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A proteomic analysis of plant programmed cell death

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Abstract

Programmed cell death (PCD) is an active cellular suicide that occurs in animals and plants throughout development and in response to both abiotic and biotic stresses. In contrast to animals, little is known about the molecular machinery that regulates plant PCD. We have previously identified transcriptomic changes associated with heat- and senescence-induced PCD in an *Arabidopsis* cell suspension culture [Plant J. 30 (2002) 431]. However, since plant PCD is also likely to involve elements that are regulated post-transcriptionally, we have undertaken a proteomic analysis in the *Arabidopsis* system. We identified 11 proteins that increased in abundance relative to total protein in both treatments despite extensive degradation of other proteins. We argue that some of these proteins are maintained during PCD and may therefore have specific functions in the PCD pathway. The increased abundance of several antioxidant proteins as well as a measured increase in free Fe^{2+} content of the cells indicates an oxidative stress in this system. Several mitochondrial proteins were identified, confirming the importance of this organelle during PCD. We also identified an extracellular glycoprotein that may function in the transmission of a 'death signal' from cell to cell. Putative roles for the identified proteins are presented.

Keywords: *Arabidopsis*; Programmed cell death; Proteomic; Mitochondria

1. Introduction

Programmed cell death (PCD) is an orderly process of cellular suicide that requires active gene expression (Lam et al., 2001). In plants, PCD plays a critical role in a number of developmental events, facilitating the programmed removal of specific cell types that are no longer required (Lam et al., 2001; Gunawardena et al., 2004). PCD is recognised to follow the senescence programme in both petals and leaves (Quirino et al., 2000), though the two processes are distinctly defined (Thomas et al., 2003). In addition, PCD has been implicated as part of the plant response to pathogen invasion, causing the formation of a localised lesion of dead cells that limits cell-to-cell transfer of the pathogen in a process known as the hypersensitive response (HR) (Heath, 2000).

PCD is characterised by a number of hallmark cytological and biochemical features, including a distinct cellular morphology and the production of characteristic DNA "ladders" resulting from the inter-nucleosomal cleavage of nuclear DNA (reviewed in Reed, 2000). The molecular and biochemical events of PCD have been extensively studied in animal cells and a number of key proteins have been identified that regulate and execute cell death. These include casapse proteases, the pro- and anti-apoptotic members of the Bcl-2 family (Kaufmann and Hengartner, 2001), components of the mitochondrial permeability transition pore (PTP) such as VDAC, ANT, and cyclophilin D (Crompton, 1999), as well as several mitochondrial intermembrane space proteins such as cytochrome *c*, Apoptosis Inducing Factor and endonuclease G (Kaufmann and Hengartner, 2001).

Despite the fundamental importance of PCD in plants, comparatively little is known about the molecular mechanisms of plant PCD. Plant genomes do not

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contain obvious homologues to the key animal cell death proteins such as the Bcl-2 family proteins (Arabidopsis Genome Initiative, 2000) (although a family of genes related to mammalian caspases have been identified in plants (Uren et al., 2000)). Biochemical studies of plants been able to establish a causal role for events such as the translocation of cytochrome *c* from the mitochondrion to cytosol (Balk et al., 1999; Sun et al., 1999; Zhao et al., 1999; Xu and Hanson, 2000). In an attempt to identify key plant PCD genes that may function universally during different types of plant PCD, we have previously undertaken a custom microarray analysis of gene expression during PCD in an *Arabidopsis* cell suspension culture (Swidzinski et al., 2002). By identifying mRNA transcripts that changed in abundance following two unrelated PCD-inducing treatments (a brief, mild heat treatment for 10 min at 55 °C and culture senescence) we have been able to discriminate between genes that may be common to a core plant cell death programme and those that are specifically related to the inducing stimulus itself. While this study was successful in identifying several candidate genes whose common up- or downregulation during PCD may indicate a role for their products in plant PCD, it was restricted to elements of the PCD process that are transcriptionally regulated and ignored post-transcriptional and post-translational regulation. Indeed, post-translational events such as proteolytic cleavage and activation and modifications such as phosphorylation are key regulatory events in animal PCD (Hengartner, 2000; Reed, 2000).

As a first step towards identifying such post-transcriptional mechanisms that function during plant PCD, we have undertaken a proteomic analysis of changes in total cellular protein content during both heat- and senescence-induced PCD in an *Arabidopsis* cell culture. Both PCD systems were accompanied by decreased protein content and increased proteolytic activity. Analysis of two-dimensional gel electrophoresis displays of proteins revealed 11 proteins whose abundance (relative to total protein) increased following both treatments. The relative increase of these proteins in both a heat- and senescence-induced PCD system suggests that they may play a general role in the plant cell death programme.

2. Results and discussion

2.1. Changes in total protein content and protease activity during heat- and senescence-induced PCD

Two different treatments were used to induce PCD in a heterotrophic *Arabidopsis* cell suspension. These were: a heat treatment (incubation of a 6-day old cell culture for 10 min at 55 °C with analysis of the cells immediately thereafter) and natural cell culture senescence (13–14-day old cell cultures) (Swidzinski et al., 2002). In both of these systems, 20–40% of cells have undergone PCD as determined by cellular morphology and absence of staining by fluorescein diacetate (data not shown).

Since proteolysis is known to occur during PCD (Beers et al., 2000; Lam and del Pozo, 2000), we compared protein content in control cells to that after the two treatments (Fig. 1(a) and (b)). Total soluble proteins were extracted (in the presence of protease inhibitors) and quantified using a Bradford assay. The total protein content per g of fresh or dry tissue was significantly decreased (*t* test; $p < 0.05$) in both heat- and senescence-induced PCD cultures in comparison to controls. In addition, protease activity (determined as the rate of breakdown of casein) was significantly increased in both heat- and senescence-induced PCD cultures in comparison to control cultures (*t* test; $p < 0.05$) (Fig. 1(c)). These results are indicative of a general proteolytic

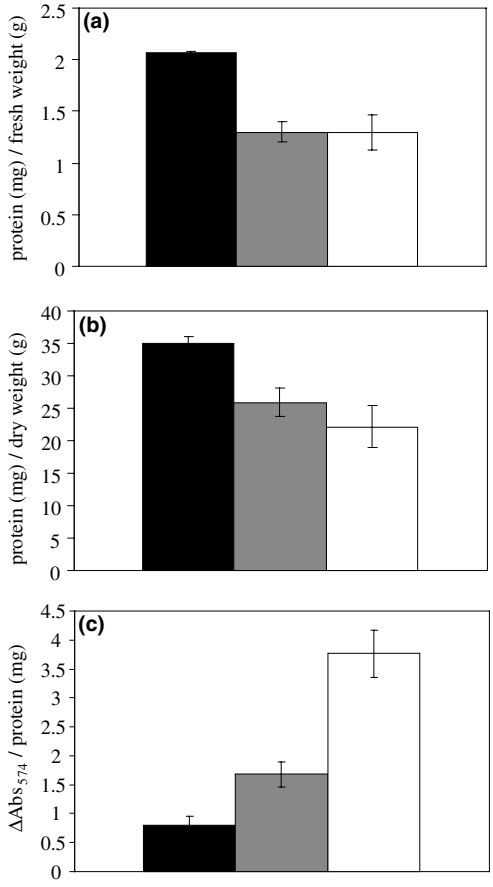

Fig. 1. Changes in total protein content and proteolytic activity during PCD. (a) and (b) Total protein was extracted from 6-day old control (black), 6-day old heat-induced PCD cultures (grey), or 13–14-day old senescence-induced PCD *Arabidopsis* cell suspension cultures (white) in the presence of EDTA and a protease inhibitor cocktail. Total soluble protein is expressed per gram fresh weight (a) and per gram dry weight (b) of each sample. (c) Protease activity in each sample was measured using a casein universal protease substrate.

degradation of protein in the cells undergoing programmed cell death.

This observation is to be expected given that protein degradation is a well recognised feature of PCD, but is worthy of special consideration in the context of the proteomic strategy employed in the present study. The rationale underlying the proteomic approach is to identify proteins that increase in abundance following two independent means of inducing PCD. Such proteins may play an important role in a core PCD pathway common to many inducing stimuli. An apparent increase in the abundance of a protein spot on a two-dimensional gel (relative to total protein) can be due to at least three mechanisms, either alone or in combination. First, increased synthesis of that protein may occur. Second, post-translational modification may alter the position of a protein on a two-dimensional gel such that it appears as a novel spot in comparison to the control gel. Third, selective degradation of other proteins may occur. Given the extent of protein degradation we have observed, it is likely that this latter possibility makes a significant contribution. A selective resistance of a protein to degradation during PCD may imply that the protein is maintained to play an important function during PCD. Post-translational modification may also be a contributing factor. In this system, however, it is unlikely that increased protein synthesis makes a significant contribution to protein abundance, since proteins were analysed after just 10 min of a heat treatment and this is insufficient time for detectable protein synthesis to have occurred.

2.2. Proteomic analysis of heat- and senescence-induced PCD

To identify proteins that are important in the PCD pathway, we fractionated equal amounts of protein from control cells, from heat-treated cells and from senescent cells using two-dimensional gel electrophoresis (Fig. 2). Protein extraction and proteomic analyses were carried out in the presence of protease inhibitors (0.1 mM PMSF; 0.3 μM pepstatin) to minimise proteolytic degradation during protein isolation or during sample preparation for gel electrophoresis. Gels were stained with colloidal coomassie blue and the protein spots quantified and normalised using Z3 software (Compugen, Tel Aviv, Israel). Sets of protein spots that increased in relative abundance (PCD/control) of at least 2-fold in comparison to the control in three replicate gels were identified. The increase in these proteins was statistically significant (t test; $p < 0.05$). From these sets, a subset of proteins that increased in abundance in both the heat-treated cells and the senescent cells was identified.

Twelve protein spots were commonly increased in relative abundance in both treatments relative to the

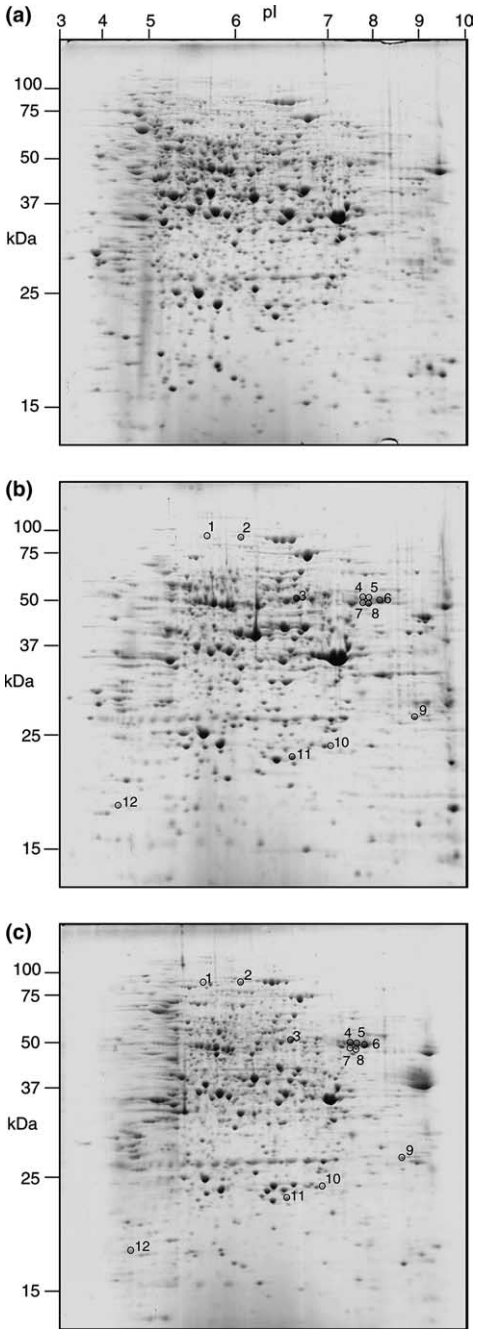

Fig. 2. Two-dimensional PAGE analysis of total cellular protein from control cell cultures and cell cultures undergoing PCD. Total soluble protein extracts from *Arabidopsis* cell suspension cultures (Swidzinski et al., 2002) were isolated from 1 to 2 g of frozen cells using a phenol method (Hurkman and Tanaka, 1986). 800 μg of protein were fractionated by isoelectric focussing (pH 3–10, non-linear gradient) and SDS–PAGE as described previously (Millar et al., 2001). Spot detection, normalised quantitation and gel matching were performed using algorithms contained within the Z3 software package (Compugen, Tel Aviv, Israel). Spots (1–12) identified as being unique to and/or increased in relative abundance in both heat-treated and senescing samples undergoing PCD were excised, digested with trypsin and analysed by MS/MS. Each gel result was repeated in triplicate: (a) untreated, 6-day old cultures; (b) 6-day old cultures incubated for 10 min at 55 °C and sampled immediately thereafter; (c) senescing, 13–14-day old cultures.

control, healthy cell cultures. These spots were excised from the gel, digested with trypsin and the proteins indentified using tandem MS/MS mass spectrometry (Table 1). Four of these spots are isoforms of catalase, while several, including lipoamide dehydrogenase, the voltage-dependent anion channel protein Hsr2, and MnSOD, are mitochondrial proteins. In addition to an EP1-like glycoprotein and a protein of unknown function, we also identified an aconitase protein (that has previously been demonstrated to be present in *Arabidopsis* mitochondria but may also be present in the cytosol (Millar et al., 2001)). The aconitase spot was increased in relative abundance by a factor of 2.9 in the senescence-induced PCD cells, but the spot matching algorithms in the Z3 software were unable to conclusively match this spot between control and heat-induced PCD gels (Table 1).

In addition, we observed the appearance of multiple spots that are the product of the same gene, suggesting that post-translational modification of these proteins had occurred. Spots 4 and 5 both are encoded by the same catalase gene, At1g20620, and spots 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 of approximately twice the molecular weight, the N-terminal domain of which also encodes a putative catalase. This suggests a misannotation of the gene sequence such that either these two domains are functionally separated in vivo or that the larger protein is cleaved to yield a C-terminal catalase enzyme.

2.3. Identification of post-transcriptional events associated with plant PCD

A comparison of the protein abundance changes reported here with transcript abundance changes in the same experimental system that we have previously published (Swidzinski et al., 2002) reveals PCD-related events that are regulated exclusively at the transcriptional level, exclusively at the post-transcriptional level, or both. Of the proteins we identified in this study, we have previously analysed the abundance of the following transcripts: *Catalase 3*, *VDAC/AtHsr2* and *MSD1* (At3g10920). The *VDAC/AtHsr2* transcript did not significantly change in abundance whereas the protein level was increased (from undetectable levels in the control experiment) indicating a post-transcriptional control of protein abundance. In contrast, *Catalase 3* transcript and Catalase 3 protein were increased suggesting a transcription-mediated change. MnSOD (At3g10920) protein levels were increased in both heat- and senescence-induced PCD but *MSD1* transcript levels increased (2.7-fold) only in heat-induced PCD (in senescence-induced PCD the transcript level actually decreased 1.5-fold). For all other proteins we identified in this study, cDNAs were not represented on the custom array presented in Swidzinski et al. (2002).

2.4. Relative increases in antioxidant enzymes are associated with plant PCD

The increased relative abundance of four catalase isoforms and two forms of mitochondrial MnSOD in both heat- and senescence-induced PCD (Table 1) is consistent with the observation that oxidative stress is implicated in the induction/execution of PCD (Swidzinski et al., 2002). Previous studies have shown that transgenic tobacco plants with reduced catalase levels show increased susceptibility to stress conditions (Willekens et al., 1997) and are hyper-responsive to pathogen attack (Mittler et al., 1999), indicating that this enzyme plays a central role in antioxidant defense. The identification of two isoforms of the same protein suggests that post-translational modification of MnSOD may be important during plant PCD, and that perhaps such modifications 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 during this time.

Changes in levels of reactive oxygen species (ROS) and oxidative stress are often observed in association with plant programmed cell death. However, it remains unclear whether increased ROS are generated actively as a signal to trigger the PCD pathway (Hildeman et al., 2003), or whether ROS are a by-product of the stress condition that induces PCD. If the former possibility is correct, then one would expect to see a down-regulation of antioxidant enzymes such that the ROS signal is not quenched. Such a down-regulation has been observed during developmental PCD in barley aleurone cells (Fath et al., 2001). The fact that we have observed increases in the abundance of antioxidant enzymes suggests that this mechanism is not operating in our system. In fact, the cells appear to be mounting an active antioxidant response to counter the oxidative stress caused by the PCD-inducing stimuli. Presumably, the response is insufficient and the extreme oxidative stress goes on to trigger PCD. As such, therefore, it is unlikely that the antioxidant enzymes identified in this study are a direct part of the PCD pathway.

2.5. Specific mitochondrial proteins are associated with PCD

It is remarkable that of the eight gene-products whose relative abundance increased following the induction of PCD, four are targeted to the mitochondrion. This may either imply that mitochondrial proteins are particularly important during PCD or that proteins within the

Table 1
Identification of increased 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	p*I* of match	MW (Da) from gel	p*I* from gel	Spot ratio H/C[c]	Spot ratio S/C[c]
1	–	–	Not identified	–	–	–	–	–	–	–	–
2	AY050431	At4g26970	Aconitate hydratase	11	12	108,311	6.85	100,000	6.10	–	2.9
3	CAA11554	At3g17240	Mitochondrial lipoamide dehydrogenase	19	48	49,987	6.00	53,000	6.60	U[d]	U
4	NM_101913	At1g20620	Expressed protein (catalase 3)	8	15	57,059	7.31	54,000	7.80	2.4	13.3
5	NP_564120	At1g20620	Expressed protein (catalase 3)	7	13	57,059	7.31	54,000	7.90	2.2	9.4
6	NP_178006	At1g78850	Putative EP1-like glycoprotein	7	12	49,020	7.82	53,000	8.10	2.0	4.4
7	NM_101914	At1g20630	Expressed protein (catalase 1)	8	15	57,068	6.95	51,000	7.80	U	U
8	AAF79625	–	Expressed protein (catalase 3)	11	10	117,407	8.32	51,000	7.90	4.6	3.2
9	NP_197013	At5g10590	Voltage-dependent anion-selective channel protein Hsr2	4	16	29,193	7.85	30,000	9.00	U	U
10	NP_187703	At3g10920	Putative (Mn) superoxide dismutase	5	19	25,428	8.47	24,000	7.00	2.8	7.4
11	NP_187703	At3g10920	Putative (Mn) superoxide dismutase	4	19	25,428	8.47	23,000	6.70	2.9	2.6
12	NP_568698	At5g48480	Unknown protein	8	41	17,603	4.80	19,000	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).

mitochondrion are protected from the proteolytic degradation that is occurring elsewhere in the cell. If the latter is true then one would expect to see an increase in abundance of all mitochondrial proteins relative to total cellular protein. To investigate whether this is the case, we assessed the abundance of several other mitochondrial proteins by Western Blotting (Fig. 3). The following proteins, representing different sub-mitochondrial compartments, were analysed: porin/VDAC (outer membrane), adenine nucleotide translocase (inner membrane), fumarase (matrix) and the E1α subunit of pyruvate dehydrogenase complex (matrix). The increase in relative abundance of VDAC in both heat treated and senescent cells observed on two-dimensional gels (Section 2.2) was confirmed, but other mitochondrial proteins did not follow the same pattern. For example, E1α is almost undetectable in heat-treated cells but appears to be increased during senescence. Conversely, fumarase levels are increased in the former and decreased in the latter. We can therefore conclude that not all mitochondrial proteins are maintained during PCD, but that specific mitochondrial proteins (including superoxide dismutase (MnSOD), a voltage-dependent anion channel (VDAC) Hsr2, aconitase and lipoamide dehydrogenase) may play important roles in the PCD pathway.

The increased relative abundance of lipoamide dehydrogenase, a subunit that is a part of several mitochondrial multienzyme complexes including pyruvate dehydrogenase complex (PDC) and 2-oxoglutarate dehydrogenase complex (2-OGDC) (Lutziger and Oliver, 2001), is interesting given that other subunits of these complexes, such as the E1α subunit of PDC, decrease in abundance during heat-induced PCD (Fig. 3(c)). This suggests the increased lipoamide dehydrogenase content is not related to the function of mitochondrial dehydrogenase complexes but may reflect an alternative function for lipoamide dehydrogenase. One possibility is that changes in the redox state of lipoamide may form part of a redox signalling mechanism.

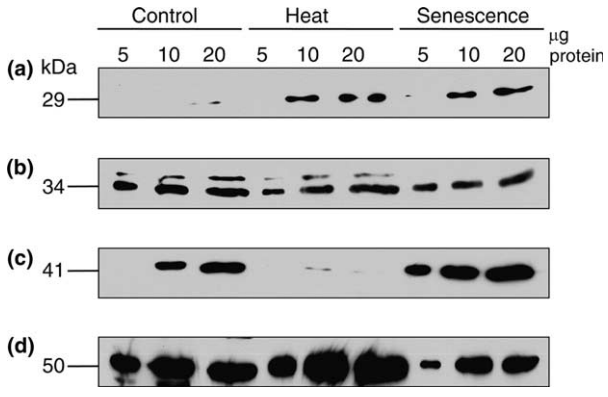

Fig. 3. Western Blot analysis of mitochondrial proteins. Total protein was isolated from 6-day old control, 6-day old heat-induced PCD, or 13–14-day old senescence-induced PCD *Arabidopsis* cell suspension cultures. Total soluble protein samples were separated by SDS–PAGE, transferred to nitrocellulose, and probed with the following antibodies: (a) 1/500 anti-VDAC; (b) 1/1000 anti-ANT; (c) 1/1000 anti-E1α subunit, PDC; (d) 1/1000 anti-fumarase.

2.6. The voltage-dependent anion channel protein, Hsr2, may play a PCD-specific role in plants

The increased relative abundance of a voltage-dependent anion channel protein in both heat- and senescence-induced PCD was unequivocally identified by mass spectrometry to be the Hsr2 isoform of VDAC (Table 1). The gene encoding Hsr2 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 Hsr2 protein did not change significantly in a microarray analysis of heat- and senescence-induced PCD (Swidzinski et al., 2002), the proteomic results indicate that, in these systems, Hsr2 abundance may be regulated at a post-transcriptional level. Transcriptional regulation of Hsr2 during the hypersensitive response and post-transcriptional regulation of Hsr2 during heat- and senescence-induced PCD strongly suggest that this protein plays a significant and general role during plant PCD.

The notion that VDAC Hsr2 may be involved in plant PCD is intriguing since this protein, along with inner membrane ANT, is a central component of the mitochondrial permeability transition pore (PTP) that functions during animal PCD (Martinou and Green, 2001). In addition, changes in VDAC configuration to the closed state (Vander Heiden et al., 2000) or the complexing of VDAC with other pro-apoptotic proteins such as Bax (Shimizu and Tsujimoto, 1999) are sufficient to cause the permeability transition in animal mitochondria. While a direct homologue of Bax does not exist in plants, it is possible that other proteins interact with plant VDAC in a similar fashion during PCD. Alternatively, changes in VDAC configuration alone may be sufficient to lead to changes in mitochondrial permeability and the increased relative abundance of VDAC protein during plant PCD may serve to augment this event. Interestingly, a recent study has demonstrated that overexpression of rice VDAC in mammalian cells is sufficient to cause cell death, and that inhibition of VDAC function protects against heat-induced cell death in cucumber (Godbole et al., 2003).While the case for the existence of a PTP in plants is preliminary and based on studies using pharmacological inhibitors of known animal PTP components (such as cyclosporin A, an inhibitor of cyclophilin D) (Arpagaus et al., 2002; Saviani et al., 2002; Tiwari et al., 2002), the presence of a mitochondrial permeability transition has certainly been detected in plant cells in response to stress leading to cell death (Arpagaus et al., 2002; Tiwari et al., 2002). The increased relative abundance of VDAC Hsr2 during plant PCD seen in this study may be a key feature of

this permeability transition and potentially involved in the release of pro-apoptotic proteins from plant mitochondria.

2.7. Identification of a potential cell-to-cell PCD signalling mechanism

One of the protein spots that increased in relative abundance during heat- and senescence-induced PCD was identified as an EP1-like protein (Table 1). This protein is 51% identical and 67% similar to an extracellular glycoprotein, EP1, initially characterised in carrot suspension cells (van Engelen et al., 1991). The protein identified in this study bears homology (40–60% similarity) to S-like glycoproteins and the S-like domain of receptor proteins kinases from several species, including *Arabidopsis*. Since *Arabidopsis*, 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 (TargetP v1.01 (Emanuelsson et al., 2000); $p < 0.05$) and therefore may be secreted in *Arabidopsis* cell cultures undergoing PCD as part of a cell-to-cell signalling mechanism.

2.8. Increased free iron is associated with PCD

The increased abundance of aconitase during heat- and senescence-induced PCD (Table 1) prompted an investigation of changes in free iron levels since mammalian aconitase, in addition to its role in the TCA cycle, functions as an iron regulatory protein (Cairo and Pietrangelo, 2000). Free cellular iron (Fe^{2+}) levels were measured in both heat- and senescence-induced PCD cultures (Table 2). The measurements were repeated with four independent cultures with similar results (Table 2). In both treatments, free iron content relative to the control was increased (from 1.5 to 5.6-fold), with an average fold-increase of 1.66 ± 0.28 after heat-treatment and 1.60 ± 0.10 during senescence.

The *Arabidopsis* aconitase we have identified possesses a significantly higher degree of sequence identity to mammalian IRE-BP (70%) than to the mammalian mitochondrial aconitase (43%), and plant aconitases are known to be inactivated by ROS (Navarre et al., 2000). Therefore, plant aconitase may be induced in the presence of ROS to function as an IRE-BP that is involved in the regulation of free iron content, or in regulating the expression of other genes.

Excess free iron has been implicated in oxidative damage and is suggested to be required for DNA damage, resulting in the production of DNA "ladders" which are a hallmark of many PCD systems (Eaton and Qian, 2002). However, this proposed role for increased free Fe^{2+} levels in generating an oxidative signal or oxidative damage must be reconciled with the observed increase in antioxidant enzymes such as catalase and MnSOD (Section 2.5) which have the potential to ameliorate the effects of free Fe^{2+}. We suggest that the significant increase in free Fe^{2+} that we have observed would generate hydroxyl radicals in such quantities as to overwhelm the antioxidant systems. Although the increase in free Fe^{2+} was relatively small (1.6-fold) in comparison to the increases in antioxidant protein levels (MnSOD increased by 2.8–7.4-fold), even a small increase in free Fe^{2+} can cause a dramatic rise in the rate of hydroxyl radical production. This is because the Fe^{2+} catalyst can be recycled by reaction of Fe^{3+} with superoxide – the superoxide-assisted Fenton reaction (Halliwell and Gutteridge, 1989). The induction of antioxidant enzymes (as a default response to increased ROS levels) is rendered redundant by the extent of the increase in free Fe^{2+} and consequent ROS production. Studies in animals have demonstrated the induction of antioxidant enzymes in response to increased free iron and the insufficiency of this induction to protect against oxidative damage – it may, in fact, promote oxidative damage (Eaton and Qian, 2002).

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

3. Conclusion

We have identified a number of proteins that are increased in relative abundance during PCD-induced in an *Arabidopsis* cell suspension culture by two independent means. These proteins appear to be maintained in the face of general and extensive protein degradation and therefore may be required to allow PCD to proceed. Several of these proteins show evidence of post-translational modifications which may alter their properties for a PCD-specific function. While the identified antioxidant proteins are most probably a response to the stress of the inducing stimuli rather than being related directly to the PCD process, plausible PCD-related roles for several of the other proteins can be hypothesised. It is particularly intriguing that we identified several mitochondrial proteins since the mitochondrion has been established to be at the heart of the PCD pathway in animals (Kroemer and Reed, 2000). These mitochondrial proteins may be involved in redox signalling

(lipoamide dehydrogenase and aconitase) that triggers PCD or in the release of pro-apoptotic mitochondrial proteins into the cytosol (VDAC). We also identified an extracellular glycoprotein that bears sequence similarity to receptor kinases and may therefore be part of a signalling mechanism that transmits a 'death signal' between cells. Such a mechanism may be important in maximising the efficiency of a localised death lesion to minimise pathogen spread or transmission of oxidative insults. The identification of such a receptor-based pathway of PCD would not only have direct biological significance, but would also constitute a much needed research tool that would allow the precise induction of PCD in the absence of a stress-stimulus. Such an approach would allow a more definitive identification of genes and proteins that play a role plant PCD.

This study demonstrates the utility of the proteomic approach in addressing a biological system in which there is little prior knowledge to form the basis of more hypothesis-driven studies. We have identified a number of proteins that are putatively involved in plant PCD and have provided a foundation for further functional studies to examine the precise roles and functions of these proteins.

4. Experimental

4.1. Maintenance of cell cultures and induction of PCD

Arabidopsis thaliana var *Lansberg erecta* cell suspension cultures were maintained as described in Swidzinski et al. (2002). Cultures were sampled for protein extraction and two-dimensional gel analysis following the induction of PCD by either a heat treatment at 55 °C for 10 min of 6-day old cultures, or in senescent cultures at day 13–14 after subculturing (Swidzinski et al., 2002). At these timepoints, approximately 20% and 40% of the cells in culture, respectively, had executed PCD as assayed by vital staining, cellular morphology, and DNA laddering. Control samples were derived from healthy, 6-day old cultures in which PCD had not been induced.

4.2. Protein isolation

Soluble protein was extracted from 1 to 2 g of frozen cells in 0.5 M Tris–HCl (pH 7.5), 10 mM EDTA, 2% (v/v) mercaptoethanol and a Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics Ltd, Lewes, UK) (1 tablet/10 ml extraction buffer). Proteins were precipitated using a phenol method (Hurkman and Tanaka, 1986).

4.3. Measurement of protease activity

Protease activity in samples was measured using the Universal Protease Substrate (Roche Diagnostics Ltd., East Sussex, UK). 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* for 5 min at 4 °C in a microcentrifuge. Protein concentration of the supernatant was measured using a Bradford assay. Protease assays were carried out in a 200 μl volume with the following: 0.1% (w/v) resorufin-labeled 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% (v/v) trichloroacetic acid with subsequent incubation at 37 °C for 10 min. Precipitated protein was pelleted by centrifugation at 20,000*g* for 5 min in a microcentrifuge, and 400 μl of the supernantant 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.

4.4. Two-dimensional gel electrophoresis

Eight hundred micrograms of total soluble protein pellets were separated by isoelectric focussing in the first dimension and SDS–PAGE in the second and stained with colloidal coomassie blue as described previously (Millar et al., 2001).

4.5. 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) two-dimensional gel analysis software was employed according to the manufacturer's instructions. Spot detection, normalised quantitation and gel matching were achieved using algorithms contained within the software (Smilansky, 2001). Gels of proteins obtained from cultures undergoing PCD (heat-treated and senescence) were matched to a control gel of 6-day old, untreated cultures and protein spots aligned. Spots that were unique and/or increased in relative abundance by more than 2-fold in both PCD-inducing treatments were excised and proteins identified by tandem MS/MS at the GARNet Proteomic Facility (Cambridge, UK). Each gel analysis was repeated in triplicate from independently isolated protein samples derived from independent mother cultures.

4.6. 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).

4.7. Western blot analysis

Protein samples were fractionated by SDS–PAGE and transferred to nitrocellulose. Proteins were visualised by brief staining in Ponceau S. Membranes were blocked with 5% (w/v) BSA or 5% (w/v) milk powder (for monoclonal and polyclonal antibodies, respectively) and incubated with antibodies overnight at 4 °C. Horseradish peroxidase secondary antibodies were visualised by chemi-luminescence (Pierce, Rockford, USA).

4.8. Measurement of free iron

Free Fe^{2+} content was determined as described previously (Li et al., 1999). Cells were washed three times in 10 mM Tris–HCl (pH 7.5) to remove any Murashige and Skoog media containing iron salts, vacuum-filtered, and snap-frozen in liquid nitrogen. Samples were ground to a fine powder in a mortar and pestle in liquid nitrogen and then resuspended in 1 volume (ml/g) of 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. Fe^{2+} concentration in aliquots of the supernatant 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_2O. The absorbance of each sample at 700 nm was subtracted from the absorbance at 540 nm, and the Fe^{2+} concentration determined from a standard curve.

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