interest were examined by all three methods to ensure that these values corresponded. Additionally, the standard deviation of the intensity ratios of all pixels within a feature was checked and confirmed to be less than 2 in all features of interest. Therefore, only features derived from high quality data, as judging by the criteria described, were considered for further analysis.

2.7.5.3. Identification of changes in steady-state transcript levels

To initially identify genes of interest whose steady-state transcript levels changed significantly in the PCD versus control samples, the ratio of each feature (normalised 635 nm/532 nm) was calculated. Background fluorescence values were automatically calculated by the GenePix program and subtracted from all feature intensities before further calculations were performed. The method used by GenePix to calculate background intensities involves measuring an area around each feature with 3 times the diameter of the feature itself, and then using this entire area to compute the background while excluding any pixels that fall within a neighbouring feature. In all calculations, median values were used since, in microarray analysis, median intensities are preferable to mean intensities as the median is less affected by extreme values that could be the result of dust, contaminants, or other artefacts. Furthermore, it was determined that the array analysis should exclude the first feature in each row of 4 replicates per gene. This is due to the fact that the first feature in each row consistently appeared to have the most variable fluorescence intensities, perhaps owing to carry-over from the gene in the row below due to inadequate washing of the pins when printing the array slides. Thus, all calculations hereafter use data derived only from features 2, 3 and 4 for each gene.

Normalisation of fluorescence intensities between channels was achieved in the following manner: The total fluorescence value for the array was summed for each wavelength, and the individual wavelength fluorescence at each feature expressed as a percentage of the total fluorescence. The ratio of the 'percentage fluorescence' at 635 nm and 532 nm was then calculated, and a mean ratio value for the 3 replicates of each DNA spot was obtained. This method intrinsically normalizes the data to account for overall differences in fluorescence between Cy3 and Cy5, owing to different efficiencies in labelling, hybridisation, etc. This method of normalisation yielded reproducible ratios in subsequent experiments.

After final ratios had been determined for each gene, those transcripts with expression ratios greater than 1.5 or less than 0.667 (PCD/control) were taken to be significant changes in gene expression. Finally, array sets for both PCD-inducing treatments (heat treatment at 55°C for 10 min and during 13 to 14 day-old culture senescence) compared to the control (6 day-old cultures), were repeated in triplicate. One repetition included a reverse labelling of the Cy3 and Cy5 probes. Ratios of <1 were transformed to a -1/ratio, and the ratio values obtained for each gene were then averaged across the 3 replicates. It was ensured that the correlation coefficient (R^2) between each replicated experiment was significant ($p<0.05$) (see Table 4.1).

2.8. Affymetrix GeneChip analysis

GeneChip hybridisations and data acquisition were carried out by the GARNet Transcriptomics Facility (NASC, York, UK). Two replicates were carried out for each of the control, heat, and senescence samples, using total RNA pooled from 3 different cultures for each sample. Hybridisations were normalised globally using a scaling factor and final normalised data was received in a Microsoft Excel spreadsheet format. For each transcript, the following data was available: Signal value, indicating the level of transcript expression; Detection Call, indicating whether the transcript was expressed or not, for which the transcript is either Present (1), Absent (-1), or Marginal/too close to say (0); and a detection p-value, giving the probability that the amount of expression occurred through random chance. The signal value is a quantitative measure of transcript expression ("how much has this transcript been expressed?"), while the detection call is a qualitative measure of transcript expression ("has this transcript been expressed or not?").

To determine the reproducibility of the data, a correlation coefficient was calculated between the duplicate experiments. Additionally, a false positive rate was calculated based on the percentage of genes showing significant fold-changes (≥2.5) in duplicate data sets (see section 5.2.3 and Table 5.1).

Fold-changes for each transcript steady-state level were calculated for each of the duplicate PCD samples (heat or senescence) compared to each of the duplicate control samples in a pairwise fashion, generating 4 comparisons for each of the PCD-inducing treatments. The logarithm (base 2) of the fold changes for each transcript were calculated and used for data analysis.

2.9. Two-dimensional gel electrophoresis

2.9.1. Protein isolation

Total cellular protein extracts were isolated using a phenol method. Cells were harvested, the medium removed by filtration and snap-frozen in liquid nitrogen before being stored at -80°C. 1.0 to 2.0 g of cells that were snap-frozen in liquid nitrogen were homogenised under liquid nitrogen into a fine powder. Cell powder was transferred to a chilled mortar and pestle and homogenised in one volume of ice-cold extraction medium (0.5 M Tris-HCl, pH 7.5; 10 mM EDTA, pH 8.0; 2% (*v/v*) β-mercaptoethanol). Cell debris was pelleted by centrifugation at 20,000 *g* at 4°C for 5 min. Protein concentration of the supernatant was measured using a Bradford's assay (Coomassie Plus Reagent (BDH)), and protein samples (800 µg) were phenol-extracted by adding an equal volume of ice-cold 0.5 M Tris-HCl (pH 8.0)-saturated phenol and vortexing for 10 sec. The sample was centrifuged at 20,000 *g* at 4°C for 1 min, and approximately 80% of the upper aqueous phase was removed by pipetting, leaving the interface intact. The organic phase was re-extracted with an equal volume of extraction medium (as above) and centrifuged at 20,000 *g* at 4°C for 1 min. After removing approximately 80% of the aqueous phase, proteins were precipitated by addition of 5 volumes of 0.1 M ammonium acetate in methanol to the interphase / organic phase and incubated overnight at -20°C. Proteins were pelleted by centrifugation at 20,000 *g* at 4°C for 5 min and the supernatant discarded. The pellet was washed with 1 ml 0.1 M ammonium acetate in methanol and subsequently with 1 ml 80% (*v/v*) acetone. The supernatant was pipetted off and samples allowed to air-dry for approximately 30 min.

2.9.2. First- and second-dimension gel electrophoresis

Protein pellets were resuspended in 330 µl IEF sample buffer (8 M urea; 2% (*w/v*) CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate); 2% (*v/v*) IPG buffer, pH 3-10 non-linear (AP Biotech); 0.001% (*w/v*) bromophenol blue). Non-resuspended debris was pelleted by centrifugation at 20,000 *g* at room temperature for 5 min. Aliquots of 330 µl were used to re-swell dried 180 mm, pH 3-10 non-linear IPG strips (Immobiline DryStrips, AP Biotech) overnight. Isoelectric focusing was performed for 19.5 h reaching a total of 49 kVh at 20°C on a flat-bed electrophoresis unit (Multiphor II, APBiotech) according to the manufacturer's directions. IPG strips were then transferred to an equilibration buffer consisting of 50 mM Tris-HCl (pH 6.8), 4 M urea, 2% (*w/v*) SDS, 0.001% (*w/v*) bromophenol blue and 100 mM β-mercaptoethanol and incubated for 20 min with gentle rocking.

The equilibrated strips were then slotted into central single wells of 4% (*w/v*) acrylamide stacking gels above 0.1 cm x 18.5 cm x 20 cm, 12% (*w/v*) acrylamide, 0.1% (*w/v*) SDS-polyacrylamide gels, prepared according to standard procedures. Strips were overlaid with 0.5% (*w/v*) low-melting-point agarose in SDS-PAGE running buffer. Gel electrophoresis was performed at constant power of 5 W for 20 min followed by 200 W for 5 h with circulating cooling to maintain a temperature of 28°C. Gels were fixed by incubation for 1 h in 500 ml 12% (*w/v*) trichloroacetic acid with gentle rocking.

A 10% (*w/v*) stock solution of Coomasie G250 Brilliant Blue was prepared by dissolving 10 g in 7.5% (*v/v*) acetic acid at 70°C. Ammonium sulphate was added slowly whilst stirring until the solution turned clear (reached before ~30% (75 g)). The solution was cooled to room temperature and the supernatant discarded. The Coomassie 'bolus' was then dissolved in 40 ml ddH_2O, 50 ml 95% (*v/v*) ethanol, and 10 ml acetic acid, to give a final stock concentration of 10% (*w/v*). Proteins were visualised by

staining gels in 500 ml diluted colloidal Coomassie (G250) stain (0.1% (*w/v*) colloidal Coomassie G250; 3% (*v/v*) orthophosphoric acid (85% *v/v*); 6% (*w/v*) ammonium sulphate) in 500 ml stain overnight with gentle rocking. Gels were rinsed for 10 min in 25% (*v/v*) methanol to clean the surface and subsequently stored in distilled water at 4°C.

2.9.3. Gel scanning and data analysis

An Epson Twain 32 scanner was used to capture the gel image at a resolution of 300 dpi. Z3 (Compugen, Tel Aviv, Israel) 2-dimensional gel analysis software was according to the manufacturer's instructions. Spot detection, normalised quantification, and gel matching were performed using algorithms contained within the software (Smilansky, 2001). Gels of cellular proteins obtained from cultures undergoing PCD (heat-treated and senescing) were matched to a control gel of 6 day-old control cultures and spots aligned. Spots identified as being upregulated and/or unique in both PCD-inducing treatments were identified and cut out for MALDI-ToF mass spectrometry peptide fingerprint analysis. Each gel was repeated in triplicate from independently isolated protein samples derived from cultures maintained on different weeks.

2.9.4. Tandem mass spectrometry for peptide sequence analysis

In-gel digestion and tandem mass spectrometry (MS/MS) (Q-ToF) were performed as a service by the GARNet Proteomics Facility (York, UK) using a Micromass ToFSpec 2E machine. Protein spots of interest were excised from gels and placed in wells of a 96 titre-plate. 50 µl destain solution (50% (*v/v*) acetonitrile, 25 mM NH_4HCO_3) was added to samples for 45 min, removed, and replenished once. Destained gel slices were dried at 50°C for 20 min and digested at 37°C overnight in 10 µl 25 mM

NH_4HCO_3 containing 12.5 µg/ml trypsin. 10 µl acetonitrile, containing 1% (*v/v*) trifluoroacetic acid, was added to each gel slice and incubated for 15 min.

2.9.5. Identification of database entry matches and sequence analysis

CID mass spectra were searched against predicted fragment ion masses derived from the translated NCBI NR genomic database using Mascot software (www.matrixscience.com). Protein identity was based on a cumulative probability-based MOWSE score for analysed peptides. All proteins were then cross-matched by comparison of the predicted MW and pI of the identified protein with the observed molecular weight and pI of the excision site on 2-D gels. Predicted molecular mass and pI were determined using the ProtParam program on the ExPASy website (http://au.expasy.org) (Bjellqvist *et al.*, 1993).

2.10. Proteins

2.10.1. Western blot analysis

Protein samples were separated by SDS-PAGE (12-15% (*w/v*) acrylamide) according to standard procedures and transferred to nitrocellulose (0.2 µm, Schleicher & Schuell) by semi-dry electroblotting (Bio-Rad). Proteins were visualised by brief staining in 1x Ponceau S and dried between two sheets of Whatmann filter paper. Prior to blocking, membranes were briefly rinsed in ddH_2O and then equilibrated for 5 min in PBS-T (137 mM NaCl; 2.7 mM KCL; 10 mM Na_2HPO_4; 1.5 mM KH_2PO_4; 0.1% (*v/v*) Tween-20). The membrane was blocked against non-specific binding by incubation in 5% (*w/v*) skimmed milk powder in PBS-T (for polyclonal antibodies) or 5% bovine serum albumin (BSA) (*w/v*) in PBS-T (for monoclonal antibodies) overnight at 4°C. The membrane was then incubated with the primary antibody, diluted as indicated, in blocking solution at room temperature for 3 h on a rotary shaker. The membrane was washed 3 times in blocking solution, with a final rinse in PBS-T. The membrane was then incubated with HRP (horseradish peroxidase)-conjugated secondary antibody (Amersham), diluted 1:10,000 in PBS-T, at room temperature for 1 h on a rotary shaker. After 4 washes in PBS-T, labelling was detected by chemi-luminescence (Pierce) according to the manufacturer's protocol. For the antibodies used in these experiments (see Figure 6.3), Thomas Elthon (University of Nebraska, USA) provided the anti-VDAC and the anti-E1α antibodies, and Bernd Müller-Röber (University of Potsdam, Germany) provided the anti-fumarase antibody. The anti-ANT antibody was from the lab of Chris Leaver.

2.10.2. Protease activity

Protease activity in samples was measured using the Universal Protease Substrate (resorufin-labeled casein; Roche Diagnostics Ltd). Briefly, cells were ground to a fine powder in liquid nitrogen and then further ground in 1 volume (ml/g) 0.1 M Tris-HCl (pH 7.5). Cell debris was pelleted by centrifugation at 20,000 *g* at 4°C for 5 min in a microcentrifuge. Protein concentration of the supernatant was measured using a Bradford's assay (Coomassie Plus Reagent (BDH)). Protease assays were carried out in a 200 µl volume with the following: 0.1% (*w/v*) resorufin-labelled casein; 50 mM Tris-HCl, pH 7.5; 5 mM $CaCl_2$; 100 µl cell extract. The reaction was incubated at 37°C for 20 h, and stopped by the addition of 480 µl 5% (*w/v*) TCA with subsequent incubation at 37°C for 10 min. Precipitated casein was pelleted by centrifugation at 20,000 *g* at room temperature for 5 min, and 400 µl of the supernatant added to 600 µl 0.5 M Tris-HCl (pH 8.8). The sample was mixed and the absorbance of the sample read at 574 nm against a blank. Protease activity was calculated as the change in A_{574} per mg of protein over 20 h.

2.11. Measurement of cellular free iron

Cell cultures were washed three times in 10 mM Tris-HCl (pH 7.5) to remove any MS media containing iron salts. Cells were harvested, the medium removed by filtration and snap-frozen in liquid nitrogen before being stored at -80°C. Samples were ground to a fine powder in a mortar and pestle under liquid nitrogen. The powder was resuspended in 1 volume 10 mM Tris-HCl (pH 7.5), 0.6% (*w/v*) SDS and vortexed briefly. Cell debris was pelleted by centrifugation at 20,000 *g* at 4°C for 5 min. The protein concentration of the supernatant was determined using a Bradford's assay (Coomassie Plus Reagent (BDH)). Free iron concentration of sample aliquots was determined with 100 µl 100 mM bathophenanthroline disulfonic acid, 20 µl 1 M dithionite, made to a final volume of 1 ml with ddH_20. The absorbance of each sample at 700 nm was subtracted from the absorbance at 540 nm, and the free iron concentration determined from a standard curve.

2.12. Protoplast preparation and transformation for transient gene expression analyses

2.12.1. Protoplast preparation

Six day-old, dark-grown cell cultures were used for protoplast preparation. 25 ml of cell culture was placed in a sterile 50 ml conical centrifuge tube and cells pelleted by centrifugation at 100 *g* at room temperature for 10 min. The supernatant was removed and replaced with 40 ml filter-sterilised digestion solution (1% (*w/v*) cellulase R-10 [Serva]; 0.25% (*w/v*) macerozyme R-10 [Serva]; 0.4 M mannitol; 3% (*w/v*) sucrose; 8 mM $CaCl_2$; adjusted to pH to 5.7 with KOH) and the cells gently resuspended by rocking. Cells were plasmolysed by incubation in the dark at room temperature for 1 h horizontally without shaking, 30 min gently rocking, followed by 1 h horizontally without shaking. A small sample of cells was removed and checked by light microscopy to ensure that the enzyme digestion had reached completion; if not, cells were incubated for another 10 to 20 min using discretion with each preparation.

Protoplasts were filtered through sterile 70 μm nylon mesh (Plastok, Wirral, UK) to remove clumps. The protoplast/enzyme solution was centrifuged at 100 *g* at room temperature for 10 min. If no pellet was formed, 10 ml sterile mannitol/W5 (0.4 M mannitol; 1 mM glucose; 30 mM NaCl; 25 mM $CaCl_2$; 1 mM KCl; 0.3 mM MES; adjusted to pH 5.7 with KOH) was added and the sample spun again. Subsequently, 20 ml of the supernatant was removed, 30 ml mannitol/W5 solution added, and the pellet resuspended by gentle rocking. Protoplasts were pelleted by centrifugation at 100 *g* at room temperature for 10 min, and as much of the supernatant was removed as possible. Protoplasts were washed twice with 30 ml sterile mannitol/Mg (0.4 M mannitol; 0.1% (*w/v*) MES; 15 mM $MgCl_2$; adjusted to pH to 5.7 with KOH) and finally resuspended

gently in ≤6 ml sterile mannitol/Mg. Samples were stored on ice for 30 to 60 min before transformation.

2.12.2. PEG-mediated protoplast transformation

Plasmid DNA prepared for each transformation was phenol-chloroform extracted and ethanol precipitated a minimum of two times to ensure the purity and cleanliness of the DNA. Transformation was carried out on a petri dish under sterile conditions. 50 µl of each DNA solution, containing 250 µg sheared, sonicated, phenol-chloroform extracted salmon sperm DNA (Life Technologies) and 10 µg of each appropriate plasmid, was placed in a 15 mm diameter ring. To the middle of the DNA ring, 300 µl protoplast suspension (10^7 protoplasts/ml) was added using a wide-bore pipette and mixed gently with the DNA using a swirling motion. After a time period of 60 sec the DNA/protoplast mixture was mixed with 350 µl of filter-sterilised PEG-CMS (0.4 M mannitol; 100 mM $CaNO_3$; 40% (*w/v*) PEG 4000) using a gentle swirling motion. Protoplasts were washed in step-wise dilution manner as follows: 30 min after transformation, 600 µl sterile mannitol/W5 (0.4 M mannitol; 1 mM glucose; 30 mM NaCl; 25 mM $CaCl_2$; 1 mM KCl; 0.3 mM MES; adjusted to pH 5.7 with KOH) was added in a small number of drops around the transformation mixture. At subsequent 15 min intervals, 1 ml, 2 ml, and finally 4 ml of sterile mannitol/W5 was added in similar fashion. The washed protoplasts were then collected in a 15 ml sterile conical tube and placed vertically on ice for 30 min to allow the protoplasts to settle to the bottom of the tube. The supernatant was removed and protoplasts resuspended in approximately 2 ml sterile protoplast culture medium (0.4 M sucrose; 250 mg/L D-xylose; 1x MS salts with vitamins; adjusted to pH 5.8 with KOH), and then cultured horizontally in the dark at 20°C at a slight angle.

2.12.3. Protoplast protein extraction

To extract total cellular protein from protoplasts for the purpose of measuring β-glucoronidase (GUS) activity, 500 µl of cultured protoplast suspension was added to 3 ml mannitol/W5 (0.4 M mannitol; 1 mM glucose; 30 mM NaCl; 25 mM $CaCl_2$; 1 mM KCl; 0.3 mM MES; adjusted to pH 5.7 with KOH) and the protoplasts were pelleted by centrifugation at 2,000 *g* at room temperature for 5 min. The supernatant was aspirated off using a Pasteur pipette and the pellet resuspended in 200 µl MUG (4-methylumbelliferyl-β-D-glucuronide hydrate) extraction buffer (50 mM $NaPO_4$, pH 7.0; 0.1% (*w/v*) sarcosyl, 0.1% (*v/v*) Triton X-100; 10 mM EDTA, pH 8.0; 10 mM β-mercaptoethanol) to disrupt the protoplasts. Cellular debris was pelleted by centrifugation at 20,000 *g* at room temperature for 2 min and the protein concentration of the supernatant determined by Bradford's assay (Coomassie Plus Reagent (BDH)).

2.12.4. Measurement of GUS activity using the fluorimetric MUG assay

For the measurement of GUS activity, 20 to 50 µg of extracted cellular protein was transferred to a 96-well microtitre plate and made up to a final volume of 80 µl with MUG extraction buffer (see section 2.12.3). To each well, 200 µl 1.4 mM MUG (in MUG extraction buffer) was added for a final volume of 280 µl. The plate was covered in foil and incubated on a 37°C plate warmer. At time intervals between 10 and 60 min, 40 µl aliquots of the reaction were removed and added to a new microtitre plate containing 200 µl 0.2 M Na_2CO_3 to stop the reaction. The stopped reactions were kept covered with foil and on ice until fluorimetric measurements were taken. GUS activity at each time point was measured using an excitation wavelength of 370 nm and an emission wavelength of 460 nm using a Perkin Elmer Luminescence Spectrometer LS 50B.

2.12.5. Plasmid constructs and DNA primers used in protoplast transformations

Plasmids used for protoplast transformation were based on the pRT100 and pRT103 plasmids (3340 bp) previously described (Topfer *et al.*, 1987). For GUS assays, a pRT103 plasmid containing an inserted *β-glucuronidase* (GUS) gene provided by Ian Moore (University of Oxford, UK) was used for transformations. Genes of interest, such as *SRG3* and *Athsr5*, were cloned into the plasmid pKI102 (3252 bp) at XhoI/BamHI sites in either the sense (pKSRG and pKATH) or antisense (pKSRG-AS and pKATH-AS) orientation. pKI102 is identical to pRT100 except that the ampicillin resistance gene has been exchanged for a kanamycin resistance gene. This plasmid was also provided by Ian Moore. The *SRG3* (At3g02040) and *Athsr5* coding sequences (At5g63790) were cloned from cDNA (see section 2.5.3). The following primers with XhoI restriction sites on the forward primer and BamHI restriction sites on the reverse primer (underlined) were used:

SRG3 Forward, 5'-AAACTCGAGATGTCTCTAAAAGCCATTCATGTC–3'

SRG3 Reverse, 5'-AAAGGATCCTTAGAGTTGTATCAACTCGGGAATG-3'

Athsr5 Forward, 5'-AAACTCGAGATGAACATGAGCAATAAACGTAATC-3'

Athsr5 Reverse, 5'-TTTGGATCCCTAAGAATTTTTCCGAGTCAAGTG-3'.

The pBax plasmid was provided by Patrick Gallois (University of Manchester, UK) and contained the murine *Bax-α* coding sequence (GenBank accession NM_007527) cloned into plasmid pDH51 (3407 bp) using the XbaI restriction sites (Pietrzak *et al.*, 1986).

For RT-PCR analysis of *Bax* gene expression, primers for the *Bax* coding sequence were used. Primers for the *β-tubulin* coding sequence were used as an RT-PCR positive control. Primer sequences were as follows:

Bax Forward, 5'-ATGGACGGGTCCGGGGAGC-3'

Bax Reverse, 5'-TCAGCCCATCTTCTTCCAGATG-3'

β-tubulin Forward, 5'-GATATCTGTTTCCGTACCTTGAAGC-3'

β-tubulin Reverse, 5'-ACCGACTGTAGCATCTTGATATTGC-3'.

2.13. Growth of *Arabidopsis* plants

2.13.1. Isolation of T-DNA knockout mutant seed lines

All *Arabidopsis thaliana* var Columbia T-DNA insertional mutants in genes of interests were obtained from the Torrey Mesa Research Institute (Syngenta, La Jolla, USA). Putative insertional mutant lines were identified by using the BLAST algorithm (Altschul *et al.*, 1997) to screen the TMRI mutant databases with genomic sequences of interest, including 300 bp of genomic sequence upstream (5') and downstream (3') of each coding sequence. Expect value (E) scores of BLAST matches of less than or equal to $1E^{-04}$ were considered to be likely insertional mutants in the genes of interest. Seed was then obtained for each of the indicated lines.

2.13.2. Growth conditions of *Arabidopsis* plants

Seeds were surface-sterilised with 70% (*v/v*) ethanol for 5 min with vigorous shaking and were then dried on Whatmann filter paper that had been sterilised with 95% (*v/v*) ethanol. Surface-sterilised seeds were plated using a toothpick on petri plates containing 0.5x MS medium and 0.7% (*w/v*) agar. The medium contained no supplemental sucrose. For Basta selection of resistant seedlings, the medium was supplemented with 10 mg/L D-phosphinothricin (PPT). Petri plates were placed in the dark at 4°C for 48 h to synchronise germination and were subsequently transferred to a growth chamber with day and night temperatures of 22 and 20°C, respectively. The average light intensity at the level of the plate was maintained at ~110 $\mu mol \cdot m^{-2} sec^{-1}$ supplied by 2-foot fluorescent tubes during a 16 h photoperiod.

At 10 to 14 days following germination when the primary roots were 2 to 3 cm in length and the second rosette leaves had begun to form, plants were transferred into individual 2.5-inch diameter pots arrayed in standard flats containing compost

(Levington Universal Extra Professional Growing Medium (The Scotts Company, Suffolk, UK)). Plants were grown in growth cabinets on wire racks with relative humidity maintained at 70%. Plants were watered by subirrigation as needed, usually every 2 to 3 days. Day length was 16 h and daytime and night time temperatures were maintained at 22 and 20°C, respectively. The average light intensity at the top of the pots was ~175 $\mu mol \cdot m^{-2} sec^{-1}$. Seeds were collected by allowing the plants to dry out approximately 8 weeks after germination when most siliques had yellowed.

2.14. Assays for cell death in *Arabidopsis* plants

2.14.1. Measurement of leaf chlorophyll content

The chlorophyll content of rosette leaves during development was measured *in situ* using a portable chlorophyll meter (SPAD-502, Minolta Corp. Milton Keynes, UK). The chlorophyll content of each leaf was estimated by taking the average of at least 3 readings per leaf, and at least 3 leaves from different plants of each line were measured for each time point. For senescing leaves, a series of measurements were taken from leaf tip to leaf base to gain an overall average of the leaf chlorophyll content.

2.14.2. Phloroglucinol staining of plant tissue

To identify lignified tracheary elements in cross-sections of *Arabidopsis* stems, a phloroglucinol staining procedure was used. Stem samples were cleared of chlorophyll by incubation in 95% (*v/v*) ethanol for 2 to 3 h at room temperature, and subsequently transferred to 70% (*v/v*) ethanol for at least 30 min. A fresh solution of 1% (*w/v*) phloroglucinol was prepared by dissolving 1 g in a minimum volume of 95% (*v/v*) ethanol and making up to volume with 6 M HCl. Cleared samples were hand-sectioned and incubated in the phloroglucinol solution for 5 min. Excess stain was removed and a mounting solution (50% (*v/v*) glycerol; 6 M HCl) was added to prevent the stain from washing off. Samples were viewed using phase contrast light microscopy and red staining indicated lignified cells.

2.14.3. Ion leakage following paraquat- and UV-C-induced cell death

To induce PCD by paraquat treatment, 9 day-old seedlings on agar plates were sprayed with 1 mM paraquat (methyl viologen or 1,1'-dimethyl-4,4'-bipyridinium dichloride) and incubated in the light for at least 12 h under normal growth conditions.

Each T-DNA knockout line was grown on the same plate as a wildtype control to account for plate-to-plate differences in paraquat treatment. Ion leakage was measured by floating 3 seedlings on ddH_2O at room temperature for 3 h with gentle shaking and measuring the final conductivity of the bathing solution (µs/cm) using a Primo Conductivity Meter (Hanna Instruments, Woonsocket, USA). Ion leakage was expressed as a function of seedling mass, and 3 replicates were undertaken for each time point.

To induce PCD by UV-C radiation, 9 day-old seedlings were irradiated with 30 kJ/m^2 UV-C radiation using a UV Stratalinker 1800 (Stratagene). Following treatment, 3 seedlings were floated on ddH_20 and the conductivity of the bathing solution was measured over time. Ion leakage (µs/cm) was expressed as a function of seedling mass, and 3 replicates were undertaken for each line. Ion leakage from controls was undertaken exactly as above but with untreated, 9 day-old seedlings.

2.14.4. Measurement of trichome cell death

To quantify cell death following the appropriate treatment, leaves were removed from seedlings and placed with water on a microscope slide with a raised cover slip. Trichomes were examined by light microscopy (100x magnification) and the percentage of dead trichomes scored by examining cellular morphology. Dead trichomes exhibited the characteristic cytoplasmic condensation indicative of PCD.

2.14.5. Hypersensitive Response (HR) assays and growth of pathogens

The plant pathogenic bacterium *Pseudomonas syringae* pv. *tomato* DC3000 (Pst) (kindly provided by D. E. Cuppels) and a derivative of Pst carrying the plasmid pAvrB1 (Pst *AvrB*) (Tamaki *et al.*, 1988) were used for plant inoculations as indicated. Plants used for inoculations were grown for 4 weeks under short-day conditions (8 h photoperiod) to prevent flowering. Bacterial strains were grown for 48 h on LB agar

with antibiotic selection, and then resuspended in sterile ddH_2O to an OD_{600} of 0.05, equivalent to 10^7 colony-forming units(cfu)/ml.

To observe the progression of HR and the bacterial concentration required to elicit HR-related cell death, Pst bacterial suspensions were adjusted to 10^5, 10^6, or 10^7 cfu/ml. The inoculum was delivered with a needleless 1 ml syringe to the underside of the leaf (Gopalan *et al.*, 1996a). Lesions and cell collapse were observed over a period of 48 h. Experiments were repeated 3 times on independent plants for each line.

To follow the population dynamics of the compatible (Pst) and incompatible (Pst *AvrB*) strain in *Arabidopsis*, leaves were inoculated with 10^5 cfu/ml of either strain, since this concentration was determined to not produce visible HR cell death. At 1 h, 24 h and 72 h post-inoculation, leaf samples were removed with a cork borer and macerated in 1 ml 10 mM $MgCl_2$. The 1 h sampling time ensured that inoculations across plants were uniform. Bacterial counts were determined by plating 10 µl of an appropriate dilution of the leaf homogenate onto LB amended with 0.5x Cetrimide-Fucidin-Cephalosprorin Selective Agent (Oxoid Companies, Hampshire, UK), 25 µg/ml rifampicin and, in the case of Pst *AvrB*, 25 µg/ml kanamycin. Plates were incubated at 25°C for 48 h and the number of colonies were counted for dilutions that gave between 1 and 20 colonies. The cfu/cm^2 was then calculated as:

$$\text{cfu/cm}^2 = (N \times D \times 100)/.785\ \text{cm}^2$$

where N is the number of colonies in the dilution factor, D, and the factor 100 takes into account that 10 µl of the original 1000 µl was plated. Each data point was represented as the mean and standard error of the decimal logarithm of three independent replicates performed on the same day, since replicates performed on different days have been shown to lead to statistically significant differences (Tornero and Dangl, 2001).

CHAPTER THREE

3. *Arabidopsis* cell suspension cultures as a model system in which to study plant programmed cell death

3.1. Introduction

In plants, PCD has been studied in association with a number of physiological processes including the hypersensitive response (HR) (Heath, 2000a) and developmental processes such as tracheary element formation (Fukuda, 2000) or leaf and organ senescence (Quirino *et al.*, 2000). However, in order to advance our understanding of the molecular events underlying PCD, it is desirable to develop a simple system in which PCD can be experimentally induced and studied. In this regard, plant cell suspension cultures provide a useful system that is easily manipulated and in which individual cellular characteristics, such as PCD, may be readily examined. Viable, necrotic, and PCD cellular morphologies are all distinguishable, and the use of a vital stain, such as fluorescein diacetate (FDA) (Persidsky and Baillie, 1977), allows for rapid quantification of the number of dead cells by fluorescence microscopy. Nuclear DNA laddering, if present, can be visualised by fractionating the DNA by gel electrophoresis and staining with ethidium bromide, eliminating the need for time-consuming Southern blot analysis (McCabe and Leaver, 2000).

Treatments that have, in the past, been used to induce and study PCD in cell cultures include elicitors of the hypersensitive response (in *Arabidopsis*, tobacco, and parsley) (Desikan *et al.*, 1996; Glazener *et al.*, 1996; Naton *et al.*, 1996; Desikan *et al.*, 1998; Dorey *et al.*, 1999), hydrogen peroxide (H_2O_2) treatment (*Arabidopsis*) (Desikan *et al.*, 1998), UV-C radiation (*Arabidopsis*) (Danon and Gallois, 1998), cold stress (tobacco) (Koukalova *et al.*, 1997), low cell density (carrot) (McCabe *et al.*, 1997), and

heat treatment (carrot) (McCabe *et al.*, 1997). It is desirable to have more than one method of inducing PCD in a system, particularly for the identification and study of any regulatory genes or common mechanisms that may be involved in all forms of plant PCD. Indeed, the literature contains many reports of studies that identify specific changes in transcript expression during senescence-related cell death or HR-induced cell death but, often, these changes are not observed consistently across the various conditions that induce PCD (Pontier *et al.*, 1999).

The objective of the work described in this chapter was to establish an *Arabidopsis thaliana* cell suspension system in which PCD could be readily induced in a reproducible manner by more than one method. *Arabidopsis* was the plant of choice primarily because of availability of the complete, sequenced genome (The *Arabidopsis* Genome Initiative, 2000), which confers a major advantage for routine cloning of genes and database homology searches. Moreover, microarray analysis of transcript expression is currently routinely and easily accessible for *Arabidopsis*, due to the availability of comprehensive expressed sequence tag (EST) stock centers and the ability to clone any target gene of interest by reference to the complete genomic sequence.

Established *Arabidopsis thaliana* cell cultures (May and Leaver, 1993) were examined for general growth characteristics to determine a suitable culture age for experimental induction of PCD and also as a 'control' for non-induced cells. Additionally, studies were undertaken to determine the susceptibility of cells at progressive days after subculturing to PCD-inducing stimuli. Various treatments by which PCD could be induced in the cell cultures were investigated, with the aim of providing an experimental system for an investigation of some of the common molecular and biochemical features that are involved in plant PCD irrespective of the inducing stimulus. Several methods of both qualitatively and quantitatively measuring

the extent of PCD in the cultures were also investigated to identify those which constitute the most robust and objective morphological and biochemical markers of PCD.

3.2. Results

3.2.1. Growth of *Arabidopsis* cell suspension cultures

The growth characteristics of the *Arabidopsis thaliana* cell suspension system (May and Leaver, 1993) employed in these studies were initially characterised by determining the packed cell volume of cultures over 12 days of growth following subculturing (Figure 3.1). It can be seen that cultures grow most rapidly between days 3 and 7 after subculturing. This is considered the log phase of culture growth, when background cell death in the culture is less than 5% as determined by FDA vital staining (see Figures 3.2 and 3.5A). Six day-old cultures therefore provide a source of viable 'control' cells to be used for comparison with those undergoing PCD.

It was generally found that young cells at the early stage of the growth curve (*ca.* 4 days) were highly susceptible to any PCD-inducing treatments tested (i.e. heat or oxidative stress) and exhibited extensive and rapid cell death. Moreover, often this death was necrotic (data not shown), indicating that these treatments were sufficient to overwhelm the cells' capacity to respond with the induction of a programmed cell death pathway. Conversely, cell cultures at about 10 days after subculturing already exhibited significant background levels of PCD associated with natural culture senescence (see Figure 3.5A). These senescing cultures were therefore unsuitable for the induction of PCD using external stimuli since it would be difficult to separate cellular changes associated with senescence from the response to the stimulus applied. It was found that 6 day-old cultures contained a high percentage of viable, growing cells which were susceptible to the application of a range of PCD-inducing stimuli in a dose-response manner.

Finally, it was observed that the healthiest cultures, defined by high levels of packed cell volume and consisting of finer cell clumps amenable to microscopic

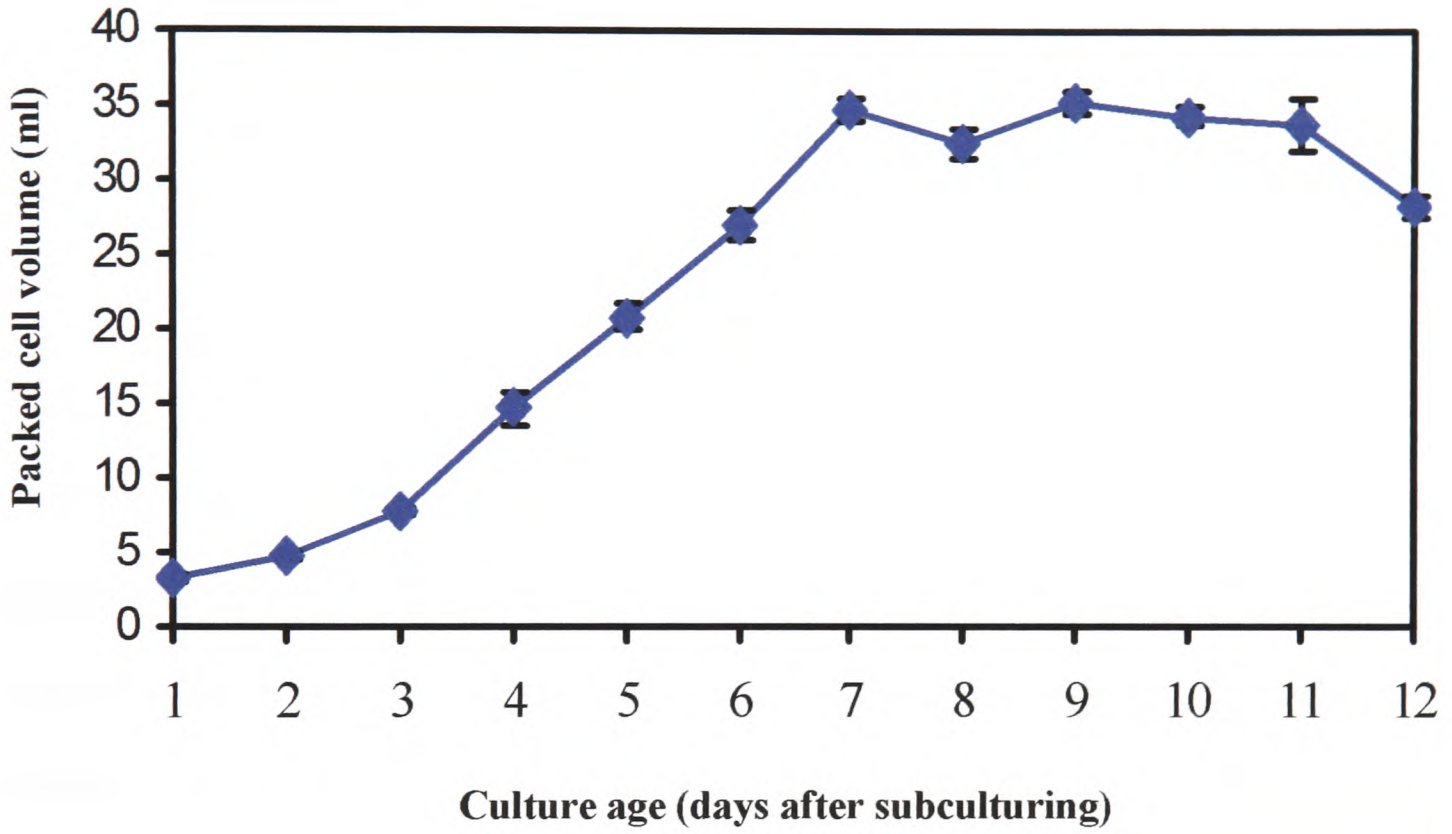


Figure 3.1. Growth curve of an *Arabidopsis* cell suspension culture.
Cultures were maintained by subculturing 10 ml into 100 ml fresh At3 media every 7 days (see section 2.1.1). Packed cell volume was measured at daily intervals by centrifugation in 2x50 ml sterile conical tubes at 500 *g* for 3 min and then returning cultures to flasks on the rotary shaker at 24°C, 150 rpm, in the dark (see section 2.1.2). Values presented are the means of at least 3 replicate samples.

analysis, were obtained when culture stocks were maintained in the light and then transferred to dark growth for 6 days prior to induction of PCD. Maintaining cultures continuously in light conditions was undesirable due to the high numbers of developed chloroplasts that interfered with microscopic examination of cellular morphology. Conversely, continued week-to-week maintenance of cultures in dark conditions eventually had a negative effect on culture viability, with a higher percentage of flasks undergoing death by day 7 in the absence of other stimuli (data not shown).

3.2.2. Morphological features of PCD in *Arabidopsis* cell suspension cultures

In order to distinguish between living cells and cells that have died by either PCD or necrosis, cell cultures subjected to various stimuli were examined by light microscopy in conjunction with the vital stain, FDA (see section 2.3.1) (Figure 3.2). In agreement with results obtained with carrot cell cultures (McCabe *et al.*, 1997), three types of cellular morphology were identifed in *Arabidopsis* cell cultures (Figure 3.3). Healthy, control cultures containing viable cells were identified by the presence of highly vacuolate cells in which the plasma membrane was appressed to the cell wall (Figure 3.3A). These cells were also confirmed to be viable by the presence of FDA staining. Conversely, cells in which PCD has occurred either as a result of heat treatment (55°C, 10 min) or senescence (13 days-old) were identified by a striking cellular morphology in which the cytoplasm had condensed and the plasma membrane had shrunk away from the cell wall (Figures 3.3B and C). These cells were confirmed to be dead by the absence of FDA staining. However, cells which failed to stain with FDA but had undergone necrotic cell death as the result of a severe heat treatment (80°C, 10 min) did not display any of the features of PCD morphology such as cellular condensation and plasma membrane shrinkage, and were thus easily distinguished from the former cell types (Figure 3.3D).

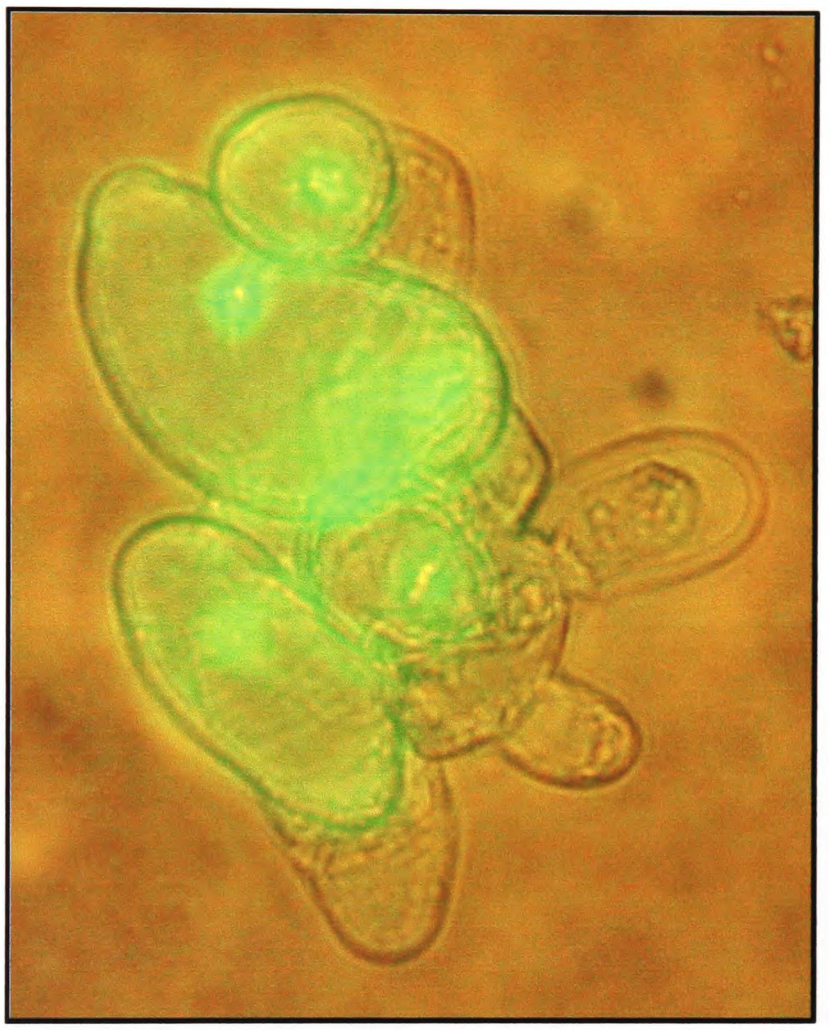


Figure 3.2. Vital staining with fluorescein diacetate (FDA) of *Arabidopsis* suspension culture cells.
Living and dead suspension culture cells are distinguished with the use of the vital stain, FDA, at a final concentration of 0.002% (*w/v*) (see section 2.3.1). Living cells, seen in the foreground, stain positively with FDA and emit a green fluorescence (emission 510 nm). Conversely, dead cells seen in the background do not hydrolyse FDA and therefore do not show staining.

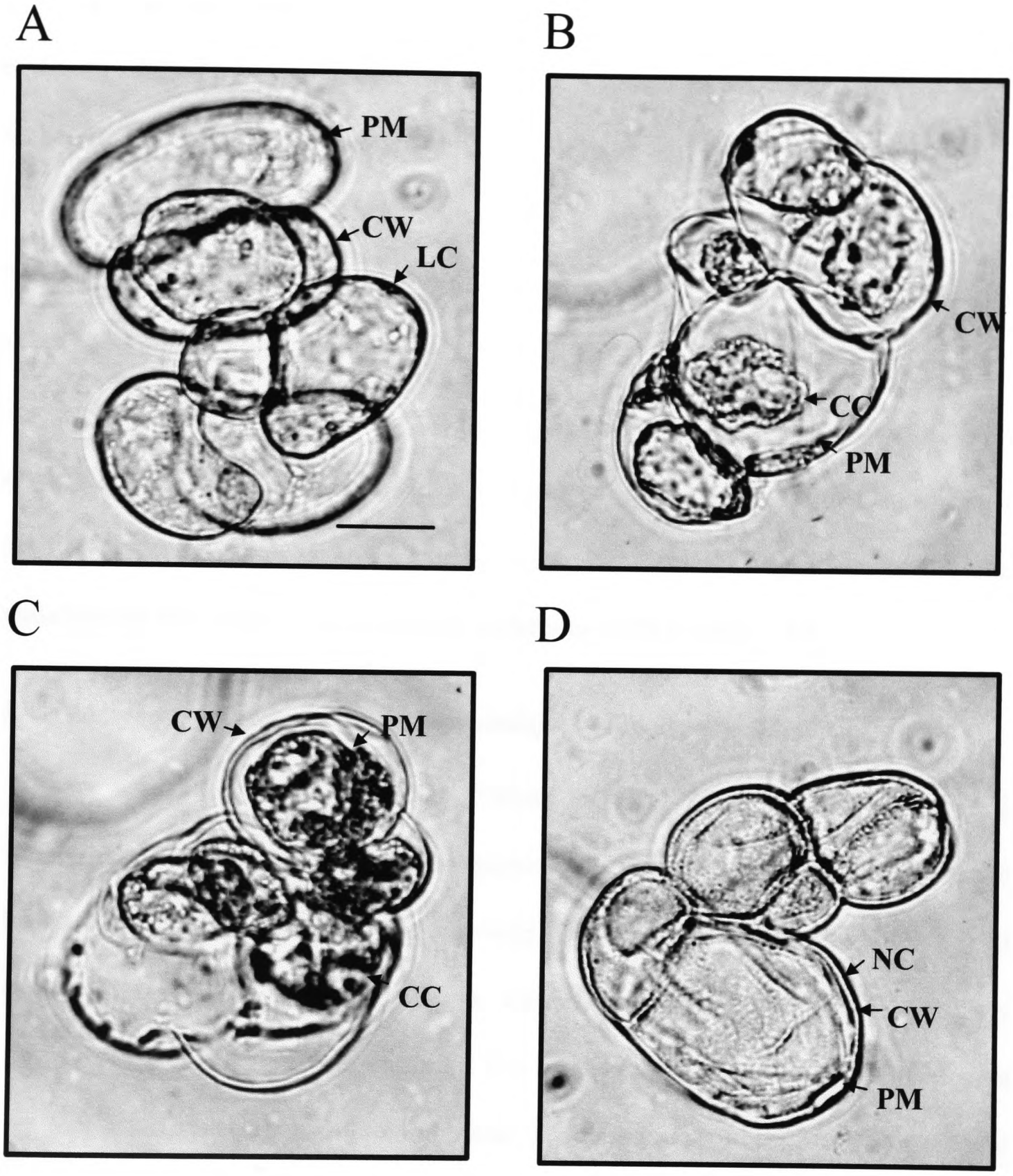


Figure 3.3. Cellular morphology of *Arabidopsis* suspension culture cells during cell death.

Cellular morphology was examined by phase-contrast microscopy (400x magnification) in the following cells: **A.** Untreated, 6 day-old cultures. **B.** Six day-old cultures incubated at 55°C for 10 min and examined immediately thereafter. **C.** Untreated, 13 day-old cultures. **D.** Six day-old cultures incubated at 80°C for 10 min and examined immediately thereafter.

PCD morphology is evident as a condensed cytoplasm and shrinkage of the plasma membrane away from the cell wall (**B** and **C**). CC, condensed cytoplasm; CW, cell wall; PM, plasma membrane; LC, living cell; NC, necrotic cell. Bar is 10 μm for A-D.

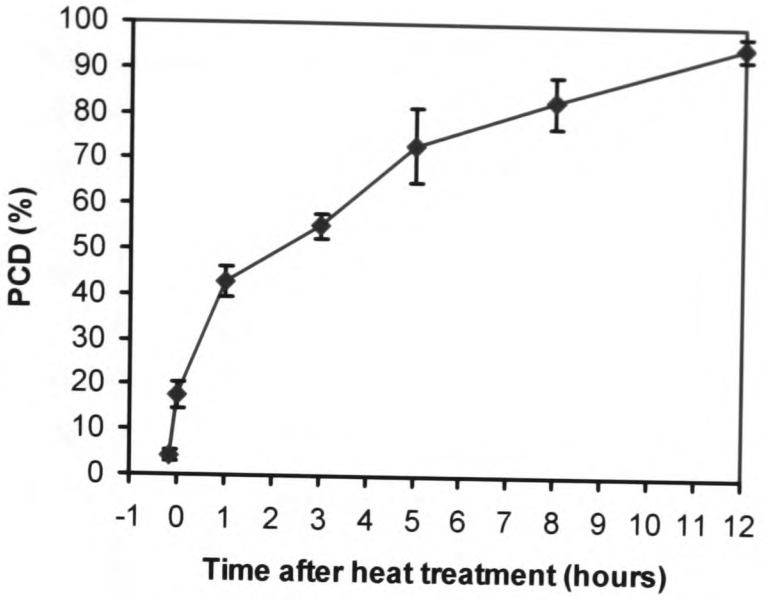
3.2.3. Heat treatment induces PCD in an *Arabidopsis* cell suspension culture

A brief heat treatment of carrot and *Arabidopsis* cell suspension cultures has previously been shown to induce PCD in the cells as characterised by the typical biochemical and morphological markers (McCabe *et al.*, 1997; McCabe and Leaver, 2000). In the present work, a heat treatment of *Arabidopsis* cell suspension cultures at 55°C for 10 min in a shaking water bath was sufficient to induce PCD in an immediate and synchronous fashion (see section 2.3.3), with 100% of the cells dying within 12 h after the heat treatment (Figure 3.4A). Morphological features of PCD were visible in the cultures immediately following heat treatment (see Figure 3.3B), and laddering of total DNA extracted from cultures became detectable at about 5 h post-treatment when approximately two-thirds of the cells had undergone PCD (Figure 3.4B).

3.2.4. Senescence induces PCD in an *Arabidopsis* cell suspension culture

As originally demonstrated by Callard *et al.* (1996), PCD is observed in *Arabidopsis* cultures which undergo senescence when left to grow in the absence of subculturing. Significant PCD (>20%) became evident in the cultures at about day 10 following subculturing and increased in subsequent days (Figure 3.5A). Notably, necrotic cell death was absent throughout. The time required for 100% PCD to occur in the culture could vary depending on the initial 'health' of the cultures used, time of year, etc., and could require anywhere from 13 to 17 days. Senescing cell cultures consistently exhibited the hallmark features of PCD, including morphological alterations (see Figure 3.3C) and detectable DNA laddering when at least two-thirds of the cells in culture had undergone PCD (Figure 3.5B). It is noteworthy that the DNA laddering that occurred in senescent cells was more apparent than that of heat-treated cells (i.e. compare laddering in Figures 3.5B and 3.4B). This may be due to the rapidity of the response in

A



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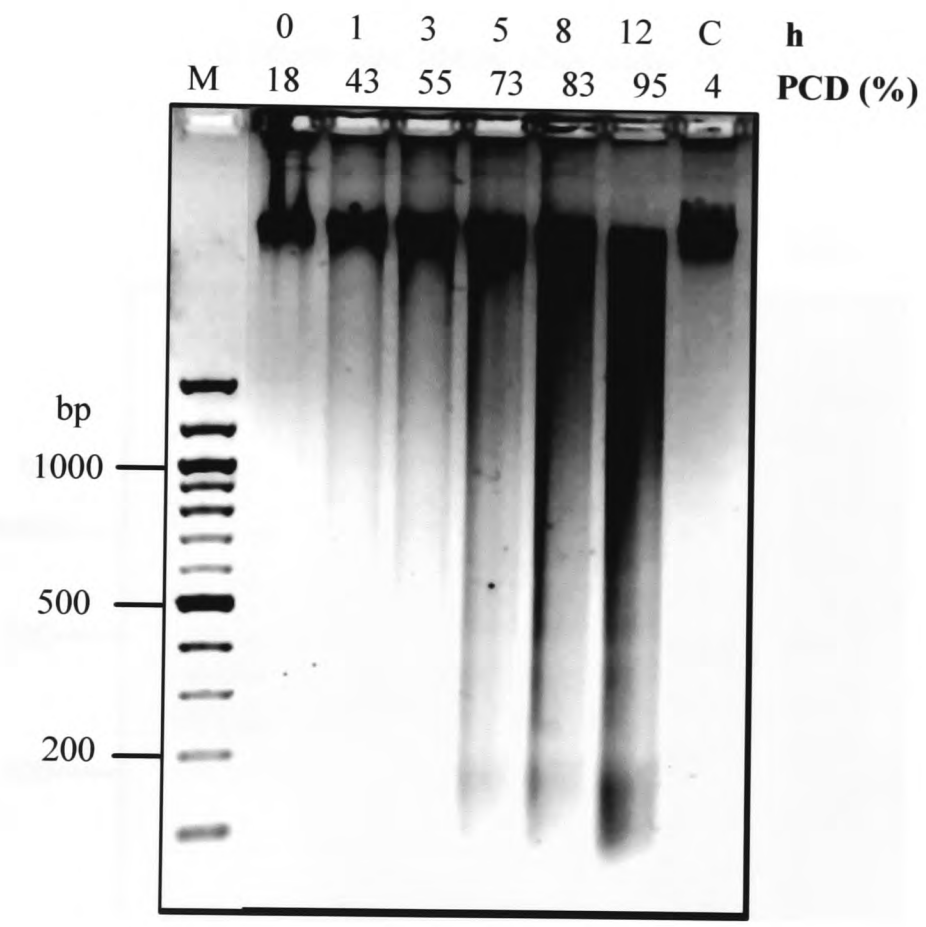
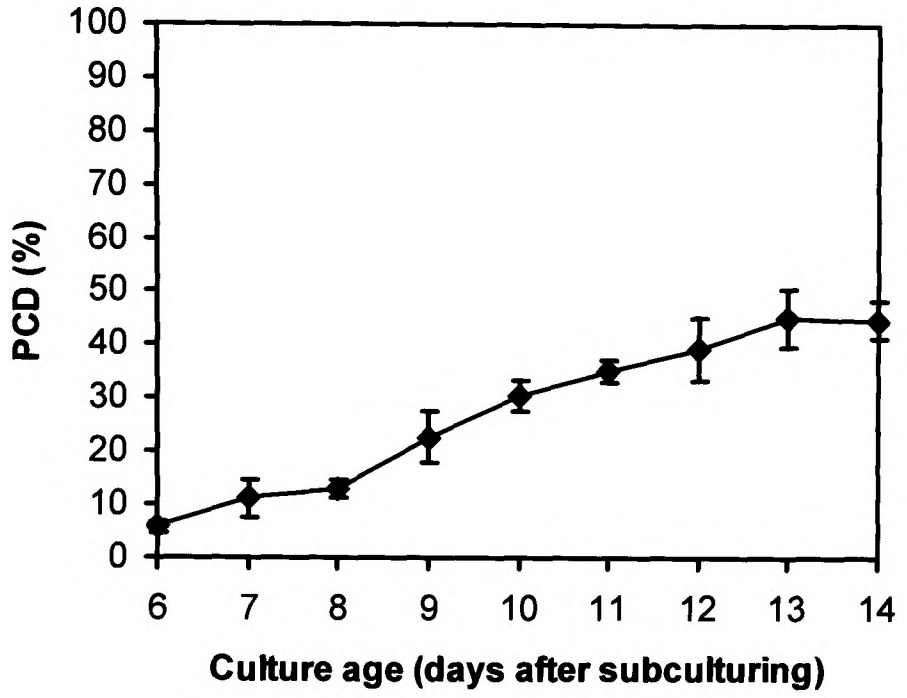


Figure 3.4. Time course of heat-induced PCD in an *Arabidopsis* cell suspension culture.

Six day-old cell cultures were induced to undergo PCD by treatment at 55°C for 10 min (see section 2.3.3). **A.** The percentage of cells exhibiting PCD in the culture was scored on the basis of a condensed cytoplasmic morphology and the absence of FDA staining by phase-contrast microscopy (see Figures 3.2 and 3.3B). Values presented are the means of at least 3 replicate samples. **B.** DNA laddering following heat treatment. DNA was extracted at various time points following treatment at 55°C for 10 min as the total percentage of PCD increased in the culture (indicated as hours post-treatment above each lane). 10 μg of genomic DNA was fractionated by gel electrophoresis on a 1.5% (*w/v*) agarose gel and stained with ethidium bromide (see section 2.3.2). Control DNA isolated before heat treatment is in lane 7 (C). The percentage of cells exhibiting PCD morphology at the time of extraction is indicated above each lane. M, marker.

A



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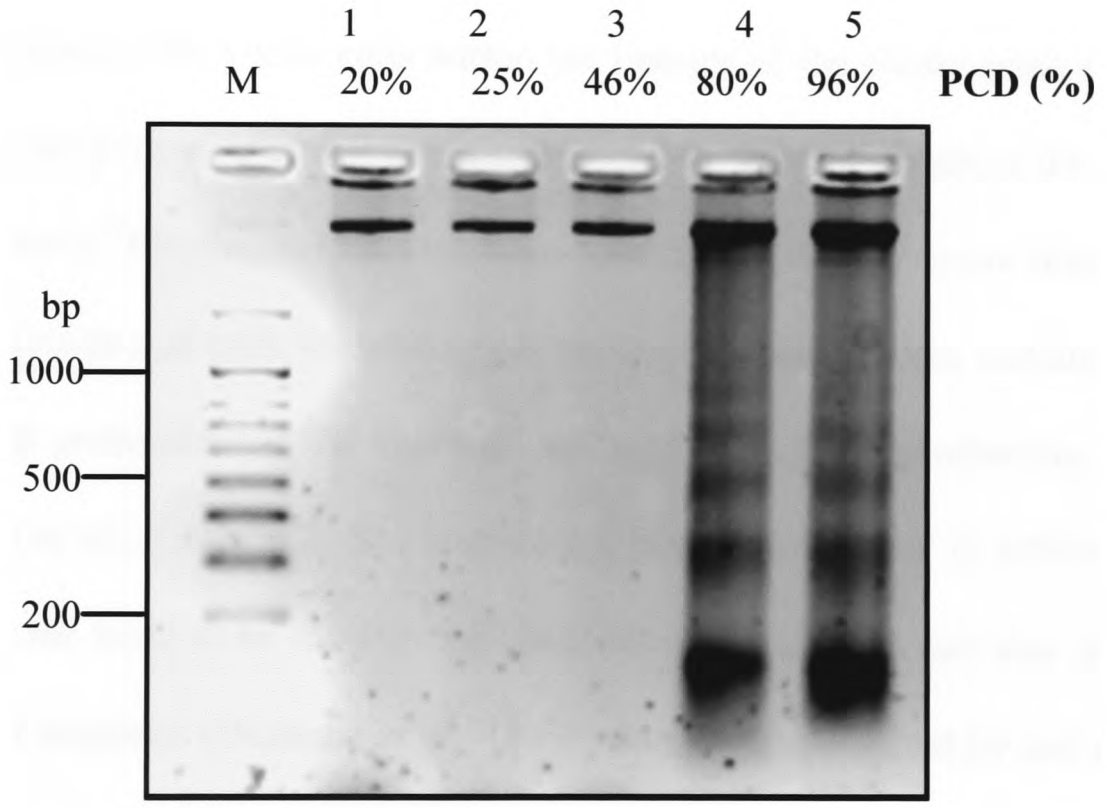


Figure 3.5. Time course of senescence-induced PCD in an *Arabidopsis* cell suspension culture.

A. Cell cultures were examined at a fixed time each day beginning at 6 days after subculturing. The percentage of cells exhibiting PCD in the culture was scored on the basis of a condensed cytoplasmic morphology and the absence of FDA staining by phase-contrast microscopy (see Figures 3.2 and 3.3C). Values presented are the means of at least 3 replicate samples. **B**. DNA laddering during culture senescence. DNA was extracted at various time points during culture senescence as the total percentage of PCD increased in the culture. 10 μg of genomic DNA was fractionated by gel electrophoresis on a 1.5% (*w/v*) agarose gel and stained with ethidium bromide (see section 2.3.2). The percentage of cells exhibiting PCD morphology at the time of extraction is indicated above each lane. M, marker.

the cultures induced to undergo PCD by heat treatment, which may have resulted in faster and perhaps less precise DNA cleavage (i.e. a mixture of oligonucleosome cleavage and general DNA breakdown).

3.2.5. Oxidative stress induces PCD in an *Arabidopsis* cell suspension culture

Oxidative stress has been used in many experimental systems to induce PCD and can be generated in a number of ways, including by the direct addition of H_2O_2, and thus these methods were investigated in the *Arabidopsis* cell suspension system. Addition of H_2O_2 (in a range of concentrations from 10 mM to 80 mM) to the cultures caused an asynchronous response, in which clumps of cells would typically contain dead cells around the periphery but viable cells within the interior of the clump (data not shown). Thus, cells on the periphery of cell clumps appeared to 'quench' much of the exogenous H_2O_2. Additionally, filtering cultures to reduce clump size did not prove feasible due to insufficient quantities of cells for subsequent nucleic acid and protein isolation. The use of the bacterial proteinacious HR elicitor, harpin, to elicit H_2O_2 production (He *et al.*, 1994; Desikan *et al.*, 1998) was also considered, but this substance is difficult to work with owing to the need to be used at high concentrations and the fact that it induces a relatively weak response (Glazener *et al.*, 1996) which is also affected by cell clumping.

The use of menadione (2-methyl-1,4-naphthoquinone), a redox-active quinone that generates intracellular ROS and has been shown by others to cause PCD in mammalian systems (Cande *et al.*, 2002), was investigated for its effectiveness in inducing PCD in *Arabidopsis* cell suspension cultures. The effectiveness of menadione in inducing PCD was investigated at a range of concentrations (200 µM to 2 mM). Menadione at a final concentration of 400 µM was sufficient to induce PCD in the cell cultures as recognised by the typical cellular morphology (Figure 3.6A). Similarily,

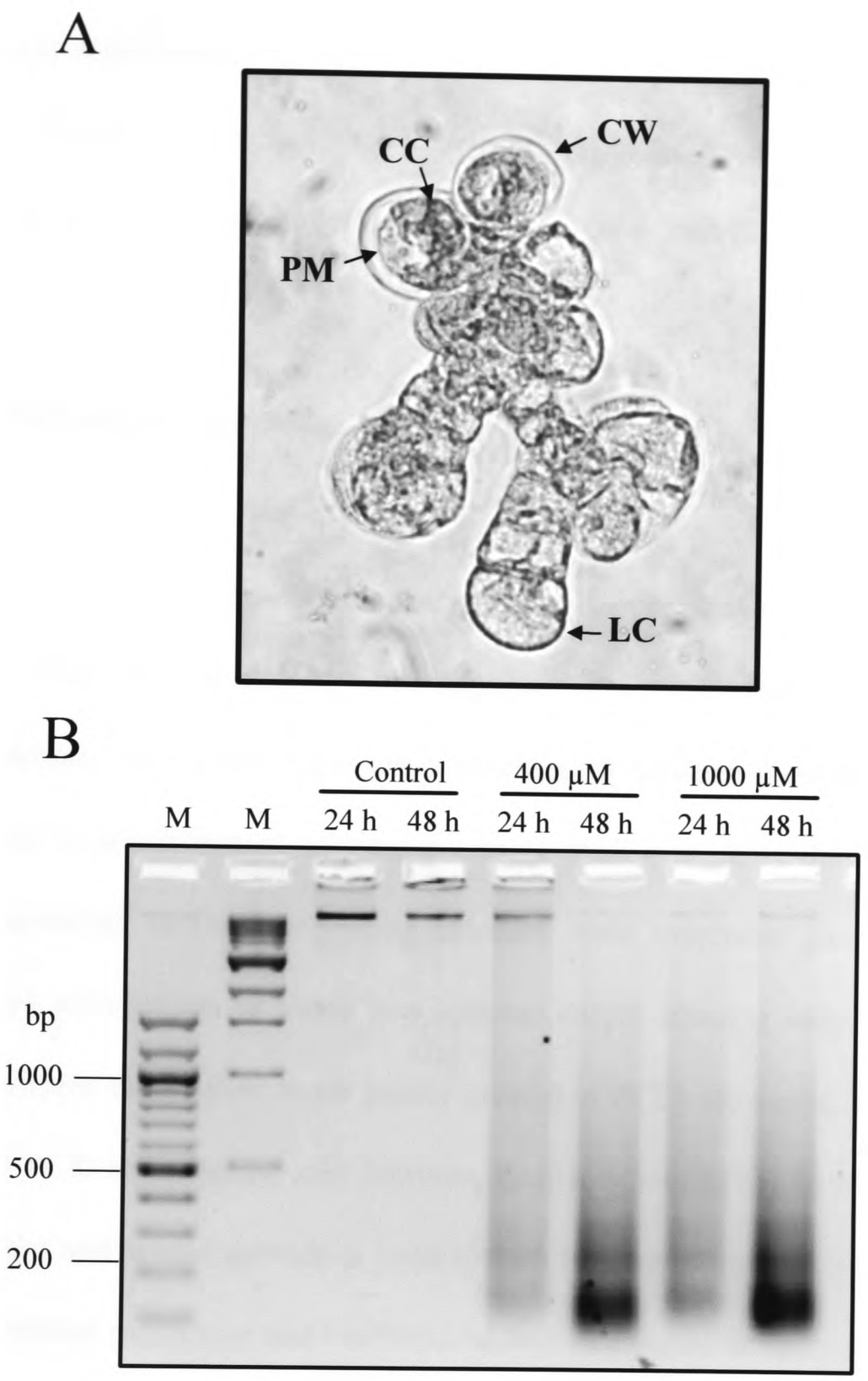


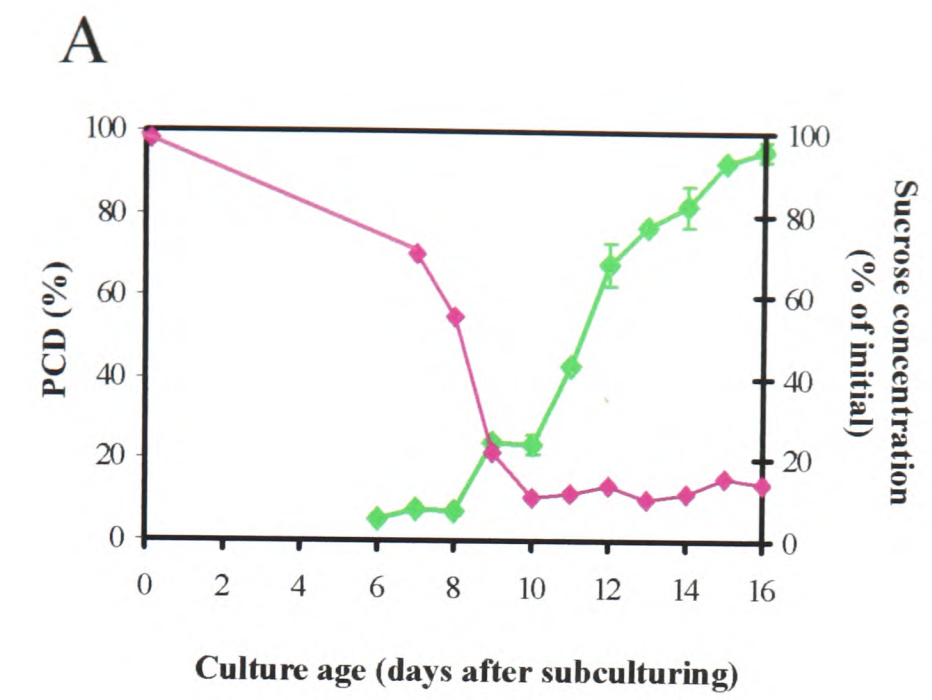
Figure 3.6. Menadione-induced PCD in an *Arabidopsis* cell suspension culture. Six day-old cell cultures were treated with menadione to induce oxidative stress and PCD (see section 2.3.4). 24 and 48 h later, cells were examined and scored for cell death on the basis of a condensed cytoplasmic morphology and the absence of FDA staining by phase-contrast microscopy (also see Figure 3.2). **A.** PCD morphology 24 h following 400 µM menadione treatment. Phase contrast microscopy, 400x magnification. CC, condensed cytoplasm; CW, cell wall; PM, plasma membrane; LC, living cell. **B.** DNA laddering following menadione treatment. DNA was extracted 24 and 48 h after induction of PCD. 10 µg of genomic DNA was separated by gel electrophoresis on a 1.5% (*w/v*) agarose gel and stained with ethidium bromide (see section 2.3.2). Laddering is evident in cells treated with both 400 µM and 1000 µM menadione beginning at 24 h, while control cells show no changes in DNA integrity over the same time period. M, marker.

DNA laddering in menadione-treated cultures was evident beginning 24 h after addition and increased significantly by 48 h post-treatment (Figure 3.6B). Repetitions of this experiment, however, revealed that the response to menadione was highly variable depending on the initial culture conditions, and the time course of PCD could vary by as much as 24 h.

3.2.6. Carbohydrate starvation induces PCD in an *Arabidopsis* cell suspension culture

PCD occurred in cell cultures that were no longer actively growing, as shown in Figure 3.5. This may be due to accumulation of toxic products or a limitation of essential nutrients, and it was important to distinguish between these two possibilities. If PCD was due to accumulation of toxic products, then it is likely that a stress response would be occurring in these senescing cultures. Heat treatment also induces a stress response, and comparison of these two systems might identify only common 'stress-response' features rather than those purely related to PCD. However, if starvation were the cause of PCD in senescing cell cultures, then it is less likely to involve a classical stress response and would provide a good system for comparison to heat-induced PCD, since any common molecular and biochemical features would be more likely to be PCD-related rather than part of a stress response.

Previous studies have shown that sucrose depletion in the medium is associated with culture senescence in both *Arabidopsis* and rice (Chen *et al.*, 1994; Callard *et al.*, 1996). To investigate this possibility, the sucrose concentration of the medium was measured during culture senescence (see section 2.3.5). In this study, sucrose concentration was seen to drop dramatically at days 7 to 10, corresponding with the onset of PCD (Figure 3.7A). To investigate whether the sucrose concentration in the medium was indeed the primary limiting factor during culture senescence, the delay of



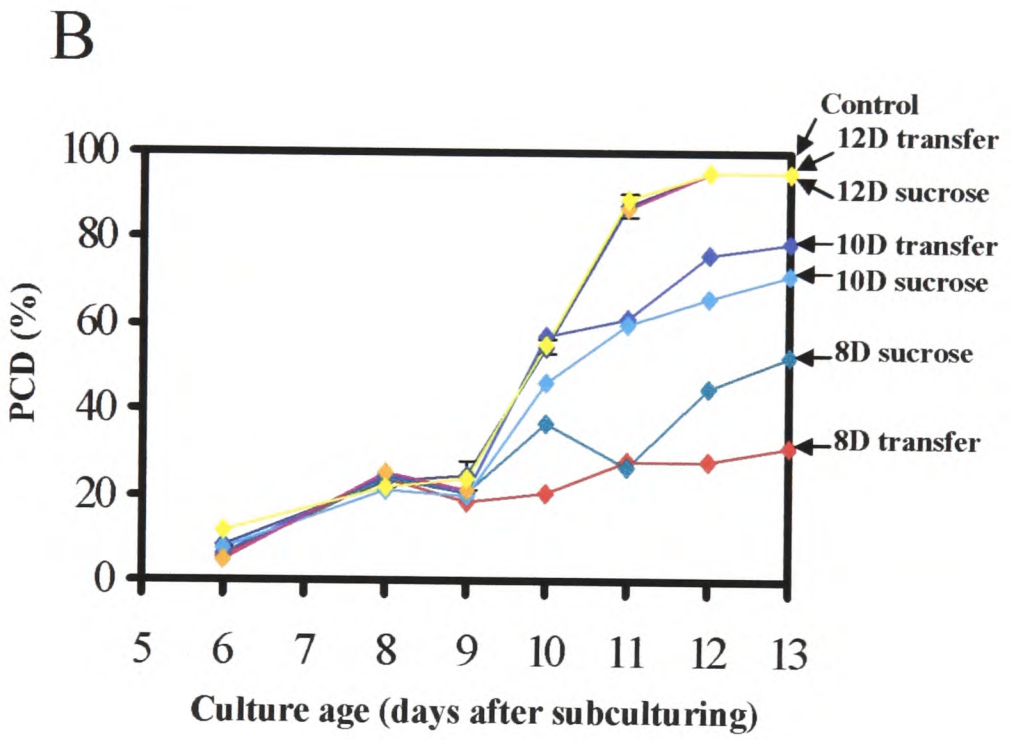
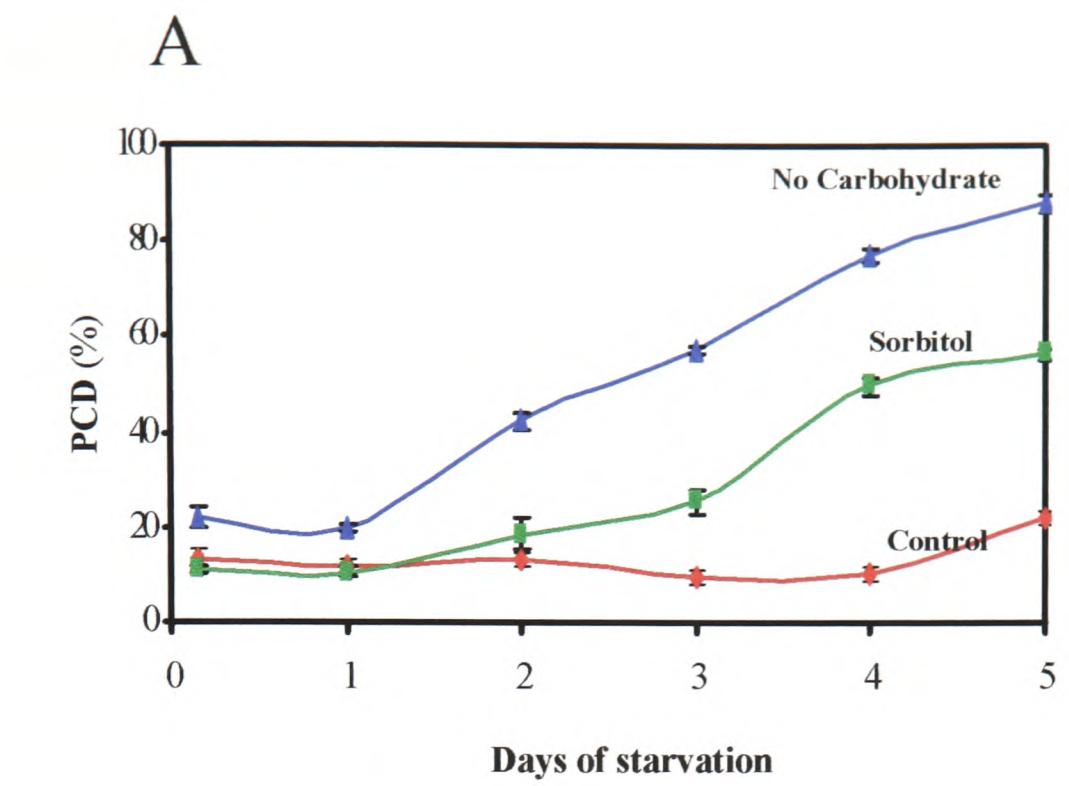


Figure 3.7. The relationship between culture senescence and medium sucrose concentration.

A. Cultures were examined every day beginning 6 days after subculturing. PCD in the culture was scored each day on the basis of a condensed cytoplasmic morphology and the absence of FDA staining by phase-contrast microscopy (green) (see Figures 3.2 and 3.3). Over the same time course, sucrose concentration in the At3 medium was measured by Anthrone Reagent (pink) (see section 2.3.5). Values for both sucrose and PCD are the means of at least 3 replicate samples. **B.** The influence of sucrose addition on the progression of senescence was investigated by either the addition of 88 mM sucrose ('sucrose') at day 8 (green), 10 (light blue), or 12 (yellow), or by transferring the cells to fresh At3 medium ('transfer') at day 8 (red), 10 (dark blue), or 12 (pink) (see section 2.3.6). Control cultures were maintained without transfer or sucrose addition (black). Cell death was scored each day on the basis of a condensed cytoplasmic morphology and the absence of FDA staining by phase-contrast microscopy.

senescence by sucrose addition was attempted at days 8, 10, or 12, by either adding sucrose to its original concentration of 88 mM in the At3 medium or transferring the cells to fresh At3 medium (see section 2.3.6). As shown in Figure 3.7B, the percentage of cells exhibiting PCD followed the same kinetics in cultures to which sucrose was added, as in cultures that were transferred into fresh media at either days 8 or 10, suggesting that sucrose was indeed the limiting factor at these stages. By day 12, however, cultures could not be rescued by either method, indicating that the majority of cells had already undergone or executed a PCD programme.

In an attempt to achieve a synchronous starvation-induced PCD response in the cultures, experiments were undertaken in which 6 day-old cultures were transferred to either At3 medium lacking a carbohydrate source or, to rule out any osmoticum effects, to At3 medium with 88 mM sorbitol replacing the sucrose component (see section 2.3.6). Control cultures were simply transferred to fresh At3 medium at this time. PCD in the starved cultures became rapidly visible at about 2 days after transfer to the medium lacking any carbohydrate source and continued to rise to almost 90% at about 5 days post-starvation (Figure 3.8A). Cells cultured in sorbitol also underwent PCD, albeit at a much slower rate and only beginning clearly at about day 3. DNA laddering was clearly evident in cultures without sucrose within 4 days, and increased in cells cultured in sorbitol from 4 to 5 days (Figure 3.8B). Conversely, control cells did not show DNA laddering over the same time.



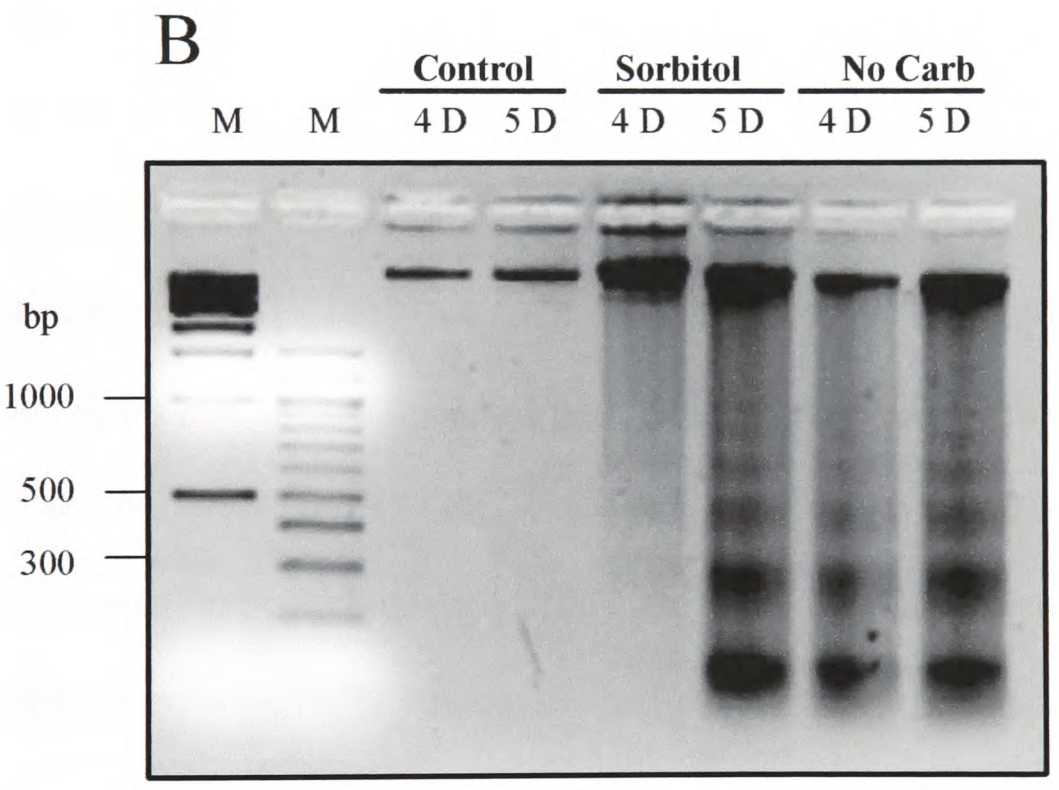


Figure 3.8. Carbohydrate starvation-induced PCD in an *Arabidopsis* cell suspension cultures.

Six day-old cultures were transferred into fresh medium containing either no carbohydrate source or 88 mM sorbitol as an osmoticum. Control cultures were transferred into fresh At3 medium (red) (see section 2.3.6). **A.** Cell death was scored each day on the basis of a condensed cytoplasmic morphology and the absence of FDA staining by phase-contrast microscopy (see Figures 3.2 and 3.3). Values presented are the means of 3 replicate samples. **B.** DNA laddering in carbohydrate-starved cell cultures. DNA was extracted at 4 and 5 days (D) after the induction of starvation and 10 µg of genomic DNA was separated by gel electrophoresis on a 1.5% (*w/v*) agarose gel and stained with ethidium bromide (see section 2.3.2). DNA laddering is evident at 4 and 5 days in both cultures containing no carbohydrate source in the medium but sorbitol as an osmoticum, and in those containing neither carbohydrate source nor osmoticum. Control cultures show no changes in DNA integrity over the same time period. M, marker.

3.3. Discussion

The *Arabidopsis thaliana* cell suspension system developed to study plant programmed cell death is advantageous in that PCD is easily scored in these cultures at both a morphological and biochemical (DNA laddering) level, and the induction of PCD can be achieved by a variety of stimuli. For routine analyses of PCD, it is desirable to apply a PCD-inducing stimulus to a culture in which the initial percentage of dead cells is minimal, since any molecular, biochemical, or cellular changes associated with PCD can then be chiefly attributed to the treatment itself. Very young cultures (4 days-old) in which background PCD is minimal are hypersensitive to PCD-inducing stimuli. In response to PCD-inducing treatments, these cultures exhibit extensive cell death that does not allow for a proper assay of PCD to be undertaken, or for comparative effects of different treatments on PCD to be investigated. Therefore, the culture age at which minimal cell death had already occurred, but which PCD-inducing treatments could still be applied and the extent of PCD measured, was determined. In this respect, 6 day-old cultures were found to be optimal for use as a control in the induction and study of PCD, as these cells are growing rapidly in the log phase, are healthy, and have recovered from the initial 'stress' of subculturing and low density that might be affecting 4 day-old cultures (McCabe *et al.*, 1997). On the other hand, they have not yet begun normal culture senescence. Moreover, 6 day-old cultures provide a sufficient quantity of cells from which to isolate reasonable quanties of DNA, RNA, and protein, while the same is not true of younger cultures.

Ideally, any methods used to induce PCD in this system must induce death in a reproducible, synchronous, minimally invasive, maximally effective, and biologically significant manner. Both heat treatment at 55°C for 10 min in a shaking water bath and the normal PCD which occurs during the progression of culture senescence were found

to be reliable and reproducible inducers of PCD (Figures 3.4 and 3.5). Since the percentage of cells exhibiting PCD progresses gradually over a time period in these treatments, it is possible to both follow the progression of PCD in the culture and also to 'select' particular time points at which a specific percentage of cells have undergone PCD. Thus, due to the ease of working with heat treatment and culture senescence and the reproducibility of results obtained in these systems, they were determined to be optimal inducers of PCD in *Arabidopsis* cell suspension cultures.

As PCD as a result of heat treatment is likely to be due to an oxidative stress response (Noctor and Foyer, 1998; Larkindale and Knight, 2002; Panchuk *et al.*, 2002), the effectiveness of the application of a direct oxidative stress in inducing PCD was also investigated. Both H_2O_2 and harpin (a bacterial elicitor) were found to yield unreproducible reponses, and thus menadione (2-methyl-1,4-naphthoquinone) was used as an alternative inducer of oxidative stress. Menadione is a quinone which, when applied to living cells, is capable of causing intracellular production of reactive oxygen species and leads to conditions of oxidative stress (Thor *et al.*, 1982). Specifically, this quinone serves as a substrate for flavoenzymes and undergoes one-electron reduction to form a semiquinone radical, which then reduces dioxygen to a superoxide anion radical (O_2^-), eventually yielding H_2O_2 and O_2. The latter two species may then react together in the presence of a metal ion catalyst to form damaging hydroxyl radicals (OH^-). Thus, menadione can be used as an alternative to H_2O_2 to induce oxidative stress and subsequent PCD (Sun *et al.*, 1999). While menadione treatment was sufficient to cause PCD in the *Arabidopsis* cell suspension cultures (Figure 3.6), the onset of PCD was found to be highly dependent on the initial culture conditions, most likely those related to cell density. The variability in response to menadione therefore made it unsuitable for routine investigations of PCD.

To further investigate the factors which lead to senescence in cell cultures, measurements of sucrose concentration in the culture medium over the time course of senescence were undertaken. It was found that the onset of PCD correlated with a drop in sucrose concentration in the medium (Figure 3.7A), suggesting that sucrose may be the primary limiting factor in this system. Support for this hypothesis was provided when it was shown that the addition of sucrose at the earlier stages of senescence was sufficient to slow the onset of PCD in the culture (Figure 3.7B). Conversely, transfer of cells to a medium lacking a carbohydrate source (a starvation medium) was found to be sufficient to induce significant PCD within 3 days (Figure 3.8). (Hereinafter, 'senescence-induced' PCD in the *Arabidopsis* cell suspension system refers to that which is most likely caused by nutrient starvation in the cultures).

The initial lag in the onset of PCD following transfer to a starvation medium (Figure 3.8A) was most likely due to the presence of intracellular carbohydrate stores sufficient to sustain the cells' metabolic needs for a short period. The difference in PCD responses between the medium lacking sucrose and that containing sorbitol could be explained in two ways. Firstly, some of the cell death in the former may be due to osmotic effects, though one would expect this fraction of cell death to be relatively low, since 88 mM sucrose is a relatively low osmolarity and is often simply omitted in cell culture starvation studies (Chen *et al.*, 1994). Secondly, it is possible that the cells are able to metabolise sorbitol, if only for a short period, as it has been demonstrated that some higher plants are able to utilise sugar alcohols such as mannitol to meet their metabolic needs in the absence of sugars (Stoop *et al.*, 1996). Similar responses in the onset and progression of PCD for cells starved in the presence of mannitol were observed (data not shown). Moreover, it is suggested that these sugar alcohols may actually act as scavengers of reactive oxygen species and serve to protect against cellular

damage (Stoop *et al.*, 1996), making this an inappropriate osmoticum to be used in a study of PCD. Thus, if starvation is to be employed in the future, an alternative osmoticum to a sugar alcohol (e.g. PEG) should be utilised.

In summary, it has been demonstrated that both a heat treatment at 55°C for 10 min and natural culture senescence are robust methods to follow the induction and progression of PCD in *Arabidopsis* cell cultures. The use of two very different and independent methods of induction should allow for the distinction to be drawn between those cellular and biochemical features which are potentially involved in PCD (i.e. those features that are common to both treatments) from features which are components of unrelated responses to the treatment (i.e. those identified in only one of the treatments). For example, while heat treatment will lead to the induction of a classical heat shock stress response, the same is not necessarily true of senescence. These systems can therefore be used together to identify common molecular, biochemical, and associated cellular changes that are involved in plant PCD.

CHAPTER FOUR

4. A custom cDNA microarray analysis of transcript expression during programmed cell death in *Arabidopsis*

4.1. Introduction

As outlined in Chapter 1, there have been several studies that have attempted to draw parallels between the cellular and molecular features of animal and plant PCD. This approach is chiefly due to the fact that there is currently a wealth of information concerning the mechanisms and pathways that underlie PCD in animals, while research into plant PCD is still in its infancy. For example, several studies have attempted to demonstrate the existence of caspases in plants, primarily by using synthetic caspase inhibitors to inhibit PCD (del Pozo and Lam, 1998). In addition, transgenic expression of pro- and anti-apoptotic Bcl-2 family proteins in plants has been shown to affect the cell death response (Lacomme and Santa Cruz, 1999; Mitsuhar *et al.*, 1999; Dickman *et al.*, 2001; Kawai-Yamada *et al.*, 2001). The effect on cell death of a recently identified *Arabidopsis* homologue to the animal *Bax-Inhibitor 1* gene, *AtBI-1* (Sanchez *et al.*, 2000), has also been studied in both plants (Kawai-Yamada *et al.*, 2001) and animals (Yu *et al.*, 2002). Finally, a number of preliminary studies have also suggested a role for cytochrome *c* in plant PCD (Balk *et al.*, 1999; Sun *et al.*, 1999; Zhao *et al.*, 1999).

Although such studies suggest that some of the molecular mechanisms of plant and animal cell death may be evolutionarily conserved, no universal plant cell death genes have been identified. Continued progress in the field of plant PCD will increasingly rely on more inclusive approaches that do not simply attempt to identify animal PCD mechanisms in plant cells. In this regard, microarray analysis provides a

means by which hundreds to thousands of genes may be screened simultaneously for changes in mRNA expression profiles during PCD, thereby potentially identifying the important regulatory genes that function in plant PCD and unravelling the molecular pathways involved in this significant, yet poorly understood, cellular event.

The aim of the work presented in this chapter was to analyse the steady-state mRNA expression profiles of approximately 100 selected genes during programmed cell death in an *Arabidopsis* cell suspension culture. Both heat treatment and senescence have been used to induce PCD in the cell cultures (see Chapter 3). The use of two very different and independent methods of induction allows for a distinction to be drawn between genes that are potentially involved in PCD (those whose expression is changed as a result of both treatments) and genes that are part of some other response to the treatment (those whose expression changes in only one of the treatments). Inevitably, expression of a number of genes will be common to both situations, but the use of more than one method of inducing PCD will be more likely to lead to the identification of PCD regulatory genes or common mechanisms that underlie all PCD events. While the literature contains reports of many studies that identify genetic markers, for example, of senescence-related cell death or HR-induced cell death, often these markers are studied only in the specific context of senescence or HR and there is no information on the role of these genes in PCD induced by other stimuli (Pontier *et al.*, 1999). The work presented in this chapter represents the first attempt to investigate whether such genes have a role in cell death irrespective of the inducing stimulus.

A cDNA microarray was constructed to measure changes in mRNA transcript abundances for genes during plant PCD. Genes examined included those that have previously been implicated in PCD-related responses, such as senescence and the HR, and also those which, based on knowledge of animal PCD and the central role of

mitochondria in this process, were hypothesised to play a role during plant PCD. A large number of the genes selected were those previously identified as senescence-activated or senescence-related genes, since PCD marks the endpoint of senescence (Gan and Amasino, 1997; Yen and Yang, 1998; Delorme *et al.*, 2000; Quirino *et al.*, 2000). Numerous studies have identified transcripts that are upregulated during senescence — a time when the majority of cellular transcripts are downregulated — although no conserved senescence regulatory elements have been discovered. Moreover, because there appears to be multiple regulatory pathways functioning during senescence, the question of whether many of these upregulated transcripts are components of senescence-specific processes or may play a more general role in plant PCD has become of central importance in plant PCD studies (Pontier *et al.*, 1999; Quirino *et al.*, 2000). The evidence thus far indicates that there are some commonly upregulated transcripts in plant cell death in response to different stimuli, but to date no consistent markers or regulatory genes involved in plant PCD have been identified.

The array used in this study included several previously identified senescence-activated genes to determine if the expression of these transcripts was also upregulated during an independent PCD-inducing heat treatment. It was anticipated that expression of these transcripts would be induced during culture senescence, but a change in the expression of the same transcripts during heat-induced PCD would suggest a common underlying molecular mechanism of PCD in the two situations. Senescence-related genes selected for array analysis included those encoding the *Arabidopsis* senescence activated gene *SEN1* (Oh *et al.*, 1996), the *Arabidopsis* homologue to tomato *SENU3* (Drake *et al.*, 1996), *SRG1*, *SRG2,* and *SRG3*, which were originally identified in senescing *Arabidopsis* cell cultures (Callard *et al.*, 1996), an *SPF1*-like protein similar to that identified in cucumber (Kim *et al.*, 1997), cysteine proteases (Solomon *et al.*,

1999), and several other ESTs identified only as 'senescence activated genes' according to the ABRC EST database annotations. Additionally, senescing cucumber cotyledons have been used in our laboratory to identify genes upregulated at the onset of PCD (Kim *et al.*, 1997; Delorme *et al.*, 2000). A number of cDNAs have been identified in this system, including transcription factors, DNA binding proteins, a matrix metalloproteinase (Delorme *et al.*, 2000), and several other genes of unknown function. *Arabidopsis* homologues of these genes were included on the array.

Gene transcripts identified as being upregulated during the HR were also arrayed. These included those identified in the *Arabidopsis* HR-related studies of Lacomme and Roby (1999) and Sanchez *et al.* (1999). The former study identified several *Athsr* transcripts upregulated during *Arabidopsis* HR, including those encoding alternative oxidase, a specific isoform of the mitochondrial voltage-dependent anion channel (VDAC/Athsr2), and several unknown proteins. In the study of Sanchez *et al.* (1999), a putative *Arabidopsis* Bax-Inhibitor homologue (*AtBI-1*) was identified as being upregulated during wounding and pathogen challenge.

As well as analysing the expression of transcripts that are associated with types of PCD during senescence and HR, the expression of transcripts associated with metabolic events that are thought to generate signals that form part of the molecular mechanism of PCD in plants was also investigated. These included genes associated with antioxidant metabolism such as superoxide dismutases, glutathione and ascorbate peroxidases, and those involved in Ca^{2+} signalling, including a number of calmodulin and calcium-dependent protein kinases. Oxidative stress can trigger PCD in plant cells and, in some cases, it has been shown that reactive oxygen or nitrogen species serve as signals to induce changes in gene expression associated with PCD (Levine *et al.*, 1994; Desikan *et al.*, 1998; Dorey *et al.*, 1999; Clarke *et al.*, 2000; Desikan *et al.*, 2000; Rao *et*

al., 2000). Additionally, the production of reactive oxygen species (ROS) may also occur as a consequence of the activation of a death pathway. The suppression of antioxidant enzyme activity (in barley and tobacco studies) may actually be necessary to allow progression of PCD (Mittler *et al.*, 1999b; Fath *et al.*, 2001). Interestingly, in a screen to identify plant suppressors of Bax-induced cell death in yeast, a glutathione-*S*-transferase/peroxidase was identified, again linking the production of ROS to cell death (Kampranis *et al.*, 2000). Similarly, changes in intracellular Ca^{2+} have also been demonstrated to be a common event during plant PCD (Levine *et al.*, 1996; Sanders *et al.*, 1999).

Finally, potential involvement of genes encoding mitochondrial proteins in plant PCD was investigated. In mammalian systems, mitochondria have been demonstrated to play a central role in the execution of the death pathway, but to date the evidence for a similar involvement of mitochondria in plant PCD is largely circumstantial. The transcript expression levels for a number of genes essential to mitochondrial function were investigated in an attempt to address the issue of involvement of mitochondria in plant PCD. These included those encoding VDAC (*Athsr2*), alternative oxidase (*AOX/Athsr3*), the adenine nucleotide translocase (*ANT1* and *ANT2*), Mn superoxide dismutase (*MSD1*), Heat shock protein 60 (*Hsp60*), and two subunits of the ATP Synthase complex (mitochondrially-encoded *Atp1* and nuclear-encoded *Atp2*).

In addition, several cDNAs encoding enzymes involved in metabolic regulation, including isocitrate lyase, thiolase, and malate synthase (Graham *et al.*, 1994), were arrayed as general markers of metabolic activity. Finally, 16 *Arabidopsis* 'housekeeping' genes (Reymond *et al.*, 2000) were arrayed as markers of general cellular activity.

4.2. Results

4.2.1. Cell culture sample collection for microarray analysis

It has been demonstrated that a brief heat treatment (55°C, 10 min) of *Arabidopsis* cell cultures is sufficient to induce PCD as characterised by typical biochemical and morphological markers (see Figure 3.4). For microarray analysis, RNA samples were isolated immediately following heat treatment (time 0). At this time point, DNA laddering in the cultures was not detectable (see Figure 3.4B) but the majority of cells were induced to undergo or were in early stages of PCD, therefore making this a suitable time for studying changes in gene expression. Approximately 20% of the cells exhibited PCD at this time (see Figure 3.4A).

As originally demonstrated by Callard *et al.* (1996), *Arabidopsis* cell cultures undergoing senescence express many senescence-specific genes which were originally identified in whole plants, suggesting that cell cultures are an appropriate system to study developmental senescence. The *Arabidopsis* cell cultures used in the present study also undergo senescence after 13 to 14 days of growth (see section 3.2.4). Samples for microarray analysis were harvested from 13 to 14 day-old cell cultures when ~ 40% of cells had undergone programmed cell death (see Figure 3.5).

In both the heat-treated and senescing cultures, samples are amenable to molecular analysis as indicated by the integrity of total RNA extracts (Figure 4.1). One hour following heat treatment proved to be unsuitable for array analysis since, due to the rapidity of the response, total RNA was already significantly degraded (data not shown). In spite of the rapid response of the cultures to the heat treatment, the response can clearly be identified as programmed cell death and not disorganised necrotic cell death. Cells treated at 80°C for 10 min undergo a necrotic cell death response in which 100% of the cells die immediately following treatment and the typical PCD morphology is

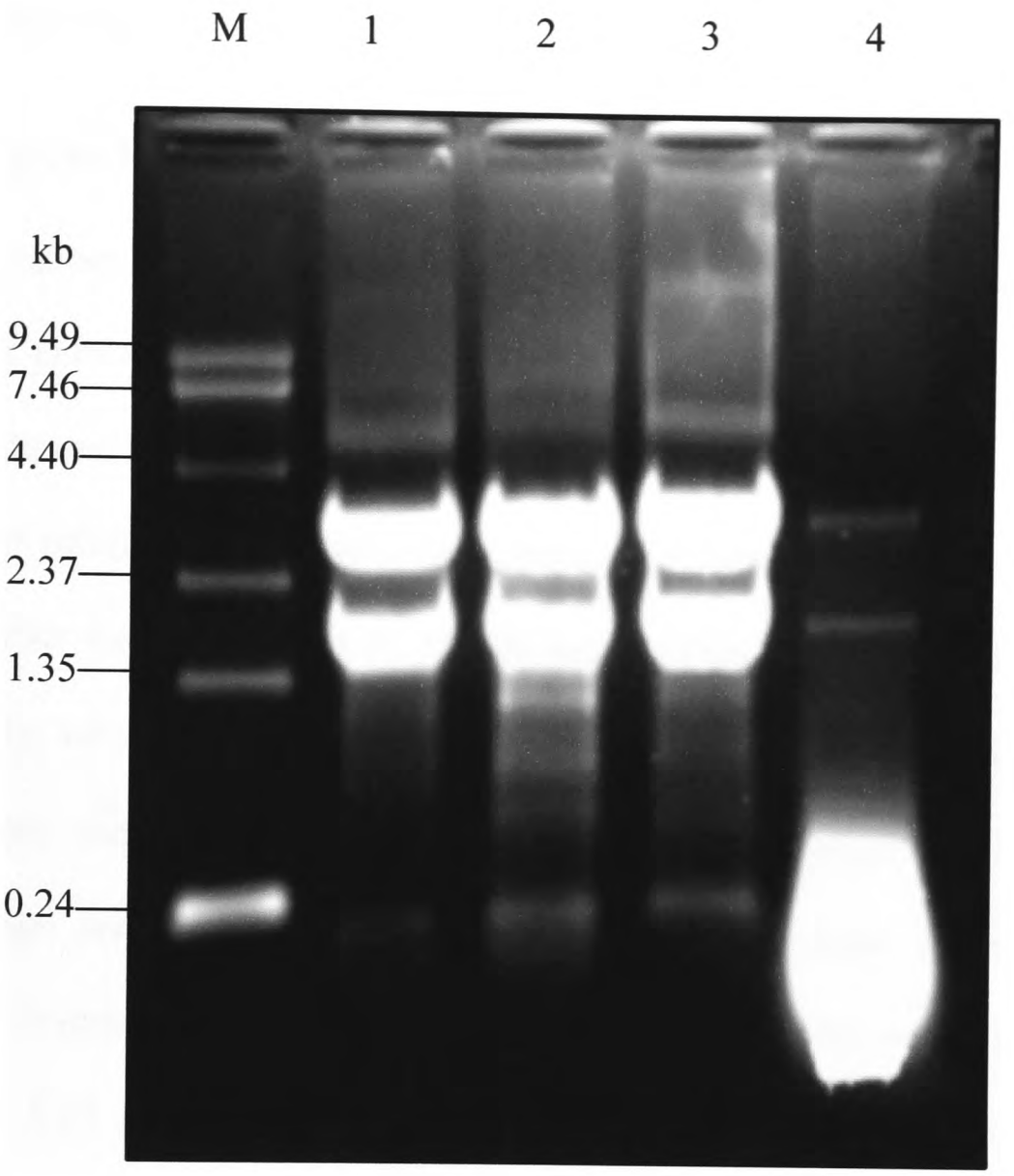


Figure 4.1. RNA integrity following induction of PCD in an *Arabidopsis* cell suspension culture.
RNA was extracted (see section 2.4.3) from control, 6 day-old cultures (lane 1), 6 day-old cultures immediately following heat treatment at 55°C for 10 min (time 0 h in Figure 3.4A) (lane 2), or senescing, 13 to 14 day-old cultures (13 to 14 days in Figure 3.5A) (lane 3) for use as a template in reverse transcription and microarray analysis. In the heat and senescence treatments which induce PCD, RNA integrity is fully maintained at the time points analysed. Conversely, 6 day-old cultures treated at 80°C for 10 min show immediate RNA breakdown following the heat shock (lane 4), indicative of necrotic cell death. M, RNA Marker 0.24-9.49 kb (Life Technologies).

clearly absent in over 95% of the cells (see Figure 3.3D). Additionally, since RNA extracted immediately following this heat shock is completely degraded (Figure 4.1) and PCD is defined by its requirement for active gene expression (Jones, 2001), only the milder treatment at 55°C allows for a PCD pathway to be invoked.

4.2.2. Microarray hybridisation experiments and expression analysis

The custom-designed cDNA microarrays used in this analysis were hybridised with labelled cDNA synthesised from RNA samples derived from each of the PCD-inducing treatments, heat or senescence, simultaneously with that isolated from control, 6 day-old cell cultures. Total RNA was isolated from heat-treated or senescing cells as described earlier (see section 2.4.3). cDNA was synthesised in a reverse transcription reaction during which the fluoresent dyes, Cy3 or Cy5, were incorporated as α-dCTP precursors into the control or test (PCD) samples, respectively (for a detailed description, see section 2.7). For each treatment, arrays were replicated 3 times, including one reverse labelling reaction in which control samples were Cy5 labelled and test samples Cy3 labelled. Transcript expression ratios (PCD/control) <1 were transformed to -1/ratio and the results from the 3 arrays were then averaged. In the final data analysis, only genes for which the transcript expression varied more than 1.5-fold compared to the control when averaged across 3 array results for each treatment were taken to represent significant changes in gene expression (see section 2.7.5).

4.2.3. Reproducibility of microarray data

This threshold ratio of ±1.5-fold used to identify significant changes in transcript expression is lower than that used in some previous array studies (Reymond *et al.*, 2000; Schenk *et al.*, 2000), but has been demonstrated to represent significant changes in expression in other microarray analyses (Desikan *et al.*, 2001; Kawasaki *et al.*, 2001;

Perez-Amador *et al.*, 2001). Nevertheless, it was important to confirm the quality of the array data both by determining the reproducibility of array data and whether changes in gene expression detected by microarray analysis could be confirmed by another experimental means.

To address the first issue, the correlation coefficient (R^2) between replicate arrays was determined. The arrays demonstrated a high correlation coefficient between replicates (Table 4.1); even the lowest R^2 value obtained, 0.876, was still well within the range of significance ($p<0.05$). Statistically, to detect differential expression of genes included in the microarrays at greater than or equal to 1.5-fold, the log (base 2) expression ratio of 1.5 (0.58) should fall at least two standard errors away from zero (no change in log (base 2) expression) across 3 replicate arrays. It is thus calculated that the log (base 2) standard deviation (SD) for each gene, averaged across 3 replicate arrays, must be less than or equal to 0.50. In fact, in 172 expression ratios included in the final analysis, 93% of the means calculated had a SD $\leq$0.50. Taken together with the correlation coefficients, this data indicated that the results gave excellent reproducibility and, using 3 replicate arrays for both heat and senescence, it can be concluded that significant changes in transcript expression could be detected at ± 1.5-fold.

To further confirm the significance in a change in expression of 1.5-fold and to demonstrate that the array results could be replicated by an independent method, competitive quantitative RT-PCR was carried out. The method of RT-PCR employed involved the reverse transcription of RNA samples derived from either control or PCD-induced cells using unique oligo(dT) RT primers for each RNA sample. Both RT primers had identical PCR primer binding sites and oligo(dT) domains, but one primer contained an extra linker of 40 nucleotides. In this manner, each cDNA in one sample was synthesised with an additional 40 bp at its 5' end. This allowed populations of

Table 4.1. Correlation coefficients (R^2) of transcript ratios for each microarray replicate from control *Arabidopsis* cell suspension cultures versus heat- or senescence-induced cultures undergoing PCD.

Replicate	C-Cy5 H-Cy3 (1)	H-Cy5, C-Cy3	C-Cy5, S-Cy3 (1)	S-Cy5, C-Cy3
H-Cy5 C-Cy3	0.883	–	–	–
C-Cy5 H-Cy3 (2)	0.876	0.884	–	–
S-Cy5 C-Cy3	–	–	0.948	–
C-Cy5 S-Cy3 (2)	–	–	0.949	0.969

C, control (6 day-old cultures); H, heat-induced PCD (55°C, 10 min); S, senescence-induced PCD (13 to 14 day-old cultures); Cy5, Cy5-deoxyCTP; Cy3, Cy3-deoxyCTP. The numbers 1 and 2 in parentheses indicate replicate 1 and replicate 2 of the same microarray slide.

cDNA from both control and either heat- or senescence-induced PCD samples to be mixed together and a single RT-PCR reaction to be carried out. The cDNA from each sample therefore competes for the amplification reagents, and the PCR product derived from each sample can be distinguished by a 40 bp difference. PCR products were separated by gel electrophoresis and stained with ethidium bromide, and the intensity of each band was measured using imaging software (for a detailed description, see section 2.5.5). This method gives a more accurate indication of the relative transcript abundance in each RNA sample and avoids the potential inaccuracies associated with comparisons across different PCR reactions.

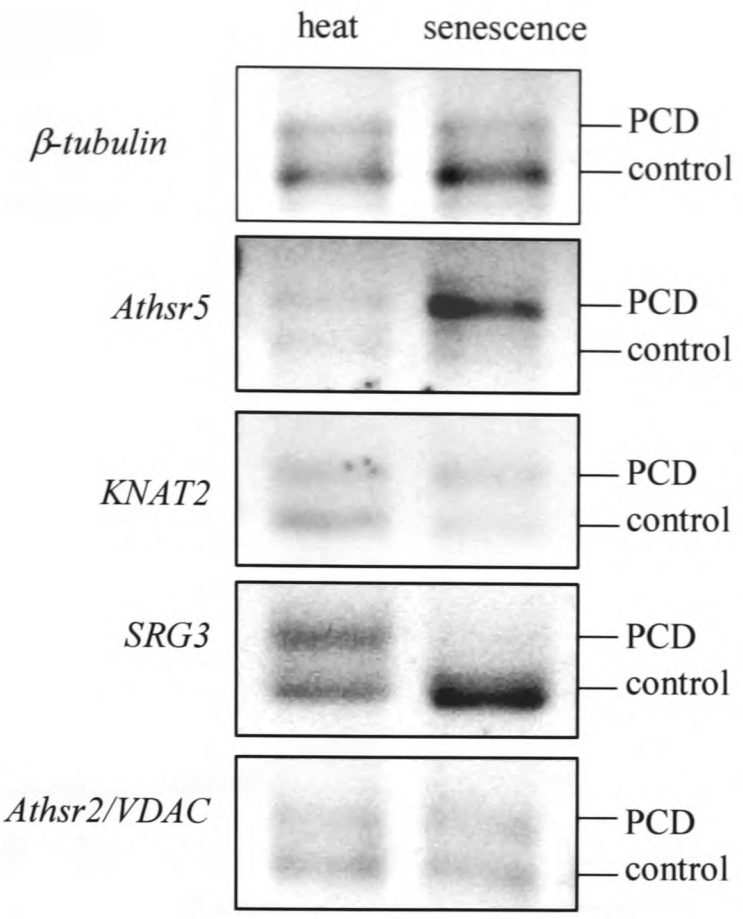
The transcript expression levels of four selected genes were analysed by RT-PCR. These genes, *Athsr5*, *KNAT2*, *SRG3*, and *VDAC/Athsr2*, were selected because they gave microarray expression ratios between ±1.5 to 2.0-fold in one or both of the PCD treatments compared to control. To normalise the RT-PCR data, each reaction was carried out in parallel with an RT-PCR reaction using primers for *β-tubulin*. Both microarray and quantitative RT-PCR data require some method of normalisation to account for differences in the efficiency of cDNA synthesis. Microarrays hybridised simultaneously with two different samples can be normalised at a global level by adjusting the total fluorescence in one channel against the total fluorescence in the second channel, thereby accounting for possible differences in the quantity of cDNA between samples. To normalise a quantitative RT-PCR reaction, however, adjusting fluorescence is not an option. While synthesised cDNA can be quantified by spiking the cDNA synthesis reaction with a radiolabelled nucleotide and then measuring the incorporated signal, it is more common practice to normalise RT-PCR against a gene whose expression level is known to remain constant (a ratio of 1.0) (DuBois *et al.*, 1999).

It can be difficult, however, to identify transcripts whose steady-state levels remain unchanged, particularly during PCD (see section 4.2.4). In light of such uncertainties, normalisation against the *β-tubulin* transcript was undertaken for each gene and, in a similar manner, the microarray ratio for each gene analysed was normalised against the microarray ratio obtained for *β-tubulin* (replicated 12 times across the microarray slide). This allowed a direct comparison between the *β-tubulin*-normalised RT-PCR ratio and the *β-tubulin*-normalised microarray ratio for each transcript investigated. In this manner, the ratio obtained by microarray analysis was tested to determine if it could be replicated by an independent method and, especially, whether the change in expression for transcripts whose ratios were between ±1.5 to 2.0 could be confirmed by RT-PCR analysis. Figure 4.2 confirms the high correlation between the expression ratios obtained for each transcript observed in the custom cDNA microarry and that observed by RT-PCR, using the ratio for *β-tubulin* for normalisation. This confirmed the interpretation of the array data and justified the use of a 1.5-fold threshold to represent a significant change in transcript expression. It is important to stress, however, that the microarray expression ratios should not be interpreted as an exact quantification of an increase or decrease in transcript levels; previous studies have failed to demonstrate a direct linear relationship between transcript abundance and fluorescence intensity (Ruan *et al.*, 1998). Nevertheless, the data was sufficiently reproducible to indicate general changes in transcript expression patterns during PCD.

4.2.4. Transcipt analysis during heat- and senescence-induced PCD

Analysis of the microarray data revealed significant changes in the steady-state levels of the transcripts of approximately 40 to 50 of the selected genes in at least one of the treatments (Figure 4.3). Of these, approximately 25% were decreased in abundance,

A



B

	Microarray[a] heat:control	RT-PCR[b] heat:control	Microarray senescence: control	RT-PCR senescence: control
Athsr5	2.0	1.7	2.6	23.3
KNAT2	1.9	2.1	3.0	4.2
SRG3	3.0	2.9	-3.8	-2.0
Athsr2/VDAC	1.2	1.3	1.5	2.0

[a]Microarray ratio relative to microarray ratio for beta-tubulin.
[b]Competitive RT-PCR ratio relative to competitive RT-PCR ratio for beta-tubulin.

Figure 4.2. Competitive RT-PCR analysis of selected gene transcripts.
A. Competitive RT-PCR was undertaken on 4 transcripts to confirm the changes in steady-state levels obtained by microarray analysis. In each lane the higher band (PCD) represents the RT-PCR product from the PCD sample (heat or senescence), while the lower band (control) represents the RT-PCR product from the control sample. The intensity of each ethidium bromide-stained band was quantified by Multi-Analyst software (Bio-Rad) and used to calculate the ratio, heat:control or senescence:control (see section 2.5.5). The ratios obtained were normalised to a *β-tubulin* competitive RT-PCR reaction run in parallel. RT-PCR reactions were carried out using primers for *β-tubulin*, *Athsr5*, *KNAT2*, *SRG3* and *Athsr2/VDAC* (for primer sequences, see section 2.5.5). **B**. Transcript ratios obtained by microarray analysis for each of the selected transcripts was normalised against the microarray expression value for *β-tubulin*. Ratios obtained by microarray analysis are compared to those obtained by competitive RT-PCR analysis.

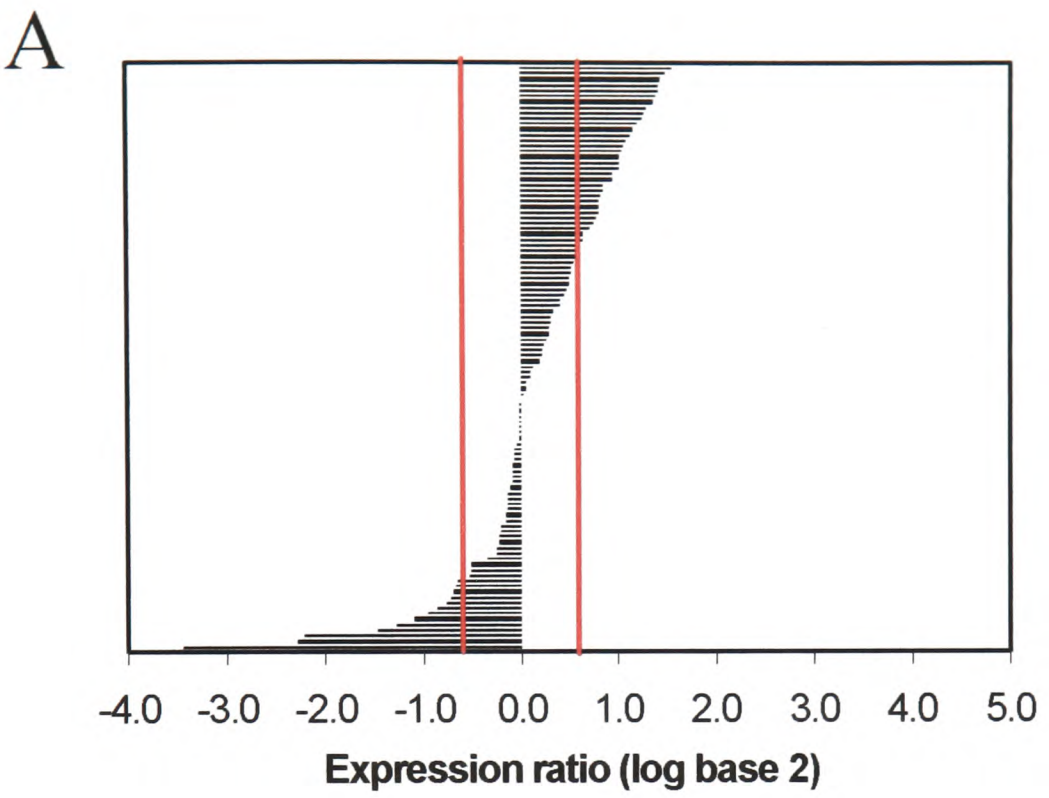


Figure 4.3. Evaluation of cDNA microarray data.
Expression ratios (PCD treatment/control) of transcripts following heat- (**A**) or senescence-induced (**B**) PCD determined by microarray analysis. The expression ratios are transformed on a logarithmic (base 2) scale. The positive expression ratios represent transcripts increased during PCD, and the negative expression ratios, transcripts decreased during PCD. In each analysis, the red lines indicate the cut-off (±1.5-fold) for genes for which the change in transcript level was not considered to be significant. Transcripts exceeding these thresholds were considered for further analysis and are highlighted in Table 4.2.

while the remainder, many of which are known senescence- or stress-related genes, were increased. Sixteen transcripts commonly increased in both heat- and senescence-induced PCD, while 9 transcripts commonly decreased in both systems (Figure 4.4).

In Table 4.2, transcripts with significant changes in expression profiles grouped by putative function are highlighted. The first group contains so-called 'housekeeping genes', often used in studies as control genes whose expression profiles are thought to remain constant throughout development. It is evident that, in this study, few such 'housekeeping genes' exist, and even *β-tubulin* expression decreased almost two-fold in senescing cultures. This was further supported by Northern blot analysis of the *β-tubulin* transcript abundance (data not shown). The second group of genes contains those with known mitochondrial-associated functions. In this study, there was no evidence that transcripts encoding mitochondrial proteins were generally upregulated during plant PCD. However, one of the two genes encoding the adenine nucelotide translocase (*ANT1*) exhibited a significant decrease in transcript levels in both treatments. Transcripts encoding cytochrome *c* and uncoupling proteins 1 and 2 increased during heat-induced PCD but not during senescence. Transcript levels of the α and β subunits of the mitochondrial ATPase were differentially regulated. Mitochondrial-encoded *Atp1* transcripts increased during senescence; levels of the nuclear-encoded *Atp2* transcript decreased in both systems.

Transcripts encoding several antioxidant-related proteins were found to be upregulated in both the heat and senescence system. These included the superoxide dismutases, *CSD1* and *CSD3*, and glutathione peroxidase (*GPX*). Several antioxidant genes were differentially regulated in the two systems. For example, expression of *MSD1* encoding the mitochondrial superoxide dismutase was increased during heat-induced oxidative stress, but was downregulated during senescence. Two of the catalase

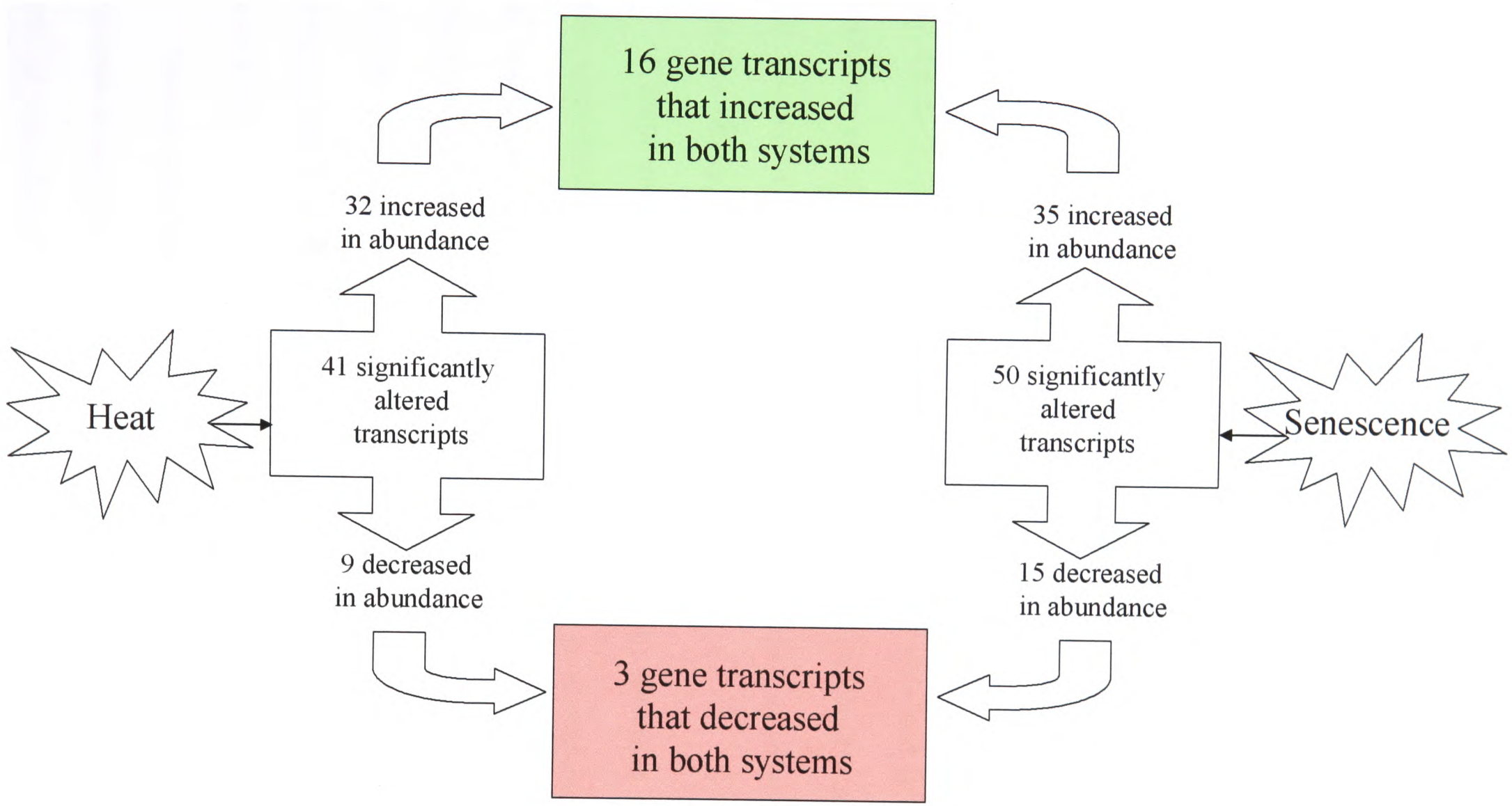


Figure 4.4. Summary of genes exhibiting significant changes in transcript levels during heat- and senescence-induced PCD.
Transcripts that changed ±1.5-fold or greater in either of the PCD-inducing treatments compared to control are summarised on the basis of whether they increased or decreased in abundance. Gene transcripts that either increased or decreased in both systems were of particular interest since these may be generally involved in plant PCD (see Table 4.2 for further details).

genes arrayed showed differential expression patterns between heat and senescence. *Catalase3* was found to be induced in both heat and senescence, albeit to a much greater degree in senescence, while the *sen2/catalase* gene was upregulated only during senescence. It was found that transcripts encoding gamma-glutamylcysteine synthetase (γ-ECS), the first enzyme in the glutathione biosynthetic pathway, but not glutathione synthetase (GSHS), the enzyme that catalyses the subsequent step in this pathway, were upregulated during senescence. Finally, glutathione-*S*-transferase (*GST6)* transcripts were significantly downregulated during senescence but unchanged during heat-induced PCD.

The expression profiles of several HR-related genes were also examined in this study. Of the *Athsr* genes originally identified by Lacomme and Roby (1999), *Athsr5* and *Athsr6* were also upregulated in this study, which is unrelated to the HR. All but one of the senescence-related genes, *SRG3*, exhibited marked increases in expression in senescence-induced cultures.

A large number of senescence-related transcripts increased in the senescence system, again confirming that ageing cell cultures are a good model system in which to study senescence. One senescence-activated transcript encoding a RING-H2 finger protein (*SAG* EST N37587) was also upregulated in heat-treated cells. It was found that transcripts encoding cysteine proteases involved in senescence, including an *Arabidopsis SENU3* homologue (Drake *et al.*, 1996), were also upregulated during heat-induced PCD.

Several transcripts, including those encoding transcription factors, were upregulated during both heat and senescence-induced PCD. These included the cytosolic isoform of monodehydroascorbate reductase, an enzyme involved in the ascorbate-glutathione cycle that detoxifies reactive oxygen species (Noctor and Foyer, 1998),

EREBP-4, and *STZ/Zat10*. Transcripts encoding the *Arabidopsis* Knotted2-like homeobox protein (KNAT2), but not those for KNAT1, KNAT3, or KNAT4, which were also included on the array, were upregulated in both heat- and senescence-induced PCD. Transcripts encoding calmodulin proteins were generally upregulated only during heat-induced PCD, while transcripts of the cytosolic *Hsp70-3* gene were downregulated in this treatment.

Table 4.2. Expression profiles of genes following PCD-inducing treatments in *Arabidopsis* cell suspension cultures.

Protein	Gene	Genbank Accession ID	ABRC Clone	Heat	Sen
Cell Maintenance ('housekeeping')					
beta-9-tubulin	*beta-9-tubulin*	M84706	198P12	-1.1	-1.9
polyubiquitin 4	*UBQ4*	X12853	103M18	1.4	2.3
ubiquitin	*UBQ*	H37347	180B9	-1.1	2.2
alpha-2-tubulin	*alpha-2-tubulin*	M84697	184O22	1.2	-2.4
actin	*actin 2*	U41998	222G17	2.0	1.2
histone H4	*histone H4*	M17132	ATTS0406	2.6	-8.9
oxygen evolving complex	*OEC*	X52428	249J13	-1.0	-2.3
translation elongation factor	*EF-1*	X16432	161F17	-1.9	1.1
translation initiation factor	*ELF4A*	X65052	184O13	1.0	1.0
elongation factor Tu	*TUFA*	X52256	205P2	1.2	1.3
HMG coA reductase	*HMG1*	J04537	185D15	1.2	1.6
phosphoglycerate kinase	*PGK*	U37701	125L3	1.8	-1.3
farnesyl pyrophosphate synthase	*FPS1*	X75789	F1A1	1.0	-1.4
Mitochondrial Function					
cytochrome *c*	*cyt c*	AF000657	N/A	2.7	-1.2
uncoupling protein	*AtPUMP /UCP1*	AJ223983	N/A	2.2	1.2
uncoupling protein	*UCP2*	AB021706	N/A	1.9	1.2
heat shock protein 60	*Hsp60*	AA067495	92A6	1.6	-3.9
adenine nucleotide translocase	*ANT 1*	X65549	N/A	-11.4	-1.6
adenine nucleotide translocase	*ANT2*	X68592	N/A	-11.0	1.2
voltage-dependent anion channel	*VDAC/Athsr2*	AJ131391	N/A	1.2	-1.2
alternative oxidase	*AOX/Athsr3*	AJ131392	N/A	1.3	-1.8
ATP synthase complex, subunit α (mitochondrial)	*Atp1*	Y08502	N/A	1.4	3.4
ATP synthase complex, subunit β(mitochondrial)	*Atp2*	T21236	93E4	-2.4	-1.7
ATP synthase complex, subunit β(mitochondrial)	*Atp2*	T22836	107I9	-4.6	-1.3

Table 4.2. *Continued*

Protein	Gene	Genbank Accession ID	ABRC Clone	Heat	Sen
Antioxidant Function					
Cu/Zn superoxide dismutase (cytosolic)	*CSD1*	X60935	N/A	2.9	1.9
Cu/Zn superoxide dismutase (peroxisomal)	*CSD3*	AF061520	N/A	1.8	1.8
Mn superoxide dismutase (mitochondrial)	*MSD1*	AF061518	N/A	2.7	-1.5
ascorbate peroxidase (cytosolic)	*APX1*	N96408	F1D10	-1.2	1.7
glutathione peroxidase (cytosolic)	*GPX*	AA067390	88H14	4.6	7.4
monodehydroascorbate reductase (cytosolic)	*MDHAR*	N38638	221C13	2.1	1.5
monodehydroascorbate reductase (chloroplast/mitochondrial)	*MDHAR*	N96254	G7B8	-1.3	-1.4
catalase	*sen2/catalase*	AA067385	88E3	-1.8	16.9
catalase	*cat3 (cat1)*	N37201	201A13	2.1	10.8
gamma-glutamylcysteine synthetase	*γ-ECS*	R90472	187L7	1.4	5.3
glutathione synthetase	*gsh2*	Z50153	N/A	1.1	1.2
glutathione-*S*-transferase	*GST6*	T45246	83B5	1.2	-3.0
Hypersensitive Response					
Athsr5	*Athsr5*	AJ243378	N/A	1.8	2.6
Athsr6	*Athsr6*	AJ243379	N/A	1.5	2.7
Athsr7	*Athsr7*	AJ243380	N/A	1.1	-2.0
At Bax Inhibitor-1	*AtBI-1*	AF208124	N/A	2.5	1.4
zinc-finger protein, LSD1	*Lsd1*	R84041	82D11	1.4	1.4
Senescence					
senescence-activated gene	*SAG*	N65251	224A3	-1.0	21.9
senescence-activated gene (ring-finger protein)	*SAG*	N37587	212B17	1.6	30.8
senescence-activated gene	*SAG*	T76398	149L5	1.4	7.2
SENU3-like protease	*SENU3-like*	H37279	179H20	1.5	4.8
cysteine protease	*cysteine protease*	N38085	218B14	1.8	13.1
senescence-related gene 1	*SRG1*	X79052	N/A	-1.1	2.5
senescence-related gene 2 (C-term)	*SRG2C*	X82624	N/A	1.3	23.0
senescence-related gene 2 (N-term)	*SRG2N*	X82623	N/A	-1.2	22.6
senescence-related gene 3	*SRG3*	X98376	N/A	2.8	-1.9

Table 4.2. *Continued*

Protein	Gene	Genbank Accession ID	ABRC Clone	Heat	Sen
Other (Metabolism, Development, etc.)					
calmodulin	*CaM6*	N65115	227I7	2.4	-1.1
calmodulin	*CaM5*	H77078	203B18	2.1	-1.2
calmodulin	*CaM3*	N38696	221M6	2.3	-1.0
calcium-dependent protein kinase	*CDPK2*	R89947	186L12	1.4	-1.3
calcium-dependent protein kinase	*CDPK4*	R30212	157K18	1.1	1.7
calcium-dependent protein kinase	*CDPK5*	N96086	G1D1	-1.6	1.5
calcium-dependent protein kinase	*CDPK6*	R65465	171D8	-1.1	1.1
calcium-dependent protein kinase	*CDPK9*	T42899	119L21	-1.4	1.7
knotted-like protein 2	*KNAT2*	N65651	240N22	1.7	1.6
knotted-like protein 3	*KNAT3*	T44387	124O9	1.5	1.2
knotted-like protein 4	*KNAT4*	N38159	220C17	1.3	1.6
zinc-finger protein	*STZ/ZAT10*	R90419	186P13	2.6	1.8
SPF1-like DNA-binding protein	*SPF1-like*	T45479	132H23	1.3	2.3
ethylene responsive element binding protein	*EREBP-4*	H36563	174N22	2.0	1.8
heat shock protein 70-3	*Hsp70-3*	N96136	G5C8	-4.8	-1.2
malate synthase	*MS*	T04620	N/A	1.9	2.2
thiolase	*Thiolase*	AB008854	N/A	-1.1	15.2
isocitrate lyase	*ICL*	Z18772	N/A	1.8	1.3
cinnimate-4-hydroxylase	*C4H*	U71081	N/A	-1.6	-6.5
alcohol deydrogenase	*ADH*	N97103	244O12	-1.1	-11.4
hexokinase 2	*AtHXK2*	AA041077	E1C7	1.7	-1.8
glycerol kinase	*glycerol kinase*	T43219	119C8	1.2	2.8
unknown	unknown	AF069442	N/A	1.6	2.1
unknown	unknown	AC005278	N/A	1.3	1.3

Arabidopsis cell cultures were induced to undergo PCD by either of two means: Six day-old cultures were subjected to a heat treatment (55°C, 10 min) and RNA extracted immediately thereafter (Heat), or untreated cultures were allowed to undergo senesce until 13 to 14 days after subculturing at which point RNA was extracted (Sen). Fluorophore-labelled cDNA synthesised from total RNA was used to screen a custom cDNA microarray (for a detailed description, see section 2.7). The fold-change for each trancript in PCD treatment/control was calculated based on an average from 3 array replicates, one of which included a 'dye-swap' of Cy3 and Cy5 for control and PCD samples. Transcript expression ratios <1 were tranformed to -1/ratio. Transcripts which increased or decreased by a factor of 1.5 or more are shaded, with transcripts that increased in green, and those decreased in red. If the cDNA was obtained as an EST clone from the ABRC (Ohio State, USA), the ABRC clone ID is given. Genes marked "N/A" were either independently cloned cDNAs or obtained as gifts from colleagues (see section 2.6). Genbank accession numbers are given for each EST clone or that corresponding to each cDNA.

4.3. Discussion

4.3.1. A custom cDNA microarray analysis of heat- and senescence-induced PCD reveals changes in gene transcript levels that are common to both systems

In this chapter, changes in the transcript expression profiles of over 90 genes that have been reported to be involved in programmed cell death and/or senescence, or which may have a role in these processes, were investigated. Very few genes have been previously identified that can be used as markers for plant PCD irrespective of the inducing stimulus. Many HR-related genes, senescence-related genes, or stress-related genes have been identified (Lacomme and Roby, 1999; Pontier *et al.*, 1999; Yoshida *et al.*, 2001). It is unclear whether changes in expression of these genes are unique to the particular developmental or physiological event, or whether a subset of these genes have a role in the PCD which is associated with these processes.

The aim of this study was to determine if genes which had previously been proposed to have direct or indirect roles in plant PCD exhibited similar transcript expression profiles when compared across two, independent PCD-inducing treatments (heat and senescence), and whether any other novel, molecular markers of plant PCD could be identified. Additionally, by identifying any putative cell death-related genes which were upregulated at the transcriptional level following PCD induction, it is possible to suggest a role for their gene products in plant PCD. Indeed, transcription is known to be necessary for the execution of PCD, and pro- and anti-apoptotic proteins have been shown to be regulated at the transcriptional level in animals (Hengartner, 2000; Meier *et al.*, 2000; Chiang *et al.*, 2001).

4.3.2. Changes in total cellular RNA content during PCD may affect microarray results

It is important to recognise that the transcript ratios calculated and reported in this microarray analysis should not be interpreted as exact numbers for fold-induction or -repression, since overall transcript abundance on a per cell basis is suggested to change markedly during PCD (see discussion below). In addition, the results obtained by this microarray analysis were influenced by the fact that the 'total fluorescence' of the microarray was calculated only on the basis of approximately 100 selected genes. This may introduce a bias when the array is normalised (against total flourescence) if a large proportion of those selected genes have altered expression profiles during PCD (a factor in any custom array that does not include the organism's entire transcriptome). Moreover, previous studies have demonstrated that a direct linear relationship between transcript abundance and fluorescence intensity does not exist (Ruan *et al.*, 1998).

The fact that putative 'housekeeping' genes showed altered expression levels in the analyses described underscores the need for caution when using a particular gene as a marker of stable (unchanged) transcript expression levels. This is particularly important since such 'housekeeping genes' are often used as normalisation factors in array analyses (Reymond *et al.*, 2000). Since the arrays analysed in this study did not contain a selection of 'control' genes whose transcript expression could be demonstrated to be unchanged in both PCD-inducing treatments compared to the control, normalisation of fluorescence was achieved by a global method in which the fluorescence intensities of individual transcripts were calculated in comparison to the total fluorescence obtained from all of the genes on the array.

In previous studies using cDNA microarray analysis of steady-state transcript levels, arbitrary ratios of ±2.0- or 3.0-fold in the level between control and experimental

mRNA transcripts have been taken to be significant (Ruan *et al.*, 1998; Aharoni *et al.*, 2000; Reymond *et al.*, 2000; Schenk *et al.*, 2000; Kawasaki *et al.*, 2001). However, what constitutes a 'significant' change in transcript expression may vary depending on the system under investigation and the quality of the array data (Brazma and Vilo, 2000). In this study and others (Desikan *et al.*, 2001), a ratio of ±1.5-fold in transcript levels between control and treatments was deemed to be significant, since this was supported by reproducibility in the results both between arrays and when changes in gene expression were investigated by a competitive RT-PCR method.

It is important to note that, during the induction and execution of PCD, total RNA decreases on a per cell basis (in senescing cucumber cotyledons) (Werner Howad, unpublished results). In fact, total RNA yield may decrease up to 10-fold (Buchanan-Wollaston, 1994; Quirino *et al.*, 2000). Against this backdrop of decreasing RNA levels, a smaller increase in a given transcript's abundance may be significant in terms of gene function. Equal amounts of total RNA were used for reverse transcription for both control and treated samples. However, due to the likely decreased RNA yields per cell in PCD-treated cultures, a gene for which the steady-state transcript level remains unchanged on a cellular basis during PCD will appear to increase up to 10-fold in copy number relative to other transcripts if the total cellular RNA decreases by 10-fold. It is important to consider that such a transcript may not be upregulated during PCD, but it may not be degraded at the same rate as the majority of cellular transcripts (Buchanan-Wollaston, 1994). Given that other researchers have suggested that the majority of transcripts are downregulated during PCD (Buchanan-Wollaston, 1994), maintained or increased steady-state expression levels for a particular gene transcript may be indicative of an important function for that gene product during PCD. While PCD is a particular case in which extensive cellular RNA degradation occurs, the expression profiles

inferred from microarray data may always be influenced by the possibility that total RNA yields per cell are not equal across control and treated cells. Researchers working with such systems should account for this possibility in their interpretation of array results.

4.3.3. Transcript profiles of genes encoding mitochondrial proteins during PCD

In light of the key role of mitochondria and mitochondrially-localised proteins in the induction and execution of animal cell death (Green and Reed, 1998), it was considered to be of interest to examine the transcript profiles for a selection of genes encoding mitochondrial proteins. The release of cytochrome *c* and other apoptotic proteins from the mitochondrial intermembrane space is a key event in the induction of PCD in mammalian systems (Parone *et al.*, 2002). However, the release of these proteins may not necessarily be transcriptionally regulated since it is thought to be mediated by either a complexing of existing proteins in the inner and outer mitochondrial membranes to form a permeability transition pore, or the recruitment of pro-apoptotic proteins such as Bax to the outer mitochondrial membrane (Parone *et al.*, 2002). However, several genes encoding mitochondrial membrane and matrix proteins are differentially expressed during mammalian cell death. During lymphoma cell death, for example, uncoupling proteins and VDAC are transcriptionally upregulated (Voehringer *et al.*, 2000). Similarly, a microarray analysis of neuronal cell death demonstrated that transcripts encoding mitochondrial proteins including the adenine nucleotide translocase, cytochrome *b*-560, cytochrome *c* oxidase, and several other oxidoreductases are modulated during the cell death programme (Chiang *et al.*, 2001).

While no general upregulation of the expression of transcripts encoding selected mitochondrial proteins was apparent from this analysis, it was of interest to observe a significant downregulation of transcripts encoding the mitochondrial adenine nucleotide

translocase, ANT1. A decrease in ANT protein, particularly if associated with mitochondrial biogenesis (as has been shown during PCD in human colon carcinoma cells (Mancini *et al.*, 1997)), would limit exchange of ATP/ADP and thus inhibit ATP synthesis. In neuronal cell death, *ANT* expression was also decreased during the early phase of cell death (Chiang *et al.*, 2001) and, in an *Arabidopsis* microarray analysis of defense responses, Schenk *et al.* (2000) demonstrated the repression of a transcript encoding ANT following treatment with salicylic acid, methyl jasmonate, and the inoculation with an incompatible fungal pathogen. Collectively, these results provide preliminary evidence that a disruption of the cell's energy supply may be a general feature of early stages of cell death in both plant and animal cells, and may be one factor that triggers the later phases of the molecular machinery of cell death. Indeed, in murine pro-B cell lines, it has been demonstrated that a disruption of ATP/ADP exchange between the mitochondrion and the cytosol is one of the earliest events following induction of apoptosis (Vander Heiden *et al.*, 1999). Moreover, this disruption of ATP/ADP exchange, which is suggested to be due to defective function in inner membrane ANT, outer membrane VDAC, or both, is hypothesised to be one of the chief inducers of downstream apoptotic events, including mitochondrial swelling, cytochrome *c* release, and the opening of the permeability transition pore. Therefore, it is tempting to suggest that, in plant cells, downregulation of ANT may contribute to a similar disruption of ATP/ADP exchange and may be one of the triggers of cell death.

The genes encoding the mitochondrial uncoupling proteins, UCP1 and UCP2, exhibited similar transcript profiles in both PCD-inducing treatments (increasing after heat treatment but not during senescence). The upregulation of *UCP1* and *UCP2* transcripts following heat treatment may play a role in limiting reactive oxygen species formation following heat treatment (Larkindale and Knight, 2002), as suggested for the

role of these proteins in potato mitochondria (Kowaltowski *et al.*, 1998). It has previously been shown that, in murine B cell lymphoma lines, there is specific upregulation of only one member of the UCP family following induction of apoptosis by ionising radiation (Voehringer *et al.*, 2000). It is possible that other UCP family members in *Arabidopsis* apart from those used in this study (and which were identified with the complete sequencing of the genome (The *Arabidopsis* Genome Initiative, 2000)) may be differentially regulated during plant PCD. The observed differential regulation of mitochondrial-encoded *Atp1* and nuclear-encoded *Atp2*, both encoding subunits of the mitochondrial ATP synthase complex, could be explained by the fact that the former transcript may be protected or maintained at steady-state levels during PCD due to its localisation within the mitochondria. As mentioned earlier, proliferation of mitochondria has been observed prior to apoptotic death in human colon carcinoma cells (Mancini *et al.*, 1997). It is of interest to note that the transcripts for a specific isoform of VDAC (originally identified as *VDAC/Athsr2*), were upregulated during the HR of *Arabidopsis* cultures inoculated with pathogenic *Xanthomonas* (Lacomme and Roby, 1999), and yet did not change significantly in either of the PCD-inducing treatments in this study. VDAC may therefore play a specific role during the HR that is not universally connected with PCD.

4.3.4. Transcript profiles of antioxidant genes during PCD

Oxidative stress clearly plays an important role in plant PCD and, undoubtedly, many transcripts induced during PCD may be part of an antioxidant response. However, whether the production of reactive oxygen species (ROS) during PCD is a consequence of PCD-inducing stimuli or is a necessary precursor to allow PCD to proceed (as suggested for barley aleurone cell death and in tobacco) remains under investigation (Mittler *et al.*, 1999b; Fath *et al.*, 2001). Changes in transcript profiles for several

antioxidant genes were seen in the microarray analysis. The transcripts for the cytosolic and peroxisomal isoforms of Cu/Zn superoxide dismutase (SOD) (*CSD1 and CSD3*) were upregulated by heat treatment and senescence, which is in accordance with their roles as general stress-response enzymes in *Arabidopsis* (Kliebenstein *et al.*, 1998). The upregulation of transcripts encoding cytosolic glutathione peroxidase (GPX) and cytosolic monodehydroascorbate reductase (MDHAR), enzymes involved in the gluathione and ascorbate cycles (Eshdat *et al.*, 1997; Noctor and Foyer, 1998), occurred both following heat treatment and during senescence. These genes may therefore be potential markers for general oxidative stress and/or PCD.

However, several of the antioxidant genes examined were differentially regulated in heat treatment and senescence, suggesting that the type of oxidative stress induced may result in specific metabolic accommodation strategies. This is supported by previous studies that have demonstrated the differential regulation of the seven *Arabidopsis* SODs during various environmental stresses, including high light treatment, ozone fumigation, and UV-B radiation (Kliebenstein *et al.*, 1998). The upregulation of catalase (*sen2/catalase*) transcripts only during senescence-induced PCD suggests that this gene, originally identified in a study of gene expression during leaf senescence in *Arabidopsis* (Park *et al.*, 1998), may be under a strict senescence-specific regulation. Such a marker for senescence may be useful in further studies to identify unique promoter elements which are specific to genes expressed only during senescence, thus helping to elucidate the senescence regulatory network. The upregulation of gamma-glutamylcysteine synthetase (*γ-ECS*) transcripts during senescence suggests that cells at this stage of development ameliorate increased oxidative stress by increasing glutathione production, since increased γ-ECS activity in plants is associated with glutathione production (May *et al.*, 1998). Finally, it is of interest that transcripts for glutathione-*S*-

transferase 6 (*GST6*) were significantly downregulated in senescence-induced PCD, since Kampranis *et al.* (2000) demonstrated that tomato glutathione-*S*-transferase was capable of suppressing mammalian Bax-induced cell death in yeast. Therefore, the downregulation of *GST6* in senescing cells may be necessary for allowing the progression of some types of PCD.

4.3.5. Transcript profiles of HR- and senescence-related genes during PCD

Several cDNAs corresponding to genes whose transcripts have been previously shown to be elevated during the HR or senescence were arrayed to determine their transcript profiles during heat- and senescence-induced PCD. Interestingly, of the HR-related genes, transcripts for *Athsr5* and *Athsr6*, which encode unknown proteins (Lacomme and Roby, 1999), were upregulated following both the heat treatment and during senescence. The common upregulation of these transcripts during PCD associated with the HR, heat treatment, and senescence, suggests that these three systems may share common molecular mechanisms. Moreover, this demonstrates that identifying a gene as 'HR-regulated' does not exclude a role for its product in other types of PCD.

All but one of the senescence-related genes on the array exhibited marked increases in transcript expression in senescing cell cultures. This suggests that the observed PCD in the cell cultures was the endpoint of senescence and that cell cultures are a suitable system for studying senescence. Expression of the one selected gene whose transcripts did not increase during senescence, *SRG3*, was previously shown to be transiently increased just prior to the onset of senescence and decreased thereafter in *Arabidopsis* cell cultures (Callard *et al.*, 1996). It was therefore not surprising that it was downregulated in the senescing samples used in this study. It was intriguing, however, that *SRG3* transcripts were upregulated following heat induction of PCD, suggesting that

this gene may play a role in the early stages or at the onset of plant cell death. Three transcripts increased during senescence-induced PCD were similarly increased following a heat treatment. These included the *Senescence-Activated Gene* (*SAG*) (N37587), which encodes a ring-finger protein of unknown function, as well as two proteases, the homologue to tomato *SENU3* (Drake *et al.*, 1996) and a general cysteine protease. The upregulation of the latter two transcripts indicates that proteolytic degradation is a feature of both PCD systems in this study.

4.3.6. Transcript profiles of other genes involved in cellular signalling, metabolism, and gene regulation during PCD

Several cDNAs arrayed in this study included those involved in Ca^{2+} signal transduction and protein phosphorylation, since Ca^{2+} signalling has been implicated in plant PCD (Price *et al.*, 1994; Levine *et al.*, 1996; Xu and Heath, 1998; Jakobek, 1999; Navarre and Wolpert, 1999). As well, transcript profiles of mRNAs encoding proteins involved in gene regulation (many of which are homologues to transcripts that were upregulated during PCD in senescing cucumber cotyledons (Werner Howad, unpublished results)) were investigated, in addition to transcripts encoding proteins involved in other aspects of general cellular metabolism.

Several of the transcripts encoding transcription factors arrayed were upregulated during both heat- and senescence-induced PCD, suggesting that the encoded proteins may play a regulatory role during this cellular event. The transcription factor STZ/Zat10, which has a known role in the regulation of salt tolerance in *Arabidopsis* (Lippuner *et al.*, 1996), may be generally involved in a wide range of stress responses including those leading to cell death. The upregulation of transcripts for KNAT2, the *Arabidopsis* homologue to maize homebox domain protein Knotted2, was also of considerable interest. While no downstream targets of this gene have been identified in

either maize or *Arabidopsis* (Lincoln *et al.*, 1994; Ori *et al.*, 1999), the results obtained here suggest that KNAT2 may play a role in plant PCD. Transcripts encoding the Ethylene Responsive Element Binding Protein-4 (EREBP-4), which were upregulated in both PCD systems, is in accordance with the role of this family of transcription factors in plant defense and stress responses (Singh *et al.*, 2002). However, transcripts encoding calmodulin proteins were generally upregulated only following heat-induced PCD.

Finally, it is of interest that transcripts for the cytosolic Heat shock protein 70-3 (Hsp70-3) were downregulated following the induction of PCD by heat treatment. In a mammalian cell-free system, Hsp70 has recently been shown to exert a negative regulatory role on cell death by binding to and inhibiting Apaf-1, a component of the apoptosome (Saleh *et al.*, 2000). It has also been suggested that Hsp70 may function to protect the mitochondrial membrane and prevent cytochrome *c* release (Polla *et al.*, 1996; Mallouk *et al.*, 1999), as well as protecting against the ATP depletion that is associated with cell death (Wong *et al.*, 1998). Increased expression of Hsp70 is associated with resistance to apoptosis in stressed cells and tumour cells (Jaattela, 1999). It is possible that, in plant cells, cytosolic Hsp70-3 acts similarly as a negative regulator of PCD, and the downregulation of this gene is one of the early events that allows for the execution of cell death.

4.3.7. Conclusions

The results of the cDNA microarray expression data using a selected set of cDNAs with proposed roles in plant PCD has allowed for the identification of a subset of genes whose transcripts are commonly up- or downregulated in cell cultures during PCD following heat treatment or during senescence. As some of these genes have also been previously implicated in the HR or stress responses, the results presented in this chapter indicate that some overlap in differential transcript expression between heat-,

HR-, and senescence-induced PCD may exist. These genes include those implicated in antioxidant responses, such as *CSD1*, *CSD3*, cytosolic *GPX*, cytosolic *MDHAR*, and *catalase3*, and genes associated with cellular degradation, such as cysteine proteases. Additionally, genes that have been previously shown to be markers of *Arabidopsis* HR, such as *Athsr5*, *Athsr6*, and *AtBI-1*, may prove to be universally involved in plant PCD. The transcription factors STZ/ZAT10, KNAT2, and EREBP-4 may be components of the plant PCD regulatory network(s).

In spite of the finding that transcripts encoding mitochondrial proteins are not generally upregulated during PCD, the relative increase in expression of the mitochondrial-encoded *Atp1* compared with the nuclear-encoded *Atp2* suggests that mitochondrial integrity may be maintained during the execution of plant PCD, thereby protecting the former transcript from degradation. The downregulation of one of the adenine nucleotide translocase gene transcripts (*ANT1*) observed in this study and other cell death arrays (Schenk *et al.*, 2000; Chiang *et al.*, 2001) suggests a role that plant mitochondria may have cellular demise by limiting energy resources in a process akin to that proposed in animal PCD (Vander Heiden and Thompson, 1999; Matsuyama and Reed, 2000). Likewise, the downregulation of *Hsp70-3* gene expression may be one of the early events in plant PCD if the Hsp70-3 protein exerts an anti-apoptotic role similar to that described in animal cells.

These observations support the need for future investigations to utilise more than one system of PCD induction before generally ascribing a role for a gene product in plant cell death. As with all microarray studies, it must be noted that, in spite of the fact that many putative cell death genes do not appear to be transcriptionally regulated during PCD, this does not preclude a role for their gene products in this process. In the future, the use of multiple PCD-inducing systems, combined with large-scale

investigations such as whole-genome microarrays and cellular and subcellular proteome analysis, will be necessary to both identify the genes and proteins involved in the induction and execution of plant cell death and to elucidate the crosstalk between the regulatory pathways involved in plant PCD. The results presented in this chapter have highlighted a number of genes which may be implicated in plant PCD, and for which further functional analyses can be undertaken to more clearly define their role in this process.

CHAPTER FIVE

5. A genome-wide analysis of changes in transcript expression during programmed cell death in *Arabidopsis*

5.1. Introduction

The custom cDNA microarray analysis described in Chapter 4 was successful in identifying transcripts that were regulated in common during both heat- and senescence-induced PCD in an *Arabidopsis* cell suspension culture. This analysis provided a starting point for a more detailed functional analysis of genes of interest. However, it was limited to only the ~100 selected cDNAs that were arrayed. Undoubtedly, the regulation and execution of plant PCD will involve many other genes that were not included on the custom array. To undertake a more comprehensive survey of changes in transcript expression during plant PCD, it was therefore desirable to screen the maximum number of genes possible (ideally, the entire *Arabidopsis* genome) to identify novel targets that may be involved in this process.

However, the preliminary custom array analysis was useful at the time at which it was conducted in that it allowed a study of changes in steady-state levels of specific transcripts of interest. Many of these are known to be under strict senescence- or stress-specific regulation, and others, such as transcription factors, may be present in low abundance. Moreover, by using cDNA probes that were specific for a particular member of a gene family (for e.g. *UCP1* and *UCP2*, *ANT1* and *ANT2*), it was possible to examine individual changes in transcript expression for highly similar genes. These advantages are not necessarily conferred by large-scale cDNA microarrays, i.e. those which are printed on a high-throughput scale using EST library collections as a source

of cDNA templates. For example, ESTs arrayed on the cDNA microarrays available through the *Arabidopsis* Functional Genomics Consortium (AFGC) included approximately 11,000 cDNAs primarily derived from an *Arabidopsis* EST collection generated from etiolated seedlings, roots, leaves, and flowering inflorescences (Newman *et al.*, 1994). An analysis of these ESTs revealed that they included only approximately half of the genes selected for the custom microarray analysis, suggesting that senescence-, stress-, or, indeed, PCD-specific genes were under-represented on the AFGC array.

In mid-2002, Affymetrix produced the first *Arabidopsis* ATH1 GeneChip. The GeneChip contained 22,500 oligonucleotide probes synthesised *in situ* and designed to detect over 24,000 gene sequences based on the complete sequence of the *Arabidopsis* genome (The *Arabidopsis* Genome Initiative, 2000). This constituted a new and exciting resource in *Arabidopsis* functional genomics, and it provided an opportunity to screen the largest number of genes possible to identify those transcripts that are similarly regulated during both heat- and senescence-induced PCD. RNA isolated from control and PCD-induced (heat and senescence) cell suspension cultures was used to probe the GeneChip through a service provided by the Genomics *Arabidopsis* Resource Network (GARNet, UK). RNA samples used for screening the GeneChip arrays were derived from different cultures than those used to screen the custom cDNA microarray, but were treated, prepared, and pooled in the same manner. The analysis of the GeneChips described in this chapter was successful in identifying additional genes that may be involved in plant PCD. Additionally, a descriptive analysis of the functional classes of gene transcripts that are commonly regulated in heat- and senescence-induced PCD may be useful in furthering our understanding of the similarities between responses to heat

and senescence/starvation, and allows for speculation on what might constitute the common signals and regulatory mechanisms in plant PCD.

5.2. Results

5.2.1. Sample collection for GeneChip analysis

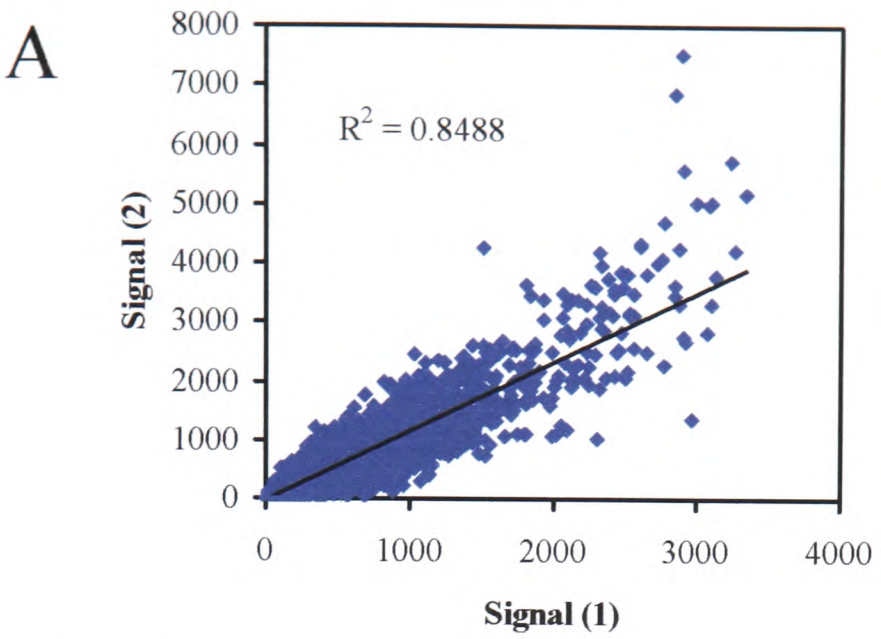
RNA samples used for Affymetrix ATH1 GeneChip analysis were derived from cultures treated exactly as those for the custom microarray analysis (see section 4.2.1). For each of the control, heat-treated, and senescence samples, total RNA was pooled from 3 independent cultures. RNA quality control procedures were carried out by the GARNet facility and included analysis of RNA and cRNA integrity using the Agilent Bioanalyser (for details, see http://nasc.nott.ac.uk/prototype/).

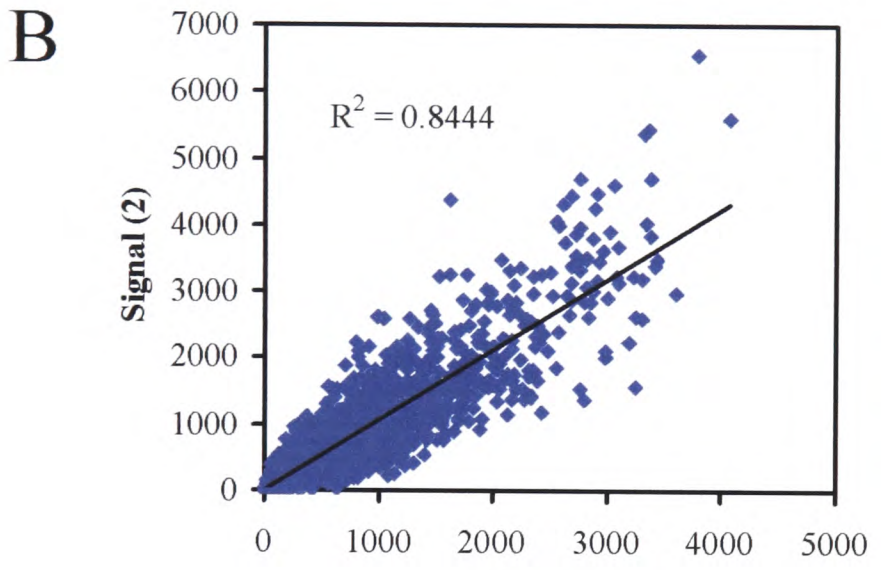
5.2.2. GeneChip hybridisation experiments and data collection

The synthesis of cRNA and hybridisation of the Affymetrix ATH1 GeneChips were carried out by the GARNet Transcriptomics Facility (Nottingham, UK). Duplicate chips were hybridised for each of the control, heat-treated, and senescence samples. Normalisation of data was achieved using Affymetrix software and involved global normalisation using a 'scaling factor' such that comparisons between different chips could be made. Transcript abundance ratios were calculated by comparing the signal value for each control sample pairwise to each PCD sample, for a total of 4 expression ratios per treatment and 8 expression ratios in total (4 heat/control and 4 senescence/control).

5.2.3. Reproducibility of GeneChip data

In order to calculate the reproducibility between duplicate GeneChips, the correlation coefficient (R^2) of signal values between duplicates was determined (Figure 5.1). The chips showed significant correlation coefficients ($p<0.05$) indicating good reproducibility. Various threshold values (2.0, 2.5 and 3.0) for fold-changes in transcript





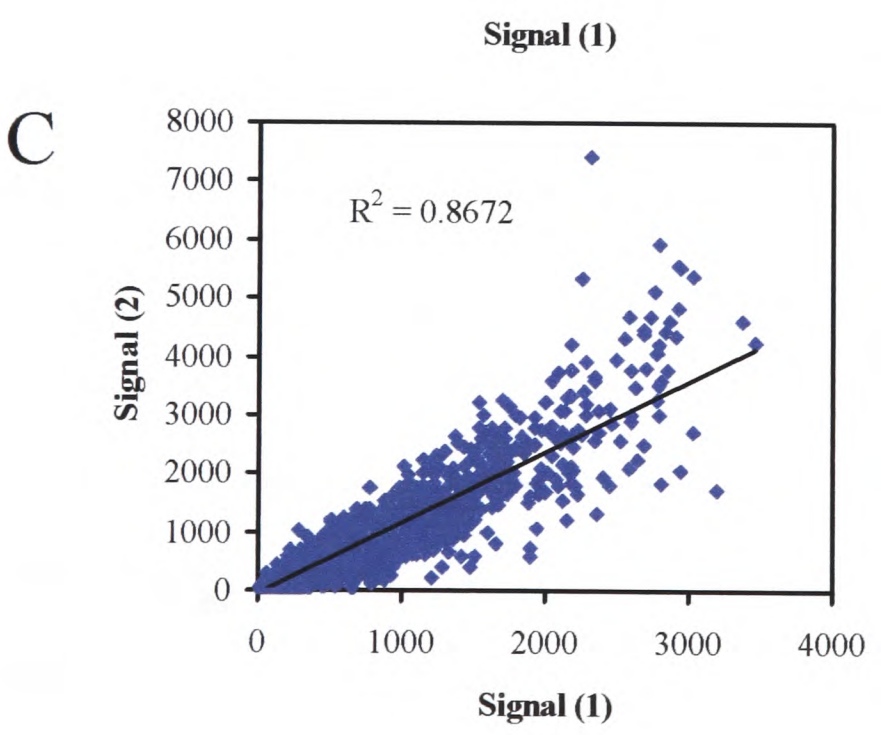


Figure 5.1. Correlation coefficients between duplicate GeneChip experiments. The signal values obtained for each probe set in one GeneChip experiment were plotted against those obtained in a duplicate GeneChip experiment for control (**A**), heat-treated (**B**), and senescence samples (**C**). The correlation coefficient (R^2) for signal values was calculated and is indicated on each graph. In all cases, the GeneChips demonstrate a significant degree of correlation between duplicates ($p<0.05$) indicating excellent reproducibility.

expression were applied to calculate false-positive rates (percentage) and the number of false positives that would be therefore be expected when comparing 4 replicate ratios for 22,500 probe sets (Table 5.1). False-positives were determined as those transcripts which showed a variation of greater than or equal to the applied threshold ratio between duplicate GeneChips for each of the control, heat-treated, or senescence samples. It was found that an acceptable false positive rate of ≤2% (Lipshutz *et al.*, 1999) was achieved with the application of a 2.5-fold threshold ratio for changes in transcript abundance in treated samples compared to control. Using a 2.5-fold ratio threshold with 4 comparisons to detect significant changes in gene expression, the false-positive rate was predicted to be <3 per 10^7 genes. Moreover, this threshold ratio and associated false positive rate is in accordance with values reported in the literature (Voehringer *et al.*, 2000; Cheong *et al.*, 2002; Fowler and Thomashow, 2002).

5.2.4. Comparison of GeneChip results with custom cDNA microarray data

It was of immediate interest to determine whether the results obtained in the custom cDNA microarray analysis (Chapter 4) were reproduced using the Affymetrix GeneChip analysis, since this would provide extra validation for both data sets. BLAST algorithm (Altschul *et al.*, 1997) was used to find the corresponding *Arabidopsis* Genome Initiative (AGI) accession number for each cDNA in the custom microarray analysis. Where a cDNA could be found to correspond to a specific AGI sequence, the GeneChip data for the probe corresponding to that AGI number was analysed.

It was difficult to compare directly across these two microarrays since different threshold levels were used for each to represent significant changes in gene expression. A 1.5-fold threshold in the custom cDNA array was found to represent real and significant changes in gene expression as confirmed by competitive RT-PCR analysis, and to be capable of representing statistically significant changes in gene expression

Table 5.1. False positive rates for GeneChip replicates with application of different threshold ratios.

	Control	Heat	Senescence
% false positive with 2.0-fold ratio	4.50	5.98	3.48
Number expected across 4 replicates	$4.09E^{-06}$	$1.28E^{-05}$	$1.47E^{-06}$
% false positive with 2.5-fold ratio	1.60	2.39	1.44
Number expected across 4 replicates	$6.56E^{-08}$	$3.26E^{-07}$	$4.33E^{-08}$
% false positive with 3.0-fold ratio	0.78	1.10	0.72
Number expected across 4 replicates	$3.63E^{-09}$	$1.49E^{-08}$	$2.67E^{-09}$

Transcript expression ratios were calculated pairwise from signal values between each duplicate set of control (6 days-old), heat-induced PCD (55°C, 10 min), and senescence-induced PCD (13 to 14 days-old) GeneChips. This gave a 'fold-change' in transcript expression between replicates (i.e. in a perfect replicate, this number should be equal to 1). False positives were calculated by applying either a 2.0-, 2.5-, or 3.0-fold threshold value and determining the percentage of transcripts that exceeded this threshold ratio across replicate GeneChips (expressed as % false positive). The number of false positives that would thus be expected when considering 22,500 probe pairs across 4 replicate GeneChips was determined. This is because, in the final GeneChip analysis, fold-changes in transcript expression were determined by comparing each PCD GeneChip pairwise to each control GeneChip, giving 4 ratios per PCD treatment/control (see section 2.8).

given the random variation in replicate microarrays (see section 4.2). However, in the GeneChip analysis, a 2.5-fold threshold was used to represent a significant change in gene expression since, when analysing over 20,000 sequences, this cut-off gave an acceptable false positive value (Table 5.1). This threshold ratio stringently selected for a small subset of genes that could be considered, with high confidence, to be commonly regulated in both PCD treatments. This does *not* mean that genes exhibiting lower (for example, 1.5- or 2.0-) fold changes in transcript steady-state levels were not significantly altered. Rather, using a higher cut-off and applying stringent selection criteria allowed for a higher degree of confidence in the results *in the absence of other experimental confirmation* and allowed for a small subset of genes to be analysed in detail.

Therefore, to compare data between the two microarray experiments, genes present in both microarrays which showed changes in transcript levels of ±1.5-fold for PCD/control were compared. It was found that 53% of transcripts which showed significant changes in the heat-induced PCD/control custom cDNA microarrays were replicated in the Affymetrix GeneChip array results, and that 75% of the senescence-induced PCD/control custom cDNA transcript changes were replicated in the GeneChip senescence array results. Across both treatments, this gave an overall replication of 64%. However, some of the changes in transcript levels that were found to be significant in the custom microarray analysis, such as for *Athsr5*, *KNAT2*, and *SRG3*, were not confirmed in the GeneChip data and yet were confirmed by competitive RT-PCR (see Figure 4.2), indicating reproducibility of the custom cDNA microarray results.

In fact, it was found that slightly less than 50% of the upregulated transcripts identified in the custom cDNA microarray analysis for heat-induced PCD were not altered by 1.5-fold or greater in the GeneChip array. This may suggest a bias against

detecting transcript expression in GeneChips probed with RNA derived from heat-treated cultures as opposed to controls. This could conceivably be due to problems with the 'scaling factor' and normalisation methods applied to these Affymetrix arrays. However, since the problem of relatively low levels of replication between the two array analyses relates to transcripts being *unchanged* in steady-state levels rather than a disproportionate number being significantly different in the heat/control GeneChips, it was unlikely to result in the selection of false positive changes. If anything, there may have been a higher number of false negative changes on the GeneChip heat/control analysis, i.e. genes which did exhibit significant changes in transcript levels but were not detected in these experiments (see section 5.3.1 for further discussion).

An analysis was also performed to determine whether any of the gene transcripts that were undetected in the custom microarray analysis yielded reliable signal values in the GeneChip analysis, since this may be an indication of which method is more sensitive for detecting low levels of transcript abundance. Seven genes for which transcripts were undetected in the custom microarray, including *KNAT1*, two *STIG*-like genes and several matrix metalloproteinases, were checked for expression in the GeneChip data. In no case was expression of these genes detected by the GeneChip. Therefore, it is likely that these genes are either not expressed in the control or treated cell cultures or, if they are expressed, that neither the custom microarray nor GeneChip array are sufficiently sensitive to detect this expression.

5.2.5. Identification of transcripts that exhibited significant changes in steady-state levels in PCD samples compared to control

Very stringent criteria were used to identify genes exhibiting changes in transcript levels (Fowler and Thomashow, 2002). The steady-state level of a transcript was only considered to increase significantly during heat- or senescence-induced PCD if

the fold increase value was ≥2.5 for all four comparisons and it had a 'present' or 'marginal' detection call in both replicate PCD treatment chips (determined by algorithms contained within the Affymetrix analysis software, meaning that the transcript was detected as being expressed (present) or was not able to be conclusively determined (marginal)). Conversely, a transcript steady-state level was designated as being decreased in either heat- or senescence-induced PCD if the fold decrease value was ≥2.5 for all four comparisons and it had a 'present' or 'marginal' detection call in both of the replicate control samples. Using the detection call criteria for both duplicate samples, it was found that samples with low signal values (approximately <20) were automatically eliminated from further analysis.

Fold-change values were compared for all replicate arrays and, in the final analysis, only those transcripts that gave consistent changes of 2.5-fold or more *across all 4 replicate chips* for each treatment were taken to represent significant changes in gene expression for that treatment compared to control. (However, the fold-changes for induced and repressed genes are not presented in the final analysis (see Table 5.2). This is because, for the majority of induced transcripts identified in this analysis, expression was absent in the control samples and therefore it was impossible to measure the level of induction). Due to the stringent criteria used for identifying significantly modulated transcripts and, in accordance with methodology employed in other studies (Voehringer *et al.*, 2000; Chen *et al.*, 2002; Cheong *et al.*, 2002; Fowler and Thomashow, 2002), the data were sufficiently reproducible to allow for an analysis of gene expression patterns without confirmation of changes in selected transcripts by an alternate means of analysis (for example, Northern blots or RT-PCR).

5.2.6. Transcript analysis during heat- and senescence-induced PCD

Analysis of the GeneChip data revealed that a greater proportion of transcripts increased or decreased in abundance during senescence than following heat treatment (Figure 5.2). Approximately 1000 transcripts significantly increased or decreased during senescence-induced PCD, while 213 and 19 transcripts significantly increased or decreased, respectively, during heat-induced PCD (Figure 5.3). Of these transcripts, 60 were identified that increased in both heat- and senescence-induced PCD, while 8 decreased. The protein sequence for each translated transcript was screened using the BLAST algorithm (Altschul *et al.*, 1997) in both The *Arabidopsis* Information Resource (TAIR) database (http://www.arabidopsis.org), and also against the protein sequence database at Entrez NCBI (http://www.ncbi.nlm.nih.gov/). The most informative description for the putative function of each protein was determined and, in cases where the protein function was only indicated by homology, the Expect Value (E) was noted (Table 5.2). The functional classification of these genes according to the Munich Information Center for Protein Sequences (MIPS) (http://mips.gsf.de/) is presented in Figure 5.4.

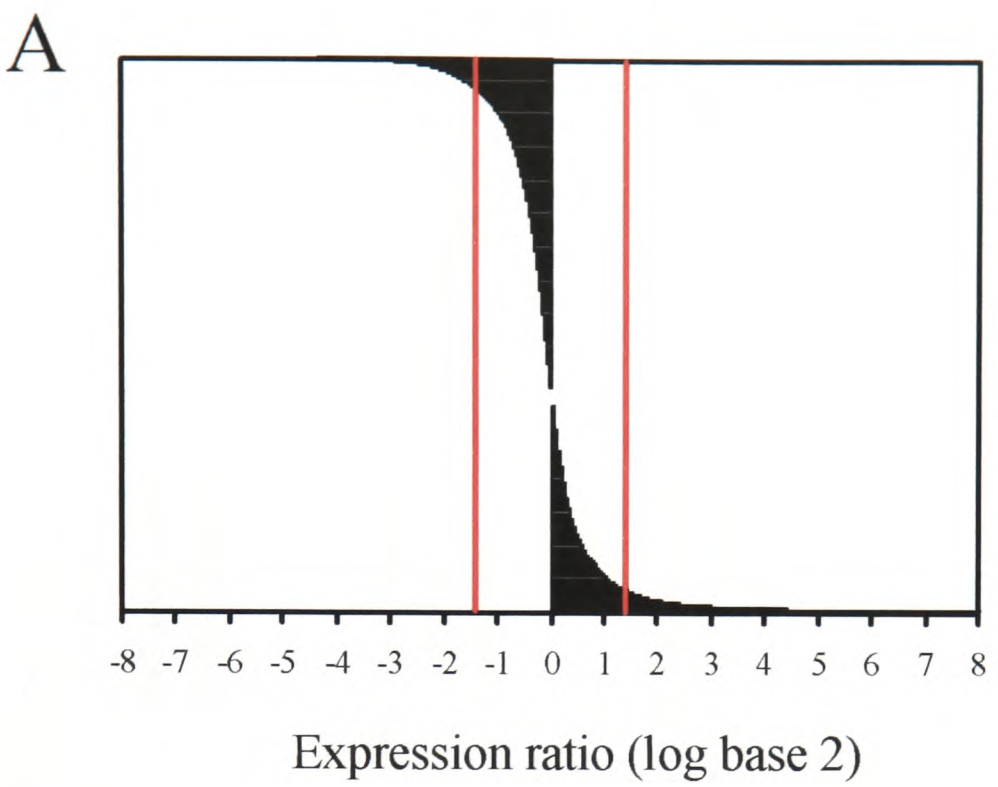


Figure 5.2. Evaluation of GeneChip microarray data.
Expression ratios (PCD treatment/control) of transcripts following heat- (**A**) or senescence-induced (**B**) PCD determined by microarray analysis. The expression ratios are transformed on a logarithmic (base 2) scale. The positive expression ratios represent transcripts increased during PCD, and the negative expression ratios transcripts decreased during PCD. In each analysis, the red lines indicate the cut-off (±2.5-fold) for genes for which the change in transcript level was not considered to be significant. Transcripts exceeding these thresholds in both systems were considered for further analysis and are listed in Table 5.2.

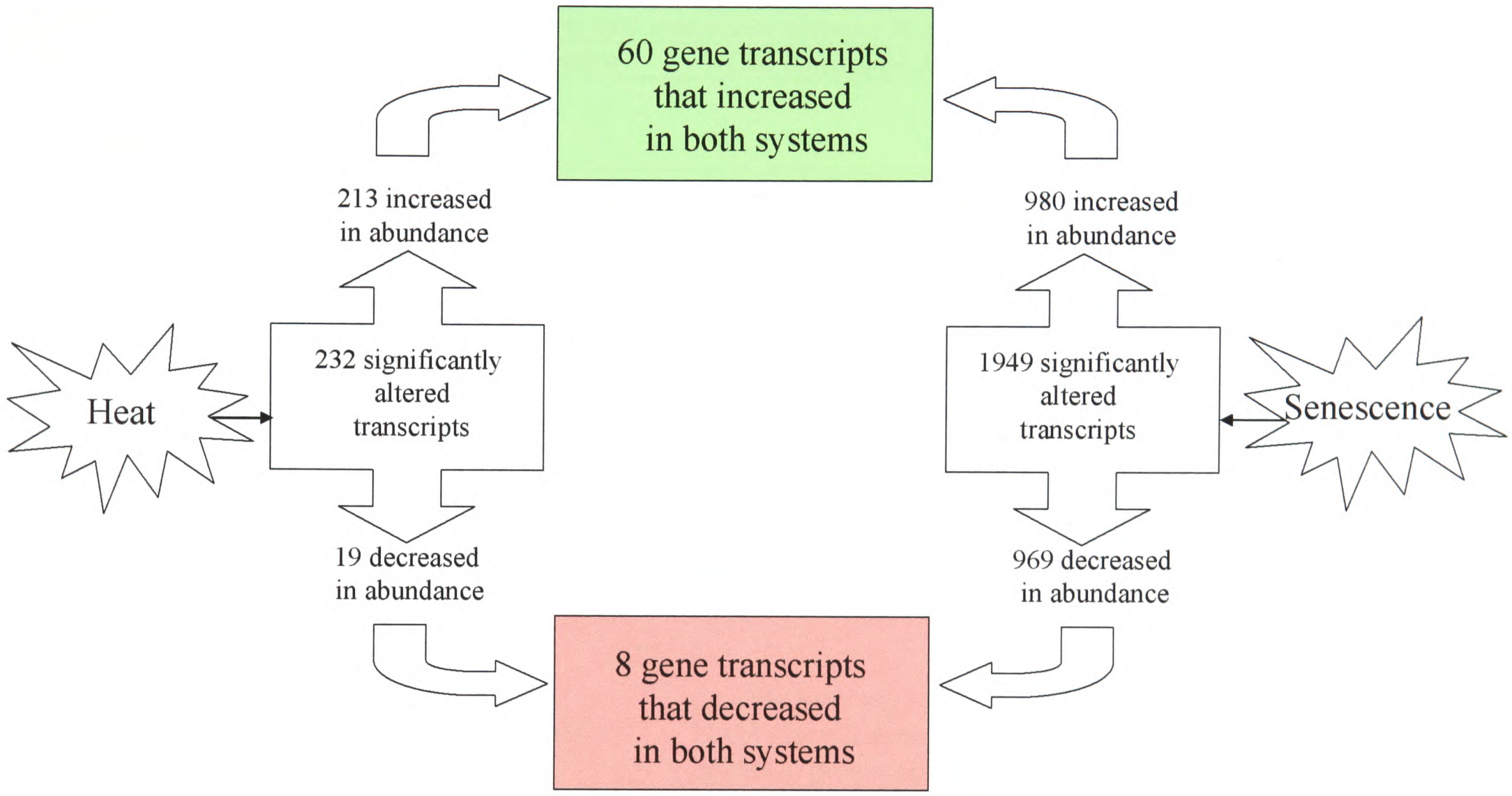


Figure 5.3. Summary of genes exhibiting significant changes in transcript levels during heat- and senescence-induced PCD.
Transcripts that changed ±2.5-fold or greater in either of the PCD-inducing treatments compared to control are summarised on the basis of whether they increased or decreased in abundance. Gene transcripts that either increased or decreased in both systems were of particular interest since these may be generally involved in plant PCD (see Table 5.2 for further details).

Table 5.2. Increased and decreased transcripts during heat- and senescence-induced PCD in *Arabidopsis* cell suspension cultures.

AGI Accession	TAIR description/*Arabidopsis* proteins	NCBI Entrez BLAST result (all organisms)	E score
Increased transcripts			
Extra- and Intracellular Signalling			
At2g39400	putative phospholipase	putative phospholipase	0
At2g20960	pEARLI 4 protein	pEARLI 4 phospholipase protein	0
At2g26560	similar to latex allergen	patatin-like protein 1 (tobacco)	E^{-141}
At1g32940	subtilisin-like serine protease	subtilisin-like serine protease	0
At5g11210	putative protein	putative ligand-gated ion channel	0
At4g23700	putative Na^+/H^+-exchanging protein	putative Na^+/H^+-exchanging protein	0
At1g10470	putative response regulator 3	two-component response regulator ARR4	0
At1g03220	unknown protein	strong similarity to extracellular dermal glycoprotein (EDGP) (carrot)	0
At5g59530	1-aminocyclopropane-1-carboxylate oxidase - like protein	1-aminocyclopropane-1-carboxylate oxidase-like protein	0
Gene Regulation			
At1g70930	putative homeobox protein	putative homeodomain leucine zipper protein	$2E^{-27}$
At2g43000	NAM (no apical meristem)-like protein	NAM (no apical meristem)-like protein	E^{-160}
At3g12910	unknown protein	unknown protein (putative NAM)	$1E^{-81}$

Table 5.2. *Continued*

AGI Accession	TAIR description/*Arabidopsis* proteins	NCBI Entrez BLAST result (all organisms)	E score
Hypersensitive Response and Pathogenesis-Related			
At1g68620	unknown protein	PrMC3 (*Pinus radiata*); cell death-associated protein (tobacco); HSR203J (tobacco)	E^{-52}
At1g65690	hypothetical protein	putative harpin-induced protein (HIN1 from tobacco)	$8E^{-9}$
At4g16260	β-1,3-glucanase class I precursor	β-1,3-glucanase class I precursor	E^{-160}
At1g02360	putative chitinase	glycosyl hydrolase family 19 (chitinase)	E^{-147}
At3g54420	class IV chitinase	class IV chitinase	E^{-163}
At2g44490	putative β-glucosidase	putative β-glucosidase	0
At1g55850	putative cellulose synthase catalytic subunit	putative cellulose synthase catalytic subunit	0
At1g32100	putative pinoresinol-lariciresinol reductase	putative pinoresinol-lariciresinol reductase	E^{-174}
At1g67980	putative S-adenosyl-L-methionine:trans-caffeoyl-Coenzyme A 3-O-methyltransferase	putative S-adenosyl-L-methionine:trans-caffeoyl-Coenzyme A 3-O-methyltransferase	E^{-116}
At1g63180	putative uridine diphosphate glucose epimerase	uridine diphosphate glucose epimerase	0
At1g19610	putative defensin AMP1	putative defensin AMP1	$9E^{-31}$
At4g11650	osmotin precursor	osmotin precursor (thaumatin-like)	E^{-125}
Oxidative Stress			
At1g03850	hypothetical protein	glutaredoxin-like protein	$6E^{-24}$
At2g29470	putative glutathione-*S*-transferase	putative glutathione-*S*-transferase	E^{-119}
At2g31570	putative glutathione peroxidase	putative glutathione peroxidase	$8E^{-92}$
At4g37530	peroxidase-like protein	putative peroxidase	E^{-179}
At4g29210	γ-glutamyltransferase-like protein	γ-glutamyltransferase-like protein	0
At1g32350	putative oxidase	strong similarity to alternative oxidase	E^{-180}
At1g09560	germin-like protein	germin-like oxidase; nectarin-like	$8E^{-72}$
At3g19010	hypothetical protein	oxidase-like protein	0

Table 5.2. *Continued*

AGI Accession	TAIR description/*Arabidopsis* proteins	NCBI Entrez BLAST result (all organisms)	E score
Oxidative Stress *Continued*			
At1g15670	unknown protein	expressed protein; contains similarity to Keap1 from Mus musculus and contains two Kelch motifs	0
At1g23120	unknown protein	major latex protein (MLP)-related; similar to ripening-induced protein	E^{-173}
Other (Metabolism, Development)			
At2g22330	putative cytochrome P450	putative cytochrome P450	0
At2g34500	putative cytochrome P450	putative cytochrome P450	0
At1g30720	putative reticuline oxidase-like protein	FAD-linked oxidoreductase family; berberine bridge enzyme	0
At2g34790	putative berberine bridge enzyme	FAD-linked oxidoreductase family; berberine bridge enzyme	0
At5g44380	berberine bridge enzyme-like protein	FAD-linked oxidoreductase family; berberine bridge enzyme	0
At4g15530	pyruvate orthophosphate dikinase	pyruvate orthophosphate dikinase	0
At3g24503	expressed protein	cytosolic aldehyde dehydrogenase	0
At5g27420	RING-H2 zinc finger protein-like	putative RING-H2 zinc finger protein ATL6	E^{-117}
At3g13790	β-fructofuranosidase 1	β-fructofuranosidase 1 (invertase)	0
At3g19930	monosaccharide transport protein STP4	monosaccharide transport protein STP4	0
At1g77210	putative sugar carrier protein	monosaccharide carrier protein STP14	0
Unknown Function			
At3g47800	aldose 1-epimerase-like protein	aldose 1-epimerase-like protein	0
At3g59690	putative protein	putative protein	$2E^{-94}$
At1g66690	unknown protein	AtPP protein (*Brassica napus*)	E^{-152}
At1g15045	hypothetical protein	hypothetical protein	E^{-177}
At4g28290	putative protein	putative protein	$4E^{-10}$
At3g18250	hypothetical protein	hypothetical protein	$1E^{-15}$
At5g62630	putative protein	unknown protein	0

Table 5.2. *Continued*

AGI Accession	TAIR description/*Arabidopsis* proteins	NCBI Entrez BLAST result (all organisms)	E score
Unknown Function *Continued*			
At2g36220	unknown protein	expressed protein	E^{-125}
At5g12420	putative protein	putative protein	0
At1g68765	expressed protein	expressed protein	$3E^{-26}$
At3g29240	unknown protein	unknown protein	0
At5g62150	putative protein	putative protein	$2E^{-45}$
At3g29240	unknown protein	unknown protein	0
At1g52200	unknown protein	expressed protein	E^{-105}
At2g20670	unknown protein	expressed protein	E^{-156}
At3g49130	putative protein	putative protein	0
Decreased transcripts			
At1g65310	putative xyloglucan endotransglycosylase	putative xyloglucan endotransglycosylase	E^{-146}
At4g14130	xyloglucan endotransglycosylase-related protein XTR-7	xyloglucan endotransglycosylase (XTR7)	E^{-158}
At5g45280	pectin acetylesterase	pectin acetylesterase	0
At4g38420	putative pectinesterase	putative pectinesterase	0
At5g12020	heat shock protein 17.6-II	heat shock protein 17.6-II	$1E^{-69}$
At5g50790	MtN3-like protein	MtN3-like protein	E^{-121}
At1g51000	hypothetical protein	hypothetical protein	$3E^{-17}$
At1g66180	unknown protein	expressed protein	0

Transcripts that were significantly modulated in expression in both heat- (55°C, 10 min) and senescence-induced (13 to 14 days-old) PCD samples compared to control (6 days-old) samples were identified using the Affymetrix ATH1 GeneChip microarray. Only those transcripts that changed by a factor of greater than or equal to ±2.5-fold in *both* PCD-inducing treatments compared to the control were considered (see section 2.8). The encoded protein sequence for each gene was screened by BLAST algorithm (Altschul *et al.*, 1997) in both The *Arabidopsis* Information Resource (TAIR) database, and also against the protein sequence database at Entrez NCBI (http://www.ncbi.nlm.nih.gov/). The most informative description for the putative function of each encoded protein was determined and, in cases where the protein function was only indicated by homology, the Expect Value (E) was noted (see section 5.2.6).

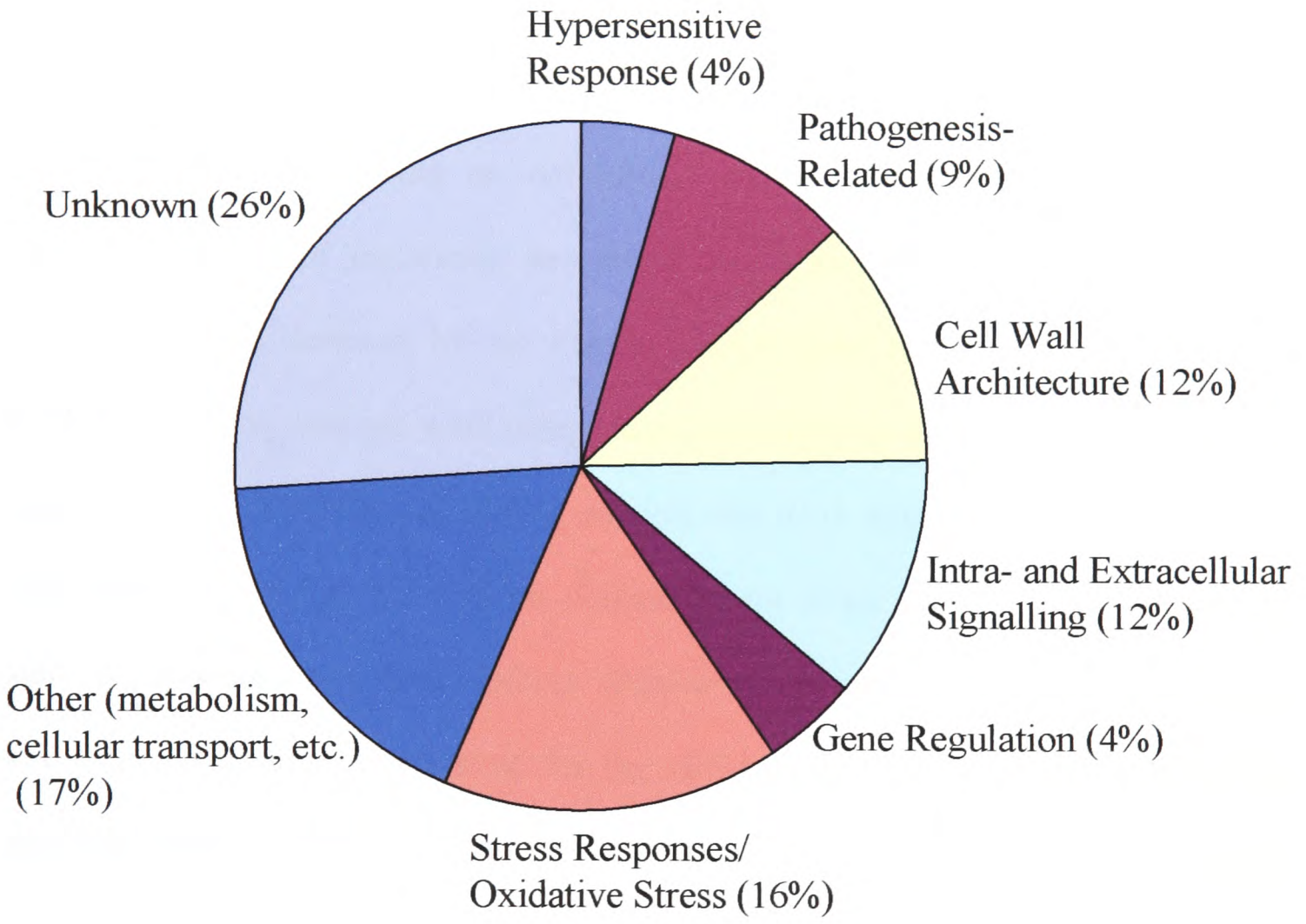


Figure 5.4. Functional classification of transcripts altered during heat- and senescence-induced PCD.
Transcripts that were signficantly changed in abundance (either increased or decreased) in both heat- and senescence-induced PCD, compared to the control, were grouped by known or putative function based on information contained in the Munich Information Center for Protein Sequences (MIPS) (http://mips.gsf.de/).

5.3. Discussion

5.3.1. A custom cDNA microarray and an Affymetrix GeneChip array are both valid methods to identify significant changes in gene transcript levels during PCD

Of the ~100 cDNAs arrayed on the custom microarray, 41 and 50 transcripts showed significant changes in steady-state levels in heat- and senescence-induced *Arabidopsis* cell suspension cultures, respectively, compared to control cultures (see Figure 4.4). It was found that approximately two-thirds of these changes in transcript levels were replicated using an Affymetrix ATH1 GeneChip. Overall, this is a satisfactory degree of replication between the two methods of analysis. However, transcript changes detected by the custom cDNA array that were not verified by GeneChip analysis, and yet were independently verified by competitive RT-PCR (for example, *Athsr5*, *KNAT2*, and *SRG3*), suggest that there will be changes detected by either analytical system that will be affected by the RNA samples analysed and the nature of the technology itself. Indeed, different RNA samples, collected and pooled more than a year apart, were used for the cDNA custom array and the Affymetrix GeneChip array. Although every attempt was made to replicate the experimental systems as closely as possible, there will undoubtedly be differences between cell samples that will contribute to differences in the steady-state levels of gene transcripts.

Additionally, it must be borne in mind that the custom cDNA microarray analysis and the GeneChip analysis are technically different methods. The former utilised cDNAs ranging from several hundred basepairs to kilobases in length as probes, and many of these represented 5' or 3' UTR sequences. Conversely, GeneChip arrays utilise probe pairs of short 25-mer oligonucleotides designed to detect specific sequences for each gene (for all genes considered in this comparison, the Affymetrix

probes were designated '_at', indicating that they represent probe sets designed to detect a unique sequence) (Lipshutz *et al.*, 1999). Furthermore, a custom cDNA array involves the use of fluorophore-conjugated nucleotides to label two different cDNA populations, which are then mixed and hybridised simultaneously to a single chip. The relative fluorescence intensity of each channel is calculated on one chip (Duggan *et al.*, 1999). In contrast, the Affymetrix technology involves using biotin-labelled cRNA for hybridisation of individual experimental samples to a single chip, followed by a comparison of signal values across chips (Lipshutz *et al.*, 1999). The principle advantage of the Affymetrix oligonucleotide arrays, as compared to cDNA microarrays, is the creation of high-density chips in which the transcripts of up to 40,000 genes can be assayed per chip.

While the underlying analytical set-up in both technologies is designed to detect changes in transcript levels, it is inevitable that there will be some differences in the results obtained. As stated earlier, fluorescence intensities achieved in cDNA microarray hybridisations do not bear a linear relationship to transcript abundance (Ruan *et al.*, 1998), and similarly the quantitative accuracy of oligonucleotide arrays in measuring RNA concentration can fall within a factor of ±2x (Lipshutz *et al.*, 1999). In the absence of any published comparisons on the quality of data from either type of microarray, it is perhaps best to analyse each experiment individually. As always, the objective of these microarray analyses is not to define regulatory circuits and the plant programmed cell death pathway, but rather to provide a starting point to identify putative targets for which expression profiles may be confirmed by alternate methods and on which further functional analyses may be undertaken.

5.3.2. Putative extra- and intracellular signalling mechanisms that may be involved in plant PCD were identified using the GeneChip analysis

The GeneChip analysis was successful in identifying several genes whose transcript levels were modulated during both heat- and senescence-induced PCD, and whose products encode putative signalling components (Table 5.2). Such genes are of considerable interest since the regulatory mechanisms involved in plant PCD are generally ill-defined (Lam *et al.*, 1999a; Lam *et al.*, 2001).

5.3.2.1. Phospholipase activity may be a component of plant PCD signalling networks

Three transcripts encoding putative phospholipases were upregulated in both heat- and senescence-induced PCD. Plant phospholipases, which cleave the phospholipids present in biological membranes, include at least three classes, A, C, or D (PLA, PLC, PLD), depending on their cleavage site and hydrolysis products (Chapman, 1998). It is only recently that the genes encoding some of these proteins have been cloned, and thus it is not immediately evident to which class of phospholipases the genes identified in this study belong. Nevertheless, there is a growing body of evidence that phospholipase activity is involved in a wide range of plant signalling events, including those related to senescence, wounding, stress, and the hypersensitive response (Chapman, 1998; Laxalt and Munnik, 2002).

It might seem obvious that phospholipase activity would be required during senescence for the recycling of membrane lipids and the breakdown of cellular membranes (Chapman, 1998). However, the products generated by phospholipase activity are known to include a variety of compounds involved in mediating downstream signal transduction cascades and signalling pathways. For example, the production of phosphatidic acid has been shown to affect the formation of reactive oxygen species, MAP kinase signal transduction cascades, and ion channel activity (Munnik, 2001). In

response to wounding in both tomato leaves and castor bean leaves, it has been proposed that activation of phospholipases produces linolenic acid, an oxylipin that is a precursor for jasmonic acid biosynthesis (Conconi *et al.*, 1996; Ryu and Wang, 1996), and eventually leads to the downstream expression of defense genes (Chapman, 1998). Rapid activation of phospholipase activity in response to pathogen challenge is also thought to generate a component of the signalling pathway(s) leading to downstream induction of defense responses (Chapman, 1998; Munnik, 2001). This may involve the production of reactive oxygen species by activation of a plasma membrane-localised NADPH oxidase.

One of the genes identified in this analysis (At2g39400) encodes a putative phospholipase and another (At2g20960) encodes a pEARLI 4 protein which may be a phospholipase. It is of interest that the third (At2g26560) phospholipase identified in this analysis bears considerable homology to the patatin-like phospholipase A_2 of tobacco. The activity of this enzyme has been shown to be induced in response to infection with tobacco mosaic virus and to precede the accumulation of oxylipins (Dhondt *et al.*, 2000), compounds with a variety of direct and indirect roles in defense gene induction. Therefore, the upregulation of transcripts for these three phospholipases in both heat- and senescence-induced PCD, while possibly related to membrane turnover, may also form part of the signalling network that is responsible for producing downstream responses. The characterisation of PCD-like responses in mutants for these genes would help to clarify their potential role in generating phospholipid second messengers in the plant PCD regulatory network.

5.3.2.2. Subtilisin-like protease activity may be a component of plant PCD signalling networks

It was of interest to observe that transcripts encoding a protein with homology to subtilisin-like serine proteases (At1g32940) increased during both heat- and senescence-induced PCD. Subtilisins are synthesised as inactive zymogen precursors that are then cleaved to produce the mature active enzyme which functions extracellularly (Taylor *et al.*, 1997). The subtilisin encoded by the transcript identified in this study is also predicted to be a part of the secretory pathway (Target P; $p<0.05$) (Emanuelsson *et al.*, 2000). This class of proteases has previously been implicated as playing a role in plant PCD since their expression has been associated with a wide range of plant cell death phenomena (Beers *et al.*, 2000).

The most well-characterised subtilisin protease, P69, was identified in tomato as being induced at the transcript level in response to pathogen attack (Tornero *et al.*, 1996a; Tornero *et al.*, 1997). P69 has been shown to function extracellularly and is suggested to play a role in modifying the extracellular matrix, which may be important in activating signal transduction pathways. One of the targets of P69 activity is LRP (a member of the leucine-rich repeat (LRR) protein family), a tomato extracellular matrix-associated protein that is proteolytically processed in pathogen-infected plants (Tornero *et al.*, 1996b). LRR-containing proteins are known to function in both protein-protein interactions and in mediating signal transduction pathways. Interestingly, however, increases in P69 transcripts do not occur during wound responses (Tornero *et al.*, 1996a), suggesting that perhaps the P69 subtilisin functions specifically in the responses to pathogen invasion. The increase in transcripts encoding a subtilisin-like serine protease observed during both heat- and senescence-induced PCD in this study, however, suggests that this protease may have a general role in plant PCD, and it would

be of great interest to identify the downstream targets in the extracellular matrix that may be cleaved by this protease.

5.3.2.3. Ion exchange protein activity may be a component of plant PCD signalling networks

Two other transcripts that increased in abundance during both heat- and senescence-induced PCD were those encoding ion exchange proteins. One of these (At5g11210) bears homology to a ligand-gated ion channel, while the other (At4g23700) encodes a putative Na^+/H^+-exchanging protein. The subcellular localisation of these two proteins could not be predicted with any degree of certainty (Target P) (Emanuelsson *et al.*, 2000). Nevertheless, there is a body of evidence demonstrating that plant ion channels of various kinds play important roles in signalling, and that the activity of these channels is rapidly modulated in response to a range of stimuli including pathogen attack and hormone perception (Zimmermann *et al.*, 1999; Krol and Trebacz, 2000). Plant ion channel activity is suggested to be a crucial feature in mediating HR cell death (Lam *et al.*, 2001).

The putative ligand-gated ion channel identified in this study belongs to a class of proteins that bind intracellular second messengers, including Ca^{2+}, H^+ ions, nucleotides, proteins, and hormones, and undergo a conformational change to transduce a cellular response (Zimmermann *et al.*, 1999; Krol and Trebacz, 2000). Although Ca^{2+} fluxes have been implicated in plant PCD (Lam *et al.*, 1999a), there is also evidence to suggest that other ion movements, including K^+ and Cl^- effluxes and H^+ influx, are important in mediating the hypersensitive response. For example, membrane depolarisation and changes in cytoplasmic pH have been shown to occur following treatment of rice suspension cultures with an oligosaccharide elicitor, and are suggested to form part of the signalling network associated with defense responses (Kikuyama *et*

al., 1997; Kuchitsu *et al.*, 1997). Likewise, defense gene induction in challenged suspension-cultured parsley cells is associated with the fluxes in Ca^{2+}, H^+, K^+, and Cl^- ions (Hahlbrock *et al.*, 1995). The induction of a MAP kinase cascade triggered by such ion movements and its potential role in gene induction were also demonstrated in the same system (Ligterink *et al.*, 1997).

Therefore, in spite of the relatively limited information on the role of ion fluxes in controlling plant signalling responses, evidence is accumulating that such channels are involved in mediating the response to a wide range of stimuli (Zimmermann *et al.*, 1999). A role for ion channel activity in plant PCD has been more directly shown by the recent identification of the *defense, no death* (*dnd1*) mutation in *Arabidopsis*. *dnd1* mutants fail to undergo HR-associated cell death and yet still show defense gene induction (Clough *et al.*, 2000). This gene was cloned to reveal that it encodes a cyclic nucleotide-gated ion channel protein, AtCNGC2, a member of a family of proteins typically activated by binding of a cytoplasmic ligand and which allow the passage of several cations. Expression of the *AtCNGC2* transcript has been shown to be upregulated during the early stages of senescence in both whole *Arabidopsis* plants and cell cultures, supporting a role for the gene product as a positive regulator of cell death (Kohler *et al.*, 2001). The two ion channels identified in this analysis may play similar roles in positively regulating PCD associated with a range of stimuli, including abiotic stress and senescence. It would be of considerable interest to determine whether mutants in these genes, like *dnd1* with respect to the HR, are similarly unable to execute PCD.

5.3.2.4. Two-component response regulators may be a component of plant PCD signalling networks

Transcripts encoding a response regulator of the two-component response regulator family (At1g10470) were upregulated during both heat- and senescence-

induced PCD. Although not annotated as such in the TAIR or NCBI databases, the encoded protein is identical to ARR4 (also called ATRR1 or IBC7 (induced by cytokinin)) (Brandstatter and Kieber, 1998; Imamura *et al.*, 1998; Urao *et al.*, 1998). Two-component response regulators include a family of proteins in *Arabidopsis* that are homologous to well-defined prokaryotic systems (Urao *et al.*, 2000; Hwang *et al.*, 2002). These systems consist of a membrane-localised histidine kinase which autophosphorylates upon perception of the appropriate environmental stimulus, and then transfers the phosphoryl group to a conserved aspartate residue on the response regulator. This process may or may not involve an intermediate phosphotransfer protein. Sequencing of the *Arabidopsis* genome has revealed the presence of a large family of genes encoding putative histidine kinases, phosphotransfer proteins, and at least 32 response regulator proteins (Hwang *et al.*, 2002). The histidine kinase proteins have been implicated in a wide range of plant cellular responses, including ethylene perception, cytokinin signalling, and osmoregulation (Urao *et al.*, 2000). Many response regulators have also been isolated and characterised, and are generally classified into two groups: Type-A response regulators (which includes ARR4) have a receiver domain only, while Type-B response regulators also have a large C-terminal extension that typically encodes an output (DNA-binding) domain that may be important in regulating gene expression.

Transcripts for *ARR4*, the response regulator upregulated in this study, have previously been shown to be induced in *Arabidopsis* within one hour following low-temperature, dehydration, and salt treatments, as well as following exogenous abscisic acid application (Urao *et al.*, 1998). Interestingly, however, the same authors found that the *ATRR1/ARR4* mRNA was completely absent following high (40°C) temperature treatment. In a separate study, Brandstatter and Kieber (1998) found that *ARR4/IBC7*

mRNA levels were marginally induced (compared to other response regulators) over one to two hours following cytokinin treatment in *Arabidopsis*, and that the mRNA was expressed at a basal level in most tissues examined. The ability of the ARR4 protein to accept a phosphoryl group from histidine kinase proteins has been demonstrated *in vitro* (Imamura *et al.*, 1999), and a yeast two-hybrid analysis has isolated two proteins, ATDBP1 and ATDBP2, which interact with ARR4 (Yamada *et al.*, 1998). These proteins may be upstream regulators of ARR4, perhaps acting to localise it to the plasma membrane, or may be downstream regulators of the ARR4 response, for example as transcriptional activators. Subsequent studies have shown that ARR4 acts as a negative regulator of cytokinin gene induction and may be involved in the integration of light and cytokinin signalling pathways, and that a GFP-tagged version of the protein localises to both the cytoplasm and the nucleus (Hwang and Sheen, 2001).

It is unlikely that, in the current system using dark-grown cell cultures undergoing heat- and senescence-induced PCD, *ARR4* transcript upregulation would be related to light signalling. Given the described stress-inducible increases in *ARR4* mRNA levels, it is conceivable that *ARR4* is similarly stress-responsive in the heat and senescence systems. However, this would be in direct contrast to the downregulation of *ARR4* in response to high temperatures that has been previously demonstrated (Urao *et al.*, 1998). Since cytokinins are known to retard the progression of senescence and to stimulate cell growth (Gan and Amasino, 1995), and since ARR4 has been shown to act as a negative regulator of cytokinin signalling (Hwang and Sheen, 2001), it is also tempting to suggest that an induction of *ARR4* during plant PCD functions to inhibit those cellular processes that would be antagonistic to cell death.

5.3.2.5. Extracellular proteins and hormone biosynthesis may be components of plant PCD signalling networks

Two other transcripts that were upregulated during both heat- and senescence-induced PCD encode proteins that may also play a role in cellular signalling. The identification of transcripts encoding a protein with high homology to the carrot extracellular dermal glycoprotein (EDGP) (At1g03220) was of interest since this mRNA was originally found to be induced in response to wounding in carrot phloem cells, and to be expressed at a high basal level in carrot cell suspension cultures (Satoh *et al.*, 1992). The mRNA was not induced in response to pathogen infection, however. The authors suggest that EDGP may encode a protein with similarity to extracellular aspartyl proteases, but did not test for activity. The EDGP protein identified in this study is also predicted to be localised to the secretory pathway (Target P; $p<0.05$) (Emanuelsson *et al.*, 2000). One possibility is that the *Arabidopsis* EDGP homologue may play a role in cellular signalling during cell death induced by a variety of stimuli.

Additionally, the observed increase in steady-state levels of transcripts for 1-aminocyclopropane-1-carboxylate-oxidase (ACC oxidase) (At5g59530), an enzyme involved in ethylene biosynthesis, was also found to be associated with wounding and senescence in tomato (Barry *et al.*, 1996). In tobacco, expression of some ACC oxidase gene family members are induced in response to a variety of stimuli, including salicylic acid treatment, wounding, and pathogen infection (Kim *et al.*, 1998). Taken together, these findings suggest that biotic and abiotic stress signalling pathways converge in causing increased ethylene biosynthesis (Solano and Ecker, 1998; Bleecker and Kende, 2000; Wang *et al.*, 2002) that may be important in mediating downstream cellular responses related to cell death. As reviewed by Beers and McDowell (2001), ethylene may in fact be the best candidate for a signalling molecule in plant PCD given its

association with a wide range of cell deaths in response to developmental and physiological events.

5.3.3. Putative gene regulatory proteins that may be involved in plant PCD were identified using the GeneChip analysis

It was of interest to observe that steady-state levels of three transcripts encoding putative transcriptional regulators were increased during both heat- and senescence-induced PCD. One of these genes (At1g70930) encodes a putative homeobox leucine zipper protein, which belongs to a class of proteins that are known transcriptional regulators involved in a wide range of plant developmental events (Schena and Davis, 1994; Tsiantis, 2001). The protein encoded by the transcript upregulated in this study, however, has not been previously characterised.

Two of the upregulated transcripts (At2g43000 and At3g12910) in this system encode similar proteins with homology to the 'no apical meristem' (NAM) family of transcriptional regulators. No Apical Meristem (NAM) is a protein originally identified in petunia that, as the name suggests, is required for development of a shoot apical meristem (Souer *et al.*, 1996). NAM homologues in *Arabidopsis* have subsequently been identified, and the proteins, including CUC2 (cup-shaped cotyledon) and NAP (NAC-like; activated by AP3/PI), are involved in the meristem development and organ identity (Aida *et al.*, 1997; Sablowski and Meyerowitz, 1998). This family of proteins is characterised by a conserved N-terminal NAC domain (for petunia NAM and Arabidopsis ATAF1, ATAF2, and CUC2) that is unique to plants (Aida *et al.*, 1997). Subsequent analyses have shown that this domain likely functions as a DNA-binding domain, supporting earlier inferences that this family of proteins, of which there are at least 90 members in *Arabidopsis*, functions as transcriptional activators (Duval *et al.*, 2002).

It is suggested that, in *Arabidopsis* and other plants such as rice, NAC-containing proteins function in a wide range of developmental events and in response to a variety of environmental stimuli, similar to other classes of transcriptional activators such as MADS-, homeodomain-, and AP2 domain-containing proteins (Kikuchi *et al.*, 2000; Duval *et al.*, 2002). Interestingly, NAC-family proteins in wheat have been shown to bind geminivirus proteins and inhibit viral replication (Xie *et al.*, 1999), while the *SEND5* mRNA that is upregulated during tomato senescence may also contain a NAC domain (John *et al.*, 1997). Recently, transcripts encoding two potato NAC domain proteins and the *Arabidopsis* NAC domain proteins, ATAF1 and ATAF2, have been shown to be upregulated in response to pathogen infection and wounding in potato and *Arabidopsis*, respectively (Collinge and Boller, 2001). The two NAC domain proteins identified in this study have not been previously described, though the protein encoded by At2g34000 is 40 to 60% similar at the amino acid level to the wound-inducible ATAF1 and ATAF2. The evidence suggesting that NAC domain proteins act as transcriptional activators, combined with the identification of two novel NAC domain proteins whose transcripts are upregulated during heat- and senescence-induced PCD, indicates that these proteins, along with the putative leucine zipper homeobox protein identified, may play a role in regulating plant PCD gene expression and regulatory networks.

5.3.4. Hypersensitive response- and pathogenesis-related proteins that may be involved in plant PCD were identified using the GeneChip analysis

A variety of transcripts encoding proteins that have previously been implicated in the hypersensitive response (HR) and other pathogenesis-related responses were identified as being upregulated during both heat- and senescence-induced PCD. A closer examination of the classes of upregulated transcripts suggests possible roles for their

products that may extend to a number of physiological instances of plant PCD apart from those associated with the HR.

5.3.4.1. HR-related proteins may be involved in plant PCD induced by a variety of stimuli

Two transcripts whose steady-state levels were increased during heat- and senescence-induced PCD (At1g68620 and At1g65690) have previously been implicated in the plant HR. One of these transcripts encodes an *Arabidopsis* protein that bears considerable homology to tobacco Hsr203J (Pontier *et al.*, 1994) and the *Pinus radiata* homologue, PrMC3 (Walden *et al.*, 1999). In the tobacco HR in response to challenge with an incompatible pathogen, but not in response to wounding or treatment with fungal elicitors, an increase in *Hsr203J* mRNA occurs (Pontier *et al.*, 1994). This initially suggested that Hsr203J function is restricted to the HR. Subsequent analysis of both tobacco *Hsr203J* and its tomato homologue revealed that expression of this gene occurs preferentially during incompatible reactions and also in response to cell death-inducing heavy metal treatment, but not in response to H_2O_2, salicylic acid, or methyl jasmonate, leading the authors to suggest that the gene product may play a general role in plant PCD (Pontier *et al.*, 1998). This notion was supported by a study of PCD-induced tomato cell suspension cultures treated with camptothecin, an inhibitor of topoisomerase I, in which *Hsr203J* mRNA levels were shown to increase 5-fold (Hoeberichts *et al.*, 2001).

The Hsr203J protein has been functionally characterised as a serine hydrolase capable of hydrolysing short-chain acyl esters (Baudouin *et al.*, 1997). The protein is thought to play a role in limiting the spread of cell death (Pontier *et al.*, 1998). Interestingly, *Hsr203J* mRNA is not upregulated during natural leaf senescence in tobacco, suggesting that this gene may not function in PCD associated with a wide range

of stimuli (Pontier *et al.*, 1998). The *Arabidopsis Hsr203J* homologue identified in the current study, however, may have a role in PCD that extends to that induced by a variety of stimuli and developmental events, including senescence.

Similar to *Hsr203J*, transcripts for the *Arabidopsis* homologue to tobacco HIN1, a harpin-induced protein (Gopalan *et al.*, 1996b), were increased in this study. In tobacco leaves undergoing the HR, the induction kinetics of *Hsr203J* and *HIN1* transcripts are very similar, and the tomato *HIN1* homologue is also induced during the HR in that species. In tobacco leaves undergoing natural senescence, induction of *HIN1* transcripts occurs during late to advanced leaf senescence when cell death is expected to occur (Pontier *et al.*, 1999). Transcripts for another *Arabidopsis* HIN1 homologue (which differed from that identified in this study) have been shown to be upregulated at the late stages of leaf senescence in response to darkness, ethylene, and abscisic acid (Yoshida *et al.*, 2001). HIN1 and its homologues do not bear any significant homology to known proteins, and the subcellular localisation of this protein cannot be predicted from its amino acid sequence (Target P) (Emanuelsson *et al.*, 2000). Thus, it is unclear what functional role HIN1-like proteins may play in plant PCD, although their transcript expression patterns are suggestive of a general role in plant PCD in response to a variety of stimuli.

5.3.4.2. Pathogenesis-related proteins may be involved in plant PCD induced by a variety of stimuli

Steady-state levels of transcripts encoding pathogenesis-related proteins, including a class I β-1,3-glucanase (At4g16260), class IV chitinases (At1g02360 and At3g54420), and a putative β-glucosidase (At2g44490) that are classically induced in response to pathogen challenge in a whole variety of plants (reviewed by Linthorst, 1991), were increased during heat- and senescence-induced PCD. In addition, steady-

state levels of transcripts for several enzymes involved in cell wall deposition and cross-linking were also increased, and these processes are known to be a component of general, or nonhost, disease resistance in plants (Heath, 2000b). These genes included those encoding cellulose synthase (At1g55850) (Richmond, 2000), pinoresinol-lariciresinol reductase (At1g32100), which is involved in lignin biosynthesis (Gang *et al.*, 1999), S-adenosyl-L-methionine:trans-caffeoyl-Coenzyme A 3-O-methyltransferase (At1g67980), which is involved in cell wall reinforcement (Schmitt *et al.*, 1991), and uridine diphosphate glucose epimerase (At1g63180), which is involved in the synthesis of UDP-galactose necessary for cell wall polysaccharide biosynthesis (Dormann and Benning, 1996). One possible explanation for these findings is that the upregulation of these genes during abiotic stress or senescence protects these vulnerable tissues against pathogen infection (Hanfrey *et al.*, 1996). This hypothesis would also serve to explain the upregulation of transcripts for a putative antimicrobial defensin peptide (At1g19610) (Thevissen *et al.*, 1996) and a PR protein, osmotin (thaumatin-like). Osmotins have been implicated in both stress and defense responses in tobacco and may serve to alter membrane permeability of the pathogen (Kononowicz *et al.*, 1992).

β-1,3-glucanases and chitinases are known to act upon pathogen (e.g. fungi) cell walls to release elicitor molecules, such as oligosaccharides, that then play a role in initiating downstream signalling responses of the HR (Shibuya and Minami, 2001). Deficiencies in class I β-1,3-glucanases result in susceptibility to pathogen infection in tobacco (Beffa *et al.*, 1996), and transcripts for a class IV extracellular chitinase are induced in *Arabidopsis* in response to pathogen challenge (Gerhardt *et al.*, 1997). Thus, while a role for these enzymes in the protection against pathogens is clear, it is of interest that class IV chitinase transcripts have also been shown to be induced during

leaf senescence in *Brassica napus* (Hanfrey *et al.*, 1996) and in *Phaseolus vulgaris* in response to salicylic acid treatment and heat stress (Margis-Pinheiro *et al.*, 1994).

Recent studies have also demonstrated that secreted chitinases from *Pinus caribaea* cells can cleave arabinogalactoproteins (AGPs) (Domon *et al.*, 2000). This finding has important implications as AGPs comprise a family of complex proteoglycans that have been suggested to play a role in a wide range of developmental events (Gaspar *et al.*, 2001), including PCD in *Arabidopsis* cell suspension cultures (Gao and Showalter, 1999). Therefore, the potential for induction of chitinases during heat- and senescence-induced PCD may indicate that they are involved in generating extracellular signals, perhaps including the cleavage of plant AGPs, that are involved in initiating downstream signalling pathways. Similarly, the upregulation of a putative β-glucosidase in this study is in accordance with previous studies that have shown such transcripts are increased during senescence of *Arabidopsis* cell suspension cultures (Callard *et al.*, 1996) and in *Arabidopsis* in response to wounding, insect chewing, jasmonic acid and ethylene treatment (Stotz *et al.*, 2000). Therefore, this enzyme could also potentially be involved in generating signals common to plant PCD pathways.

5.3.5. Oxidative stress-related proteins that may be involved in plant PCD were identified using the GeneChip analysis

The increased steady-state levels of transcripts for a variety of genes encoding oxidative stress-related proteins following heat treatment and during senescence are similar to those seen in the custom microarray analysis (see Chapter 4) and are in accordance with the suggestion that oxidative stress is generated in these experimental systems. In addition to the antioxidant roles of the majority of the proteins encoded by these genes, including glutaredoxin (At1g03850), glutathione-*S*-transferase (At2g29470), glutathione peroxidase (At2g31570), peroxidase (At4g37530), and γ-

glutamyltransferase (At4g29210) (Noctor and Foyer, 1998), it was also interesting to observe the upregulation of an alternative oxidase-like transcript (At1g32350). Alternative oxidase mRNAs are upregulated during the *Arabidopsis* hypersensitive response (Lacomme and Roby, 1999), and transgenic tobacco lacking alternative oxidase have increased susceptibility to cell death induced by H_2O_2, salicylic acid, and the protein phosphatase inhibitor, cantharidin (Robson and Vanlerberghe, 2002).

The increased steady-state transcript levels of a germin-like oxidase (At1g09560) and an oxidase-like protein (At3g19010) is of interest, since this class of proteins encode oxalate oxidases that produce H_2O_2 (Lane *et al.*, 1993). The production of H_2O_2 may have a role in triggering an oxidative burst that plays a role in cell wall crosslinking and in the induction of downstream signalling responses associated with cell death (Lane *et al.*, 1993). Additionally, one transcript upregulated during heat- and senescence-induced PCD (At1g15670) encodes an unknown protein which, at its C-terminus, bears homology to the double glycine repeat (DGR) domain of murine Keap1 (Itoh *et al.*, 1999). The mammalian Keap1 protein plays a role in repressing the antioxidant response by binding to and suppressing the activity of the transcriptional activator Nfr2, and the interaction between the two proteins is mediated through the DGR domain. It would therefore be of interest to undertake a yeast two-hybrid analysis of the *Arabidopsis* homologue of this protein, since identification of its binding partners may lead to the elucidation of a conserved antioxidant regulatory network that functions during plant PCD.

5.3.6. Other proteins that may be involved in plant PCD were identified using the GeneChip analysis

A variety of other transcripts that were increased in steady-state levels during heat- and senescence-induced PCD may encode proteins that are generally involved in

plant PCD. However, many of these encode only unknown proteins or enzymes whose specific cellular function is not well defined. These include cytochrome p450s (At2g22330 and At2g34500) and FAD-linked oxidoreductases (At1g30720, At2g34790, and At5g44380). It is difficult to speculate on their possible roles in the heat- and senescence-induced PCD systems. Transcripts for pyruvate orthophosphate dikinase (PPDK) (At4g15530), an enzyme whose function in C3 plants (such as *Arabidopsis*) is ill-defined (Chastain *et al.*, 2002), were also upregulated in both systems. The encoded protein is likely to be cytosolic (Target P; $p<0.05$) (Emanuelsson *et al.*, 2000) and, in rice, an induction of PPDK has been shown to occur in response to low-oxygen stress and water deficit. Likewise, steady-state levels of cytosolic alcohol dehydrogenase (ADH) transcripts (At3g24503) increased in this study, which is in accordance with previous studies in rice and *Arabidopsis* that have shown the induction of this gene in response to low-oxygen stress, dehydration, abscisic acid, and cold stress (Dolferus *et al.*, 1994; de Bruxelles *et al.*, 1996; Moons *et al.*, 1998). Therefore, PPDK and ADH gene induction may be related to a more general stress response that occurs in both heat-treated and senescing cell cultures. Additionally, transcripts for *ATL6* (At5g27420) encode a protein that belongs to a family of highly similar RING-H2 finger proteins in *Arabidopsis*, and *ATL6* transcripts have been previously shown to be upregulated in *Arabidopsis* seedlings treated with fungal elicitors (Salinas-Mondragon *et al.*, 1999). In rice, an *ATL* homologue has been shown to encode a ubiquitin-conjugating enzyme involved in protein degradation, and is suggested to be involved in cellular signalling mechanisms that occur during the defense response (Takai *et al.*, 2002a; Takai *et al.*, 2002b). The function of ATL6 in *Arabidopsis* may extend more generally to cellular signalling mechanisms common to defense responses and PCD.

Transcripts for β-fructofuranosidase I (invertase) (At3g13790) and two genes encoding sugar transporters, STP4 (At2g19930) and STP14 (At1g77210), were upregulated in both systems. The predicted invertase identified in this study has a basic pI (>9) (as determined using the ProtParam program on the ExPASy website; http://au.expasy.org (Bjellqvist *et al.*, 1993)), is targeted to the secretory system (Target P; $p<0.05$) (Emanuelsson *et al.*, 2000), and is therefore likely to therefore be an apoplastic invertase (Sturm, 1999). These enzymes function to cleave sucrose into glucose and fructose monosaccharides and, in some plant species, invertase gene expression is regulated by sugar availability (Sturm, 1999). In carrot, transcripts for extracellular invertase are upregulated in response to wounding and pathogen challenge, suggesting that these tissues require additional carbohydrate sources to meet their metabolic demands (Sturm and Chrispeels, 1990). Monosaccharide transporters include a family of at least 14 members in *Arabidopsis*. The expression patterns of these transporters throughout development, which in many instances is under transcriptional regulation, seem to play a key role in source-sink interactions (Williams *et al.*, 2000). Similar to extracellular invertase, the *STP4* transporter has been shown to be increased in *Arabidopsis* in response to wounding, and also induced in elicited suspension cultures (Truernit *et al.*, 1996). The authors suggest that the increased metabolic demands placed on wounded or infected tissues may require the import of carbohydrates from other tissues and, additionally, may serve to recover sugars from the apoplast to minimise losses to the invading pathogen. The upregulation of genes encoding monosaccharide transporters during leaf senescence in *Arabidopsis* has also been demonstrated (Quirino *et al.*, 2001).

Combined with the evidence that decreased sucrose concentration in the medium is correlated with senescence in *Arabidopsis* cell suspension cultures (see Figure 3.7A),

the upregulation of transcripts encoding extracellular invertase and two monosaccharide transporters seen in this study suggests that heat-treated and senescing cell cultures are experiencing a limitation in carbohydrate supply relative to their metabolic demands. It is also interesting to consider the alternate explanation put forth by Strum (1999) that suggests that increased hexoses released into the cells under these conditions may function in a cellular signalling pathway.

There were 8 genes for which transcript steady-state levels were decreased during both heat- and senescence-induced PCD, including those encoding xyloglucan endotransglycosylases (XET) (At1g65310 and At4g14130) and pectin acetylesterases (At5g45280 and At4g38420). These enzymes are generally classified as cell wall-modifying enzymes, acting on specific components of the cellulose, hemicellulose, pectins, and structural proteins that compose the plant cell wall (Hadfield and Bennett, 1998; Campbell and Braam, 1999). The former group acts upon hemicellulose xyloglucan polysaccharides present in the cell wall which are bonded to cellulose, cleaving and reattaching the xyloglucan chains to permit wall loosening and cell expansion. Not surprisingly, XET activity is highly correlated with regions of cell expansion and growth. In *Arabidopsis*, transcripts for seven *XET* family members have been shown to be differentially regulated in response to a range of hormonal and environmental treatments, including heat shock, suggesting that different XETs may be required in response to different stimuli (Xu *et al.*, 1996). Likewise, enzymes that cleave and modify cell wall pectin polysaccharides are associated with a variety of cellular modifications, including those occurring during cell wall softening, cell separation, and pollen tube growth (Hadfield and Bennett, 1998). In a study of transcriptional changes following wounding in *Arabidopsis*, a large number of transcripts encoding cell wall-modifying enzymes, including XETs and pectinesterases, were found to be upregulated

(Cheong *et al.*, 2002). This suggests that, unlike other abiotic stresses and defense responses, which also result in the induction of cell wall-modifying enzymes (Schenk *et al.*, 2000), the downregulation of XET and pectin acetylesterase expression may be a feature that is unique to PCD and reflects a cessation of cellular growth and associated metabolic activity.

Finally, it was of interest to observe a downregulation of transcripts encoding the cytosolic small Heat shock protein (Hsp) 17.6-II (At5g12020). This is in contrast to a study of the *Arabidopsis* transcriptome in response to oxidative stress in cell suspension cultures, in which *Hsp17.6* transcripts were found to be upregulated (Desikan *et al.*, 2001). The downregulation of cytosolic Hsp expression may be one of the regulatory mechanisms of PCD that is shared between plants and animals (see section 4.3.6 for a detailed discussion).

5.3.7. Conclusions

The ability to survey genome-wide transcriptional changes that occur during heat- and senescence-induced PCD has only recently been made possible by the production of the Affymetrix *Arabidopsis* ATH1 GeneChip. An analysis of transcript expression in these experimental systems and the application of stringent criteria to identify significant changes in transcript levels has allowed for the identification of 60 genes whose transcript steady-state levels are increased in both systems, and 8 that are decreased in both systems. The shared changes in the relative abundance of these transcripts may indicate a general role for their gene products in plant PCD. Many of these genes encode unknown proteins and therefore could not have otherwise been predicted to play a potential role in PCD without the use of such a non-hypothesis driven approach.

A large proportion of the upregulated transcripts are for genes which have previously been implicated in the hypersensitive response to pathogens and abiotic stress responses (such as wounding or oxidative stress). These results suggest that there is substantial crosstalk between the regulatory pathways induced by both biotic and abiotic stresses, and that the expression of these genes may be regulated by common mechanisms, such as reactive oxygen species or hormones, that are common to all these situations (see general discussion in Chapter 9). However, possible functions for some of these gene products, such as chitinases, glucanases, invertases, and ring-finger proteins, may also relate to extra- and intracellular signalling that is important during PCD. Thus, a more specific role for these genes in plant PCD cannot be excluded.

Finally, this study was successful in identifying several regulatory components that may function during plant PCD. These included three phospholipases, a subtilisin-like protease, two ion channel proteins, a two-component response regulator, and an extracellular glycoprotein, in addition to two NAC-domain proteins and a leucine zipper homeobox protein that likely function as transcriptional activators. The regulatory mechanisms that function during plant PCD are ill-defined at present, although ion fluxes, changes in cellular energy and redox states, and cell-surface glycoproteins have all been suggested to play potentially important roles (Lam *et al.*, 2001). The results presented in this analysis thus provide a new resource to study the functional effects of the identified genes on plant PCD in greater detail.

CHAPTER SIX

6. Proteomic analysis of programmed cell death in *Arabidopsis*

6.1. Introduction

In an attempt to identify key genes that may play a universal role in the induction and execution of plant PCD, a custom cDNA microarray analysis and Affymetrix GeneChip analysis of transcript expression during PCD in an *Arabidopsis* cell suspension system were undertaken (Chapters 4 and 5). By screening for transcripts that were similarily regulated in two unrelated PCD-inducing treatments, heat and senescence, it was possible to discriminate genes that may be common to a core plant cell death programme from those that are specifically related to the inducing stimulus itself. However, a transcriptomic analysis of changes in mRNA levels does not reveal anything about post-transcriptional or post-translational control of gene expression — regulatory mechanisms that may be equally important in PCD.

In addition to changes in the rate of protein turnover, post-transcriptional regulatory mechanisms may also include proteolytic cleavage and activation, and post-translational modifications such as protein phosphorylation (Tomlinson and Holt, 2001). Microarray screens of changes in mRNA steady-state levels are obviously incapable of identifying any of these mechanisms, and therefore can provide only limited information about changes in total protein profiles during a given cellular process. Indeed, a study comparing the correlation between specific mRNA and protein levels in mid-log phase yeast cells found that protein levels did not necessarily reflect transcript levels (Gygi *et al.*, 1999). While increased mRNA levels were *generally* found to correlate with increased protein levels, particularly for the most abundant proteins, specific mRNA and

protein levels relative to one another sometimes varied by as much as 20-fold. Studies such as these underscore the need for analyses of protein expression profiles, in addition to mRNA expression profiles, to fully investigate the patterns of gene expression in a given system.

In order to identify such post-transcriptional mechanisms that may be functioning during plant PCD, a proteomic analysis of changes in total cellular protein content during both heat- and senescence-induced PCD in an *Arabidopsis* suspension cell culture was undertaken. The aim of the work in this chapter was to identify those proteins that were increased in relative abundance in both heat- and senescence-induced PCD. Again, those proteins that change in both systems are more likely to be involved in PCD generally, rather than related to the specific stimulus (heat) or developmental stage (senescence). The identification of these proteins may contribute to our understanding of their role during plant PCD.

6.2. Results

6.2.1. Changes in total cellular protein during heat- and senescence-induced PCD

Total cellular protein was isolated from 6 day-old *Arabidopsis* cell suspension cultures induced to undergo PCD by a heat treatment at 55°C for 10 min and isolated immediately thereafter, or following senescence at 13 to 14 days. It is anticipated that the protein profile in senescing cell cultures will be different from that of control, 6 day-old cultures, due to the fact that the two cultures are in different developmental phases and therefore unique proteins will be expressed. However, it is acknowledged that a 10 min heat treatment may not be a sufficient time during which *de novo* protein synthesis will occur. Proteins that appear, by 2-D PAGE, to increase in abundance in these PCD-induced cells may, in fact, simply be increased relative to a background of decreasing total cellular proteins. Therefore, it was important to first determine the total extractable protein content of control, heat-treated, and senescing cell cultures. As shown in Figures 6.1A and B, the total soluble protein content per gram of fresh or dry weight was significantly decreased in both heat- and senescence-induced PCD cultures when compared to controls (t-test; $p<0.05$). These changes were observed when proteins were isolated in the presence of EDTA and a protease inhibitor cocktail. Protease activities per mg of total protein in both PCD samples were significantly higher than the control as measured using a universal protease substrate (Roche Diagnostics Ltd, East Sussex, UK) (t-test; $p<0.05$) (Figure 6.1C).

6.2.2. Proteomic analysis during heat- and senescence-induced PCD

A two-dimensional (2-D) SDS-PAGE analysis of equal amounts of protein extracted from heat- and senescence-induced PCD cells was used to identify proteins increased in relative abundance by a factor of at least 2-fold following both treatments

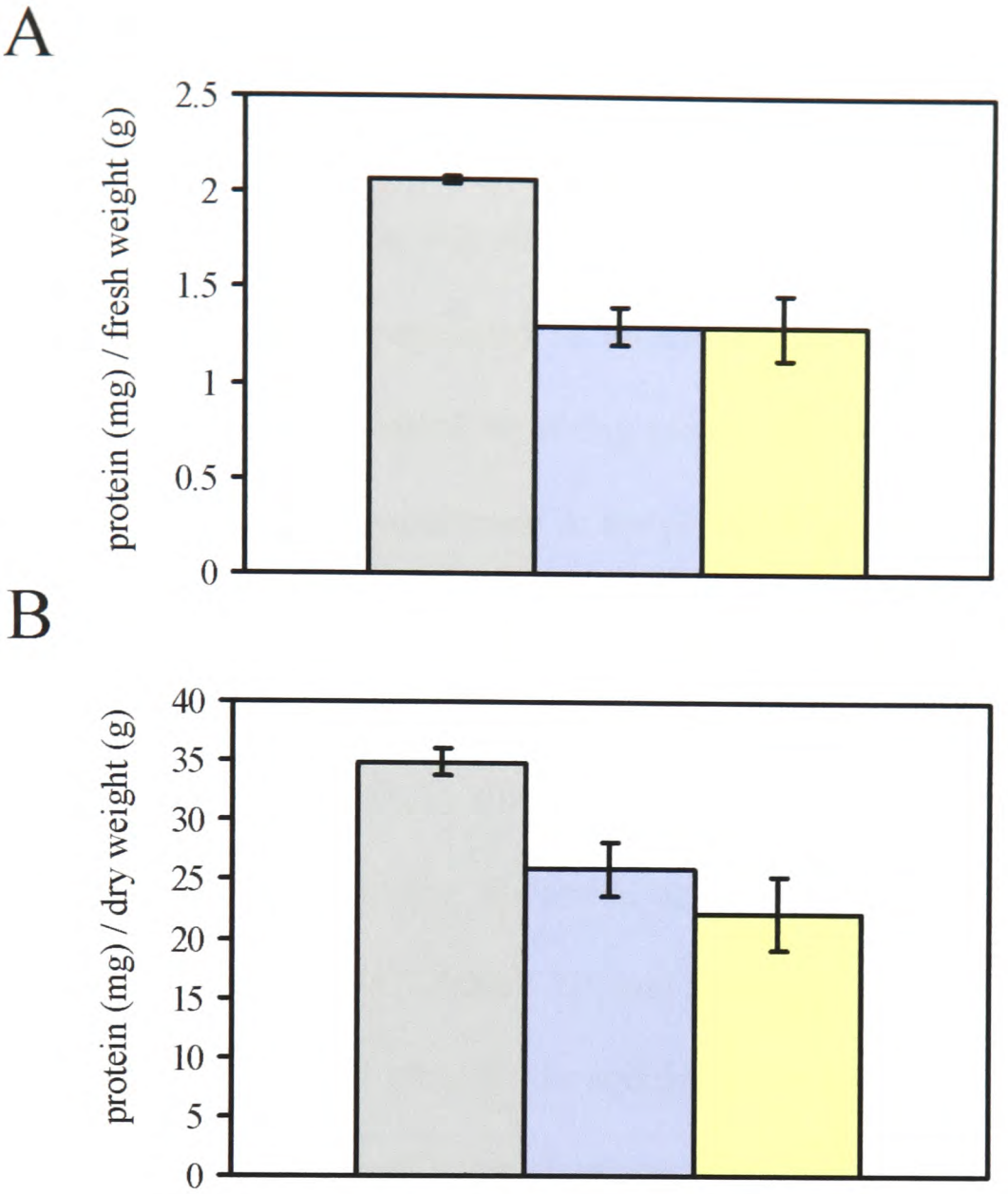


Figure 6.1. Changes in total cellular protein and protease activity during PCD.
A and **B.** Total protein was extracted from control (6 days-old) (grey), heat-treated (55°C, 10 min) (blue), and senescing (13 to 14 days-old) (yellow) cell cultures in the presence of EDTA and a protease inhibitor cocktail (see section 2.9.1). The total protein extracted is expressed per gram of fresh weight (**A**) and dry weight (**B**) of each sample. **C**. Protease activity in each sample (as in **A** and **B**) was measured using a universal casein protease substrate (see section 2.10.2). Values presented are the means of 3 replicate samples.

when compared to the control profile (6 day-old, untreated cells) (Figure 6.2). Using Z3 (Compugen, Tel Aviv, Israel) 2-D gel analysis software, 12 spots were identified that increased in relative abundance in both treatments or were unmatched in the control gel (Table 6.1). Protein extraction was also carried out in the presence of protease inhibitors (0.1 mM PMSF; 0.3 μM pepstatin) to ensure that proteolytic degradation was not occurring during protein isolation or during sample preparation for gel electrophoresis. Samples prepared and electrophoresed in the presence or absence of protease inhibitors gave similar results (data not shown).

Tandem MS/MS was used to unequivocally identify spots 2-12, but was not successful for spot 1 (Table 6.1). Four of these spots (4, 5, 7 and 8) were isoforms of catalase, while several, including lipoamide dehydrogenase (3), the voltage-dependent anion channel protein (VDAC) Athsr2 (9), and Mn superoxide dismutase (MnSOD) (10 and 11), were mitochondrial proteins. In addition to an EP1-like glycoprotein (6) and a protein of unknown function (12), an aconitase was identified (that has previously been demonstrated to be present in *Arabidopsis* mitochondria but may also be present in the cytosol (The *Arabidopsis* Genome Initiative, 2000; Millar *et al.*, 2001)). The aconitase spot increased in relative abundance by a factor of 2.9 during senescence-induced PCD, but the spot-matching algorithms in the Z3 software were unable to conclusively match this spot between control and heat-induced PCD gels (Table 6.1).

The appearance of multiple spots that are the product of the same gene suggests that post-translational modification of these proteins had occurred. Proteins 4 and 5 are both encoded by the same catalase gene, At1g20620, and proteins 10 and 11 are both products of the same gene, At3g10920, encoding MnSOD. Interestingly, one of the catalases (spot 8) matches the C-terminal domain of a predicted protein with approximately twice the molecular weight, the N-terminal domain of which also

A

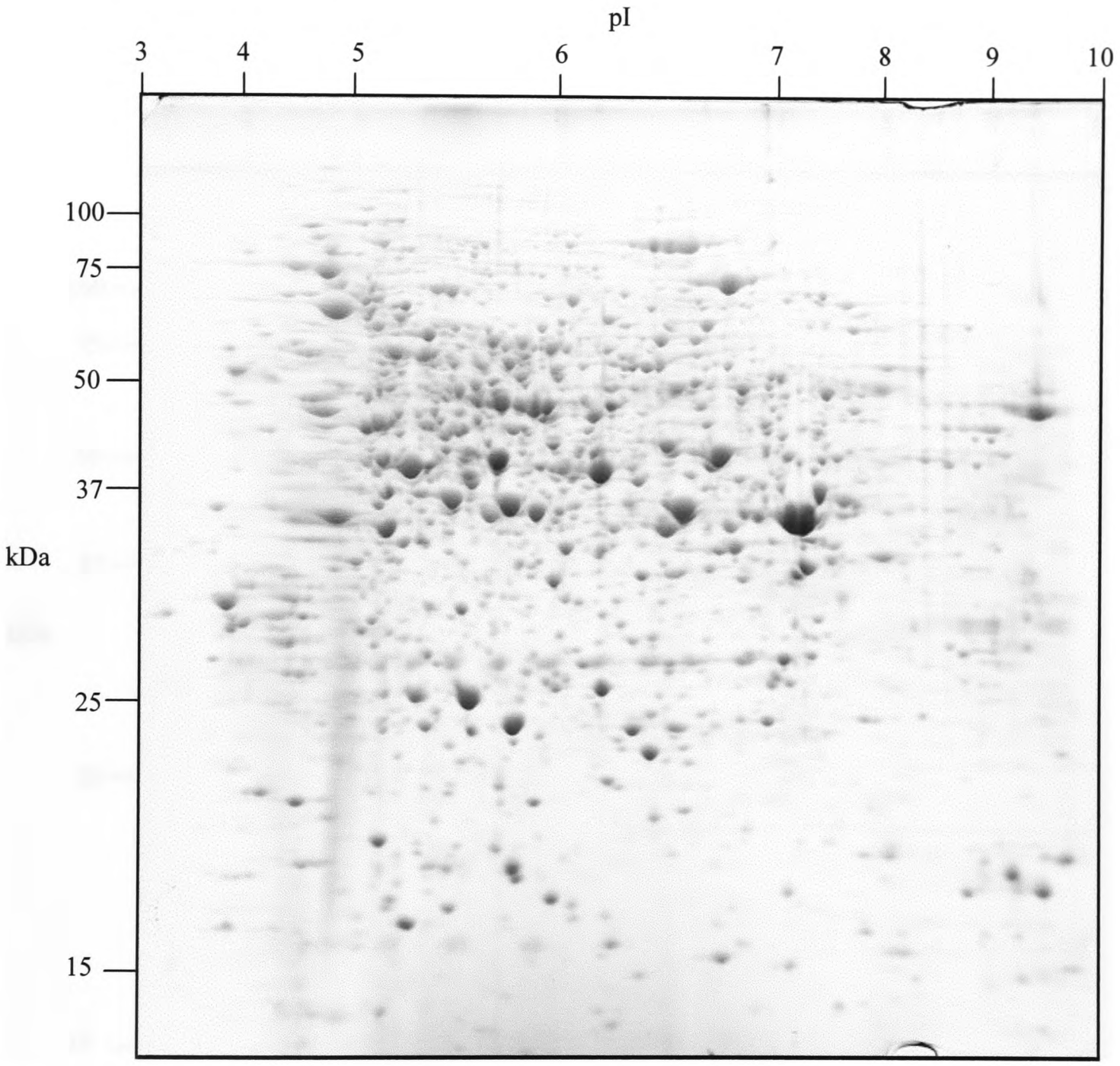
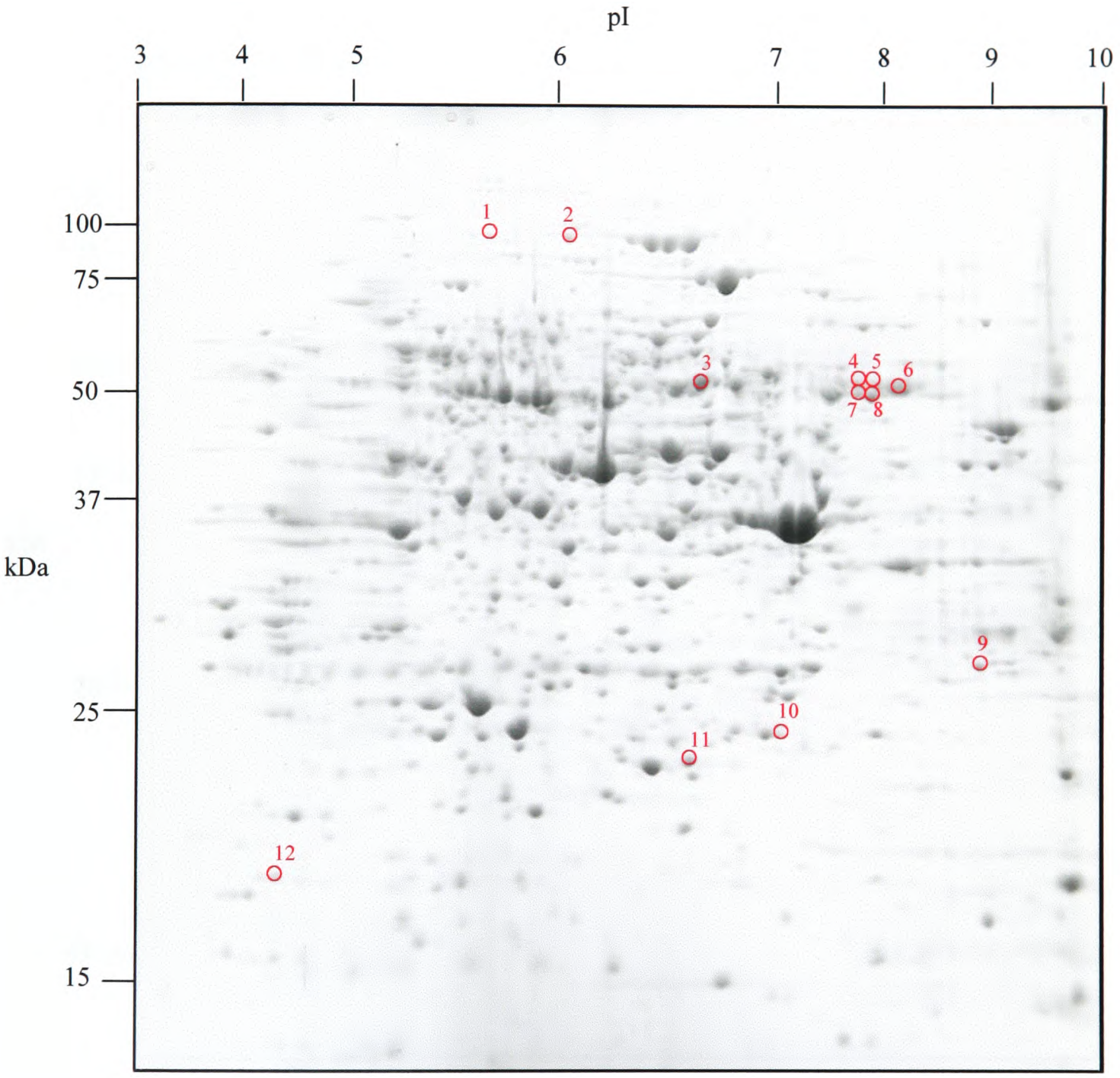


Figure 6.2. Two-dimensional SDS-PAGE analysis of total cellular protein from *Arabidopsis* cell suspension cultures undergoing PCD.
Total protein extracts (800 μg) of *Arabidopsis* cell suspension cultures were analysed by two-dimensional SDS-PAGE with a non-linear pI gradient 3-10. Spots (1-12) identified as being unique to and/or upregulated in both heat-treated and senescing samples were cut out and protein sequence obtained by tandem MS/MS analysis. **A.** Untreated, 6 day-old cultures. **B.** Six day-old cultures incubated at 55°C for 10 min and sampled immediately thereafter. **C.** Untreated, 13 to 14 day-old senescing cultures.

B



C

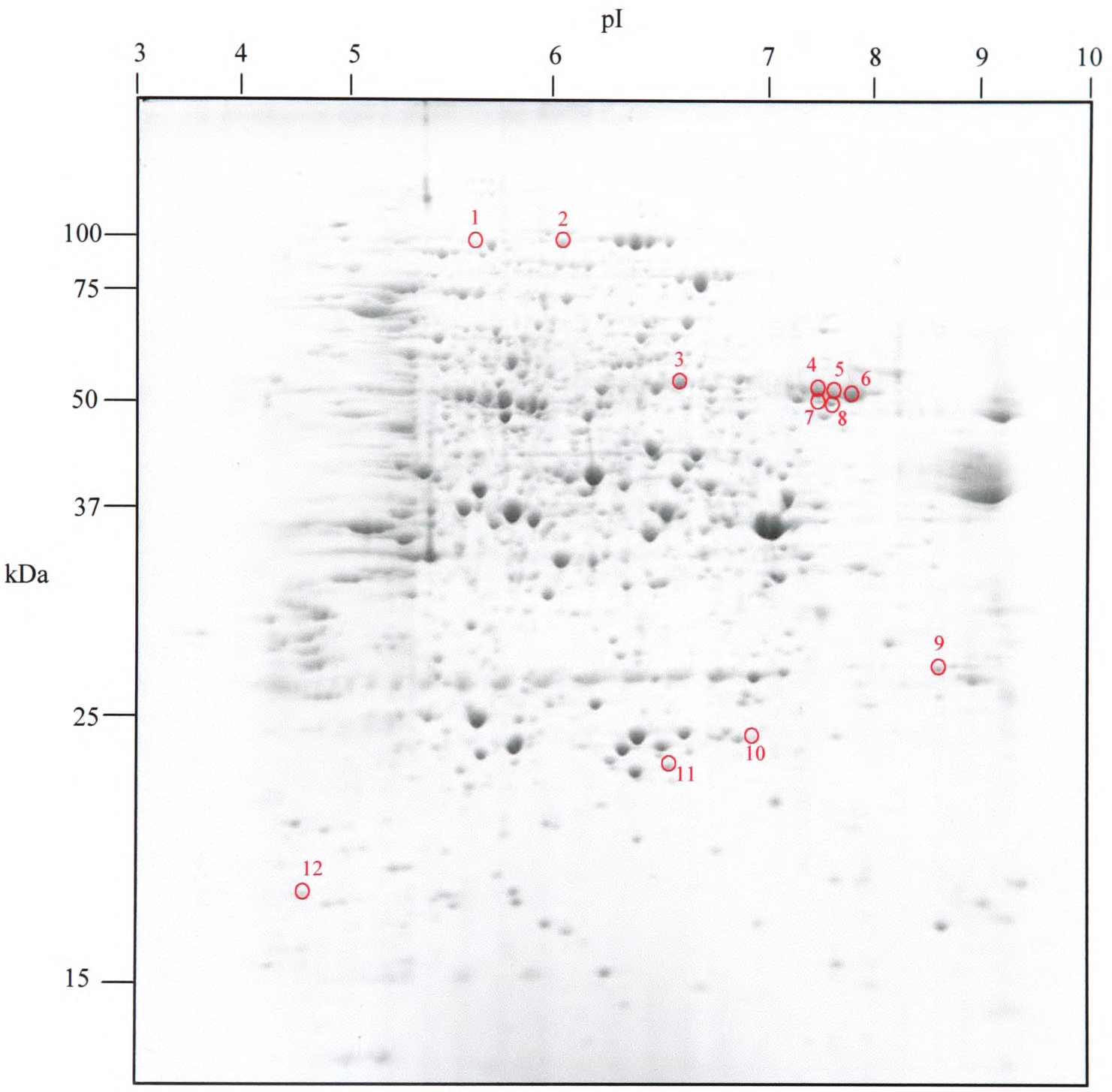


Table 6.1. Identification of induced proteins in heat- and senescence-induced PCD *Arabidopsis* cell cultures.

Spot No	Accession (NCBI)	AGI number	AGI Protein Description	No. MP[a]	% cover[b]	MW (Da) of match	pI of match	MW (Da) from gel	pI from gel	Spot ratio H/C[c]	Spot ratio S/C[c]
1	—	—	not identified	—	—	—	—	—	—	—	—
2	AY050431/ AAM97080	At4g26970/ At2g05710	aconitate hydratase	11	12	108311	6.85	100000	6.10	—	2.9
3	CAA11554	At3g17240	mitochondrial lipoamide dehydrogenase	19	48	49987	6.00	53000	6.60	U[d]	U
4	NM_101913	At1g20620	expressed protein (catalase 3)	8	15	57059	7.31	54000	7.80	2.4	13.3
5	NP_564120	At1g20620	expressed protein (catalase 3)	7	13	57059	7.31	54000	7.90	2.2	9.4
6	NP_178006	At1g78850	putative EP1 glycoprotein	7	12	49020	7.82	53000	8.10	2.0	4.4
7	NM_101914	At1g20630	expressed protein (catalase 1)	8	15	57068	6.95	51000	7.80	U	U
8	AAF79625	—	expressed protein (catalase 3)	11	10	117407	8.32	51000	7.90	4.6	3.2
9	NP_197013	At5g10590	voltage-dependent anion-channel protein Athsr2	4	16	29193	7.85	30000	9.00	U	U
10	NP_187703	At3g10920	putative (Mn) superoxide dismutase	5	19	25428	8.47	24000	7.00	2.8	7.4
11	NP_187703	At3g10920	putative (Mn) superoxide dismutase	4	19	25428	8.47	23000	6.70	2.9	2.6
12	NP_568698	At5g48480	unknown protein	8	41	17603	4.80	19000	4.30	U	U

[a] MP, matching peptides; [b] Percentage cover of matching peptides of full-length predicted protein; [c] H, heat-induced PCD; C, control; S, senescence-induced PCD; [d] Spot unique to treatments (i.e. not present in control)

Total protein extracts of *Arabidopsis* cell suspension cultures were analysed by two-dimensional SDS-PAGE with a non-linear pI gradient 3-10. Spots (1-12) identified as being unique to and/or upregulated in both heat-treated (55°C, 10 min) and senescing (13 to 14 days-old) samples compared to the control (6 days-old) were cut out and protein sequence obtained by tandem MS/MS. The GenBank and AGI accession number for each identified protein is presented, as well as the AGI protein description. The molecular weight and pI of each protein, as determined by the 2-D gel analysis, is compared to that predicted for the encoded protein using the ProtParam program on an ExPASy website (http://au.expasy.org) (see section 2.9.5)

encodes a putative catalase. This suggests that these two domains are cleaved post-translationally.

6.2.3. Western blot analysis of selected mitochondrial proteins

To further determine whether an upregulation of mitochondrial proteins in relation to total protein levels may be a general feature of plant PCD, one-dimensional SDS-PAGE was carried out on the same protein samples. Western blots of these gels were used to estimate the abundance of several mitochondrial proteins in control, heat-treated, and senescing samples (see section 2.10.1). Proteins from different mitochondrial sub-compartments were chosen for Western blot analysis. Outer membrane VDAC (that was identified as being upregulated in PCD samples compared to the control; see spot 9 on Table 6.1), inner membrane adenine nucleotide translocase (ANT), and the matrix-localised fumarase and E1α subunit of pyruvate dehydrogenase complex (PDC) were analysed. The blots suggest that there is no general increase or conservation of mitochondrial proteins during heat- and senescence-induced PCD (Figure 6.3), and that the relative increases in mitochondrial proteins identified by 2-D gel analysis likely represent real changes in specific protein abundance. For example, the E1α subunit of PDC is almost undetectable in heat-treated cells but increases in relative abundance during senescence while, conversely, fumarase increases in the former and decreases in the latter.

6.2.4. Increased free cellular iron is associated with PCD

The identification of an aconitase protein in the 2-D gel analysis prompted an investigation of cellular iron concentrations during PCD. This is because aconitase is suggested to function as an Iron Regulatory Protein that may function to affect the translatability of mRNAs involved in iron homeostasis (Cairo and Pietrangelo, 2000),

Alterations in aconitase levels may therefore serve to effect changes in cellular iron concentrations. Free cellular iron (Fe^{2+}) was measured in control, heat-treated, and senescing cultures using a colourimetric assay (see section 2.11). Measurements of Fe^{2+} during heat- and senescence-induced PCD of a single culture were compared to that of the same culture under control conditions, since the initial Fe^{2+} concentration of the control, 6 day-old culture can be variable and it is thus important to examine changes in Fe^{2+} concentration within the same culture. It was found that Fe^{2+} relative to cellular protein content was increased following both treatments. Fe^{2+} measurements repeated with 4 independent cultures gave similar results. The average Fe^{2+} concentration expressed relative to that of control cells (pmol Fe^{2+}/μg total protein) was: after heat-treatment, 1.66 ± 0.28; during senescence, 1.60 ± 0.10 (Table 6.2).

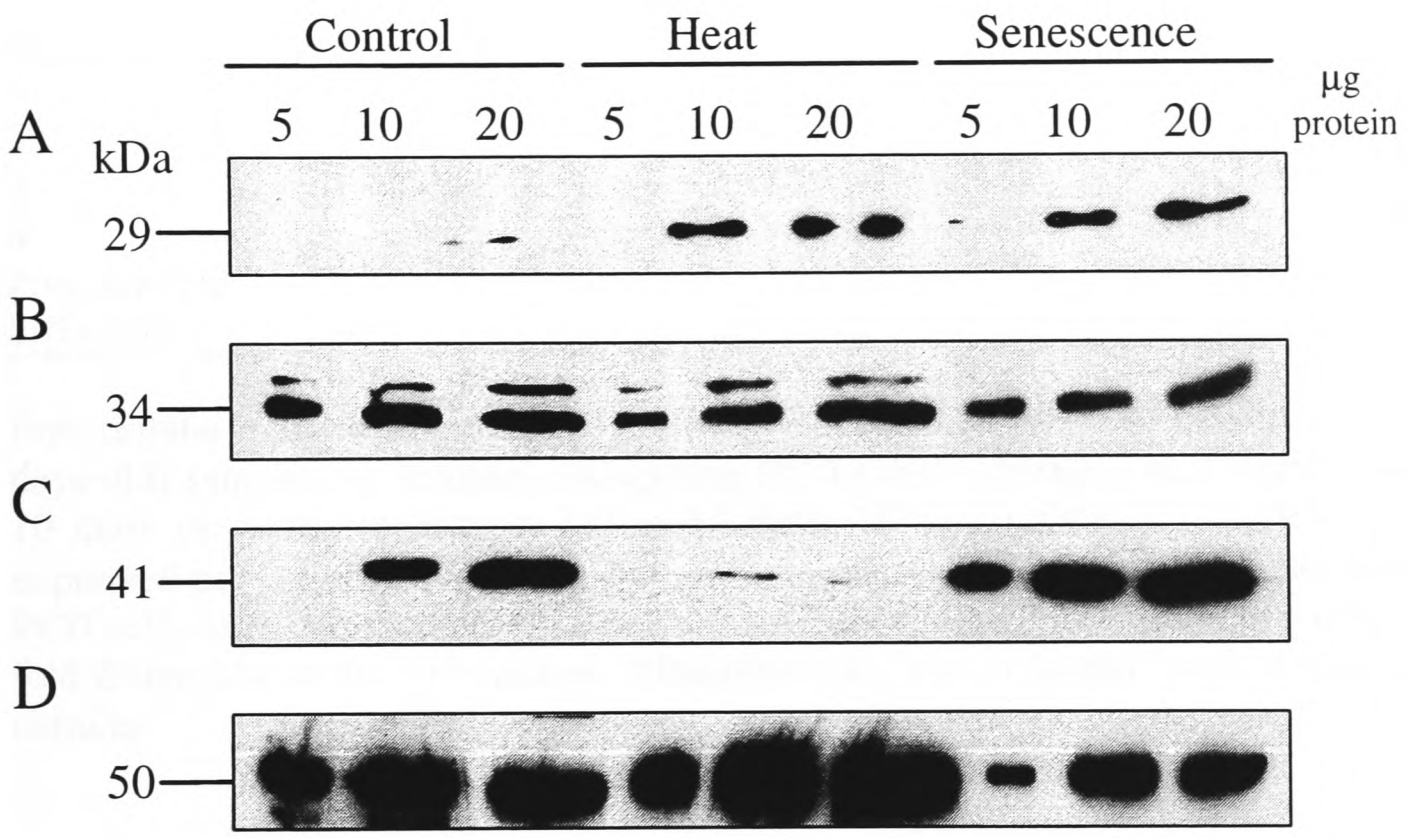


Figure 6.3. Western blot analysis of mitochondrial proteins.
Total protein was isolated from *Arabidopsis* cell suspension cultures from control (6 days-old) samples, or samples undergoing PCD either following heat treatment (55°C, 10 min), or during senescence (13 to 14 days-old). Protein samples were separated by SDS-PAGE and probed with the following antibodies (see section 2.10.1): **A.** 1/500 anti-VDAC. **B.** 1/1000 anti-ANT. **C.** 1/1000 anti-E1α subunit, PDC. **D.** 1/1000 anti-fumarase.

Table 6.2. Free cellular iron (pmol Fe^{2+}/μg total protein) in control and PCD-induced *Arabidopsis* cell suspension cultures.

Replicate batch	Control	Heat	Senescence
1	1.24	1.50 (1.21-fold)	2.08 (1.68-fold)
2	1.29	1.70 (1.32-fold)	2.13 (1.65-fold)
3	1.68	2.79 (1.68-fold)	2.18 (1.30-fold)
4	2.29	5.57 (2.43-fold)	4.03 (1.76-fold)
Average fold difference	—	1.66 ± 0.28	1.60 ± 0.10

Free cellular iron was measured in *Arabidopsis* cell suspension cultures from control (6 days-old) samples, or samples undergoing PCD either following heat treatment (55°C, 10 min), or during senescence (13 to 14 days-old) (see section 2.11). Fe^{2+} content is expressed per μg of total protein. Fe^{2+} concentrations for heat- and senescence-induced PCD cells were compared to the same culture under control conditions, allowing for the fold-difference to be determined. Measurements were repeated with 4 independent cultures.

6.3. Discussion

6.3.1. Mitochondrial proteins are not generally upregulated or conserved during heat- and senescence-induced PCD

The identification of four of the twelve spots as mitochondrial proteins in the proteomic analysis (Table 6.1) raised the possibility that this apparent upregulation may not reflect the *in vivo* situation. It is conceivable that mitochondrial structure and integrity may be maintained during the initiation and execution of PCD, as has been reported in a human colon carcinoma cell line (Mancini *et al.*, 1997). Against a background of general cytosolic proteolysis and/or downregulation of protein synthesis, total protein extracts from such dying cells might therefore be enriched for mitochondrial proteins relative to other cellular proteins. Such an apparent 'upregulation' of mitochondrial proteins may be a consequence of analysing equal amounts of total protein between control and PCD cells, and may not reflect a specific upregulation of the identified proteins *per se*.

Such an interpretation would be supported by results obtained in the cDNA microarray analysis that revealed that the steady-state levels of the mitochondrial-encoded *Atp1* transcript were maintained (heat) or upregulated (senescence) during PCD, while the levels of the nuclear-encoded *Atp2* gene were decreased in both treatments (see Table 4.2). Since both of these transcripts encode subunits of the mitochondrial ATP synthase complex and their gene products are assembled in equal stoichiometries, it is possible that the observed maintenance/upregulation of the *Atp1* transcript reflects its protection within intact mitochondria during PCD. However, for the mitochondrial proteins analysed by Western blot analysis, no general upregulation

during PCD is evident (Figure 6.3). Thus, this analysis has identified *specific* mitochondrial proteins that are maintained and therefore may play a role in PCD.

Furthermore, the Western blot analysis does support the data obtained in both the 2-D gel and cDNA microarray analyses. A specific isoform of VDAC, Athsr2, was identified as being upregulated in both heat- and senescence-induced PCD cells (Table 6.1). Similarly, an antibody raised against mitochondrial VDAC demonstrates an upregulation of this protein in both samples, thus supporting the interpretation of the 2-D gel data (Figure 6.3A). Microarray analysis revealed a downregulation of transcripts encoding ANT during both heat- and senescence-induced PCD (see Table 4.2). Western blot analysis indicates that the level of the inner membrane ANT protein might also be decreased in both samples (Figure 6.3B), supporting the hypothesis that a general downregulation of ANT at both the transcript and protein level may be a general feature of plant PCD (see section 4.3.3 for a detailed discussion).

6.3.2. Upregulation of the antioxidant proteins catalase and MnSOD is associated with plant PCD

The apparent upregulation of four isoforms of catalase and two isoforms of mitochondrial MnSOD in both heat- and senescence-induced PCD samples was not surprising given the fact that oxidative stress is associated with PCD (reviewed by Fleury *et al.*, 2002). Moreover, the increased steady-state levels of transcripts encoding several antioxidant enzymes, such as the superoxide dismutases CSD1 and CSD3 and glutathione peroxidase (GPX), identified by the microarray analysis (see Table 4.2), suggest that oxidative stress is associated with both systems. The relative increase of four catalase isoforms suggests that this enzyme figures prominently in scavenging H_2O_2 produced during plant PCD. Indeed, previous studies have shown that transgenic tobacco plants with reduced catalase levels show increased susceptibility to stress

conditions (Willekens *et al.*, 1997) and are hyperresponsive to pathogen attack (Mittler *et al.*, 1999b), indicating that these enzymes play a central role in antioxidant defense. Since four different catalase isoforms were identified in the 2-D gel analysis, it seems likely that unique roles for each isoform in combatting oxidative stress may exist. Orendi *et al.* (2001) have demonstrated the differential regulation of *catalase2* and *catalase3* genes in *Arabidopsis* following stress treatments, indicating a complex regulation of catalases. Interestingly, the authors suggest that a decline in the expression of *catalase3* — which is normally induced following stress — during leaf senescence may reflect a specific mechanism to reduce the responsiveness of the plant to oxidative stress at this stage and, in turn, may promote senescence.

If, as has been inferred above, an increase in reactive oxygen species (ROS) occurs in heat- and senescence-induced cell cultures undergoing PCD, it is not surprising that increased levels of mitochondrial MnSOD were observed by 2-D gel analysis. Superoxide dismutases (SODs) catalyse the conversion of O_2^- to H_2O_2 and these enzymes are defined by the metal cofactor at the active site (Fe, Mn, or Cu and Zn) (Kliebenstein *et al.*, 1998). In plant cells, the various isozymes also differ in their subcellular location, with MnSOD being mitochondrial, FeSOD plastidic, and Cu/ZnSOD plastidic or cytosolic. Seven SOD enzymes have been isolated and characterised in *Arabidopsis*, though only one of these is an MnSOD (MSD1). Therefore, it is of interest that the 2-D gel analysis has revealed two isoforms of this enzyme, which seem to be the same protein based on peptide fingerprint analysis but clearly differ in their pI and very slightly in their molecular weight.

However, in response to several stress treatments including various light regimes, ozone fumigation, and UV-B radition, Kliebenstein *et al.* (1998) were unable to detect changes in the MSD1 mRNA, protein, or activity level in *Arabidopsis*. Therefore,

the results presented here are novel in demonstrating changes in two MSD1 isoforms in response to heat treatment and senescence which may, perhaps, be due to a post-translational modification (i.e. phosphorylation). Such modifications perhaps occur only under severe conditions of oxidative stress, i.e. those sufficient to cause PCD. This may be particularly important in preventing widespread mitochondrial damage during the initiation and execution of PCD, since maintenance of mitochondrial function may be required for PCD. Transcript levels of *MSD1* were not increased in both heat- and senescence-induced PCD in an *Arabidopsis* cell suspension system (see Table 4.2).

Finally, the relative increase of MnSOD and catalase in both systems is consistent with the notion that increased MnSOD activity will result in higher intracellular H_2O_2 concentrations. Since H_2O_2 is freely diffusible within the cell (Willekens *et al.*, 1997), an upregulation of catalase enzymes will contribute to the balancing mechanism needed to reduce ROS levels.

6.3.3. Possible roles of ROS and antioxidant enzymes in plant PCD

Collectively, the findings of this study and others point to a central role for catalase and possibly MnSOD in plant antioxidant defense. On the other hand, it might seem contradictory that, in some cases, a downregulation of catalase expression or activity results in cell death in barley aleurone cells and transgenic tobacco (Mittler *et al.*, 1999b; Fath *et al.*, 2001) while, in the current analysis, the opposite correlation is seen. This apparent inconsistency may be explained, however, by considering that perhaps increased catalase levels during heat- and senescence-induced PCD maintain a delicate balance of H_2O_2 that allows for PCD, but prevents uncontrolled oxidative damage. Very high levels of intracellular H_2O_2 in yeast have been shown to destroy cellular architecture to the point where apoptotic features, such as chromatin condensation and membrane blebbing, are no longer apparent and, instead, necrotic cell

death ensues (Madeo *et al.*, 1999). Similarly, in transgenic *Arabidopsis* compromised for antioxidant defense resulting from altered levels of fatty acid dioxygenase, severe cellular necrosis, rather than typical HR lesions, is observed in response to pathogen infection (de Leon *et al.*, 2002). This suggests that, above some threshold ROS concentration, PCD is no longer invoked and instead the overwhelming level of ROS results in necrosis. Increasingly high concentrations of H_2O_2 added to carrot cell suspension cultures have been shown to cause a switch in the cellular response from PCD, accompanied by the characteristic cellular condensation and shrinkage, to an outright necrotic cell death (McCabe *et al.*, 1997).

Thus, the balance of ROS levels in the plant cell following stress and during senescence may be obviously important. Increased ROS production may contribute to the induction of PCD, as the downregulation of antioxidant enzymes prior to cell death and analysis of transgenic plants with reduced antioxidant enzyme levels indicates. However, an uncontrolled increase in ROS production may not be compatible with a regulated PCD, since the resulting severe cellular damage would lead to cellular necrosis. It is also important to recognise that the relative levels of antioxidant enzymes may differ depending on the plant system under investigation and the time point sampled, and thus may lead to apparently contradictory interpretations in the literature.

However, one may consider a subtly different explanation for the apparent upregulation of some antioxidant genes in the current study. The argument presented above views the cell's antioxidant defense as an active 'control' mechanism to allow the production of sufficient ROS to cause PCD, but avoid overwhelming ROS production that may result in necrosis. Alternatively, when the production of ROS is a consequence of biotic or abiotic stress (as is the case following heat-treatment or during nutrient starvation that occurs in senescence), cellular responses such as the induction of

antioxidant enzymes may always be deployed to combat the oxidative stress. Studies such as those of Fath *et al.* (2001) show the downregulation of antioxidant enzymes prior to a developmental PCD in barley aleurone cells. This may reflect the existence of a specific developmental or hormonal mechanism that triggers PCD. In this developmentally progammed event, an active cellular process leads to a downregulation of ROS scavenger enzymes, such as catalase, ascorbate peroxidase, and superoxide dismutase, leading to an accumulation of ROS in the cell linked to the induction of PCD. Similarily, studies which show that antisense repression of antioxidant genes causes cell death in tobacco (Mittler *et al.*, 1999b) may demonstrate that higher than normal levels of ROS are capable of causing cell death. The conclusions from these studies do not imply that downregulation of antioxidant genes prior to PCD is a general mechanism in plants; rather, they only confirm that high levels of ROS are capable of causing cell death.

6.3.4. Cytosolic aconitase may function to increase free cellular iron during PCD under conditions of oxidative stress

One protein spot that increased in relative abundance in the senescence-induced PCD samples (but was not conclusively matched between the control and heat-induced PCD samples) was identified as a cytosolic aconitase protein (Table 6.1). This raises interesting questions about the possible role for this protein as a regulatory molecule in plant oxidative stress and/or PCD. Aconitase (or citrate (isocitrate) hydrolase or aconitase hydrolase) is an Fe-S cluster-containing protein known to be present in both the mitochondria and cytosol in plants and animals (Peyret *et al.*, 1995). In the mitochondria, aconitase participates in the TCA cycle, catalysing the isomerisation of citrate to isocitrate. The role of cytosolic aconitase in plants is less clear, though it has been suggested to play a role in the glyoxylate cycle (Courtois-Verniquet and Douce,

1993). In mammals, however, cytosolic aconitase has been shown to have two very different but important functions: In one case, cytosolic aconitase has enzymatic activity (the role of which is not obvious in animal cells) but, under certain physiological conditions, aconitase adopts an Iron Responsive Element-Binding Protein (IRE-BP) activity that is crucial in the post-transcriptional regulation of various mRNAs involved in iron homeostasis and pathobiology (reviewed by Cairo and Pietrangelo, 2000).

The enzymatic and RNA-binding activities of cytosolic aconitase are mutually exclusive and under the control of an Fe-S cluster-dependent switch (Cairo and Pietrangelo, 2000). Low cellular iron concentration causes a conversion of the cluster from the 4Fe-4S state to the 3Fe-4S state, which confers IRE-BP activity to the protein. Target mRNAs of the IRE-BP, of which there are two forms (IRP-1 and IRP-2) in animals, include various transcripts encoding proteins involved in iron storage and uptake, such as ferritin and the transferrin receptor. The binding of IRPs to specific IRE sequences in the UTRs of these transcripts either inhibits or enhances their translatability, respectively, and it is in this manner that iron homeostasis is maintained under normal cellular conditions.

Free iron also plays a crucial role in the generation of intracellular reactive oxygen species, most notably in the Fenton reaction which converts superoxide (O_2^-) and H_2O_2 to the hydroxyl radical (OH^-) (Wendehenne *et al.*, 2001). While the former two ROS play a role in normal signal transductions, signalling, and redox control pathways that regulate cell proliferation and death, OH^- is a highly reactive and toxic ROS. Therefore, in considering a potential role for free iron in regulating ROS levels, it is perhaps not surprising that enzymatic activity versus the IRP activity of the aconitase protein in mammalian cells is modulated by the presence of ROS. In particular, both O_2^- and nitric oxide (NO) have been demonstrated to cause the reversible inactivation of

aconitase activity by promoting the loss of the Fe-S cluster, thus converting aconitase into an IRP and linking cellular redox state to post-transcriptional regulation (Gardner *et al.*, 1995; Gardner *et al.*, 1997).

The evolutionary conservation of aconitases as RNA-binding proteins is supported by work which demonstrated that the aconitases of both *Bacillus subtilis* and *Xanthomonas campestris* pathovar *campestris* (*Xcc*) possess such activity. In *B. subtilis*, the RNA-binding activity of aconitase was found to be dependent on low iron levels and to be sequence-specific for RNAs containing IREs, for which many candidate sequences exist in the bacterial genome (Alen and Sonenshein, 1999). It was further suggested that the non-enzymatic RNA-binding activity of *B. subtilis* aconitase may be important in post-transcriptional regulation during sporulation. In the plant pathogen *Xcc*, the regulatory role of the aconitase protein in gene expression was found to be related to the production of pathogenicity factors and was also linked to changes in intracellular iron concentration (Wilson *et al.*, 1998).

In *Arabidopsis* (Peyret *et al.*, 1995) and tobacco plants (Navarre *et al.*, 2000), aconitase activity has been detected in all tissues analysed but found to be highest in flowers, during seed and pollen maturation, and during germination. In tobacco, the level of the transcript is not increased following oxidative stress treatment (Navarre *et al.*, 2000). Interestingly, the cDNA corresponding to the *Arabidopsis* aconitase analysed by Peyret *et al.* (1995) possesses a higher degree of sequence identity to mammalian IRE-BP (70%) than to the mammalian mitochondrial aconitase (43%) and contains putative IRE-BP sequences that are known to crosslink to IRE sequences from animals. However, the identification of *Arabidopsis* sequences containing IRE elements has not been reported. Similarly, a tobacco aconitase also possesses a high degree of sequence similarity to human IRP-1 (76%) and contains putative IRE binding sequences (Navarre

et al., 2000). Although no investigation of the possible role of plant aconitases in gene regulation has been undertaken, the evolutionary conservation of this protein suggests that this is a very real possibility.

Navarre *et al.* (2000) have demonstrated that NO inactivates tobacco aconitase and suggest that this inactivation could be related to plant defense responses in two ways. Firstly, the inactivation of mitochondrial aconitase would most likely result in an increase in citrate levels. Increased citrate has been previously shown to induce alternative oxidase expression (Vanlerberghe and McIntosh, 1996) that may be involved in defense responses. Secondly, and more relevant to this study, aconitase inhibition by NO may effectively lead to an increase in cellular iron levels if plant aconitases, like their mammalian counterparts, possess an IRP activity that is involved in increasing free iron concentration. Excess free iron has been implicated in oxidative damage, such as lipid peroxidation and DNA damage, and cell death by promoting formation of the toxic hydroxyl radical (Wendehenne *et al.*, 2001). Moreover, free intracellular iron has been suggested to be required for DNA damage and preferential scission into nucleosome-sized fragments in mammals (Eaton and Qian, 2002), resulting in the production of DNA 'ladders' that hallmark many PCD systems. Thus, aconitase inhibition and subsequent IRP activity in the presence of ROS may be a part of the plant cell death programme.

The apparent increase in a cytosolic aconitase seen in a proteomic analysis of cells undergoing both heat- and senescence-induced PCD suggests that this protein may play a role in oxidative stress or PCD-related mechanisms. The protein identified in this study by peptide fingerprint analysis bears equal identity (42%) to two putative *Arabidopsis* aconitases (see Table 6.1), one of which contains a predicted mitochondrial targetting sequence (Target P; $p<0.05$) (Emanuelsson *et al.*, 2000). Unfortunately, the

designation of plant aconitases as mitochondrial or cytosolic is unclear in the current literature and in gene databases. However, the protein that showed an apparent increase in this study does not match either of the two aconitase proteins previously identified as being present in the mitochondria (Millar *et al.*, 2001), lending support to the hypothesis that the identified protein may be a cytosolic isoform.

It is intriguing to suggest that the increase of this protein in plant cells following PCD-inducing stimuli may be related to a mechanism that leads to an increase in free iron levels that may contribute to downstream cell death. In this model, increased cellular ROS levels following heat treatment and during senescence may induce the stabilisation and/or expression of aconitase protein but, due to the presence of ROS in the cell, the cytosolic aconitase protein would be modulated such that it can only function as an RNA-binding protein. A role in the subsequent post-transcriptional regulation of iron-related genes may allow free iron concentrations to increase to the point of promoting downstream cell death. This hypothesis is supported by measurements of Fe^{2+} levels during heat- and senescence-induced PCD, which indicate that increased free iron relative to total cellular protein content is associated with both systems. It is of immediate interest to identify transcripts that the plant cytosolic aconitase may be capable of binding to and modulating their translatability, as this may include mRNAs encoding proteins involved in iron homeostasis (or perhaps even other transcripts involved in stress or cell death-related mechanisms). Given the evidence for such a regulatory role of aconitase in both mammalian and bacterial systems, combined with the demonstration that plant aconitase activity is modulated by NO and the fact that the relative levels of this protein are increased following PCD induction, it is certainly an interesting possibility to consider.

Finally, it is important to reconcile the apparently contradictory observation of the relative increase in antioxidant enzymes such as catalase and MnSOD with the proposed role for increased free iron levels in generating oxidative damage. As discussed above, at a fundamental level, the induction of antioxidant genes may always occur in the presence of ROS. PCD may result when the cell can no longer cope with the level of oxidative stress. The highly toxic effects of increased free Fe^{2+} are likely to outweigh any antioxidant activity of enzymes such as catalase or MnSOD. Previous studies in animals have demonstrated the induction of antioxidant enzymes in response to increased free iron and the failure of this induction to protect against oxidative damage — it may, in fact, promote oxidative damage (Eaton and Qian, 2002). Once again, those studies in plants which show that a downregulation of antioxidant enzymes is associated with PCD (Mittler *et al.*, 1999b; Fath *et al.*, 2001) may represent a developmental event, and only confirm that increased ROS can cause cell death.

6.3.5. The voltage-dependent anion channel protein, Athsr2, may play a PCD-specific role in plants

The relative increase of the voltage-dependent anion channel protein Athsr2 in heat- and senescence-induced PCD was of interest for several reasons. First, a BLAST analysis (Altschul *et al.*, 1997) of the *Arabidopsis* genome indicates that it encodes at least five VDAC-like proteins. However, the protein identified by 2-D gel analysis can be unequivocally identified as the Athsr2 protein by peptide fingerprint analysis. This is of particular interest since the transcript encoding Athsr2 was originally identified as being preferentially expressed during the *Arabidopsis* hypersensitive response (Lacomme and Roby, 1999). Since the steady-state levels of the transcript encoding the Athsr2 protein did not change significantly in the custom cDNA microarray analysis of heat- and senescence-induced PCD (see Table 4.2), the proteomic data suggests that, in

these systems, the relative abundance of the Athsr2 protein may be regulated at a post-transcriptional level. Transcriptional control of Athsr2 expression may therefore be a feature unique to the hypersensitive response. Nevertheless, the combined results of the present study and the upregulation of Athsr2 transcripts during the HR strongly suggest that this specific VDAC isoform may play a cell death-specific role.

The notion that VDAC/Athsr2 may be involved in plant PCD is intriguing since it has been established that this protein is a central component of the mitochondrial permeability transition pore (PTP) that functions during animal PCD (reviewed by Martinou and Green, 2001). The PTP is thought to form and function during PCD, leading to the permeabilisation of the outer mitochondrial membrane in response to conditions of oxidative stress, high Ca^{2+}, or low ATP, allowing low-molecular weight solutes (up to ~1.5 kDa) to diffuse into the matrix across the inner mitochondrial membrane. The ensuing mitochondrial swelling then ruptures the outer mitochondrial membrane, leading to the release of 'death-promoting factors', including cytochrome *c*, primarily from the intermembrane space. In animals, the PTP is thought to comprise a complex of existing mitochondrial proteins, including outer membrane VDAC, inner membrane ANT, and matrix cyclophilin D. However, many studies have suggested that alterations (in concentration or conformation) in either VDAC or ANT alone are sufficient to cause the permeability transition (see section 1.2.2). While overexpression of ANT1 has been demonstrated to induce apoptosis in mammalian cells (Bauer *et al.*, 1999), the downregulation of ANT mRNA (see Table 4.2) and possibily also protein levels (Figure 6.3B) seen in the present study makes it unlikely that an analogous role for ANT exists in plant PCD.

Conversely, changes in VDAC configuration to the closed state in mice (Vander Heiden *et al.*, 2000) or the complex of VDAC with other pro-apoptotic proteins such as

Bax in reconstituted mammalian liposomes (Shimizu, 1999) are sufficient to cause the permeability transition. While Bax does not exist in plants, it is possible that other proteins interact with plant VDAC in a similar fashion during PCD. However, changes in VDAC configuration alone may be sufficient to lead to changes in mitochondrial permeability, and an increase in VDAC protein during plant PCD may serve to augment this event. While the case for the existence of a PTP in plants is preliminary and based mostly on studies using pharmacological inhibitors of known animal PTP components (such as cyclosporin A as an inhibitor of cyclophilin D in potato, *Citrus*, and *Arabidopsis*) (Arpagaus *et al.*, 2002; Saviani *et al.*, 2002; Tiwari *et al.*, 2002), the presence of a mitochondrial permeability transition has been detected in potato mitochondria and in *Arabidopsis* cells in response to stress leading to cell death (Arpagaus *et al.*, 2002; Tiwari *et al.*, 2002). The relative increase in VDAC/Athsr2 during plant PCD observed in this study may be a feature of this permeability transition and potentially involved in the release of pro-apoptotic proteins from the plant mitochondria.

6.3.6. Identification of additional proteins potentially involved in plant PCD and related aspects of cellular signalling

Several other proteins increased in relative abundance during both heat- and senescence-induced PCD, suggesting that they play a role in plant cell death. The mitochondrial lipoamide dehydrogenase, a subunit of enzyme complexes including the pyruvate dehydrogenase complex (PDC) and the 2-oxoglutarate dehydrogenase complex (2-OGDC) (Lutziger and Oliver, 2001), increased in abundance. This may indicate that increased TCA cycle activity is a feature of PCD, perhaps as a source of NADH reducing power for ATP synthesis necessary for the execution of cell death. However, results obtained by Western blot analysis of the E1α subunit of PDC and fumarase

indicated no consistent upregulation of TCA cycle enzymes during PCD (Figures 6.3C and D). Moreover, it has been shown that the activity of the E2 subunit of potato 2-OGDC is rapidly inhibited in the presence of ROS due to the production of 4-hydroxy-2-nonenal (HNE) resulting from lipid peroxidation. In the presence of HNE, the activity of the potato 2-OGDC complex is reduced to 19% of the control (Millar and Leaver, 2000). This suggests that changes in the redox state of lipoamide may have a role in PCD, possibly as part of a redox signalling mechanism. Since this proteomic analysis does not allow for determination of the subcellular localisation of proteins, it is possible that this enzyme may be upregulated for a PCD-specific role in the cytoplasm or nucleus. Proteomic or Western blot analysis of subcellular fractions of plant cells undergoing PCD would help to determine the localisation of this enzyme subunit during PCD and therefore indicate whether it may be playing some TCA cycle-independent role.

The proteomic analysis of cell cultures undergoing PCD also identified one protein of unknown function and an EP1-like protein which were increased in relative abundance. While several EP1-like proteins are encoded in the *Arabidopsis* genome, a specific isoform (At1g78850) was upregulated during heat- and senescence-induced PCD. This protein is 51% identical and 67% similar to an extracellular cell wall glycoprotein initially characterised in carrot suspension cells (van Engelen *et al.*, 1991). The function of the EP1 protein in carrot is unclear; the protein is known to be encoded by a single transcript but to give rise to multiple products of different molecular weights that differ in post-translational modifications, particularly *N*-glycosylation. In carrot cell cultures, EP1 is only expressed in vacuolated, nonembryogenic cells. In the whole plant, the protein is most highly expressed in the basal hypocotyl and root, with lower

expression in apical parts of the root and hypocotyls and in cotyledons. Interestingly, the MW of the protein is characteristically different in each organ.

While the expression pattern of EP1 in carrot initially suggested that the protein may play a role in cell elongation, subsequent sequence analysis of the cDNA encoding the protein revealed homology to *Brassica* S-locus glycoprotein genes, an *Arabidopsis* S-like gene and putative S-like receptor kinases from maize and *Arabidopsis* (van Engelen *et al.*, 1993). Similarly, the protein identified in *Arabidopsis* cell suspension cultures in this study (At1g78850) also bears an equivalent (40 to 60% similarity) homology to S-like glycoproteins (SLG) and the S-like domain of receptor proteins kinases from several species such as beet (*Beta vulgaris*), rice (*Oryza sativa*), maize (*Zea mays*), and *Brassica* (*Brassica rapa*), as well as *Arabidopsis* (as determined by BLAST algorithm analysis (Altschul *et al.*, 1997)). The MW of this protein (~50 kDa), its predicted glycosylation sites in the N-terminal region (as determined by domain predictions using Expasy Tools; http://www.expasy.ch/prosite/), and the presence of a cysteine-rich C-terminal domain, further indicate its similarity to both carrot EP1 and SLG and SLR proteins (van Engelen *et al.*, 1993).

However, since *Arabidopsis thaliana*, like carrot, does not possess a genetic self-incompatibility system (Bi *et al.*, 2000), the EP1-like protein may be involved in other receptor kinase activation pathways and signal transduction. The encoded EP1-like protein is predicted to be part of the secretory pathway (Target P; $p<0.05$) (Emanuelsson *et al.*, 2000). It is tempting to suggest that the identifed EP1 homologue is secreted in *Arabidopsis* cell suspension cultures undergoing PCD as part of a cell-to-cell signalling mechanism. The purification and biochemical analysis (such as potential protein-protein interactions) of the EP1-like protein from *Arabidopsis* cell suspension cultures, expression analysis of the protein in whole plant tissues, as well as studies of the cell

death responses in plants either overexpressing or deficient in this protein, may help to determine whether it plays a role in cell signalling during plant PCD.

6.3.7. Conclusions

The preliminary proteomic analysis presented in this chapter was successful in identifying 11 proteins that were commonly increased in relative abundance and/or unique to both heat- and senescence-induced PCD in an *Arabidopsis* cell suspension culture. The increased abundance of these proteins in both a heat- and senescence-induced PCD system during a period when total cellular protein is decreasing suggests that these proteins may play a general role in the plant cell death programme. Identification of these proteins has revealed that the specific increased abundance of several mitochondrial proteins as well as antioxidant enzymes is associated with plant PCD. The identification of an EP1-like glycoprotein suggests a possible cellular signalling mechanism that may function in plant PCD. Finally, the identification of cytosolic aconitase combined with a demonstrated increase in free cellular iron levels relative to cellular protein content suggests an iron regulatory mechanism that may be important in generating oxidative damage during plant PCD.

It is important to bear in mind the changes in total protein content when interpreting protein profiles by 2-D SDS-PAGE, as well as acknowledging the many possible mechanisms that may be responsible for the appearance of a novel spot or one that is increased in intensity. Individual proteins can increase in abundance (relative to total protein) by at least one of three mechanisms: First, *de novo* synthesis of that protein may occur. Second, post-translational modification may alter the position of a protein on a 2-D gel such that it appears as a novel spot in comparison to the control gel. Third, general degradation of the majority of cellular proteins may occur. Given that changes after heat treatment occur after just 10 min, it is unlikely that significant *de novo* protein

synthesis has occurred, and so it is more likely that the changes observed are probably the result of post-translational modification or degradation of other proteins. Widespread protein degradation is known to occur during plant PCD (Beers *et al.*, 2000; Lam and del Pozo, 2000), and there was a significant decrease in the amount of protein in cells undergoing PCD in this study (Figures 6.1A and B). This decreased protein content was associated with an increased proteolytic activity (Figure 6.1C). It is important to appreciate that the 2-D gels contained an equal loading of protein and thus only allowed for the identification of proteins whose abundances changed *relative to the total protein profile*. It would therefore be informative to undertake a proteomic analysis of plant PCD and examine changes in specific proteins on a per cell basis.

CHAPTER SEVEN

7. Establishment of a transient gene expression system in *Arabidopsis* protoplasts

7.1. Introduction

Numerous genes and proteins were identified by transcriptomic and proteomic analyses, respectively, that were hypothetical candidates for involvement in a plant cell death programme. However, while the expression pattern of a transcript or protein may be suggestive of a role for its product in a given process, the assertion that the gene product plays a role requires a demonstration of its functional effect on that process (Krysan *et al.*, 1999). In this respect, a reverse genetics approach in which a gene of interest is either overexpressed, underexpressed, or knocked-out allows for a phenotypic characterisation and the determination of whether such abberant expression does indeed affect the developmental process of interest. Thus, to determine whether any of the candidate plant PCD genes identified in these studies play a functional role in this process, it was desirable to establish a reverse genetics screen for these genes.

Since plant PCD is likely to involve overlapping or redundant signalling and execution pathways, a loss or decrease in function in any one gene may be insufficient to affect the cell death response. In contrast, the overexpression of a positive regulator of plant PCD may result in increased cell death or sensitisation to cell death-inducing stimuli. Conversely, the overexpression of a negative regulator may reduce sensitivity to cell death-inducing stimuli, if not preventing cell death altogether. Therefore, it was decided that overexpression of candidate PCD genes coupled to a phenotypic analysis would be the most useful approach to investigate a functional effect for the gene product in plant PCD. Given the large number of genes of interest, however, it was desirable to

establish a routine 'screen' for functional effects, rather than undertaking laborious and time-consuming whole-plant transformations to generate stable overexpressing lines. In this regard, a transient expression system in plant protoplasts was selected as a potentially useful approach for routinely screening genes of interest before investing further time and effort on a more detailed functional characterisation.

Transient gene expression analyses using PEG-mediated transformation in protoplasts, including those isolated from *Arabidopsis* cell suspension cultures (Axelos *et al.*, 1992), have been described previously and used to monitor the expression of reporter genes within 6 h post-transformation (Abel and Theologis, 1994). Maximum *β-glucuronidase* (*GUS*) reporter gene expression has been demonstrated to occur anywhere from 18 to 60 h post-transformation (Axelos *et al.*, 1992; Abel and Theologis, 1994), but functional assays on genes of interest have been successfully undertaken within 4 h post-transformation in *Arabidopsis* protoplasts (Kovtun *et al.*, 2000). Of relevance to this study, both untransformed protoplast systems and those involving transient gene expression assays have been employed to study stress signalling (*Arabidopsis*) (Kovtun *et al.*, 1998; Kovtun *et al.*, 2000), pathogen-induced cell death and hormone signalling (*Arabidopsis* and tomato) (Wang *et al.*, 1996a; Asai *et al.*, 2000), and physiological and developmental cell death responses (tobacco, *Arabidopsis*, and barley aleurone) (Danon and Gallois, 1998; Fath *et al.*, 1999; Chen *et al.*, 2000). The aim of the work presented in this chapter was to investigate the effect of genes of interest on the timing and extent of plant cell death by transiently overexpressing (or antisensing) them in protoplasts. The cell death response (i.e. % dead cells against time) of the transformed cells was measured and compared to control, untransformed cells in the presence or absence of death-inducing stimuli.

7.2. Results

7.2.1. Monitoring protoplast viability using a fluorimetric assay

The aim of this study was to routinely screen the effect of genes of interest on protoplast cell death in an efficient and reproducible manner. To this end, counting individual protoplasts by microscopy was not a suitable method, since the time required to count several hundred protoplasts per replicate can be considerable with many replicates, and also can be a very subjective method of quantification. Protoplast cell death morphology is not nearly as obvious as that in whole cells; dead protoplasts can range in appearance from shrivelled to normal to lysed. Moreover, following PEG-mediated transformation, individual protoplasts have a tendency to clump together, often making the analysis of individual cell morphologies impossible. Therefore, it was decided that a more objective and high-throughput method of monitoring protoplast viability would be the use of a vital stain in a fluorimetric assay, in which the intensity of fluorescence in a given protoplast culture volume could be measured and compared across other samples in equal volumes.

Fluorescein diacetate (FDA) vital staining has been used successfully to measure the number of living cells in a sample using a fluorimeter (Persidsky and Baillie, 1977). The number of living cells bears a linear relationship to the amount of fluorescein detected and is unaffected by the presence of dead cells in the sample. To determine if the fluorescence intensity of a protoplast sample incubated with FDA bears a similar linear relationship to the number of living cells, varying volumes of protoplast culture (10^7 protoplasts/ml), each therefore containing an increasing number of live protoplasts, were incubated with FDA in a microtiter plate. The fluorescence intensity of the hydrolysed, free fluorescein measured at 515 nm (using a Perkin Elmer Luminescence Spectrometer LS 50B) was found to increase in a linear fashion over time in a manner

proportional to the number of protoplasts present (Figure 7.1). Therefore, provided the sample volume and the incubation times of the sample with FDA are consistent, a measure of the fluorescein intensity is an accurate indicator of the total number of viable cells. To further determine whether the variability in replicate readings is affected by either the volume of the sample used or the incubation time with FDA, two different protoplast sample volumes were incubated for either 10 or 20 min with FDA, and 3 replicate readings of each were taken. As shown in Table 7.1, the average coefficient of variation for each set of 3 replicates was less than 10% in all cases, indicating that this system provides a robust measurement of living cells that is relatively unaffected by the exact sample volume or incubation time.

7.2.2. Measurement of transformation efficiency using *GUS* reporter gene expression

In order to assay the efficiency of protoplast transformation, it was necessary to devise a quantitative reporter system that could be used routinely. The *GUS* gene system has been repeatedly demonstrated to be an excellent method of monitoring the efficiency of protoplast transformation (Topfer *et al.*, 1988; Axelos *et al.*, 1992; Abel and Theologis, 1994) using both X-gluc staining or the much more sensitive and versatile MUG fluorigenic assay (Jefferson, 1987). MUG assays have the added advantage of being amenable to microtiter plate volumes, thus allowing for routine and rapid measurements of GUS activity using a fluorimeter.

To confirm that transformation of the protoplasts was efficient, MUG assays on pRT103 *GUS* (see section 2.14.5 for plasmid details) transformants were undertaken (see section 2.12.4) (Figure 7.2). Consistent values for GUS activity (per total protein) were obtained in all replicate experiments and were found to be in good accordance with

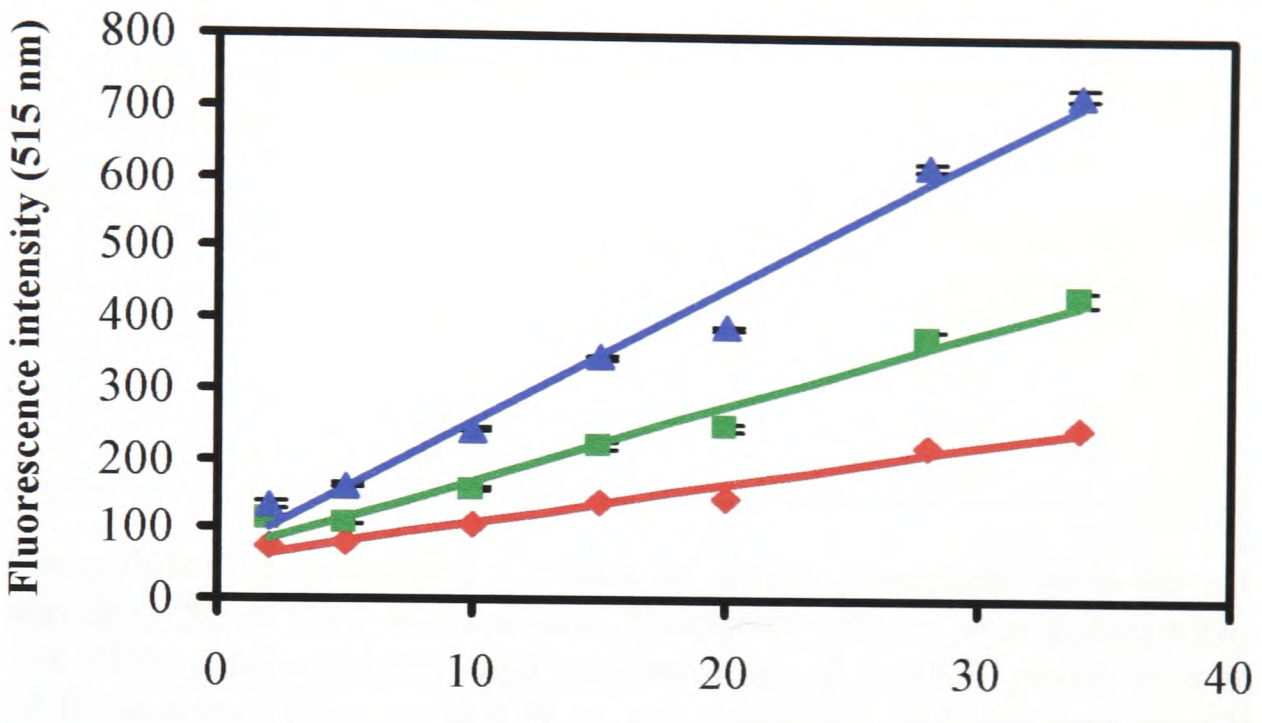


Figure 7.1. Linear relationship between FDA fluorescence and protoplast number. 30 µl (red), 60 µl (green), or 90 µl (blue) of protoplast suspensions (10^7/ml) were incubated with FDA and the fluorescence intensity (515 nm) measured over time. Values presented are the means of 3 replicate samples.

Table 7.1. Variation of fluorescein diacetate fluorescence intensity readings in different protoplast volumes and with different incubation times.

Protoplast volume (µl)	Incubation time (min)	Average coefficient of variation (%)
30	10	9.57
30	20	6.71
50	10	5.62
50	20	3.89

The reproducibility of measuring numbers of viable protoplasts with the vital stain, fluorescein diacetate (FDA), was assessed. Protoplast cultures were mixed with an equal volume of FDA solution (FDA final concentration of 0.002% (*w/v*)) in a microtitre plate, and fluorescence intensity at 515 nm measured using a fluorimeter after 10 and 20 min. Each measurement was repeated in triplicate and the coefficient of variation determined as an indicator of reproducibility.

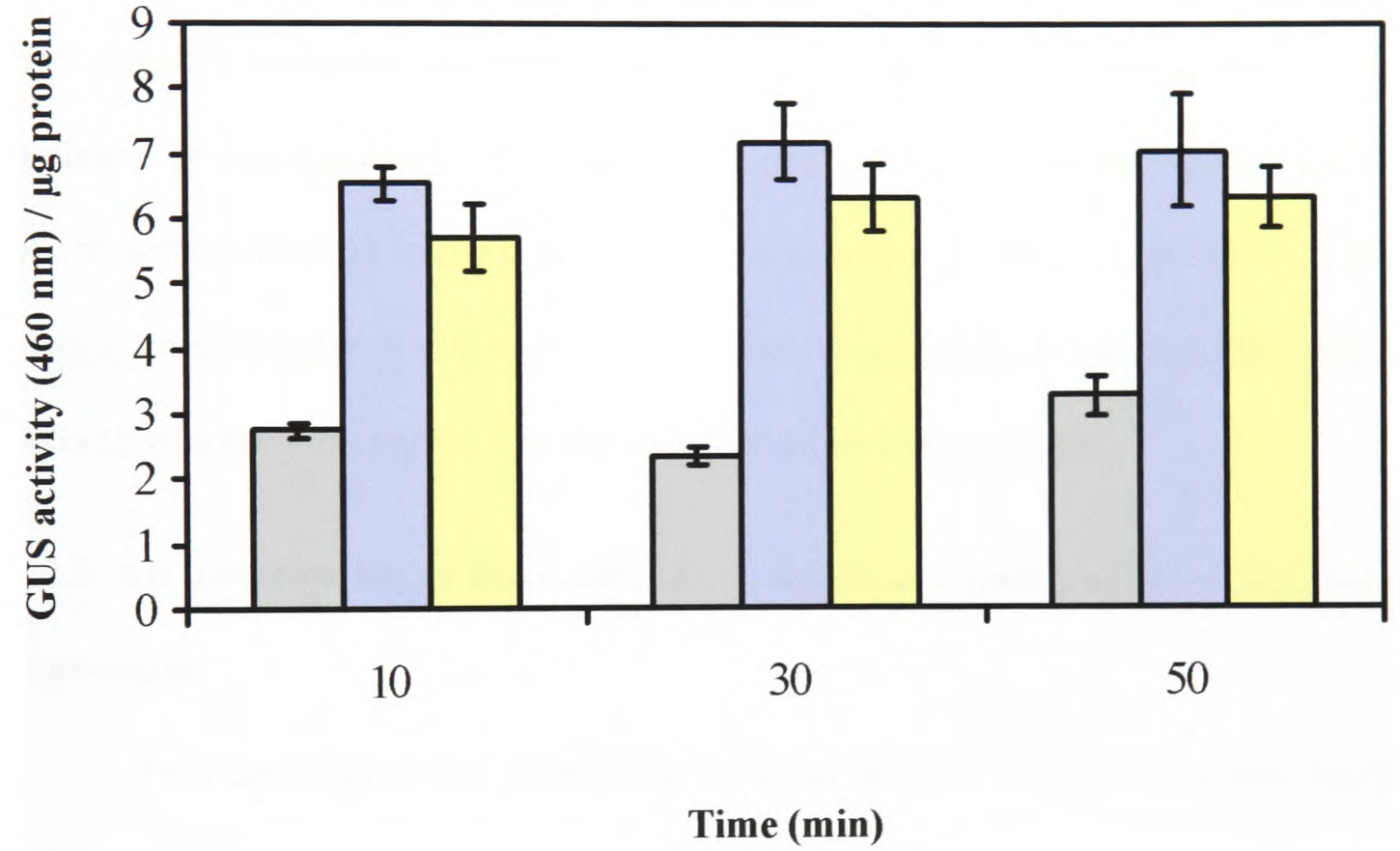


Figure 7.2. GUS activity in transformed protoplasts.
Protoplasts were transformed using procedures described in section 2.12.2. Control protoplasts were transformed with 250 µg salmon sperm DNA (grey). For GUS activity assays, protoplasts were transformed with 250 µg salmon sperm DNA and either 10 µg of plasmid pRT103 containing the *β-glucoronidase* (*GUS*) gene (blue) or 10 µg pRT103 *GUS* in conjunction with 10 µg of plasmid pKI102 (yellow) (see section 2.12.5). The GUS activity (emmission at 460 nm) of each transformed sample was then measured using the fluorigenic MUG assay (see section 2.12.4). Values presented are the means of at least 3 replicate samples.

other systems (Steve Rutherford, personal communication). However, assays on genes of interest would involve co-transformation of both the *GUS* reporter gene plasmid, pRT103, and the plasmid containing the gene of interest. Therefore, it was important to confirm that GUS activity, and therefore transformation of any individual plasmid, was not markedly reduced when co-transformed with an additional plasmid. As seen in Figure 7.2, co-transformation of the pRT103 *GUS* plasmid with a second plasmid, pKI102, did not reduce the measured GUS activity, indicating that co-transformation of two plasmids is feasible and that measured GUS activity in co-transformants is a useful indicator of the efficiency of transformation (for plasmid details, see section 2.12.5). In all protoplast transformations undertaken in subsequent analyses, pRT103 *GUS* was always co-transformed with any plasmids containing genes of interest. Measured GUS activity was used to confirm that the transformation was successful.

7.2.3. An investigation of Bax-induced cell death as a positive control for transient expression

Two candidate genes potentially involved in plant PCD as identified by cDNA microarray analysis (see Chapter 4), *SRG3* and *Athsr5*, did not affect the rate of protoplast cell death within 48 h when either overexpressed or introduced as antisense constructs (see section 2.12.5) compared to the control (Figure 7.3). This experiment was repeated over several weeks with similar results (data not shown). Other selected putative cell death-related genes (e.g. *Hsp70-3*; see Chapter 4) also did not affect the rate of protoplast cell death when expressed transiently in either the sense or antisense orientations in a series of repeated experiments (data not shown). However, the aim of this study was to determine whether overexpression of genes of interest had a detectable effect on the rate of cell death. It cannot be concluded if overexpression of these genes simply did not affect cell death, or if the system itself was ineffective in detecting such

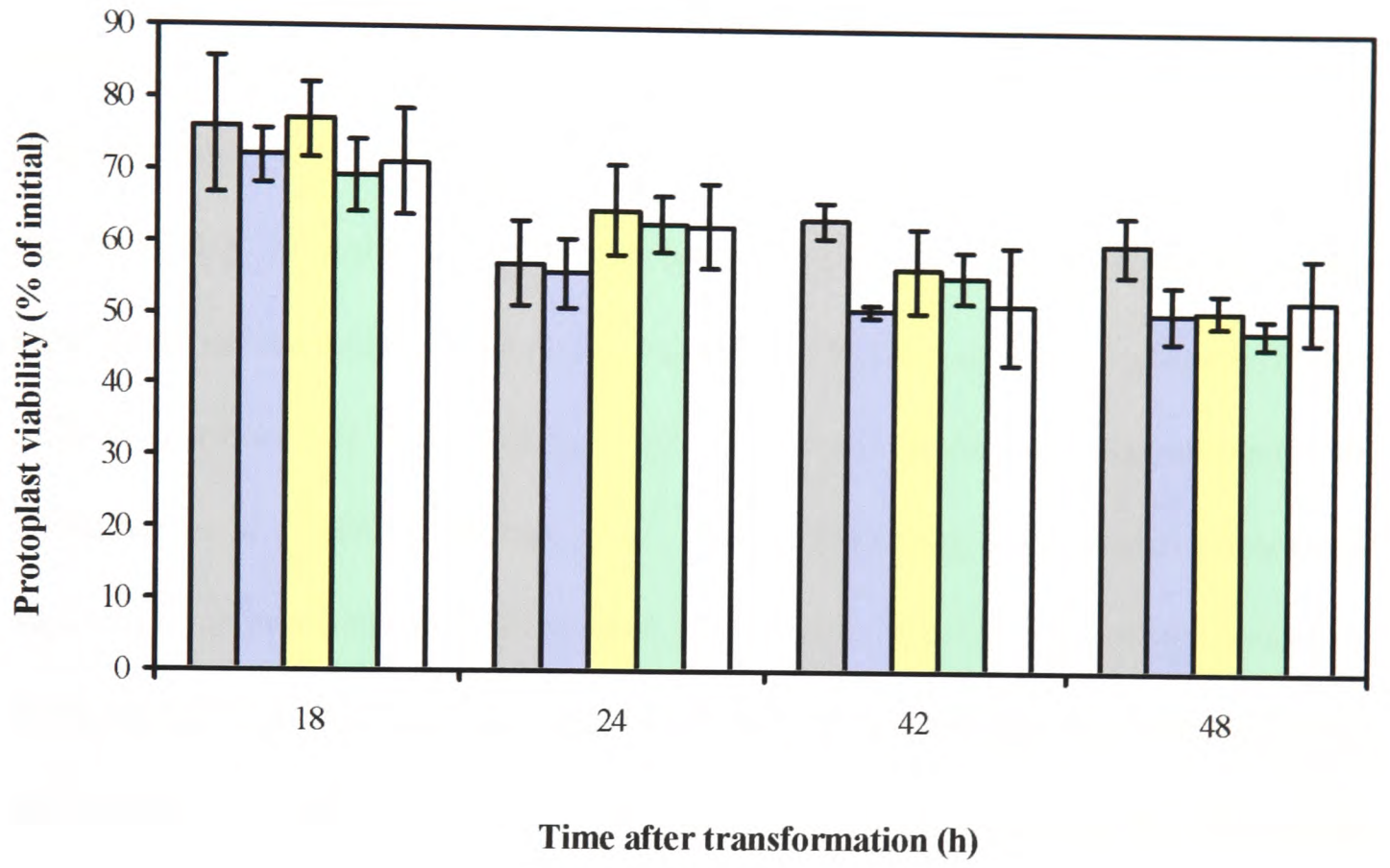


Figure 7.3. Effect of putative cell death-related genes on protoplast viability. Protoplasts were transformed (see section 2.12.2) with 10 µg of each indicated plasmid containing either a putative cell death gene in either the sense or antisense orientation. All transformants involved co-transformation with 10 µg pRT103 *GUS*. Protoplast viability was estimated (FDA fluorescence) over time. Transformants were as follows (see section 2.12.5): pRT103 *GUS* control (grey); *SRG3* sense (pKSRG) (blue); *Athsr5* sense (pKATH) (yellow); *SRG3* antisense (pKSRG-AS) (green); *Athsr5* antisense (pKATH-AS) (white). Values presented are the means of 3 replicate samples.

differences. Thus, a positive control affected in the rate of cell death was required for comparison.

As a positive control, it is ideal to use a gene that, when expressed alone, can consistently affect the timing or extent of cell death in a developmental- and tissue-independent manner. Although such 'master control switches' of plant PCD have not been identified, the pro- and anti-apoptotic members of the Bcl-2 family of proteins in animals are key regulators of animal cell death and their misexpression is sufficient to alter cell death responses (Vander Heiden and Thompson, 1999). While plants do not encode Bcl-2 homologues (The *Arabidopsis* Genome Initiative, 2000), transgenic expression of the animal cell-death suppressor Bcl-X_L or the *C. elegans* homologue CED-9 in tobacco is sufficient to inhibit cell death in response to pathogen infection (Mitsuhar *et al.*, 1999; Dickman *et al.*, 2001), indicating a functional conservation of these proteins in a heterologous system. Conversely, the inducible overexpression of the pro-apoptotic mammalian Bax protein in *Arabidopsis* is sufficient to cause an immediate and marked cell death response at the whole-plant level, indicating that Bax expression alone can trigger cell death in plants (Kawai-Yamada *et al.*, 2001).

The rapid response to Bax transgenic expression in plants and its ability to trigger cell death in a non tissue-specific manner makes this an ideal positive control for a cell death assay. In an effort to establish Bax as a positive regulator of cell death following transient overexpression in protoplasts, the mammalian *Bax* gene was used in a transient expression assay. Transformation with a pBax plasmid (see section 2.12.5) using 10 µg or 50 µg of plasmid DNA had no detectable effect on protoplast viability compared to the control within 42 h post-transformation (Figure 7.4A), despite the fact that these quantities of plasmid DNA have been used with success in other *Arabidopsis* protoplast systems (Abel and Theologis, 1994; Kovtun *et al.*, 1998; Kovtun

et al., 2000; Kawai-Yamada *et al.*, 2001). Moreover, by 42 h post-transformation, the viability of control protoplasts had declined by approximately 50%, rendering any comparisons in the rate of cell death between controls and transformants difficult. An RT-PCR analysis of the Bax and control transformants was undertaken to determine whether the *Bax* gene was being expressed. As a positive control for the RT-PCR analysis, primers designed for the *β-tubulin* gene were used to detect *β-tubulin* expression in all samples (see section 2.12.5). Figure 7.4B clearly shows the expression of the *Bax* gene in cells transformed with pBax. This indicates that, while Bax expression was occurring in the transformants, it was insufficient to cause cell death at a detectable level in the current system.

7.2.4. Bax expression does not sensitise cells to death-inducing stimuli

It is possible that Bax expression in protoplasts is not sufficient to cause cell death alone, but rather sensitises the cells to death-inducing stimuli. If this were the case, then Bax transformants would be expected to undergo cell death at a more rapid rate in response to cell death-inducing stimuli, or would undergo cell death in response to a sub-threshold level of the stimulus that is insufficient to cause cell death in control cells. To establish a cell death-inducing system in protoplast cultures, the effectiveness of both heat treatments and H_2O_2 addition were investigated. While various heat treatments of protoplast cultures were sufficient to induce cell death in the protoplasts when examined visually by microscopy and FDA staining, the fluorescein intensity when measured fluorimetrically was consistently higher in cells following the heat treatment (data not shown). It was eventually determined that this was likely an artefact of the heat treatment, in which heat may cause an increase in plasma membrane permeability. This would lead to the leakage of esterases into the medium where they would be free to cleave the fluorescein diacetate to fluorescein in an uncontrolled manner, resulting in an

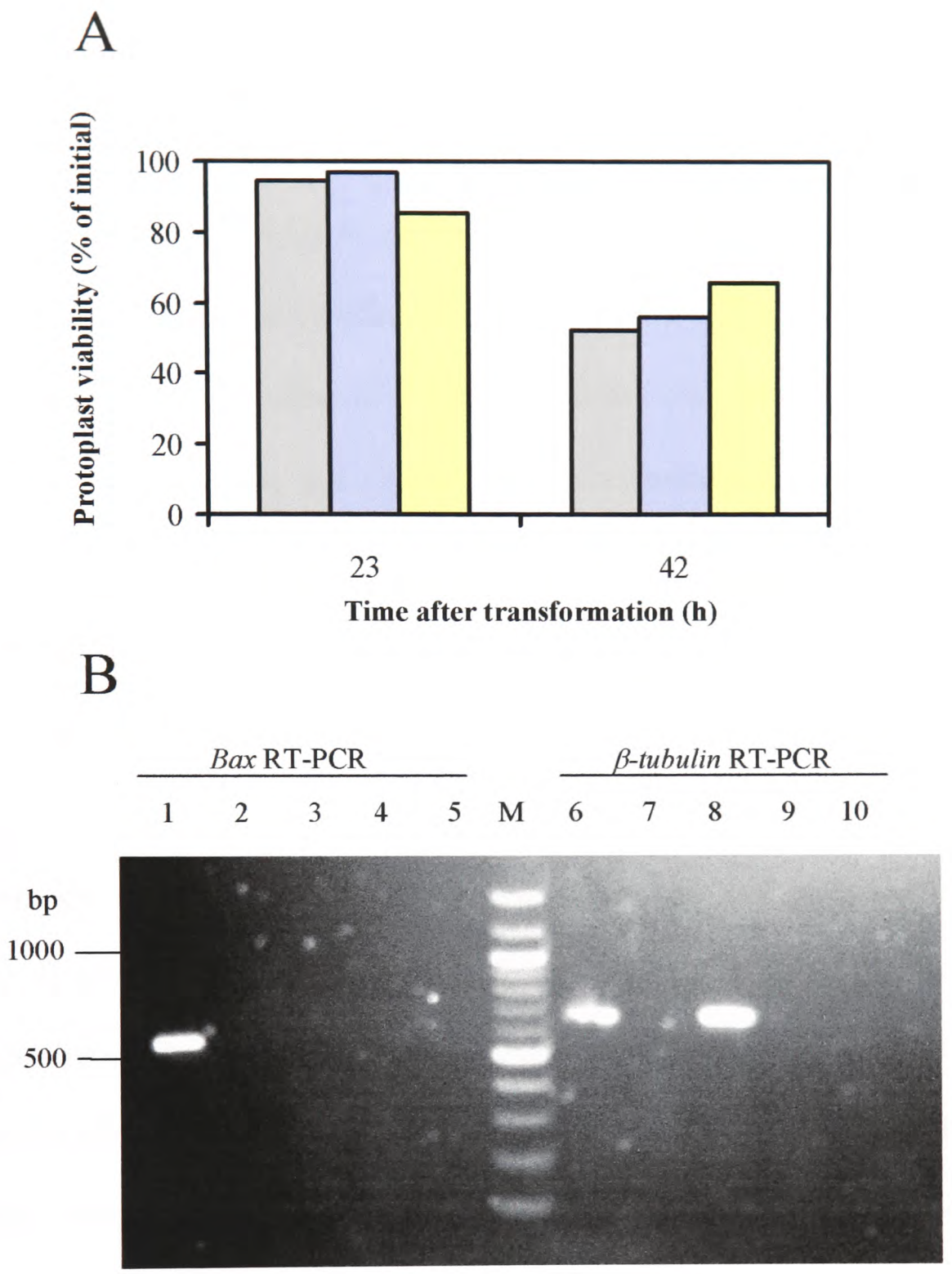


Figure 7.4. Transformation of protoplasts with mammalian *Bax*.
A. Effect of pBax plasmid quantity on protoplast viability. Protoplasts were transformed (see section 2.12.2) with either 10 µg pRT103 *GUS* (grey), 10 µg pBax (blue), or 50 µg pBax (yellow). All transformants involved co-transformation with 10 µg pRT103 *GUS*. Protoplast viability was estimated (FDA fluorescence) over time. Values presented are the average of 2 samples. **B.** RT-PCR to demonstrate expression of transgene in protoplast transformation. RNA was isolated from protoplasts 42 h after transformation with 10 µg pBax plasmid (lanes 1 and 6) or pRT103 *GUS* controls (lanes 3 and 8). cDNA synthesised from this RNA was used as a template for RT-PCR. Primers were used for either the *Bax* gene (lanes 1-5) or a *β-tubulin* positive control (lanes 6-10) (see section 2.12.5). Only the protoplasts transformed with pBax show expression of the *Bax* mRNA. Negative controls included the reverse transcription reaction with no reverse transcriptase added to confirm the absence of DNA contamination (pBax transformant negative controls, lanes 2 and 7; pRT103 transformant negative controls, lanes 4 and 9). PCR negative controls were performed without the addition of a template (lanes 5 and 10). M, marker.

increase in total fluorescein produced. This situation contrasts with the normal detection method of FDA, in which the detected fluorescein is that which has been hydrolysed inside living cells (Persidsky and Baillie, 1977).

The addition of H_2O_2 to protoplast cultures at 18 h post-transformation was investigated as a method of inducing protoplast cell death. The addition of ≥1 mM H_2O_2 to protoplast cultures resulted in a marked (~50%) decrease in viability within 7 h (Figure 7.5A), making the use of any H_2O_2 concentration ≥1 mM unsuitable for an investigation of Bax-induced cell death sensitisation. Conversely, H_2O_2 between 100 and 800 μM did not affect the viability of control protoplasts, similar to Bax expression alone (10 μg pBax plasmid). If Bax expression sensitises cells to death-inducing stimuli, these sub-threshold concentrations of H_2O_2 that do not affect viability under normal conditions should result in decreased viability in the *Bax* transformants. However, the addition of sub-threshold and minimally invasive 100 μM H_2O_2 to cells transformed with either 10 or 50 μg of Bax plasmid had no effect on protoplast viability compared to the controls within 19 h post-H_2O_2 treatment (42 h post-transformation) (Figure 7.5B). Therefore, either Bax expression did not sensitise cells to death-inducing stimuli or the resultant increase in cell death was insufficient to be detected in the current system.

7.2.5. The effect of light on protoplast viability following Bax transformation

The inability of Bax expression to effect a detectable change in the rate of protoplast cell death prompted an investigation of whether light has an influence on Bax-induced cell death in plants. Bax expression has been studied in whole *Arabidopsis* plants maintained under normal light regimes (Kawai-Yamada *et al.*, 2001), and therefore light could be a necessary but unrecognised requirement for this cell death. Fungal toxin-induced cell death in *Arabidopsis* protoplasts is light-dependent, and is

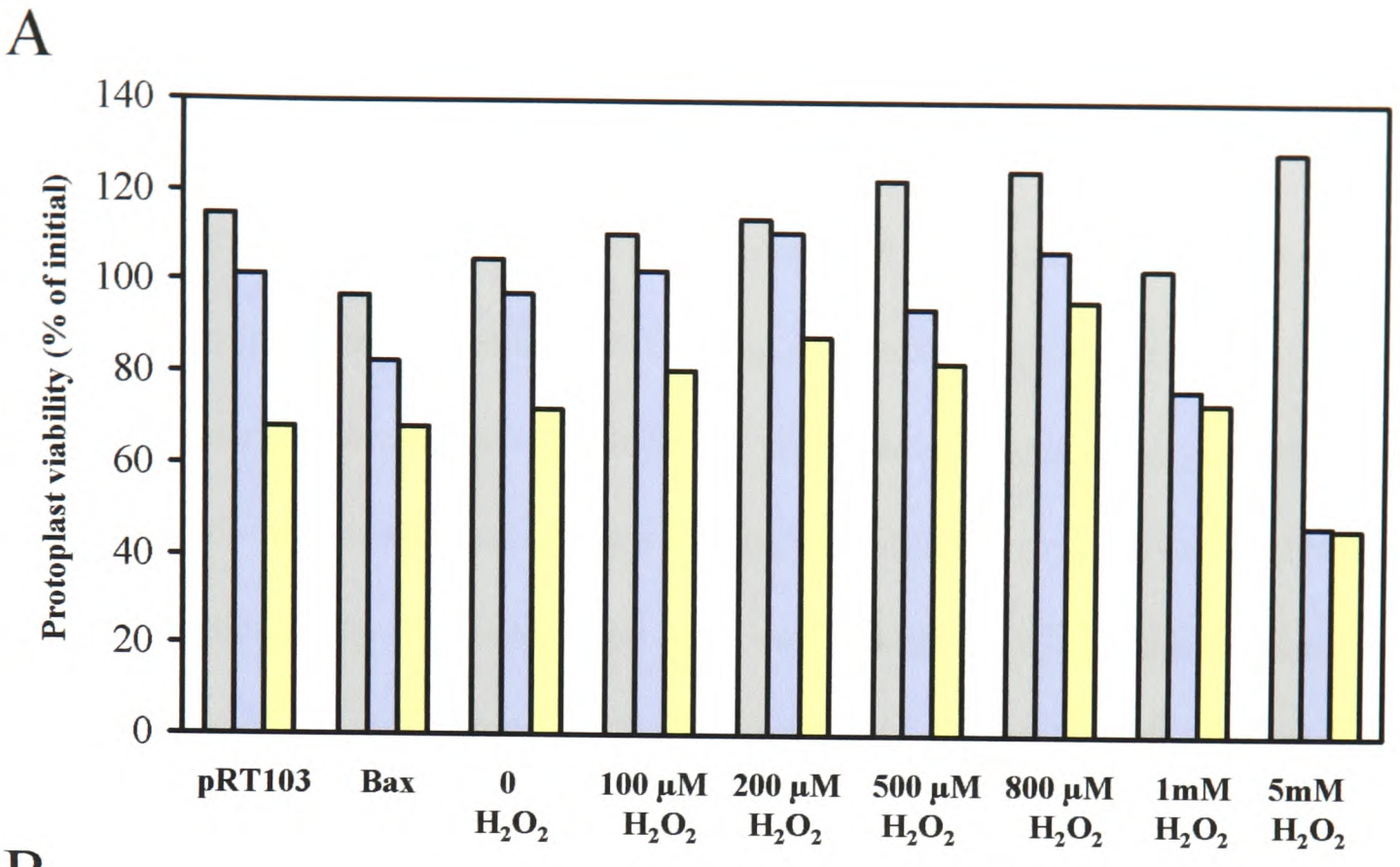


Figure 7.5. The effect of H_2O_2 on protoplast viability.
A. Protoplasts were transformed (see section 2.12.2) with either 10 µg of pRT103 *GUS* or pBax, as indicated, or were transformed with 250 µg salmon sperm DNA alone and H_2O_2 was added at the concentrations indicated. All transformants involved co-transformation with 10 µg pRT103 *GUS*. Protoplast viability was estimated (FDA fluorescence) at 18 (grey), 25 (blue), or 42 (yellow) h post-transformation. H_2O_2 was added following the 18 h viability measurement. Values presented are the average of 2 replicate samples. **B.** Protoplasts were transformed with 10 µg pRT103 *GUS* (grey and blue), 10 µg pBax (yellow and green), or 50 µg pBax (white and orange). All transformants involved co-transformation with 10 µg pRT103 *GUS*. Protoplast viability was estimated (FDA fluorescence) at the indicated time points. One of each type of transformant was treated with 100 µM H_2O_2 following the 23 h viability measurement (blue, green, and orange) to determine the effect of Bax expression on sensitivity to H_2O_2. Values presented are the average of 2 replicate samples.

suggested to be due, directly or indirectly, to a requirement for ROS generated during photosynthesis or to the induction of light-dependent gene activity (Asai *et al.*, 2000).

As described in section 2.12.1, protoplasts used in this study were typically derived from dark-grown cell suspension cultures and were maintained in continuous darkness following transformation. To investigate whether light is required for Bax-induced cell death in *Arabidopsis* protoplasts, protoplast cultures were generated from either 6 day-old, dark-grown cell cultures lacking chloroplasts, or 6 day-old cell cultures maintained under a 16 h photoperiod and containing abundant chloroplasts. Bax transformation was undertaken in both protoplast cultures and, subsequently, transformed protoplast cultures were maintained in either continuous darkness or continuous light conditions. Therefore, this experiment aimed to determine whether the presence of chloroplasts (and other cellular physiologies associated with maintenance in light conditions) and/or the presence of light, post-transformation, affected the rate of Bax-induced cell death. As seen in Figures 7.6A and B, there was no difference in the rate of cell death between Bax-transformed and control protoplasts over 48 h for those cultures derived from dark grown cell cultures, whether they were maintained in the light or dark, post-transformation. In comparing protoplasts derived from light-grown cell cultures (Figures 7.6 C and D), there appeared to be a 15 to 20% increase in cell death over 48 h post-transformation in Bax-transformed protoplasts that were then maintained in the light as compared to the dark (Figure 7.6C).

Several weeks later, replication of the result obtained in Figure 7.6C was attempted, again using protoplasts from light-grown cultures and maintaining the protoplasts in the light following transformation. The results of the repeated experiment are shown in Figure 7.6E. Evidently, while the rate of Bax-induced cell death may be increased compared to the controls at 42 h post-transformation, the results do not clearly

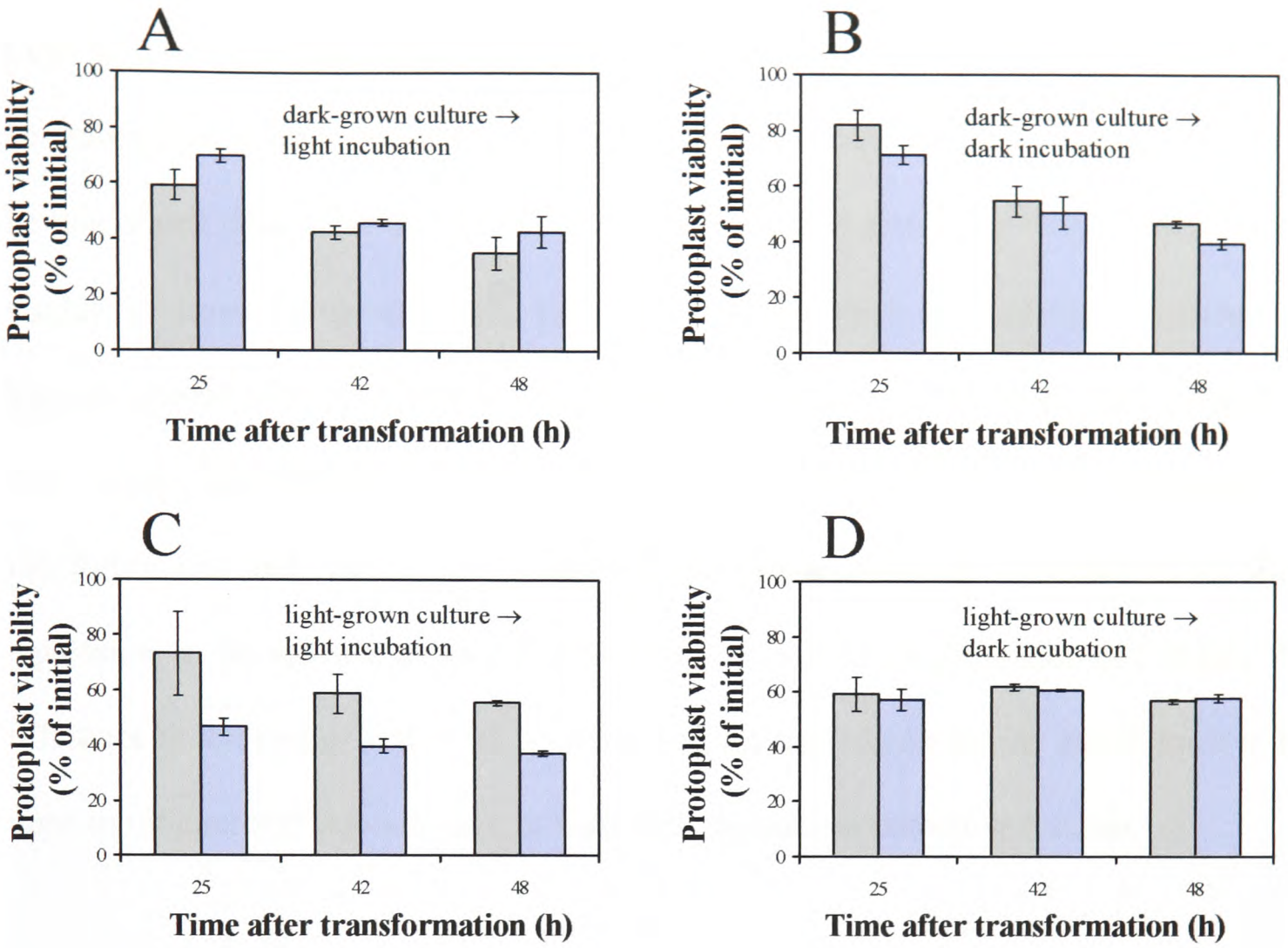


Figure 7.6. The effect of light on protoplast viability.
Protoplasts were transformed (see section 2.12.2) with either 10 µg pRT103 *GUS* (grey) or 10 µg pBax (blue). All transformants involved co-transformation with 10 µg pRT103 *GUS*. Protoplast viability was estimated (FDA fluorescence) over time. Protoplast cultures were derived either from dark-grown cell cultures and maintained (post-transformation) in the light (**A**) or the dark (**B**), or were derived from light-grown cell cultures and maintained (post-transformation) in the light (**C**) or the dark (**D**). The experiment illustrated in **E** also used protoplasts derived from light-grown cell cultures that were then maintained (post-transformation) in the light, but was independently performed several weeks after that illustrated in **C**. Values presented are the average of 3 replicate samples.

support those shown in Figure 7.6C and this experiment was therefore not reproducible. It can be concluded that, while Bax expression in chloroplast-containing protoplasts that are maintained in the light (post-transformation) may serve to effect a small increase in the rate of cell death, this increase may be limited to, and detectable within, only a small window of time. Furthermore, the period of time at which this increase is detectable does not appear to be reproducible. Differences in the rate of cell death may depend on even small perturbations in the initial state of the cultures, the efficiency of transformation, and the culture conditions post-transformation. Ultimately, for Bax expression to be utilised as an effective positive control in protoplast cell death, the difference in the rate of cell death between transformants and controls would have to be more significant and reproducible, at least with the current detection systems.

7.3. Discussion

The purpose of this study was to establish a system in which genes of interest could be routinely screened for functional effects on plant cell death during transient gene expression in *Arabidopsis* protoplasts. Protoplast transformation was effectively carried out (Figure 7.2) and the fluorimetric assays using a fluorimeter were capable of measuring differences in the number of viable protoplasts (Figure 7.1). However, the expression of a known inducer of plant cell death, the mammalian *Bax* gene, failed to either induce a consistent and significant increase in protoplast cell death (Figure 7.4) or to sensitise protoplasts to oxidative stress-induced cell death (Figure 7.5). It was shown that, while light-grown cells maintained under light conditions may be more susceptible to Bax-induced cell death, perhaps owing to increased ROS production generated during photosynthesis, this effect was not easily detected or reproducible (Figure 7.6).

The failure of Bax expression to induce detectable protoplast cell death may be explained by expected transformation efficiencies and the limitations of the fluorimetric assays used. Transient expression in *Arabidopsis* protoplasts has been shown to be an effective system for measuring changes in reporter gene activity, such as GUS or luciferase, when fused to a promoter sequence of interest (Kovtun *et al.*, 1998; Kovtun *et al.*, 2000). Similarly, it was shown in the present study that GUS activity following transformation with a *GUS*-containing plasmid was easily detectable with the sensitive fluorimetric MUG assay (Figure 7.2). However, reported transformation efficiencies of *Arabidopsis* protoplasts, using methods of transformation identical to the current study, range from 40 to 60% as detected by GUS staining (Axelos *et al.*, 1992; Abel and Theologis, 1994). Thus, it is reasonable to assume that, in any given transformation, approximately 50% of the protoplasts will have been successfully transformed. Of the transformed protoplasts, a proportion may be expected to be susceptible to Bax-induced

cell death within the time period studied, owing to differences in initial physiological state, age of the cell, etc. It could be conservatively estimated that half of the transformed protoplasts might be expected to undergo cell death in response to Bax expression, or 25% of the total number of protoplasts.

The detection system utilised may be incapable of fully measuring the presence of 25% dead cells, perhaps owing to weak FDA fluorescence, autofluorescence in the dead and dying cells, or compromised protoplast membranes that allow esterases to leak into the culture medium and hydrolyse FDA in an uncontrolled manner. (For example, an examination of Figure 7.5A shows that FDA fluorescence detected by the fluorimeter method at 18 and 23 h can sometimes increase relative to the 0 h time point in the same sample). It is therefore reasonable to estimate that an increase in protoplast cell death of approximately 15% may detectable as a decrease in FDA fluorescence. These values would be in accordance with some of the differences seen in this study (Figure 7.7C). However, the variable nature of the system and its poor efficiency in quantifying protoplast cell death, especially at low levels, will undoubtedly have contributed to lack of consistent results. Therefore, in its current form, this appears to be an unsuitable system for the routine screen of functional effects of cell death-related genes, and other approaches, such as an analysis of stable transformants, should be considered.

CHAPTER EIGHT

8. Analysis of *Arabidopsis* T-DNA insertional mutants in putative cell death-related genes

8.1. Introduction

The difficulties associated with routinely screening the effects of overexpression or antisense of putative cell death-related genes using a transient expression protoplast system (Chapter 7) necessitated the use of a whole-plant transgenic system to investigate the role of these genes in plant development. Several approaches were considered in this regard. Overexpression of genes of interest can be achieved constitutively, for example by transforming plants with the gene under the control of a 35S promoter, or inducibly, using transformation with the gene under the control of a dexamethansone-inducible promoter (Aoyama and Chau, 1997). Owing to the possibility that constitutive expression of a positive regulator of cell death may result in lethality, the latter approach of inducible overexpression was considered. The widely used and currently available glucocorticoid-inducible vector for plant gene expression mentioned above has, however, recently been demonstrated to cause severe growth defects and the induction of defense-related genes in *Arabidopsis* (Kang *et al.*, 1999). Because growth defects may be linked to cell death, and the induction of defense-related genes could be indicative of the occurrence of an HR-like response, the use of this system for assaying possible cell death-related effects of genes of interest could be problematic.

The inducible promoter system described above has been used to show that expression of mammalian Bax in *Arabidopsis* causes widespread chlorosis and severe etiolation within 3 days (Kawai-Yamada *et al.*, 2001). However, as also seen in tobacco (Lacomme and Santa Cruz, 1999), the phenotype caused by Bax expression in plants is

particularly severe and causes widespread cell death. It is possible, therefore, that any minor growth defects caused by the dexamethasone-inducible system were masked in the presence of Bax effects. The genes identified in the transcriptomic and proteomic analyses in the current work may only cause subtle cell death phenotypes upon overexpression. Thus, any growth defects or HR-like responses caused by glucocorticoid induction would be likely to confuse the analysis of transgenic plants and the effects of genes of interest on cell death-related phenotypes. Therefore, after careful consideration and consultation (Ian Moore, personal communication), the widely available dexamethasone-inducible overexpression vector system was rejected as an experimental approach.

In contrast to overexpression, insertional mutagenesis of genes of interest using T-DNA transposons has become a routine and popular method to obtain gene knockouts (Parinov and Sundaresan, 2000). T-DNA sequences insert into the plant genome in a random fashion and in low-copy number (estimated to be 1.5 per diploid genome) (Azpiroz-Leehan and Feldmann, 1997) and are inherited as a single Mendelian trait (Walden, 2002). These features have made T-DNA insertional mutagenesis a commonly employed tool to study the effect of complete inactivation of gene activity — which is, in the eyes of some investigators, the most direct way to understand the function of the gene product (Parinov and Sundaresan, 2000). T-DNA insertion can result in modification of the target plant DNA, including small-scale sequence changes or entire chromosomal rearrangements that can complicate further analysis and introduce unlinked mutations in the host genome (Walden, 2002). Despite this, recent advances in genome sequencing and high throughput transformations have made T-DNA mutagenesis a popular method to study the effects of knockouts in genes of interest (Parinov and Sundaresan, 2000).

Several laboratories have generated insertion lines on a large scale, resulting in available knockout lines for at least half of the genes in the *Arabidopsis* genome (Parinov and Sundaresan, 2000). Many of these lines, including those available from the *Arabidopsis* knockout facility at the University of Wisconsin (Sussman *et al.*, 2000) and the SINS (Sequenced Insertion Sites) collection (Tissier *et al.*, 1999), are available to public users as pools of thousands of DNA samples from transposon lines. These DNA pools can then be screened, using a PCR-based strategy, to identify those containing potential knockouts in genes of interest. The insertional mutant line of interest can subsequently be isolated from these pools. However, the rapid identification of knockouts in genes of interest has been made possible by systematic sequencing of genomic DNA flanking T-DNA inserts, such that a database of sequenced DNA can simply be screened by BLAST algorithm (Altschul *et al.,* 1997) for matches to a gene of interest (Parinov and Sundaresan, 2000). While this approach is gradually being adopted for all insertion lines currently available, including the SIGnAL lines available at the Salk Institute (http://signal.salk.edu/tabout.html), the latter half of 2001 saw the release of a completely sequenced collection of T-DNA insertion lines from the Syngenta *Arabidopsis* Insertion Library (SAIL) at the Torrey Mesa Research Institute (La Jolla, USA; http://www.tmri.org). An insertion collection consisting of 100,000 T-DNA mutagenised *Arabidopsis* var Columbia lines have been sequenced at genomic DNA flanking each T-DNA insertion. This has resulted in the creation of a database of insertional mutant DNA sequences that can be screened within seconds to identify single lines carrying T-DNA insertions in genes of interest. Owing to the rapidity and ease of utilising this resource and the ability to obtain M2 seed samples that should contain hemizygous and homozygous mutant individuals for genes of interest, this system was

selected as the most efficient way to screen for the effects of knockouts in putative cell death-related genes.

The experimental approach designed for this analysis involved screening the SAIL database by BLAST algorithm (Altschul *et al.*, 1997) for knockouts in several genes of interest. The genes screened were selected as those whose transcripts or proteins were signficantly modulated in both heat- and senescence-induced PCD, or for which roles in PCD could be hypothesised based on previous studies. Unfortunately, since it was only possible to perform the Affymetrix GeneChip analysis (Chapter 5) after the functional analysis presented in this chapter, putative PCD-related genes identified in these microarray experiments were not known at the time of selecting the T-DNA insertional mutant lines. The selected genes were thus based upon the results of the custom microarray (Chapter 4) and the proteomics study (Chapter 6). The genes screened for T-DNA insertional mutants included: *Voltage-Dependent Anion Channel /Athsr 2* (*VDAC/Athsr2)* (At5g15090), increased in relative protein abundance during heat- and senescence-induced PCD but unchanged steady-state mRNA levels; cytosolic *Heat shock protein 70-3* (*Hsp70-3*) (At3g09440), decreased steady-state mRNA levels during heat-induced PCD and homologous to a negative regulator of mammalian PCD; *Senescence-Related Gene 3* (*SRG3*) (At3g02040), increased steady-state mRNA levels during heat-induced PCD and upregulated transcript levels during early senescence (Callard *et al.*, 1996); *Senescence-Activated Gene* ring-H2 finger protein (*SAG*) (At1g15100), increased steady-state mRNA levels during both heat- and senescence-induced PCD; an *Unknown Protein* (*UNK*) (At5g48480), increased in relative protein abundance during heat- and senescence-induced PCD; *Adenine Nucelotide Translocase 1 (ANT1*) (At3g08580) and *ANT2* (At5g13490), decreased in steady-state mRNA levels during heat- and senescence-induced PCD; an *EP1-like glycoprotein* (At1g78850),

increased in relative protein abundance during heat- and senescence-induced PCD; and *Athsr5* (At5g63790), increased steady-state mRNA levels during heat- and senescence-induced PCD.

M2 seed of the putative insertional mutants in genes of interest were obtained from the SAIL collection as 'GARLIC' seed lines (Table 8.1). T-DNA homozygous mutant individuals were identified on the basis of inheritance of resistance to Basta (the selectable marker included in the T-DNA sequence) and a lack of detectable expression of the gene into which the T-DNA had inserted (see section 2.13). Homozygous mutants were then screened for cell death-related phenotypes, including the timing of senescence, the development of xylem vasculature, and the response to abiotic and biotic stresses. This reverse-genetics strategy was used to determine if knockouts in genes which exhibited altered expression patterns during PCD resulted in an altered cell death phenotype, thereby linking gene expression patterns to a functional effect.

Table 8.1. SAIL T-DNA insertion lines identified in putative cell death-related genes of interest.

AGI number	GenBank Accession	Annotation	Transcript expression during PCD (Chapter 4)	Protein expression during PCD (Chapter 6)	SAIL Seed line #	E value[a]
At5g15090	15237134	VDAC/Athsr2 – anion-selective channel protein 2	Unchanged in heat and senescence PCD	Increased in heat and senescence PCD	Garlic_238_A01	E^{-87}
At3g09440	Y17053	Hsp70-3 – heat-shock protein 70-3	Decreased in heat PCD	Not detected	Garlic_301_G03	0
At3g02040	AC011664	SRG3 – putative glycerophosphoryl diester phosphodiesterase	Increased in heat PCD and early senescence (Callard *et al.*, 1996)	Not detected	Garlic_1166_C02	E^{-148}
At1g15100	AC007591	SAG – putative ring-H2 zinc finger protein	Increased in heat and senescence PCD	Not detected	Garlic_879_E04	E^{-101}
At5g48480	3985934	np_199569 – unknown protein	Not detected	Increased in heat and senescence PCD	Garlic_1257_D07	E^{-136}
At3g08580	AC012562	ANT1 – adenine nucleotide translocase 1	Decreased in heat and senescence PCD	Not detected	Garlic_247_G08	0
At5g13490	9955539	ANT2 – adenine nucleotide translocase 2	Decreased in heat and senescence PCD	Not detected	Garlic_415_G09	0
At1g78850	AY054501	EP1-like glycoprotein	Not detected	Increased in heat and senescence PCD	Not available	
At5g63790	AY045843	Athsr5 (unknown protein)	Increased in heat and senescence PCD	Not detected	Not available	

[a] Acceptable Expect value cut-offs are based on the alignment of the gene sequence with the SAIL database sequences by BLAST algorithm (Altschul *et al.*, 1997) . $1E^{-04}$ or less is a good starting point to begin analysing for T-DNA insertions (see http://www.tmri.org/).

Genes selected for study as T-DNA insertional mutants were chosen on the basis of their significant transcript or protein modulation during heat- and senescence-induced PCD (Chapters 4 and 6), or for which roles in PCD could be hypothesised. Each gene sequence and several hundred basepairs 5' and 3' of the coding sequence was screened for the availability of putative insertional mutants in the SAIL T-DNA insertional mutant lines.

8.2. Results

8.2.1. Identification of T-DNA insertional mutant lines in putative cell death-related genes

Genomic DNA sequences spanning genes of interest and including approximately 300 bp upstream (5') and downstream (3') to the coding sequences were screened by BLAST algorithm (Altschul *et al.*, 1997) against the SAIL collection database to identify T-DNA insertions in these genes. The genes shown in Table 8.1 were available as T-DNA insertional mutant GARLIC seed lines, and only insertional mutants for the *EP1-like glycoprotein* and *Athsr5* were not available (Table 8.1). The location of the T-DNA insert was then determined using the sequencing data retrieved from the SAIL database, and the results of this analysis are summarised in Figure 8.1.

8.2.2. Identification of homozygous mutant individuals in M2 seed

Approximately 100 M2 seeds for each T-DNA insertional mutant line were obtained from the SAIL collection. To initially identify hemizygous and homozygous mutant individuals, approximately 15 seeds from each batch were grown for 3 to 4 weeks. A PCR-based strategy using T-DNA specific primers and gene-specific primers was initally attempted to identify wildtype (PCR products only with gene-specific primers), hemizygous (PCR products with both T-DNA/gene-specific and gene-specific primers), and homozygous mutant (PCR products only with T-DNA/gene-specific primers) individuals using crude leaf genomic DNA preparations as a template (see section 2.4.2). While this approach was successful in yielding the expected sizes of PCR products and therefore confirming the predicted insertion site for each T-DNA in the plant genomic DNA, the combination of PCR bands for each individual often gave differing results upon repetition. This could be due to cross-contamination of small

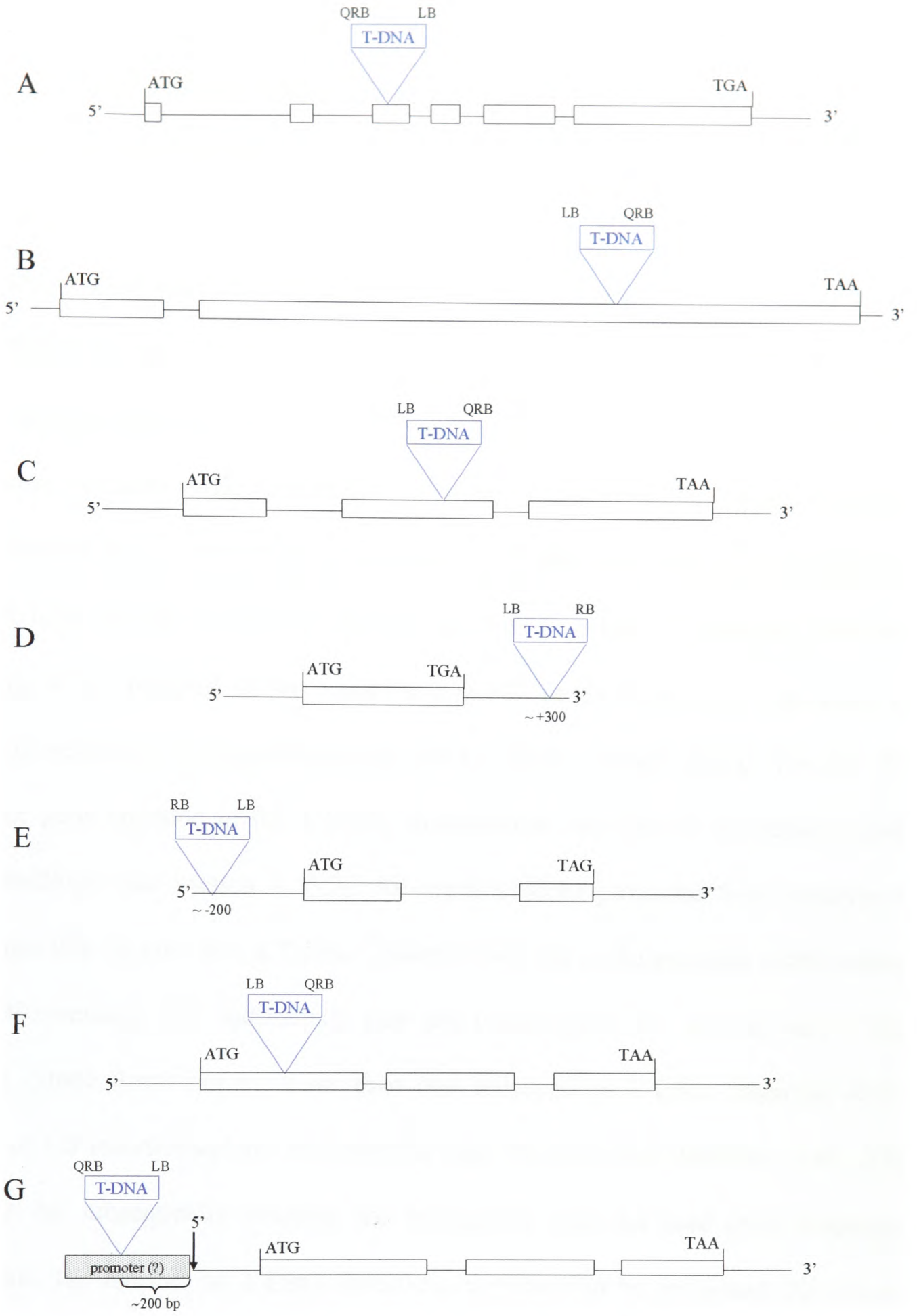


Figure 8.1. Gene models of *Arabidopsis* T-DNA insertional mutants.
The proposed structure of each gene for which putative T-DNA insertional mutants were obtained is shown. Open boxes represent exons and lines represent introns and 5' and 3' untranslated regions. Start translation codons are indicated by ATG and the stop translation codon is shown for each gene. For each gene, the predicted site of the T-DNA insertion, based on sequencing data obtained from the SAIL database, is indicated. The gene names and AGI accession numbers represented are: **A**, *VDAC/Athsr2* (At5g15090); **B**, *Hsp70-3* (At3g09440); **C**, *SRG3* (At3g02040); **D**, *SAG* (At1g15100); **E**, *UNK* (At5g48480); **F**, *ANT1* (At3g08580); **G**, *ANT2* (At5g13490). The models are drawn to relative scale for intron and exon sizes. QRB, Q T-DNA right border; RB, T-DNA right border; LB, T-DNA left border.

amounts of DNA between individuals when handling up to 15 samples at a time or, more likely, due to the fact that the PCR itself was problematic. For example, poor primer combinations between T-DNA-specific primers and gene-specific primers may have lead to a PCR reaction that was not consistently effective.

While the latter problem could potentially have been overcome by redesigning primers and testing each combination for optimal performance each time, a simpler co-segregation analysis was undertaken to identify homozygous mutant individuals (Budziszewski *et al.*, 2001). For this analysis, seed collected from 6 to 10 plants (M2) that had been grown to maturity during the prior 8 weeks of attempted PCR-based screening were collected. Approximately 100 M3 seeds from each individual were plated on selective D-phosphinothricin (PPT) media, which selects for the Basta resistance gene encoded in the T-DNA, to determine the ratio of resistant:susceptible (R:S) seedlings (see section 2.13.2). All seeds (100%) produced from wildtype M2 individuals that do not carry a T-DNA insertion will die in the presence of the selective media. Conversely, M2 individuals that are homozygous for at least one T-DNA insertion (since there can be more than one independent T-DNA insertion with an average of 1.5 insertions/plant) will produce only resistant seed (McElver *et al.*, 2001). However, by subsequently studying the segregation ratio for seed from hemizygous individuals, the number of T-DNA insertions per line can be estimated. As noted by Budziszewski *et al.* (2001), when approximately 75 seeds are analysed in this manner, R:S ratios <6.0 are indicative of a line with a single insertion locus.

It is important to note that, if mutant phenotypes were later found to be associated with a particular T-DNA insertional line, Southern blot analysis using a T-DNA probe to definitively determine the number of T-DNA inserts would be required (Zhu *et al.*, 2001). If more than one insertion was identified, backcrossing to wild-type

would be undertaken until a single T-DNA insertion in the gene of interest was obtained, thus ensuring that the mutant phenotype was only associated with the insert in the gene of interest. For the present analysis, however, the following strategy was adopted: Homozygous mutant individuals were first identified by segregation ratios on selective media, and the number of T-DNA inserts per line estimated by segregation ratios of the M3 seeds from the hemizygotes. T-DNA homozygotes were then analysed using RT-PCR to verify that the gene of interest was not being expressed. If, after subsequent phenotypic analysis, aberrant phenotypes were uncovered, a more detailed genetic analysis of the T-DNA insertion line could be undertaken.

Plating M3 seedlings on selective PPT media was successful in identifying putative homozygote T-DNA mutants in *VDAC/Athsr2* (individuals 6 and 13), *Hsp70-3* (individuals 2 and 3), *SRG3* (individual 6), *SAG* (individuals 5, 6 and 9), and *UNK* (individuals 2 and 7) (Table 8.2). In the M2 generation of a line containing a single T-DNA insertion, 25% of individuals will be homozygous mutant (or 1.5 to 2.5 plants out of 6 to 10 M2 plants). Therefore, the number of putative homozygotes obtained in each line was in agreement with the expected values. Moreover, segregation ratios of the remaining hemizygous individuals revealed that, in all cases, R:S was <6.0 and, in most cases, closer to 3.0 (chi-squared test). Therefore, it was likely that all lines analysed contained only a single T-DNA insertion.

Homozygous mutant individuals were not identified for either *ANT1* or *ANT2*. This could be explained by the fact that, during the growth of 10 M2 seedlings in each of these lines, seedlings exhibited massive growth defects and 3 to 5 individuals of each line were dead by 3 to 4 weeks. Some of these compromised individuals were likely homozygous mutants but, as they did not reach maturity, this could not be confirmed. The progeny of remaining hemizygotes seemed to give R:S ratios close to 3.0, and

Table 8.2. Genotypes of individuals from *Arabidopsis* T-DNA insertional mutant lines (M2) as determined by D-phosphinothricin (PPT) segregation ratio or PCR analysis.

Gene	#	R[a]	S[b]	R:S ratio	Chi-test for 3:1 R:S ratio	Genotype
VDAC/ Athsr2	1	77	29	2.7:1	0.57	hemizygous
	2	0	>75	0		wildtype
	3	83	26	3.2:1	0.78	hemizygous
	6	75	0			homozygous mutant
	9	0	>75	0		wildtype
	10	117	32	3.7:1	0.32	hemizygous
	11	0	>75	0		wildtype
	13	78	0			homozygous mutant
Hsp70-3	1	0	>75	0		wildtype
	2	>75	0			homozygous mutant
	3	>75	0			homozygous mutant
	4	90	20	4.5:1	0.10	hemizygous
	6	0	>75	0		wildtype
	9	82	15	5.5:1	0.03	hemizygous
	10	89	25	3.6:1	0.45	hemizygous
	11	0	>75	0		wildtype
	12	92	25	3.7:1	0.36	hemizygous
	13	0	>75	0		wildtype
SRG3	1	0	>75	0		wildtype
	2	0	>75	0		wildtype
	3	55	22	2.5:1	0.47	hemizygous
	4	0	>75	0		wildtype
	5	111	22	5.1:1	0.02	hemizygous
	6	86	0			homozygous mutant
SAG	1	62	27	2.3:1	0.24	hemizygous
	2	52	21	2.5:1	0.46	hemizygous
	3	62	17	3.6:1	0.47	hemizygous
	4	64	22	2.9:1	0.90	hemizygous
	5	>75	0			homozygous mutant
	6	>75	0			homozygous mutant
	7	57	21	1.3:1	0.69	hemizygous
	9	52	0			homozygous mutant
	10	0	65	0		wildtype
	11	60	20	3.0:1	1.0	hemizygous
UNK	1	71	24	3.0:1	0.95	hemizygous
	2	>75	0			homozygous mutant
	3	0	>75	0		wildtype
	5	0	>75	0		wildtype
	6	111	33	3.4:1	0.56	hemizygous
	7	>75	0			homozygous mutant
	8	40	15	2.7:1	0.70	hemizygous
	10	0	>75	0		wildtype
	11	60	20	3.0:1	1.0	hemizygous

Table 8.2. *Continued*

Gene	#	R[a]	S[b]	R:S ratio	Chi-test for 3:1 R:S ratio	Genotype
ANT1	1	75	25	3.0:1	1.00	hemizygous
	2	61	15	4.1:1	0.29	hemizygous
	3	0	>75	0		wildtype
	4	40	17	2.4:1	0.40	hemizygous
	5	60	20	3.0:1	1.00	hemizygous
	6	0	>75	0		wildtype
	7	71	20	3.6:1	0.51	hemizygous
	9	22	22	3.3:1		hemizygous
ANT2	1	0	>75	0		wildtype
	2	0	>75	0		wildtype
	3	0	>75	0		wildtype
	4	45	20	2.3:1	0.28	hemizygous
	5	0	>75	0		wildtype
	6	56	22	2.5:1	0.51	hemizygous
	7	94	35	2.7:1	0.58	hemizygous

[a] resistant to PPT
[b] susceptible to PPT

Arabidopsis T-DNA insertional mutants in putative cell death-related genes were obtained from TMRI as M2 individuals. Approximately 70 to 100 M3 seeds obtained from each of these individuals were plated on selective (PPT-containing) medium to determine the number of resistant seedlings (see section 2.13.2). If there were both resistant and susceptible seedlings (and therefore not the progeny of either a wildtype or homozygous mutant individual), the ratio of resistant:susceptible seedlings was calculated. This ratio was compared (chi-squared test) to that which would be expected for a 3:1 ratio, indicating a single T-DNA insert in hemizygous individuals. The chi-test value is presented and values closest to 1.0 indicate ratios most likely to represent a 3:1 segregation. This allowed for M2 individuals to be classified as either wildtype (no T-DNA insertion), hemizygous (one or more independent T-DNA insertions), or homozygous mutant for a T-DNA insertion (see section 8.2.2).

thus it does not seem likely that the *ANT* homozygous mutants are embryo or seedling lethal (In this case, a 2:1 R:S ratio would be expected between viable hemizygous and wildtype seeds). Rather, the weak growth phenotype and early death of the *ANT* insertional mutant lines suggests that the development of these lines is compromised and therefore it is difficult to grow homozygous mutant individuals to maturity and collect seed. This is not surprising given the major role of ANT in normal mitochondrial function (Belzacq *et al.*, 2002), and once again underlines the difficulty in differentiating the importance of a gene product in maintaining cell viability from an active role for the product in a cell death pathway (see section 1.4.1).

A robust and reliable PCR-based screen of 2 to 3 week-old seedlings in the *ANT1* and *ANT2* insertional lines would be useful in identifying putative homozygous mutant individuals and then following the subsequent growth defects in these plants. In any case, the difficulty of working with and obtaining homozygous mutant *ANT* insertional mutants in either *ANT1* or *ANT2* excluded these lines from further study. The localisation of the *ANT2* insertion at least 200 bp upstream (5') of the transcription start site suggests that this insertion is in the promoter and is sufficient to disrupt gene expression (Figure 8.1G). It is of interest, however, that insertional mutants in either *ANT* gene appeared to be equally deleterious, thus suggesting that *Arabidopsis* ANT1 and ANT2 do not function redundantly. Perhaps, as is the case in animals (Stepien *et al.*, 1992; Levy *et al.*, 2000), each ANT protein is uniquely required in different tissues and developmental stages. If ANT does prove to play a specific role in plant cell death, it will therefore be of interest to determine if one isoform plays a more significant role in this event, as shown in mammalian cells (Bauer *et al.*, 1999).

8.2.3. Transcript analysis of homozygous T-DNA insertional mutants for selected genes

The next step in this study entailed confirmation that the insertional mutants in genes of interest resulted in a decrease in expression of the specific gene. Sequencing errors, seed contamination at source, or an unlinked insertion could result in a T-DNA homozygote not demonstrating altered expression of the gene of interest. Additionally, two of the insertions (for *SAG* and *UNK*) were localised to several hundred basepairs downstream (3') or upstream (5') of the coding region, respectively (Figures 8.1D and E), and it was therefore uncertain whether this would disrupt gene expression. RT-PCR analysis using cDNA synthesised from total RNA of 9 day-old seedlings was undertaken to determine whether the level of specific transcripts was reduced in the putative homozygotes. DNA primers used in this analysis allowed, in all cases but for *SAG*, for a distinction between genomic DNA and cDNA PCR products to be made, since the primers spanned intron regions (Table 8.3). This RT-PCR approach, rather than Northern blotting, was used since, especially in the case of *VDAC/Athsr2*, closely-related family members can be more precisely studied using gene-specific primers.

The primer combinations used in each RT-PCR analysis are outlined in Table 8.3. As shown in Figures 8.2 and 8.3, specific transcript in the *hsp70-3* homozygous individuals #2 and #3 and in the *srg3* homozygous individual #6 were undetectable compared to the wildtype control. *vdac/athsr2* (hereinafter, *vdac*) individuals #6 and #13 exhibited almost undetectable levels of transcripts, and the weak band obtained in these RT-PCRs is almost certainly due to very closely-related *VDAC* family members (Figure 8.4). The sequencing data available for this SAIL line was unequivocally matched only the *Athsr2* isoform and not any other members of the *VDAC* gene family. Homozygous

Table 8.3. Primer combinations for RT-PCR analysis of *Arabidopsis* SAIL lines.

Gene	5' Primer (5' → 3')	3' Primer (5' → 3')	gDNA product (bp)	cDNA product (bp)
VDAC/ Athsr2	TGCTACCCAAGTGAA GAAC	ACAATCGGGGTAGCTGTG	450	250
Hsp70-3	CTCCACTGATAGCGA AC	TGTCATCTCCAGCAAGC	>2000	1900
SRG3	AGCCCTTCTTGTCAG GAAGTTGC	GTTGTATCAACTCGGGAA TGAGC	550	450
SAG	CTACAAGGTCAGCTA AGTGACGTC	CAGTGGAGAGAGAAACA CGAGATC	300	300
UNK	ATGGCTCAAGAAGAT GTTACTG	CTAAACCTCTTTGTTCTC GTC	900	550

Gene-specific primers were used in an RT-PCR analysis of putative homozygous *Arabidopsis* T-DNA insertional mutants in genes of interest. This strategy was used to determine if the specific gene for which an insertional mutant was predicted showed decreased transcript expression. The predicted sizes of genomic DNA (gDNA) and cDNA PCR products based on primer annealing sites are indicated.

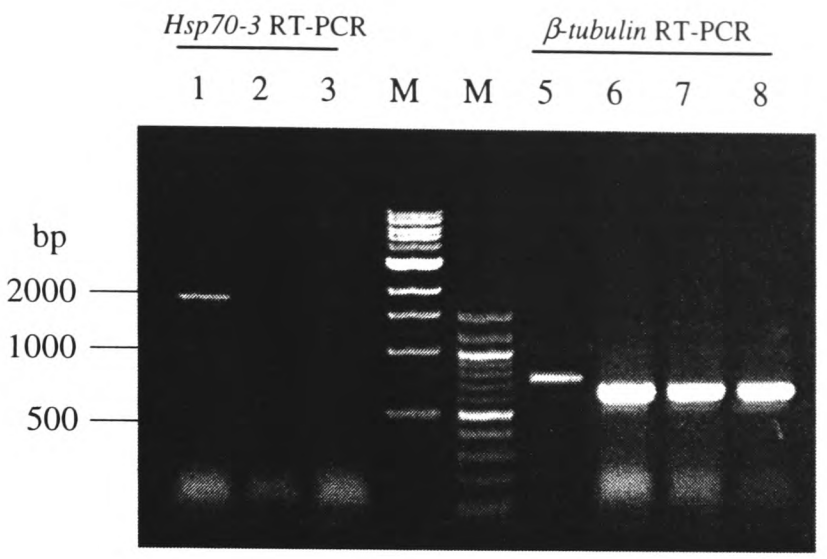


Figure 8.2. RT-PCR analysis of T-DNA *hsp70-3* (At3g09440) *Arabidopsis* knockout lines.

cDNA was synthesised from 10 µg total RNA isolated from wildtype (lanes 1 and 6), *hsp70-3* T-DNA # 2 (lanes 2 and 7), or *hsp70-3* T-DNA # 3 (lanes 3 and 8) 9 day-old *Arabidopsis* seedlings. 1 µl of undiluted cDNA was used in a PCR reaction with either *Hsp70-3*-specific primers (lanes 1-3) or *β-tubulin*-specific primers (lanes 5-8). A control reaction used 100 ng genomic DNA as a template (lane 5). While all individuals exhibit RT-PCR products for *β-tubulin*, the *hsp70-3* T-DNA #2 and #3 individuals do not exhibit the expected 1900 bp band for *Hsp70-3* compared to wildtype, indicating reduced expression of this gene. M; marker.

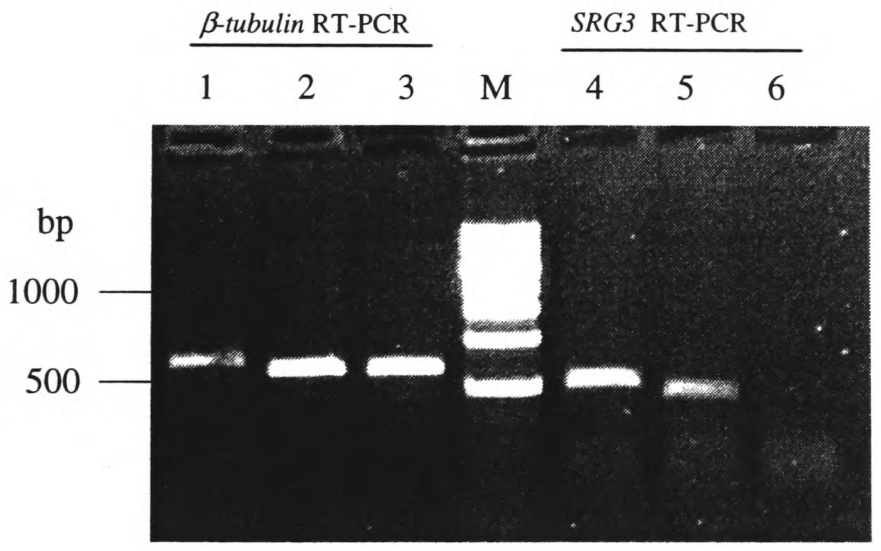


Figure 8.3. RT-PCR analysis of the T-DNA *srg3* (At3g02040) *Arabidopsis* knockout line.

cDNA was synthesised from 10 µg total RNA isolated from wildtype (lanes 2 and 5) and *srg3* T-DNA #6 (lanes 3 and 6) 9 day-old *Arabidopsis* seedlings. 1 µl of 10-fold diluted cDNA was used in a PCR reaction with either *SRG3*-specific primers (lanes 4-6) or *β-tubulin*-specific primers (lanes 1-3). Control reactions used 100 ng genomic DNA as a template (lanes 1 and 4). While all individuals exhibit RT-PCR products for *β-tubulin*, the *srg3* T-DNA #6 individual exhibits only a very weak band of 500 bp for *SRG3* compared to wildtype, indicating reduced expression of this gene. M; marker.

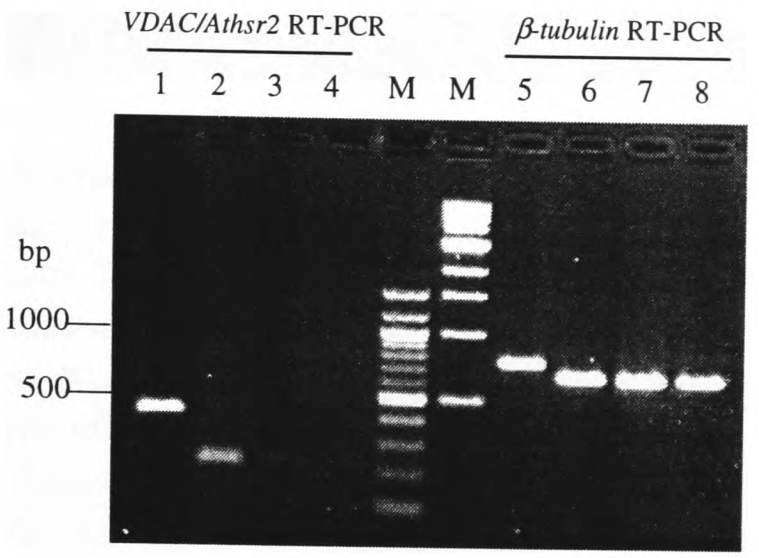


Figure 8.4. RT-PCR analysis of T-DNA *vdac/athsr2* (At5g15090) *Arabidopsis* knockout lines.

cDNA was synthesised from 10 µg total RNA isolated from wildtype (lanes 2 and 6), *vdac/athsr2* T-DNA #6 (lanes 3 and 7), or *vdac/athsr2* T-DNA #13 (lanes 4 and 8) 9 day-old *Arabidopsis* seedlings. 1 µl of 10-fold diluted cDNA was used in a PCR reaction with either *Athsr2*-specific primers (lanes 1-4) or *β-tubulin*-specific primers (lanes 5-8). Control reactions used 100 ng genomic DNA as a template (lanes 1 and 5). While all individuals exhibit RT-PCR products for *β-tubulin*, the *vdac/athsr2* T-DNA #6 and #13 individuals do not exhibit the expected 250 bp band for *Athsr2* compared to wildtype, indicating reduced expression of this gene. The very weak bands visible in these lanes are likely due to a contaminating RT-PCR product arising from a closely related (75% identical) putative *VDAC* gene (At3g01280). M; marker.

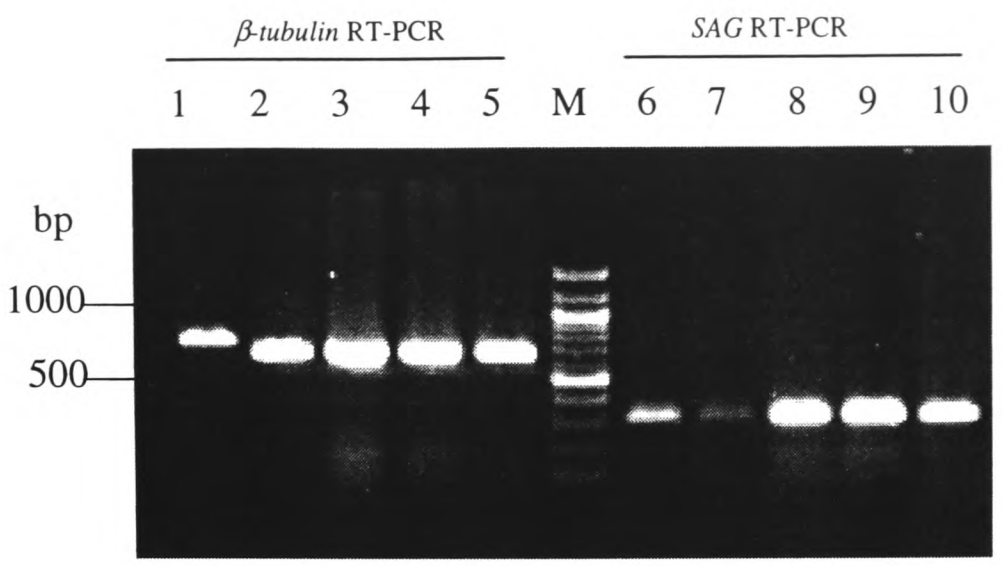


Figure 8.5. RT-PCR analysis of T-DNA *SAG* (At1g15100) *Arabidopsis* lines.
cDNA was synthesised from 10 µg total RNA isolated from wildtype (lanes 2 and 7), *SAG* T-DNA #5 (lanes 3 and 8), *SAG* T-DNA #6 (lanes 4 and 9), or *SAG* T-DNA #9 (lanes 5 and 10) 9 day-old *Arabidopsis* seedlings. 1 µl of cDNA was used in a PCR reaction with either *β-tubulin*-specific primers (lanes 1-5) or *SAG*-specific primers (lanes 6-10). Wildtype cDNA was diluted 1/10. A control reaction used 100 ng genomic DNA as a template (lanes 1 and 6). All individuals exhibit RT-PCR products for both *β-tubulin* and the *SAG* cDNA, indicating that the T-DNA insertion does not knockout expression of the *SAG* gene. M; marker.

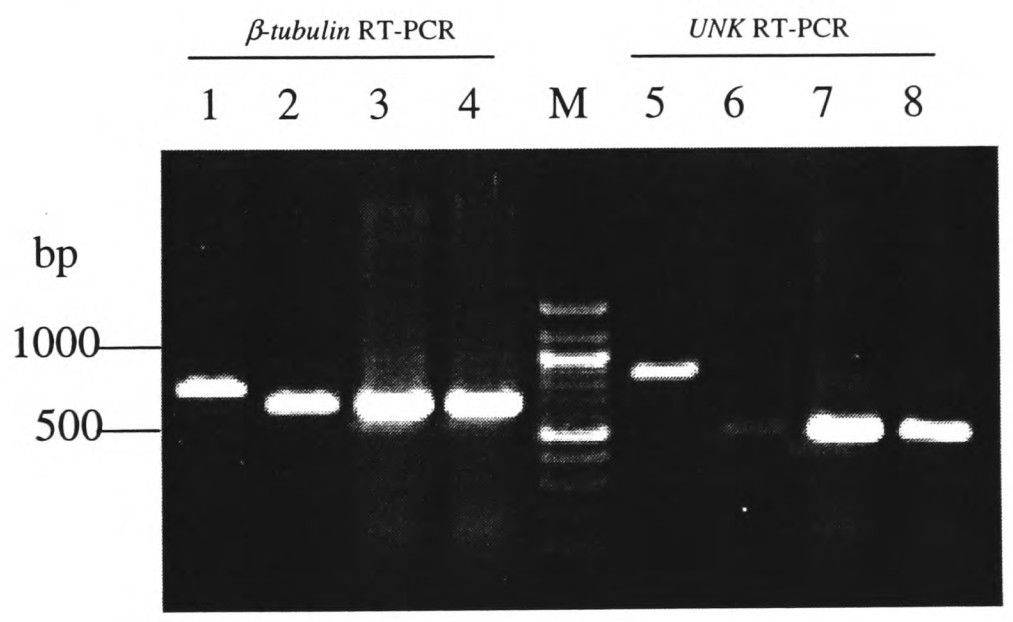


Figure 8.6. RT-PCR analysis of T-DNA *Unknown Protein* (*UNK*) (At5g48480) *Arabidopsis* lines.
cDNA was synthesised from 10 µg total RNA isolated from wildtype (lanes 2 and 6), *UNK* T-DNA #2 (lanes 3 and 7), or *UNK* T-DNA #7 (lanes 4 and 8) 9 day-old *Arabidopsis* seedlings. 1 µl of cDNA was used in a PCR reaction with either *β-tubulin*-specific primers (lanes 1-4) or *UNK*-specific primers (lanes 5-8). Wildtype cDNA was diluted 1/10. A control reaction used 100 ng genomic DNA as a template (lane 1 and 5). All individuals exhibit RT-PCR products for both *β-tubulin* and the *unknown protein* cDNA, indicating that the T-DNA insertion does not knockout expression of the *UNK* gene. M; marker.

mutants in the *SAG* and *UNK* genes, however, did not exhibit reduced transcript levels (Figures 8.5 and 8.6). Therefore, it can be concluded that the insertions downstream (3') or upstream (5') of the coding sequences do not disrupt expression of these genes and that insertional mutants in these specific genes do not exist in the SAIL collection.

8.2.4. T-DNA insertional mutants in selected genes do not exhibit altered growth, development, or senescence

It was only possible to identify homozygous T-DNA insertional mutants in genes for *SRG3*, *VDAC*, and *Hsp70-3*. To determine whether knockouts in these putative cell death-related genes affected growth and development of whole plants, the growth phenotypes of homozygous mutant individuals (one for *srg3* and two each for *vdac* and *hsp70-3*) were examined on a weekly basis over the life cycle of the plant. All seedlings grown on agar plates for two weeks exhibited normal germination, cotyledon and rosette leaf development, and root growth compared to wildtype (data not shown). After transfer to soil at two weeks, plants were examined at weekly intervals (Figure 8.7). Each line was examined for observable phenotypes and compared to wildtype plants grown under identical conditions (see section 2.13.2), and the analysis was carried out on four independent individuals. Growth characteristics studied included rosette leaf size, shape and number, timing of bolting or flowering, height and branching, final seed set, and the onset and progression of senescence. Particular attention was paid to those developmental processes that are known to involve PCD, such as reproductive development (Gray and Johal, 1998; Wang *et al.*, 1999; Balk and Leaver, 2001) and senescence (Pennell and Lamb, 1997; Yen and Yang, 1998; Delorme *et al.*, 2000). To this end, relative chlorophyll content of the fourth rosette leaf was measured as a quantitative indication of senescence progress (see section 2.14.1) (Figure 8.8). Compared to wildtype, mutant plants exhibited no aberrant phenotypes (Figure 8.7),

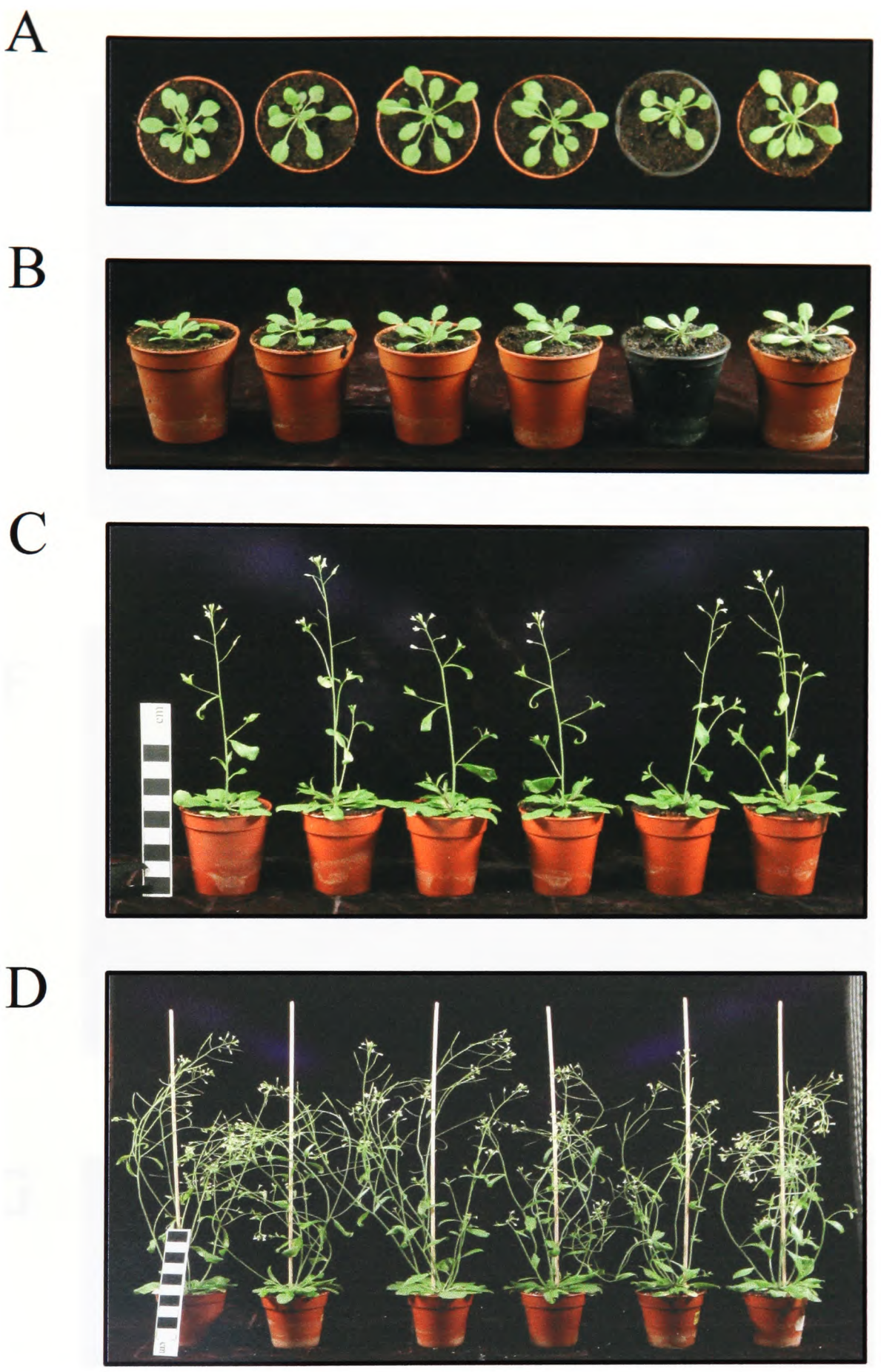
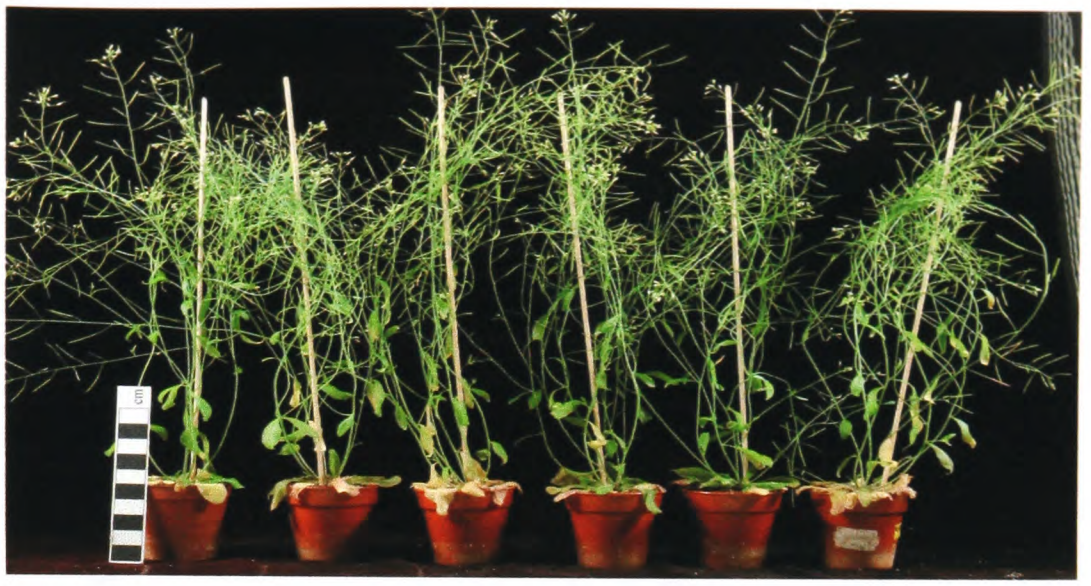


Figure 8.7. Growth characteristics of wildtype and T-DNA knockout *Arabidopsis* lines.

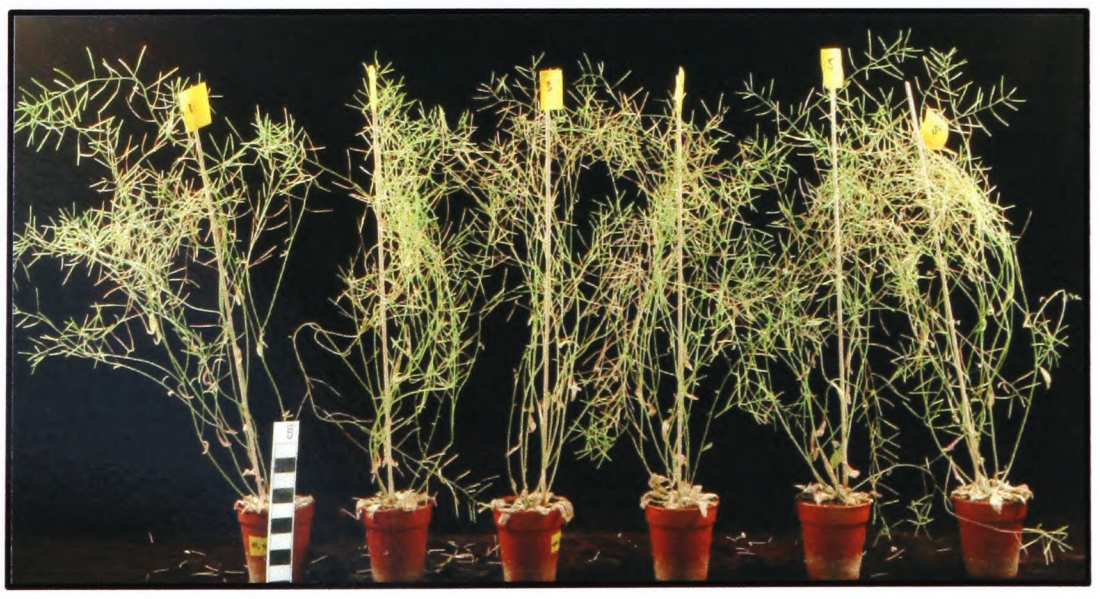
Growth characteristics of homozygous T-DNA insertional mutants were compared to wildtype plants at weekly intervals (see section 2.13.2). When 4 different repetitions of the analysis were undertaken with different plants, mutant plants did not differ from wildtype in any observable phenotype, including leaf size, shape and number, timing of bolting or flowering, height and branching, onset and progression of senescence, or final seed set. Representative plants at each stage are shown. From left to right, each panel shows the following individuals: wildtype; *srg3*; *vdac* #6; *vdac* #13; *hsp70-3* #2, *hsp70-3* #3. Developmental age is: **A** and **B**, 3 weeks; **C**, 4 weeks; **D**, 5 weeks; **E**, 6 weeks; **F**, 7 weeks; **G**, 8 weeks.

E



F

G



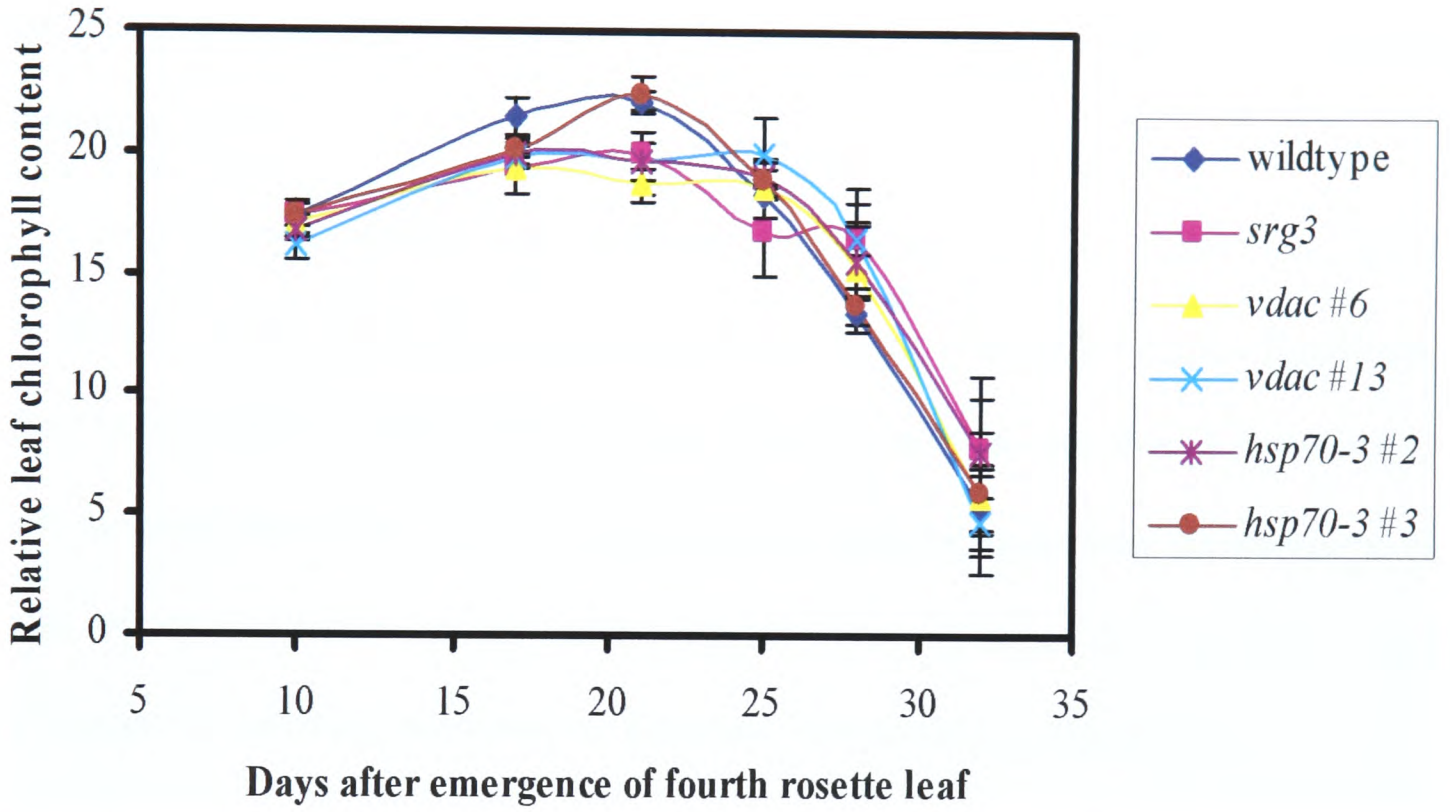


Figure 8.8. Age-dependent senescence of wildtype and T-DNA knockout *Arabidopsis* lines.
Relative chlorophyll content of rosette leaves were measured from 10 days after emergence when the fourth rosette leaves were just fully grown. Measurements were taken with a Minolta Spad-502 Chlorophyll Meter (see section 2.14.1). Values presented are the means of at least 4 leaves from different individuals.

underwent senescence at the same time and rate (Figure 8.8), and yielded equivalent amounts of seed. Therefore, knockouts in the genes of interest do not appear to cause an observable phenotype, and it can be concluded that, generally, these genes are not essential for normal growth and development.

8.2.5. T-DNA insertional mutants in selected genes do not exhibit altered vascular patterning

Homozygous mutant individuals for knockouts in *SRG3*, *VDAC*, or *Hsp70-3* were examined for the stem vascular patterning, since tracheary element formation is known to be a form of plant PCD (Fukuda, 2000). Stem cross-sections of four week-old plants revealed that there were no differences in vascular patterning formation in four plants of each line examined and compared to wildtype (see section 2.14.2). Representative vascular patterning for each line is illustrated in Figure 8.9.

8.2.6. T-DNA insertional mutants in selected genes do not exhibit altered responses to abiotic stress

It was of interest to determine whether insertional mutations in *SRG3*, *VDAC*, or *Hsp70-3* affected the response of the plant to abiotic stresses sufficient to cause cell death in wildtype plants. To this end, it was first necessary to establish reproducible, appropriate treatments that were capable of causing chlorosis and cell death within a suitable time period. Since heat treatment was originally used with cell suspension cultures to induce PCD, this was tested as a method of inducing cell death in whole seedlings. Heat treatment of *Arabidopsis* seedlings described previously has involved removing seedlings from agar plates into water-filled microfuge tubes and then heating in a heating block (Larkindale and Knight, 2002). However, it was desirable in the current study to avoid removing seedlings from plates and introducing hypoxic effects,

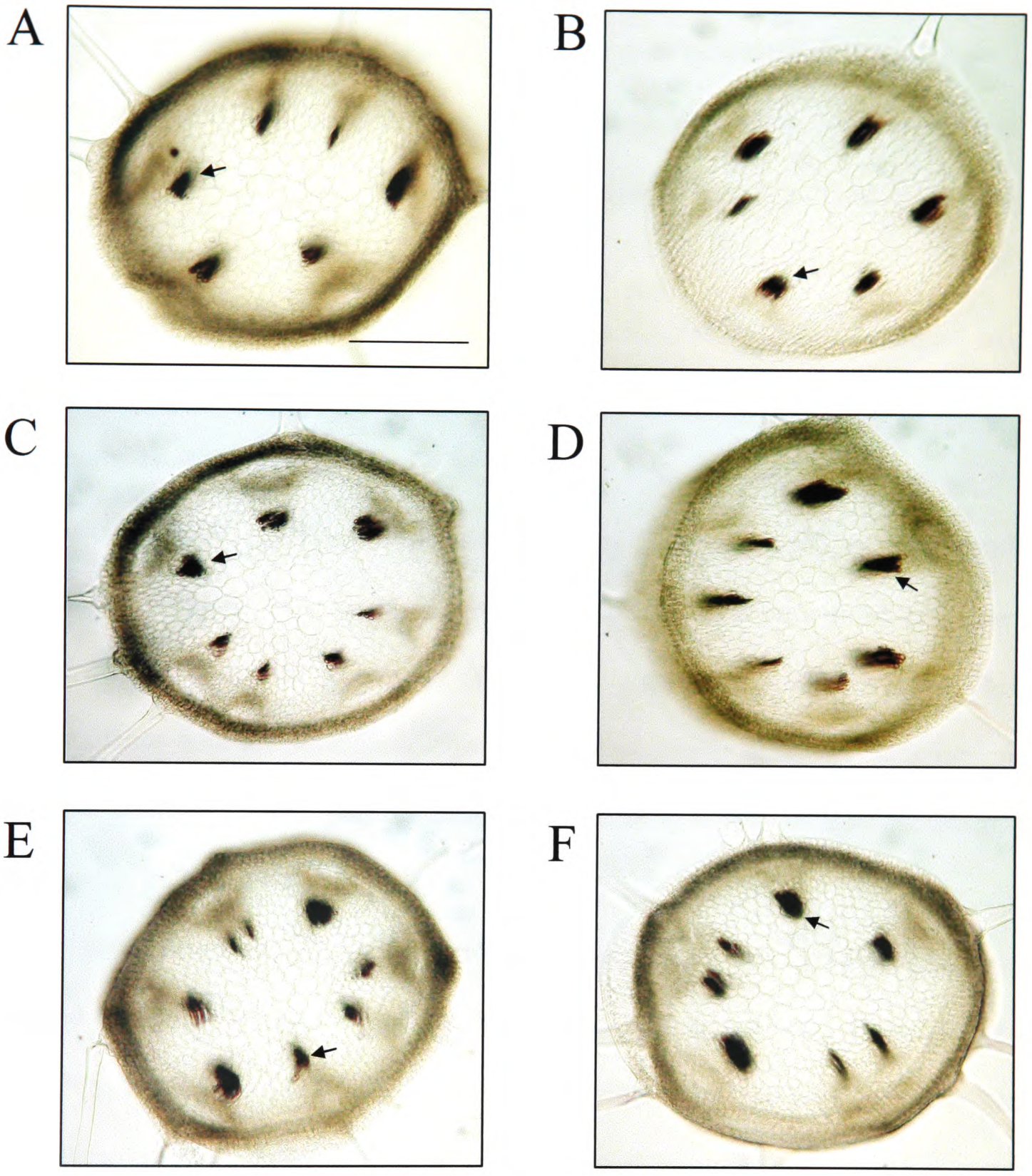


Figure 8.9. Stem vascular structure of wildtype and T-DNA knockout *Arabidopsis* lines.

Cross-sections of influorescence stems at 4 weeks of development (approximately 10 cm in length). The lignified secondary wall of tracheary elements (arrow), formed as a result of a programmed cell death pathway, are indicated by phloroglucinol staining (see section 2.14.2). **A**, wildtype; **B**, *srg3*; **C**, *vdac* #6; **D**, *vdac* #13; **E**, *hsp70-3* #2; **F**, *hsp70-3* #3. Scale bar 100 μm, A-F.

not only so that seedlings could be examined over a period of days post-treatment, but also so that it could be ensured that each line was treated identically to wildtype. Therefore, any method of abiotic stress induction must be amenable to treating wildtype and mutant plants identically on the same agar plate. All abiotic stress treatments were initially tested by treating plates of 30 to 40 9 day-old wildtype seedlings to ensure a uniform response.

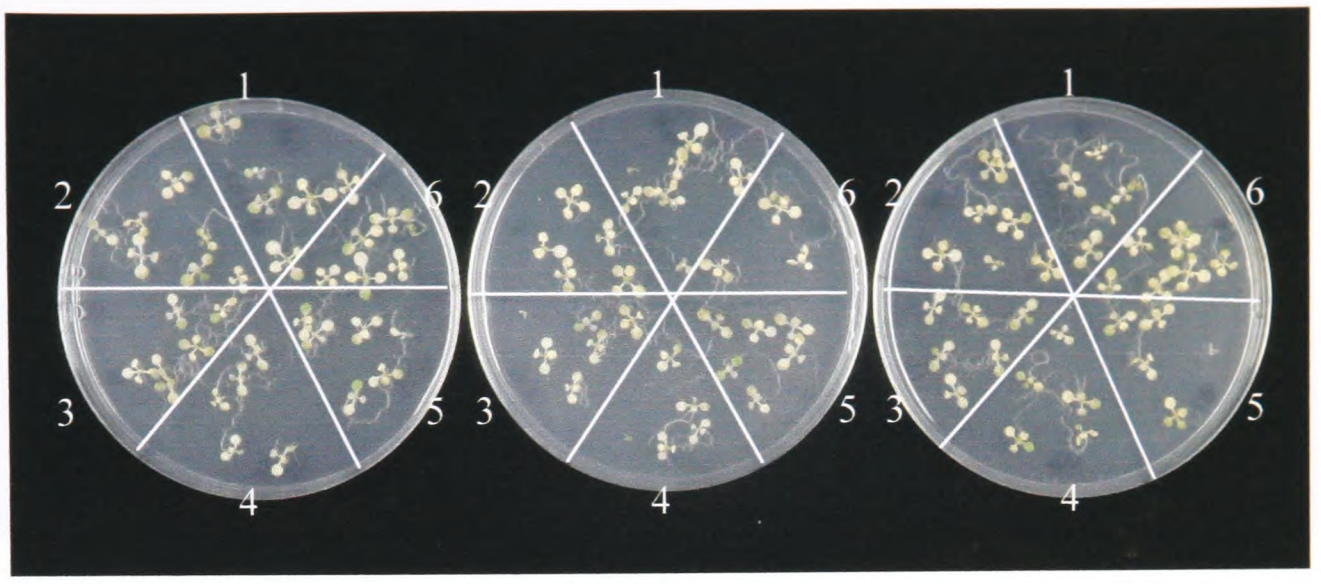
Heat treatment of wildtype seedlings in an oven at 40°C for 1 h had no effect on seedling viability as judged by the complete lack of chlorosis over one week post-treatment. However, when treated at 45°C for 1 h, seedlings began to show mild chlorosis beginning 48 h post-treatment leading to death (as judged by chlorosis) by 72 h (data not shown). However, there were considerable variations in the response both between and within plates, indicating that heating in the oven was not uniform. This was in accordance with previous observations using *Arabidopsis* seedlings (Jane Larkindale, personal communication), and therefore heat treatment was not a reproducible method of inducing PCD in whole plants.

H_2O_2 treatment as a method of inducing cell death was investigated, since this method has been used with success in *Arabidopsis* cell suspension cultures (Desikan *et al.*, 1998; Desikan *et al.*, 1999). However, spraying 9 day-old seedlings with various concentrations of H_2O_2 up to 0.175 M was ineffective in producing a visible effect up to one week post-treatment, most likely due to rapid 'quenching' of the H_2O_2. To elicit a stress response and obvious chlorosis, seedlings required immersion in 0.15 M H_2O_2 for approximately 6 h (data not shown). This method of treatment was technically difficult because some seedlings became uprooted from the agar when immersed in liquid and could therefore be affected by additional stresses. Thus, H_2O_2 treatment was rejected as a reproducible and uniform method of inducing cell death.

Paraquat treatment (Abarca *et al.*, 2001; de Leon *et al.*, 2002) and UV-C radiation of *Arabidopsis* (Danon and Gallois, 1998) have both been shown to be effective methods of causing cell death in whole *Arabidopsis* plants, and have the added advantage that the treatment can be applied at a specific time point without the need for continuous incubation. It was found that 1 mM paraquat, when sprayed at the beginning of the light period and with subsequent incubation in long-day conditions (16 h light), was sufficient to induce chlorotic effects and cell death within 48 h post-treatment (Figure 8.10). At lower concentrations, chlorotic effects were minimal. UV-C radiation of 20 to 50 kJ/m^2 was sufficient to cause chlorosis within 3 days post-treatment, with 30 kJ/m^2 providing optimal results (Figure 8.11 and 8.12). These results are in accordance with previous observations of other researchers (David Day and Patrick Gallois, personal communication).

While the visible phenotype between control wildtype and T-DNA insertional mutants did not differ in response to either paraquat or UV-C radiation, it was possible that slight effects were occurring which required a more quantitative method of detection. To this end, several methods of attaching a quantitative value to the level of cell death were investigated. Unlike cell suspension cultures, whole plant tissues are not amenable to single-cell counting and a quantification of the percentage of cells that have undergone PCD. However, trichomes can be viewed under low magnification with a light microscope and can therefore potentially be scored for PCD morphology (see section 2.14.4). Unfortunately, scoring trichome cell death proved extremely difficult since, without being able to see the entire trichome in the field of view, the presence of cytoplasmic condensation as a characteristic morphological feature of PCD could be not be consistently determined. When several replicate measurements of trichome PCD were undertaken on the same sample, results were highly inconsistent (data not shown).

A



B

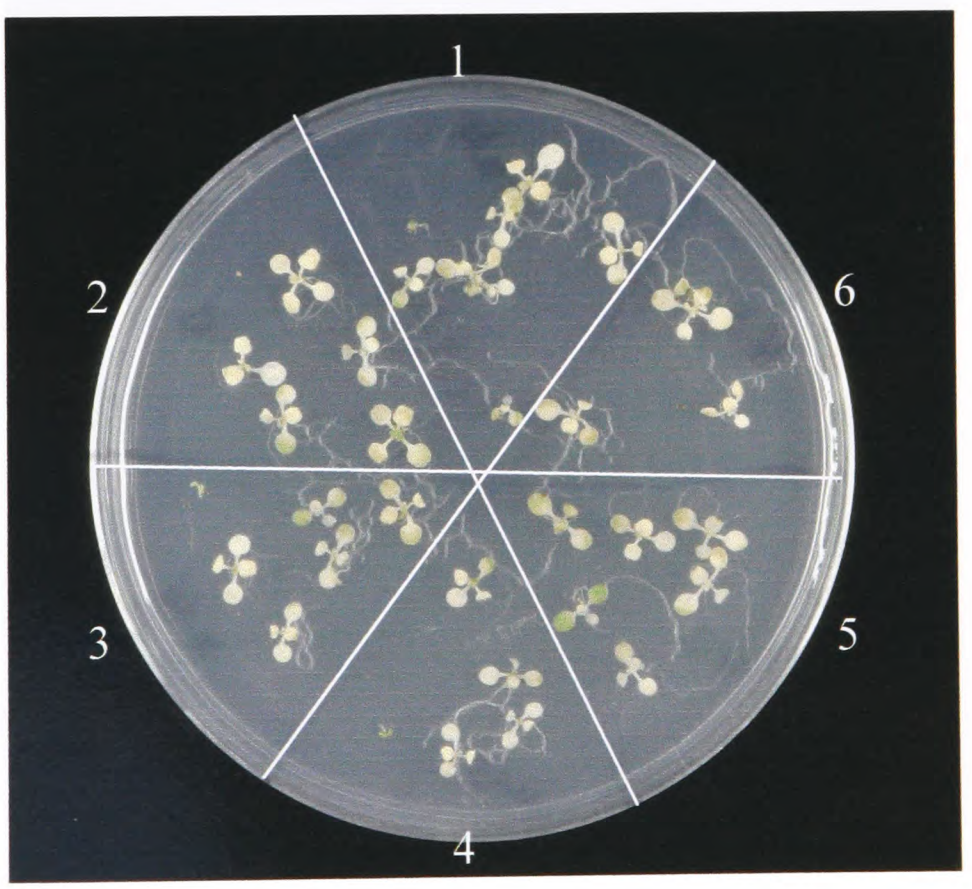
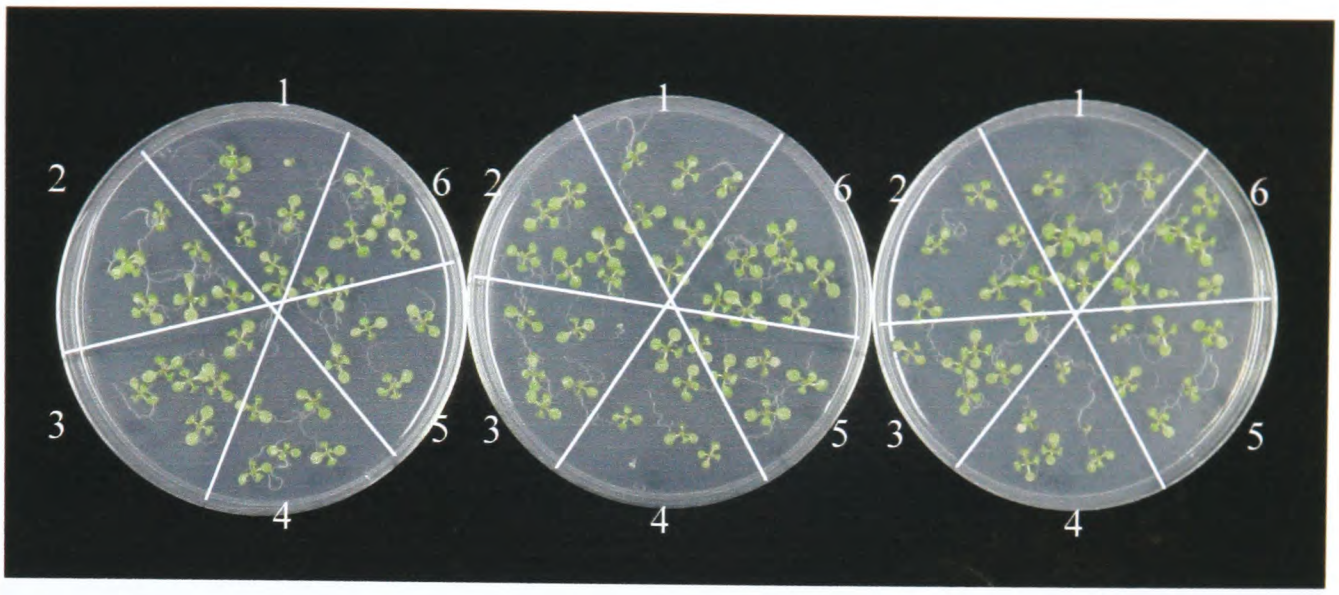


Figure 8.10. Phenotype of wildtype and T-DNA knockout *Arabidopsis* lines 48 h after paraquat treatment.
9 day-old seedlings were sprayed with 1 mM paraquat and grown under long-day (16 h light) conditions for 48 h. After this time, leaves and cotyledons displayed extensive chlorosis. **A.** 3 replicate plates. **B.** Higher magnification of one plate. Lines are: 1, wildtype; 2, *srg3*; 3, *vdac* #6; 4, *vdac* #13; 5, *hsp70-3* #2; 6, *hsp70-3* #3.

A



B

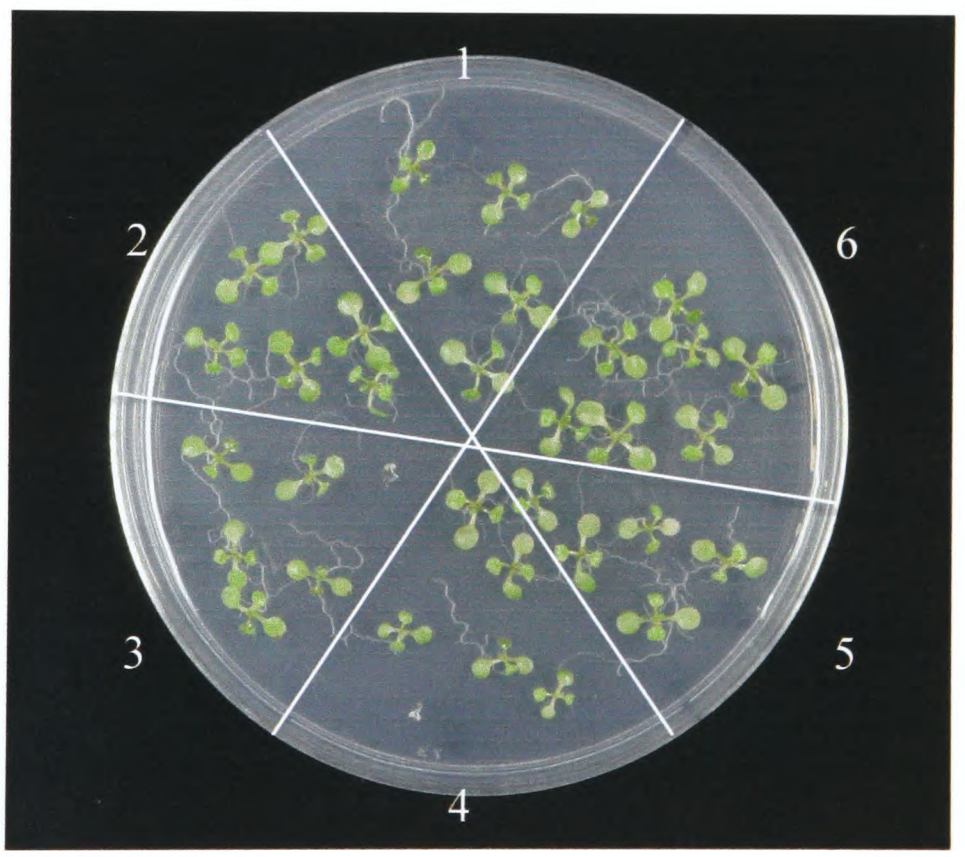
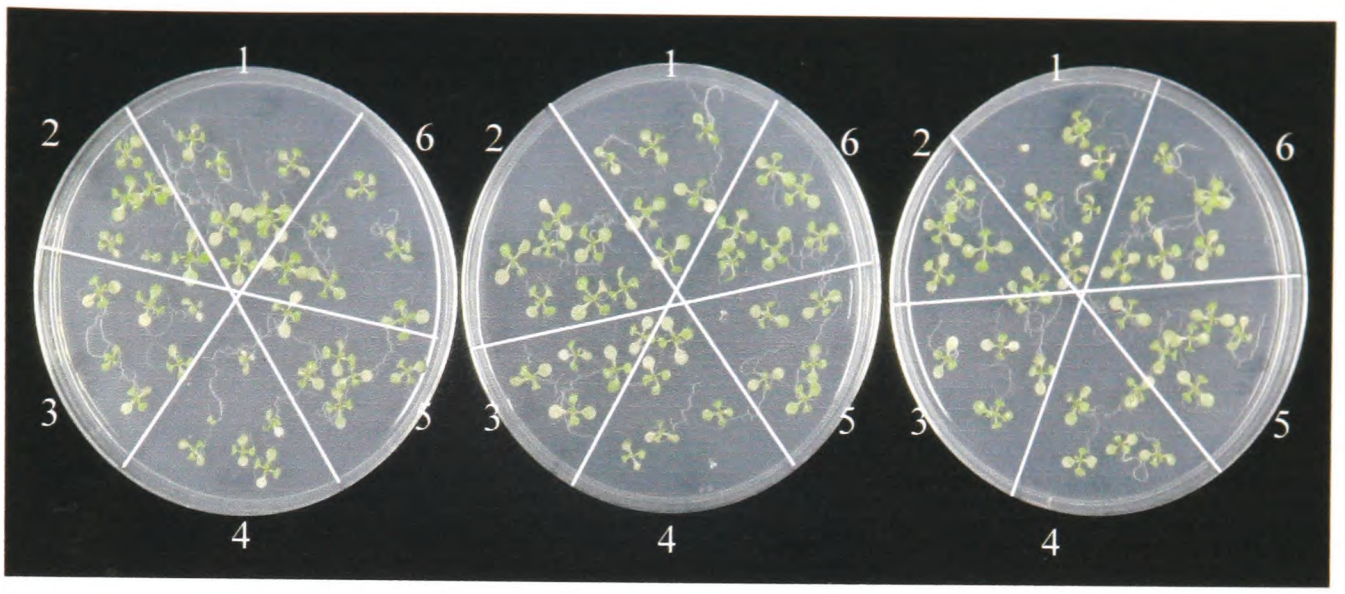


Figure 8.11. Phenotype of wildtype and T-DNA knockout *Arabidopsis* lines 48 h after UV-C radiation.

9 day-old seedlings were treated with 30 kJ/m^2 UV-C radiation and grown under long-day (16 h light) conditions for 48 h. After this time, leaves and cotyledons displayed only mild chlorosis that was not visible at 24 h. **A.** 3 replicate plates. **B.** Higher magnification of one plate. Lines are: 1, wildtype; 2, *srg3*; 3, *vdac* #6; 4, *vdac* #13; 5, *hsp70-3* #2; 6, *hsp70-3* #3.

A



B

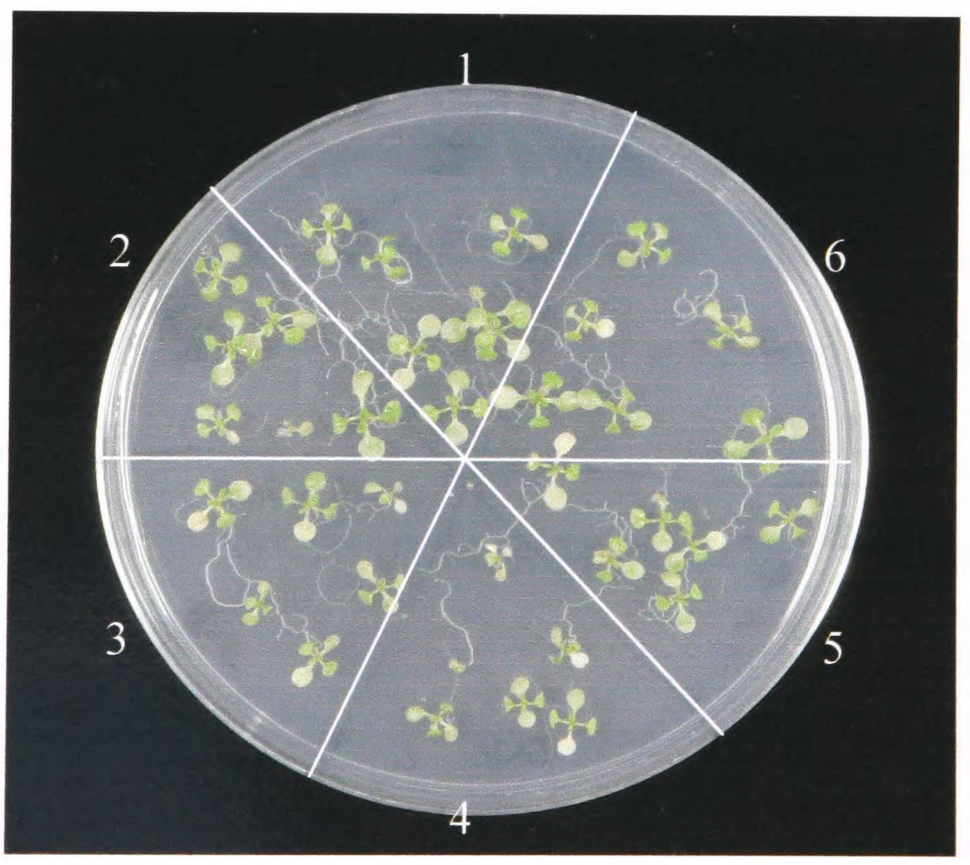


Figure 8.12. Phenotype of wildtype and T-DNA knockout *Arabidopsis* lines 72 h after UV-C radiation.
9 day-old seedlings were treated with 30 kJ/m^2 UV-C radiation and grown under long-day (16 h light) conditions for 72 h. After this time, leaves and cotyledons began to display severe chlorosis. **A.** 3 replicate plates. **B.** Higher magnification of one plate. Lines are: 1, wildtype; 2, *srg3*; 3, *vdac* #6; 4, *vdac* #13; 5, *hsp70-3* #2; 6, *hsp70-3* #3.

Thus, scoring of trichomes was neither an objective nor reliable method by which to quantify PCD in whole plants.

The rate of ion leakage from plant tissue has been successfully used in the past as a quantitative measure of cell death, since dying *Arabidopsis* and tobacco cells exhibit increased electrolyte leakage and therefore increase the conductivity of their bathing solution (Mitsuhar *et al.*, 1999; de Leon *et al.*, 2002). This method of cell death quantification has previously been shown to be effective in detecting differences between wildtype and transgenic lines in these studies. Therefore, electrolyte leakage from whole seedlings was measured at various times following 1 mM paraquat treatment (see section 2.14.3). As seen in Figure 8.13, the electrical conductivity of the seedling wash (µs/cm/mg FW) in the T-DNA insertional mutants was not different from that of wildtype seedlings at 24 or 48 h post-treatment. The reduction in electrical conductivity of the bathing solution at 48 h compared to 24 h is most likely explained by the fact that the total ion content of seedlings at the later time point is reduced (due to cell damage and death) and therefore fewer ions are available for leakage into the bathing solution. The ion leakage of seedlings which were untreated or exposed to 30 kJ/m^2 UV-C radiation was also measured. In this case, since the seedlings do not require an extended incubation in the light before the treatment is effective (as is the case with paraquat treatment), the electrical conductivity of the bathing solution of the same seedlings could be measured over a series of time points. As shown in Figure 8.14, the rate of ion leakage from untreated seedlings or following UV-C radiation was not significantly different between wild-type and T-DNA insertional mutant seedlings.

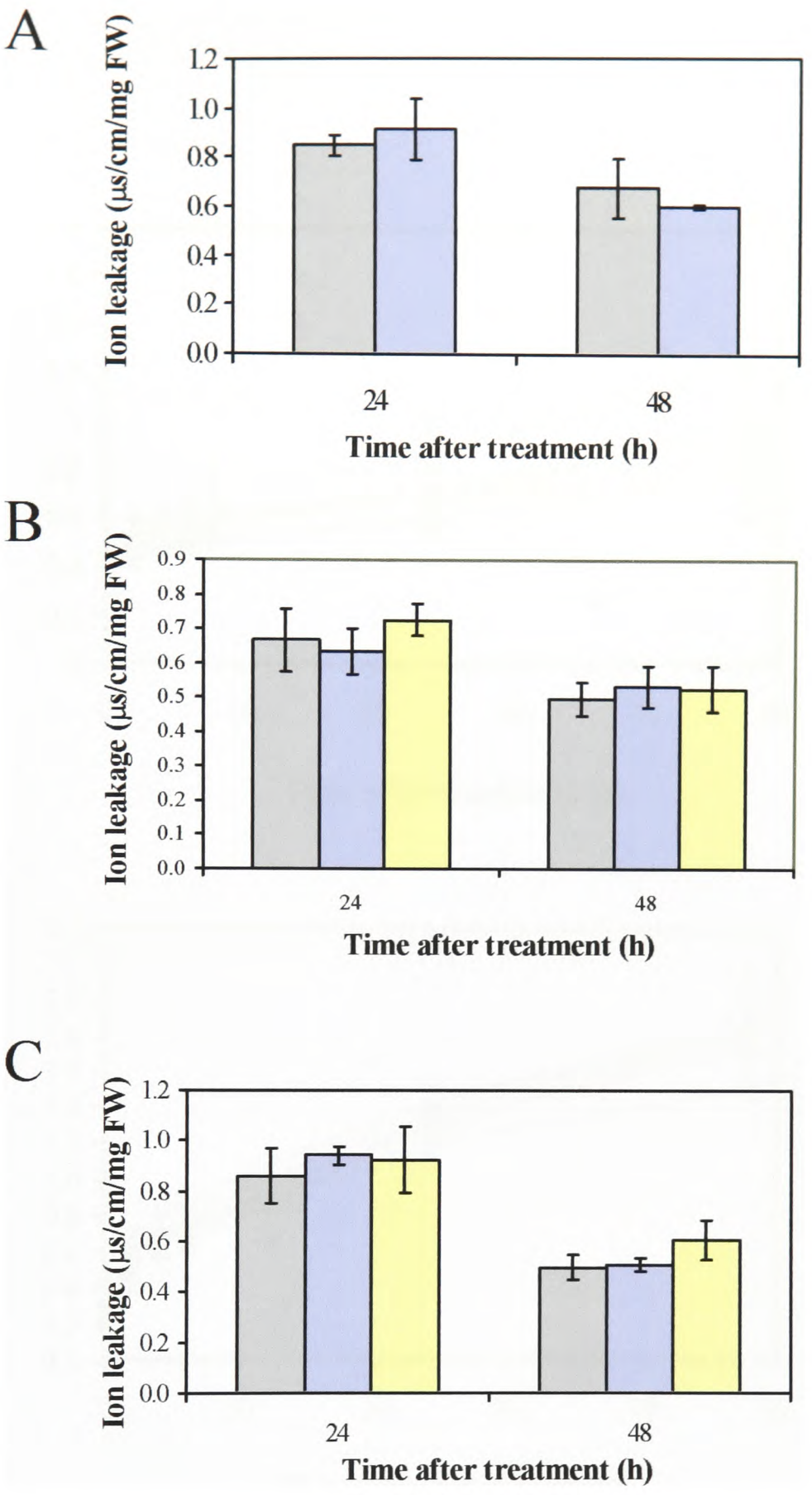
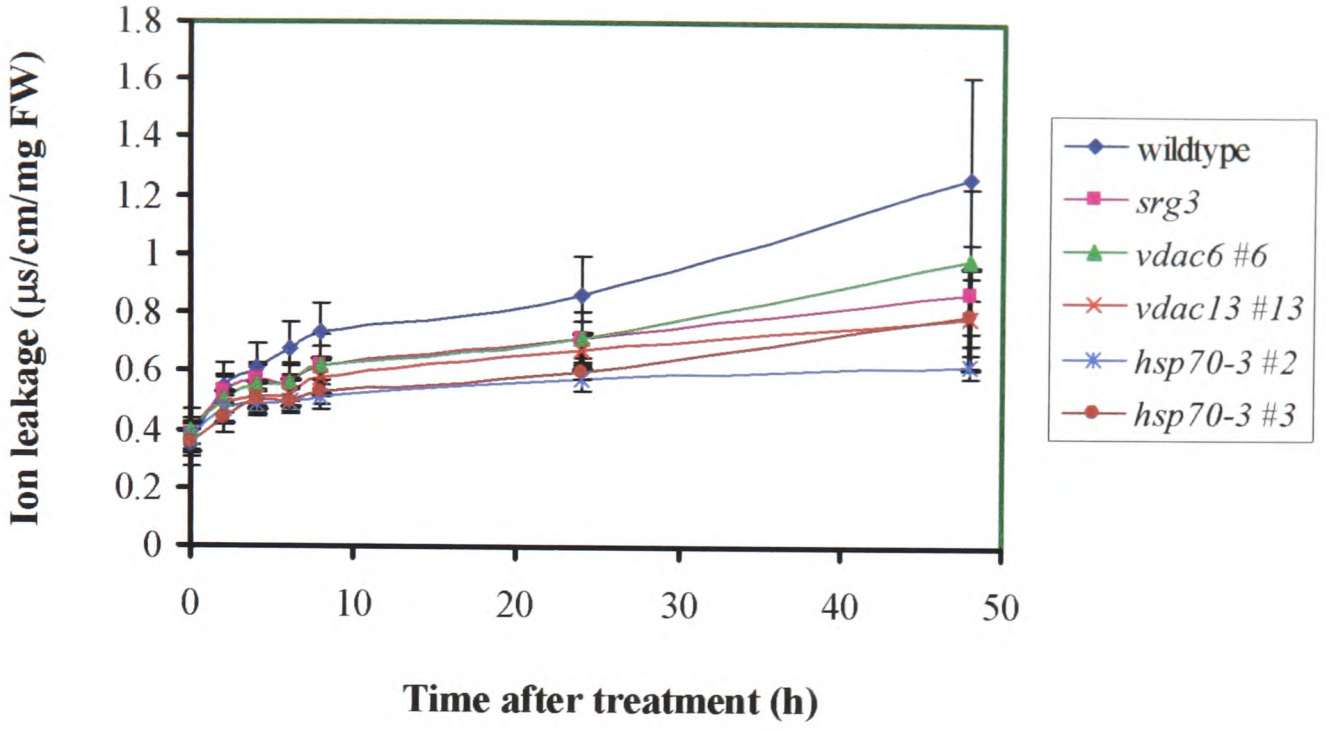


Figure 8.13. Ion leakage from wildtype and T-DNA knockout *Arabidopsis* lines following paraquat treatment.

9 day-old seedlings were sprayed with 1 mM paraquat and grown under long-day conditions (16 h photoperiod). T-DNA knockout plants in each line were grown together with wildtype plants on the same petri plates to ensure uniformity of treatment. Ion leakage was measured by floating 3 seedlings on ddH_2O for 3 h at room temperature with gentle shaking and measuring the final conductivity of the solution (µs/cm) (see section 2.14.3). Final ion leakage was expressed as a function of seedling mass (mg FW). Values presented are the means of 3 replicates. **A.** wildtype (grey); *srg3* (blue). **B.** wildtype (grey); *vdac* #6 (blue); *vdac* #13 (yellow). **C.** wildtype (grey), *hsp70-3* #2 (blue); *hsp* 70-3 #3 (yellow).

A



B

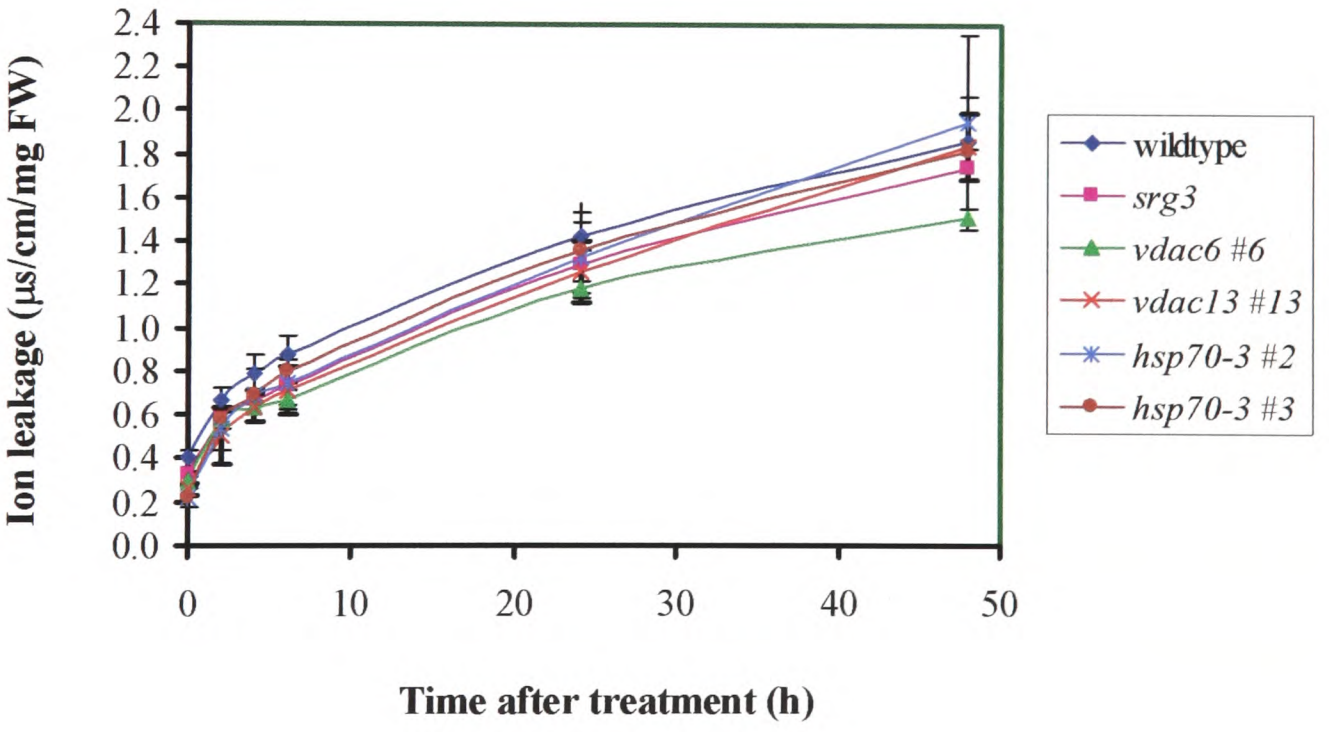


Figure 8.14. Ion leakage from wildtype and T-DNA knockout *Arabidopsis* lines.
9 day-old seedlings were floated on ddH_20 and the conductivity of the solution (µs/cm) was measured over time (see section 2.14.3). Final ion leakage was expressed as a function of seedling mass (mg FW). Seedlings were either untreated (**A**) or were initially treated with 30 kJ/m^2 UV-C radiation (**B**). Wildtype and T-DNA knockout lines were grown together on the same petri plates to ensure uniformity of treatment. Values presented are the means of 3 replicates.

8.2.7. T-DNA insertional mutants in selected genes do not exhibit an altered hypersensitive response

PCD is a component of the hypersensitive response (HR) (Heath, 2000a) and one of the T-DNA insertional mutants was in *VDAC/Athsr2*, a gene whose transcript was originally described as being expressed during *Arabidopsis* HR (Lacomme and Roby, 1999). Thus, it was of interest to determine whether the insertional mutants differed in their HR and related cell death compared to wildtype. To this end, two types of analyses were undertaken. In the first, the induction of HR-related cell death, as assayed by visible chlorosis and cell collapse (that occurs when at least ~25% of cells have actually undergone cell death (Klement, 1982; Ercolani, 1984)), was measured in wildtype and knockout lines. The HR was induced by inoculation with the incompatible pathogen, *Pseudomonas syringae* pv. *tomato* DC3000 (Pst) carrying the plasmid pAvrB1 (Pst *AvrB*) (see section 2.14.5). As shown in Figure 8.15, visible HR was apparent at 48 h post-inoculation. In all of the wildtype and T-DNA insertional mutant lines, HR-related cell collapse and chlorosis was most clearly evident with the highest concentration of pathogen inoculum, 10^7 cfu/ml, and visible HR progression occurred up to one week following inoculation (Figure 8.16). The HR cell death in the knockout lines was identical to that seen in wildtype and did not differ in terms of the concentration of pathogen capable of eliciting the HR.

As a further method of comparing the HR of wildtype and knockout mutants, the characteristics of both compatible (Pst) and incompatible (Pst *AvrB*) pathogen growth within the plant tissue were measured at zero, one, and three days post-inoculation. Past this time, desiccation of plant leaf tissue can lead to erroneous measurements of pathogen numbers per area of leaf tissue (Tornero and Dangl, 2001). It is important to actually measure pathogen growth as opposed to only looking for visible HR, since

certain 'defense, no death' mutants have been described in *Arabidopsis* which mount a HR defense response but do not undergo visible HR cell death (Yu *et al.*, 2000) (see section 1.4.1) Therefore, this experiment is complementary to that described above. Briefly, rosette leaves were inoculated with bacterial suspensions at 10^5 cfu/ml. Immediately following inoculation and at one and three days post-inoculation, leaf samples were homogenised and the growth of the pathogen in the leaf was measured by plating the leaf homogenate on selective media (see section 2.14.5). As expected, the growth of the compatible pathogen exceeded that of the incompatible pathogen over the time course analysed (Figure 8.17), since the latter elicits the HR within the plant host and leads to an inhibition of bacterial growth (Preston, 2000). There were, however, no differences in either compatible or incompatible bacterial population growth over three days when comparing wildtype to any of the T-DNA insertional mutants.

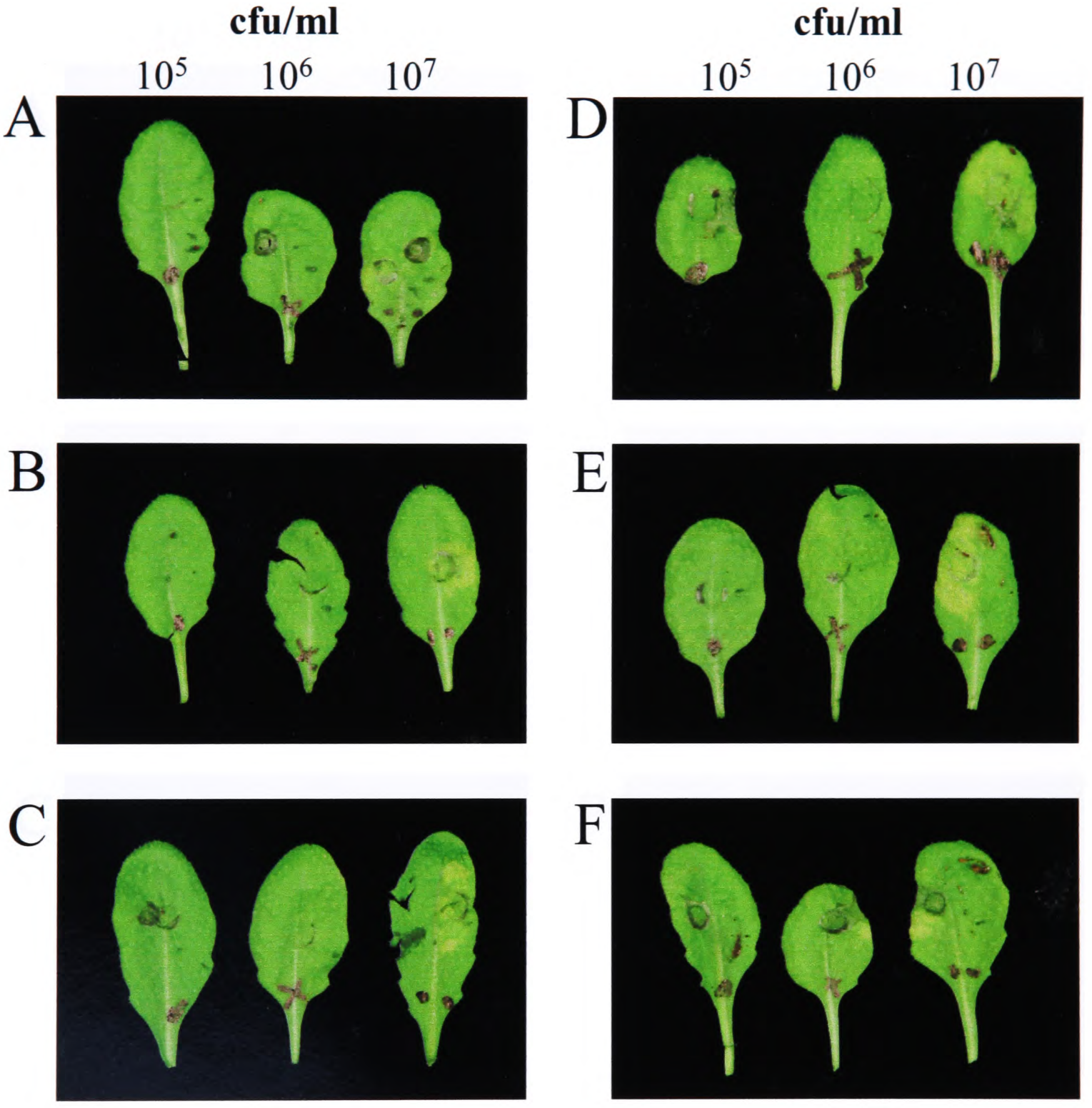


Figure 8.15. Development of HR cell death in wildtype and T-DNA knockout *Arabidopsis* lines after 48 h.

4 week-old rosette leaves were inoculated with the indicated bacterial suspension concentrations (cfu/ml) of the incompatible pathogen, *Pseudomonas syringae* pathovar *tomato* DC3000, *AvrB* (see section 2.14.5). After 48 h, HR-related cell collapse and chlorosis were evident in the highest inoculum, 10^7 cfu/ml, in all lines. Results were replicated 3 times and representative leaves are shown. **A**, wildtype; **B**, *srg3*; **C**, *vdac* #6; **D**, *vdac* #13; **E**, *hsp70-3* #2; **F**, *hsp70-3* #3.

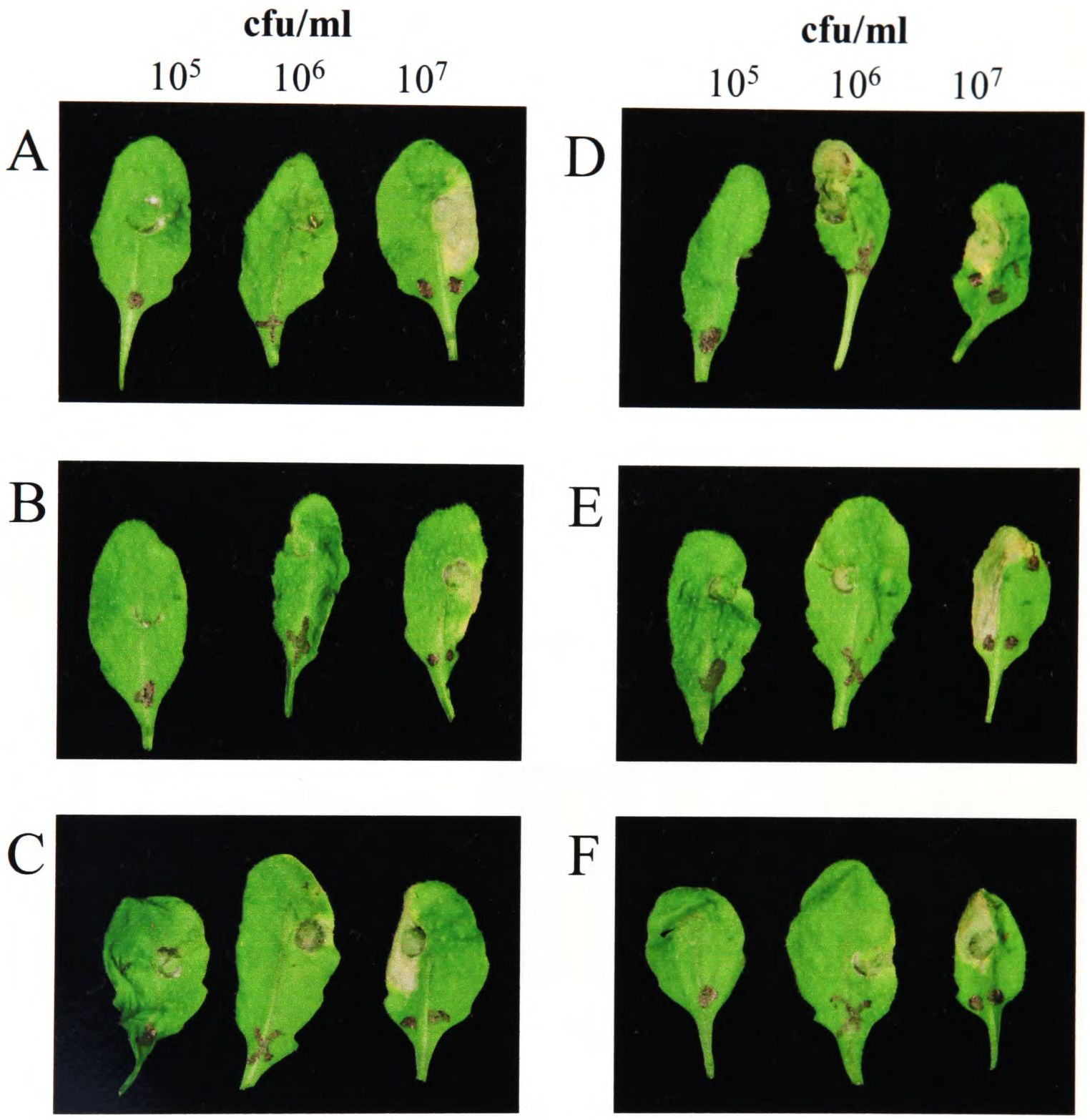
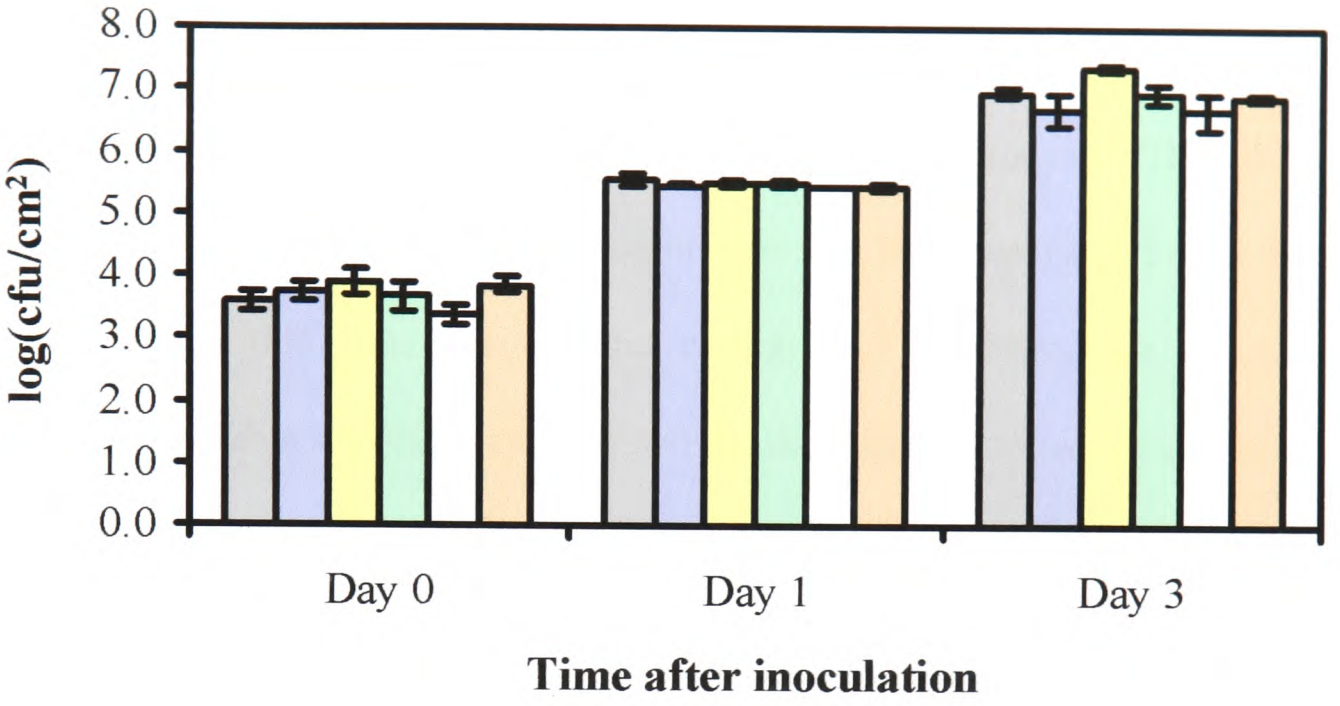


Figure 8.16. Development of HR cell death in wildtype and T-DNA knockout *Arabidopsis* lines after 7 days.
4 week-old rosette leaves were inoculated with the indicated bacterial suspension concentrations (cfu/ml) of the incompatible pathogen, *Pseudomonas syringae* pathovar *tomato* DC3000, *AvrB* (see section 2.14.5). After 7 days, noticeable HR-related cell collapse and chlorosis were evident in the highest inoculum, 10^7 cfu/ml, in all lines. Milder HR responses were evident in the intermediate inoculum, 10^6 cfu/ml. Results were replicated 3 times and representative leaves are shown. **A**, wildtype; **B**, *srg3*; **C**, *vdac* #6; **D**, *vdac* #13; **E**, *hsp70-3* #2; **F**, *hsp70-3* #3.

A



B

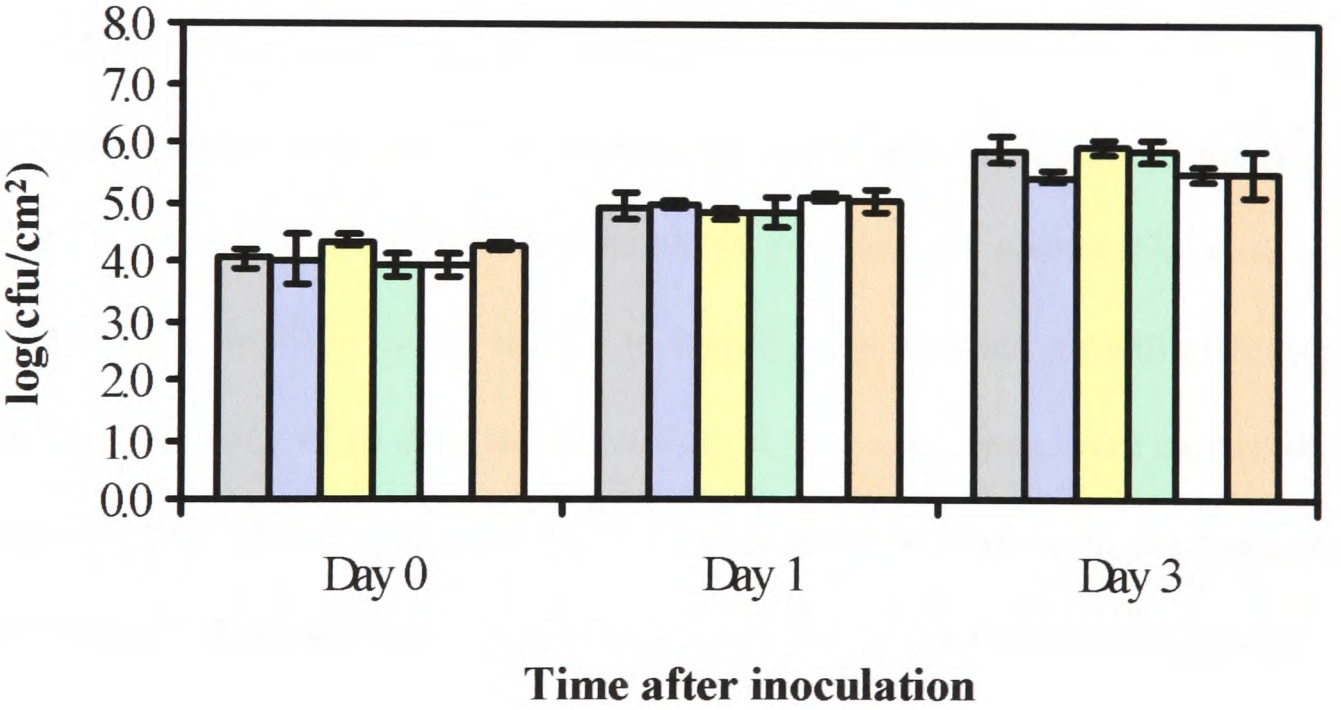


Figure 8.17. Population dynamics of compatible (Pst) and incompatible (Pst *AvrB*) pathogens in wildtype and T-DNA knockout *Arabidopsis* lines.
4 week-old rosette leaves were inoculated with 10^5 cfu/ml of the compatible pathogen, Pst (**A**), or the incompatible pathogen, Pst *AvrB* (**B**). Immediately following inoculation (Day 0) and at 1 and 3 days post-inoculation, equal areas of leaf samples were extracted and the population growth of the pathogen in the leaf was determined by plating dilutions of the leaf homogenate on selective media (see section 2.14.5). Values presented are the means of 3 replicates carried out on independent plants for each line. Wildtype, grey; *srg3*, blue; *vdac* #6, yellow; *vdac* #13, green; *hsp70-3* #2, white; *hsp70-3* #3, orange.

8.3 Discussion

The purpose of the analysis described in this chapter was to isolate *Arabidopsis* homozygous mutant knockout lines for a loss-of-function in putative cell-death related genes identified in previous transcriptomic and proteomic analyses. The screening of publicly available T-DNA insertional mutant lines was the simplest and most direct way of accomplishing this aim, and was not complicated by difficulties which may be associated with stable overexpression of cell death-related genes or the use of currently available inducible overexpression systems. Although several genes were selected for screening of T-DNA insertional mutant lines, only three of these lines were shown to contain an insertional mutation in the gene of interest that disrupted gene expression and which were amenable to a phenotypic analysis. These lines included insertional mutants in *SRG3*, *VDAC/Athsr2*, and cytosolic *Hsp70-3*.

One homozygous mutant individual for *SRG3* and two each for *VDAC/Athsr2* and *Hsp70-3* were identified and subjected to a variety of phenotypic analyses and responses to cell death-inducing stimuli to identify any aberrant growth phenotypes or differential responses when compared to wildtype. These analyses were carried out with the intention that, should cell death-related phenotypes be evident in the lines, a more detailed genetic characterisation would be undertaken to confirm the association of the insertional mutation in the gene of interest with the phenotype and the heritability of the trait. Nevertheless, segregation ratios on a selective agent indicated that each line probably contained only one T-DNA insert.

The insertional mutants analysed did not exhibit any altered growth patterns or visible phenotypic defects (including stem vasculature), and the onset and progression of senescence in each line did not differ from that of wildtype. The response of the T-DNA knockout lines to abiotic stresses, including paraquat treatment and UV-C radiation, did

not differ from wildtype as assayed by visible cellular collapse and ion leakage. Finally, the appearance and progression of the hypersensitive response in the mutants did not differ from wildtype. Taken together, these results demonstrate that the T-DNA insertional mutants studied were not apparently affected in the execution or progression of cell death in response to a range of abiotic and biotic stimuli, and were capable of completing the normal life cycle compared to wildtype plants. It is possible that the induction or execution of PCD was affected in the knockout mutants and that cell death by necrosis (disorganised cell death that is not controlled by a genetic programme, see Chapter 1) occurred in these lines in response to abiotic and biotic stress. In the absence of a definitive experimental test that can distinguish between programmed cell death and necrotic cell death in whole plants (i.e. since single cell morphological analysis is not possible), this possibility cannot be discounted.

The three genes that were studied in this analysis, *SRG3*, *VDAC/Athsr2*, and cytosolic *Hsp70-3*, were selected because they exhibited altered expression levels at either the transcript or protein level during PCD in *Arabidopsis* cell suspension cultures. Specifically, *SRG3* transcripts are induced at the onset of senescence (Callard *et al.*, 1996), and the steady-state levels of these transcripts were increased during heat-induced PCD, as detected by microarray analysis and confirmed by competitive RT-PCR analysis (see Chapter 4). An analysis of the SRG3 protein sequence reveals that it bears highest homology to glycerophosphoryl diester phosphodiesterases, enzymes that catalyse the breakdown of phospholipids (Tommassen *et al.*, 1991) and are induced in response to phosphate starvation in *Bacillus subtilus* (Nilsson *et al.*, 1994; Antelmann *et al.*, 2000). Recently, the activities of both intracellular and extracellular glycerophosphoryl diester phosphodiesterases have been characterised in carrot cells, and the authors suggest that these enzymes may be involved in membrane disruption and

turnover during germination, starvation, senescence, and tracheary element cell death (van der Rest *et al.*, 2002). Intriguingly, the expression of a cytosolic lipase gene was recently shown to be upregulated at the onset of carnation petal senescence and was therefore suggested to be a key event in mediating the onset of senescence (Hong *et al.*, 2000). The protein encoded by *SRG3* is also predicted to be cytosolic, as indicated by the absence of signal peptides or transit sequences (PSORT; $p<0.05$) (http://psort.ims.u-tokyo.ac.jp/). The upregulation of *SRG3* transcript expression at the onset of *Arabidopsis* senescence (Callard *et al.*, 1996) and following heat-induced PCD may indicate that the *SRG3* gene product functions universally in plant PCD in the turnover of membrane phospholipids during cellular breakdown. In any case, a loss-of-function mutation in this gene is not sufficient to affect cell death, indicating that its function can be complemented by other genes or that it is not essential to cellular function.

Similarly, the steady-state levels of cytosolic *Hsp70-3* transcripts were identified as being downregulated following heat-induced PCD by microarray analysis. The availability of a T-DNA insertional mutant in *Hsp70-3* prompted an investigation into whether a loss-of-function of this gene affected plant PCD. Cytosolic Hsp70 is known to be a negative regulator of mammalian cell death, serving to bind and inhibit both Apaf-1 and AIF, and a downregulation in expression of this gene is necessary for the progression of cell death (Saleh *et al.*, 2000; Ravagnan *et al.*, 2001). Moreover, these authors demonstrated that overexpression of Hsp70 is sufficient to prevent cell death in mammalian cells. Given the downregulation of the Hsp70-3 steady-state mRNA transcript levels following heat-induction of PCD — which seems counter-intuitive to a normal heat response — it was tempting to speculate that this protein may also act as a negative regulator of plant cell death and that its downregulation allows the cell death programme to be executed. If this were the case, then knockouts in *Hsp70-3* would be

expected to be sensitised to cell death-inducing stimuli, as in mammalian systems (Ravagnan *et al.*, 2001). However, the cell death-related phenotypes and responses of an insertional mutant line in this gene did not appear to be significantly different from wildtype. There are several possible explanations of this result : (1) Cytosolic Hsp70-3, specifically, does not play a similar inhibitory role in PCD in plants as in animals; (2) Another member of the cytosolic Hsp70 gene family may be involved in plant PCD (Sung *et al.*, 2001); (3) Other plant heat shock proteins, but not of the Hsp70 family, may be involved in regulating plant PCD (for example, Hsp17.6 identified in as being downregulated during PCD; see Chapter 5); or (4) The role of heat shock proteins as regulators of cell death is not conserved between animals and plants. It is not possible to differentiate between these explanations with the preliminary results obtained in this analysis, and evidence for a potential role of heat shock proteins in regulating plant PCD awaits further investigation.

VDAC/Athsr2 transcript abundance has been shown to increase during the *Arabidopsis* HR (Lacomme and Roby, 1999). In spite of the existence of several other *VDAC* genes in the *Arabidopsis* genome, and although the steady-state transcript levels of this gene were not modulated during heat- and senescence-induced PCD (see Chapter 4), this specific isoform increased in relative abundance at the protein level during PCD in *Arabidopsis* cell suspension cultures (see Chapter 6). Taken together, these results strongly suggested that this specific VDAC isoform may play a cell death-specific role, similar to that described in animal systems (Tsujimoto and Shimizu, 2002). However, an insertional mutation in this specific isoform did not appear to affect the onset or progression of cell death, even during the HR and senescence — systems in which the expression of this gene or protein, respectively, are specifically modulated. Therefore, these results once again suggest that, if VDAC/Athsr2 does play some PCD-related role

in plants, its function may be redundant and perhaps provided by another member of this gene family in the insertional mutant line. Alternatively, as in mammalian cells, there may be several pathways leading to the induction and execution of cell death (Hengartner, 2000), and a disruption in a component of one of these pathways may not be sufficient to affect the cell death response.

CHAPTER NINE

9. General discussion

In order to identify and investigate the role of genes and proteins that may be involved in the induction and execution of plant programmed cell death, an *Arabidopsis* cell suspension system was established in which PCD could be induced by two unrelated stimuli, heat treatment and senescence. These experimental systems were used as a source of RNA for both a custom cDNA microarray analysis of changes in select transcript profiles during plant PCD, and a genome-wide analysis of changes in steady-state transcript levels with the Affymetrix ATH1 GeneChip array. As a first step towards investigating post-transcriptional and post-translational mechanisms that may also be important in plant PCD, a 2-D gel analysis was used to examine changes in the total cellular protein profile in cells undergoing PCD. Collectively, the transcriptomic and proteomic analyses of plant PCD were successful in identifying numerous genes and proteins that may be involved in this process, from potential signalling components to enzymes involved in cellular breakdown and the execution phase of PCD.

It is recognised that these approaches, while instructive, are not exhaustive. Other genes and proteins involved in the induction and execution of plant PCD may not be identified by these studies. However, an analysis of the functional classes of genes and proteins identified in this work reveals that many of them have previously been implicated in defense, oxidative stress, abiotic stress (such as wounding), and senescence responses. This suggests that common regulatory mechanisms are involved in all of these responses that can lead to PCD, and may include both reactive oxygen species (ROS) signalling and crosstalk between plant hormone signalling pathways. Based on the genes identified in these approaches, a transient expression system using

Arabidopsis protoplast cultures and an analysis of T-DNA knockout mutants were used to further investigate the potential role of selected genes of interest in plant PCD.

9.1. Building a molecular model of plant PCD

The genes and proteins identified in this study, which exhibited significant changes in expression following the induction of PCD, include components that may function in the initial generation and perception of a PCD-inducing cellular signal, to those that transmit the cellular signal and eventually lead to a controlled destruction of the cell. The putative roles of each of these proteins in plant PCD has been discussed in detail in the appropriate chapters and will not be elaborated on here. An integrative, speculative model of how these proteins may be involved in the perception, transmission, and downstream responses of plant PCD is presented in Figure 9.1.

The perception of an intracellular (e.g. nutrient starvation) or extracellular (e.g. heat) stress that serves to trigger PCD is likely to be followed by changes in the expression and/or activity of a variety of genes and proteins that serve to both transmit the stress signal and attempt to mitigate its effects. It is important to recognise that the degree of stress must also be perceived by the cell, since milder stresses may be accommodated while more severe stresses may induce a cell death programme. The perception of a stress associated with increased levels of ROS is likely to be followed by the induction of antioxidant enzyme activity (see sections 4.3.4, 5.3.5, and 6.3.3), such as catalase, MnSOD, and various components of the glutathione/ascorbate cycle. Ca^{2+} signal transduction is also likely to play a role in transmitting the stress response throughout the cell in some instances (see section 4.3.6) (Lam *et al.*, 1999a). In addition, the induction of defense responses can occur, including the synthesis of pathogenesis-related proteins and genes that have been traditionally associated with the HR, but which have also been shown to be induced in non-HR systems where PCD occurs (such as during senescence and following abiotic stress). These gene products may function both

in protecting vulnerable tissues from pathogen infection and also in the regulation of the cell death programme (see section 5.3.4).

The induction of PCD is likely to involve additional signal transduction mechanisms and other second messengers. These may include the activity of two-component response regulators, such as ARR4 (see section 5.3.2.4), and ion fluxes and associated changes in intracellular pH (see section 5.3.2.3). Additionally, the import of hexoses via monosaccharide transporters, such as STP4 and STP14, may be important in both providing a source of energy for the increased metabolic activity in stressed or dying cells, but may also serve as a modulator in the regulation of gene expression (see section 5.3.6). Phospholipase activity, in addition to functioning in the breakdown of membrane lipids, will also release phospholipid molecules that then can play a role in intracellular signalling and provide substrates for hormone biosynthetic pathways, for example that of jasmonic acid (see section 5.3.2.1).

Stress response pathways, extracellular signals, and signal transduction cascades may indirectly or directly signal in the following ways: To the nucleus to effect changes in gene expression; to the mitochondrion, which may then serve as an integrator of the cellular response; and/or to affect the activity of cytosolic enzymes and proteins. In the first case, transcriptional activators may be required to induce PCD-associated processes that are under transcriptional control. Such transcription factors may include KNAT2, STZ/ZAT10, EREBP family members, homeobox leucine zipper proteins, and NAC-domain proteins (see sections 4.3.6 and 5.3.3). With respect to mitochondrial signalling, changes in specific mitochondrial protein abundance or form may be important in regulating and executing PCD (see section 6.3). Mitochondrial MnSOD may serve to prevent oxidative damage to this organelle during PCD, and lipoamide dehydrogenase possibly functions in a redox-sensitive signalling mechanism. Alterations in VDAC

abundance and/or conformation may be one of the means by which the mitochondrial permeability transition and/or the release of mitochondrial proteins, such as cytochrome *c*, is mediated during plant PCD. Additionally, a relative decrease in ANT protein abundance and/or activity may serve to decrease the cellular ATP/ADP ratio, which could function as an important signalling mechanism in the regulation of plant PCD (see section 4.3.3).

A variety of cytosolic processes are likely to be important in the induction, execution, and degradation phases that occur during PCD. The adaptation of aconitase function as an Iron Regulatory Protein to increase free cellular iron concentrations relative to cellular protein content could be an important mechanism by which cellular destruction is mediated (see section 6.3.4). Additionally, the induction of cysteine protease activity may play a role in inhibiting or activating other proteins, as well as playing a general role in cellular degradation (see section 4.3.5). Finally, a downregulation of cytosolic heat shock protein expression, such as that of Hsp70 or Hsp17 family members, may be necessary to allow the execution and progression of PCD in plants (see section 4.3.6 and 5.3.6).

Finally, PCD induction and execution in plants possibly involves extracellularly-generated molecules that signal to neighbouring cells to amplify and/or transmit the cell death response. Such extracellular ligands may include a variety of extracellular glycoproteins and oligosaccharide molecules (see sections 6.3.6 and 5.3.2.5). These may be generated, in part, by the action of extracellular subtilisin-like proteases (see section 5.3.2.2), chitinases, and glucanases (see section 5.3.4.2), that serve to cleave and release components of the cell wall and extracellular matrix-associated proteins.

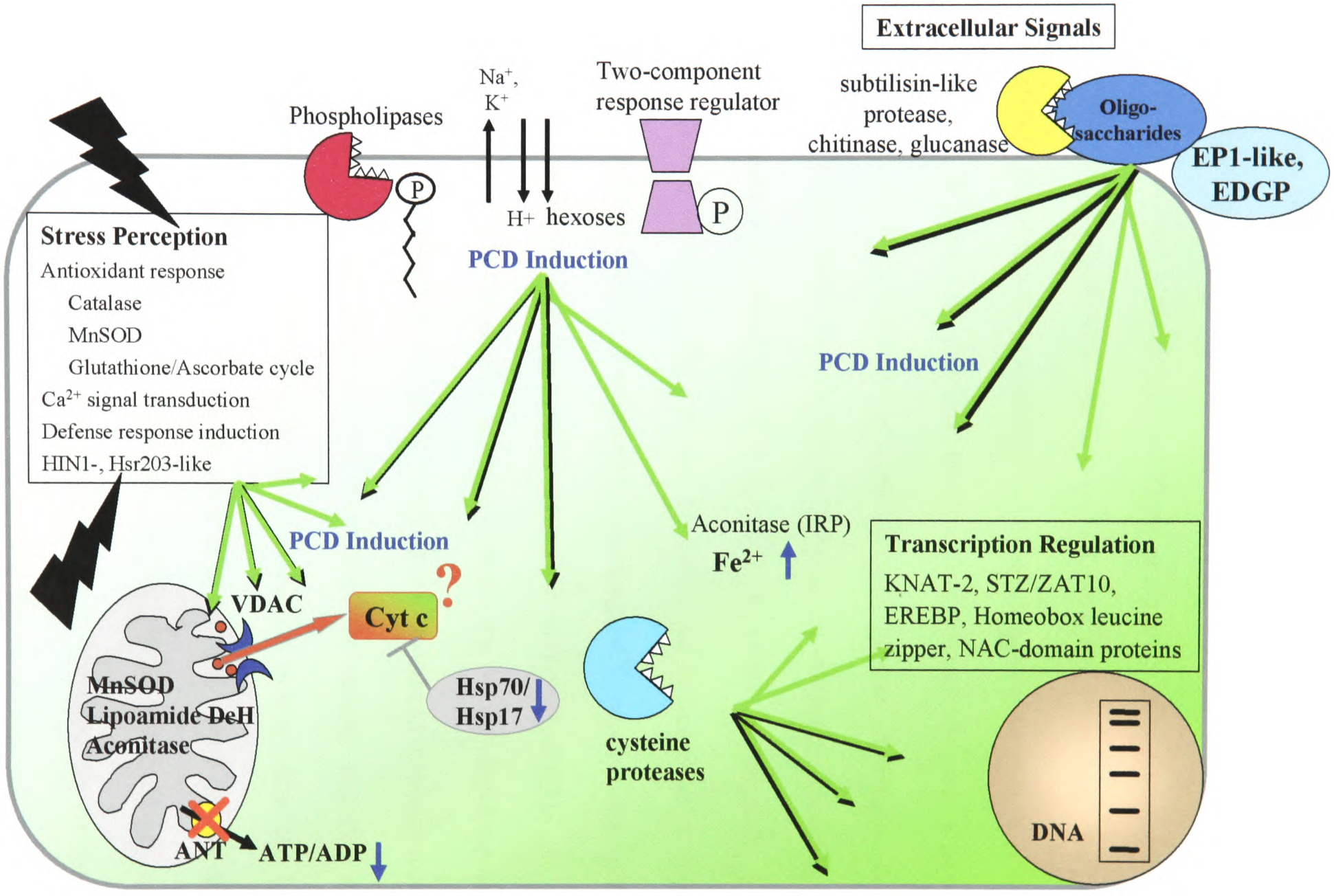


Figure 9.1. An integrative, speculative model of plant PCD.
Gene products identified in transcriptomic (Chapters 4 and 5) and proteomic (Chapter 6) analyses of heat- and senescence-induced PCD in *Arabidopsis* cell suspension cultures that may function in the induction and execution of plant PCD. For a detailed description, see section 9.1.

9.2. Crosstalk between pathways leading to PCD may involve hormone signalling and reactive oxygen species

It was striking to note that many of the genes whose steady-state transcript levels were upregulated during heat- and senescence-induced PCD (Chapters 4 and 5), and some of the proteins identified by a 2-D gel analysis in these systems (Chapter 6), have previously been implicated in antioxidant defense responses, the hypersensitive response, or general pathogen defense. These observations are similar to those described in a study of transcript profiles in senescing tobacco leaves, which showed the upregulation of many defense- and pathogenesis-related transcripts (Obregon *et al.*, 2001). In UV-irradiated *Arabidopsis*, the induction of transcripts encoding pathogenesis-related proteins was also observed (Surplus *et al.*, 1998). Additionally, a disruption of the mitochondrial cytochrome pathway by antimycin A treatment in cultured tobacco cells caused the induction of transcripts encoding ACC oxidase and lipases that have been traditionally associated with senescence (Maxwell *et al.*, 2002).

In addition to the studies described above, the use of cDNA or oligonucleotide microarrays to investigate changes in transcript expression in response to a whole range of environmental and physiological treatments or during specific developmental transitions has become increasingly adopted in plant biology over the last few years (Harmer, 2000). Such studies have demonstrated that many genes are commonly regulated in response to oxidative stress, abiotic stress, and pathogen challenge. For example, in *Arabidopsis* cell suspension cultures exposed to oxidative stress by incubation with H_2O_2, 18% of the induced transcripts have been previously implicated in cell rescue/defense (Desikan *et al.*, 2001). Similarly, in tobacco leaves acclimated to oxidative stress, many of the differentially expressed genes included pathogenesis-related proteins such as chitinases, and those encoding enzymes involved in

phenylpropanoid and lignin biosynthesis (Vranova *et al.*, 2002). With respect to wounding and abiotic stress, a microarray analysis of wounded *Arabidopsis* plants has revealed the induction of many genes traditionally associated with pathogen responses and with cell wall modification, as well as antioxidant responses (Cheong *et al.*, 2002). Conversely, a microarray analysis of gene expression in *Arabidopsis* following pathogen challenge or treatment with salicylic acid, methyl jasmonate, or ethylene, showed overlap in the induction of oxidative stress-, defense-, and senescence-related transcripts (Schenk *et al.*, 2000). A detailed survey of *Arabidopsis* stress-responsive transcription factors has revealed that stress responses, senescence, and defense responses may all share overlapping pathways of transcriptional activators (Chen *et al.*, 2002).

Taken together, these studies suggest that considerable overlap in gene expression exists between stress, defense, and senescence pathways. It can be argued that the common denominator in all of these processes, whether specifically examined in a particularly study or not, is the capacity to induce programmed cell death (Pennell and Lamb, 1997). Indeed, it is possible that, in many of the stress and HR-related studies referenced above, PCD was occurring in the experimental system under study but was not recognised or acknowledged by the investigator. Therefore the common induction of these genes may relate to a possible role in the cell death programme. This may include roles not only in the regulation or execution of cell death, but also in the associated processes of cellular destruction and degradation (Obregon *et al.*, 2001). Additionally, as mentioned earlier (see section 5.3.4), the induction of defense-related transcripts during senescence or wounding may reflect a mechanism to protect vulnerable or dying tissues from pathogen infection (Hanfrey *et al.*, 1996).

The substantial overlap in gene expression between these PCD-associated processes raises the obvious question of what the common inducing signal(s) might be.

In this respect, the possibilities are complex and, as noted by Reymond *et al.* (2000), similar gene expression profiles in response to wounding or abiotic stress do not necessarily imply that they are regulated by the same signal. There have been several reviews aiming to unravel the crosstalk between signalling pathways involved in plant defense and cell death (Genoud and Metraux, 1999; Beers and McDowell, 2001; Loake, 2001). The hormones salicylic acid (SA), ethylene, and jasmonic acid (JA) have all been implicated as playing a role in these processes, sometimes synergistically and sometimes independently of one another. Certainly, the upregulation of transcripts for ACC oxidase during heat- and senescence-induced PCD indicates that ethylene signalling may play a role in these systems (see section 5.3.6), and the action of phospholipases to generate phospholipids for oxylipin/JA synthesis may also support a role for this hormone in *Arabidopsis* PCD (see section 5.3.2.1). These hormones have also been implicated, again either synergistically or independently, in signal transduction during defense responses (reviewed by Pieterse and van Loon, 1999; McDowell and Dangl, 2000). Intriguing evidence in support of the notion that SA may play a role in many types of cell death in plants can be inferred from a recent study of *Arabidopsis* mutants defective in SA signalling. In these plants, leaves undergo normal senescence as indicated by chlorosis, but display delayed visible cell death (Morris *et al.*, 2000), suggesting that SA may be a trigger necessary for PCD as the endpoint of senescence.

There is also a considerable amount of evidence that ROS play a key role in mediating both animal (Fleury *et al.*, 2002) and plant PCD (Lam *et al.*, 1999a). ROS have also been implicated in plant defense and the hypersensitive response (reviewed by Sandermann, 2000; Beers and McDowell, 2001). Therefore, it is possible that the generation of ROS — which can be inferred to be a component of the heat- and senescence-induced PCD systems from the upregulation of antioxidant genes and

proteins (see Chapters 4, 5, and 6) — may also be a common signalling mechanism associated with PCD in response to abiotic stress, as the endpoint of senescence, and the hypersensitive response. It is also important to note that synergistic action between ROS and plant hormones, particularly salicylic acid, may be especially important in regulating plant PCD (Beers and McDowell, 2001).

It is interesting to consider whether ROS constitute a common regulator of plant PCD, as has been suggested to be the case in yeast cell death (Madeo *et al.*, 1999), or if a plant cell death programme independent of ROS activation also exists. In the former case, the common induction of genes during stress-, senescence-, and HR-associated PCD could be attributed to their regulation by ROS. In the latter case, however, the induction of genes that function universally in plant PCD would be expected to occur independently of the presence of ROS. To differentiate between these possibilities, it would be useful to examine changes in transcript and protein expression during the induction and execution of plant PCD in response to a stimulus that does not involve the production of ROS. As the many examples of antioxidant gene induction in response to abiotic stress, senescence, and defense would indicate, however, it is difficult to design an experimental system capable of separating out the antioxidant and PCD responses. Even the induction of senescence and PCD by nutrient starvation (as occurs in 13 to 14 day-old *Arabidopsis* cell suspension cultures, see section 3.2) has been shown to induce oxidative stress in glucose-deprived human cells (Blackburn *et al.*, 1999) and in starved maize root cells (Basset *et al.*, 2002). An alternative, therefore, might be to undertake an investigation of gene and protein expression under a mild oxidative stress. A comparison of modulated transcripts and proteins in this system to that of a PCD-induced system could conceivably identify those transcripts and proteins altered in response to oxidative stress alone.

There were several reasons why the above approach, while considered, was not adopted in this study. First, it is entirely possible that ROS are a core component of the plant cell death programme, perhaps mediating signals through the mitochondrion or affecting mitochondrial function leading to PCD (reviewed by Beers and McDowell, 2001; Lam *et al.*, 2001). A separation of ROS- and PCD-associated changes may not only prove futile, but may falsely eliminate many genes and proteins that are involved in plant PCD and yet also associated with oxidative stress. Secondly, mild oxidative stress treatments have been demonstrated to repress the expression of many classes of genes in mammalian systems, including those that are associated with cell death (Morel and Barouki, 1999). Thirdly, and related to the previous point, even oxidative stress-related gene induction that is unrelated to PCD may require a relatively severe oxidative stress. This can be seen, for example, in contrasting the apparent non-responsiveness of *Arabidopsis* MnSOD transcript, protein, and activity levels to a wide range of oxidative stress-inducing stimuli in whole plants (Kliebenstein *et al.*, 1998), with the apparent upregulation of MnSOD transcript and protein levels following a heat treatment or during senescence that were sufficient to induce PCD (see Chapters 4 and 6). Therefore, effecting changes in expression of all genes associated with ROS may require more than a mild stress treatment — it may require a treatment of sufficient severity such that PCD will be inevitably be induced.

9.3. An assessment of experimental techniques to study plant programmed cell death

9.3.1. The use of an *Arabidopsis* cell suspension system to study plant PCD

The use of an *Arabidopsis thaliana* cell suspension system to investigate genes and proteins that may be involved in plant PCD involved the establishment of more than one method of inducing PCD, such that responses that may be common to plant PCD could be distinguished from those that were specific to an inducing stimulus. In this respect, a variety of treatments were investigated. Incubation of 6 day-old cell cultures at 55°C for 10 min or natural culture senescence that occurred due to nutrient exhaustion at 13 to 14 days after subculturing were both accompanied by a quantitatively reproducible occurrence of programmed cell death (see section 3.3). Cell suspension cultures provide a model system to work with in that the number of cells that have undergone PCD can be quantified using morphological observations and vital staining, and the occurrence of PCD confirmed by biochemical indicators such as DNA laddering.

There are some disadvantages to working with the system described that must be acknowledged. First, the reponses that occur in cells in suspension may not necessarily reflect those that occur *in planta*. Secondly, the induction and execution of PCD following heat treatment and during senescence were not synchronous within the culture. This means that samples taken at a particular time point represented an average of the cellular responses in the culture and, as such, included healthy cells, cells in which PCD has just been induced, cells in the process of executing PCD, and dead cells. It is difficult, therefore, to ascribe a role for the identified genes and proteins in either the upstream or downstream responses of PCD. Ideally, a treatment that leads to a

synchronous response from which various time points could be sampled would be established. This would allow for a more detailed understanding of the cascade of molecular events that occur during the induction and execution of plant PCD. Finally, it is acknowledged that stress responses are likely to be associated with both a heat treatment and nutrient starvation leading to senescence (as inferred, for example, by the increased expression of antioxidant enzymes in both systems). Although these stresses are sufficient to lead to PCD in both systems, one cannot exclude the possibility that identified genes and proteins play a role only in the response of the cells to a common stress and are not directly involved in the pathway of PCD.

9.3.2. Identifying PCD-related genes and proteins through transcriptomic and proteomic analyses

The work presented in Chapters 4, 5, and 6 demonstrated the use of cDNA and oligonucleotide microarrays to investigate changes in transcript expression profiles during plant PCD, and a proteomic 2-D gel analysis to identify proteins increased in relative abundance following the induction of PCD by heat and senescence in an *Arabidiopsis* cell suspension system. These approaches are complementary to one another in allowing for the detection of transcriptional, post-transcriptional, and post-translational regulation of gene expression (see section 6.1). In animals, the existence of both types of gene regulation have been demonstrated to be important in the induction and execution of PCD (Hengartner, 2000). Nevertheless, it must be acknowledged that the activity of proteins involved in plant PCD may not necessarily be regulated at the level of transcript or protein expression. There are many examples in animal PCD in which regulation and execution of this process occurs independently of gene expression or protein modification (see Chapter 1) (Hengartner, 2000). The formation of complexes of existing cellular proteins (e.g. the 'apoptosome' consisting of cytochrome *c*, Apaf-1,

and caspase-9), the interaction of pro- and anti-apoptotic proteins with one another (e.g. that of Bcl-2 family members with one another or of Smac/DIABLO with IAPs), and the movement of proteins between subcellular compartments (e.g. the release of mitochondrial intermembrane space proteins into the cytosol or their translocation to the nucleus) are all mechanisms involved in animal PCD. Similar mechanisms of regulating the activity of proteins involved in plant PCD may exist. Therefore, while transcriptomic and proteomic analyses are appropriate to investigate aspects of the mechanisms involved in the induction and execution of PCD, other approaches, such as the identification of PCD-inducing molecules using cell-free systems and cell fractionation studies to investigate changes in protein localisation during PCD, will be required to fully elucidate the molecular mechanisms underlying this process.

9.3.3. Functional analyses of genes of interest using transient expression systems and gene knockout mutants

The expression pattern of a transcript or protein during plant PCD is suggestive of a role for the gene product in this process. However, proof of a functional role for a gene product in PCD requires the demonstration that PCD can be affected by the expression of this gene. To this end, an attempt to investigate the effect of putative cell-death related genes on plant PCD was undertaken with both transient gene expression analyses in *Arabidopsis* protoplasts (Chapter 7) and T-DNA insertional mutants in genes of interest (Chapter 8).

9.3.3.1. Transient gene expression in protoplasts as a high-throughput screen to investigate the role of PCD-related genes

The use of transient expression of genes of interest in protoplasts allows for the routine and relatively rapid screening for functional effects of genes of interest prior to a

more detailed biochemical and genetic investigation. This method was therefore investigated as a means to study the functional role of genes of interest in plant PCD. However, the system was unsuccessful in that the quantification of dead protoplasts was inconsistent and that even the expression of a positive regulator of plant PCD, mammalian Bax, did not cause a detectable increase in protoplast cell death (see section 7.3). One of the difficulties of this approach is that only a proportion of the protoplasts are transformed, such that typically only between 40 to 60% of protoplasts actually express the transgene (Axelos *et al.*, 1992; Abel and Theologis, 1994). Ideally, one would only examine the transformed protoplasts for phenotypic effects of transgene expression. This could be achieved by co-transformation of protoplasts with a selectable marker such as yellow fluorescent protein (YFP), whose emission spectra does not overlap with the vital stain, fluorescein diacetate (FDA). Using confocal microscopy, this would allow simultaneous assessment of which cells are transformed and the viability of those cells following PCD-inducing stimuli.

However, the system described above has the drawback of not lending itself easily to high throughput analysis — the original objective behind developing this screen. An alternative appraoch would be to use fluorescence activated cell sorting (FACS) (Herzenberg *et al.*, 2002), a technique that has previously been applied in the study of tobacco PCD (O'Brien *et al.*, 1997; O'Brien *et al.*, 1998b). FACS analysis would allow for the rapid and automated counting of YFP-positive (transformed) cells and FDA-positive cells in an efficient and accurate manner. If this system could be successfully established, it would represent a novel technology that could be adapted for high-throughput screening of functional effects of genes of interest on plant PCD.

9.3.3.2. The use of gene knockouts to investigate the role of PCD-related genes

The analysis of *Arabidopsis* T-DNA knockout mutants in three genes of interest, *SRG3*, *VDAC/Athsr2*, and *Hsp70-3*, did not lead to the detection of any cell death-related phenotypes in these plants. One explanation for this is that the genes whose expression has been disrupted may be members of a functionally redundant gene family (see section 8.3). Alternatively, as in mammalian cells, there may be several pathways leading to the induction and execution of cell death (Hengartner, 2000), and disruption of a component of one of these pathways may not be sufficient to affect the cell death response. These latter points are salient when considering what constitutes an appropriate experimental system to assay the functional role of specific genes in plant PCD. It is acknowledged that the expression pattern of a transcript or a protein, though suggestive of a role for it in a given process, does not provide a causal link (Krysan *et al.*, 1999). Therefore, it remains possible that the three genes studied in these analyses, which were chosen primarily on the basis of changes in their expression profiles during plant PCD, do not actually function in this cellular process, hence the absence of phenotypic effects in the T-DNA insertional mutant lines. Perhaps T-DNA knockouts in other cell death-related genes would exhibit altered sensitivity to cell death-inducing stimuli.

Nevertheless, in spite of the increasing popularity of utilising T-DNA insertional mutants as experimental tools in functional genomics (Parinov and Sundaresan, 2000), there is also a growing body of evidence demonstrating that the majority of these insertional mutant lines do not give rise to visible and informative phenotypes (Bouche and Bouchez, 2001). In a recent analysis of 200 random *Arabidopsis* knockout lines, less than 2% displayed morphological alterations. Importantly, it is generally felt that hypothesis-driven examination for a knockout phenotype, based on the expression

pattern of a transcript or protein, is likely to be the most successful method of identifying a mutant phenotype. Though this was precisely the nature of the analysis undertaken in this study, this method was unsuccessful in establishing a link between a PCD-related expression pattern and functional effect for the genes of interest.

It has been suggested that examining mutant lines grown under an exhaustive range of environmental conditions may reveal conditional phenotypes since, generally, higher plants are remarkable in their ability to adapt their physiology and growth to a wide range of stresses and limitations (Bouche and Bouchez, 2001). More often than not, however, the vast amount of functional redundancy that is likely to exist in *Arabidopsis* genes means that the combination of independent knockouts in similar genes (for example, crossing mutants in all of the *VDAC* genes) may be necessary to uncover a phenotype (this may, however, result in lethality). Indeed, the fact that approximately 70% of *Arabidopsis* genes are present in more than one copy leads to a potentially vast array of functional redundancy (The *Arabidopsis* Genome Initiative, 2000; Thorneycroft *et al.*, 2001).

Notwithstanding the accessibility and ease of screening for T-DNA knockout lines in any gene of interest, future studies aimed at demonstrating a functional role for a specific gene in plant PCD will undoubtedly require additional approaches. Knockouts in any single gene may not give rise to phenotypic effects due to either redundancy of the gene or the molecular pathways of PCD. Thus, an overexpression system, similar to those that are routinely employed in studies of mammalian cell death, may be necessary to uncover functional effects. Stable transformants expressing genes of interest could be considered, though may be hampered by lethality associated with the overexpression of positive cell death regulators. Thus, the development of a reliable, inducible overexpression system which does not in itself lead to any cell death-related effects

(Kang *et al.*, 1999) would be of considerable value. In this regard, such an inducible overexpression vector has been recently described (Pautot *et al.*, 2001), and this may be an ideal experimental system for an analysis of putative PCD-related genes. Additionally, molecular and biochemical assays on proteins of interest, including yeast-two hybrid assays to uncover protein-protein interactions and DNA-binding analyses on putative transcription factors, may lead more directly towards an understanding of the functional role of a putative plant PCD-related protein. Specifically, future strategies to investigate the molecular mechanisms of plant PCD and identify the relevant proteins may include the following:

- Detailed proteomic analyses of cell samples undergoing PCD, undertaking sub-cellular fractionation prior to protein isolation. This would help to identify proteins that move between cellular compartments (e.g. the mitochondrion, cytosol, and nucleus) during the induction and execution of PCD. The use of narrower pI ranges (e.g. 4 to 7) would be useful to achieve better resolution of proteins present in lower abundance. Finally, the identification of post-translational modifications (e.g. phosphorylation) on proteins of interest may aid in understanding mechanisms that regulate protein function during PCD.

- The use of a cell-free system (Newmeyer *et al.*, 1994) to fractionate and identify proteins that either promote or inhibit plant PCD, as assayed by a 'read-out' such as nuclear DNA laddering. The addition of purified recombinant proteins to a cell-free system can be used to help confirm their role in PCD, and also to identify interacting proteins by affinity purification.

- An understanding of the mechanism by which mammalian Bax promotes PCD when expressed transgenically in plant cells could be achieved by identifying plant proteins with which Bax associates (for example, by using affinity chromatography).

- To better understand the molecular basis underlying plant PCD mutant phenotypes (for example, that of *lsd1*, *acd11*, *cpr5*, and others; see section 1.4.1), a genetic approach may be employed. This would primarily involve genetic screens for suppressors of these mutant genes to elucidate the biochemical pathways in which the encoded proteins function. A genetic screen for suppressors of the PCD-protective effects of overexpressed *Arabidopsis* Bax Inhibitor-1 (Kawai-Yamada *et al.*, 2001) would enable a better understanding the function of this protein in plant PCD. Finally, *in planta* genetic screens for suppressors of ectopically-expressed mammalian Bax (as opposed to the described screens in yeast systems using plant cDNA libraries; see section 1.4.4) may further help in understanding the mechanism of Bax action in plant cells.

In summary, the work presented in this thesis has identified a number of genes and proteins that may be involved in the induction and execution of plant PCD. A function-based classification of these genes and proteins reveals that common signalling pathways may operate during a range of circumstances leading to PCD. In addition, different approaches to validate the function of these genes and proteins in plant PCD were investigated. In the future, the application of an inducible overexpression system and biochemical and cell-free analyses based on the identified genes and proteins will significantly advance our understanding of the molecular events underlying this essential process in plant development and defense.

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