

PROGRAMMED CELL DEATH IN PLANTS: NEW INSIGHTS INTO REDOX REGULATION AND THE ROLE OF HYDROGEN PEROXIDE

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Abstract

Programmed cell death (PCD), the highly regulated dismantling of cells, is essential for plant growth and survival. PCD plays key roles in embryo development, formation and maturation of many cell types and tissues, and plant

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reaction/adaptation to environmental conditions. Reactive oxygen species (ROS) are not only toxic by products of aerobic metabolism with strictly controlled cellular levels, but they also function as signaling agents regulating many biological processes and producing pleiotropic effects. Over the last decade, ROS have become recognized as important modulators of plant PCD. Molecular genetic approaches using plant mutants and transcriptome studies related to ROS-mediated PCD have revealed a wide array of plant-specific cell death regulators and have contributed to unraveling the elaborate redox signaling network. This review summarizes the biological processes, in which plant PCD participates and discusses the signaling functions of ROS with emphasis on hydrogen peroxide.

Key Words: Programmed cell death, Reactive oxygen species, Hydrogen peroxide, Antioxidants, Plant development, Stress responses, Cell signaling.

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1. INTRODUCTION

Programmed cell death (PCD) is an active, genetically controlled process leading to selective elimination of unwanted or damaged cells in eukaryotes. PCD is essential for growth and development of multicellular organisms as well as for proper response to environment (Gechev *et al.*, 2006; Lam, 2004). Coordination between cell death and proliferation, growth, and differentiation is of fundamental importance for the maintenance of tissue and organ homeostasis (Van Breusegem and Dat, 2006). Plant PCD is associated with a number of developmental processes including embryo formation, degeneration of the aleurone layer during monocot seed germination, differentiation of tracheary elements in water-conducting xylem tissues, formation of root aerenchyma and epidermal trichomes, anther tapetum degeneration, floral organ abscission, pollen self-incompatibility, remodeling of some types of leaf shape, and leaf senescence (Gechev *et al.*, 2006; Thomas and Franklin-Tong, 2004). PCD is also connected with plant immunity to biotrophic pathogens. In all these examples, PCD is essential or beneficial for plants. However, necrotrophic pathogens can cause disease by triggering PCD in healthy tissues (Coffeen and Wolpert, 2004; Navarre and Wolpert, 1999; Wang *et al.*, 1996b). Unwanted PCD can also be instigated by many abiotic factors like extreme temperatures, salinity, and pollutants (Koukalova *et al.*, 1997; Overmyer *et al.*, 2000; Swidzinski *et al.*, 2002).

Whereas PCD is well-studied in animals, our knowledge of the genetic mechanisms that regulate and execute plant cell death is limited. Recent biochemical and molecular genetic studies have revealed parts of the

complex plant cell death network and broadened our understanding of the machinery controlling cell death in plants. Hydrogen peroxide (H_2O_2) and other reactive oxygen species (ROS) have become recognized to be key modulators of PCD as well as many other biological processes such as growth, development, and stress adaptation (Gechev *et al.*, 2006). Although specific ROS receptors/sensors remain largely elusive, downstream components of H_2O_2 and ROS signal transduction networks controlling plant PCD have been identified, including protein kinases, protein phosphatases, and transcription factors. The majority of these are restricted to plants, with only a few genes having close homologues in animals. The notion that the plant cell death network is genetically different from the animal cell death machinery was further supported by identification of plant-specific proteases and nucleases involved in execution of some types of plant PCD. Thus, despite some functional similarities with animals and fungi, many aspects of the plant PCD gene network appear to be unique.

Hydrogen peroxide is produced in all cellular compartments as a result of reactions of energy transfer, electron leakage from saturated electron transport chains, and the activities of various oxidases and peroxidases (Apel and Hirt, 2004). Because of its low reactivity compared with other types of ROS, it has relatively long life, low toxicity, and ability to readily cross biological membranes. Thus, although the majority of hydrogen peroxide is generated in chloroplasts, peroxisomes, and the apoplast, it can quickly migrate into neighboring compartments to provoke a myriad of biological responses distant from the site of generation. Further complicating the picture are the interconversions and interactions between different types of ROS. A highly dynamic and redundant network of low molecular weight antioxidants, ROS-scavenging, and ROS-producing proteins adjusts ROS levels in different subcellular locations according to the cellular needs at that particular time (Gechev *et al.*, 2006; Mittler *et al.*, 2004). The consequent biological responses depend on the chemical identity of the ROS, its site of production, the timing and the intensity of the signal, the developmental stage of the plant, the plant stress history, and the interactions with other signaling agents. In particular, interactions between H_2O_2 and nitric oxide (NO) are of primary importance for the control and the realization of several types of cell death (Delledonne, 2005; Delledonne *et al.*, 2001; Zago *et al.*, 2006). Plant hormones and the link between ROS and lipid messengers are equally as important for tuning the cell death response (Gechev and Hille, 2005).

This chapter summarizes our current knowledge about plant PCD with respect to the biological processes associated with PCD and the cell death network. In the second part, we focus on hydrogen peroxide and other ROS as signaling molecules that modulate plant PCD and other plant processes, with emphasis on the factors that determine particular plant responses and discuss the emerging hydrogen peroxide cell death network.



2. PCD IN PLANTS

All eukaryotes possess active genetic cell death programs that have become integral parts of their growth, development, and reactions with the environment (Lam, 2004). In plant biology the term “programmed cell death” is widely used to describe genetically controlled forms of cell death, which share common morphological and biochemical features with animal cell apoptosis, including shrinkage of the cytoplasm, condensation and aggregation of the chromatin, cleavage of the nuclear DNA into inter-nucleosomal fragments, and formation of apoptotic bodies in some cases (Wang *et al.*, 1996b). These are active processes requiring “*de novo*” protein synthesis and are distinct from necrosis—cell death caused by extrinsic factors independent of specific genetic control and cellular activities (Van Breusegem and Dat, 2006).

Processes showing analogy to PCD in multicellular organisms have been described in prokaryotes (*Bacillus*, *Streptomyces*, *Myxobacteria*) and unicellular eukaryotes (Ameisen, 2002; Pennell and Lamb, 1997). Several unicellular species belonging to different branches of the eukaryote phylogenetic tree have been reported to exhibit apoptotic-like cell death programs (Ameisen, 2002). The controlled cell death in *Volvox* and *Marsilea* is thought to share similarities with PCD in both plants and animals (Pennell and Lamb, 1997). The common origin of *Dictyostelium*, green algae, ferns, plants, and animals suggests that the mechanisms of PCD are to a certain degree conserved throughout eukaryotes (Ameisen, 2002; Pennell and Lamb, 1997). The chains of events leading to controlled cellular death in different branches of Eukaryota not only share common elements in their executionary and regulatory systems but are also characterized by a variety of unique features typical of each eukaryotic group. Plant PCD differs morphologically and genetically from the suicidal mechanisms in fungi and animals. There are no universal hallmarks of plant cell death and no universal proteases dedicated to PCD analogous to the animal caspases.

The specific morphological attributes associated with plant PCD are in part limited by the prominent plant cell wall, which precludes engulfment of cell compartments by surrounding cells. Plant-specific organelles such as the vacuole and the chloroplasts are important to the plant cell death network and contribute to the unique aspects of plant PCD. In most manifestations of plant PCD, the *cell wall* remains after the decomposition of the protoplast and the reutilization of its components. The hypersensitive response (HR) to pathogens, for instance, is marked by destruction of the organelles, collapse of the plasma membrane, and its separation from the cell wall, which is left deformed after the leakage of the protoplast’s contents into the apoplast (Gunawardena *et al.*, 2004; Lam, 2004; Lamb and Dixon, 1997). During tracheary elements differentiation, the cell wall not only remains but also

undergoes reinforcement and thickening that is coordinated with the vacuole swelling and rupture. Only the fragment of the primary cell walls located between adjacent tracheary elements is hydrolyzed to form a channel (Nakashima *et al.*, 2000). In contrast, there are examples of total lysis of the cell wall during the formation of aerenchyma and the remodeling of the leaf shape (Gunawardena *et al.*, 2004).

In recent years, a number of studies have outlined the importance of the *vacuole* in plant cell death. Vacuoles have emerged as crucial sources for factors that mediate cellular lysis in addition to being depots for a variety of metabolites, and functioning in defense and in recycling of cellular components. Collapse of the tonoplast and release of accumulated nucleases and proteases are considered common phenomena associated with the lytic events in all forms of PCD in plants, but a key role of the vacuole has been reported for the processes of tracheary elements differentiation, formation of lysigenous aerenchyma, leaf fenestration, elimination of the aleurone layer, senescence, interaction with mycotoxins, as well as in the HR (Gunawardena *et al.*, 2004; Hatsugai *et al.*, 2004; Kuroyanagi *et al.*, 2005; Lam, 2005; Obara *et al.*, 2001; Pennell and Lamb, 1997). The tonoplast invaginates, releases vesicles toward the interior of the vacuole, gradually fragments, and becomes difficult to distinguish in the early stages of the PCD associated with the remodeling of the lace plant leaf shape. These events are followed by changes in the nuclear morphology, chromatin condensation, and DNA fragmentation (Gunawardena *et al.*, 2004). Nuclear DNA cleavage has been observed before the vacuole collapse during the HR (Mittler *et al.*, 1997). Using the HR induced by TMV infection as a model, Hatsugai *et al.* (2004) have demonstrated that the previously characterized vacuole-localized protease vacuolar processing enzyme (VPE) is a fundamental mediator in plant PCD. VPE is a caspase-like cysteine protease that cleaves a peptide bond at the C-terminal side of asparagine and aspartate. The catalytic diads and the substrate pockets of VPE and caspase-1 are conserved but there is little overall similarity between the sequences of the two proteins (Hatsugai *et al.*, 2006). Of the four VPE genes in the genome of *Arabidopsis*, three (αvpe , γvpe , and δvpe) appear to contribute to different types of PCD in plants (Kuroyanagi *et al.*, 2005). Vacuoles also accumulate other important hydrolytic enzymes like the S1-type Zn^{2+} -dependent nuclease ZEN1 (*Zinnia* endonuclease-1), which has been found to be a major participant in nuclear DNA degradation during the developmental cell death associated with xylem formation (Ito and Fukuda, 2002; Kuriyama and Fukuda, 2002). Vacuoles in plants and lysosomes in animals play essential roles in *autophagy*, a conserved mechanism in all eukaryotes for degradation of cellular contents to recycle nutrients or break down damaged proteins or toxic material (Bassham, 2007). In plants, autophagy is important for nutrient remobilization during sugar and nitrogen starvation and leaf senescence, degradation of oxidized

proteins during oxidative stress, disposal of protein aggregates, and possibly even removal of damaged proteins and organelles during normal growth conditions as a housekeeping function (Bassham, 2007; Xiong *et al.*, 2007). Many types of plant PCD exhibit typical morphological features of autophagic cell death, including an increase in vacuole and cell size, uptake of organelles into the vacuole followed by organelle degradation, and eventual lysis of the vacuole resulting in cell death (Filonova *et al.*, 2000; Gaffal *et al.*, 2007). However, paradoxically autophagy is necessary to prevent excessive spreading of cell death during pathogen-triggered HR (Liu *et al.*, 2005). Silencing of BECLIN1 gene, required for autophagy, results in accelerated senescence and lesions expanded beyond the infection site of the pathogen (Liu *et al.*, 2005). Although the exact mechanism by which autophagy prevents the spread of cell death is still unknown, this reveals potentially antagonistic roles for autophagy with respect to HR-associated PCD.

Chloroplasts are important sources of signals that can initiate plant PCD. Examples of chloroplast-derived molecules involved in chloroplast-to-nucleus retrograde signaling and initiation of stress responses or PCD include hydrogen peroxide, singlet oxygen, and Mg-protoporphyrin IX (Koussevitzky *et al.*, 2007; Lee *et al.*, 2007; Liu *et al.*, 2007; Wagner *et al.*, 2004). Photosynthetic electron transport chains produce hydrogen peroxide and superoxide anion radicals, especially under conditions leading to over-energization of the electron transfer chains (Gechev *et al.*, 2006). Although excessive production of ROS is potentially dangerous, in this case the ability of oxygen to accept electrons prevents overreduction of the electron transport chains, thus minimizing the chance of singlet oxygen production. Many abiotic and biotic stress conditions inhibit carbon fixation reactions in the stroma, which in combination with the increased ROS production may lead to severe oxidative stress. During the tobacco HR, a mitogen-activated protein kinase (MAPK) cascade shuts down carbon fixation in chloroplasts, and hydrogen peroxide, generated by light in the chloroplasts, triggers PCD (Liu *et al.*, 2007). In another example, the conditional *flu* (*fluorescent*) mutant of *Arabidopsis thaliana*, defective in the negative feedback control of chlorophyll biosynthesis, accumulates the chlorophyll precursor protochlorophyllide. The protochlorophyllide is a potent photosensitizer and upon dark-to-light shift singlet oxygen ($^1\text{O}_2$) rapidly accumulates as a result of energy transfer from the excited protochlorophyllide to O_2 (Meskauskiene *et al.*, 2001). The biological effects of $^1\text{O}_2$ are due to switching on a genetic cell death program, not cytotoxicity, as evidenced by the identification of *EXECUTER1* and *EXECUTER2* genes involved in the relaying of the signal (Lee *et al.*, 2007; Wagner *et al.*, 2004). As $^1\text{O}_2$ is a very short-lived and reactive, it is possible that other messengers, most likely lipid-derived molecules, mediate the transmission of the signal to the nucleus. Singlet oxygen is detoxified by carotenoids located in the thylakoids. *Arabidopsis* mutants defective in carotenoid biosynthesis or treated

with the carotenoid biosynthetic inhibitor norflurason bleach and undergo cell death (Strand *et al.*, 2003). Norflurason-treated plants accumulate Mg-protoporphyrin IX, which may function as a signal released from the chloroplasts to repress nuclear-encoded genes in another retrograde chloroplast-to-nucleus signaling pathway (Strand *et al.*, 2003). The *gun* mutants with reduced Mg-protoporphyrin IX levels display increased tolerance to norflurason-induced cell death. Further studies suggest that the chloroplast-localized pentatricopeptide-repeat protein GUN1 and the *Apeta2*-type transcription factor ABI4 participate in the same signaling pathway (Koussevitzky *et al.*, 2007). The interaction between GUN1 and EXECUTER-regulated pathways remains unclear. A number of other studies on chloroplast-localized genes also support a critical role for the chloroplasts in plant PCD (Hu *et al.*, 1998; Ishikawa *et al.*, 2001, 2003; Seo *et al.*, 2000).

Chloroplast signals appear to coordinately regulate plant PCD with mitochondria. *Mitochondria* are prominent players in animal cell death. Many lethal agents targeted at them cause release of cytochrome *c* and other proapoptotic proteins, which can initiate caspase activation and apoptosis. In plants, mitochondria may not play such a universal role. Nevertheless, involvement of mitochondria and release of cytochrome *c* have been reported in different types of plant PCD like heat-shock induced cell death, cell death associated with formation of tracheary elements, or self-incompatibility (Balk *et al.*, 1999; Thomas and Franklin-Tong, 2004; Yu *et al.*, 2002).

Accelerated cell death2 (ACD2) is an *A. thaliana* chloroplast protein that modulates the amount of PCD triggered by *Pseudomonas syringae* and protoporphyrin IX treatment (Yao and Greenberg, 2006). Upon infection with avirulent bacterial pathogen, ACD2 redistributes in cells undergoing PCD and their neighboring cells from being primarily chloroplast localized to being more broadly localized in mitochondria and cytosol where the protein protects cells from mitochondrial H₂O₂-dependent PCD. Further substantiating the role of mitochondria in some forms of PCD, *acd2* mutant plants undergoing spontaneous cell death suffer a loss of mitochondrial membrane potential which appears to be a common event that precedes nuclear morphological changes during PCD induced by ceramides, protoporphyrin IX, and the HR elicitor AvrRpt2 (Yao *et al.*, 2004). The genetically controlled death of the tapetal cells shows morphological and biochemical similarities to apoptosis like nuclear shrinkage, chromatin condensation, nuclear DNA fragmentation, and release of cytochrome *c* from mitochondria (Balk *et al.*, 2003). ROS and the open state of the mitochondrial permeability pores are necessary for the development of salt stress-induced PCD in tobacco protoplasts (Lin *et al.*, 2006). An Mg²⁺-dependent DNase with a potential role in plant PCD is localized in the mitochondrial intermembrane space (Balk *et al.*, 2003). H₂O₂ burst in

mitochondria was detected also in victorin-induced PCD in oat, and the possible involvement of H_2O_2 was suggested by delaying the cell death with scavengers such as catalase and *N*-acetyl-L-cysteine (Yao *et al.*, 2002). Finally, mitochondria-associated hexokinases appear to play an important role in the regulation of plant PCD, similar to their function in the control of apoptotic cell death in animals (Kim *et al.*, 2006b).

2.1. Role of PCD in plant biology: Biological processes and functions associated with PCD

PCD is indispensable for plant survival and development. It accompanies various processes starting as early as embryogenesis. PCD can be observed in the tissues of germinating seeds, in the root cap, during the formation of aerenchyma, during the differentiation of tracheary elements, in various tissues of the reproductive organs, during leaf shape remodeling, some types of trichome development, and leaf senescence (Fig. 3.1). PCD can also be observed during interactions with biotrophic and necrotrophic plant pathogens and as a result of exposure to unfavorable abiotic factors. Here we summarize examples of developmental and environmentally related PCD. Whereas PCD can be found during both plant and animal embryogenesis or formation of body shape, most of the above-listed processes are specific to plants and reflect unique aspects of plant architecture and physiology.

2.1.1. Developmental PCD

Developmental PCD is a terminal stage of plant cell differentiation. In some cases, the dead cells play specific functions (e.g., tracheary elements, fiber cells, trichomes), in other cases, cells must die to form organs with proper functions or shapes (e.g., unisexual reproductive organs in dicots, leaf shape in some plants, aerenchyma tissue), or cells die because they accomplished their function and/or are no longer required (e.g., petals and nectaries in some flowers after pollination, leaf senescence, Fig. 3.1).

The life cycle of terrestrial plants and some algae alternates between haploid gametophyte and diploid sporophyte phases. In flowering plants, the sporophyte phase begins with the formation of a diploid zygote and persists through development of the embryo and the adult plant with its flowers. The gametophyte phase, produced by meiosis from the sporophyte, is limited only to small reproductive structures in the flowers—male gametophyte (microgametophyte) in the pollen grains and female gametophyte (megagametophyte) in the ovules.

Developmental PCD occurs in various tissues of *reproductive organs* and in some cases, the organs themselves undergo abscission (Fig. 3.1) (Rogers, 2006). By removing the organs and tissues that are no longer necessary, the plant preserves its energy resources, eliminates possible entry sites for pathogens,

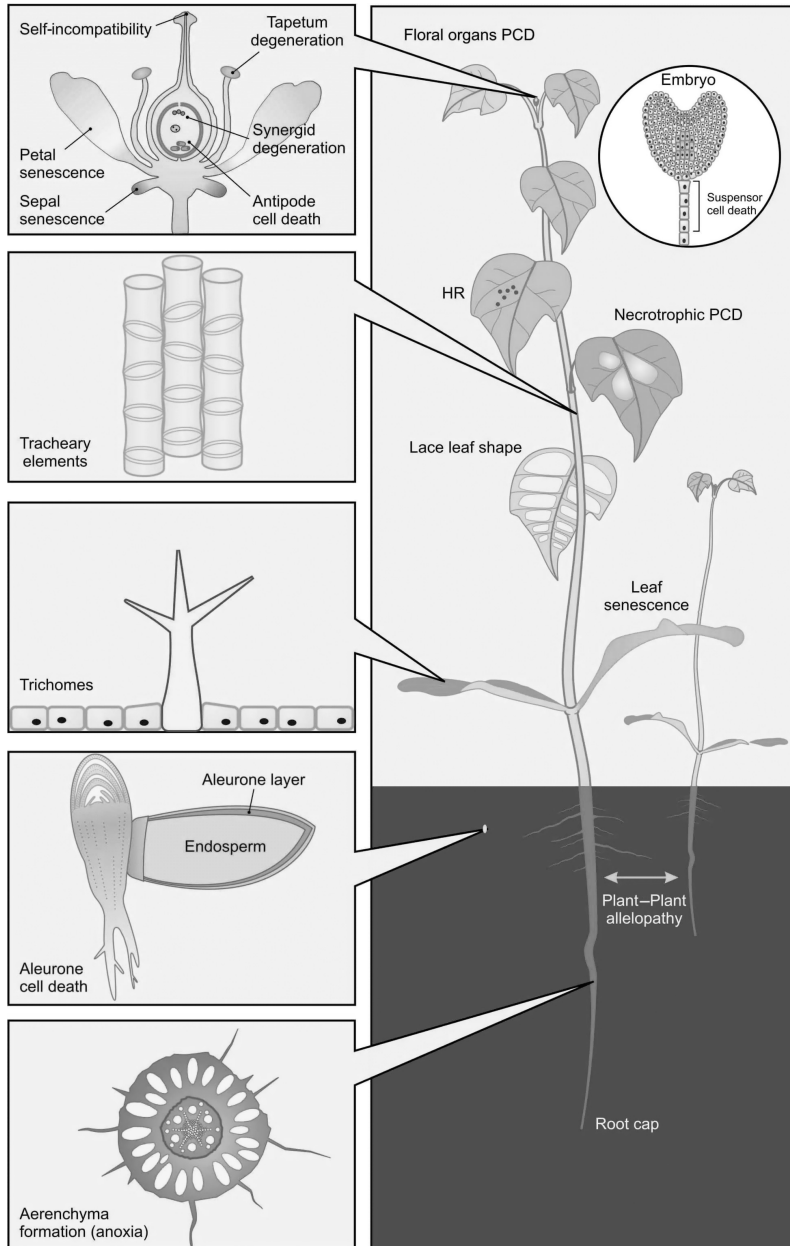


Figure 3.1 Examples of programmed cell death (PCD) in plant development and in response to environmental fluctuations. PCD occurs during embryogenesis (suspensor elimination), tapetum degeneration, pollen self-incompatibility, organ senescence (petals, sepals, leaves), formation of lace leaf shape, synergid and antipode cell death in the female gametophyte, tracheary element differentiation, some types of trichome maturation, cell death of the aleurone layer in germinating monocot seeds, aerenchyma formation under oxygen deprivation (anoxia), cell death of root cap cells, cell death during allelopathic plant–plant interactions, and plant attack by necrotrophic or hypersensitive response (HR)-triggering pathogens.

and excludes competition of nonpollinated flowers for pollinators. After having accomplished their role, *sepals* and *petals* may senesce and either abscise or remain *in situ*, protecting the initial growth of the ovule. Pollination is often accompanied by increased levels of ethylene, a major signal for the petal senescence, especially in the long-lived flowers like orchids. In short-lived flowers, senescence can be controlled independently from pollination and is modulated by other hormones (Rogers, 2006). Petal cells in tobacco are histologically homogenous and their senescence follows an acropetal gradient, culminating in death of the entire corolla at the last stages of the process (Della Mea *et al.*, 2007). Senescence and PCD of corolla are characterized by gradual decrease in chlorophyll, activation of proteases, DNA laddering, nuclear blebbing, rupture of the tonoplast membrane, pigment depletion and modification of the cell walls.

The *Digitalis purpurea* floral nectary tissue undergoes PCD with hallmarks including increased vacuolization, progressive incorporation of plasmatic components into the vacuole reminiscent of autophagy, degradation of the plastids and the nucleus with gradual disappearance of chromatin, loss of tonoplast integrity, and subsequent autolysis of the remaining cellular content (Gaffal *et al.*, 2007). In contrast, the neighboring phloem cells of the nectary remain intact beyond the secretory phase. Abortion of *stamen* or *carpel primordia* is a form of developmental PCD utilized to produce unisexual flowers. In maize, the abortion of stamen primordia in female flowers begins near the apex of the primordium and then spreads basipetally, forming an abrupt border with the living cells adjacent to the base of the primordium (Calderon-Urrea and Dellaporta, 1999; Gunawardena *et al.*, 2004).

The haploid female gametophyte (megagametophyte) develops in the diploid tissues of the ovule, either a structure enclosed within the ovary of the gynoecium in flowering plants or naked and not hemmed in a carpel in gymnosperms (Yadegari and Drews, 2004). The nucellus, located in the middle of the ovule inside the integuments, is functionally equivalent to the megasporangium. The megasporocyte in the immature ovules undergoes sporogenesis through meiosis to produce one surviving haploid megaspore and three other haploid cells that eventually die in a programmed manner (Coimbra *et al.*, 2007). After fertilization, the nucellus, surrounding the embryo, usually starts functioning as a nutritive tissue—perisperm. However, at later stages, *nucellus* degenerates in a PCD fashion (Dominguez *et al.*, 2001). In wheat grains, the process is characterized with internucleosomal cleavage of the nuclear DNA, fragmentation of the cytoplasm, vacuolization of the nuclear envelope, degradation of the organelles, and disruption of the plasma membrane (Dominguez *et al.*, 2001).

The megaspore, enveloped by the nucellus, undergoes mitotic cycles and develops into the haploid megagametophyte (embryo sac), which in most flowering plants (more than 70% of all species) consists of seven cells,

one of them with two nuclei (Yadegari and Drews, 2004). The cells at the micropylar end differentiate into the egg cell and the two synergid cells. The chalazal cells develop into the three antipodal cells. The large central cell of the embryo sac contains two polar nuclei (Yadegari and Drews, 2004). The *synergid cells*, producing signals that attract and guide the growth of the pollen tube, eventually degenerate in a programmed manner (Christensen *et al.*, 2002; Punwani *et al.*, 2007). In some plants, synergid cell death is the final step of the megagametogenesis developmental program, whereas in other species, synergid cell death occurs only after pollination (Yadegari and Drews, 2004). In *Arabidopsis*, cell death is triggered after direct contact with the pollen tube (Sandaklie-Nikolova *et al.*, 2007). The synergid cell death requires mitochondrial J-domain-containing protein GFA2, orthologue of the yeast mitochondrial chaperone Mdj1p (Christensen *et al.*, 2002). The three *antipodal cells* on the opposite end of the ovule in *Arabidopsis* undergo cell death immediately before fertilization. In contrast, in maize and other grasses the antipodal cells proliferate into as many as 100 cells in the mature embryo sac (Yadegari and Drews, 2004).

PCD is observed also in the much larger female gametophyte of gymnosperms (He and Kermode, 2003a,b). The megagametophyte is a living tissue in mature seeds of white spruce but undergoes PCD after germination. This PCD is characterized by vacuolization, nuclear fragmentation, internucleosomal cleavage, and activation of serine and cysteine proteases (He and Kermode, 2003a,b).

During the late developmental stages of male sexual organs, *tapetum* cells must degenerate in order to release pollen. The tapetum degeneration is believed to be executed via PCD with such hallmarks as loss of mitochondrial membrane integrity, vacuolization, nuclear blebbing, and DNA fragmentation (Rogers, 2006). Tapetum degeneration retardation (*tdr*) gene plays a key role in this type of PCD with likely targets a cysteine protease and a protease inhibitor (Li *et al.*, 2006).

Self-incompatibility is a plant-specific mechanism to prevent inbreeding by rejection of incompatible pollen. Self-incompatibility in *Papaver* is associated with activation of a Ca^{2+} signaling cascade followed by release of cytochrome *c* from mitochondria into the cytosol and caspase-3-like enzyme activity (Bosch and Franklin-Tong, 2007; Thomas and Franklin-Tong, 2004). The process is mediated by the activity of MAPK signaling cascade (Li *et al.*, 2006). These events trigger actin depolymerization essential for the cell death (Thomas *et al.*, 2006).

During pollination, the pollen tube enters the female gametophyte through the micropyle of the ovule and releases its contents including the two male gametes. In a process unique to flowering plants, one of the sperm cells migrates to the egg cell and the other one fertilizes the binuclear central cell to form a triploid *endosperm* (Yadegari and Drews, 2004). Later the endosperm, functioning as a storage tissue, undergoes PCD mediated by

ethylene and abscisic acid and accompanied by an increase in nuclease activity and DNA laddering (Young and Gallie, 2000). Nonfertilized *ovules* are also eliminated by a type of PCD (Sun *et al.*, 2004). The endosperm is surrounded by a single cell thick aleurone layer. During seed germination, the plant embryo produces gibberellic acid (GA) triggering the *aleurone* cells to release α -amylase, which in turn hydrolyzes and mobilizes starch from the endosperm in the seeds providing the embryo with energy. The aleurone cells are eliminated by PCD when the germination is completed. The cytoplasm and the nucleus shrink accompanied by nuclear DNA fragmentation into oligonucleosome fragments. ROS and GA play central roles in the control and execution of aleurone PCD (Fath *et al.*, 2001; Yadegari and Drews, 2004). GA represses transcription of antioxidant enzymes [i.e., catalase, ascorbate peroxidase (APX), and superoxide dismutase (SOD)], leading to the dramatic increase in ROS responsible for the cell death (Fath *et al.*, 2002). GA-stimulated repression of antioxidant enzymes and enhanced ROS production is realized through destruction of DELLA proteins (Achard *et al.*, 2008). DELLA proteins are positive regulators of antioxidant enzymes gene expression, thus alleviating stress and delaying H₂O₂-induced PCD. At the same time, DELLA - reduced ROS production represses root growth in *Arabidopsis* (Achard *et al.*, 2008). A nuclear-localized GA-induced nuclease was found to be active just prior to the appearance of DNA laddering in wheat aleurone cells undergoing PCD (Dominguez *et al.*, 2004).

The division of the *zygote* into two cells, apical and basal, is a crucial moment in plant morphogenesis that sets the polar pattern in plant development. The fate of the two cells is tightly coordinated and leads to the formation of an embryo (from the apical cell) and a *suspensor* (from the basal cell). The suspensor channels growth factors to the embryo for a short time and is subsequently eliminated by genetically controlled cell death exhibiting similarities with apoptotic cell death in animals (Bozhkov *et al.*, 2005a).

Postembryonic development is also dependent on PCD. The lateral *root cap cells* are constantly eliminated as a result of well-documented genetically regulated cell death associated with H₂O₂ accumulation, not simply by the abrasive influence of the soil (Pennell and Lamb, 1997; Van Breusegem and Dat, 2006).

A well-characterized model system for the study of plant PCD is the development of *tracheary elements* in the xylem of vascular plants (Kuriyama and Fukuda, 2002). The differentiation of mesophyll into xylem in *Zinnia elegans* cell cultures is connected with accumulation of nucleases and proteases in the vacuole and with reticulation of the cell walls. The swelling of the vacuole is followed by its destruction and by fragmentation of the organelle and nuclear DNA in the course of the ongoing autolysis. Brassinosteroids and ROS play key roles in the initiation of the terminal stages of tracheary elements differentiation including the autolytic program. Some

apoptotic morphologies are not observed and the process shares many analogies with autophagic cell death associated with microtubule rearrangement (Lam, 2005; Obara *et al.*, 2001). A similar scenario occurs in other forms of developmental cell death—aerenchyma formation in the root and the senescence-associated cell death (Lam, 2004).

The programmed elimination of cells plays a crucial role in the sculpting of the plant body. PCD participates in the developmental remodeling of *lace leaf shape*, which can be seen as a process analogous to the elimination of the interdigital tissue during digit formation in the vertebrate limb (Gunawardena *et al.*, 2004). While in many species, the pinnately or palmately dissected leaf shape is achieved through localized growth suppression or stimulation in early leaf morphogenesis, the complex leaf shapes of some monocots arise solely through localized developmental cell death in the early stages of leaf expansion (Gunawardena *et al.*, 2004). In the lace plant (*Aponogeton madagascariensis*), the fenestrated leaf shape appears as a result of a cytologically well-characterized PCD, which initiates with tonoplast rupture and nuclear DNA cleavage. Cell shrinkage and nuclear and organellar degradation are observed in the later stages of the process (Gunawardena *et al.*, 2004). This type of developmental cell death resembles to a certain degree the formation of tracheary elements.

Trichomes of many plant species are dead in their fully differentiated stage. The trichome development follows a switch from mitosis to endoreduplication, cell branching, expansion, and eventual cell death preceded by a burst in hydrogen peroxide (Hulskamp, 2004). These processes are interconnected as misexpression of the cyclin-dependent kinase inhibitor. ICK1/KRP1 reduces the endoreduplication and cell size and induces cell death (Schnittger *et al.*, 2003). Enhanced hydrogen peroxide levels are observed in trichomes of knockout plants of succinic semialdehyde dehydrogenase, a key enzyme of γ -aminobutyrate metabolic pathway (Bouche *et al.*, 2003). These plants are more sensitive to UV and heat stress and their trichomes rapidly undergo cell death when transferred to elevated light intensities supplemented with UV radiation.

Plant *senescence* describes the spectrum of terminal events in plant vegetative and reproductive development connected with active turnover and reutilization of cellular material from tissues and organs to be eliminated eventually followed by cell death (Pennell and Lamb, 1997). Senescence of leaves and other organs is dependent on genetic and hormonal control. Analysis of various mutants and senescence-regulated genes revealed a highly complex molecular regulatory network in which receptors, transcription regulators, and metabolism regulators coordinate this process at molecular, cellular, organellar, and organismal levels (Lim *et al.*, 2007). Both internal (plant hormones) and external factors (abiotic or biotic stresses) can modulate plant senescence. Ethylene is an important regulator of this process, as indicated by delayed leaf senescence in experiments using

ethylene antagonists (compounds blocking its synthesis and perception) and mutants defective in ethylene biosynthetic or signaling pathways. Ethylene stimulates the expression of senescence-associated genes (in *Arabidopsis*: *SAGs*, senescence-associated genes; in tomato: *SENUs*, senescence-upregulated genes) encoding proteins such as cysteine proteases, RNases, and a glutamine synthetase (Della Mea *et al.*, 2007). However, transgenic plants constitutively overproducing ethylene do not exhibit premature senescence, indicating that other signals in combination with ethylene are required to initiate the process (Lim *et al.*, 2007). Polyamines are involved in the control of apoptosis in animals and PCD in plants where they influence senescence of different organs (Della Mea *et al.*, 2007). ROS like superoxide radicals and hydrogen peroxide are also key coordinators of senescence (del Rio *et al.*, 2006). In contrast to ethylene, cytokinins are known as negative regulators of senescence (Lim *et al.*, 2007). However, both ethylene and cytokinin signals appear to be transmitted through classic two-component relay systems. Cytokinins are perceived by three receptors that transfer the signal to histidine phosphotransfer proteins which in turn translocate to the nucleus and relay the signal to response regulator proteins that either induce or repress the expression of cytokinin-regulated genes. *Arabidopsis* cytokinin receptor histidine kinase 3 (AHK3) transmits the cytokinin signal to positively control leaf longevity (Kim *et al.*, 2006a). Missense mutation or overexpression of AHK3 leads to delayed senescence, while loss-of-function mutation leads to accelerated senescence symptoms. The cytokinin signal is relayed via the response regulator ARR2 leading to suppression of nuclease, protease, and lipase *SAGs* (Kim *et al.*, 2006a). Interestingly, high concentrations of cytokinins induce PCD with typical apoptotic features in both *Arabidopsis* and *Daucus carota*, including chromatin condensation, DNA laddering, and release of cytochrome *c* from mitochondria (Carimi *et al.*, 2003). The biological relevance of this phenomenon remains unclear.

Reverse genetics studies identified a number of transcription factors that are positive regulators of leaf senescence. *WRKY53*, belonging to the plant-specific WRKY family of transcription factors, is highly induced at the early stages of leaf senescence but its expression decreases at the later stages coinciding with the cell death symptoms, suggesting that it may play a regulatory role and govern the global transcriptional reprogramming during senescence (Hinderhofer and Zentgraf, 2001). Indeed, null mutants of *WRKY53* display delayed leaf senescence, whereas *WRKY53* overexpression accelerated the process (Miao *et al.*, 2004). Interestingly, *WRKY53* interacts with the jasmonic acid-inducible protein ESR/ESP, and the two proteins mutually antagonize each other (Miao and Zentgraf, 2007). In addition to *WRKY53*, transcription factors from the NAC family have been identified as positive regulators of leaf senescence in *Arabidopsis* (Lim *et al.*, 2007). Knockout *AtNAP* mutants exhibit significantly delayed leaf senescence (Guo and Gan, 2006).

2.1.2. PCD in the interactions between plants and the environment

A number of plant adaptation processes, including the HR to pathogens, some plant–plant allelopathic interactions, and aerenchyma formation in response to oxygen deprivation, require PCD. In contrast, many unfavorable abiotic stress factors as well as necrotrophic pathogens trigger unwanted PCD. Thus, PCD both serves as a positive and negative aspects of plant adaptation to the environment.

2.1.2.1. Interactions with pathogens The invasion of an avirulent pathogen leads to a localized HR, characterized by rapid collapse of the tissue at the immediate and surrounding regions of the site of infection, and formation of dry lesions clearly distinguishable from the surrounding healthy tissue (Lam, 2004). In this way, HR protects plants from potential propagation and development of the pathogen. This localized response is frequently associated with the establishment of *systemic acquired resistance*, manifested in distant organs, and accompanied by the induction of pathogenesis-related genes, the synthesis of secondary metabolites with protective functions, and cell wall reinforcement (Dangl and Jones, 2001).

Clear evidence for the genetic control of HR-associated cell death comes from identification of mutant *Arabidopsis*, maize, and tomato plants that spontaneously undergo HR-like cell death in absence of pathogen attack (Overmyer *et al.*, 2000). Some of these “initiation” mutants exhibit localized lesions typical of the HR, whereas the cell death in other “propagation” mutants is massive. The existence of these two classes of mutants reflects the complexity of the genetic control and indicates that genetically distinct processes are responsible for the different stages during HR lesion formation—the initiation and the confinement of the cell death (Overmyer *et al.*, 2000). HR is a type of PCD showing some similarities to apoptosis (Lam, 2004). The process starts with rapid ion fluxes through the plasma membrane and a burst of H₂O₂ and superoxide anion radicals, leading to increased cytosolic Ca²⁺ levels, activated protein kinase cascades, global transcriptional reprogramming, and rapid cell death. The oxidative burst is biphasic where the first wave is shorter, having signaling functions, and the second phase is longer with sustained ROS production that initiates PCD (Lamb and Dixon, 1997). Key generators of ROS in *Arabidopsis* are the apoplastic peroxidases and the membrane-localized NADPH oxidases, composed of AtrbohD- and AtrbohF-encoded subunits (Bindschedler *et al.*, 2006; Torres *et al.*, 2002). Hydrogen peroxide produced by these enzymes is essential not only for PCD in cells surrounding the invading pathogen but also for prevention of cell death in neighboring cells and tissues (Torres *et al.*, 2005). The HR is characterized by nuclear DNA cleavage, rapid cytoskeletal reorganization, and organelle dismantling. The caspase-like protease γ VPE emerged as an important executor in this type of cell death. Vacuolar processing enzyme (VPE) is synthesized

as an inactive larger precursor, which after processing is transported to and subsequently fully activated in the vacuole (Hatsugai *et al.*, 2004; Kuroyanagi *et al.*, 2005). In *Arabidopsis*, the cells undergoing PCD during the HR condense and shrink resembling apoptotic bodies (Lam, 2004; Pennell and Lamb, 1997). In addition to ROS, HR-associated cell death is modulated by different phytohormones. Pharmacological and genetic experiments showed the importance of salicylic acid (SA) in a number of HR systems. It has been proposed that depending on its concentration, SA stimulates or suppresses PCD thus contributing to the establishment of the boundary between the lesion and the living tissue (Alvarez, 2000). The interaction based on a feedback control between the levels of ROS and SA as well as the balance between the intracellular NO and H₂O₂ are of primary importance (Delledonne *et al.*, 2001; Zago *et al.*, 2006; Zaninotto *et al.*, 2006). Recently, processes similar to autophagy in animals have been shown to act as negative regulators of the HR-associated cell death by inhibiting the growth of the lesions in the healthy tissue through possible prevention of the effect of the progressing cell death signal (Patel *et al.*, 2006). A model has been proposed which suggests that in response to pathogen recognition, autophagy-associated genes are upregulated leading to the formation of autophagosomes in the healthy cells adjacent to the HR PCD. These autophagosomes can sequester VPE pre- and proproteins that will be degraded after the fusion with the vacuole, thus preventing its dismantling and the development of PCD (Liu *et al.*, 2005; Patel *et al.*, 2006; Seay and Dinesh-Kumar, 2005).

Necrotrophic fungi secrete so-called *host-selective toxins* to trigger cell death in the host plant to feed on the dead tissue (Stone *et al.*, 2000; Wang *et al.*, 1996a). Examples of such phytotoxic molecules are the AAL-toxin and fumonisin B1, synthesized by *Alternaria alternata* f. sp. *lycopersici* and *Fusarium verticillioides* (formerly *moniliforme*), respectively. The two toxins share similar chemical structures and act as sphingosine analogues to inhibit ceramide synthase, a key enzyme in sphingosine metabolism, leading to decreased levels of complex ceramides, increased levels of their precursors, and eventual cell death (Gechev *et al.*, 2004; Spassieva *et al.*, 2002). The cell death induced by AAL-toxin and fumonisin B1 is the end result of active, genetically controlled processes showing morphological and biochemical analogies to animal apoptosis, including nuclear condensation, DNA fragmentation, and requiring *de novo* protein synthesis (Asai *et al.*, 2000; Stone *et al.*, 2000; Wang *et al.*, 1996a). The process is accompanied by oxidative burst and is regulated by ROS (Gechev *et al.*, 2004). The caspase-like VPE is a key regulator of fumonisin-induced cell death in *A. thaliana* (Kuroyanagi *et al.*, 2005). Victorin, another host-selective toxin produced by *Cochliobolus victoriae*, triggers PCD in oat (Navarre and Wolpert, 1999). Victorin-induced PCD displays morphological and biochemical hallmarks of animal apoptosis, including chromatin condensation, DNA laddering, and cell shrinkage (Coffeen and Wolpert, 2004). Victorin binds to and inhibits

mitochondrial glycine decarboxylase complex, resulting in changes of mitochondrial potential with a concomitant H₂O₂ burst (Yao *et al.*, 2002). Calcium fluctuations, ethylene, and subtilisin-like serine proteases with caspase-like activity seem to play role in victorin-induced cell death (Coffeen and Wolpert, 2004). Recently, screening for victorin-insensitive mutants in *Arabidopsis* identified thioredoxin h5 (ATTRX5, coiled-coil nucleotide-binding site leucine-rich repeat protein) as required for the cell death (Sweat and Wolpert, 2007). Victorin responses seem specific for ATTRX5 as the closely related ATTRX3 only partially compensates for the loss of function (Sweat and Wolpert, 2007). Interestingly, ATTRX5 is highly induced by AAL-toxin- and H₂O₂-dependent cell death (Gechev and Hille, 2005; Gechev *et al.*, 2004).

2.1.2.2. Plant–plant allelopathic interactions PCD plays a role in some interesting types of allelopathic plant–plant interactions (Bais *et al.*, 2003). *Centaurea maculosa* displaces native species from their habitat by secreting the phytotoxin catechin into the soil. The catechin triggers H₂O₂ accumulation in root meristems of neighboring species, activates a Ca²⁺-dependent signaling cascade, transcriptome reprogramming, and eventually cell death (Bais *et al.*, 2003).

2.1.2.3. Plant PCD triggered by abiotic factors Many abiotic stress factors, including pollutants, UV-light, salinity, and extreme temperatures, can result in oxidative stress and subsequently ROS-dependent PCD. *Ozone* (O₃) is an important air pollutant, a potent toxin, and component of the photochemical smog in urban areas (Overmyer *et al.*, 2003). O₃ enters the apoplast where it elicits generation of ROS, mainly hydrogen peroxide (Pellinen *et al.*, 2002). The ROS burst is biphasic with a smaller peak at 4 h and a larger peak at 16 h, similar to what is seen with pathogen attack (Mahalingam *et al.*, 2006). This is accompanied by a burst of NO at 9 h. These ROS and reactive nitrogen species (RNS) act not simply as destructive toxic agents but also as signals unlocking the PCD program (Overmyer *et al.*, 2003). Hallmarks of O₃-induced cell death include intensive ion fluxes across membranes, shrinkage of the nuclei, chromatin condensation, nuclear DNA fragmentation, lesion formation, induction of pathogenesis-related genes, and reinforcement of the cell wall (Overmyer *et al.*, 2005). These reactions are dependent on *de novo* transcription and protein synthesis, ATPase, kinase, and caspase-like proteolytic activities, and are modulated by ethylene and SA levels (Overmyer *et al.*, 2005; Sandermann, 2004).

Other pollutants like *heavy metals* can also initiate H₂O₂-induced PCD. Cadmium initiates an oxidative burst and eventual cell death in tobacco (Garnier *et al.*, 2006). The oxidative burst is observed in three waves localized mainly to the apoplast and mitochondria. Calcium release from

internal pools leads to NADPH oxidase-dependent hydrogen peroxide accumulation preceding the cell death. Membrane peroxidation and mitochondrial superoxide radical production are also essential for cadmium-induced cell death (Garnier *et al.*, 2006). Distinct MAPKs are activated in response to cadmium or copper-induced heavy metal stress (Jonak *et al.*, 2004). Other heavy metals like aluminum or mercury can also cause perturbations in redox homeostasis and ROS-dependent cell death (Ortega-Villasante *et al.*, 2007; Pan *et al.*, 2001).

Exposure of plants to *high light* intensities and/or *UV-light* can result in activation of PCD (Davison *et al.*, 2002; Danon *et al.*, 2004). Overexpression of β -carotene hydroxylase, an enzyme in the zeaxanthin biosynthetic pathway, enhances high light-induced cell death in *Arabidopsis* (Davison *et al.*, 2002). UV-C overexposure triggers a rapid burst of ROS followed by loss of mitochondrial membrane potential and cell death (Gao *et al.*, 2008). The cell death can be retarded by antioxidants (ascorbic acid), inhibitors of photosynthetic electron transport (DCMU), inhibitors of mitochondrial permeability transition pores (cyclosporin), or caspase inhibitors (Danon *et al.*, 2004; Gao *et al.*, 2008). The burst of hydrogen peroxide and superoxide radicals is followed by induction of metacaspase 8 (AtMC8), which is involved in mediating UV-C and ROS-induced cell death (He *et al.*, 2008).

Salt stress causes PCD with hallmarks of TUNEL staining and DNA laddering in different plant species (Huh *et al.*, 2002; Lin *et al.*, 2006). In *Arabidopsis*, salt stress induces PCD in primary roots of both wild-type and salt overly sensitive (*sos1*) mutant seedlings. However, whereas wild-type plants survived salt stress levels due to formation of secondary roots from the shoot/root transition zone, *sos1* mutants failed to do so and died (Huh *et al.*, 2002). Salt stress-induced PCD in rice root tip cells and tobacco protoplasts is associated with disturbed Ca^{2+} , K^{+} , and H^{+} ion equilibrium and increased production of hydrogen peroxide (Huh *et al.*, 2002; Shabala *et al.*, 2007). High H_2O_2 level together with the open state of the mitochondrial permeability pores are compulsory for the realization of the cell death program, showing direct analogies with apoptosis in animals (Lin *et al.*, 2006). Interestingly, expression of animal antiapoptotic gene CED-9 in tobacco maintains K^{+} homeostasis and protects from salt- and oxidative stress-induced cell death (Shabala *et al.*, 2007).

Exposure to *heat shock* can lead to PCD in a number of species, including *Arabidopsis* and tobacco (Swidzinski *et al.*, 2002). Heat shock leads to a burst of H_2O_2 , which in turn leads to cytochrome *c* release from mitochondria followed by activation of proteases with caspase-3-like and proteasome activities (Coffeen and Wolpert, 2004; Vacca *et al.*, 2007). The ROS scavenging enzymes catalase and SOD or specific caspase-3 inhibitors block the activation of caspase-3-like activities and cell death (Vacca *et al.*, 2007). The *Arabidopsis* Bax-1 inhibitor protein seems to be a negative regulator of heat-shock induced cell death (Watanabe and Lam, 2006).

Low temperature induces cell death in tobacco BY-2 cells with typical PCD hallmarks as chromatin condensation and DNA laddering (Koukalova *et al.*, 1997). Such cell culture retained 11% living cells even after prolonged cold treatment. Although the mechanism of cold-induced PCD is not clear, heterologous overexpression of the animal antiapoptotic genes *bcl-xL* and *ced-9* improves the tolerance to low-temperature-induced necrotic lesions in tomato (Xu *et al.*, 2004), supporting functional conservation of PCD components between plants and animals.

Formation of lysigenous aerenchyma tissue in response to labored gas exchange and *hypoxia* occurs in a number of species, including *Arabidopsis* and maize (Kuriyama and Fukuda, 2002; Muhlenbock *et al.*, 2007; Pennell and Lamb, 1997). Typical for this tissue, the intercellular spaces filled with air to facilitate gas exchange and oxygen transfer, appear as a result of PCD. Protein phosphorylation and Ca^{2+} fluxes, disruption of cytoplasmic streaming, and tonoplast rupture that releases vacuolar lytic enzymes are among the first processes observed during this type of PCD, followed by shrinkage of the nucleus, chromatin condensation, nuclear DNA fragmentation, and decomposition of the organelles and the cell wall (Gunawardena *et al.*, 2001). In *Arabidopsis*, aerenchyma formation under hypoxic conditions is associated with ethylene and hydrogen peroxide production (Muhlenbock *et al.*, 2007). The cell death regulator LESION STIMULATING DISEASE1 (LSD1) negatively regulates ethylene and hydrogen peroxide production as well as PCD, as evidenced by increased ACC (ethylene precursors) and H_2O_2 levels and two-fold greater aerenchyma formation in *lsd1* mutants. At the same time, in absence of functional LSD1, ethylene- and hydrogen peroxide-dependent PCD during hypoxia is positively regulated by ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1) and PHYTOALEXIN DEFICIENT4 (PAD4) defense regulators (Muhlenbock *et al.*, 2007).

2.2. Plant cell death machinery: Plant-specific regulators, transcription factors, proteases, and nucleases

In the last few years, numerous studies identified different components of the versatile plant cell death network. Despite this progress, the cell death pathways in plants still remain enigmatic with most of the genes and proteins involved yet to be discovered or, for most of the identified players, yet to be placed in a specific position within the complex network. For example, there are no core universal regulators and executioners of plant PCD analogous to the members of the animal Ced-9/Bcl-2, Ced-4/APAF1, and caspase families (Lam, 2004). From the emerging picture, it is becoming increasingly clear that most of the genes involved in the regulation of plant PCD are specific to the plant kingdom and this is most likely a reflection of the specific morphology and physiology of plants. Nevertheless, there are structural and functional similarities underlined by the ability of some

animal or yeast pro- and antiapoptotic proteins to function as cell death regulators in plants (Lam, 2004). Expression of the proapoptotic Bcl-2 member Bax induces PCD in plants, whereas the antiapoptotic protein Bcl-xL or its *C. elegans* homologue Ced-9 suppresses pathogen-induced cell death (Dickman *et al.*, 2001; Kawai-Yamada *et al.*, 2001; Lacomme and Santa Cruz, 1999; Lam, 2004; Xu *et al.*, 2004). Research data suggest connection between the function of Bcl-xL/Ced-9 and redox homeostasis. Transgenic plants expressing the protein accumulate anthocyanins and exhibit better resistance to UV irradiation, paraquat treatment, TMV infection, salt-stress, low-temperature exposure, and wounding (Mitsuhashi *et al.*, 1999; Qiao *et al.*, 2002; Xu *et al.*, 2004). As previously mentioned, the expression of CED-9 conferred increased salt and oxidative stress tolerance to *Nicotiana benthamiana* plants by altering ion flux patterns across plasma membrane (Shabala *et al.*, 2007). The inhibitor of Bax-induced PCD—Bax Inhibitor 1 (BI-1)—functions as cell death suppressor in fungi, plants, and animals. This is a transmembrane protein with predominant localization to the endoplasmatic reticulum (ER). AtBI-1 has been found to modulate ER stress-mediated PCD associated with accumulation of H₂O₂ in *Arabidopsis* (Watanabe and Lam, 2008). The *BAP1* and *BAP2* genes of *Arabidopsis* together with their partner *BON1* function as general inhibitors of PCD in plants and yeast, induced by various stimuli including ROS (Yang *et al.*, 2007).

Many of the newly identified genes are presumably involved in modulating the initial signaling events. These include genes that modulate the intensity of the cell death stimulus, participate in the interaction with other signaling molecules, or perceive the cell death signal, thus playing roles as receptors. Other genes relay and amplify the cell death signal by MAPK- or Ca²⁺/Calmodulin (CAM)-dependent cascades, ultimately regulating cell death-specific transcription factors to govern the global transcriptional reprogramming observed during cell death. Finally, as a result of this reprogramming, sets of genes including proteases and nucleases orchestrate the orderly executed cell suicide and remobilization of resources. Interestingly, as it is with animal caspases, some of these plant proteases may not be simple executioners of cell death but are also involved in regulating the initial steps of the process by proteolytically activating pro-death factors or degrading antiapoptotic proteins.

Alterations in *ion fluxes* are one of the primary events of PCD initiation. The *Arabidopsis* lesion mimic mutant gene *hlm1* and its barley homologue *nec1* encode cyclic nucleotide-gated channels (CNGC) (Balague *et al.*, 2003; Rostoks *et al.*, 2006). The *Arabidopsis* CNGC4 is permeable to both K⁺ and Na⁺ and is activated by both cGMP and cAMP. The gene is induced by pathogens and is believed to be involved in the HR (Balague *et al.*, 2003). Hydrogen peroxide-induced oscillations in Ca²⁺ ions are essential signals for many biological responses, including stomatal closure, stress adaptation, and several types of PCD (Allen *et al.*, 2001; Lecourieux

et al., 2002; Pei *et al.*, 2000; Rentel *et al.*, 2004). It appears that the Ca^{2+} signature is quite specific for the different responses; increased cytosolic Ca^{2+} can be short-lived or sustained and oscillations may vary in amplitude and phase. Other cyclic nucleotide-gated channels, CNGC1 and CNGC2, also known as DND1 and DND2, respectively, are also induced by pathogens and conduct Ca^{2+} into cells during HR, but have opposite phenotypes to *hlm1* and *nec1* when mutated (Ali *et al.*, 2007; Clough *et al.*, 2000). The Ca^{2+} wave induces a burst of NO essential for PCD. Interestingly, *dnd1* plants retain characteristic responses to avirulent pathogens and are resistant against a broad spectrum of virulent pathogens (Yu *et al.*, 1998). Targets of Ca^{2+} can be Ca^{2+} /CAM-binding proteins, Ca^{2+} -dependent protein kinases, various EF-hand containing proteins (e.g., NADPH oxidase related to the oxidative burst), and also proteins without EF-hands (Lecourieux *et al.*, 2006). The plant-specific MLO family transmembrane proteins possess CAM-binding domains. Mutations in *mlo* gene of barley lead to spontaneous cell death without any obvious inducing signals (lesion mimic mutants of the initiation type) and enhanced disease resistance to powdery mildew (Kim *et al.*, 2002). However, the powdery mildew-resistant plants are more susceptible to necrotrophic pathogens that utilize PCD to feed on dead tissues (Jarosch *et al.*, 1999).

Hydrogen peroxide-induced Ca^{2+} oscillations can in turn amplify ROS signals by activating the CAM-binding protein NAD kinase and EF-hand motif-containing NADPH oxidase (Harding *et al.*, 1997; Lecourieux *et al.*, 2006). Both H_2O_2 and $\text{NO}^\bullet$ orchestrate pathogen-induced PCD (Delledonne, 2005; Delledonne *et al.*, 2001). Ca^{2+} can also induce $\text{NO}^\bullet$ essential for pathogen-induced cell death (Lamotte *et al.*, 2004). Indeed, plant $\text{NO}^\bullet$ synthase contains CAM-binding motif and both Ca^{2+} and CAM are required for its full activation (Guo *et al.*, 2003). In turn, $\text{NO}^\bullet$ participates in Ca^{2+} release, thus amplifying the initial signal (Lamotte *et al.*, 2004). Plant genes involved in Ca^{2+} responses and modulation of H_2O_2 or $\text{NO}^\bullet$ levels are important determinants of PCD. More on these genes, particularly on the antioxidant gene network, will be discussed in Section 3.

Plant hormones like ethylene, brassinosteroids, and cytokinins together with other signaling molecules regulate PCD in a complex manner. Studying the genetic components of the hormone pathways identified plant-specific receptors and other proteins that are involved in various types of PCD. Brassinosteroids are plant hormones that regulate growth and development (Vert and Chory, 2006). In *Arabidopsis*, they are perceived outside the cell by two receptor-like protein kinases BRI1 and BAK1 (Gendron and Wang, 2007). The group of receptor-like kinases in plants has expanded and diversified to more than 600 members in *Arabidopsis* and 1000 in rice to regulate various aspects of development and stress responses (Morillo and Tax, 2006). Interestingly, BAK1 can also contain the spread of pathogen-induced PCD and this function can be uncoupled from brassinosteroid

regulation of plant development (Kemmerling *et al.*, 2007). *Bak1* mutants show enhanced production of hydrogen peroxide and spreading necrosis upon infection with necrotrophic fungal pathogens, while brassinosteroid-insensitive and -deficient mutants do not exhibit increased sensitivity and spreading lesions in response to such pathogens (Kemmerling *et al.*, 2007). BAK1 can also physically associate with the flagellin receptor-like protein kinase FLS2 to regulate plant immunity (Chinchilla *et al.*, 2007). *Bak1* mutants challenged with the bacterial flagellin-derived peptide elicitor do not mount an H_2O_2 burst and flagellin-dependent stress responses (Chinchilla *et al.*, 2007). Thus, BAK1 has multiple roles in plant growth, development, and cell death.

Cytokinins are also plant-specific hormones involved in plant growth and development (Sakakibara, 2006). As discussed earlier, cytokinins negatively regulate leaf senescence-associated PCD in *Arabidopsis* and AHK3 is the receptor involved in perception of the signal (Kim *et al.*, 2006b).

Other specific plant cell death regulators are revealed by identification and cloning of genes responsible for lesion-mimic mutants *acd1*, *acd2*, *acd5*, *acd6*, and *acd11*. While ACD1 and ACD2 are connected to chlorophyll catabolism, ACD5 and ACD11 are related to lipid metabolism (Brodersen *et al.*, 2002; Liang *et al.*, 2003; Tanaka *et al.*, 2003). As indicated earlier, ACD2 may not only be involved in chlorophyll metabolism but also shields chloroplasts and mitochondria from excess ROS production (Yao and Greenberg, 2006). The earlier-mentioned chloroplast proteins EXECUTER1 and EXECUTER2 are also restricted to plants.

Cell death signals are relayed by a complex network of Ca^{2+} and MAPK cascades to activate PCD transcriptional reprogramming via cell death-specific *transcription factors*. LOL1 and LSD1 are positive and negative regulators of superoxide-induced cell death, respectively, acting as a molecular rheostat to control PCD in *Arabidopsis* (Dietrich *et al.*, 1997; Eppele *et al.*, 2003). The negative cell death regulator LSD1 retains the basic leucine zipper transcription factor AtbZIP10 in the cytosol, thus preventing it from initiating PCD transcriptional reprogramming (Kaminaka *et al.*, 2006). The phenotype of *lsd1* mutants is uncontrolled, spreading cell death, initiated by $\text{O}_2^{\bullet-}$ (Jabs *et al.*, 1996). Interestingly, a triple mutant between *lsd1* and two ROS-generating NADPH oxidase subunit homologues, *atrbohD* and *atrbohF*, showed uncontrolled cell death even under growth conditions that normally repress *lsd1* cell death (Torres *et al.*, 2005). The *lsd1* phenotype was restored by overexpression of *AtrbohD*, demonstrating that $\text{O}_2^{\bullet-}$ produced by NADPH oxidase and its subsequent dismutation to H_2O_2 is somehow able to antagonize the $\text{O}_2^{\bullet-}$ -induced cell death in the neighboring cells (Torres *et al.*, 2005). AtMYB30 is another positive regulator of pathogen-induced PCD and has an important role in disease resistance (Vailleau *et al.*, 2002). Silencing of AtMYB30 strongly inhibits HR-like cell death in response to avirulent pathogens, which corresponds with decreased defense gene expression and decreased resistance to

virulent pathogens. Targets of MYB30 are genes from the acyl-CoA elongase complex and the action of MYB30 seems to modulate very-long-chain fatty acid content (Raffaele *et al.*, 2008). Another transcriptional regulator, AtSPL14, suppresses fumonisin B1-induced cell death when misregulated (Stone *et al.*, 2005).

WRKY is a large plant-specific family of transcription factors that governs transcriptional reprogramming during pathogen responses and developmental processes such as leaf senescence and some types of trichome development/maturation (Eulgem and Somssich, 2007). Members of the WRKY family are highly upregulated during abiotic and biotic stresses. Transcriptome analysis of PCD induced by hydrogen peroxide, superoxide anion radicals, and singlet oxygen identified WRKY75 as commonly induced by all three types of ROS (Gadjev *et al.*, 2006). WRKY53 is induced during senescence-associated PCD, and functional studies with knockouts and WRKY53-overexpressing plants confirmed its role in leaf senescence (Miao and Zentgraf, 2007).

The global transcriptional reprogramming results in a complete switch to a cell death program in which sets of proteases and nucleases play roles in the orderly destruction of the plant cell (Lam, 2004, 2005). Plant proteases can be involved in both the regulatory and the executionary phases of cell death, as is the case with caspases in animal apoptosis. *Caspases*, a family of cysteine proteases highly conserved from nematodes to humans, are the core signaling and execution proteases in animals (Chichkova *et al.*, 2004; He *et al.*, 2008). They are normally expressed in a dormant form and/or are localized in an isolated cellular compartment, which allows them to play the role of major regulatory switches initiating the irreversible processes of controlled cell death (Lam, 2004, 2005). In plants, both cysteine and serine protease activities have been reported to be important factors in the attainment of ROS-driven PCD (Levine *et al.*, 1996). Inhibitors of cysteine proteases have been shown as an agent suppressing the HR induced by pathogens and ROS (Solomon *et al.*, 1999). Other pharmacological studies with inhibitors of the total caspase activity and with specific inhibitors of different initiator and effector animal caspases revealed the presence of caspase-like activities in the plant cell (Chichkova *et al.*, 2004; Danon *et al.*, 2004; Urquhart *et al.*, 2007). The plant-specific vacuolar protease VPE, structurally unrelated to caspases, possesses caspase-like activity essential for PCD induced by fumonisin B1 or during HR (Hatsugai *et al.*, 2004; Kuroyanagi *et al.*, 2005). However, direct structural orthologues of canonical caspases have not been found in plants. The absence of these key regulators from the sequenced plant genomes together with the fact that apparently most instances of plant PCD are associated with the induction of caspase-like activities imply that alternative mechanisms must control PCD in plants (Lam, 2004; Sanmartin *et al.*, 2005). This role may be served by the caspase-like vacuole processing enzymes and/or the more distantly related metacaspases, which are also

found in *Protozoa*, *Fungi*, and *Chromista* (Vercammen *et al.*, 2007). Caspases, metacaspases, and paracaspases (found in *Animalia*) are seen as phylogenetically equidistant from each other (Vercammen *et al.*, 2007). The three groups share the caspase-specific catalytic diad of histidine and cysteine but the overall sequence similarity among the representatives of these three major branches is very low (Bozhkov *et al.*, 2005b). Paracaspase activity has not been implicated in the execution of cell death, but metacaspases have been found to play an important role in ROS-induced PCD in yeast and plants (Madeo *et al.*, 2002; Suarez *et al.*, 2004; Vercammen *et al.*, 2007). Plant metacaspases have been classified as type I and type II based on their sequence and structural features. *Arabidopsis* contains three type I (*AtMCP1a-1c*) and six type II (*AtMCP2a-2f*) metacaspases (Sanmartin *et al.*, 2005; Vercammen *et al.*, 2007). Metacaspases do not have caspase activities; instead, these cysteine-dependent proteases cleave their substrates after arginine and lysine and can be inhibited by the serine protease inhibitor Serpin1 (Vercammen *et al.*, 2004, 2006). It has been demonstrated that a type II metacaspase from *Picea abies*, mcII-Pa, is essential for PCD during somatic embryogenesis (Bozhkov *et al.*, 2005b). This cysteine-dependent arginine-specific protease translocates from cytoplasm to nuclei in terminally differentiated suspensor cells, destined for elimination, where it colocalizes with the nuclear pore complex and chromatin and participates in the nuclear envelope disassembly and the DNA fragmentation. Recently, *Arabidopsis* metacaspase 8 (AtMC8) has been implicated in cell death induced by UV-C, H₂O₂, and paraquat (He *et al.*, 2008). Overexpressing AtMC8 enhances PCD, while knocking out the gene retards the cell death symptoms.

Not only cysteine but also serine-dependent protease activities participate in plant PCD. Serine proteases with specificity to aspartate have been reported to be associated with the control of cell death in *Avena sativa* (Coffeen and Wolpert, 2004). A nuclear-localized serine protease and a Ca²⁺/Mg²⁺ nuclease were recently isolated from wheat grain nucellar cells undergoing PCD (Dominguez and Cejudo, 2006).

Proteases are not simply executioners but also important regulators of the initial events in the PCD signaling cascade. This is underlined best by studying the complex role of the proteasome and the COP signalosome in cell death. Plant proteasomes degrade specific substrates targeted by ubiquitination via a series of reactions catalyzed by ubiquitin-activating (E1), conjugating (E2), and ligating (E3) enzymes (Smalle and Vierstra, 2004). The COP9 signalosome is a multiprotein complex interacting with and regulating E3 ubiquitin ligases, thus influencing the specificity of protein degradation (Serino and Deng, 2003). This is one of the many cases when an important complex is first discovered in *Arabidopsis* and later reported in other kingdoms. Proteasome-dependent proteolytic degradation is involved in myriad of plant processes related to development, hormone signaling,

stress responses, and cell death (Chini *et al.*, 2007; Moon *et al.*, 2004; Tan *et al.*, 2007). Proteasomal degradation has a dual role in PCD, as different proapoptotic or negative cell death regulators can be degraded by this proteolytic complex in particular types of cell death. This may explain why proteasome function is required for some types of PCD (e.g., cell death induced by heat shock in tobacco), while in other cases, inhibition of proteasome function triggers cell death accompanied by cytochrome *c* release from mitochondria and caspase-like activities (Kim *et al.*, 2003; Vacca *et al.*, 2007). Interestingly, manipulation of tomato ubiquitin-proteasome system by the E3 ubiquitin ligase from the bacterial pathogen *P. syringae* can suppress immunity-associated cell death (Janjusevic *et al.*, 2006).

Induction of nucleases and nuclear fragmentation are common events in PCD (Dominguez *et al.*, 2004; Zaina *et al.*, 2003). A 40 kD Zn²⁺-dependent S1 type endonuclease 1 named ZEN1 (*Zinnia* endonuclease-1) is responsible for nuclear DNA degradation (Ito and Fukuda, 2002). Silencing of *ZEN1* suppressed the degradation of nuclear DNA in tracheary elements undergoing PCD but did not affect vacuole collapse in *Zinnia* cell suspension culture. BFN1 – a nuclease showing similarities to ZEN1 – has been associated with senescence, abscission and programmed cell death-related processes in plants (Farage-Barhom *et al.*, 2008; Pérez-Amador *et al.*, 2000).

3. HYDROGEN PEROXIDE AND OTHER ROS AS SIGNALS MODULATING PLANT PCD

3.1. Production and removal of ROS

ROS are produced in plants and other aerobic organisms as a result of O₂ reduction during a number of normal metabolic processes. These harmful and highly reactive intermediates of O₂ reduction can damage biological molecules and structures and have been considered by many as unwelcome by-products of metabolism (Gechev *et al.*, 2006; Moller *et al.*, 2007). Plants initially developed an antioxidant system composed of antioxidant enzymes and small antioxidant molecules as a means of protection against excessive ROS production. The evolution of this antioxidant system into an elaborate network of ROS-producing and -detoxifying enzymes permitted ROS to be co-opted as signaling molecules that regulate various cellular processes, including growth, development, stress adaptation, and cell death. To modulate so many and such diverse processes, the biological response to altered ROS levels needs to be very specific. The specificity of the biological response to altered ROS levels depends on the type of ROS, the intensity of the signal, and the sites of ROS production. These multiple factors, provided by the ROS network, interact with other factors such as plant

developmental stage, previous stress encounters, plant hormones, RNS, and lipid messengers to determine the final outcome of ROS signaling.

The most important types of ROS are superoxide ($\text{O}_2^{\bullet-}$) and hydroxyl ($\text{HO}^\bullet$) radicals, hydrogen peroxide (H_2O_2), and singlet oxygen ($^1\text{O}_2$), reviewed in a number of recent articles (Apel and Hirt, 2004; Gechev *et al.*, 2006; Moller *et al.*, 2007). Their biochemical properties together with those of the most important RNS are summarized in Table 3.1. Hydrogen peroxide is perhaps the most prominent signaling molecule characterized by its relative stability and significant mobility. The half-life of H_2O_2 is 1 ms, the longest of all ROS types. In contrast, $\text{O}_2^{\bullet-}$ and $^1\text{O}_2$ have much shorter half-lives of about 1–4 μs and $\text{HO}^\bullet$ has an extremely short half-life of only 1 ns (Gechev *et al.*, 2006; Moller *et al.*, 2007). The reactivity and half-lives of different ROS are linked with their mobility in the cell: H_2O_2 can migrate from the sites of its synthesis to adjacent compartments and even neighboring cells, while the highly destructive $\text{HO}^\bullet$ reacts with any biomolecule it contacts and is therefore not very mobile (Bienert *et al.*, 2006, 2007; Henzler and Steudle, 2000). $\text{O}_2^{\bullet-}$ can inactivate important metabolic enzymes containing Fe-S clusters to alter enzyme activities (Halliwell, 2006; Van Breusegem *et al.*, 2001). Its protonated form, $\text{HO}_2^\bullet$, is found mainly in acidic cellular environments. It can cross biological membranes and initiate lipid oxidation by extracting protons from polyunsaturated fatty acids. In most biological systems, $\text{O}_2^{\bullet-}$ is rapidly converted to H_2O_2 by the enzyme SOD (Halliwell, 2006). H_2O_2 can inactivate enzymes by oxidizing their thiol groups (Halliwell, 2006). The destructive properties of $\text{O}_2^{\bullet-}$ and H_2O_2 are more prominent when they interact in the presence of metal ions to form $\text{HO}^\bullet$ during the so-called Haber-Weiss reaction (Kehrer, 2000). Because $\text{HO}^\bullet$ is highly reactive, cells do not possess enzymatic mechanisms for $\text{HO}^\bullet$ detoxification and rely on mechanisms that prevent its formation. These mechanisms include elimination of $\text{O}_2^{\bullet-}$ and H_2O_2 and/or sequestering $\text{Fe}^{3+}/\text{Cu}^{2+}$ metal ions that catalyze the Haber-Weiss reaction with metal-binding proteins, such as ferritins or metallothioneins (Hintze and Theil, 2006; Mittler *et al.*, 2004). In addition to reacting with H_2O_2 and forming $\text{HO}^\bullet$, $\text{O}_2^{\bullet-}$ can react with $\text{NO}^\bullet$ to form peroxynitrite (ONOO^-). Peroxynitrite is rapidly protonated to peroxynitrous acid (ONOOH), which is a powerful oxidizing agent. It can damage all biomolecules and initiate reactions leading to formation of several other destructive radical- and nonradical reactive species (Halliwell, 2006). $^1\text{O}_2$ is a nonradical ROS produced by spin reversal of one electron of the ground state triplet oxygen ($^3\text{O}_2$) (Laloi *et al.*, 2006). Such spin reversals occur under input of energy and are most often caused by reaction with the highly energized triplet-state chlorophyll (Laloi *et al.*, 2006). If not quenched by carotenoids, $^1\text{O}_2$ can in turn transfer its energy to other molecules and damage them, like the rapid peroxidation of polyunsaturated fatty acids (Halliwell, 2006). As other types of ROS, $^1\text{O}_2$ can have either

Table 3.1 Most important reactive oxygen species (ROS) and reactive nitrogen species (RNS), their properties and scavengers

Type of ROS	Half-life and mobility	Mode of action	Main scavengers	References
Superoxide radical ($O_2^{\bullet -}$)	1 μ s, 30 nm	Reacts with double bond-containing compounds, Fe-S clusters of proteins; reacts with $NO^{\bullet}$ to form $ONOO^-$	Superoxide dismutases (SOD)	Halliwell, 2006; Moller <i>et al.</i> , 2007
Hydrogen peroxide (H_2O_2)	1 ms, 1 μ m	Oxidizes proteins (cysteine residues); reacts with $O_2^{\bullet -}$ in a Fe-catalyzed reaction to form $HO^{\bullet}$	Catalases, various peroxidases, peroxiredoxins, flavonoids	Halliwell, 2006; Moller <i>et al.</i> , 2007
Hydroxyl radical ($HO^{\bullet}$)	1 ns, 1 nm	Extremely reactive with proteins, DNA, lipids, and other biomolecules	Flavonoids, prevention of $HO^{\bullet}$ formation by sequestering Fe	Gechev <i>et al.</i> , 2006; Halliwell, 2006; Moller <i>et al.</i> , 2007
Singlet oxygen (1O_2)	1 μ s, 30 nm	Directly oxidizes proteins, polyunsaturated fatty acids, DNA	Carotenoids, α -tocopherol	Gechev <i>et al.</i> , 2006; Halliwell, 2006; Moller <i>et al.</i> , 2007
Nitric oxide ($NO^{\bullet}$)	Limited due to reactivity	Nitrosylates proteins via S-nitrosothiols	Hemoglobins, glutathione	Halliwell, 2006; Neill <i>et al.</i> , 2008
Peroxynitrite ($ONOO^-$)	Not determined	Very reactive with lipids, DNA and proteins		Moller <i>et al.</i> , 2007; Neill <i>et al.</i> , 2008

signaling or cytotoxic effects depending on the endogenous levels accumulated (Przybyla *et al.*, 2008).

ROS and RNS are produced by multiple sources during a variety of cellular processes at different sites in all cellular compartments (Fig. 3.2) (Gechev *et al.*, 2006). In general, ROS are maintained at constant basal levels in healthy cells, but their levels transiently or persistently increase under different stress conditions or upon developmental signals.

H₂O₂ is synthesized mainly in chloroplasts, peroxisomes, and glyoxysomes but also in the apoplast, cytosol, mitochondria, endomembrane system, and nucleus (Ashtamker *et al.*, 2007; Gechev *et al.*, 2006). Chloroplasts are a major site of ROS generation in plants (Asada, 2006). Photosynthetic electron transfer chains produce O₂^{•−}, especially under conditions leading to overenergization of the electron transfer chains (Dat *et al.*, 2000). O₂^{•−} is formed by electron leakage from Fe-S centers of photosystem I, reduced ferredoxin (Mehler reaction), or the acceptor side of photosystem II to O₂ and is then quickly metabolized to H₂O₂ by SOD. Although excessive production of ROS is dangerous, in this case the ability of oxygen to accept electrons prevents overreduction of the electron transport chains, thus minimizing the chance of ¹O₂ accumulation (Dat *et al.*, 2000). ¹O₂ is produced by energy transfer to ³O₂ from the excited triplet state chlorophyll in photosystem II, especially under high light intensities (Laloi *et al.*, 2006). Carotenoids can quench ¹O₂ directly, a role which is shared with tocopherols or prevent ¹O₂ formation by quenching the excited triplet state chlorophyll (Asada, 2006). Peroxisomes and glyoxysomes are other major sites of H₂O₂ generation during photorespiration and fatty acid oxidation, respectively (del Rio *et al.*, 2006). Photorespiration is a complex process tightly linked to photosynthesis. Under conditions that impair CO₂ fixation in chloroplasts, the ribulose-1,5-bisphosphate carboxylase oxygenase activity increases and the produced glycolate moves to peroxisomes, where it is oxidized by glycolate oxidase forming H₂O₂. Fatty acid oxidation in glyoxysomes of germinating seeds generates H₂O₂ as a by-product of the acyl-CoA-oxidase enzyme activity. Mitochondrial respiration is another process leading to H₂O₂ and O₂^{•−} formation (Moller, 2001). The main sources of ROS production in mitochondria are NADH dehydrogenase, ubiquinone radical, and complex III (Moller, 2001). Although mitochondrial ROS production is much lower compared to chloroplasts (no ¹O₂ production, lack of light energy-absorbing chlorophyll pigments), mitochondrial ROS are important regulators of a number of cellular processes, including stress adaptation and PCD (Robson and Vanlerberghe, 2002). The estimated H₂O₂ production in mitochondria may be 20 times slower than in chloroplasts, at least in C₃ plants (Foyer and Noctor, 2003). Plasma membrane-bound NAD(P)H oxidases as well as cell wall-associated extracellular peroxidases are the main H₂O₂- and O₂^{•−}-producing enzymes in the apoplast (Bindschedler *et al.*, 2006; Choi *et al.*, 2007; Sagi and Fluhr,

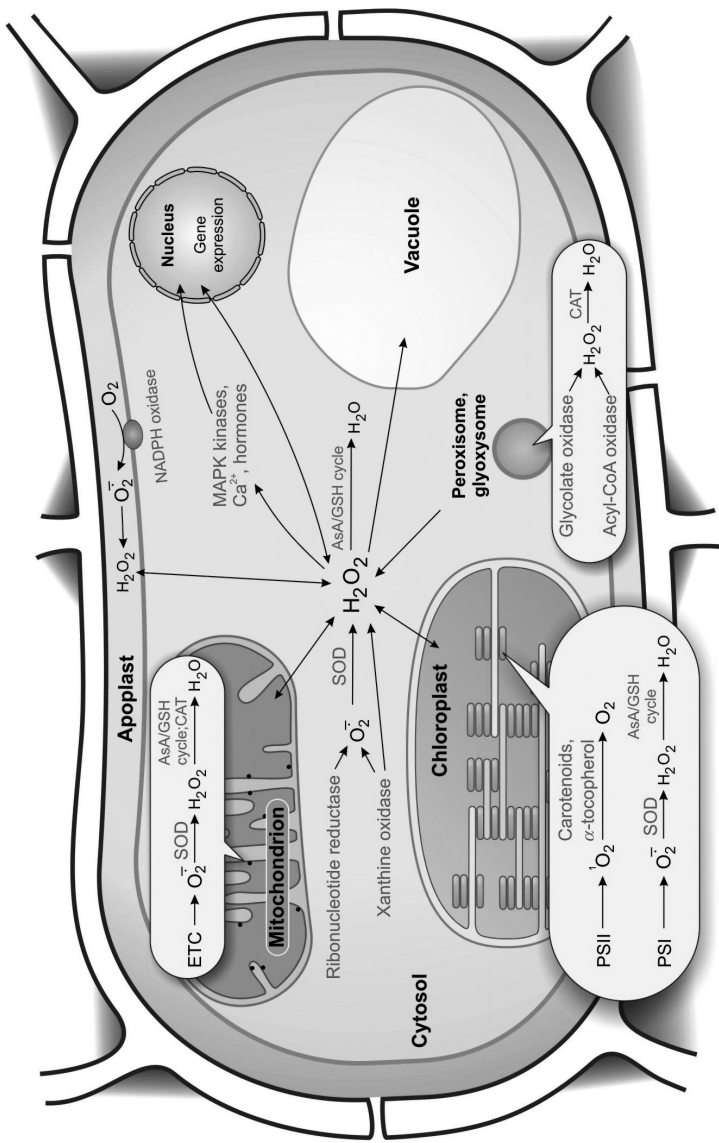


Figure 3.2 Production and metabolic fate of various ROS (hydrogen peroxide, superoxide anion radical, singlet oxygen) in different cellular compartments. Major enzymes and nonenzymatic components involved in ROS homeostasis are indicated. Plants have many different sources of ROS and have evolved elaborate mechanisms to scavenge and utilize the various forms of ROS. Abbreviations are as follows: ROS, reactive oxygen species; PSI, photosystem I; PSII, photosystem II; ETC, electron transport chain; SOD, superoxide dismutase; CAT, catalase; AsA/GSH, ascorbic acid/glutathione.

2006). These enzymes are regulated by various developmental and environmental stimuli. Most notably, ROS produced by them participate in the so-called oxidative burst observed as a part of the pathogen-induced HR but also regulate cell growth, development, and cell death (Foreman *et al.*, 2003; Gapper and Dolan, 2006; Laloi *et al.*, 2006; Sagi and Fluhr, 2006; Torres *et al.*, 2002). $O_2^{\bullet-}$ and H_2O_2 are produced also by xanthine oxidase during purine catabolism, ribonucleotide reductase during deoxyribonucleotide synthesis, and various other oxidases induced by biotic and abiotic stresses. The nucleus has also recently been reported as a site of H_2O_2 production (Ashtamker *et al.*, 2007). Studies on cryptogeiin-induced ROS production during cell death of *Nicotiana tabacum* BY-2 suspension cells with Amplex Red/Amplex Ultra Red reagents, which report real time H_2O_2 accumulation, revealed that internal signals develop more rapidly than the external apoplastic signals. Using 2',7'-dichlorofluorescein diacetate as a fluorescent probe, subcellular accumulation of H_2O_2 was first detected in the nucleus and then, after a short delay, in the endomembrane system and cytoplasm (Ashtamker *et al.*, 2007). The authors report that even isolated nuclei are capable of producing H_2O_2 in a calcium-dependent manner.

Balancing H_2O_2 and other ROS levels is essential to ensure an accurate execution of their signaling functions and to prevent their toxicity. Therefore, plants have evolved an elaborate antioxidant system, consisting of enzymes and nonenzymatic antioxidants, which together with the ROS-producing enzymes maintain ROS homeostasis in all cellular compartments and adjust ROS levels according to the cellular need at a particular time (Table 3.2) (Mittler *et al.*, 2004). SODs are the only plant enzymes capable of scavenging $O_2^{\bullet-}$, while H_2O_2 can be catabolized directly by catalases or with the aid of various reductants by APXs, peroxiredoxins, glutathione peroxidases (GPX), and the heterogeneous group of guaiacol peroxidases (Dat *et al.*, 2000). Nonenzymatic antioxidants also contribute to ROS homeostasis, with ascorbate, glutathione, tocopherol, and carotenoids as the most abundant water- and lipid-soluble antioxidants (Table 3.3) (Dellapenna and Pogson, 2006). As catalase degrades H_2O_2 without any reducing power, this enzyme provides plants with an energy-efficient way to remove H_2O_2 . However, catalase is active only at relatively high H_2O_2 concentrations. Lower H_2O_2 levels are eliminated by APX and other peroxidases with the aid of various reductants like ascorbate and glutathione. Whereas some of the ROS network enzymes such as SOD, catalase, and APX are entirely dedicated to ROS homeostasis, others like guaiacol peroxidases, thioredoxins, ferritins, and glutathione-S-transferases are involved also in various other processes related to control of development, redox regulation of target proteins, and detoxification reactions. Some of the ROS-associated enzymes, like guaiacol peroxidases, thioredoxins, glutaredoxins, and glutathione-S-transferases, have evolved into very large

Table 3.2 Most important plant enzymes and antioxidants involved in reactive oxygen species (ROS) and reactive nitrogen species (RNS) homeostasis

Enzyme/antioxidant	Function	Localization	References
NADPH oxidases	Generate ROS ($O_2^{\bullet -}$ and eventually H_2O_2). Localized production of ROS by NADPH oxidases is involved in plant growth and pathogen-induced cell death	pm	Gechev <i>et al.</i> , 2006
Guaiacol peroxidases (POX)	Detoxify H_2O_2 with various substrates as reductants; can also produce ROS ($O_2^{\bullet -}$, $HO^{\bullet}$, $HOO^{\bullet -}$). Involved in lignin biosynthesis, hormone metabolism, cross-linking of cell wall polymers, pathogen defense, plant development, senescence, and symbiotic interactions	cw, vac, cyt, mit	Bindschedler <i>et al.</i> , 2006; Passardi <i>et al.</i> , 2004; Prasad <i>et al.</i> , 1995
Glycolate oxidase	Generates significant amounts of H_2O_2 during conversion of photorespiratory glycolate to glyoxylate	per	Dat <i>et al.</i> , 2000
Acyl-CoA-oxidase	Produces H_2O_2 during fatty acids oxidation in glyoxysomes	gly	Dat <i>et al.</i> , 2000
Ribonucleotide reductase	Involved in DNA synthesis. Generates $O_2^{\bullet -}$ as by-product in cytosol	cyt	Fontecave <i>et al.</i> , 1987
Xanthine oxidase	Generates H_2O_2 as by-product from purine catabolism	cyt	Dat <i>et al.</i> , 2000
Nitric oxide synthase/ nitric oxide associated	Involved in production of $NO^{\bullet}$	cyt, myt, chl	Neill <i>et al.</i> , 2008
Nitrate reductase	Converts nitrate to nitrite. Produces $NO^{\bullet}$	cyt	Neill <i>et al.</i> , 2008

(continued)

Table 3.2 (continued)

Enzyme/antioxidant	Function	Localization	References
Superoxide dismutase (SOD)	Dismutation of $O_2^{\bullet -}$; leads to H_2O_2 formation	chl, cyt, mit, per	Mittler <i>et al.</i> , 2004
Catalase	Detoxifies H_2O_2 ; no reductant required	per, gly, mit	Dat <i>et al.</i> , 2000
Ascorbate peroxidase (APX)	Detoxifies H_2O_2 with ascorbate as reductant	chl, cyt, per, mit	Asada, 2006
Monodehydroascorbate reductase (MDHAR)	Reduces monodehydroascorbate radicals with NAD(P)H as reductant	chl, mit, cyt	Mittler <i>et al.</i> , 2004
Dehydroascorbate reductase (DHAR)	Reduces dehydroascorbate radicals with GSH as reductant	chl, mit, cyt	Mittler <i>et al.</i> , 2004
Glutathione reductase (GR)	Reduces oxidized glutathione with NADPH as reductant	chl, mit, per, cyt	Mittler <i>et al.</i> , 2004
Glutathione peroxidases (GPX)	Detoxify H_2O_2 and lipid hydroperoxides with GSH as reductant	chl, cyt, mit, er	Mittler <i>et al.</i> , 2004
Glutathione-S-transferases (GST)	Detoxification reactions (glutathionation); can detoxify lipid hydroperoxides and exhibit DHAR activity; and can act as noncatalytic carriers that facilitate the distribution and transport of various biomolecules	cyt, chl, mit, nuc, apo	Moons, 2005
Peroxiredoxins (Prx)	Thiol-containing peroxidases, detoxify H_2O_2	chl, mit, nuc	Mittler <i>et al.</i> , 2004
Thioredoxins (Trx)	Redox-control of enzymes and transcription factors, electron donor to Prx and GPX	chl, cyt, mit, nuc	Mittler <i>et al.</i> , 2004
Glutaredoxins (Grx)	Deglutathionylation, redox-control of enzymes and transcription factors, electron donor to DHA and Prx. Protection against oxidative damage, reacts with $NO^{\bullet}$, and regulation of plant development	chl, cyt, mit, plasmalemma, er	Roubier <i>et al.</i> , 2004

Ferritin	Binds iron, thus sequestering it in a bioavailable, nontoxic form and preventing formation of HO•. Iron homeostasis	chl, mit	Hintze and Theil, 2006
Alternative oxidases (AOX)	Channel electrons from electron transfer chains of mitochondria and chloroplasts directly to oxygen, thus minimizing O ₂ •– production under conditions that favor electron transport chain overenergization. The chloroplastic AOX homologue immutants also participates in carotenoid biosynthesis	chl, mit	Mittler <i>et al.</i> , 2004
Ascorbate	Substrate for APX. Detoxifies H ₂ O ₂	chl, cyt, mit, per, vac, apo	Asada, 2006
Glutathione	Substrate for various peroxidases, glutathione transferases, and glutathione reductases. Detoxifies H ₂ O ₂ , other hydroperoxides, and toxic compounds	chl, cyt, mit, per, vac, apo	Dat <i>et al.</i> , 2000
α-Tocopherol	Protects membrane lipids from peroxidation, detoxifies lipid peroxides, and quenches ¹ O ₂	Membranes	Dellapenna and Pogson, 2006
Carotenoids	Quench ¹ O ₂ . Photosystem assembly, key components of the light harvesting complex, precursors of ABA	chl, chromo-, elaio-, and amyloplasts	Dellapenna and Pogson, 2006
Flavonoids	Can directly scavenge H ₂ O ₂ and HO•	Vac	Edreva, 2005; Gould <i>et al.</i> , 2002
Hemoglobins	Involved in NO• scavenging	Cyt	Neill <i>et al.</i> , 2008

The abbreviations are chl, chloroplasts; cyt, cytosol; myt, mitochondria; nuc, nucleus; er, endoplasmatic reticulum; vac, vacuole; per, peroxisomes; gly, glyoxysomes; cw, cell wall; apo, apoplast.

Table 3.3 Plant-specific proteins involved in H₂O₂ network and PCD

Gene/protein	Function	References
BAK1	LRR protein kinase, coreceptor with FLS2 for brassinosteroid signaling and cell death; BAK1 has also brassinoid-independent role in PCD. <i>Bak1</i> mutants challenged with flagellin do not mount H ₂ O ₂ burst but when challenged with necrotrophic pathogens have enhanced production of ROS and PCD	Chinchilla <i>et al.</i> , 2007; Kemmerling <i>et al.</i> , 2007
AtPep1	Upregulated by H ₂ O ₂ . AtPep1 can induce H ₂ O ₂ synthesis and defense gene expression as well as its own precursor gene	Huffaker and Ryan, 2007; Huffaker <i>et al.</i> , 2006
LCB1	Subunit of serine palmitoyltransferase, mutants fail to generate ROS, and execute PCD after FB1 treatment	Shi <i>et al.</i> , 2007
LOH2	<i>Loh2</i> mutants exhibit perturbations in sphingolipid biosynthesis, accumulation of H ₂ O ₂ and PCD upon AAL-toxin treatment	Gechev <i>et al.</i> , 2004
ACD2	Protects from PCD that requires early oxidative burst in mitochondria	Yao and Greenberg, 2006
ANP1	H ₂ O ₂ -inducible MAPKKK, activates Arabidopsis kinases MPK3 and MPK6 and eventually transcription of GST6 and HSP18.2. Overexpression of ANP1 induces multiple stress tolerance	Kovtun <i>et al.</i> , 2000
OXI1	H ₂ O ₂ inducible, needed for full activation of MPK3 and MPK6 and normal root hair growth	Rentel <i>et al.</i> , 2004
OMTK1	MAPKKK in alfalfa specific to H ₂ O ₂ , activates downstream MAP kinase MMK3 to channel the H ₂ O ₂ cell death signal	Nakagami <i>et al.</i> , 2004
AtNDK1	Interacts with Arabidopsis catalases; overexpression leads to increased resistance to paraquat and ability to eliminate H ₂ O ₂	Fukamatsu <i>et al.</i> , 2003
NDPK2	Reduces accumulation of H ₂ O ₂ , enhances abiotic and oxidative stress tolerance	Moon <i>et al.</i> , 2003
GSTs	Marker for H ₂ O ₂ accumulation; various roles in protection against abiotic and oxidative stress tolerance	Gechev <i>et al.</i> , 2006; Kovtun <i>et al.</i> , 2000

HSPs	Marker for H ₂ O ₂ ; protective role against stress; possible role as H ₂ O ₂ sensor	Kovtun <i>et al.</i> , 2000; Miller and Mittler, 2006
Thioredoxin	Participates in H ₂ O ₂ homeostasis. Required for victorin-induced PCD	Sweat and Wolpert, 2007
h5ATTRX5		
LSD1	Negative regulator of ROS-induced cell death. Mutations lead to increase in H ₂ O ₂ and ethylene production and enhanced hypoxia-induced PCD during lysigenous aerenchyma formation	Dietrich <i>et al.</i> , 1997; Muhlenbock <i>et al.</i> , 2007
LOL1	Positive regulator of ROS-induced cell death; antagonizes LSD1	Epple <i>et al.</i> , 2003
WRKY53	WRKY53 can be induced by H ₂ O ₂ and can regulate its own expression in a negative feed back loop. Involved in senescence-induced cell death	Miao <i>et al.</i> , 2004
Zat12	Induced by H ₂ O ₂ and other ROS. ZAT12 overexpressors have elevated levels of oxidative stress-related transcripts, while ZAT12-deficient plants are more sensitive to H ₂ O ₂ -induced cell death	Davletova <i>et al.</i> , 2005b
AtMC8	Protease mediating UV and H ₂ O ₂ -induced cell death. Overexpression of MC8 enhances, while knockout reduces, H ₂ O ₂ -dependent cell death in protoplasts	He <i>et al.</i> , 2008
Proteasome subunits	Many proteasome components upregulated during H ₂ O ₂ -induced PCD. Involved in several types of cell death	Kim <i>et al.</i> , 2003; Vacca <i>et al.</i> , 2007; Vandenabeele <i>et al.</i> , 2004
ZEN1	Nuclease involved in nuclear DNA fragmentation during PCD in tracheary elements differentiation	Ito and Fukuda, 2002

multigene families with diverse functions to facilitate the adaptation of the photosynthesizing organisms to terrestrial life in elevated oxygen concentrations and different stressful environments (Meyer *et al.*, 2005; Moons, 2005; Passardi *et al.*, 2004; Prasad *et al.*, 1995; Rouhier *et al.*, 2004). These and other antioxidant enzymes together with the ROS-producing enzymes compose a highly sophisticated and redundant network, which in *A. thaliana* consists of at least 289 genes (Table 3.2) (Gechev *et al.*, 2006).

All cellular compartments that produce ROS, including chloroplasts, mitochondria, peroxisomes, and the cytosol, are well-equipped with anti-oxidant enzymes and antioxidants (Fig. 3.2, Table 3.2). ROS are normally scavenged at the sites of their production by the local antioxidant systems. However, when the local antioxidant capacity cannot cope with the ROS generation levels (e.g., during high ROS production under stress or reduced antioxidant enzyme activities upon developmental signals), H_2O_2 can leak from those compartments into the cytosol and other compartments. As a next line of defense, plants may have evolved mechanisms to deal with excess H_2O_2 by transporting and detoxifying it in vacuoles (Gould *et al.*, 2002). Vacuoles are very rich in flavonoids, powerful antioxidants that can scavenge hydrogen peroxide, singlet oxygen, superoxide, hydroxyl, peroxy radicals, and peroxynitrite (Edreva, 2005; Tsuda *et al.*, 2000). They also contain high levels of ascorbate, glutathione, and peroxidases localized at the tonoplast inner surface (Gould *et al.*, 2002).

3.2. Biological functions modulated by ROS: Plant growth, development, stress responses, and PCD

Hydrogen peroxide modulates a number of biological functions including seed dormancy, pollen tube and root hair growth and elongation, tuber development in potato, numerous stress responses, and PCD (Gechev *et al.*, 2006; Kim *et al.*, 2007; Oracz *et al.*, 2007).

Inability of mature seeds to germinate under favorable conditions, referred to as seed dormancy, is an important adaptive phenomenon enabling seeds to delay germination until conditions are more appropriate for seedling growth (Oracz *et al.*, 2007). Breaking dormancy in most species occurs during storage in dry conditions or during imbibition at low temperature (stratification). Releasing seed dormancy in sunflower is associated with marked production of H_2O_2 and $O_2^{\bullet-}$ in cells of embryonic axes (Oracz *et al.*, 2007). Moreover, treating seeds with ROS-generating herbicide paraquat was able to release the dormancy, and specific embryo proteins became oxidized.

ROS generated by NADPH oxidases are involved in pollen tube growth. Transgenic tobacco plants with antisense constructs to silence pollen-specific NADPH oxidase by decreasing specific mRNA levels displayed inhibited pollen tube growth (Potocky *et al.*, 2007). Normal growth

of the pollen tube was restored by exogenous application of H_2O_2 . Genetic evidence for the role of ROS in plant growth is provided by the double mutant of two *Arabidopsis* NADPH oxidase homologues, *atrbohD* and *atrbohF*, which not only has decreased ROS accumulation after pathogen challenge but also has reduced ABA-mediated seed germination and root elongation inhibition (Kwak *et al.*, 2003; Torres *et al.*, 2002). Furthermore, studies on the *atrbohC* mutant revealed that it has low ROS levels and defective root hair growth (Foreman *et al.*, 2003). Consistent with these findings, knockout mutation of the *A. thaliana* serine/threonine kinase *oxi1* results in reduced root hair growth (Rentel *et al.*, 2004). H_2O_2 production may have, on the other hand, an inhibitory effect on growth, as suggested by the inhibition of auxin responses by ANP1, the MAPK kinase kinase that relays the H_2O_2 signal in *A. thaliana* (Kovtun *et al.*, 2000). The balance between $\text{O}_2^{\bullet -}$ and H_2O_2 is also believed to regulate plant growth and tuber development in potato (Kim *et al.*, 2007).

Hydrogen peroxide is an important modulator of plant stress responses. Significant increases in endogenous H_2O_2 levels are observed during a number of adverse environmental conditions as a result of increased ROS production and/or impaired ROS detoxification (Dat *et al.*, 2000; Mittler *et al.*, 2004). Extreme temperatures, drought, UV and high light intensities, and many pollutants can cause oxidative stress and cell death (Dat *et al.*, 2000; Gechev *et al.*, 2004). In addition, H_2O_2 is a secondary messenger during wounding responses and various biotic interactions (Bais *et al.*, 2003; Orozco-Cardenas *et al.*, 2001). Redox changes are sensed by the plant cell as a “warning” message, and depending on the situation, genetic programs leading to stress acclimation or PCD are switched on (Gechev *et al.*, 2002). Small transient increases in H_2O_2 serve as signals triggering stress acclimation against subsequent more severe abiotic or oxidative stress. Protective roles for H_2O_2 have been demonstrated against chilling, salt, high light, heat, and oxidative stress (Karpinski *et al.*, 1999; Lopez-Delgado *et al.*, 1998). H_2O_2 -induced acclimation can be very durable; for example, the tolerance to high temperature lasts more than a month after the initial H_2O_2 treatment (Lopez-Delgado *et al.*, 1998). H_2O_2 can initiate acclimation not only in local leaves but also in distant nonacclimated leaves, a process referred to as systemic acquired acclimation (Karpinski *et al.*, 1999). H_2O_2 is also involved in initiating cell death-protective responses in the neighboring cells that surround the sites of the HR to pathogens and triggers systemic acquired resistance in distant tissues (Alvarez *et al.*, 1998; Torres *et al.*, 2005). Consistent with its signaling role, H_2O_2 can alter resistance against a number of pathogens (Chamnongpol *et al.*, 1998). H_2O_2 -induced stress tolerance can be explained by activation of defense mechanisms such as antioxidant enzymes, MAPKs, stress-specific transcription factors, dehydrins, low-temperature-induced, heat-shock, and pathogenesis-related proteins (Gechev *et al.*, 2002; Karpinski *et al.*, 1999; Moon *et al.*, 2003; Vranova

et al., 2002). Genes involved in both induction and maintenance of stress acclimation are part of these defense mechanisms (Charnng *et al.*, 2006).

H₂O₂-induced cell death is implicated in a number of developmental processes and stress responses. These include the already mentioned aleurone cell death, lysigenous aerenchyma formation, tracheary elements maturation, trichome development, formation of lace leaf shape, organ senescence, plant–plant allelopathic interactions, the HR to pathogens, and some types of necrotrophic cell death (Fig. 3.1). Thus, paradoxically, H₂O₂-induced cell death is essential for plant growth, development, and proper responses to the environment (Gechev *et al.*, 2006). At the same time, cell death can be an unwanted event during many unfavorable environmental conditions, including heat, cold, salt and xenobiotic stresses, and compatible or disease-causing plant–pathogen interactions (Koukalova *et al.*, 1997; Swidzinski *et al.*, 2002).

PCD can be initiated also by other types of ROS, including singlet oxygen and superoxide radicals (Dat *et al.*, 2003; Op den Camp *et al.*, 2003; Vranova *et al.*, 2002). In addition, HO•-initiated lipid peroxidation is a rich source of oxidized lipids that can trigger PCD on their own or in concert with other ROS (Montillet *et al.*, 2005; Mueller, 2004). Singlet oxygen-induced enzymatic lipid peroxidation, however, is likely a part of stress response pathway rather than a cell death or growth inhibition pathway as double *flu/aos* mutants, producing singlet oxygen but with compromised jasmonate biosynthesis, have the same growth inhibition and cell death as the single *flu* mutant (Przybyla *et al.*, 2008).

3.3. Specificity of ROS signals: How different responses like stress acclimation or PCD are achieved?

One of the intriguing questions is how H₂O₂ and other ROS, being such simple molecules, can regulate so many different processes in different cell types and organs at different developmental stages. It is now accepted that the biological outcome of ROS signaling depends on multiple factors including ROS chemical identity and sites of production, duration and intensity of the signal (Gechev *et al.*, 2006; Queval *et al.*, 2007), developmental stage of the plant, previous stress encounters, and interaction with other signaling molecules such as NO, lipid messengers, and plant hormones (Kwak *et al.*, 2006; Zaninotto *et al.*, 2006). H₂O₂ is the most prominent ROS signal. In recent years, a number of microarray- or AFLP-based studies identified many genes that respond to elevated H₂O₂ levels (Desikan *et al.*, 2001; Gechev *et al.*, 2005; Levine *et al.*, 1994; Vandenabeele *et al.*, 2003, 2004; Vanderauwera *et al.*, 2005). In addition, mutants presumably involved in H₂O₂ signaling were identified (Table 3.3). Signaling properties and distinct transcriptional responses were also confirmed for the other ROS (Demidchik *et al.*, 2003; Op den Camp *et al.*,

2003; Vranova *et al.*, 2002). Comparing the transcriptional responses to different ROS, specific transcription footprints for $O_2^{\bullet-}$, 1O_2 , and H_2O_2 have been identified (Gadjev *et al.*, 2006). This specificity can be determined by promoter modules specific for the various types of ROS (Shao *et al.*, 2007).

The fate of the ROS signaling is to a large extent related to the chemical properties of different ROS and their doses (Gechev *et al.*, 2002; Op den Camp *et al.*, 2003; Vranova *et al.*, 2002). In general, low doses of $O_2^{\bullet-}$ and H_2O_2 protect against oxidative and abiotic stress, while high doses trigger cell death (Gechev *et al.*, 2002; Vranova *et al.*, 2002). This cell death is genetically programmed, although extremely high doses of ROS can cause necrosis (Laloi *et al.*, 2006; Montillet *et al.*, 2005; Van Breusegem and Dat, 2006).

The site of ROS production is of particular importance to the biological outcome of the initial signal (Carol *et al.*, 2005; Mullineaux *et al.*, 2006). Localized production of $O_2^{\bullet-}$ by NADPH oxidase in the root hair tip triggers Ca^{2+} peaks at the hair tip necessary for the root hair growth (Foreman *et al.*, 2003). This spatial regulation of NADPH oxidase activity is regulated by the Rho-like GTPases (Carol *et al.*, 2005). These GTPases also control tracheary element differentiation through localized ROS production (Nakanomyo *et al.*, 2002).

Although H_2O_2 is relatively mobile, there may be “hot spots” of hydrogen peroxide within the cell. Similar hot spots are even more likely to occur for the other, less mobile, types of ROS: 1O_2 , $O_2^{\bullet-}$, and especially $HO^{\bullet}$. In contrast with others, H_2O_2 can migrate quite a distance from the site of its production and even cross biological membranes through specialized aquaporins (Bienert *et al.*, 2006, 2007; Henzler and Steudle, 2000). This transport is another way of adjusting the local concentration of H_2O_2 , modulating the biological effect. Example of cross-compartment communication associated with H_2O_2 mobility is the increased levels of H_2O_2 produced in cytosol in the absence of the cytosolic APX, which leads to inhibition of chloroplastic APX and collapse of the chloroplastic antioxidant system (Davletova *et al.*, 2005a). It has also been shown that peroxisomal catalase can act as a sink for H_2O_2 produced in peroxisomes or elsewhere (Willekens *et al.*, 1997).

3.4. Hydrogen peroxide signaling network: Perception, transduction, and transcription factors

In recent years, a variety of forward and reverse genetics studies revealed a number of components in the H_2O_2 signaling network, including protein kinases, protein phosphatases, and ROS-responsive transcription factors (Table 3.3). Among these genes, many are involved in the generation of H_2O_2 and are required for PCD and other biological responses (e.g., NADPH oxidases, extracellular peroxidases), whereas others are involved

in the modulation of H₂O₂ and other ROS levels (e.g., catalase, APXs, and other antioxidant enzymes, Table 3.2).

One of the earliest events that follow elevation in H₂O₂ levels is alteration in sodium, potassium, and calcium ion fluxes discussed earlier. The transient Ca²⁺ oscillations are stress-specific and can lead to various downstream effects through the numerous Ca²⁺-interacting proteins, including calmodulins and calcium-dependent protein kinases or/and over-amplification of the H₂O₂ signal. In addition to calcium-dependent protein kinases, a vast network of MAPKs is involved in relaying the H₂O₂ signal. With its 20 MAPKs, 10 MAPK kinases and 60 MAPK kinase kinases in *Arabidopsis*, the versatile MAPK network offers many convergence and divergence points for different stress signals (Ichimura *et al.*, 2002). MAPK kinase cascades mediate PCD triggered by pathogens or chloroplast-derived H₂O₂ (Asai *et al.*, 2002; Liu *et al.*, 2007). The MAPK kinase kinase MEKK1 is regulated by different stresses and H₂O₂ in a proteasome-dependent manner (Nakagami *et al.*, 2006). It activates the downstream MAPK MPK4. Compromising MEKK1 results in impaired H₂O₂-induction of MPK4. Surprisingly, MEKK1 can interact directly with WRKY53, transcription factor involved in senescence-induced PCD, thus bypassing downstream kinases (Miao *et al.*, 2007). The H₂O₂-inducible MAPK kinase ANP1 activates two downstream MAPKs, AtMPK3 and AtMPK6, to eventually regulate gene expression of specific H₂O₂-inducible transcripts (Kovtun *et al.*, 2000). Two of the upregulated genes, GST6 and HSP18.2, as well as the whole heat-shock regulon are both reliable markers for H₂O₂ production and protectors against abiotic and oxidative stress (Gechev and Hille, 2005; Vanderauwera *et al.*, 2005). In addition, heat-shock proteins have been implicated as possible H₂O₂ sensors (Miller and Mittler, 2006). Overexpression of ANP1 in transgenic plants resulted in increased tolerance to heat shock, freezing, and salt stress (Kovtun *et al.*, 2000). The serine/threonine kinase OXI1 (*oxidative signal-inducible 1*) is another essential component of the H₂O₂ signaling network in *A. thaliana* (Rentel *et al.*, 2004). It is inducible by abiotic stress and H₂O₂. OXI1 activity is required for full activation of AtMPK3 and AtMPK6. *Oxi1* mutants have abnormal root hair growth and enhanced susceptibility to pathogen infection, demonstrating once more the importance of ROS in plant development and stress responses (Rentel *et al.*, 2004). Another H₂O₂-inducible kinase is OMTK1 in alfalfa (Nakagami *et al.*, 2004). In contrast to OXI1, OMTK1 is specific to H₂O₂. OMTK1 activates MMK3, downstream MAPK that can also be activated by ethylene and elicitors (Nakagami *et al.*, 2004).

Nucleotide diphosphate kinases are other components of the H₂O₂ signaling network. *Arabidopsis* NUCLEOTIDE DIPHOSPHATE KINASE2 (NDK2) is also inducible by H₂O₂ and its overexpression reduces the accumulation of H₂O₂ and enhances tolerance to cold, salt,

and oxidative stresses (Moon *et al.*, 2003). *Arabidopsis* NDK1 interacts with the three *A. thaliana* catalases; its overexpression results in enhanced ability to detoxify H_2O_2 and resistance to paraquat (Fukamatsu *et al.*, 2003).

The H_2O_2 gene network eventually transmits the signal to ROS-specific transcription factors. Some of these are the previously described zinc finger proteins LSD1 and LOL1, negative and positive regulators of ROS-induced cell death, the senescence-specific WRKY53, and the ROS-inducible WRKY75 and heat-shock transcription factors (Table 3.3) (Dietrich *et al.*, 1997; Epple *et al.*, 2003; Gechev *et al.*, 2005; Miao *et al.*, 2004; Vanderauwera *et al.*, 2005). Two other zinc finger transcription factors, Zat11 and Zat12, are induced by H_2O_2 and other ROS (Gadjev *et al.*, 2006). Overexpressing Zat12 results in elevated transcript levels of oxidative- and light stress-responsive transcripts, while compromising Zat12 results in increased sensitivity to H_2O_2 -induced oxidative stress (Rizhsky *et al.*, 2004; Davletova *et al.*, 2005b).

Transcriptional reprogramming resulting from the activation of cell death-specific factors eventually leads to activation of execution components of the H_2O_2 cell death network (Table 3.3). Among these are various proteases (AtMC8, proteasome pathway) and nucleases (ZEN1) that take part in the orderly dismantling of the plant cell.

3.5. ROS interaction with other signaling molecules modulates plant PCD

Interaction with other signaling molecules such as $\text{NO}^\bullet$, lipid messengers, or plant hormones determines the outcome or fine-tune the biological response to altered ROS levels (Kwak *et al.*, 2006; Zaninotto *et al.*, 2006). $\text{NO}^\bullet$ itself controls growth and development in a complex manner through modulation of Ca^{2+} , calcium-dependent protein kinases, cGMP, and MAPKs (Neill *et al.*, 2008). For example, $\text{NO}^\bullet$ interacts with H_2O_2 and $\text{O}_2^{\bullet-}$ to regulate cell death during HR (Delledonne *et al.*, 2001). It has been proposed that ROS are key mediators in channeling $\text{NO}^\bullet$ into the death pathway. *Arabidopsis* overexpressing the H_2O_2 -detoxifying enzyme thylakoid APX has increased resistance toward NO-induced cell death (Murgia *et al.*, 2004).

Lipid-derived messengers also interplay with ROS to modulate PCD. Sphingolipids are bioactive lipids that regulate plant growth and PCD (Liang *et al.*, 2003). Fumonisin B1 and AAL-toxin inhibit ceramide synthase, resulting in accumulation of free sphingoid bases and depletion of complex ceramides, followed by H_2O_2 accumulation and subsequent cell death (Gechev *et al.*, 2004). The resistance to the two toxins in tomato and *Arabidopsis* is controlled by genes most likely parts of the ceramide synthase (Brandwagt *et al.*, 2000; Gechev *et al.*, 2004). The accumulation of free sphingoid bases seems to be crucial for triggering PCD as inhibiting serine

palmitoyl transferase (SPT), enzyme that catalyzes the first, rate-limiting step of sphingolipid biosynthesis, results in reduced cell death symptoms (Spassieva *et al.*, 2002). A recently isolated mutant of long-chain base 1 (LCB1), component of SPT, was indeed compromised in accumulation of sphingoid bases in response to FB1, ROS burst, and cell death (Shi *et al.*, 2007). Supporting this scenario, direct feeding with sphingoid bases was able to induce ROS accumulation and cell death. Hydroxylation of sphingoid long chain bases is also related to plant growth and cell death, as *Arabidopsis* compromised simultaneously in the two long-chain-base hydroxylase genes *Sphingoid Base Hydroxylase1* and *Sphingoid Base Hydroxylase2* exhibit dwarfism and PCD (Chen *et al.*, 2008). The interplay between sphingolipids and ROS during cell death was further substantiated by isolation of mutants more tolerant to fungal toxin- and ROS-induced cell death (Gechev *et al.*, 2008; Gechev and Hille, 2005; Stone *et al.*, 2000).

Other lipids capable of modulating H₂O₂-induced cell death are oxylipins and phospholipids (Loeffler *et al.*, 2005; Meijer and Munnik, 2003). The oxylipin phytoprostane B1, for example, can trigger detoxification and defense responses, and plants primed with phytoprostane B1 are more tolerant to oxidative stress-induced cell death (Loeffler *et al.*, 2005). H₂O₂ can activate phospholipase D in *Arabidopsis* and the released phosphatidic acid can inhibit H₂O₂-induced cell death (Zhang *et al.*, 2003). Plants with compromised phospholipase D are unable to release phosphatidic acid and are more susceptible to H₂O₂-induced cell death.

H₂O₂-induced cell death and stress responses are influenced by a variety of plant hormones. H₂O₂ is in complex interaction with a number of plant hormones to regulate PCD. Ethylene and SA are positive regulators of several types of H₂O₂-induced cell death, including PCD during lysigenous aerenchyma formation and the HR (Muhlenbock *et al.*, 2007; Wang *et al.*, 2002). Abiotic, biotic, and oxidative stress can stimulate ethylene biosynthesis through activation of ACC synthase and ACC oxidase; in turn, the elevated levels of both ethylene and SA can overamplify the H₂O₂ signal (Wang *et al.*, 2002). Interactions of ethylene and H₂O₂ are not confined to regulation of cell death. These two signaling molecules, together with ABA, have recently been reported to regulate stomatal closure in *Arabidopsis* (Desikan *et al.*, 2005). Ethylene and ROS are also positive regulators of leaf senescence (Lim *et al.*, 2007). Jasmonic acid, on the other hand, is a negative regulator of leaf senescence. The jasmonate-inducible protein ESR/ESP interacts in an antagonistic fashion with the senescence-specific, H₂O₂-inducible transcription factor WRKY53 (Miao and Zentgraf, 2007). GA is stimulating H₂O₂ burst through inhibition of antioxidant enzymes to trigger H₂O₂-dependent cell death in aleurone layer of monocots (Fath *et al.*, 2001). Small polypeptide hormones, including systemin and the recently identified AtPep1, can stimulate H₂O₂ synthesis and activate defense gene expression in *Arabidopsis* (Huffaker and Ryan, 2007; Huffaker *et al.*, 2006;

Orozco-Cardenas *et al.*, 2001). AtPep1 itself is regulated by H₂O₂ and can induce its own precursor gene *propep1*, suggesting a possible amplification of the H₂O₂ signal (Huffaker *et al.*, 2006). PROPEP1 and PROPEP2 are elevated during H₂O₂ and AAL-toxin-induced cell death (Gechev *et al.*, 2004; Huffaker *et al.*, 2006).

4. CONCLUDING REMARKS

We have reviewed the latest body of literature related to ROS-mediated PCD in plants. Given the intensive interest in this area of research, as it relates to both general aspects of plant development and responses to abiotic and biotic stress, we were unable to include all of the vast information currently available and apologize for omission of relevant citations. Further characterization of the genes identified and their physiological functions in different aspects of plant development and response to environmental fluctuations will help to delineate the intricate network and elucidate the detailed mechanisms of specific checks and balances determined by levels and localization of various forms of ROS in all aspects of plant growth and development.

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