Journal of Fruit and Ornamental Plant Research Vol. 13, 2005: 135-158

REVIEW

HYPERSENSITIVE CELL DEATH IN PLANTS – ITS MECHNISMS AND ROLE IN PLANT DEFENCE AGAINST PATHOGENS

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(Received November 11, 2005/Accepted February 2, 2006)

A B S T R A C T

This review is a recent update in the understanding of the hypersensitive response (HR) of plants with special consideration to the physiological and biochemical determinants in different model systems. Hypersensitive response is reviewed as a form of programmed cell death (PCD) representing one of the mechanisms of plant defence against diseases. Major signalling pathways and molecules that accompany the HR, such as proteolytic cascades, oxidative events and ethylene that are supposed to play a key role in the plant's cell death machinery are discussed. Special attention is paid to the HR in fruit species. Studies on plant PCD are shown to provide a clue to better understanding disease resistance processes in plants and to establish the evolutionary aspects of PCD similarities through animal and plant kingdoms.

Key words: fruit plants, hypersensitive response, programmed cell death

Abbreviations: AOA, aminooxyacetic acid; APX, ascorbate peroxidase; AVG, aminoethoxyvinylglycine; CAT, catalase; CMV, cucumber mosaic virus; CPT, camptothecin; DHAR, dehydroascorbate reductase; DHMC, 2,5-dihydroxymethyl-cinnamate; DPI, diphenyl iodonium; GR, glutathione reductase; JA, jasmonic acid; LAR, local acquired resistance; MAPK, mitogen-activated protein kinase; MDHAR, monodehydroascorbate reductase; MeJA, methyljasmonate; NO, nitric oxide; PAL, phenylalanine ammonia-lyase; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulphonylfluoride; PPV, plum pox virus; ROS, reactive oxygen species; SA,

salicylic acid; SAR, systemic acquired resistance; SOD, superoxide dismutase; STS, silver thiosulphate; TCV, turnip crinkle virus; TDFs, transcript-derived fragments; TFP, trifluoperazine; TMV, tobacco mosaic virus; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling; TVTV, turnip vain clearing tobamo-virus; VPE, vacuolar processing enzyme; W-7, N-[6-aminohexyl]-5-chloro-1-naphthalene-sulfonamide

INTRODUCTION

The term hypersensitive response (HR) historically comes from Stakman (1915) who used it to describe a rapid host cell death in plants (oat, wheat and barley) infected with fungal pathogen *Puccinia graminis.* Modern studies of this phenomenon were initiated in early 1960' by Klement et al. (1964), who observed rapid, localised necrosis on tobacco leaves infiltrated with plant pathogenic bacteria *Pseudomonas syringae* pv. *syringae.* Although since that time numerous works have been done on pathogen-induced cell death in plants (for reviews see: Greenberg et al., 1994; He 1996; Dangl et al., 1996; Morel and Dangl, 1997; Gilchrist, 1998; Heath, 2000; Shirasu and Schulze-Lefert, 2000; Goldbach et al., 2003; Greenberg and Yao, 2004; Khurana et al., 2005), it is far from being fully understood. Because it is associated with disease resistance, HR remains the focus of interest for plant biologists. Many studies are devoted to this process in model plants such as *Arabidopsis thaliana,* tobacco, rice, wheat, spruce, tomato, carrot, pepper, etc. The exploration of plant cell death response to pathogens led to an assumption for a common players, cross talking in the struggle of the plant to survive. Thus, the devlopment of an effective hypersensitive response in any plant system relies, on an effective and rapid signal transduction system.

This review aims to summarize some of the recent findings on the physiological, biochemical and molecular machineries of the HR. As the HR is a process of programmed cell death (PCD) associated with plant reaction to pathogens, a description of main morphological and biochemical determinants of PCD is also discussed.

1. Programmed cell death – definition and morphological determinants

Programmed cell death is functionally conserved and genedirected well-orchestrated process of cell self-destruction (Gilchrist, 1998). In animal and plant kingdoms, it is an active process of cell suicide leading to controlled elimination of cells that are harmful, unwanted or misplaced in specific structures and organs, and plays an essential role during development and morphogenesis. PCD is involved in defence mechanisms against infected or mutated cells and in response to environmental stresses, including abiotic and biotic stimuli. Deregulation of PCD is implicated in various human diseases, including birth defects, ischemic vascular diseases, neurodegenerative diseases (e.g. Alzheimer's and Parkinson's

diseases), autoimmune diseases, AIDS, cancer, *Diabetes mellitus* type I.

There are various mechanisms by which PCD is executed and this diversity reflects the definitions. According to Martin et al. (1994), PCD is a functional term used to describe "cell death as a normal part of the life of multicellular organisms" emphasising the role of developmental cell death. Priority to the genome is given by Zhivotovsky et al. (1997), who describe the PCD as "genetically controlled cell deletion process". Biochemistry has been stressed by Laytragoon (1998), who defined PCD as a process whereby developmental or environmental stimuli activate "a specific set of events that culminate in cell death". Morphological similarities are found between animal cells undergoing apoptosis and suicidal plant cells, including condensation and shrinkage of the cytoplasm and nucleus, nuclear DNA fragmentation and formation of apoptotic-like bodies (Wang et al., 1996ab; de Jong et al., 2000). This implies an evolutionary conservation of aspects of programmed cell death between animals and plants.

Three major types of programmed cell death, based mainly on morphological changes, are recognised so far: apoptosis, autophagy and non-lysosomal cell death (reviewed in van Doorn and Woltering, 2005). Apoptosis, a term originally introduced by Kerr et al. (1972), is characterised by a specific set of cellular morphological features (Steller, 1995) such as plasma membrane blebbing, disintegration of cytoskeletal elements, condensation of the nucleus and the cytoplasm, internucleosomal cleavage of DNA and fragmentation of the cell into membrane confined, DNA containing vesicles (apoptotic bodies). A distinguishing feature of apoptosis is that fragments of the dying cell are engulfed and digested by the lysosomes of neighbouring (living) cells (e.g. phagocytes). This latter process has, so far, not been observed in any plant system, which indicates that true apoptosis does not occur in plants. Autophagy (self-eating) is a major degradation and recycling system in eukaryotic cells (Lum et al., 2005). It is featured by degradation of cellular components by formation of autophagolysosome (Yoshimori, 2004). Autophagy is associated with cell survival as well as cell death and it may be a strategy to remobilise part of the cell contents prior to cell death. Autophagy was found to play a role in plant immune response, especially in preventing of death and pathogen spread in cells adjacent to HR lesion (Greenberg, 2005). Developmental PCD (which is associated with the normal developmental processes) is involved in degeneration of specific cells and most of the examples of developmental PCD in plants conform to the definition of autophagic PCD (van Doorn and Woltering, 2005). Many morphological features that were earlier considered typical for apoptosis also occur in autophagic cell death. The third morphological type of PCD is called non-lysosomal (or necrosis-like) PCD. It does not involve a role of the lysosome of the dying cell itself nor of other cells. It is associated with swelling of organelles and formation of "empty spaces" in the

cytoplasm, and has similarities to necrosis (Baehrecke, 2005). Although not much detailed information is available on cell morphology during the plant HR, mostly this seems to conform to this non-lysosomal type of PCD (van Doorn and Woltering, 2005). Necrosis is a cell death that is not accompanied by chromatin condensation (Leist and Jäätelä, 2001) and is often classified as aborted apoptosis. Although necrosis has been defined as an accidental and uncontrolled cell death, there is growing evidence that necrotic and apoptotic forms of cell death may share similarities (Guimaraes and Linden, 2004).

2. Plant PCD

Apart from the specificity of morphological characteristics, the process of PCD is accompanied by typical biochemical events and similarities have been reported between animal and plant programmed cell death signalling.

In plants, as in animals, programmed cell death is essential machinery for growth, development and plays a role in the response to stresses (Danon et al., 2000; Jones, 2001; Lam, 2004). Cellular suicide is involved in, for example, xylogenesis (Fukuda, 2000), aerenchyma formation (Drew et al., 2000), plant reproduction (Wu and Cheung, 2000), leaf and petal senescence (Quirino et al., 2000; Rubinstein, 2000), endosperm cell death during germination (Fath et al., 2000; Young and Gallie, 2000). Furthermore, PCD plays an important role in plant response to pathogens (Heath 2000; Shirasu and Schulze-Lefert, 2000; Greenberg and Yao, 2004), and to diverse abiotic stresses such as heat shock (Tian et al., 2000; McCabe and Leaver, 2000; Zhang et al., 2004), exposure to toxic chemicals (Sun et al., 1999), ozone (Overmyer et al., 2000; Rao et al., 2000; Pasqualini et al., 2003, Overmyer et al., 2005), UV radiation (Danon and Gallois, 1998) and hypoxia (Xu et al., 2004). Biochemical changes involving production of reactive oxygen species (Jabs, 1999; Levine et al., 1994; Moeder et al., 2002), Ca release (Levine et al., 1996; Zuppini et al., 2003), proteolysis (Beers and Freeman, 1997; Xu and Chye, 1999; de Jong et al., 2000), and ethylene biosynthesis (Lund et al., 1998; Spencer et al., 2003; Woltering et al., 2003) are found to actively participate in the plant PCD signal transduction.

The classic form of apoptosis involves key cell death executioners, which are cysteinyl-aspartic-proteases named caspases (Grütter, 2000). A sequence of caspase-activated proteolytic cascade, involving initiator caspases and down-stream activation of executioner caspases, leads to apoptotic phenotype (Hengartner, 2000). Although true structural homologues of animal caspases have not yet been identified in plants, there are accumulating evidences that cysteine proteases, showing functional similarity to caspases, participate in the programmed cell death in plants. Caspases can be selectively inhibited by small peptides mimicking the substrate recognition site and such

peptides can also be used to determine caspase activity. Using such an approach, caspase-like activity has been detected following the infection of tobacco with bacteria and a virus (Del Pozo and Lam, 1998; 2003). By administration of specific inhibitors, a role of caspase-like proteases in apoptotic-like cell death of tomato suspension cells in response to treatment with camptothecin (CPT, an alkaloid from *Camptoteca acuminata*) and the fungal toxin Fumonisin B1 (de Jong et al., 2000; Woltering et al., 2002) is established.

Two groups of proteases are suggested to function in caspase-like manner in plants: vacuolar processing enzymes (VPEs) and metacaspases (Woltering, 2004; Sanmartin et al., 2005). In addition, two subtilin-like Ser proteases (SAS-1 and SAS-2), named saspases, were purified and characterized to exhibit caspase-like hydrolytic activity (Warren and Wolpert 2004). VPEs play a role in cell death and are important factors affecting plant susceptibility to pathogens. A study with *Arabidopsis thaliana* showed that VPE-gamma has caspase-like activity and that mutants with reduced activity showed lesser cell death rate and increased susceptibility to pathogens (*Pseudomonas syringae*, *Botrytis cinerea*, turnip mosaic virus) (Rojo et al., 2004). The involvement of VPE is also reported in TMV-induced hypersensitive cell death in tobacco (Hatsugai et al., 2004) and mycotoxin-induced cell death (Kuroyanagi et al., 2005).

3. HR definition, morphological features and elicitation

Plants have elaborated sophisticated and efficient system to counteract the spread of pathogen invasion. The hypersensitive response is a form of cell death associated with plant resistance to pathogen infection and occurs at incompatible and sometimes in compatible plant-pathogen interactions (Morel and Dangl, 1997). HR is characterized by a rapid, localized death of tissues at the site of infection, limiting further pathogen multiplication and spread (Gilchrist, 1998; Heath, 2000; Shirasu and Schulze-Lefert, 2000). During this response, the plant sacrifices some of its cells to circle the invading pathogen with a layer or a ring of dead plant cells thus inhibiting the growth of the pathogen by killing of infected and non-infected cells and by producing a physical barrier.

During the HR, dying plant cells strengthen their cell walls by depositing different phenolic compounds, synthesizing diverse toxic compounds called phytoalexins, and accumulating proteins with antimicrobial activity called pathogenesis-related proteins (Dangl et al., 1996). The understanding of the pathogen-plant interactions that result in the HR may lead to the discovery of effective methods for disease control (Dangl et al., 1996).

Hypersensitive cell death is genetically controlled and occurs when the plant genotype recognizes a specific isolate of a pathogen. The reaction involves activation of host defence-related genes (Suh et al., 2003) and

various defence responses (Greenberg et al., 1994; Lamb and Dixon, 1997).

The morphological and ultra-structural changes that accompany PCD during plant-microorganism interactions have been studied in only a few model pathogens and plants. Cessation of cytoplasmic streaming followed by protoplast dismantling and by cleavage of nuclear DNA has been observed as an early event during HR in the epidermis of cowpea (*Vigna unguiculata*) that had been infected by the rust fungus *Uromyces vigna* and in cell death induced by the barley powdery mildew fungus. PCD in plant-pathogen interactions does show some features of apoptosis but no clear evidence for the formation of apoptotic-like bodies has been found and no phagocytosis has been observed in the primary lesion. It seems that HR cell death mostly belongs to the non-lysosomal category of cell death (Woltering and van Doorn, 2005). In the light of the recent understanding of the role of autophagy in plant immune responses, Lui et al. (2005) have observed formation of autophagosomes in cells adjacent to the primary infection and in distant systemic tissues. The blocking of autophagy resulted in increased local virus propagation, which demonstrates that autophagy plays a prominent role in plant immune response.

Evidences suggesting that HR results from PCD process are: the activation of cell death in the absence of pathogens by mutations in certain genes thought to be involved in the cell death pathway, the activation of cell death upon recognition of elicitors produced by the pathogen, and the activation of the HR by expression of transgenes in plants (Heath, 2000). The fact that cell death resembling the HR can be activated in the absence of a pathogen strongly suggests that this type of cell death is not directly caused by the invading pathogen but rather results from the activation of a host-encoded pathway for PCD. Perhaps the most persuasive evidence that the HR is a PCD process is the existence of mutants that spontaneously activate the HR in the absence of a pathogen (Dangl et al., 1996). These mutants are often referred to as "disease lesion mimics" and the mutations that cause the appearance of HR lesions in the absence of a pathogen are thought to occur in plant genes that control PCD, thus presenting a powerful tool for the study of HR in plants. *Disease lesion mimic* mutants have been isolated from tomato, maize, barley, rice, and Arabidopsis.

PCD can be induced in plants by toxins produced by a number of pathogens – harpins from *Pseudomonas syringae, Erwinia amylovora*, *Xanthomonas campestris*, the fungal toxin victorin, xylanase from *Trichoderma viridae* (He, 1996; Grant and Mansfield, 1999; Lam et. al., 1999), *Alternaria alternata* AAL toxin, the fungal toxin Fumonisin B1 (FUM) from *Fusarium moniliforme* (Wang et al., 1996a), fungal toxin cryptogein from *Phytophthora cryptogea* (Hirasawa et al., 2005). Also, plant viruses such as *tobacco mosaic virus* (TMV) are reported to elicit PCD (del Pozo and Lam, 2003). Purified elicitors, such as Fumonisin

B1 or harpins can trigger HR, inducing physiological changes associated with disease resistance (Numberger et al., 1994).

For colonizing and parasitizing their hosts, pathogens have evolved multiple mechanisms, including virulence factors that compromise the integrity and function of the plant cell. One of them are harpins, which are cell membrane associated proteins, involved in type III secretion system of pathogens and are encoded by genes of the *hrp* clusters (Samuel et al., 2005). Bacterial genes encoding the components of type III secretion system are induced when bacteria penetrate the plant and type III proteins are believed to promote the disease by altering the normal physiology of the plant to benefit the pathogen (Abramovitch and Martin, 2004). It has been shown that spraying plants with harpin coordinately induces systemic resistance to pathogens and micro-HR and that both signal components EDS1 and NDR1, which are required for the function of some *R* genes are necessary for the development of resistance (Peng et al., 2003).

4. Biochemical features of the HR

Results of molecular and biochemical studies support the hypothesis that caspase-like enzymes are involved in the HR. These include the suppression of HR by synthetic peptide caspase inhibitors and the observed increase of caspase-like protease activity in plant cells undergoing HR-PCD (Lam et al., 2001). Additional players that may be similar to some of those controlling PCD in animals are small GTP-binding proteins of the Ras class and cystein-sensitive proteases. Study of Hatsugai et al. (2004) has shown that VPE and the cellular vacuole control the cellular suicide that is essential for HR in response to TMV.

Major players involved in the activation of the HR are reactive oxygen species (ROS), nitric oxide (NO), calcium and proton pumps, mitogen-activated protein kinases (MAPKs), and salicylic acid (SA). It is believed that the initial recognition of the pathogen by a plant receptor (part of the gene-for-gene response) activates a signal transduction pathway that involves the translocation of Ca^{2+} and protons across the plasma membrane into the cytosol, protein phosphorylation/dephosphorylation, activation of enzymes that generate ROS such as NADPH-oxidase and peroxidases, and accumulation of NO and SA. From challenged, HR-developing cells, some particular diffusible molecules known as stress phytohormones (SA, jasmonic acid (JA) and ethylene), may play an important signalling function in establishing resistance both locally (local acquired resistance, LAR) and systemically (systemic acquired resistance, SAR).

When attacked by incompatible pathogens, plants respond by activating a variety of defence responses, including ROS-generating enzyme complex (Bolwell, 1999). The increase of cellular concentration of ROS is a key event in plant and animal PCD and occurs as a result of many stresses (Laloi et al., 2004; Neill et al., 2002),

but an oxidative burst is an essential prerequisite for induction of plant hypersensitive cell death (Levine et al., 1994; Lamb and Dixon, 1997; Jabs, 1999). Under normal conditions, ROS are cleared from the cell by the action of superoxide dismutase (SOD), catalase, or glutathione peroxidase. Under stress conditions, ROS enhance the lipid catabolism resulting in peroxidation of polyunsaturated fatty acids in the cell membranes that in turn leads to structural decomposition, change in permeability, and induces damage by alterations of essential proteins, and DNA. CPT-induced PCD in tomato cell culture is accompanied by a release of ROS into the culture medium and it has been demonstrated that accumulation of H_2O_2 and cell death can be effectively suppressed by addition of NADPH oxidase inhibitor diphenyl iodonium (DPI) and caspase inhibitors (de Jong et al., 2002; Woltering et al., 2003). In addition, the finding that catalase inhibits CPT-induced cell death suggests that H_2O_2 is responsible for the induction of PCD (de Jong et al., 2002).

Upon pathogen recognition, one of the earlier cell reactions is opening of specific ion channels, and the formation of superoxide and H_2O_2 (Kasparovsky et al., 2003). It has been shown that H_2O_2 participates in the cross-linking of cell wall structural proteins and functions as a local trigger of PCD in pathogen challenged cells. In addition, H_2O_2 acts as a diffusible signal (Hung et al., 2005) and induces genes encoding cellular protectants in adjacent cells when soybean cells have been elicited by glucan elicitor isolated from mycelial walls of the fungal pathogen *Phytophthora megasperma f. sp. glycinea* (Levine et al., 1994).

Calcium is an important element in elicitor-mediated cell suicide signalling. Manipulations of proton and calcium homeostasis are shown to activate the HR. These include biochemical studies and the expression of a proton channel that is localized to the plasma membrane and is reported to activate the HR (Dominique et al., 2002). La^{3+} exerts inhibitory effect on Ca channels and can completely block the cytoplasm shrinkage of cultured carrot cells triggered by ethanol, heat shock and H_2O_2 (McCabe et al., 1997). In tomato suspension cells La^{3+} treatment prevents Fumonisin B1- and CPT-stimulated cell death, thus indicating that Ca^{2+} is implicated in CPT-triggered suicidal cascade (de Jong et al., 2002; Iakimova et al., 2004). Crosstalk between pathways of proteolysis, ROS, calcium and ethylene is suggested at apoptotic-like cell death studied in two model systems: in chemical-treated suspension tomato cells and in *Pseudomonas syringae* pv. *tabaci* challenged tobacco leaves (de Jong et al., 2002; Iakimova et al., 2004) and in tobacco leaves simultaneously infiltrated with *Pseudomonas syringae* pv *tabaci* and caspase inhibitors (Richael et al., 2001).

The combination of enhanced production of ROS and NO is shown to activate the HR in the absence of a pathogen, and SA is demonstrated to facilitate the formation of ROS as well as to inhibit two of the key ROS removal enzymes in plants: catalase

and ascorbate peroxidase (Conrath et al., 1995; Delledonne et al., 2001; Durner et al., 1997; Durner and Klessig, 1995). These processes are thought to be orchestrated by a cascade of MAPKs (del Pozo et al., 2004; Pedley and Martin, 2004; Menke et al., 2005). The examination of a non-host hypersensitive response and a disease induced by *Pseudomonas syringae* pv. *phaseolicola* and *P. syringae* pv. *tabaci* in tobacco has revealed an increase of NO 40 min following the challenge with the pathogen, which is some 5 h before the initiation of visible tissue collapse (Mur et al., 2005).

Many plant species are resistant to the majority of microbial invaders, a phenomenon that is termed non-host resistance. At non-host resistance, the importance of signalling pathways involving ROS, NO, alteration of cytoplasmic Ca^{2+} levels and post-translationally regulated MAPK activity is also demonstrated, in addition to their role at incompatible plant-pathogen interactions. It has been shown that activation of inducible plant defence responses is probably brought about by the recognition of pathogen-associated molecular pat-terns (PAMP) that are characteristic of a whole class of microbial organisms (Nürnberger and Lipka, 2005). By characterisation of expressed sequence tags (ESTs), 14 HR-specific ESTs involved in the execution of the HR at β-megaspermin elicitation of tobacco cells are established. Half of the ESTs exhibit homology with genes encoding a receptor-like kinase protein, proteins involved in the regulation of plasma membrane structure, proteins of the ubiquitin/16S proteasome proteolytic system, RNA binding proteins, and a protein hypothesized to be a true regulator of the HR (Ghannam et al., 2005).

cDNA–AFLP is a technique that is extensively used for construction of genetic linkage maps and is an efficient method for identification of stress-induced genes. It allows localization of genes that confer resistance to viruses, nematodes, fungi or bacteria (Savelkoul et al., 1999). By cDNA–AFLP, transcripts, whose expression is rapidly altered during infection with fungal pathogen *Cladosporium fulvum* expressing the Avr9 gene at the defence response of tobacco cell culture, are displayed. cDNA clones have been obtained for ACRE (Avr9/Cf-9 rapidly elicited) genes. The amino acid sequence of some ACRE proteins shows homology to known proteins such as calcium binding protein, 13-lipoxygenase, and a RING-H2 zinc finger protein (Durrant et al., 2000).

Study on the involvement of NO in plant resistance to pathogens has revealed that key features, such as defence gene expression and the activation of a HR in synergy with ROS are dependent on NO production. By using cDNA-AFLP, the investigation of the changes in the expression profiles of *Arabidopsis thaliana* following infiltration with the NO donor sodium nitroprusside, has detected altered expression patterns for 120 of the approximately 2,500 cDNAs examined. Sequence analysis shows homologies with genes involved in signal transduction, disease resis-

tance and stress response, photosynthesis, cellular transport, and basic metabolism. Comparison of the expression profiles with data obtained by microarray has revealed that many of the identified genes, modulated by NO, have been previously reported to be modulated during plant diseases (Polverari et al., 2003).

Cassava genes differentially expressed during the hypersensitive reaction of leaves in response to an incompatible *Pseudomonas syringae* pathovar have also been isolated by the application of cDNA-AFLP. Seventy-eight transcript-derived fragments (TDFs) showing differential expression (75% up-regulated, 25% down-regulated) have been identified. Many encode putative homologues of known defence-related genes involved in signalling (e.g. calcium transport and binding, ACC oxidases and a WRKY transcription factor), cell wall strengthening (e.g. cinnamoyl coenzyme A reductase and peroxidase), programmed cell death (e.g. proteases, 26S proteosome), antimicrobial activity (e.g. proteases and β -1,3-glucanases) and the production of antimicrobial compounds (e.g. DAHP (3-deoxy-arabinoheprulosonate-7-phosphate) synthase and cytochrome P450s) (Kemp et al., 2005). The regulation of defence gene expression is involved in induced disease resistance. These include the plant-specific WRKY family of transcription factors defined by a DNA binding domain that contains the highly conserved amino acid sequence WRKYGQK. WRKY factors have been implicated in plant defence, plant senescence, and response to various environmental stresses. WRKY70 appears to play a central role in controlling which type of defence response (jasmonate- or salicylate-mediated) is activated in response to pathogen attack. High WRKY70 levels activate expression of systemic acquired resistance-related genes while repressing jasmonic acid-responsive gene expression and vice versa (Li et al., 2004).

Sphingolipid signalling is another important player in the HR. Fumonisin B1 and AAL toxin are shown to disrupt sphingolipid metabolism by inhibiting ceramide synthase, thus inducing subsequent PCD (Spassieva et al., 2002). The HR is also associated with altered mitochondrial functions. Inhibition of ATP synthesis, blockage of cytochrome C release, conversion of electron transport from cytochrom oxidase pathway to alternative oxidase pathway, uncoupling of oxidative phosphorylation, depolarization of mitochondrial membranes and opening of mitochondrial pores are among devastating changes leading to cell death at pathogen attack (Lam et al., 2001). Such changes have been found in HR induced by *Erwinia amylovora*, by T-toxin of *Colchliobolus heterostrophus* and by victorin from *C. victoriae* (see Xie and Chen, 2000).

5. Role of ethylene in the HR

Although the processes of plant PCD share similarity to animal PCD, the control of cell death in plants involves plant-specific regulators. In addition to common suicidal cascades, it has been demonstrated in a number of experimental plant systems that the

plant hormone ethylene plays an important role in programmed cell death and senescence.

The role of ethylene in pathogen-induced cell death is evaluated in ethylene insensitive (*never-ripe*) NR-tomatoes. Following infection of these mutants, greatly reduced cell death is observed, indicating ethylene involvement in programmed cell death (Lund et al., 1998). Ethylene signalling is found to play a role in the cell death induced by the mycotoxin Fumonisin B1 in Arabidopsis and tomato (Asai et al., 2000; Moore et al., 1999; Mur et al., 2003).

Formation of HR-like lesions and enhanced production of ROS are found to occur in response to abiotic cell death inducer such as ozone (O_3), which interplays with ethylene (Overmyer et al., 2000; 2005; Neill et al., 2003). Evidence exist that O_3 triggers HR in Bel W3 tobacco plants, which is accompanied by H_2O_2 accumulation (Pasqualini et al., 2003). Ethylene synthesis and perception have been found necessary for active H_2O_2 production and cell death resulting in visible injury in tomato (*Lycopersicon esculentum*) leaves exposed to ozone and a selective response is supposed in the regulation of the spread of cell death (Moeder et al., 2002).

ROS and ethylene are also recently reported to participate in susceptibility to *cauliflower mosaic virus* (CaMV), *turnip crinkle virus* (TCV), *cucumber mosaic virus* (CMV), *turnip vein clearing tobamovirus* (TVTV) (see Love et al., 2005).

Cell death in maize endosperm could be hastened by treatment with ethylene and blocked by ethylene inhibitors (Young and Gallie, 2000). In oat mesophyll cells, the administration of inhibitors of ethylene biosynthesis and action – aminooxyacetic acid (AOA) and silver thiosulphate (STS) effectively inhibited victorin-induced PCD, involving RUBISCO cleavage, DNA laddering and changes in mitochondrial permeability (Curtis and Wolpert, 2004). Microarray study of AAL toxin-treated tobacco reveals that genes responsive to reactive oxygen species, ethylene and a number of proteases are among the earliest to be up-regulated, suggesting that an oxidative burst, production of ethylene and proteolysis play a role in the activation of the cell death (Gechev et al., 2004). In *Taxus chinensis* cell suspension ethylene enhances cell death induced by a fungal elicitor from *Aspergillus niger* (Qin and Lan, 2004). Overproduced ethylene correlates closely with expression of lethal symptoms and apoptotic-like changes in hybrid tobacco seedlings and the lethality can be suppressed by ethylene synthesis inhibitors AOA and amino-ethoxyvinylglycine (AVG) (Yamada and Marubashi, 2003).

Additional evidence supporting the participation of ethylene in cell death signalling cascade comes from the study on *Arabidopsis thaliana* double mutants. Crosses of the lesion mimic mutant *accelerated cell death 5 (acd 5)* and *ethylene insensitive 2 (ein 2)*, in which ethylene signalling is blocked, show decreased cell death (Greenberg et al., 2000). A study with tomato cell suspension showed that ethylene is an essential factor in CPT-

induced PCD (de Jong et al., 2002). Simultaneous application of CPT and ethylene greatly enhances CPT-induced cell death, apparently as a result of the increased production of H_2O_2. Two partly overlapping cell death pathways are proposed. These comprise one pathway involving caspase-like proteases that requires low levels of ethylene and one caspase-independent pathway operative at high ethylene levels. The latter pathway presumably acts through MAPK-like proteins that are not essential in PCD at basal ethylene concentrations (de Jong et al., 2002).

6. HR in fruit species

The physiological and biochemical events that participate in the HR of fruit species exposed to pathogen attack are still poorly investigated, although many studies focus on molecular resistance mechanisms and biotechnology of resistant cultivars.

In order to analyze mechanisms leading to compatible or incompatible interactions, early plant molecular events have been investigated in two genotypes of *Malus* with contrasting susceptibility to fire blight, after confrontation with either *E. amylovora* or the incompatible tobacco pathogen *Pseudomonas syringae* pv. *tabaci* (Venisse et al., 2002). Defence mechanisms, such as generation of an oxidative burst and accumulation of pathogenesis-related proteins, have been elicited in both resistant and susceptible genotypes by the two pathogens at similar rates. This elicitation is linked to the functional hypersensitive reaction and pathogenicity (*hrp*) gene cluster of *E. amylovora*. A delayed induction of several genes of various branch pathways of the phenylpropanoid metabolism is recorded in tissues of the susceptible genotype challenged with the virulent wild-type strain of *E. amylovora*. In other plant-bacteria interactions, including interactions with the *hrp* secretion mutants these genes have been quickly induced. The authors suggest the existence of *hrp*-independent elicitors of defence in the fire blight pathogen as well as *hrp*-dependant mechanisms of suppression of these non-specific inductions (Venisse et al., 2002).

In susceptible pear seedlings *E. amylovora* is also shown to generate an oxidative stress similar to the one induced by the incompatible *P. syringae* pv. *tabaci* (Venisse et al., 2001). Typical HR lesions and oxidative burst are observed in leaves of non-host tobacco plants infiltrated with Asian pear pathogen *Erwinia pyrifoliae* as well. This indicates that ROS generation is necessary for the pathogen to kill the plant cell.

Grapevine (*Vitis vinifera* L., cv. Limberger) leaves and cell suspension have been induced to undergo a form of cell death that mimics the HR by treatment with methyl jasmonate (MeJA) (Repka, 2002). Characteristic features of apoptosis in animal and plant cells, such as typical changes in nuclear morphology, fragmentation of the nucleus and protoplast collapse have been observed to accompany the chemical-induced cell death. Local and ectopic treatment of grapevine leaves

with phenylmethylsulphonylfluoride (PMSF), leupeptin, and especially with a specific inhibitor of cysteine proteases, E-64, have been found to inhibit MeJA-induced cysteine protease activity and to block PCD triggered by MeJA. These results indicate that proteolysis plays a crucial role in MeJA-induced apoptotic-like cell death in grapevine leaves.

HR induction is reported in lemon seedlings infected with *Alternaria alternata* and a participation of phenylpropanoid pathway, which results in *de novo* biosynthesis of the phytoalexin scoparone, is found to be a part of the HR. For elucidating some of the signalling elements that participate in the development of HR in these plants, several compounds that are known as activators or inhibitors of signal transduction elements in plants or in animal cells have been administrated. Treatment of lemon seedlings with either cholera toxin or phorbol 12-myristate 13-acetate (PMA), in the absence of *A. alternata* induce phenylalanine ammonia-lyase (PAL) and the synthesis of phytoalexin scoparone, suggesting the participation of G-protein and of serine/threonine kinase, respectively, in signal transduction. The use of trifluoperazine (TFP), W-7 (N-[6-aminohexyl]-5-chloro-1-naphthalenesulfonamide), staurosporine, lavendustin A or 2,5-dihydroxymethyl-cinnamate (DHMC) have prevented PAL induction as well as scoparone biosynthesis in response to the fungal inoculation, thus suggesting the participation of calmodulin, serine/threonine and tyrosine protein kinases in signal transduction in *Citrus limon* in response to *A. alternata* (Ortega et al., 2002).

According to Lee and Hwang (2005), the inoculation of primary pepper leaves with an avirulent strain of *Xanthomonas campestris* pv. *vesicatoria* induces systemic acquired resistance (SAR) in the non-inoculated, secondary leaves and this SAR response is accompanied by the systemic expression of the defence-related genes, a systemic micro-oxidative burst generating H_2O_2, and the systemic induction of both ion-leakage and callose deposition in the non-inoculated, secondary leaves. Some defence-related genes including those encoding PR-1, chitinase, osmotin, peroxidase, PR10, thionin, and SAR8.2 have been markedly induced in the systemic leaves. The conspicuous systemic accumulation of H_2O_2 and the strong increase in peroxidase activity in the pepper leaves is suggested to play a role in the cell death process in the systemic micro-HRs, leading to the induction of the SAR.

An association of the antioxidant enzyme machinery with the resistance of apricot (*Prunus armeniaca* L.) to sharka disease caused by *plum pox virus* (PPV) is reported. The activity of catalase (CAT), dehydroascorbate reductase (DHAR), total superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR) and monodehydroascorbate reductase (MDHAR) have been affected. The different behaviour of SODs (H_2O_2-generating enzymes) and APX (an H_2O_2-removal enzyme) in resistant and susceptible cultivars suggests an

important role for H_2O_2 in the response to PPV of the resistant cultivar Goldrich, in which no change in APX activity is observed. The ability of the inoculated resistant cultivar to induce SOD1 and SOD2 as well as the important increase of DHAR suggests a relationship between these activities and resistance to PPV (Hernández et al., 2001). Plum K4 hybrid is reported hypersensitive against PPV isolate CG (Kegler et al., 2001) but there are almost no published results about the mechanism of the HR at PPV infection. By inoculation with two PPV strains (D- and M- strain), phenotypic features associated with the hypersensitivity of European plums (*Prunus x domestica* L.) against PPV have recently been described. Biochemical and histochemical investigations were used to explain the macroscopic visible characteristics of hypersensitivity. In the graft union of combination of the hypersensitive cultivar Jojo with budwood infected with PPV, cell collapse has been detected at the barrier between the two partners. An accumulation of phenolic compounds has been shown to precede the cell death (Neumüller et al., 2005).

Cytological differences have been found in postharvest (detached) and *in planta* (attached) fruits of pepper plants (*Capsicum annuum)* cv. Jejujaerae (susceptible) and *Capsicum baccatum* cv. PBC80 (resistant), inoculated with the anthracnose pathogen *Colletotrichum gloeosporioides.* Cytological features of PCD are observed in the resistant pepper fruit with postharvest inoculation and, these have been characterized by positive responses to terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) and DNA laddering (hallmarks of apoptosis). The PCD-positive responses occurred around the inoculation sites early in *in planta* wound inoculation of the resistant pepper. Nuclear modifications and structural changes of hypersensitivity are also observed in the resistant fruits, including separation of the plasma membrane from the cell wall, dilation of the endoplasmic reticulum, accumulation of electron-dense inclusions in vacuoles, and cytoplasmic vacuolization accompanying fragmentation of the cytoplasm (Kim et al., 2004).

Using a differential hybridization technique, pathogen-induced pepper cyclophilin (CACYP1) cDNA clone was isolated from a pepper cDNA library from HR lesions of leaves infected with *Xanthomonas campestris* pv. *vesicatoria*. The deduced amino acid sequence of this CACYP1 shows a high level of homology with those of cyclophilin proteins from tomatoes, potatoes, beans and Arabidopsis. In addition, the accumulation of CACYP1 mRNA has been strongly induced in pepper plants by *Colletotrichum gloeosporioides* infection. At the incompatible interactions, a drastic induction of the CACYP1 mRNA has been demonstrated to occur in the bacteria inoculated leaves compared to that of the compatible interaction (Kong et al., 2001).

7. Manipulation of the HR is an "open door" towards to the control of diseases in plants.

The interest for improving the resistance of crops to pathogens has remarkably stimulated the research on the identification of signals produced in plant-pathogen interactions and on the biochemical and molecular steps required for the activation of defence mechanisms. At pathogen invasion, the process of PCD is linked to initiation of resistance (Yu et al., 1998). The identification and characterization of genes and metabolic pathways involved in the HR provides a clue to better understanding the process of PCD in plants and helps to establish the similarities between animal and plant kingdoms. Selective inhibition or induction of HR can be successfully employed for therapeutic modulation of plant diseases caused by necrotrophic or biotrophic pathogens. Transgenic expression of animal anti-apoptotic genes in plants provides broad-spectrum disease resistance against compatible obligate pathogens (Dickman et al., 2001). Similarly, plant- and animal-derived pro-apoptotic genes can be engineered to be expressed in plants for activating HR-linked resistant disease response against biotrophic pathogens (Khurana et al., 2005). In addition, the use of selective synthetic pathogen-inducible promoters has a high potential for engineering enhanced and durable disease resistance thus avoiding so called "runaway" cell death (Gurr and Rushton, 2005). HR is involved in incompatible plant-pathogen interactions but besides its role in the infected cells, the HR may coordinate the defence response in neighbouring cells and local acquired resistance. Since the cell deaths associated with disease symptoms and HR presumably share common mechanisms, the investigation on underlying biochemical pathways and molecular mechanisms in wild type and transgenic plants may shed more light into resistance mechanisms in plants.

Acknowledgements. The work on this paper was partially supported by a grant from the Ministry of Education and Science, National Science Fund, Bulgaria, Project: VU-B-9/05 (for literature search).

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NADWRAŻLIWA ŚMIERĆ KOMÓREK ROŚLINNYCH – JEJ MECHANIZMY I ROLA W REAKCJI OBRONNEJ ROŚLIN PRZED PATOGENAMI

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S T R E S Z C Z E N I E

W pracy tej przedstawiono najnowsze osiągnięcia w badaniach nad reakcją nadwrażliwości (hypersensitive response – HR) u roślin, ze szczególnym uwzględnieniem fizjologicznych i biochemicznych uwarunkowań w różnych systemach modelowych. Reakcja nadwrażliwości jest rozpatrywana jako jedna z form programowanej śmierci komórki (programmed cell death – PCD), będącą jednym z mechanizmów obrony roślin przed chorobami. Omawiane są główne drogi transdukcji sygnału oraz związki chemiczne związane z reakcją nadwrażliwości, takie jak kaskada proteolityczna, reakcje oxydacyjne i etylen, które przypuszczalnie mają podstawowe znaczenie w mechanizmie śmierci komórki. Specjalną uwagę poświęcono reakcji nadwrażliwości w roślinach sadowniczych. Badania nad programowaną śmiercią komórki pozwalają lepiej zrozumieć mechanizm odporności roślin na choroby.

Słowa kluczowe: rośliny sadownicze, reakcja nadwrażliwości, programowana śmierć komórki