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Analysis of programmed cell death in Tobacco Bright Yellow-2 cells

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INTRODUCTION

1. PROGRAMMED CELL DEATH (PCD).

The suicide of individual cells is an efficient and conserved mechanism to achieve and maintain homeostasis in multicellular organisms as a response to pathogen attack and abiotic stress, as well as in normal development (Gilchrist *et al.*, 1998). The selective elimination of certain cells is carried out by a gene-directed process called programmed cell death (PCD). This is an energy-dependent asynchronic process that comprises: loss of cell-to-cell contacts, cytoplasmic shrinkage, membrane blebbing, DNA fragmentation, disassembly of the nuclei and formation of apoptotic bodies. The execution of PCD requires the participation of a complex cell suicide machinery that involves several molecules regulated by the expression of a certain set of genes. The self-contained nature of PCD contrasts with necrosis, which is an unregulated process of traumatic destruction, followed by the release of intracellular components without the active participation of the cell (Okada *et al.*, 2004).

In animals (Fig. 1), the active study of PCD began in 1972 the term apoptosis as "a basic biological phenomenon with a wide range of implications in tissue kinetics" was introduced by Kerr *et al.*, 1972. However, it took more than a decade to realize the biological importance of PCD in plant pathogenesis and development (Pennel *et al.*, 1997). As in animals, PCD plays a key role in numerous vegetative and reproductive phases of plant (Fig. 2) development, including the senescence of leaves, xylogenesis, death of petals after fertilization, postembryonic decay of aleuronic layers, root cap development, somatic and zygotic embryogenesis and sex determination. PCD in plants occurs in response to biotic and abiotic stimuli. Avirulent infections are usually characterized by a localized cell death known as hypersensitive response (HR) which results in the formation of necrotic lesions around the infection sites (Goodman *et al.*, 1996). On the other hand, there is the

abiotic stress response, and the best example is aerenchyma development under low oxygen conditions, in which root cortical cells are induced to die and form larger airspaces, enabling a greater diffusion of air from the upper parts of the plant (Drew *et al.*, 2000). PCD in plants has also been characterised in response to high temperature (McCabe *et al.*, 2000). Some of the morphological features of apoptosis as well as transduction pathways and signal molecules have been shown to be similar in both animals and plants. However, differences in the execution of PCD have also been observed.

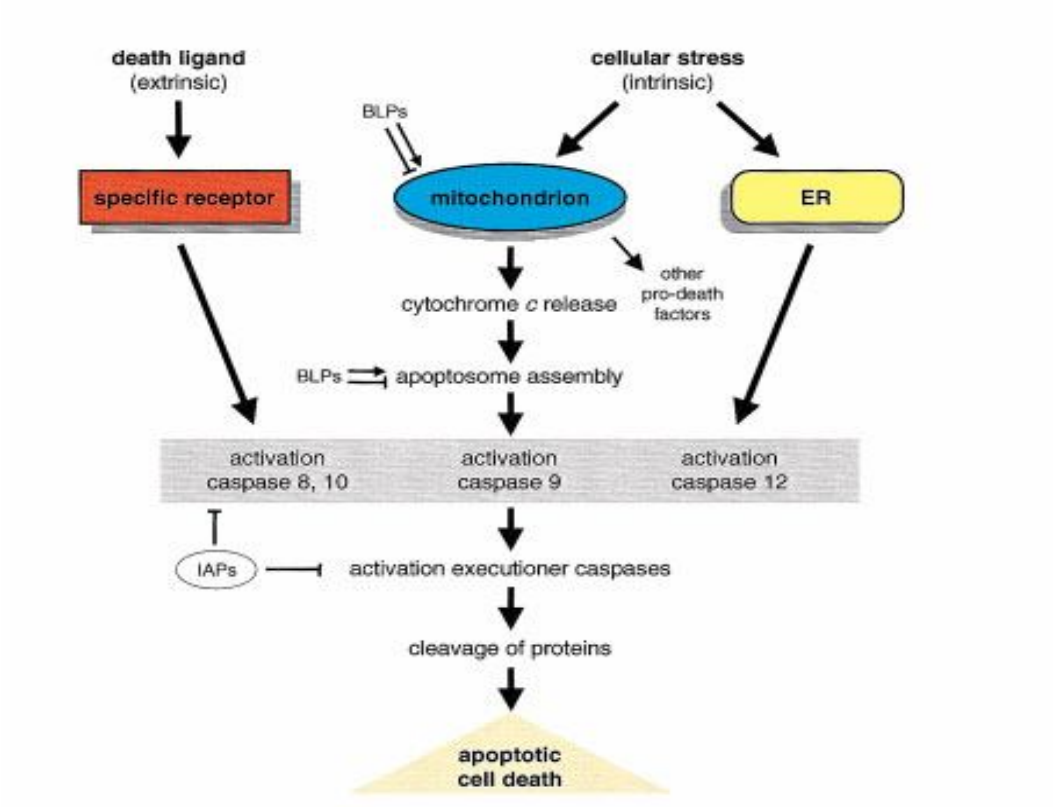

Fig. 1 Animal Apoptosis

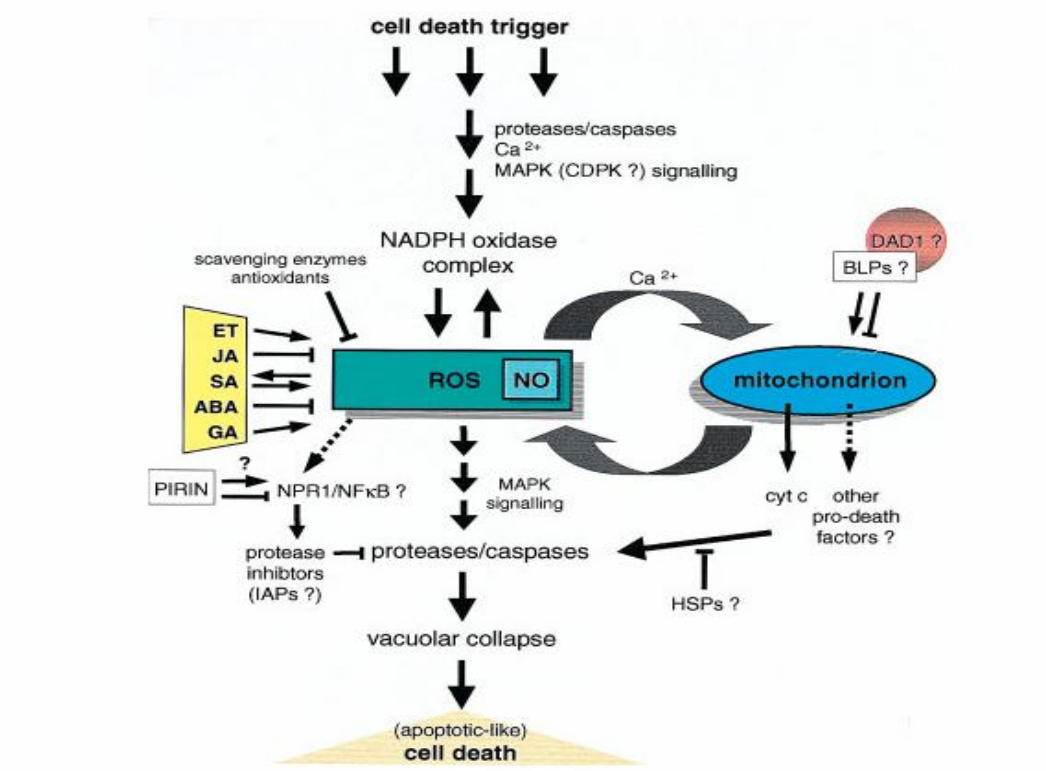

Fig. 2 Plant PCD

2. MORPHOLOGY OF APOPTOSIS IN ANIMALS AND PCD PLANTS

Morphological features of apoptosis may be detected by various cytochemical and microscopic methods. The use of intercalatory agents, fluorescent microscopy and the comet assay enables the detection of the condensation of chromatin and show DNA degradation; in plants, in addition to these techniques, the use of Green Fluorescent Protein-Nitrilase 1 permits to see the formation of nuclear lobes and the release of nuclear contents into the cytosol.

2.2 DNA cleavage

The fragmentation of the DNA occurs at the nucleosomal linker sites and the fragments are reported to be of 140-180 base pairs in animals (Cohen *et al.*, 1994). Electrophoretic separation exhibits DNA fragments as a ladder formation of which

the rungs are multiples of 180 bp. The DNA fragments can be cytochemically determined by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) of the 3'OH groups (Gavireli *et al.,* 1992). The DNA processing reported for animal PCD is also believed to exist in the dying cells of plants. Nuclear condensation as well as oligonucleosome sized DNA fragments have been detected by TUNEL and electrophoresis analysis in aleurone cells of barley (Pennel *et al.,* 1997), in dying tobacco roots cap cells (Mitler *et al.,* 1997), and also in plants exhibiting HR resistance such as cowpea leaf cells infected with *Uromyces vignae* and Tobacco Mosaic Virus (TMV) infected tobacco (Ryerson *et al.,* 1997). DNA ladders were also observed during cell death in *Alternaria Alternata* (AAL) toxin treated tomato protoplasts and leaflets. The intensity of the DNA ladders was enhanced by Ca^{2+} and inhibited by Zn^{2+} (Wang *et al,.* 1996). The DNA fragments have been seen to range from sizes as high as 50000 bp in some cases and of 140 bp in others (O'Brien *et al.,* 1998). Moreover, DNA fragmentation may be a marker feature of certain cell deaths of plants such as root cap cells, aleurone cells, etc; but it is not likely to be involved either in tracheary elements and fibers (Mitler *et al.,* 1995) or in epidermal and mesophyl cells of lace plant leaves during development (Gunawardena *et al.,* 1995).

2.3 Cell membrane components

Another well characterized morphological feature of apoptosis is the lost of membrane phospholipid asymmetry that results in phosphotidylserine (PS) exposure on the outer and the inner surface of the plasma membrane. Externalized PS appears to serve as an important signal for targeting the recognition and elimination of apoptotic cells by macrophages. PS externalization has been suggested to originate from the balance between its inward and outward translocations driven by two enzymatic activities: aminophospholipid translocase

(APT) and a non-specific phospholipid scramblase (PLSCR). Changes in PS asymmetry, analyzed by measuring Annexin V bound to the cell membrane, were detected in tobacco PCD induced by a number of chemical agents (O'Brien *et al.,* 1998) and in apple suspension cells under a low oxygen culture (Xu *et al.* 2004). The physiological role of PS exposure in plants is still unknown since phagocytosis does not occur.

2.4 Cytoplasmic events

Morphological changes in the cytoplasm of animal cells during apoptosis include condensation, shrinkage and fragmentation. However, cell membrane integrity is preserved allowing the packaging of nuclear and cytoplasm components in apoptotic bodies. Condensation and shrinkage of the cytoplasm were reported in dying aleurone cells, onion and tomato root cap cells (Wang *et al.,* 1996), HR lesion cells in *Arabidopsis* (McCabe *et al.,* 1996) and wound and herbicide induced PCD (Cutler *et al.,* 2005). The cytoplasm of differentiating treachery elements (TE) is reported to become lobed, condensed, shrunken and finally broken into small packages (Lai *et al.,* 1976). The formation of membranebound structures or apoptotic-like bodies in tomato has been observed in response to AAL toxin from *Alternaria alternata* f. sp. *lycopersiti*, and also to arachidonic acid, an inducer of HR (Wang *et al.,* 1996). In contrast to animals, hallmarks of a typical HR-PCD involve membrane dysfunction, vacuolization of the cytoplasm, vacuolar disruption (oncosis) and changes in gross mitochondrial morphology characterized by swelling and cristae disorganization (Greenberg *et al.,* 2004). The final, preeminent step of TE PCD as well as in the formation of lace plant leaf perforations is a rapid collapse of the vacuole occurring after completing of secondary cell wall synthesis (Greenberg *et al.,* 2004).

2.5 Corpse management in plant cells

Corpse management is a feature that is remarkably different between plants and animals since there is a cell wall in plant cells in contrast to animal cells and plants do not have an immune system. The cell wall precludes phagocytosis, the process of engulfing apoptotic bodies by neighboring cells or macrophages in animals, preventing their lysis and the release of toxic or immunogenic intracellular components to the nearby tissue with a consequent inflammation (Kam *et al.,* 2000). Instead of that, corpse processing in plants is autolytic, it is carried out by vacuoles. Decisions on corpse management based on the integration of various signals such as auxins , cytokinins, ethylene and elicitors, are probably made by the living cell long before death takes place and probably even well before the point of no return in the plant cell. The ability to make these decisions is especially relevant in plant cells due to the absence of macrophages and neutrophils to decide for them (Jones *et al.,* 2001). The way the cell corpse is managed is a function of the profile of hydrolases and toxins that are loaded into the vacuole and these profiles are established by the original set of signals. The cell must be metabolically active to synthesize the destructive hydrolases it needs to process its corpse; it therefore sequesters these hydrolases and toxins into the vacuole and releases them when the vacuole collapses. This collapse is an irreversible step towards death which results in the immediate cessation of cytoplasmic streaming and requires a calcium flux (Jones *et al.,* 2001).

3. MOLECULAR BASIS OF PCD

3.1 Caspase-like proteolytic activity regulates plant cell death

In plants, proteolytic enzymes are known to be associated with both developmental PCD and pathogen- and stress-induced PCD. They are generally assumed to

function in the autolysis of intracellular proteins, rather than as regulators. However, the evident participation of proteases, specifically caspases, in the regulation of animal PCD implies that proteases could also be involved in the regulation of plant PCD. Indeed, there are several reports that link protease activity to the regulation of plant PCD. Proteasome inhibitors can prevent TE differentiation in *Zinnia* cell cultures when added at the time of culture initiation, whereas proteasome inhibition following commitment to differentiation only results in a delay. This suggests that proteasome function is required for induction of TE differentiation, but not for bulk autolysis during the final phases of TE differentiation (Woffenden *et al.,* 1998). Furthermore, the appearance of a secreted protease is co-ordinated with secondary cell wall synthesis and cell death during TE differentiation. Protease activity and cell death are both inhibited by soybean trypsin inhibitor, while exogenous application of another serine protease prematurely triggers cell death. These observations lead to the hypothesis that extracellular proteolysis triggers cell death (Groover *et al.,* 1999). Inhibitor studies also implicate serine proteases in signal transduction during elicitin-induced HR cell death (Sasabe *et al.,* 2000). In soybean cells, PCD activating oxidative stress induces a set of cysteine proteases. Inhibition of the induced cysteine protease activity by ectopic expression of cystatin, a cysteine protease inhibitor gene, can block PCD triggered either by an avirulent pathogen or by ROS (Solomon *et al.,* 1999). These data suggest that the interplay between proteases and endogenous protease inhibitors is a way for plants to regulate cell death. It remains to be seen if this can be compared to the pivotal role that caspases and IAP proteins play in animal PCD.

To date, evidence for the existence of caspase-like proteins (CLPs) in plants is still indirect and mainly based on the inhibitory effects of caspase-specific inhibitors in

plant cells. Such caspase-specific inhibitors can abolish bacteria-induced PCD in tobacco (Del Pozo *et al.,* 1998). In addition, chemical-induced PCD in tomato suspension cells can be inhibited by caspase-specific inhibitors (De Jong *et al.,* 2000). Caspase-like activity has also been demonstrated in barley cell extracts and could only be inhibited by a specific caspase 3 inhibitor, not by cysteine protease inhibitors (Korthout *et al.,* 2000).

Microinjection of caspase 3 substrate into living plant cells revealed that caspase-like activity is mainly present in the cytosol rather than in the vacuole (Korthout *et al.,* 2000). Proteolytic activity in plant cells undergoing PCD has also been studied using poly (ADP-ribose) polymerase (PARP), a well-characterized substrate for human caspase 3. Cleavage of endogenous PARP occurs during menadione-induced PCD in tobacco protoplasts (Sun *et al.,* 1999) and in heat-shock-treated tobacco suspension cells (Tian R *et al.,* 2000). Exogenous (bovine) PARP is endoproteolytically cleaved in extracts of fungus-infected cowpea plants, and cleavage can be inhibited by caspase 3-inhibitor. Interestingly, a polypeptide (GDEVDGIDEV) mimicking the PARP human caspase-3 cleavage site (DEVD-G) partially inhibited PARP cleavage, whereas a modified peptide in which the essential aspartate was replaced by alanine (GDEVAGIDEV) did not affect PARP cleavage (D'Silva *et al.,* 1998). However, cleavage of exogenous PARP in cowpea extracts results in fragments that are different from the fragments that remain after cleavage by an animal caspase (D'Silva *et al.,* 1998). As the proteolytic activity detected in plants may have some different specificities to animal caspases, interpretation of these data requires some caution.

In animals, the IAP protein family has been postulated to play its regulating role by inhibiting caspases. IAP proteins, which are conserved between numerous organisms, are distinguished both by their ability to suppress apoptosis and by the

presence of at least one baculoviral IAP repeat (BIR) domain, which is required for their anti-death activity. It has been reported that Agrobacterium-induced PCD in maize cells can be suppressed by ectopic expression of an IAP from baculovirus (Hansen *et al.,* 2000). Likewise, transgenic expression of the baculovirus IAP in tobacco conferred resistance to several necrotrophic fungal pathogens that normally result in necrotic lesions (Dickman *et al.,* 2001). The macromolecule p35 is another highly specific caspase inhibitor from baculovirus that is effective in inhibiting Agrobacterium-induced PCD in maize (Hansen *et al.,* 2000). Tobacco plants expressing p35 are partially inhibited inHRcell death, whereas mutated versions of the p35 protein, which are impaired in caspase inhibition, are ineffective (Lam *et al.,* 2000). These data point towards the existence of plant proteases that are able to recognize caspase specific inhibitors, and their involvement in cell death. Recently, sequence comparison has revealed a group of CLPs, designated metacaspases, in fungi and plants. The universally conserved catalytic cysteine and histidine diad required for catalysis by cysteine proteases is present in these metacaspases (Uren *et al.,* 2000). It has been shown that the only metacaspase present in *Saccharomyces cerevisiae* displays a caspase-like proteolytic activity that is activated when yeast is stimulated by H_2O_2 to undergo apoptosi (Madeo *et al.,* 2002). A second subgroup of caspase-related proteases are legumains, cysteine endopeptidases first identified in plants. Although legumains have a strict specificity for an asparagine (and not aspartate) residue immediately N-terminal to the substrate's cleavage site, they possess a protein fold similar to animal caspases and are believed to be evolutionarily related (Chen *et al.,* 1998). Taken together, the effectiveness of animal caspase inhibitors in blocking plant PCD, the observed cleavage of animal as well as endogenous PARP by activated plant proteases, and the functioning of animal IAP proteins in plants strongly suggest that caspase-like proteolytic activity

plays a role during plant PCD. Whether these plant CLPs display sequence and secondary structure similarities with animal caspases remains to be seen. It will be interesting to see what proteins are cleaved during plant PCD. Besides PARP, lamin like proteins have been reported to be cleaved during menadione-induced PCD in tobacco protoplasts (Sun *et al.,* 1999). Degradation of lamins is an important event in apoptosis, playing an essential role in chromatin condensation and breakdown of the nuclear envelope.

3.2 Role of mitochondria, cytochrome c, and BLPs

In animal systems, changes in mitochondrial membrane permeability, subsequent release of cytochrome c and the formation of the apoptosome play an important role in apoptosis. BLPs can act as regulators of apoptosis both by interference with caspase activation or through their effect on mitochondrial membrane integrity. In various plant systems, the release of cytochrome c from mitochondria into the cytosol precedes cell death (Sun *et al.,* 1999, Hansen *et al.,* 2000, Balk *et al.,* 1999). Furthermore, HR-induced PCD is associated with the disruption of mitochondrial functions (Xie *et al.,* 2000). Cytochrome c is not released during petal cell death in (pollinated) petunia flowers, (Xu *et al.,* 2000) establishing at least one form of plant PCD in which cytochrome c release is not required. Nevertheless, the release of cytochrome c from plant mitochondria as caused by ROS, elevated calcium levels, or inhibition of electron transport, has been postulated to be a common means for integrating cellular stress and activating plant PCD (Jones *et al.,* 2000).

Evidence for a function of BLPs in plant PCD is accumulating. Initial indications, such as the detection of a BCL2 homologue in plant cells by immunoblotting, and the capability of animal BLPs to modify cell death processes in plants, (Dickman *et al.,* 2001, Lam *et al.,* 1999) are now supported by the isolation of homologues of human Bax inhibitor-1 from Arabidopsis thaliana and rice (Bax is a proapoptotic

member of the BCL2 family). Both clones, AtBI1 and OsBI1, are capable of suppressing Bax-induced cell death in yeast, (Kawai *et al.*, 1999, Sanchez *et al.*, 2000) whereas AtBI1 is rapidly upregulated during wounding or pathogen challenge (Sanchez *et al.*, 2000). In addition, overexpression of AtBI1 can rescue plants expressing mammalian Bax from cell death (Kawai-Yamada *et al.*, 2001). Furthermore, the A. thaliana genome contains two AtBI1 homologues, AtBI2 and AtBI3, and a newly identified family of 13 AtBI2-related (ABR) genes encoding putative transmembrane proteins that could form macromolecular channels (Lam *et al.*, 2001). Although their function remains to be elucidated, it has been suggested that these genes might represent functional equivalents of the mammalian BCL2 family (Lam *et al.*, 2001).

4. PCD SIGNALLING

4.1 The role of ROS and NO

Although ROS used to be regarded merely as toxic by products of cellular metabolism, it is now recognised that molecules such as hydrogen peroxide (H_2O_2), superoxide ($\cdot O_2^-$), and hydroxyl radicals ($\cdot OH$), have a signalling role in many biological systems. Experimental data indicating that ROS can activate cell death programs, both in animal and plants, are accumulating. In plant tissue, various conditions lead to accelerated generation and/or accumulation of ROS and subsequent PCD, for example ozone (O_3) fumigation, cold stress, UV radiation and senescence. The role of ROS in plant PCD has been most extensively studied during the hypersensitive response (HR) to pathogen attack, when ROS are generated rapidly and transiently at the site of infection. This process is generally referred to as the oxidative burst. During the HR, ROS may possess direct antimicrobial activity and function in cell-wall reinforcing processes. As signal

molecules, they are believed to induce PCD, and activate defence gene expression and systemic acquired resistance (SAR). Plant responses to ROS are dose dependent. High doses of ROS trigger HR-related PCD, whereas low doses induce antioxidant enzymes, and block cell-cycle progression (Vranova´ *et al.,* 2002). It has been postulated that, through this dose-dependent action, ROS act as a trigger for PCD locally and as a diffusable signal for the induction of cell defences in neighbouring cells (Vranova´ *et al.,* 2002). Despite the recognition of ROS as signalling molecules in PCD, little is known about how these signals are perceived and transduced in plant cells. It has been reported that H_2O_2 is a potent activator of a MAPK cascade that induces specific stress-responsive genes in A. thaliana leaf cells, but represses auxin-inducible promoters (Kovtun *et al.,* 2000). The activation of a redox signalling pathway possessing a MAPK module has also been reported in response to avirulent pathogen infection in *A. thaliana*. This signalling network functioned independently of the plant hormones ethylene, salicylic acid and jasmonic acid (Grant *et al.,* 2002). However, ethylene plays a critical role in the release of H_2O_2 during PCD in tomato suspension cells, as inhibitors of ethylene biosynthesis or perception block H_2O_2 production and cell death (De Jong *et al.,* 2002). The free radical gas nitric oxide (NO), well characterised as a mammalian signalling molecule, has also been recognized as a signal in plants. *A. thaliana* suspension cultures generate elevated levels of NO in response to avirulent bacteria. In this system, these elevated levels of NO were sufficient to induce cell death that involves caspase-like activity (Clarke *et al.,* 2000). Recently, it was demonstrated that the HR is triggered only by balanced production of NO and ROS. More specifically, dismutation of $\cdot O_2^-$ to H_2O_2 is required to activate cell death, which depends on synergistic interactions between NO and H_2O_2. Scavenging of $\cdot O_2^-$ by superfluous NO (or vice versa) disturbs the NO / H_2O_2 ratio, resulting in reduced

cell death (Delledonne *et al.*, 2001). Little is known about signalling pathways downstream of NO / H_2O_2. It has been shown that NO signalling during both PCD and defence responses requires cyclic GMP and cyclic ADPribose, two molecules that can serve as secondary messengers for NO signalling in mammals. Furthermore, NO activates MAP kinases in both A. thaliana and tobacco (Neill *et al.*, 2002). Collectively, these data confirm that NO is a ubiquitous signal in plants. However, understanding of NO signalling in plant PCD is still at an early stage. ROS, particularly H_2O_2, have been implicated in activation of the NF-kB signalling pathway that plays an essential role in regulating both immune and inflammatory responses, and tumour necrosis factor (TNF)-induced apoptosis in animal cells. Once activated and translocated to the nucleus, NF-kB can induce various anti-apoptotic factors, including IA proteins and BLPs.

4.2 Calcium signaling

Calcium (Ca^{2+}) is an almost universal intracellular messenger, controlling a broad range of cellular processes, including animal apoptosis. In plant PCD, calcium has also been recognised as a ubiquitous signal. Elevated calcium levels have been observed during tracheary element differentiation, aerenchyma formation, wheat aleurone differentiation, the HR, and leaf senescence. Furthermore, the plasma membrane Ca^{2+} channel blocker lanthanum chloride can inhibit H_2O_2-induced cell death in soybean cells, bacteria-induced PCD in A. thaliana, and camptothecin-induced PCD in tomato cells. However, this inhibitor does not suppress the induction of more general stress or defence pathways, suggesting that Ca^{2+} fluxes are involved in signalling the activation of PCD, but not the activation of general stress or defence responses (Hoeberichts *et al.*, 2001, Levine *et al.*, 1996). Additional data confirm a role for calcium signalling in pathogen defence. The A. thaliana dnd1 mutant has been isolated as a line that failed to produce HR cell death

in response to avirulent pathogen infection. Cloning of the corresponding DND1/CNGC2 gene revealed that it encodes a cyclic nucleotide-gated ion channel that allows passage of Ca^{2+}, K^{+} and other cations (Clough *et al.,* 2000). Expression studies have led to speculation on an additional role for DND1/CNGC2 during developmentally regulated PCD (Kőhler *et al.,* 2001). Another elicitor activated Ca^{2+} permeable ion channel has been identified in parsley by patch-clamp analysis (Zimmermann *et al.,* 1997). Calcium-binding proteins interpret information contained in the temporal and spatial patterns of Ca^{2+} fluxes and accordingly bring about changes in metabolism and gene expression. Interestingly, plants contain a unique superfamily of calmodulin-like domain protein kinases (CDPKs) capable of activating protein phosphorylation cascades, a widely used mechanism by which extracellular stimuli are transduced into intracellular responses. Various (putative) calcium-binding proteins, among them several CDPKs, are induced during plant defence responses. It has been suggested that CDPKs, in response to elevated cytosolic Ca^{2+} levels, can induce NADPH oxidase activity (Blumwald *et al.,* 1998). Present data indicate that calcium signalling is an important mediator of plant PCD. The existence of the CDPK protein family indicates that plants have incorporated certain plant specific factors into this universally present signal transduction system.

4.3 Hormones in PCD

It is likely that, in addition to the putative regulators of PCD conserved throughout the animal and plant kingdoms, there are plant-specific mediators of PCD. Various plant hormones, such as salicylic acid, ethylene, abscisic acid and jasmonic acid, are strong candidates, and supporting evidence is starting to accumulate.

Salicylic acid (SA) is a key-signalling molecule in pathogeninduced disease resistance, but its function in relation to cell death is still poorly understood. The epistatic relationship between cell death and SA accumulation has been analysed in

crosses between various A. thaliana mutants and the transgenic nahG line (depleted in SA). Whereas several mutants retain their spontaneous lesion phenotype in the nahG background, others display a reduction, delay or even abolition of their mutant phenotype. These data can only be explained if SA accumulation is placed both upstream and downstream of cell death, presumably as part of a feedback amplification loop. Biochemical evidence suggests that the function of SA upstream in the HR might affect the phosphorylation status of a signalling pathway component that regulates the activation of a sustained oxidative burst (Alvarez, 2000). Fumonisin B1-induced cell death in A. thaliana protoplasts requires SA signaling (Asai *et al.,* 2000), and transgenic nahG tobacco displays decreased lesion formation after O3-treatment (Örvar *et al.,* 1997), confirming a role for SA upstream of cell death. Conversely, ROS are capable of inducing SA accumulation (Chamnongpol *et al.,* 1998) or even of directly stimulating SA synthesis (Leo´n *et al.,* 1995), supporting the idea of a feedback amplification loop. One of the two MAPKs implicated in the activation of the HR in tobacco can be induced by SA (Zhang *et al.,* 1997, Zhang *et al.,* 2000), providing a means by which SA could induce downstream phosphorylation. In TMV-infected tobacco tissue that accumulates SA, a gradient of SA is established along with lesion formation, with the highest levels of SA present in and around the necrotic lesions (Enyedi *et al.,* 1992). It has been postulated that low concentrations of SA might be inadequate for the induction of cell death but sufficient to activate survival signals and defence responses.

This suggests that low levels of SA regulate lesion limitation, whereas high levels of SA actually induce cell death (Alvarez, 2000). Interestingly, high levels of SA can rapidly inhibit mitochondrial functions by a mechanism that requires ROS (Xie

et al., 1999). Given the function of mitochondria in animal apoptosis, this effect of SA might contribute to the induction of cell death in response to pathogens.

Ethylene is a plant hormone well known for its role during plant senescence and cellular responses to numerous forms of stress. In addition, a regulatory role during plant PCD is now emerging. Aerenchyma formation in hypoxic roots, one of the earliest examples of PCD recognised in plants, requires ethylene (Drew *et al.,* 2000). Epidermal cell death at the site of adventitious root formation, a response to submergence of the deepwater rice Oryza sativa, can be induced by application of the natural ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) and it can be suppressed by inhibiting ethylene perception (Mergemann *et al.,* 2000). During the development of cereal endosperm, ethylene is produced in two discrete peaks. Application of exogenous ethylene throughout seed development results in earlier and more extensive cell death and DNA fragmentation. Conversely, treatment with inhibitors of ethylene biosynthesis or ethylene perception reduces cell death and DNA fragmentation (Young *et al.,* 2000). There are also reports describing ethylene as a stimulant of senescence-associated PCD (Orzáez *et al.,* 1997, Navarre *et al.,* 1999) Furthermore, cell death induced by the mycotoxin fumonisin B1 seems to involve ethylene-mediated signalling pathways in both A. thaliana and tomato (Asai *et al.,* 2000, Moore *et al.,* 1999). Although ethylene alone is not sufficient to trigger PCD in tomato cell suspensions, camptothecin-induced cell death and the associated oxidative burst can be blocked by inhibition of ethylene signalling. Exogenous ethylene greatly stimulates camptothecin-induced H_2O_2 production and cell death (De Jong *et al.,* 2002). Studies of *A. thaliana* double mutants have provided additional evidence supporting a role for ethylene signalling in cell death. Crosses of the lesion-mimic mutant accelerated cell death 5 (acd5) and ethylene insensitive 2 (ein2), in which ethylene signalling is blocked, show

decreased cell death (Greenberg *et al.,* 2000). Ethylene insensitivity in double mutants of ein2 and the O3-sensitive radical-induced cell death 1 (rcd1) blocks ROS accumulation that is required for lesion propagation, whereas exogenous ethylene increases ROS-dependent cell death in rcd1 (Overmyer *et al.,* 2000). In line with the suggested positive role for ethylene during propagation of ROS-dependent lesions (Overmyer *et al.,* 2000), it has been proposed that limiting the spread of pathogen-induced cell death in tomato involves a downregulation of ethylene sensitivity (Ciardi *et al.,* 2000). These data show that various forms of plant PCD require ethylene signalling and, moreover, are accelerated by exogenous applied ethylene. It seems plausible that ethylene is required, though not sufficient, for regular PCD and that its function is linked to controlling the extent of cell death. The stimulation of PCD by ethylene seems associated with an increased production of ROS.

Abscisic acid (ABA) has been implicated as a key regulator in cereal endosperm development. Cell death is accelerated in developing endosperm of ABA-insensitive or deficient maize mutants. It is believed that a balance between ABA and ethylene establishes the appropriate onset and progression of PCDduring maize endosperm development (Young *et al.,* 2000). During germination, cell death in barley aleurone layers is induced by gibberellin (GA), whereas ABA antagonises this effect (Fath *et al.,* 2002). It has been found that ROS are mediators of this hormonally regulated cell death pathway. Incubation of aleurone layers or protoplasts in H_2O_2-containing media results in death of GA-treated but not ABA-treated aleurone cells. Cells that are programmed to die are therefore less able to withstand ROS than cells that are programmed to remain alive, supposedly because ROS scavenging enzymes are strongly downregulated in aleurone layers treated with GA, whereas ABA-treated cells maintain their ability to scavenge ROS (Fath

et al., 2002). A similar protective role of ABA against cell death has been observed during androgenesis in developing barley anthers.

Jasmonic acid (JA) is a well-known signalling molecule in plant defence and stress responses. It has been implicated in O_3-induced hypersensitive cell death since O_3 induces JA biosynthesis within several hours of treatment (Rao *et al.,* 2001). Furthermore, treatment with exogenous methyl jasmonate inhibits propagation of O_3-induced cell death in O_3-sensitive A. thaliana plants. Accordingly, the jasmonate-insensitive mutant jar1 displays increased spreading of cell death following exposure to O_3 (Overmyer *et al.,* 2000). The highly O_3-sensitive A. thaliana ecotype Cvi-0 has greatly reduced JA sensitivity, whereas various other JA mutants show a similar high sensitivity to O_3 (Rao *et al.,* 2001). Together, these studies indicate that JA is an important component of a pathway that negatively regulates cell death and lesion formation. Interestingly, JA is believed to cause this effect by attenuating the O_3-induced ROS production, as wounding or treatment of plants with JA has been shown to reduce O_3-induced cell death and O_3-induced ROS levels (Örvar *et al.,* 1997, Overmyer *et al.,* 2000). However, the precise mechanisms by which JA signalling regulates cell death are far from understood.

Contradictory results show that the viability of jar1 mutant A. thaliana protoplasts is only marginally affected by fumonisin B1, whereas this fungal toxin induces apoptosis-like PCD in wild-type protoplasts, suggesting that JA-mediated signaling increases fumonisin B1-induced cell death (Asai *et al.,* 2000).

5. PCD IN RESPONSE TO DEVELOPMENT

In plants, selective cell death is necessary for growth and survival and can occur on a local or large scale (Fig. 3).

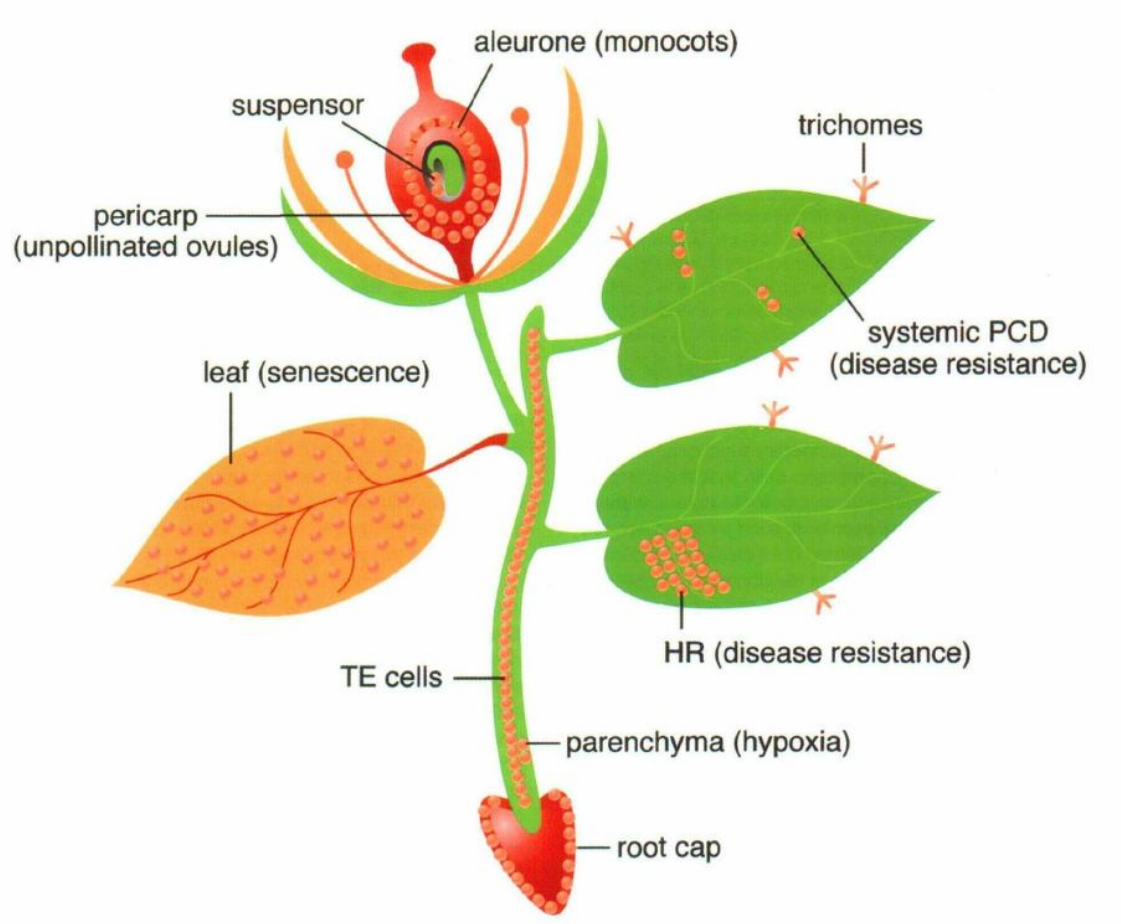

Figure 3 Sites of PCD in Vascular Plant.

5.1 Aleurone Cells

In seeds of monocots, aleurone cells form a secretory tissue that releases hydrolases to digest the endosperm and nourish the embryo. Aleurone cells are unnecessary for postembryonic development and die as soon as germination is complete (Kuo *et al.*, 1996). Several lines of physiological evidence suggest that their death involves PCD. For example, in the aleurone, secretory processes and cell death are stimulated by gibberellin (GA), whereas abscisic acid (ABA) blocks the effects of GA and retards seed germination and cell death (Jones and Jacobsen, 1991). Moreover, an elevation in cytosolic Ca^{2+} occurs in aleurone cells treated with GA

(Bush *et al.*, 1989; Gilroy and Jones, 1992), suggesting that a signal transduction pathway controls secretion and cell death.

5.2 Root Cap Cells

A cap of cells protects the root apical meristem during seed germination and seedling growth. Root cap cells are formed by initial cells in the meristem and are continually displaced to the root periphery by new cells (Laux and Jürgens, 1997; Schiefelbein *et al.*, 1997). After severa1 days, the peripheral cells die (Harkes, 1973). Cell death occurs in root caps when roots are grown in water, showing that cell death is a normal part of development and not a consequence of abrasion during soil penetration. Dying root cap cells shrink and adopt irregular profiles, and DNA staining shows that the nuclei in dying onion root cap cells become condensed (H. Wang *et al.*, 1996).

5.3 TE Cells

Vascular plants transport water in columns of specialized dead cells termed TEs. Differentiation of TEs involves cell elongation, the deposition of cell wall components, including lignin, and autolysis (Fukuda, 1997). Autolysis begins as the cytoplasm and nuclei become lobed, condensed, and shrunken and ends as the cytoplasm breaks into small packets (Wodzicki and Humphreys, 1973, 1974; Lai and Srivastava, 1976). *Zinnia* elegans mesophyll cells can be cultured and induced to redifferentiate into TEs so that the cell death process can be studied biochemically as well as morphologically (Fukuda, 1994, 1997). Treatment of differentiating *Zinnia* cells with actinomycin D or cycloheximide blocks cell death (Fukuda and Komamine, 1983). This suggests that cell death in TEs requires protein synthesis, and it is possible that among the proteins synthesized are the effector proteases and nucleases necessary for cell disassembly.

5.4 Somatic Embryogenesis

Cultured cells of some plant species can be induced to develop into somatic embryos. In embryogenic suspension cultures, totipotent cells divide asymmetrically into cell pairs, one member of which stops synthesizing DNA and dies, whereas the other member goes on to establish an embryo (Nomura and Komamine, 1985, 1986). In some dead cells, the cytoplasm is broken into small, membrane-sealed packets, which suggests that the cells have undergone a form of PCD similar to apoptosis (Havel and Durzan, 1996; McCabe and Pennell, 1996). This contention is supported by the accumulation of 3'-OH groups in the DNA of these cells (McCabe *et al.*, 1997).

5.5 Senescence

Senescence is the final phase of plant vegetative and reproductive development, preceding the widespread death of cells and organs. Senescence involves the active turnover and recapture of cellular material for use in other organs (Noodén, 1988; Bleecker and Patterson, 1997). Membrane integrity and cellular compartmentalization are maintained until late into the senescence process, suggesting that there is little or no leakage of cellular contents (Noodén, 1988). Senescence, which can be induced by ethylene (Grbic and Bleeker, 1995), requires nuclear functions (Thomas *et al.,* 1992) and involves an increase in the generation of O_2^- and H_2O_2 (Pastori and del Rio, 1997). Also, natural senescence can be blocked by mutations in the gthylene-iesponsive *(n R)* gene, by ethylene anatgonists, and by cytokinins (Bleecker *et al.,* 1988). These observations suggest that senescence and cell death during senescence are under the control of a coordinated signaling pathway, consistent with the view that senescence involves PCD (Gan and Amasino, 1997).

6. PCD IN RESPONSE TO BIOTIC STRESS

6.1 Pathogenesis

Many studies have demonstrated the induction of PCD in plants in response to pathogen attack, indicating that PCD plays central role in pathogenesis (Goodman and Novacky, 1994). Plants can recognize certain pathogens and activate defenses (called the resistance response) that result in the limitation of pathogen growth at the site of infection. One dramatic hallmark of the resistance response is the induction of a localized cell death response (the hypersensitive response or HR) at the site of the infection. The HR is likely to be important for limiting a pathogen's nutrient supply, since the dying tissue rapidly becomes dehydrated. The HR appears to be a form of PCD in plants. Firstly, the appearance of the HR is genetically controlled and second, purified HR-inducing factors from bacteria called harpins will not induce the HR unless the plant tissue is transcriptionally active (He SH *et al.,* 1993). In addition, HR-inducing bacteria will not cause the HR if protein synthesis is blocked in the plant. In the search for a signal for HR induction, several groups have determined that H_2O_2 is rapidly produced by plant cells in culture during the HR in a phenomenon termed the oxidative burst (Mehdy *et al.,* 1994). Levine *et al.* showed that enhancing H_2O_2 production during the HR led to dramatic increases in the amount of cell death observed in a soybean cell culture system. The effects on cell death after trying to block H_2O_2 production were more modest. This observation in combination with the fact that bacterial mutants that fail to induce cell death in tobacco suspension cells yet still induce the oxidative burst has led to the suggestion that H_2O_2 is not sufficient to trigger the HR but may act in conjuction with other factors to activate cell death (Glazener *et al.,* 1996). The induction of the HR by some pathogens and elicitors (molecules secreted by pathogens) may be mechanistically similar to apoptosis in animals, since apoptotic

features such as DNA breaks with 3'OH ends, blebbing of the plasma membrane as well as nuclear and cytoplasmic condensation are present in some cells undergoing the HR (Levine *et al.*, 1996, Ryerson *et al.*, 1996)). In some cases the HR is also accompanied by internucleosomic DNA cleavage, another apoptosis-associated event (Wang *et al.*, 1996). The HR is also correlated with the activation of K^+/H^+ exchange across the plasma membrane of plant cells in culture, an event which might lead to cell death and/or defense signaling. Introduction of a gene which encodes a bacterial proton pump into tobacco plants causes the plants to undergo an apparent HR (Mittler *et al.*, 1995). If the bacterial protein really is functioning to translocate protons across the plasma membrane of plants, this suggests that the protein causes the HR by mimicking the K^+/H^+ exchange that occurs during the HR. This would provide the first compelling evidence that the exchange of ions is causally related to HR control.

Many plant-pathogen interactions can lead to plant cell death that appears to be distinct from the HR either because it is not associated with resistance or it occurs late after infection and is not accompanied by tissue dehydration. It is not known if cell death that occurs in such susceptible interactions generally occurs by a programmed process. However, the existence of maize mutants that resemble different diseases and the recent discovery of Arabidopsis mutants that mimic specific diseases such as bacterial leaf spot and soft rot (unpublished observations) suggests that cell death associated with various diseases might be genetically programmed processes. It is not known whether any of the genes identified in Arabidopsis or maize act in the same pathway to control cell death. As these Arabidopsis and maize mutants become better characterized and some of the genes are cloned, it should become clearer how many pathogen triggered cell death pathways there are. Interestingly, it has also been shown that certain fungal toxins

can induce apoptosis in both animal and plant cells, although the gene products that control the cell death process in toxin-susceptible plants have not been identified (Wang *et al.*, 1996).

7. PCD IN RESPONSE TO ABIOTIC STRESS

Plant cells and tissues exposed to variety of abiotic stresses that ultimately may result in their death. Abiotic stresses include toxins such as salinity, metals, herbicides and gaseous pollutants, including reactive oxygen species (ROS), as well as water deficit and water logging, high and low temperature and extreme illumination. Plants show adaptations to the stress including mechanisms to tolerate the adverse conditions, to exclude the toxins or to avoid conditions where the stress is extreme. Abiotic stress may also result in stunted growth, followed by death of part or all of the plant. Cell death in abiotic stress may therefore be part of a regulated process to ensure survival. Alternatively, it may be due to the uncontrolled death of cells or tissues killed by unfavorable conditions. PCD may be a part of an adaptive mechanism to survive the stress. Adaptation of plants to environmental conditions such as high light intensity or low humidity often involves covering their surfaces with layer of dead unicellular hairs. These cells are thought to go through PCD resulting in the formation of a protective layer
that functions to block high irradiance and trap humidity (Greenberg, 1996).

7.1 Heat stress

Due to the global increase of atmospheric temperature, plants must cope with heat stress conditions in wider and wider areas of our planet. Transitory or constantly high temperatures affect plant growth and development and are a serious threat to crop production worldwide. Heat stress response appears to be one of the most conserved defense mechanisms present in all living organisms. Heat stress induces

different kinds of metabolic responses some of which seem to be activated under a plethora of unfavourable conditions and are aimed at maintaining cellular functionality (Desikan *et al.*, 2001; Baniwal *et al.*, 2004, Baena-González & Sheen, 2008). It has been reported recently that also heat shock induces PCD in plants (Vacca *et al.* 2004).

Heat stress was shown to cause impairments in mitochondrial functions and result in the induction of oxidative damage that manifested in lipid peroxidation (Vacca *et al.*, 2004). The steady-state transcript and protein level of many ROS-scavenging enzymes were found to be elevated by heat stress (Rainwater *et al.*, 1996, Rizhsky et al., 2002). Heat stress-response signal transduction pathways and defense mechanisms, involving heat shock transcription factors (HSFs) and heat shock proteins (HSPs), are thought to be intimately associated with ROS (Pnueli *et al.*, 2003). Several studies have indicated that HSFs are involved in the sensing of ROS. These results indicate a central role of HSFs in early sensing of H_2O_2 and expression of APX1, APX2, and Zat12. Recent studies demonstrated that protection against heat stress-induced oxidative damage involves calcium, abscisic acid (ABA), ethylene, and salicylic acid (SA) (Larkindale and Huang, 2004). Calcium channel blockers and calmodulin inhibitors induced oxidative damage to membrane, and pretreatment with calcium, SA, ABA, and 1-aminocyclopropane-1-carboxylic acid (ethylene precursor) increased survival rate of plants following a lethal heat stress (Larkindale and Knight, 2002).

7.2 Hydrogen peroxide stress

Several lines of evidence support the evidence that, among ROS, hydrogen peroxide (H_2O_2) plays a crucial role in defining PCD in plants. Firstly, H_2O_2 increases remarkably in the early events of the PCD process (Locato *et al.*, 2008) and the different concentrations of exogenously applied H_2O_2 may induce different

cell death pathways (Houot *et al.*, 2001, Gechev *et al.*, 2006). Perturbation of H_2O_2 homeostasis through alteration of catalase activity is able to induce PCD (Dat *et al.*, 2003, Palma and Kermode, 2003). The overexpression of the H_2O_2-detoxifying enzyme ascorbate peroxidase suppresses the H_2O_2-induced PCD (Murgia *et al.*, 2004), while the overexpression of genes involved in biotic response increases H_2O_2 levels following pathogen. Changes in H_2O_2 homeostasis are sensed by the plant cell and, depending on the situation, genetic programs leading to stress acclimation or PCD are triggered (Gechev and Hille, 2005).

Although there has been rapid progress in recent years, there are still many gaps in our understanding of how H_2O_2 affects PCD response of plants.

8. APPLICATION OF PCD

With an increase in the understanding of PCD mechanisms, genetic based and signal molecule genespecific therapies have become a strong alternative for combating diseases in animals and plants. Degenerative diseases such as Alzheimer's, Parkinson's and AIDS as well as proliferative diseases such as cancer that involve apoptosis in one way or another, are recent targets for the selective manipulation of PCD (Bossy-Wetzel *et al.,* 2004). Plants may also be modified for resistance to a wide range of pathogens, considering the fact that there are no specialized cells as in mammals, dedicated exclusively to defense regulation. Therefore, the modulation of PCD to alter the development and progression of diseases in plants must be site-specific at the location of the infection (Kurana SMP *et al.,* 2005). The modulation of apoptosis for disease control in plants can be achieved by either the inhibition or induction of PCD. For compatible obligate pathogens (necrotrophic) the inhibition of apoptosis may result in a broad-spectrum disease resistance. For example, the expression of the anti-apoptotic gene p35 from

baculovirus in transgenic tomato plants provided resistance to *Alternaria alternata, Colletotrichum coccode* and *Phytophtora syringae* pv. tomato (Lincoln *et al.,* 2002). Transgenic tobacco plants expressing negative regulators of apoptosis (as human Bcl-2 and Bcl-xl, nematode CED-9, or baculovirus Op-IAP) exhibited heritable resistance to several necrotrophic fungal pathogens (Dickman MB *et al.,* 2001) . For incompatible-obligate pathogens (biotrophic), disease resistance can be achieved through the induction of HR-linked cell death. Genes with distinct roles in the induction of apoptosis during HR have been identified and characterized. For example, an *Arabidopsis thaliana* gene, AtMYB30, has been identified as a positive regulator of HR-linked cell death following incompatible interactions in response to bacterial pathogens (Vailleau *et al.,* 2004). Another strategy to control biotrophic pathogens is to introduce avr/R gene pairs as two-component sensor systems which could be introduced into crop plants with the avr gene under the control of a pathogeninducible promoter so that infection by any pathogen will trigger an HR, or to fuse such a promoter to gene coding for a non-specific death elicitor. Another possibility is to use such gene pairs to induce low levels of cell death that trigger SAR.

Furthermore, the manipulation of signal transduction pathways that lead to the HR such as those related to second messengers, such as calcium, is an attractive strategy that has been used. The expression of three types of tobacco calmodulins lead to the activation of HR in response to wound and infection with TMV.

Therefore we can say that we should not think of this phenomenon as a degenerative invariably process, in fact the study of the evolution process of human aging showed a series of cellular phenomena associated with apoptosis, which are manufactured in an evolutionary sequence defined: such as DNA and DNA-helicase

alteration, mitochondrial aging, apoptotic atrophy of female reproductive tissues and the death of osteoclasts.

The decay of trophic and hormonal stimuli can be correlated with many of these events, though the relations of cause and effect are not always to clarify; a better understanding of the phenomenon would lead to clear advantages, in clinical field too.

MATERIALS

and METHODS

1. CELL CULTURE, GROWTH CONDITIONS AND HEAT TREATMENTS

In my work I used the cells line Tobacco Bright Yellow 2 (TBY-2); this cell line is important, because basic plant cell features have been well visualized with these. Other features of this cells are the high synchrony, high uniformity and stability and that are non-photosynthetic (the cells have not the Rubisco); the synchrony attained with the TBY-2 cell line is 60-70% MI starting from the S phase, however, it should be remembered, in this context, that this high synchrony may be attainable when the cell line is properly maintained and handled.

The suspension of TBY-2 cells was cultured at 27°C as previously described (de Pinto *et al.* 2002). For heat shock, a stationary culture was diluted 4:100 (v/v; 100 mL), cultured for 4 days and then transferred for 10 min into a water bath at 35°C or 55°C, with constant shaking. After this treatment, the cells were returned at 27°C for 3 and 6 hours and then collected, frozen in liquid nitrogen and stored at -80°C. Where indicated, spermidine 0.5–1.5 mM was added to the cell suspension 15 min before the HS exposition.

For H_2O_2 treatment, a stationary culture was diluted 4:100 (v/v; 100 mL), cultured for 4 days and then incubated with 50mM H_2O_2 with constant shaking. After this treatment, the cells were collected at 30 min and 3h, frozen in liquid nitrogen and stored at -80°C.

Cell viability was measured by Trypan Blue staining as previously described (de Pinto *et al.* 2002).

2. PROTEIN EXTRACTION

Frozen cell pellets were homogenised, by using a mortar and pestle, in liquid nitrogen with addition of quartz sand. Soluble proteins were extracted in four volumes (w/v) of buffer containing 500 mM Tris–HCl pH 8, 700 mM sucrose, 10

mM EDTA, 4 mM ascorbic acid, 0.4% 2-mercaptoethanol, 0.2% Triton X-100 10%, 1 mM PMSF (Sigma, St. Louis, MO, USA), 1 µM Leupeptin (Fluka, Stenheim, Germany), 0.1 mg/mL Pefabloc (Fluka, Stenheim, Germany), and subsequently stirred for 30 min at 4˚C. After sonication, three times for 10 s with intervals of 30 s, the samples were centrifuged at 13,000g for 20 min at 4˚C. An equal volume of phenol saturated with Tris–HCl 0.1 M pH 8 was added to the supernatant, and the phases were separated by centrifugation at 5000g for 20 min at 4˚ C. The proteins were precipitated by adding five volumes of cold 0.1 M ammonium acetate in methanol to the phenol phase, vortexing and incubating overnight at -20˚ C. The precipitates were recovered by centrifuging at 13,000g for 30 min, washed with ammonium acetate in methanol and with acetone 80%, and then resuspended in the IEF solubilization buffer [7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 50 mg/mL dithiothreitol (DTT)]. After sample clarification at 13,000g for 10 min, protein concentration was measured by the Bio-Rad protein assay (Hercules, CA, USA), using bovine serum albumin as a standard. The samples were directly loaded for isoelectrofocusing (IEF) or stored in aliquots at -80˚C until use.

EXTRACTION BUFFER	
	[final]
TRIS-HCl 1.5 M pH 8	**0.5 M**
Saccarosio (Mw=342.3)	**0.7 M**
EDTA (Mw=372.2)	**10 mM**
KCl (Mw=58.44)	**0.1 M**
A. Ascorbico (Mw=176.1)	**4 mM**
MSH-beta mercapto (14.3M)	**0.4 %**
Triton X-100 10% (W/V)	**0.2 %**
H_2O	

PROTEASE INHIBITOR	
	[final]
PMSF (200 mM)	**1 Mm**
Leupeptina (stock 10 mM)	**1 µm**
Pefabloc	**0.1 mg/ml**

BUFFER IEF	
	[final]
CHAPS	**4 %**
UREA	**7 M**
TIOUREA	**2 M**
DTT	**50 mg / ml**

3. 2-D GEL ELECTROPHORESIS

The washed pellets were air-dried and recovered in 7 M urea, 2 M thiourea, 4% CHAPS, 50 mg/mL DTT, 0.5% of carrier ampholyte. Protein concentration was determined by Bradford assay (Sigma-Aldrich Italia, Milan, Italy). Three independent protein extractions were performed from each sample**.** 600 μg of total proteins were loaded onto an 18 cm and pH 4-7 linear gradient IPG strips (GE Healthcare, Uppsala, Sweden). Then IEF was performed at 16°C in the IPGphor system (Amersham Biosciences, Uppsala, Sweden) as the following: for 4 h at 200 V, from 200 to 3500 V in gradient during 30 min, 3h at 3500 V, from 3500 to 8000 V in gradient during 30 min, after which the run was continued at 8000 V to give a total of 70 kVh. Each focused strip was equilibrated for 30 min against 6 M urea, 30% glycerol, 2% SDS, 50 mM Tris-HCl pH 8.8, 2% DTT and then a further 30 min with the substitution of the DTT with 2.5% iodoacetamide. The separation of proteins in the second dimension was performed with SDS polyacrylamide gels (12.5%) on an Ettan DALT System (GE Healthcare). The SDS-PAGE gels were visualized by the modified Colloidal Coomassie Brilliant Blue (CCBB) staining

method (Aina *et al.* 2007). Each separation was repeated 3 times for each biological replicate to ensure the protein pattern reproducibility.

LOADING BUFFER	
	[final]
SAMPLE	**X**
IPG buffer	**1.25 µl**
Blu Bromophenol	**2.5 µl**
Buffer IEF	**q.b.**

EQUILIBRATION BUFFER	
	[final]
Tris-HCl pH 8.8, 1.5M	**50 mM**
Urea	**6 M**
Glyicerol	**30 %**
SDS	**2 %**
H_2O	**q.b.**

RUNNING BUFFER (Laemmli, pH 8.3)	
	[final]
Trizma	**50 mM**
Glycin	**6 M**
SDS	**30 %**
H_2O	**q.b.**

GEL SOLUTION	
	[final]
MONOMER STOCK SOLUTION	**30% T, 2.67% C**
Tris-HCl pH 8.8, 1.5M	
TEMED	**0.5 %**
APS 10%	**10 %**
H_2O	**q.b.**

STAINING SOLUTION	
	[final]
Ortho phosphoric acid	**1,6%**
Ammonium Sulfate	**8%**
Blu G-250 5 %	**0,08%**
Methanol	**20%**
H_2O	**q.b.**

4. IMAGE ACQUISITION AND SPOT DETECTION

The gels were analyzed by using the Image Master 2D Platinum software version 5.0 (Amersham Biosciences). Data were normalized by expressing protein abundance as percent spot volume relative to volume of total protein in the gel (%Vol). The 2D gels for each biological replicate were averaged and the resulting gel (master gel) contains only the spots present in all of the gels. Statistical analysis (Student's *t*-test at a level of 95%) identified proteins that significantly increased or decreased (at least 1.5-fold in relative abundance) after the different treatments with respect to the control. These spots were selected for MS/MS analysis.

5. STATISTICAL ANALYSIS

For all analysis at least three replicates were performed for each heat treatment and the values represent the means (± SD). Statistical analysis was done using a two-tailed Student's *t* test and with values that are significantly different with $p < 0.05$ and $p < 0.01$ respectively.

The gels and X-ray films were analyzed by using the Image Master 2D Platinum software version 5.0 (Amersham Biosciences).

6. IN GEL DIGESTION AND MASS SPECTROMETRY ANALYSIS

Selected spots were manually excised from the 2D-gels, washed twice and stored in 50% ethanol at 4°C until digestion. Spots digestion was performed as previously described (Marsoni *et al.* 2008). The extracted tryptic fragments were analyzed by MS/MS after reverse phase separation of peptides (Liquid Chromatography-ElectroSpray Ionization Mass tandem Spectrometry, LC-ESI-MS/MS). For all experiments, a Finningan LXQ linear ion trap mass spectrometer, equipped with a Finningan Surveyor MS plus HPLC system (Thermo Electron Corporation, CA,

USA) was used. Chromatography separations were conducted on a BioBasic C18 column (150 µm I.D. x 150 mm length and 5 µm particle size; Thermo Electron Corporation), using a linear gradient from 5 to 75% acetonitril, containing 0.1% formic acid with a flow of 2 µL/min. Acquisitions were performed in the data-dependent MS/MS scanning mode (full MS scan range of 400 – 1400 m/z followed by Zoom scan for the most intense ion from the MS scan and full MS/MS for the most intense ion from the zoom scan), thus enabling a dynamic exclusion window of 3 min. Protein identification was performed by searching in the National Center for Biotechnology Information (NCBI) viridiplantae and/or EST-viridiplantae protein database using the MASCOT program (http://www.matrixscience.com). The following parameters were adopted for database searches: complete carbamidomethylation of cysteines, partial oxidation of methionines, peptide mass tolerance 1.2 Da, fragment mass tolerance 0.8 Da and missed cleavage 1. For positive identification, the score of the result of [- 10 × log(P)] had to be over the significance threshold level ($p > 0.05$). Unsuccessful protein identifications were submitted to de novo analysis by PepNovo software using default parameters (http://peptide.ucsd.edu/pepnovo.py). Only those PepNovo results were accepted that received a mean probability score of at least 0.5. Peptides sequences candidates were edited according to MS BLAST rules and MS BLAST search was performed against NCBI non redundant database at http://www.dove.embl-heidelberg.de/Blast2/msblast.html. Statistical significance of hits was evaluated according to MS BLAST scoring scheme. Other than Mowse and MS BLAST scoring system to assign correct identification we make a point of a minimum of two matched peptides and a molecular weight predicted in good agreement with that estimated from SDS-PAGE.

For the sub cellular localization we used CELLO v.2.5: subCELlular LOcalization predictor (Yu *et al.* 2006).

7. WESTERN BLOTTING

Total soluble proteins were extracted by phenol method, as previously described, but after acetone precipitation the pellet was re-suspended in Laemmli sample buffer. 75 µg of proteins were loaded onto 14% SDS-PAGE gel and transferred to PVDF membranes (Westran CS, 0,45 µm, Whatman). Membranes were probed with 1:2000 Anti-cAPX monoclonal antibody (AP6 from Saji *et al.*, 1990) using the Supersignal West Dura Extended Duration Chemiluminescent Substrate for HRP system (Pierce). Protein loading was verified by Ponceau staining of the membrane. The analysis was done in triplicate; however, only one representative Western blot is shown.

TRANSFER BUFFER	
	[final]
Trizma	**50 mM**
Glycin	**6 M**
SDS	**30 %**
MetOH	**20 %**
H_2O	**q.b.**

8. SEMIQUANTITATIVE RT-PCR EXPERIMENTS

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First strand cDNA was synthesized from 2 µg of total RNA by using Superscript III reverse transcriptase (Invitrogen). The 18S RNA was used as the inner control. The PCR products were isolated by 1% gel electrophoresis. The data were analysed with ImageJ 1.41 (http://rsb.info.nih.gov/ij/). All experiments were repeated three times independently for each biological replicate.

AIM OF THE WORK

The main objective of the research described in this thesis is the identification of the molecular factors associated with the early phases of PCD in (*Nicotiana tabacum*) tobacco cells Bright-Yellow 2 (TBY-2).

To achieve this, I used a proteomic approach which allows the global analysis of gene products in various tissues and cells in different physiological states. With the completion of genome sequencing projects and the development of analytical methods for protein characterization, proteomics has become a major field of functional genomics. The usefulness 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 has been already demonstrated.

For this study, I chose to use TBY2 cells, not only because data obtained using these cells are highly reproducible, but also because the PCD process has already been extensively studied in this system. Cells were exposed to two different heat shocks (HSs): 55 °C n or 35 °C, for 10 min. HS at 55 °C triggers PCD without the addition of any PCD inducer (Burbridge *et al.* 2007; Vacca *et al.* 2004). In TBY-2 cells exposed to a temperature of 55 °C, cell viability starts to decrease after 2 to 3 h of recovery at 27 °C, falls to 50% after 24 h, and is negligible after 72 h (Vacca *et al.* 2004). Evidence that cell death occurs by PCD is provided by cytological hallmarks: cytoplasm shrinkage in 72% of 24 h-PCD cells, DNA laddering, and cytochrome c release from mitochondria (Locato *et al.* 2008). Moreover, HS at 55 °C induces a biphasic production of H_2O_2 and a rapid increase in nitric oxide, both of which are necessary events for the activation of PCD (Delledonne *et al.* 1998). By contrast, 35°C HS leads to only a transient increase in H_2O_2 and a consequent alteration in Reactive Oxygen Species (ROS) scavenging systems aimed at maintaining the cell redox homeostasis (Locato *et al.* 2008). We performed a proteomic analysis of TBY-2 cells collected at 3 and 6 h after HS exposure. These

time intervals were chosen on the basis of previous results obtained in the same experimental setup (Locato *et al.* 2008). Three hours after HS at 55°C (3 h-PCD), the biochemical pathways leading to PCD begin to be activated and cytological evidence of PCB starts to emerge; 6 h after the HS at 55°C (6 h-PCD), cell death is much more evident, even at the cytological level. In the TBY-2 cells exposed to 35 °C HS, the homeostatic response (HRE) is at its highest level between 3 and 6 h after treatment (3 h- and 6 h-HRE) and then returns to the control level (Locato *et al.* 2008).

We analyzed the overlap of differentially-accumulated proteins between HRE and PCD, and identified the modulation of specific proteins belonging to different categories, some of which are responsible for the two different cell fates.

In TBY-2 cell suspension, the PCD process can be induced by several stimuli, such as the direct addition of 50 mM H_2O_2. However, in this treatment, the mortality trend is steeper than HS at 55°C: the cell viability reaches 30% after 24 h. Also, H_2O_2 induces cytoplasm shrinkage, a typical marker of plant PCD, in almost 80–90% of the Trypan blue-dyed cells (de Pinto *et al.* 2006). We analyzed the proteins from cells treated with 50 mM H_2O_2 for 30 min and 3 h to gain information about the proteins induced by oxidative stress and involved in PCD pathway. The results were compared with those previously obtained from HS PCD cells. The two systems are quite similar because the mortality rate is 20–25% in both 3 h H_2O_2 and 3 h HS PCD cells, although the physiological statuses of the remaining 85% of cells in the two treatments may not be directly comparable.

The PCD pathways in plants not only remain enigmatic, but the nature and activities of core regulators of plant PCD are poorly understood. Proteins that are differentially and commonly accumulated during both types of PCD may function as a core regulator of plant PCD.

The overall picture to emerge from the proteomic study was a sophisticated functional network for the regulation of oxidative stress in TBY-2 cells undergoing PCD. The role of selected members of this network was corroborated by physiological and biochemical analysis. In addition, immunological experiments were performed to show that post-translational modifications play important regulatory roles during the early phases of PCD.

Exploring the soluble proteome of Tobacco Bright Yellow-2 cells at the switch towards different cell fates in response to heat shocks

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The soluble proteome of TBY-2 cells towards PCD induced in response to Hydrogen peroxide

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SUBMITTED

RESULTS

and DISCUSSION

The soluble proteome of Tobacco Bright Yellow-2 cells towards programmed cell death induced in response to hydrogen peroxide

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ABSTRACT

We have used a comparative proteomic approach to investigate the changes elicited in Tobacco (*Nicotiana tabacum*) Bright-Yellow 2 (TBY-2) cell cultures by 50 mM H_2O_2 treatments for 30' and 3h. This strong oxidative stress is able to induce programmed cell death (PCD) in TBY-2 cells. Of the 1300 protein spots reproducibly resolved, 230 changed in abundance after H_2O_2 stress and 156 were successfully identified by LC–MS/MS analysis. The sorting of the identified proteins in different functional categories revealed that they mainly belong to metabolism (75) disease/defence (20) and protein synthesis and fate (45). For several differentially expressed proteins there is the intriguing possibility that they may play unappreciated roles in addition to the already known functions.

Some of the identified proteins must be responsible of the PCD pathway induction. To identify proteins that are putatively important in the PCD pathway, proteomic data from H_2O_2-PCD cells were compared with proteomic data obtained previously from cells undergoing PCD 3h after heat shock at 55°C. The two systems were judged to be similar because the mortality was 25% in both cases. 19 protein spots exhibited similar regulation during both types of PCD. Although studies are necessary to confirm their direct involvement in PCD regulation, they may represent a core mechanism of plant PCD.

Keywords: H_2O_2, redox homeostasis, PCD, TBY-2 cells, 2-DE, mass spectrometry

Introduction

Programmed cell death (PCD) is a genetically regulated process active during cellular differentiation, organ abortion, and cellular senescence (Kuriyama and Fukuda, 2002; Souter and Lindsey, 2000; Young and Gallie, 2000; Gunawardena *et al.*, 2004) as well as in response to biotic and abiotic stresses (Williams and Dickman, 2008; Langebartels *et al.*, 2002). Hallmarks of plant PCD include condensed cell morphology, a shrunken nucleus, fragmented DNA, mitochondrial swelling and condensation.

Several lines of evidence indicate that hydrogen peroxide (H_2O_2) plays a role in initiating PCD in plants. First, H_2O_2 increases early on in the process (Locato *et al.*, 2008), and different concentrations of exogenous H_2O_2 induce different cell death pathways (Houot *et al.*, 2001; Gechev *et al.*, 2006; Rentel *et al.*, 2004). Decreasing catalase activity causes perturbations of H_2O_2 homeostasis and induces PCD (Dat *et al.*, 2003, Palma and Kermode, 2003). The overexpression of the H_2O_2-detoxifying enzyme ascorbate peroxidase suppresses H_2O_2-induced PCD (Murgia *et al.*, 2004) while the overexpression of genes involved in responses to biotic stresses increases H_2O_2 levels (Choi *et al.*, 2007). Plant cells sense changes in H_2O_2 homeostasis and trigger genetic programs that promote either stress acclimation or PCD (Gechev and Hille, 2005).

The direct addition of 50 mM H_2O_2 to the TBY-2 cell suspension induced cell death, as about 30% of the cells were viable after 24 h. In addition, H_2O_2 induced cytoplasm shrinkage, a marker of plant PCD, in almost 80–90% of the Trypan blue-dyed cells (de Pinto *et al.*, 2006).

To investigate the early phases of H_2O_2-induced PCD, changes in protein expression were analyzed in TBY-2 cells treated with 50 mM H_2O_2 for 30 min (30'-H_2O_2) and 3 h (3h-H_2O_2) and cell viability was 100% and 85%, respectively.

Using two-dimensional electrophoresis (2-DE) in combination with MS/MS analysis, we isolated 152 H_2O_2-responsive proteins. Identification of these proteins and their mapping to various cellular processes gave a global view of the changes elicited in TBY-2 cells by oxidative stress. To identify a putative PCD core mechanism, we compared the data with that previously obtained from cells undergoing PCD induced by heat shock (Vannini *et al.,* 2010). The results provide a framework for further functional studies of several members of the PCD network.

MATERIALS AND METHODS

Cell culture, growth conditions and treatments

The suspension of TBY-2 cells was cultured at 27°C as previously described (de Pinto *et al.,* 2002). For H_2O_2, a stationary culture was diluted 4:100 (v/v; 100 mL), cultured for 4 days and then with constant shaking. After this treatment, the cells were collected, frozen in liquid nitrogen and stored at -80°C.

For heat shock, a stationary culture cultured for 4 days and then transferred for 10 min into a water bath at 35°C or 55°C, with constant shaking. After this treatment, the cells were returned at 27°C for 3 and 6 hours and then collected, frozen in liquid nitrogen and stored at -80°C.

Where indicated, spermidine 2mM was added to the cell suspension 15 min. before H_2O_2 exposition. Cell viability was measured by Trypan Blue staining as previously described (de Pinto *et al.*, 2002). Three biological replicates were done.

Protein extraction and 2-D gel electrophoresis

The total proteins were extracted by phenol as previously described (Marsoni *et al.,* 2008). The washed pellets were air-dried and recovered in 7 M urea, 2 M thiourea, 4% CHAPS, 50 mg/mL DTT, 0.5% of carrier ampholyte. Protein concentration was determined by Bradford assay (Sigma-Aldrich Italia, Milan, Italy). Three independent protein extractions were performed from each sample**.** 600 *µ*g of total proteins were loaded onto an 18 cm and pH 4-7 linear gradient IPG strips (GE Healthcare, Uppsala, Sweden). Then IEF was performed at 16 °C in the IPGphor system (Amersham Biosciences, Uppsala, Sweden) as the following: for 4 h at 200 V, from 200 to 3500 V in gradient during 30 min, 3h at 3500 V, from 3500 to 8000 V in gradient during 30 min, after which the run was continued at 8000 V to give a total of 70 kVh. Each focused strip was equilibrated for 30 min against 6 M urea, 30% glycerol, 2% SDS, 50 mM Tris-HCl pH 8.8, 2% DTT and then a further 30 min with the substitution of the DTT with 2.5% iodoacetamide. The separation of proteins in the second dimension was performed with SDS polyacrylamide gels (12.5%) on an Ettan DALT System (GE Healthcare). The SDS-PAGE gels were visualized by the modified Colloidal Coomassie Brilliant Blue (CCBB) staining method (Aina *et al.*, 2007). Each separation was repeated 3 times for each biological replicate to ensure the protein pattern reproducibility.

Image acquisition and spot detection

The gels were analyzed by using the Image Master 2D Platinum software version 5.0 (Amersham Biosciences). Data were normalized by expressing protein abundance as percent spot volume relative to volume of total protein in the gel (%Vol). The 2D gels for each biological replicate were averaged and the resulting gel (master gel) contains only the spots present in all of the gels. Statistical analysis (Student's *t*-test at a level of 95%) identified proteins that significantly increased or decreased (at least 1.5-fold in relative abundance) after the different treatments with respect to the control. These spots were selected for MS/MS analysis.

In gel digestion and mass spectrometry analysis

Selected spots were manually excised from the 2D-gels, washed twice and stored in 50% ethanol at 4°C until digestion. Spots digestion was performed as previously described (Marsoni *et al.* 2008). The extracted tryptic fragments were analyzed by MS/MS after reverse phase separation of peptides (Liquid Chromatography-ElectroSpray Ionization Mass tandem Spectrometry, LC-ESI-MS/MS). For all experiments, a Finningan LXQ linear ion trap mass spectrometer, equipped with a Finningan Surveyor MS plus HPLC system (Thermo Electron Corporation, CA, USA) was used. Chromatography separations were conducted on a BioBasic C18 column (150 µm I.D. x 150 mm length and 5 µm particle size; Thermo Electron Corporation), using a linear gradient from 5 to 75% acetonitril, containing 0.1% formic acid, for 50 min. with a flow of 2 µL/min. Acquisitions were performed in the data-dependent MS/MS scanning mode (full MS scan range of 400 – 1400 m/z followed by Zoom scan for the most intense ion

from the MS scan and full MS/MS for the most intense ion from the zoom scan), thus enabling a dynamic exclusion window of 3 min. Protein identification was performed by searching in the National Center for Biotechnology Information (NCBI) viridiplantae and/or EST-viridiplantae protein database using the MASCOT program (http://www.matrixscience.com). The following parameters were adopted for database searches: complete carbamidomethylation of cysteines, partial oxidation of methionines, peptide mass tolerance 1.2 Da, fragment mass tolerance 0.8 Da and missed cleavage 1. For positive identification, the score of the result of [- 10 × log(P)] had to be over the significance threshold level ($p > 0.05$). Unsuccessful protein identifications were submitted to de novo analysis by PepNovo software using default parameters (http://peptide.ucsd.edu/pepnovo.py). Only those PepNovo results were accepted that received a mean probability score of at least 0.5. Peptides sequences candidates were edited according to MS BLAST rules and MS BLAST search was performed against NCBI non redundant database at http://www.dove.embl-heidelberg.de/Blast2/msblast.html. Statistical significance of hits was evaluated according to MS BLAST scoring scheme. Other than Mowse and MS BLAST scoring system to assign correct identification we make a point of a minimum of two matched peptides.

For the sub cellular localization we used CELLO v.2.5: subCELlular LOcalization predictor (Yu *et al.*, 2006).

Western blotting

Total soluble proteins were extracted by phenol method, as previously described, but after acetone precipitation the pellet was re-suspended in Laemmli sample buffer. 75 µg of proteins were loaded onto 14% SDS-PAGE gel and transferred to PVDF membranes (Westran CS, 0,45 µm, Whatman). Membranes were probed with 1:2000 Anti-cAPX monoclonal antibody (AP6 from Saji *et al.*, 1990), using the Supersignal West Dura Extended Duration Chemiluminescent Substrate for HRP system (Pierce). Protein loading was verified by Ponceau staining of the membrane. The analysis was done in triplicate; however, only one representative Western blot is shown.

Statistical analysis

For all analysis at least three replicates were performed for each heat treatment and the values represent the means (± SD). Statistical analysis was done using a two-tailed Student's *t* test and (*) and (**) indicates values that are significantly different with $p < 0.05$ and $p < 0.01$ respectively.

RESULTS

H_2O_2 response

TBY-2 cell cultures exposed to 50mM H_2O_2 were analysed for cell viability. The decrease of cell viability 30 minutes and 3 hours was of 0-5% and 15% respectively, and a further decrease occurred in the following hours as already reported in literature (Vacca *et al.*, 2004; Locato *et al.*, 2008). In a substantial number of cells, cytoplasmic shrinkage was also evident confirming that PCD was triggered, consistently with data obtained previously under the same experimental conditions (Vacca *et al*., 2006).

2-D separation and identification of differentially accumulated proteins of control and H_2O_2-treated TBY-2 cells.

After phenolic extraction and during the time of the analysis here performed, the amount of total extracted protein was not significantly different between the control and H_2O_2 exposed cells (data not shown). The protein extracts were subjected to IEF on linear gradient ranging from pH 4 to 7 and they were subsequently separated on 12.5% SDS-PAGE gels. Image analysis revealed an average of about 1300 reproducible protein spots in each gel stained with colloidal CBB (Figure 1).

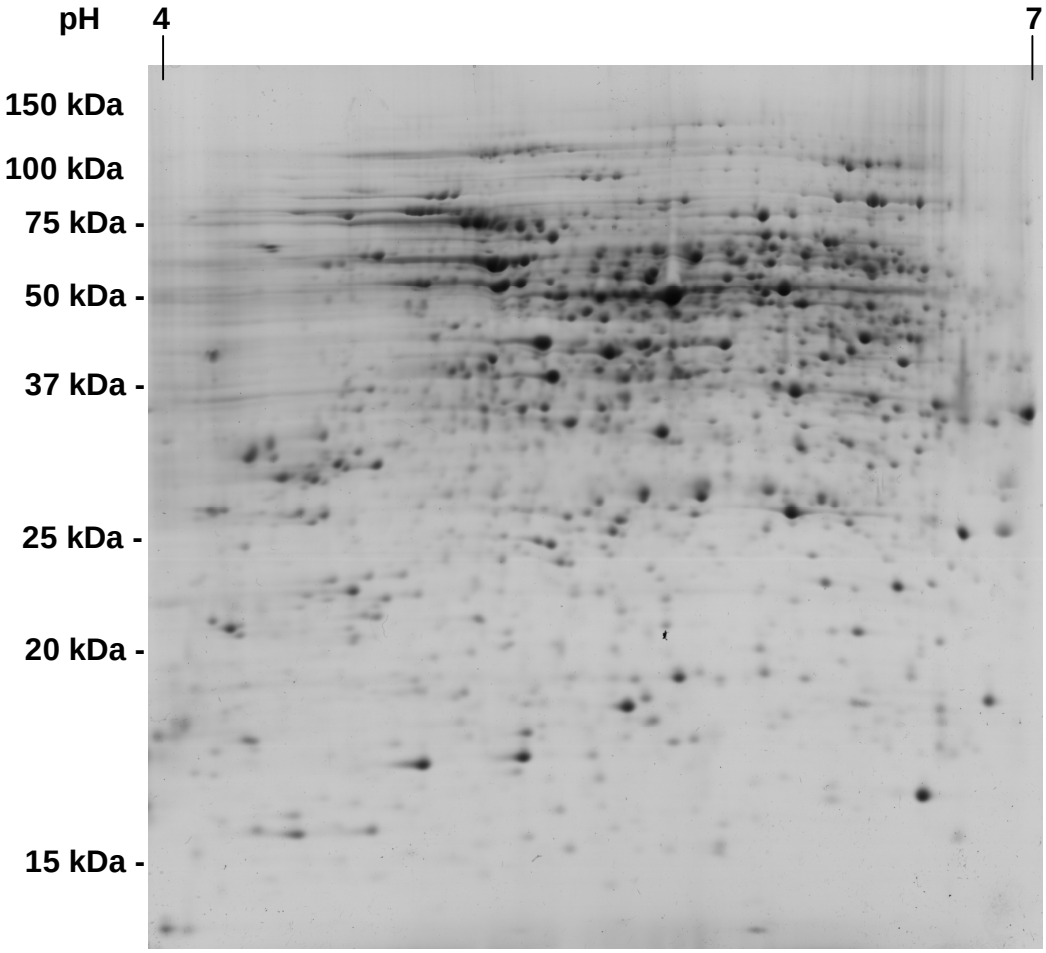

Figure 1. Images of CCB-stained 2D isoelectric focusing sodium dodecyl sulphate polyacrylamide gel electrophoresis (IEF SDS–PAGE) gels.

Image Master 2D Platinum software showed 230 protein spots that change significantly (*t* test; $p<0.05$) in relative abundance of at least 1.5 fold between control and H_2O_2 treated cells. 165 of these spots were successfully identified by LC–MS/MS analysis (Table1).

			Fold-of variation versus		MW (Da)/pI		
Spot	**Protein name**	**NCBI accession number**	**30'**	**3h**	**Theor.**	**Exp.**	**EST code**
Disease/defence							
1	thioredoxin peroxidase *Nicotiana tabacum*	gi|21912927	-6	<0.01	30.0/8.2	24.0/5.0	
2	thioredoxin peroxidase *Nicotiana tabacum*	gi|21912927	-2,4	-1,7	30.0/8.2	23.0/4.9	
3	thioredoxin peroxidase *Nicotiana tabacum*	gi|21912927	1	-1,9	30.0/8.2	32.0/6.6	
4	peroxiredoxin-2E, chloroplastic; Thioredoxin reductase 2E *Arabidopsis thaliana*	gi|143360522	1	-1,7	22.5/7.6	17.0/5.1	
5	peroxiredoxin, putative *Ricinus communis* [a]	gi|255575353	<0.01	1	23.7/7.6	17.0/5.4	gi|225317455
6	peroxiredoxin *Ipomoea batatas* [a]	gi|37783267	3,9	8	20.7/8.8	19.0/5.9	gi|52834488
7	thioredoxin H-type 1 *Nicotiana tabacum*	gi|267124	-1,9	1	14.1/5.6	12.0/5.6	
8	disulfide oxidoreductase, putative *Ricinus communis* [a]	gi|255575237	-1,6	1	39.3/8.2	33.0/5.7	gi|761563

9	ascorbate peroxidase *Nicotiana tabacum*	gi|559005	1,5	2,6	27.3/5.4	27.0/5.4	
10	ascorbate peroxidase *Nicotiana tabacum*	gi|559005	-1,5	-1,5	27.3/5.4	27.0/5.7	
11	ascorbate peroxidase *Nicotiana tabacum*	gi|76869309	1	-1,5	27.0/5.4	27.0/5.9	
12	thylakoid-bound ascorbate peroxidase *Nicotiana tabacum*	gi|4996602	-2,4	-4,1	47.4/8.5	32.0/5.7	
13	thylakoid-bound ascorbate peroxidase *Nicotiana tabacum*	gi|4996602	-1,7	-2,4	47.4/8.5	32.0/6.0	
14	Ferritin-1 *Nicotiana sylvestris*	gi|48754322	<0.01	<0.01	28.2/5.7	26.0/5.6	
15	superoxide dismutase [Fe]chloroplastic *Nicotiana plumbaginifolia*	gi|134642	<0.01	<0.01	23.0/5.5	23.0/5.9	
16	probable glutathione-S-transferase *Capsicum annuum* [a]	gi|60459397	4,7	6	25.4/5.5	26.0/6.0	gi|76871463
17	glutathione S-transferase *Solanum commersonii* [a]	gi|148616162	-1,9	1	23.8/5.9	24.0/6.75	gi|83420783
18	glyoxalase I *Solanum lycopersicum* [a]	gi|2494844	-1,5	1	20.7/5.3	20.0/5.0	gi|190145651
19	flavodoxin-Like Quinone Reductase *Arabidopsis thaliana* [a]	gi|15239652	-1,8	1	21.7/5.9	22.0/6.7	gi|83420109

20	chilling-responsive protein *Nicotiana tabacum*	gi|153793260	-2	2,3	35.7/4.9	35.0/4.9	
Protein synthesis and fate							
21	eukaryotic translation initiation factor 3 subunit, putative *Ricinus communis* [a]	gi|255550315	-1,8	1,7	26.9/5.8	25.0/5.6	gi|76867840
22	eukaryotic initiation factor 4A-11 *Nicotiana tabacum*	gi|2500518	1	3,1	47.2/5.4	47.0/5.3	
23	eukaryotic initiation factor 4A-11 *Nicotiana tabacum*	gi|2500518	1,6	3,2	47.2/5.4	45.0/5.3	
24	eukaryotic initiation factor 4A-9 *Nicotiana tabacum* [a]	gi|2500517	1	-1,8	47/5.5	46.0/5.5	gi|190874337
25	eukaryotic initiation factor 4A-9 *Nicotiana tabacum*	gi|2500517	1,7	2,5	47.0/5.5	47.0/5.2	
26	eukaryotic initiation factor 4A-15 *Nicotiana tabacum*	gi|2500521	1	2	46.9/5.4	47.0//5.35	
27	eukaryotic translation initiation factor 5A-2 *Nicotiana plumbaginifolia*	gi|124226	1	<0,01	17.6/5.6	17.0/5.65	
28	eukaryotic translation elongation factor, putative *Vitis vinifera* [a]	gi|225454579	2	1	94.0/5.9	110.0/6.4	gi|92027234
29	elongation factor 1-beta / EF-1-beta *Arabidopsis thaliana* [a]	gi|145324076	1	-1,7	28.7/4.6	31.0/4.4	gi|76867651

30	ribosomal protein L2-like *Solanum tuberosum*	gi|81074776	7,3	1	28.7/10.6	29.0/6.8	
31	glycyl-tRNA synthetase, putative *Ricinus communis* [a]	gi|255543218	2,6	1	77.3/6.6	62.0/6.2	gi|190806778
32	glycyl-tRNA synthetase, putative *Ricinus communis* [a]	gi|255543218	<0,01	1	77.3/6.6	70.0/6.5	gi|190878443
33	mitochondrial small heat shock protein *Solanum lycopersicum* [a]	gi|3492854	1	1,9	23.8/6.5	21.0/4.8	gi|92010828
34	heat shock protein, putative *Ricinus communis*	gi|255581792	1	-1,5	90.0/5.2	85.0/5.1	
35	heat shock protein *Solanum lycopersicum*	gi|68989120	<0.01	-2,9	110/6.2	90.0/6.0	
36	heat shock 70 kDa protein, putative *Ricinus communis* [a]	gi|255574576	1	-1,9	93.6/5.2	110.0/5.5	gi|190723847
37	hsp70-binding protein, putative *Ricinus communis* [a]	gi|255581500	1	-1,6	39.2/5.2	42.0/5.0	gi|224704536
38	heat shock 70 kDa protein putative *Ricinus communis* [a]	gi|255574576	1	-1,6	94.0/5.2	110.0/5.6	gi|92037393
39	heat shock 70 kDa protein, putative *Ricinus communis* [a]	gi|255574576	1	-1,8	94.0/5.2	110.0/5.5	gi|92027886
40	molecular chaperone Hsp90-1 *Nicotiana benthamiana*	gi|38154482	1	-2,2	80.0/4.9	80.0/4.9	

41	heat shock protein, putative *Ricinus communis*	gi|255554571	-5,2	1	71.0/6.1	70.0/5.4	
42	heat shock protein 70 (HSP70)-interacting protein, putative *Ricinus communis* [a]	gi|255537027	1,5	1	47.0/5.5	60.0/5.4	gi|83419786
43	hypothetical protein cpn60 *Vitis vinifera*	gi|225442531	-2	-1,7	65.3/5.6	58.0/5.1	
44	putative luminal binding protein *Corylus avellana* [a]	gi|10944737	<0.01	-2	73.0/4.9	73.0/5.1	gi|190753562
45	chaperonin containing t-complex protein 1, epsilon subunit, tcpe, putative *Ricinus communis* [a]	gi|255547962	2,3	1,6	59.2/5.5	60.0/5.7	gi|92040148
46	chaperonin containing t-complex protein 1, gamma subunit, tcpe, putative *Ricinus communis* [a]	gi|255577568	1	1,7	60.0/5.9	61.0/6.0	gi|76867096
47	chaperonin containing t-complex protein 1, epsilon subunit, tcpe, putative *Ricinus communis* [a]	gi|255547962	<0.01	<0.01	59.2/5.5	56.0/5.4	gi|92040148
48	chaperonin T-complex protein 1 subunit epsilon *Zea mays*	gi|226506102	1	2,5	59.6/5.7	60.0/5.7	
49	chaperonin 21 precursor *Solanum lycopersicum* [a]	gi|7331143	-1,9	1	26.5/6.8	26.0/5.3	gi|52839170
50	ubiquitin *Nicotiana benthamiana*	gi|213868277	1	2,7	7.5/5.7	7.5/6.1	

51	SUMO *Nicotiana benthamiana* [a]	gi|213868279	-2	1	11.0/4.9	13.8/4.9	gi|51462312
52	20S proteasome subunit beta-6 *Petunia hybrida* [a]	gi|17380185	1	1,6	24.6/6.3	26.0/5.7	gi|92037305
53	20S proteasome alpha 6 subunit *Nicotiana benthamiana*	gi|22947842	<0.01	<0.01	30.0/5.0	33.0/5.2	
54	20S proteasome alpha 6 subunit *Nicotiana benthamiana*	gi|22947842	1	<0,01	29.8/5.0	31.0/5.2	
55	26S proteasome non-ATPase regulatory subunit, putative *Ricinus communis*	gi|255538376	1	<0.01	27.2/6.1	18.0/5.9	
56	26S protease regulatory subunit, putative *Ricinus communis*	gi|255570523	1,6	-1,7	49.5/5.9	53.0/6.2	
57	putative alpha7 proteasome subunit *Nicotiana tabacum*	gi|14594925	-1,6	1	27.2/6.1	27.3/6.1	
58	cysteine proteinase aleuran type *Nicotiana benthamiana* [a]	gi|71482942	-4,7	<0,01	39.2/6.9	29.0/6.3	gi|76866797
59	ATP-dependent Clp protease ATP-binding subunit *Solanum lycopersicum*	gi|399213	-2,4	-2	102.5/5.7	85.0/5.6	
60	oligopeptidase A *Ricinus communis* [a]	gi|255572579	-8	1	88.0/5.2	80.0/5.7	gi|190802613
61	oligopeptidase A, putative *Ricinus communis* [a]	gi|255572579	-5,6	1	88.0/5.2	85.0/5.6	gi|190876913

62	oligopeptidase A, putative *Ricinus communis* [a]	gi|255572579	1,9	-1,8	88.0/5.2	78.0/5.5	gi|76868752
63	mitochondrial processing peptidase *Solanum tuberosum*	gi|587566	1	-1,8	59.9/6.2	60.0/6.0	
64	mitochondrial processing peptidase *Solanum tuberosum*	gi|587564	-2,6	1	59.4/6.2	57.0/5.7	
65	cytochrome c reductase-processing peptidase subunit II *Solanum tuberosum* [a]	gi|410634	1	-2	59.3/6.2	59.0/5.9	gi|224697460
Signal transduction/regulation							
66	BTF3 *Nicotiana benthamiana*	gi|90823167	<0.01	<0.01	17.3/6.3	17.0/6.6	
67	BTF3 *Nicotiana benthamiana*	gi|90823167	2,4	2	17.3/6.3	18.0/6.6	
68	nucleic acid binding protein, putative *Ricinus communis* [a]	gi|255558037	<0.01	-1,7	28.6/4.9	30.0/5.5	gi|190847542
69	DNA-binding protein GBP16 *Oryza sativa Japonica*	gi|2511541	<0.01	<0.01	43.4/6.5	25.0/6.2	
70	EBP1 *Solanum tuberosum* [a]	gi|116292768	1	-2,3	42.8/6.3	45.0/6.5	gi|94324881
71	RAN *Nicotiana sylvestris* [a]	gi|48249480	1	1,7	25.5/6.3	26.0/6.8	gi|92027217

72	SGT1-like protein *Nicotiana tabacum*	gi|29468339	<0,01	<0,01	41.4/5.2	33.0/5.4	
73	SGT1-like protein *Nicotiana tabacum*	gi|29468339	<0.01	<0.01	41.4/5.2	33.0/5.0	
74	SGT1-like protein *Nicotiana tabacum*	gi|29468339	2,4	1,6	41.4/5.2	43.0/5.0	
75	heterogeneous nuclear ribonucleoprotein 27C, putative *Ricinus communis* [a]	gi|255574941	1,6	-1,8	47.4/6.4	48.0/5.8	gi|39851535
76	hypothetical protein RNA binding *Vitis vinifera* [a]	gi|296081884	-1,7	1	43.7/5.8	60.0/5.3	gi|190733368
77	hypothetical protein DNA helicase, putative *Vitis vinifera* [a]	gi|147858961	2,5	1	51,4/5,3	50.0/5.8	gi|190806396
78	DNA helicase, putative *Ricinus communis*	gi|255565715	2,1	1	50.2/5.8	50.0/5.9	
Metabolism							
79	GAPDH *Nicotiana tabacum*	gi|120676	-1,8	-2,2	35.5/6.14	36.0/6.8	
80	GAPDH *Nicotiana langsdorffii* x *Nicotiana sanderae*	gi|120676	4,8	6,4	35.5/6.14	35.5/6.6	
81	glyceraldehyde 3-phosphate dehydrogenase *Nicotiana langsdorffii x Nicotiana sanderae*	gi|120676	>100	1	35.5/6.14	30.0/6.5	

82	enolase *Nicotiana tabacum*	gi|238814974	2,3	2	48.0/5.4	54.0/5.9	
83	enolase *Nicotiana tabacum*	gi|119354	2,2	2	48.0/5.6	50.0/5.9	
84	phosphoenolpyruvate carboxylase *Glicine max*	gi|399182	1	-2,7	110.6/5.7	110/5.8	
85	phosphoglycerate kinase, cytosolic *Nicotiana tabacum* [a]	gi|2499498	1	1,5	42.4/5.7	40.0/5.5	gi|190134894
86	phosphoglycerate kinase, chloroplastic Precursor *Nicotiana tabacum*	gi|2499497	1	-1,7	50.3/8.5	40.0/5.9	
87	cytosolic aconitase *Nicotiana tabacum*	gi|11066033	1	5	98.7/5.8	100/6.1	
88	alcohol dehydrogenase *Nicotiana tabacum*	gi|551257	-1,8	-2,7	41.9/6.6	42.0/6.4	
89	alcohol dehydrogenase *Nicotiana tabacum*	gi|551257	>100	>100	41.9/6.6	42.0/6.2	
90	alcohol dehydrogenase *Nicotiana tabacum*	gi|551257	2,1	1	41.9/6.6	42.0/6.1	
91	alcohol dehydrogenase class III *Solanum lycopersicum* [a]	gi|283825505	2,2	8	40.7/6.3	42.0/6.9	gi|285193921
92	pyruvate decarboxylase isozyme 1 *Nicotiana tabacum*	gi|1706327	2,3	1	45.7/6.6	60.0/6.8	

93	succinate dehydrogenase, putative *Ricinus communis* [a]	gi|255579273	<0,01	<0,01	68.5/6.2	80.0/5.5	gi|190782074
94	NADH-ubiquinone oxidoreductase, putative *Ricinus communis*	gi|255582280	1	-1,7	80.8/5.9	85,0/6,1	
95	putative aconitase *Capsicum chinense*	gi|171854675	1	<0.01	108/7.0	48/6.0	
96	NAD-dependent isocitrate dehydrogenase *Nicotiana tabacum*	gi|3790188	1	7.08	40.6/7.2	40.6/ 6.2	
97	diaminopimelate epimerase, putative *Ricinus communis* [a]	gi|255584553	1	-2	40.1/6.0	35.0/5.3	gi|83422108
98	semialdehyde dehydrogenase family protein *Arabidopsis thaliana* [a]	gi|15223910	6,1	9	40.7/6.5	39.0/5.5	gi|39863720
99	aspartate semialdehyde dehydrogenase, putative *Nicotiana sylvestris* [a]	gi|255584961	-1,8	-1,7	41.2/8.2	40.0/5.6	gi|190769575
100	aspartate-semialdehyde dehydrogenase, putative *Arabidopsis thaliana*	gi|75161484	6,1	7	40,7/6,5	38/5,5	
101	betaine-aldehyde dehydrogenase* *Nicotiana tabacum*	gi|92037527	1	-1,6	59.7/5.4	57.3/5.6	
102	putative 3-isopropylmalate dehydrogenase large subunit *Capsicum annuum*	gi|193290700	<0,01	1	43.7/5.9	43.0/5.3	
103	Isovaleryl-CoA dehydrogenase 2, mitochondrial *Solanum tuberosum* [a]	gi|25453061	1,8	1	43.9/6.1	43.0/6.1	gi|83422448

104	glutamine synthetase *Nicotiana plumbaginifolia*	gi|121373	1,8	2,3	39.0/5.5	39.0/5.2	
105	d-3-phosphoglycerate dehydrogenase, putative *Ricinus communis* [a]	gi|255555301	-5,6	1	63.0/7.7	58.0/6.3	gi|39856030
106	EDA9 (embryo sac development arrest 9) ATP binding *Arabidopsis thaliana*	gi|15235282	>100	1	63.5/6.2	60.0/6.3	
107	cvhain A structure threonine synthase *Arabidopsis thaliana*	gi|15825882	<0.01	1	53.5/5.6	45/6,6	
108	adenosine kinase isoform 1T *Nicotiana tabacum*	gi|51949796	1	1,6	37.8/5.1	36.0/5.0	
109	adenosine kinase isoform 1T *Nicotiana tabacum*	gi|51949796	1	-1,5	37.8/5.1	38.0/5.0	
110	5'-aminoimidazole ribonucleotide synthetase *Solanum tuberosum* [a]	gi|37983566	-1,9	-1,8	42.9/5.2	34.0/4.9	gi|190730691
111	putative carbamoyl phosphate synthase small subunit *Nicotiana tabacum*	gi|21535793	-2,7	1	47.7/6.0	47.0/5.9	
112	ATP synthase subunit delta', mitochondrial *Ipomoea batatas*	gi|2493046	-1,8	2,2	21.3/5.9	20.0/4.9	
113	mitochondrial ATPase beta subunit *Nicotiana sylvestris*	gi|11228579	<0.01	<0.01	59.6/5.2	50.0/5.0	
114	vacuolar H+-ATPase B subunit *Nicotiana tabacum*	gi|6715512	1,6	1	53.8/5.1	55.0/5.1	

115	electron carrier/ oxidoreductase *Arabidopsis thaliana* [a]	gi|15232542	-1,8	-2,3	37.2/5.7	34.0/6.6	gi|92032774
116	N-carbamoylputrescine amidase *Solanum Tuberosum* [a]	gi|118572820	1,5	2,2	33.4/5.9	33.0/6.6	gi|92015448
117	N-carbamoylputrescine amidase *Solanum Tuberosum* [a]	gi|118572820	-5,4	<0,01	33.4/5.9	33.0/6.8	gi|92015448
118	Spermidine synthase *Nicotiana sylvestris*	gi|6094336	1,7	1	34.5/5.2	33.0/5.4	
119	spermidine synthase *Nicotiana sylvestris*	gi|6094336	2,4	1	34.5/5.2	33.5/5.0	
120	spermidine synthase *Nicotiana sylvestris*	gi|6094336	<0,01	1	34.5/5.2	33.5/5.1	
121	putative pyridoxine biosynthesis protein isoform A *Nicotiana tabacum*	gi|46399269	2,1	1,7	33.0/5.9	31.0/5,8	
122	putative pyridoxine biosynthesis protein isoform A *Nicotiana tabacum*	gi|46399269	-1,9	1	33.0/5.9	31.0/6.1	
123	ADH-like UDP-glucose dehydrogenase *Nicotiana tabacum*	gi|48093455	-1,9	-2,2	42.0/6.2	42.0/6.8	
124	ADH-like UDP-glucose dehydrogenase *Nicotiana tabacum*	gi|48093455	3,7	2,2	42.0/6.2	42.0/6.6	
125	UTP-glucose-1-phosphate uridylyltransferase *Solanum tuberosum*	gi|17402533	2,1	1,1	52.0/5.4	50.0/6.0	

126	UDP-glucose:protein transglucosylase-like *Solanum tuberosum* [a]	gi|77416931	1,7	1	41.1/5.6	38.0/6.1	gi|123218663
127	putative cinnamyl alcohol dehydrogenase *Nicotiana tabacum* [a]	gi|156763848	1	1,8	38.9/6.6	39.0/6.5	gi|190749158
128	NAD dependent epimerase/dehydratase, putative *Ricinus communis*	gi|255537241	>100	>100	34.0/6.2	32.0/6.6	
129	NAD dependent epimerase/dehydratase, putative *Ricinus communis* [a]	gi|255537241	3,5	2,9	34.0/6.2	32.0/6.5	gi|92028282
130	NAD dependent epimerase/dehydratase, putative *Ricinus communis* [a]	gi|255537241	-8,1	-7	34.0/6.2	32.0/6.8	gi|92028282
131	type 2 proly 4-hydroxylase *Nicotiana tabacum* [a]	gi|215490181	>100	>100	32.7/6.3	32.0/6.4	gi|190760427
132	Gibberellin 20 oxidase, putative *Ricinus communis*	gi|255556243	-2,5	<0.01	42.0/5.7	38.0/5.5	
133	1-aminocyclopropane-1-carboxylate oxidase *Solanum lycopersicum* [a]	gi|50830975	1,7	-1,6	34.4/6.1	32.0/6.1	gi|39853392
134	S-adenosylmethionine synthase 2 *Solanum lycopersicum*	gi|1170938	<0.01	1	43.0/5.4	44.0/6.1	
135	acireductone dioxygenase *Solanum tuberosum* [a]	gi|158325159	<0.01	<0.01	23.3/4.8	22.5/5.0	gi|76867763
136	acireductone dioxygenase *Solanum tuberosum* [a]	gi|158325159	1	<0.01	23.3/4.8	23,0/5,3	gi|76866491

137	putative 4-methyl-5(b-hydroxyethyl)-thiazol monophosphate biosynthesis enzyme *Capsicum chinense* [a]	gi|171854671	<0,01	<0,01	41.7/5.4	38.0/5.9	gi|190775218
138	putative 4-methyl-5(b-hydroxyethyl)-thiazol monophosphate biosynthesis enzyme *Capsicum chinense* [a]	gi|171854671	>100	>100	41.7/5.4	40.0/5.5	gi|47004010
139	putative 4-methyl-5(b-hydroxyethyl)-thiazol monophosphate biosynthesis enzyme *Capsicum chinense*	gi|171854671	1	<0,01	41.7/5.4	40.0/6.2	
140	rubisco subunit binding-protein alpha subunit *Ricinus communis* [a]	gi|255587664	-1,5	-1,5	53.2/5.2	60.0/5.0	gi|190794529
141	rubisco subunit binding-protein alpha subunit *Ricinus communis* [a]	gi|255587664	-1,6	-1,6	53.2/5.2	60.0/4.9	gi|190805933
142	stem-specific protein TSJT1, putative *Ricinus communis* [a]	gi|255552037	1	-2,4	27.0/6.0	27.5/6.4	gi|92026938
143	hypothetical protein Stem-specific protein TSJT1, putative *Populus trichocarpa* [a]	gi|224099853	3,8	1	27.2/5.8	28.0/6.7	gi|92026938
144	SAL1; 3'(2'),5'-bisphosphate nucleotidase/ inositol or phosphatidylinositol phosphatase *Arabidopsis thaliana* [a]	gi|145359623	1	1,6	43.4/6.0	40.0/5.4	gi|190143393
145	patatin homolog *Nicotiana tabacum*	gi|1546817	-1,9	-1,8	42.5/5.1	42.0/5.2	

146	acyl-[acyl-carrier-protein] desaturase, chloroplastic *Solanum commersoni* [a]	gi|94730426	<0,01	-1,9	44.8/6.3	38.0/6.0	gi|92012186
147	DH putative beta-hydroxyacyl-ACP dehydratase *Capsicum annuum* [a]	gi|193290688	>100	>100	23.9/9.4	22.0/6.4	gi|52834057
148	putative pyruvate dehydrogenase E1 alpha subunit *Capsicum annuum*	gi|193290722	<0,01	<0,01	48.0/6.3	38.0/6.75	
149	glutamate-cysteine ligase, chloroplastic *Nicotiana tabacum*	gi|122194121	-2,5	-1,9	59.4/6.2	45.0/6.8	
150	L-galactono-gamma-lactone dehydrogenase *Nicotiana tabacum*	gi|6519872	<0.01	-2	67.1/7.7	54.0/6.8	
151	putative ketol-acid reductoisomerase *Capsicum annuum*	gi|193290660	-2,6	1	63.7/6.5	59.0/5.8	
152	prolyl endopeptidase, putative *Ricinus communis* [a]	gi|255539116	-2,3	-1,9	80.0/5.3	80.0/5.4	gi|190876295
153	short chain dehydrogenase *Solanum tuberosum* [a]	gi|77403673	<0.01	1	27.2/6.2	27.0/6.2	gi|254640456
Cell structure							
154	actin isoform B *Mimosa pudica* [a]	gi|6683504	<0.01	<0.01	41.7/5.3	36.0/5.7	gi|39877476

155	actin *Nicotiana tabacum*	gi|197322805	<0.01	<0.01	41.7/5.3	35.0/5.7	
156	actin-binding protein ABP29 *Lilium longiflorum* [a]	gi|117553552	-2,7	-5,5	29.4/6.0	38.0/5.6	gi|190829672
157	actin *Nicotiana tabacum*	gi|50058115	2,5	2,6	41.8/5.3	43.0/5.2	
158	alpha tubulin *Nicotiana tabacum*	gi|11967906	1	-2	50.4/4.9	49.0/6.5	
159	alpha tubulin *Nicotiana tabacum*	gi|11967906	<0.01	-2,1	50.4/4.9	49/6.4	
160	tubulin beta-2 chain *Anemia phyllitidis*	gi|464851	<0.01	1	46.8/4.9	55/6.8	
161	predicted protein *Populus trichocarpa* [a]	gi|224125262	1,5	1,5	26.0/5.1	30.0/5.7	gi|39875797
162	villin 3 fragment de novo *Arabidopsis thaliana*	gi|6735320	<0.01	1,2	64.8/5.5	38.0/5.4	
Unknown							
163	hypothetical protein stomatin-like protein *Vitis vinifera* [a]	gi|225442194	1,7	1	45.6/9.0	39.0/6.9	gi|92027149
164	hypothetical protein isoform 2 *Vitis vinifera* [a]	gi|225454579	3,2	1	17,0/4,7	24.0/6.0	gi|190805443

165	hypothetical protein coatomer delta subunit *Vitis vinifera* [a]	gi|270239956	<0.01	<0.01	61.5/5.6	33.0/5.0	gi|190738155

Table 1. a) Protein founded in the EST DataBase. The name, the molecular weight and the isoelectric point of the protein were annotated by BLAST search.

Some of the identified proteins showed a discrepancy with their theoretical *M*r or p*I*. However, these kinds of phenomena are commonly found in 2-D gels for several reasons including modification of proteins during the extraction or the separation procedure, different isoforms derived from various genes, proteolytic cleavage, post translational modifications (PTMs). Cross-species protein identification also can produce this variation. As shown in Table1 and Figure 2, among the 165 proteins identified 43 and 45 proteins were specifically expressed comparing with the control in 30'-H_2O_2 and 3h-H_2O_2 respectively, while 77 were common to both treatments.

The identified proteins were sorted in different functional categories as follows: disease/defence (20), protein synthesis and fate (45), signal transduction/regulation (13), metabolism (75), cell structure (9), unknown (3). As shown in Figure 2, in PCD cells the down-accumulated proteins are the are more numerous than of the up-regulated. 15 unique proteins (unipros), representing 38 isoforms, exibited opposite expression pattern within each set of isoforms. This result suggests that isoforms of certain unipros may play the same or different roles in modulating H_2O_2 response.

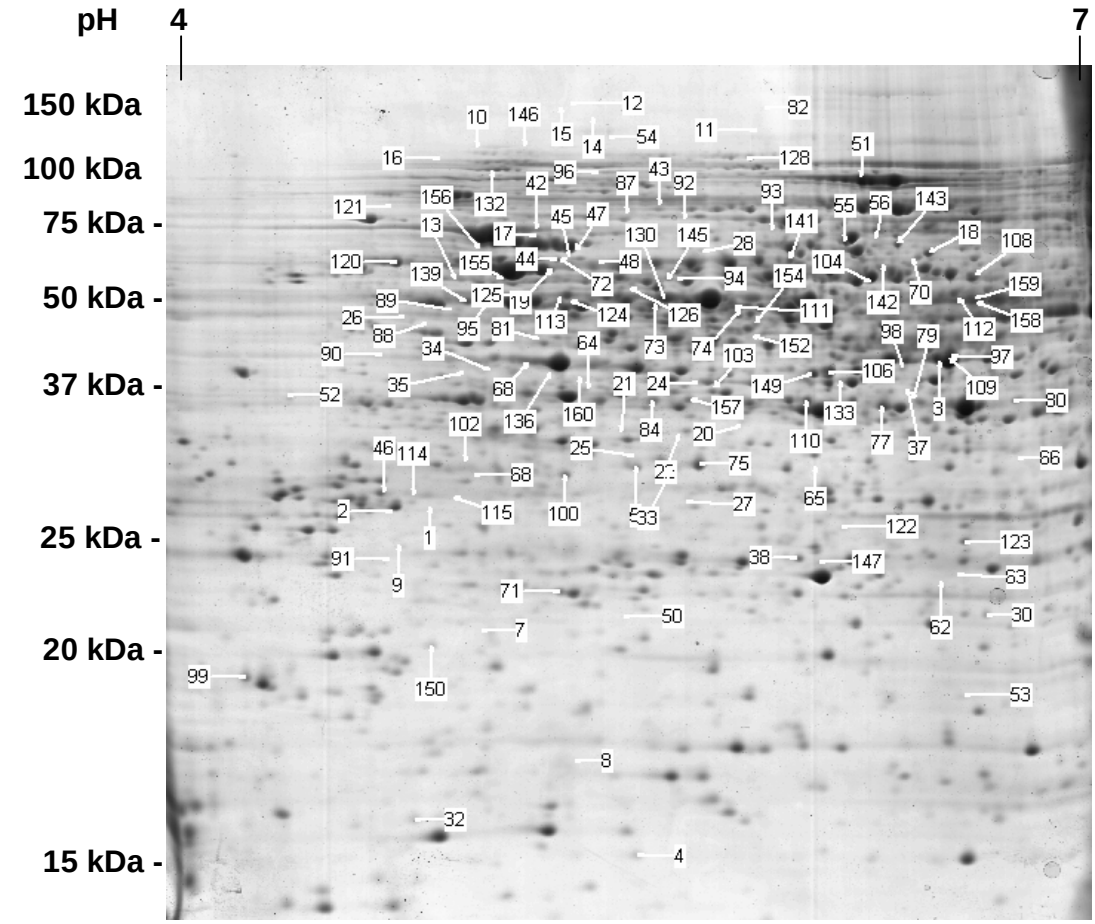

Figure 2. Image of a representative gel: spots differently expressed between Control and TBY-2 cells treated with H_2O_2 are indicated by their relative numbers.

Validation of proteomic results with other approaches

Ascorbate and Glutathione content

Proteomic results indicated a early decrease of L-galactono-gamma-lactone dehydrogenase (GLDH, spot 144) and gamma-glutamylcysteine synthetase (γ-ECS, spot 28) involved in Ascorbate (ACS) and glutathione (GSH) biosynthesis, respectively. The ASC and GSH pools (reduced plus oxidized forms) were determined starting from 30' up to 8 h in cells undergoing PCD induced by H_2O_2 and 55°C treatments. Results

indicated that already in the very early phases of PCD, the decrease of the ASC and GSH levels were more drastic in H_2O_2 treated cells (Figure 3).

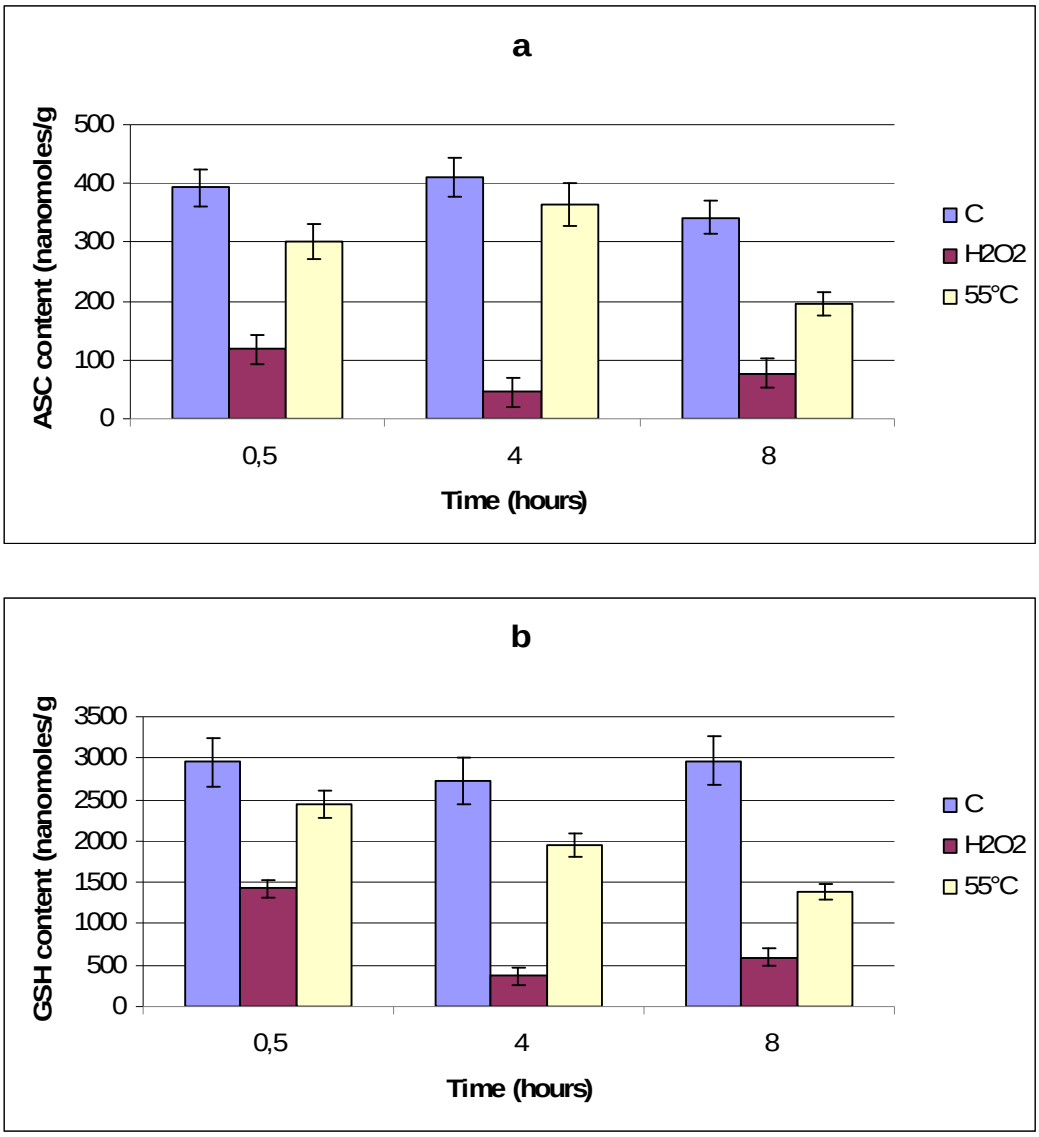

Figure 3. Changes in ascorbate (ASC) and glutathione (GSH) content induced by H_2O_2 treatments. Control and treated cells (50 mM H_2O_2 and 55°C for 10 minutes) were collected at the times indicated and used for the determination of the total ASC (a) or GSH (b) pools (reduced plus oxidized forms) as reported in Experimental procedures. Values represent means (±SE) of three experiments.

Proteasome activity

Due to the alterations of some proteasome subunits revealed by proteomic analysis, we investigated whether and how proteasome activity

changes in H_2O_2-exposed cells. Moreover, a comparison was made between cells H_2O_2- and HS-undergoing PCD with respect to proteasome activity as measured as a function of time. In both cases, proteasome activity decrease already after 30' of treatment and more rapidly in HS-cells (Figure 4).

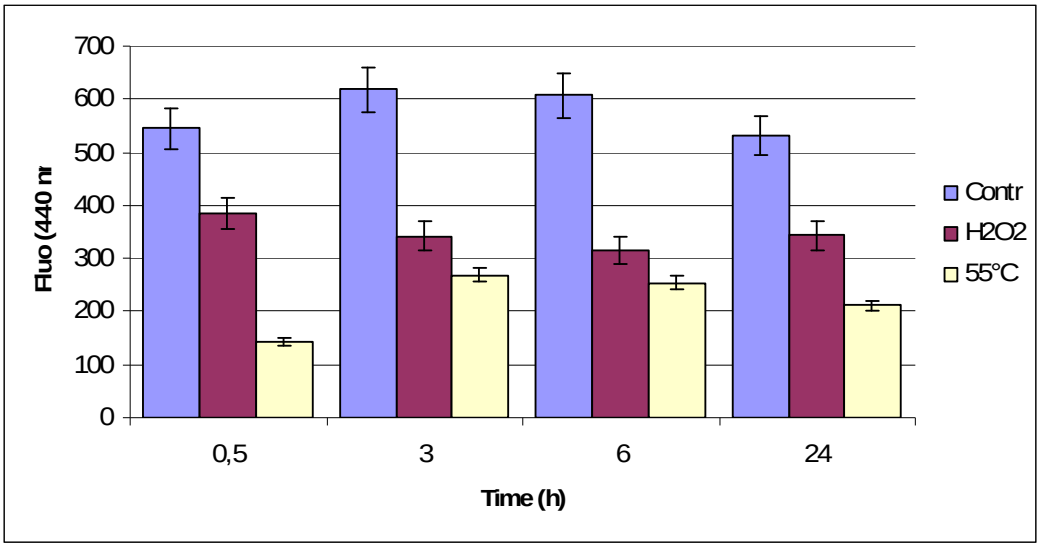

Figure 4. Effects of H_2O_2 treatments and heat stress on Proteasome activity; specific activity of Proteasome was measured in control and treated cells (50 mM H_2O_2 and 55°C for 10 minutes) during time. Values represent means (±SE) of three experiments.

Effects of the recovery by spermidine on PCD occurrence.

Since the proteomic study show changes of enzymes involved in polyamine biosynthesis, the effects of exogenous addition of spermidine on PCD were also analyzed. Our results show that 2mM spermidine protected the H_2O_2-exposed cells from PCD during the first hours of treatment (Figure 5).

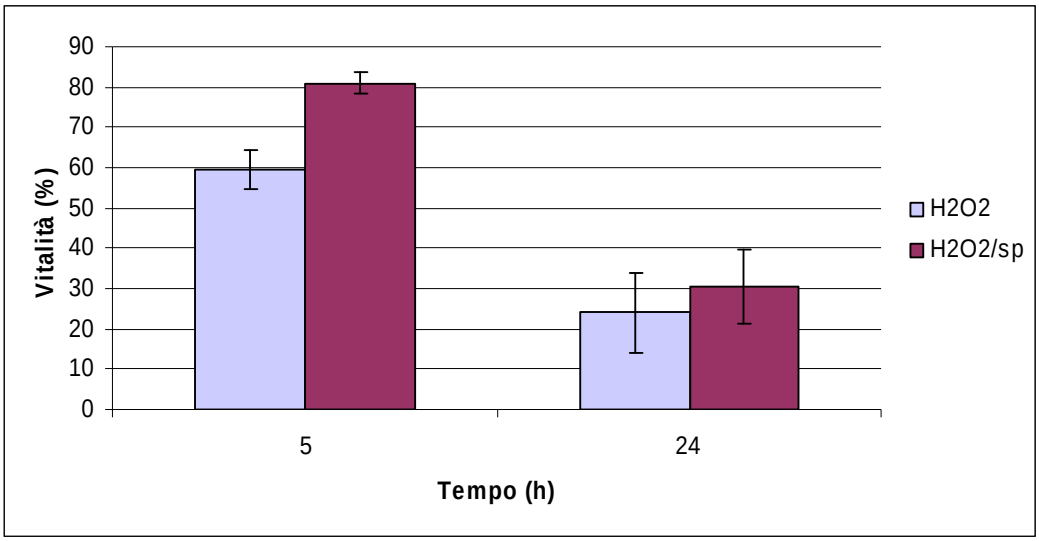

Figure 5. Effect of spermidine pre-treatment on cell viability in PCD-induced by 50 mM H_2O_2. The values are the means of three different experiments ± SD.

Western blot analysis of APX proteins

2-DE data indicate the decrease of a Ascorbate Peroxidase protein (APX) in 3h and 6h-PCD cells (heat-treatment) and an increase in 3h-PCD cells (H_2O_2-treatment). We used immunoblot analysis with Anti-cAPX antibody to detect free and conjugated forms in soluble cellular extracts separated by 2-D Electrophoresis (Figure 6).

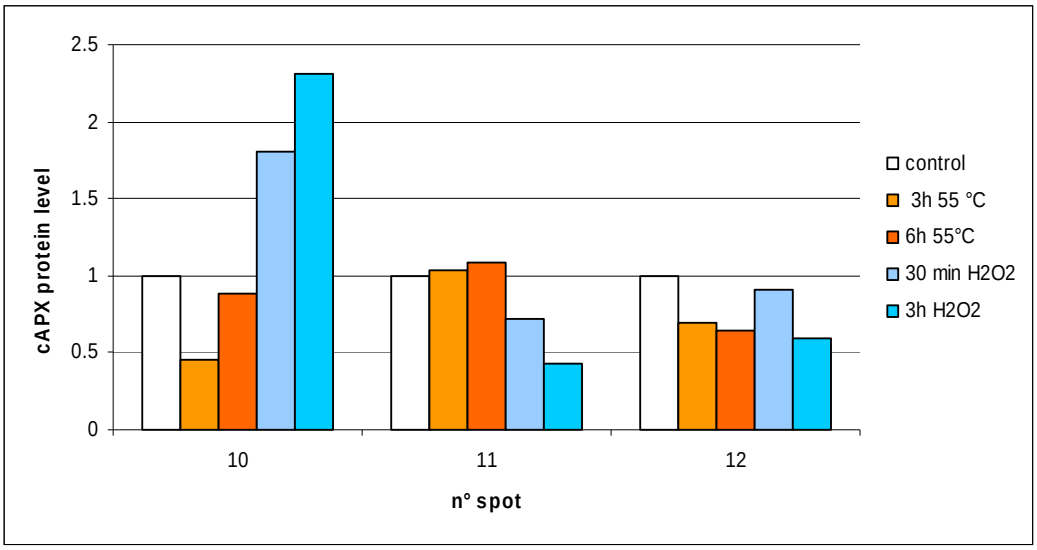

Figure 6. Western blot analysis with cytosolic-APX antibody detecting APX in control and treated sample; in cells treated with 50 mM H_2O_2 for 30 min and 3 h in cells and exposed to 55°C for 10 min and then recovered for 3 h and 6 h at 27 °C.

DISCUSSION

The present study investigated the changes found in the TBY-2 cell proteome after cells were exposed to 50 mM H_2O_2 for 30 min or 3 h to induce PCD. A number of proteins that differentially accumulated under these conditions were identified.

Detoxifying and antioxidant enzymes.

The metabolism of ascorbate (ASC) and glutathione (GSH) are important for the removal of ROS in plants (Mittler, 2002; Noctor and Foyer, 1998; Shigeoka *et al.*, 2002). Previous studies using Arabidopsis mutants deficient in ASC (*vtc1* and *vtc2*) showed that low ASC levels trigger PCD (Pavet *et al.*, 2005). While the redox states of TBY-2 cells are not necessarily important, ASC and GSH levels are important signals in TBY-2 cells undergoing PCD induced by 50 mM H_2O_2 and heat shock at

55°C (de Pinto *et al.*, 2006). A decrease in the activity of the enzyme GLDH has been observed in TBY-2 cells undergoing heat shock-induced PCD (Valenti *et al.*, 2006). Cells treated with H_2O_2 have a lower content of γ-ECS (spot 149), the rate-limiting enzyme in the synthesis of GSH, and of GLDH (spot 150), the last enzyme of the ASC biosynthesis pathway. These data could explain, at least in part, the different levels of ASC and GSH found in the H_2O_2 and HS-induced PCD. Moreover, it has been suggested that GLDH is an integral part of plant mitochondrial complex I, and that the redox state affects GLDH catalysis (Millar *et al.*, 2003). The downregulation of the Complex I (NADH-ubiquinone oxidoreductase, spot 98) in cells treated with H_2O_2 for 3 hours suggests that decreased GLDH level correlates with Complex I inhibition.

Of the 17 proteins differentially expressed in PCD cells and implicated in redox homeostasis, 14 were downregulated. Four protein spots corresponded to thioredoxin peroxidases (spots 1, 2, 3, 4), which are members of the peroxiredoxin (PRX) family. PRXs are potentially involved in a variety of cellular functions including apoptosis. In mammalian cells, PRX5 overexpression prevents p53-dependent ROS generation and apoptosis (Zhou *et al.*, 2000), and overexpression of PRX1 and PRX2 leads to the removal of H_2O_2, thereby protecting thyroid cells from apoptosis (Kim *et al.*, 2000). Furthermore, depletion of PRX3 by RNA interference in HeLa cells, or suppression of 1-Cys PRX in rat lung epithelial cells, leads to sensitized cells that are susceptible to peroxide-induced apoptosis (Chang *et al.*, 2001; *Pak et al.*, 2002).

Four of the identified PRXs showed MW values smaller than the theoretical values. In *S. Pombe*, H_2O_2 treatment caused a C-terminal

truncation and the inactivation of PRX (Koo *et al.*, 2002). All of the protein spots identified as PRXs had a more acidic experimental pI than the theoretical value. Oxidative modifications of PRX caused an acidic shift of the relative spot position and inactivated the protein (Rabilloud *et al*., 2002, Lee *et al.*, 2008). The decrease in the PRX amount revealed by proteomic analysis could be explained by the acceleration of PRX turnover under conditions that induce high oxidative damage to proteins. To our knowledge, no information is available to indicate that protein oxidation accelerates PRX turnover. However, site-specific oxidation of proteins has been reported to act as a signal for ubiquitination, which triggers protein degradation (Iwai *et al.*,1998). Moreover, our analysis showed that the level of a thioredoxin (spot 7), which regenerates the active form of PRX, was decreased.

Among the spots identified as cytosolic ascorbate peroxidases (c-APXs), the intensities of spots 9 and 11 decreased in the presence of H_2O_2, while the intensity of spot 11 increased relative to the control. Both thylacoidal APX spots (t-APX, spots 12 and 13) decreased in intensity. Previous results reported a decrease in the abundance of c-APX under experimental conditions similar to those used in the present study (de Pinto *et al.*, 2006), while a decrease of t-APX activity was observed in TBY-2 cells undergoing heat shock induced by PCD (Locato *et al*., 2009). Changes in APX content could correlate with both the observed decrease in ASC content (De Gara *et al.*, 1997) and the H_2O_2 level.

The apparent lowering of antioxidant defenses could allow H_2O_2 to trigger the oxidative burst that is characteristic of PCD.

Protein synthesis and degradation

A total of 38 proteins (30%) whose levels changed in response to H_2O_2 treatment were identified and found to participate in protein metabolism (Table 1), indicating that active control of protein biosynthesis, folding, and degradation is crucial in PCD. These proteins belonged to three different groups. The first group consisted of nine proteins that function in protein biosynthesis. The second group consisted of 16 proteins that function in protein folding and assembly. In particular, the lower intensities of four HSP70 and four HSP90 spots in cells undergoing PCD suggests that HSPs have an antiapoptotic function. Until now, there has been no suggestion that plant HSPs proteins have this function; however, the ability of HSPs to suppress apoptosis in animal systems is well established (Parcellier *et al.*, 2003; Beere, 2005; Didelot *et al.*, 2006). The inhibition of early apoptotic events caused by HSP70, such as mitochondrial depolarization and cytochrome *c* release suggests that it functions at an early stage in the apoptotic pathway (Creagh *et al.*, 2000). Interestingly, in cells treated with H_2O_2 for 30', we found a decrease in the amount of a luminal binding protein that is localized to the RE (spot 44). This protein shares high homology with the well-studied human protein, GRP78, and its role in promoting cell growth and antagonizing apoptosis has been demonstrated in several tumor cell lines (Zhao *et al.*, 2010).

The third group of proteins identified comprised 13 proteins involved in protein degradation, five of which were proteasome subunits. Changes in proteasome activity are crucial for the regulation of PCD. In animal cells, proteasome inhibitors induce apoptosis (Wojcik, 1999) while proteasome malfunction leads to PCD in plants (Kim *et al.*, 2003). The 26S regulatory

subunit (spot 55) was found to be homologous to yeast Rtp2 protein. Köhler *et al.* (2001) showed that Rpt2 functions to "gate" proteasome activity, as mutation of that subunit controlled both the entry of substrate into the proteasome and the release of proteolytic products from the proteasome.

Other proteins involved in protein degradation decreased in abundance. These included Oligopeptidase A (spots 60, 61 and 62), mitochondrial processing peptidase (spot 63 and 64), a cysteine proteinase (spot 58), and two proteins (spots 70 and 69) belonging to the metallopeptidase family M24 that were similar to human EBP1, a protein that induces apoptotic DNA fragmentation when depleted from cells (Ahn *et al.*, 2006). Other results obtained with transgenic plants expressing high and low levels of EBP1 suggest that EBP1 is a dose-dependent regulator of cell growth with functions in meristematic competence and cell proliferation (Horvath *et al*., 2006).

Metabolism

The level of carbamoyl phosphate synthetase II (CPSII, spot 111), a cytosolic enzyme that catalyzes the first step in pyrimidine biosynthesis, was decreased. Huang *et al.* (2002) showed that CPSII is a target for caspase-dependent regulation during apoptosis. Moreover, it has been demonstrated that alterations in pyrimidine nucleotide synthesis and utilization represent a metabolic signal that precedes and accompanies the NO and H_2O_2-induced PCD in tobacco BY-2 cells (Stasolla *et al.*, 2004). In cells undergoing PCD, differentially expressed proteins that are important in purine metabolism were identified. These included 5'-aminoimidazole ribonucleotide synthetase (AIR) involved in purine biosynthesis, and two isoforms of

adenosine kinase (ADK) involved in both the phosphorylation of adenosine and the purine salvage pathway. Stasolla *et al.* (2005) showed that increases in purine nucleotide synthesis are an early metabolic event to ensure high energy levels for the proper execution of PCD induced by NO and H_2O_2 in TBY-2 cells. The decrease of AIR in the present system is in agreement with intracellular energy depletion observed during petal PCD senescence in tulip (Azad *et al.*, 2008).

Purines, especially derivatives of adenosine, are potent inducers of apoptosis in animal cells. The results of Mlejnek and Prochazka (2002) and Mlejnek *et al.* (2003) suggest that intracellular phosphorylation of isopentenyladenosine (iPA) is required for the activation of caspase-like proteases and the induction of apoptosis in tobacco BY-2 cells. Among the four TBY-2 adenosine kinase (ADK) isoforms, ADK1T displays a 10-fold higher affinity for iPA than it does for other substrates. These data, together with the accumulation of adenosine kinase isoform 1T in H_2O_2-PCD cells, support the hypothesis that ADK1T is an element of the apoptosis machinery.

H_2O_2 participates in complex interactions with plant hormones to regulate PCD. An initial increase and subsequent decrease in the amount of ACC oxidase was found in H_2O_2-PCD cells. Ethylene is a positive regulator of several types of H_2O_2-induced cell death, including PCD, during lysigenous aerenchyma formation and the hypersensitive response (HR) (Muhlenbock *et al.*, 2007; Wang *et al.*, 2002). Abiotic, biotic, and oxidative stresses can stimulate ethylene biosynthesis through activation of ACC synthase and ACC oxidase, and elevated levels of ethylene can amplify the H_2O_2 signal (Wang *et al.*, 2002). The decreased amounts of two isoforms of

acireductone dioxygenase (ARD, spots 135 & 136) involved in methionine salvage may be related to ethylene synthesis and signaling. Similar results were obtained for *TaARD* in response to biotic and abiotic stresses (Xu *et al.,* 2010). The decrease in GA20 oxidase observed in H_2O_2-PCD cells confirms that giberellic acid (GA) signaling is also involved in regulating PCD, as previously reported (Fath *et al.,* 2001; Wang *et al.,* 2007).

Proteomic data confirmed that oxidative stress affects central metabolic pathways such as glycolysis, the tricarboxylic acid cycle (TCA), fermentation, and amino acid metabolism. The treatment of TBY-2 cells with H_2O_2 decreased the abundance of some key mitochondrial proteins. In particular, two enzymes involved in the electron transport chain (ETC) were downregulated, including succinate dehydrogenase (spot 93), which is involved in the ECT and TCA cycle, and NADH-ubiquinone oxidoreductase (75 kDa subunit, spot 94), which is a core component of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I). The levels of two enzymes involved in the TCA cycle were also altered. Aconitase is sensitive to ROS and its downregulation may affect the overall efficiency of the TCA cycle. However, the effect of lower levels of aconitase in H_2O_2-PCD cells was mitigated by the upregulation of NAD-dependent isocitrate dehydrogenase. The upregulation of this enzyme permits degradation of citrate, even if the aconitase level is decreased, thereby maintaining mitochondrial NADPH activity and amino acid metabolism. Enzymes of the glycolytic and fermentation pathways including glyceraldehyde 3-phosphate dehydrogenase (GAPDH, spots 81), phosphoglycerate kinase (spot 85), enolase (spots 82 & 83), alcohol dehydrogenase (spots 88, 89, 90, 91), and pyruvate decarboxylase (spot 92) were differentially regulated in H_2O_2-PCD

cells compared to the control. The upregulation of some of the enzymes involved in the fermentation of ethanol suggests that fermentation may compensate for mitochondrial energy dysfunction. Similar results were obtained under conditions of abiotic stress (Levitt, 1980; Kürsteiner *et al.*, 2003). Some enzymes involved in polyamine biosynthesis (spots) were significantly changed in H_2O_2-treated cells. Results from previous investigations suggest that exogenous application of spermidine and glycine betaine is able to alter the expression or activity of some scavenging enzymes as well as the cellular levels of ROS, thus modifying the oxidative stress intensity (Park *et al.*, 2004; He *et al.*, 2008). A similar effect of spermidine was also evident in our experimental system: spermidine treatment protected the H_2O_2-exposed cells from PCD although the effect is less evident than that obtained on TBY-2 cells in which PCD was induced by 55°C (Vannini *et al.*, 2010).

Cell wall

Changes in the accumulation of three enzymes important for biosynthesis of cell wall polysaccharides and lignin were identified in cells undergoing H_2O_2-PCD. These included UDP-glucose dehydrogenase (spot 123), UTP-glucose-1-phosphate uridyltransferase (spot 125), and cinnamoyl-CoA dehydrogenase.

UTP-glucose-1-phosphate urydyltransferase produces UDP-glucose, which can be used in the biosynthesis of cellulose (Kleczkowski *et al.*, 2004). UDP-glucose can then be used by the UDP-glucose dehydrogenase to form UDP-glucoronate, a key precursor in hemicellulose and pectin formation (Seitz *et al.*, 2000). In PCD induced during tracheary elements

differentiation, the cell wall undergoes reinforcement and thickening. Also, when corolla senescence begins, cell wall modification takes place in the corolla, as indicated by the appearance of autofluorescence, which is purported to be due to wall-bound phenolic substances.

Prolyl 4-hydroxylase (P4H), an enzyme responsible for the conversion of proline (Pro) to 4-hydroxyproline (4-Hyp) during the post-translational modification of ER arabinogalactan proteins (AGPs), accumulates in apoptotic cells (Cohen *et al.,* 1983; Cooper and Varner, 1983, Schmidt *et al.,* 1991; Serpe and Nothnagel, 1994). The highly regulated expression of AGP during xylem development (Majewska-Sawka and Nothnagel, 2000) suggests that AGPs might have roles in cell elongation, cell signaling, and programmed cell death, as they are thought to mark cells that are destined for PCD. Moreover, perturbation of AGPs by Yariv reagent induces PCD in *Arabidopsis* suspension-cultured cells (Gao *et al.,* 1999).

Crosstalk between H_2O_2- and heat shock-induced PCD pathways.

The PCD pathways in plants are enigmatic as the functions of most of the genes and proteins involved in this complex network have not been assigned while other genes and proteins involved have yet to be discovered. Moreover, the nature and activities of core regulators of plant PCD are poorly understood. Plant proteases with functional similarities to proteases involved in mammalian apoptotic cell death (caspases) are thought to be an integral part of the core mechanism of most PCD responses in plants.

To identify proteins that are important in the PCD pathway, proteomic data from H_2O_2-PCD cells were compared with proteomic data

obtained previously from cells undergoing PCD 3 h after heat shock at 55°C (3h-PCD cells) (Vannini *et al.*, 2010). The two systems were judged to be similar because the mortality was 25% in both 3h-H_2O_2 and 3h-PCD cells.

The results indicated that there are functional differences between the PCD pathways activated by HS and H_2O_2, suggesting that distinct downstream components underlie these two pathways. However, as shown in Table 1, 19 protein spots exhibited similar regulation during both types of PCD: the intensity of 16 spots was decreased while the intensity of three spots increased in both of these PCD pathways. These 19 proteins, which belong to several functional classes, may represent a core mechanism of plant PCD.

Six proteins were involved in determining protein fate: in particular, a decrease in the levels of proteasome alpha 6 and alpha 7 subunits and proteasome activity were observed. There exists previous experiments support for the involvement of these proteins in the PCD pathway. Virus-induced gene silencing of the α6 subunit of the 20S proteasome activated the PCD program, which was accompanied by reduced proteasome activity and the accumulation of polyubiquitinated proteins (Kim *et al.*, 2003). In Arabidopsis, the genes corresponding to α6 and α7 proteasome subunits are down-coexpressed in plant cell death during HR experiments, as indicated by ATTED-II (Obayashi *et al.*, 2009). Interestingly, this suggests that the different proteasome subunits may fulfill specific functions during apoptosis. The stability of individual proteasome subunits may be a potential target for the regulation of the PCD program. In conclusion, some plant PCD pathways may include signaling molecules that modify proteasome activity to activate PCD.

Among the core proteins, five proteins were related to cytoskeletal proteins, which suggests that PCD activation causes remodeling of the cytoskeleton. Swidzinski *et al.* (2002) reported that after PCD-inducing treatments in cultures of *Arabidopsis thaliana*, the expression of the alpha tubulin gene and the overexpression of the actin2 gene were inhibited. Studies using drugs that affect actin turnover suggest that either actin stabilization or depolymerization can induce PCD in yeast and mammalian cells (Gourlay and Ayscough, 2005; Thomas *et al.*, 2006).

For some of core proteins listed in Table 1 we cannot exclude the intriguing possibility that during plant PCD process they may play unappreciated roles in addition to the already known functions. For example, Hsp70 proteins play an important role in the maintenance and survival potential of mammalian cells by acting as anti-apoptotic proteins, a function that appears to be independent of their chaperoning activity (Beere *et al.*, 2000). Similarly, GAPDH is a multifunctional enzyme with a role in glycolysis and other less well understood roles, such as a sensor of H_2O_2 (Hancock *et al.*, 2005). Further studies are necessary to confirm the direct involvement of the core proteins identified here in PCD regulation and to investigate the potential of employing these core proteins as cell death markers.

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