1. **Hypersensitive response (HR) is a strong resistance reaction of plants against pathogens**

The plant ‘hypersensitive response’ (HR), a defense mechanism, involves interaction between products of an ‘avr’ gene of the pathogen and a matching ‘R’ gene of the plant (Dodds *et al.*, 2006). Plant HR is the result of an ‘incompatible reaction’, in which the ‘R’ gene of the non-host plant corresponds to the ‘avr’ gene of the pathogen, where as in a ‘compatible reaction’ the ‘R’ gene of the host plant does not match with the ‘avr’ gene of the pathogen resulting in the spread of pathogen through out the plant and disease occurs. The HR is one kind of programmed cell death (PCD) associated with the death of a small number of cells at and around the site of infection (Fig 1.1). HR serves to inhibit the growth of the invading pathogen by killing infected and uninfected cells, producing a physical barrier composed of dead cells. During HR, the dying plant cells strengthen their cell walls and accumulate certain toxic compounds like phenols and phytoalexins (Dangl *et al.*, 1996) (Fig 1.2). An HR also occurs when pathogen-derived molecules or unique proteinaceous bacterial elicitors like ‘harpin’ interact with non-host plants (Fig 1.3). Membrane damage, necrosis, and collapse of challenged cells are the common features in a highly orchestrated form of genetically-regulated PCD in plants (Greenberg *et al.*, 1994), which is similar to ‘apoptosis’ in response to viruses or pathogenic bacteria in animal cells. The mechanism of cell death activation seems to be common in all living beings, while the precursors and the processes involved might have little variations.

**Fig 1.1 Hypersensitive response on a tobacco leaf during pathogen infection**

Hypersensitive response (HR) manifested with the development of necrotic lesions (stained with Evans blue) followed by localized desiccation and browning of the affected cells. A) initial hours of infection and B) late hours of infection (adapted from Wright *et al.*, 2000).
Fig 1.2 Accumulation of phenolic compounds during HR
Autofluorescence of phenolic compounds and cell wall thickenings in plant tissues during HR, observed under UV light. A) initial hours of pathogen infection (absence of phenols shown with asterix) and B) late hours of infection (phenol compounds shown with arrows) (adapted from Soylu, 2006).

Fig 1.3 HarpinPst infiltration induces HR on tobacco leaves
Reaction of tobacco leaves with (A) harpinPst and (B) control leaf (buffer alone).

A variety of bacterial or fungal products elicit defensive plant responses in both host and non-host plants and these non-specific elicitors are the prime inducers of defense responses in non-host plant-pathogen interactions. An example of non-specific elicitors from bacteria are the ‘harpins’, which are heat stable proteins and encoded by members of ‘hrp’ (hypersensitive response pathogenicity) gene cluster of some Gram-negative phytopathogenic bacteria (Collmer et al., 2000). Generally phytopathogenic bacteria have limited host ranges, often confined to members of a single plant species or genus. Most of the phytopathogenic bacteria are specialized in colonizing the apoplast and trigger diseases in plants causing rots, spots, vasucular wilts, cankers and blights (Alfano and
Collmer, 1996). The majority of these pathogens are Gram-negative rod shaped bacteria from the genera *Erwinia, Pseudomonas, Xanthomonas* and *Ralstonia*. These bacteria reside within the intercellular spaces of various plant organs or in the xylem, causing plant tissue damage by secreting toxins, extracellular polysaccharides or cell wall degrading enzymes at some stage during pathogenesis. Several bacterial genes referred to as the *hrp* cluster are required for bacterial pathogenesis. These *hrp* genes are similar to genes of pathogenic bacteria from animals suggesting that, distinct pathogens have similar virulence strategies, emphasizing the conserved mechanisms during the process of evolution (Alfano *et al.*, 2000; Cao *et al.*, 2001).

### 1.1 Plants operate programmed cell death during development and defense

Programmed cell death (PCD) plays an important role in development of the organism, in defense against pathogens and environmental stresses. The molecular details of the signaling pathways underlying PCD, in particular, have been well studied in animals. Cells undergo apoptosis through two major pathways controlled by complex regulatory networks: the extrinsic pathway (death receptor pathway) or the intrinsic pathway (mitochondrial pathway). Caspases are the key executioners of apoptosis in animals. In the past few years, the understanding of PCD mechanisms in plants has greatly increased.

In plants, PCD plays an important role in development, response to various pathogens and abiotic stress but the key proteases involved in the execution of PCD in plants still remains unanswered. The PCD in plants plays normal physiological roles in a variety of processes including, deletion of cells with temporary functions such as the aleurone cells in seeds and the suspensor cells in embryos; removal of unwanted cells, such as the root cap cells found in the tips of elongating plant roots and the stamen primordia cells in unisexual flowers; deletion of cells during sculpting of the plant body and formation of leaf lobes and perforations; death of cells during plant specialization, leaf senescence; and responses to plant pathogens and abiotic stresses (Pennell and Lamb 1997; Danon *et al.*, 2004; Kuriyama and Fukuda 2002). PCD in plants is an active suicidal process that removes unwanted or severely damaged cells (Dangl and Jones 2001; Kuriyama and Fukuda 2002), that resembles type II or autophagic cell death in animals (Liu *et al.*,).
Some common features of type I cell death or apoptosis are conserved in both plants and metazoa (Danon and Gallois 1998; Yao et al., 2004) which include cytoplasm shrinkage, cytochrome c leakage out of mitochondria, chromatin condensation, altered nuclear morphology and DNA laddering.

PCD can be induced in plants by elicitors or toxins produced by a number of pathogens that include ‘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., 1996), 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).

While the signalling events and cell death cascades have been well studied in animals, little is known about the regulation and execution of PCD in plants (Hoeberichts and Woltering 2003). However, animal Bcl-2 members have been found to modify cell death processes in plants (Lam et al., 1999; Baek et al., 2004), indicating a possible identical apoptotic machinery in plants. Caspase-like activities have been measured in plant extracts and implicated in PCD processes, even though no sequence homologues have been found at the molecular level. Identification of such caspase-like proteases is essential to reveal the molecular mechanism that operates in plant PCD and to provide some insights into differences between plant and animal PCD. The identification, in plants, of a class of putative proteases related to animal caspases and termed metacaspases (Uren et al., 2000) has stimulated research in this protein family. New types of subtilisin-like proteases (named saspases-A and -B) from oats and a vacuolar processing enzyme (VPE) from tobacco may play important roles as caspase-like proteases in the execution of PCD in plants (Cofféen and Wolpert 2004; Hatsugai et al. 2004).

1.2 HR is a form of PCD

Plant defense responses are frequently expressed in part as the so-called hypersensitive response (HR), which is characterized by necrosis at the sites of infection (resembling
animal PCD) and restriction of pathogen growth and spread. The HR is associated with the induction of defense-related genes which play important roles in containing pathogen growth either indirectly by helping to reinforce the plant cell walls, or directly by providing antimicrobial enzymes and phytoalexins (Dangl and Jones, 2001; Scheel, 1998). HR in plants is characterized by an increase of reactive oxygen species (ROS) such as hydrogen peroxide ($H_2O_2$) and nitric oxide (NO) and is often followed by a systemic response called systemic acquired resistance (SAR) (Strobel et al., 1996; Baker et al., 1993). 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 referred to as “disease lesion mimics”, were isolated from maize, rice, barley & Arabidopsis. 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.

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 (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’ family and cysteine-sensitive proteases. Hatsugai et al., (2004) showed 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 ROS, NO, calcium and proton pumps, mitogen-activated protein kinases (MAPKs), and salicylic acid (SA). The initial recognition of the pathogen by a plant receptor (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. Specific diffusible molecules known as stress phytohormones (salicylic acid, jasmonic acid and ethylene) from challenged HR developing cells, play an important signalling function in establishing resistance both
locally (local acquired resistance, LAR) and systemically (systemic acquired resistance, SAR).

1.3 Type III secretion system (TTSS) delivers bacterial proteins into host milieu

Several Gram-negative pathogens of both plant and animals (Hueck, 1998; Galan and Collmer, 1999; Cornelis and Gijsegem, 2000) use type III protein secretion systems (TTSS). These secretion systems are particularly noteworthy because they can translocate effector proteins directly into eukaryotic cells (Cornelis and Wolf-Watz, 1997). In bacterial plant pathogens belonging to the genera *Erwinia*, *Pseudomonas*, *Ralstonia* and *Xanthomonas*, TTSS (also referred to as Hrp systems) are encoded by *hrp/hrc* genes (Lindgren, 1997; He, 1998). *Pseudomonas syringae* uses a TTSS system encoded by the Hrp pathogenicity island (Pai) to translocate effector proteins into plant cells (Alfano *et al.*, 2000). A small open reading frame (ORF), named shcA, precedes the *hopPsyA* gene in the Hrp Pai of *P. s. pv. syringae* 61. The HopPsyA protein is secreted in culture by *P. syringae* and, when expressed transiently in tobacco, it elicits an HR, indicating that its site of action is inside plant cells (Alfano *et al.*, 1997; van Dijk *et al.*, 1999; Collmer *et al.*, 2000). The predicted product of ORF shares several of the general characteristics of chaperones used in the TTSS of animal pathogens (Wattiau *et al.*, 1996; Cornelis *et al.*, 1998).

Successful parasitism appears to require multiple TTSS effectors. Genomic searches for TTSS effector genes in *P. syringae pv. tomato* DC 3000 revealed 33 confirmed effectors and several effector candidates (Buell *et al.*, 2003; Collmer *et al.*, 2002; Guttman *et al.*, 2002). A few of the type III effectors from *Pseudomonas syringae pv. tomato* were also shown to inhibit growth in yeast and cause cell death during ectopic expression (Munkvold *et al.*, 2008). Indeed, several *P. syringae* effectors were shown to suppress plant defenses (Abramovitch *et al.*, 2003; Bretz *et al.*, 2003). Several of the effectors that suppress the HR can also suppress Bax-triggered PCD in yeast and plants. AcrPphE*pto*, AvrPpiB*pto*, AvtPtoB, HopPtoF and HopPtoG effectors were found to possess such suppressor activity (Jamir *et al.*, 2004).
1.4 Harpins are the most studied type III effectors of phytopathogenic bacteria

Harpins are effector proteins secreted by TTSS of bacterial pathogens. Although harpins were originally defined as elicitors of HR, some other biological activity e.g. induction of disease resistance has been reported (Dong et al., 1999). Beside the important biological activities, harpins also attract considerable interest due to their potential application as pesticides. Harpins elicit a protective response in the plant which imparts resistance to it from wide range of fungal, bacterial, and viral infections. Since harpins do not directly interact with the disease-causing organisms, pathogens are not expected to develop resistance to harpins. As harpins are biodegradable and have no adverse effects on human health, use of harpins can substantially reduce use of more toxic chemical pesticides. The correlation of structure with activity in harpin may lead to the development of improved, engineered pesticides.

_Pseudomonas syringae_ pv. _syringae_ 61 _hrpZ_ gene encodes harpin_Pss_, a 34.7 kDa extracellular protein, which elicits HR in several plants, including tobacco (He et al., 1993). _HrpN_ of _Erwinia amylovora_ was the first harpin protein shown to elicit HR in tobacco (Wei et al., 1992), secreted via the TTSS system. Harpins are hydrophilic in nature, rich in leucine & glycine, heat stable and elicit HR when infiltrated into the apoplast of certain plants (Bauer et al., 1995). Harpin proteins, isolated from a variety of Gram–ve phytopathogenic bacteria such as _P. syringae_ pv. _syringae_ (_hrpZ_), _Xanthomonas axonopodis, E. chrysanthemi_ (_hrpN_), _E. carotovora_ (_hrpN_), have been characterized (He et al., 1993; Kim et al., 2003; Bauer et al., 1995; Mukherjee et al., 1997). In _Ralstonia solanacearum_, a harpin-like protein, PopA, has been shown to induce HR in non host tobacco plants (Arlat et al., 1994).

1.5 Harpins possess unique biochemical features and activate host cell machinery

Harpin_Pss_ exists as a polydisperse protein in nature, the multimeric forms include, but are probably not restricted to – dimer, trimer, tetramer and octamer (Chen et al., 1998). Partial deletion mutation revealed that several truncated peptides e.g., _N-terminal_ 153
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Like other harpins the mechanism of HR elicitation by harpin\textsubscript{Pss} and its truncated peptides is not clear. Exploiting the high thermal stability of this protein He \textit{et al.}, (1993) developed a simple method to purify it from a mixture of mesophilic proteins. Biochemical mechanism of harpin-elicited HR in non-host plants is far from being completely understood. Harpin\textsubscript{Ea}-induced responses in tobacco suspension cells such as oxidative burst (Baker \textit{et al.}, 1993), pH change, K\textsuperscript{+} efflux, extracellular alkalinization and membrane depolarization are blocked by lanthanum chloride, a Ca\textsuperscript{2+} channel blocker and K252a, a protein kinase inhibitor (Baker \textit{et al.}, 1993, He \textit{et al.}, 1993, Popham \textit{et al.}, 1995). Though structurally different, harpin\textsubscript{Pss} and harpin\textsubscript{Ea}, both cause immediate K\textsuperscript{+} efflux and extracellular alkalinization in tobacco suspension-cultured cells (Wei \textit{et al.}, 1992; He \textit{et al.}, 1993; Popham \textit{et al.}, 1995) which suggest that harpin\textsubscript{Pss} triggers a signal transduction pathway that involves active oxygen species production, protein phosphorylation, and Ca\textsuperscript{2+} influx. Alkalinization was induced immediately after addition of different concentrations of full-length harpin\textsubscript{Pss}. The pH change caused by the same concentrations of truncated harpin\textsubscript{Pss} is similar in magnitude to the changes caused by the full-length protein. At the same protein concentration, truncated protein and protease inactivated harpin\textsubscript{Pss} caused similar but smaller alkalinization that harpin\textsubscript{Pss} contribute to the residual pH change in the protoplast medium and suggest that the cell wall is important for the pH change induced by either harpin\textsubscript{Pss} or \textit{P. syringae pv. syringae} (Hoyos \textit{et al.}, 1996). A wall-associated kinase (Wak 1) that could function in cell wall-to-membrane signal transduction has been reported and the crucial role of plant cell wall in harpin\textsubscript{Pss}-mediated plant HR has been established (Hoyos \textit{et al.}, 1996). Mitochondria play a central role in apoptosis, and as well, harpin\textsubscript{Ea} treatment in tobacco suspension cells lead to altered mitochondrial functions (Xie and Chen, 2000).

Harpins do not have structural homology with known proteins or among themselves. Absence of homology makes it hard to establish structure-activity relationship for this class of proteins. In spite of the absence of structural homology in harpins, they share some common properties i.e. they contain a high proportion of glycine, they lack cysteine, and are thermally stable. A large number of harpins act on plant cell walls and
form amyloid fibrils in apoplastic fluid (Oh et al., 2007). Harpin\textsubscript{psh} from \textit{P. syringae} pv. \textit{phaseolicola} associates with liposomes and synthetic bilayer membranes and mediates the formation of an ion-conducting pore (Lee et al., 2001). Immunolocalisation revealed Ca\textsuperscript{2+}-dependent association of \textit{P. syringae} pv. \textit{syringae} harpin with plant cell walls (Hoyos et al., 1996). Additionally, HrpN from \textit{E. amylovora} and \textit{Pantoea stewartii} subsp. \textit{stewartii} was reported to depolarize plant cell membranes (Pike et al., 1998; Ahmad et al., 2001), and PopA from \textit{R. solanacearum} exhibits pore-forming activity and integrates into liposomes and membranes of \textit{Xenopus laevis} (Recapé et al., 2005). As the unusual membrane permeability was proposed to cause cellular dysfunction and death, it may be possible that destabilization of membranes induces HR in plants. A molecular level understanding about how harpins destabilize membranes to cause HR still remains unclear.

1.6 PCD in animal cells has been widely studied

Cell death is divided in animal species into PCD and necrosis. PCD is an important process for multicellular organisms. As it removes superfluous, damaged or infected cells in an organized manner, PCD plays an important role in development, in tissue homeostasis, in the immune responses and to cope with adverse environmental stresses (Steller, 1995; Meier et al., 2000; Lawen, 2003; Jin and El-Deiry, 2005). PCD was held synonymous with apoptosis. The term apoptosis is derived from the Greek word for the process of leaves falling from trees or petals falling from flowers. It was introduced in the 1970s to differentiate a morphologically distinctive form of cell death associated with normal physiology (Kerr et al., 1972). Apoptosis is associated with activation of caspases, executioners of cell destruction. Four mechanistic classes of proteases, recognized by the International Union of Biochemistry and Molecular Biology in 1984, including serine, cysteine, aspartic and metallo proteases. Caspases belong to an evolutionarily conserved family of cysteine proteases (Kroemer and Martin, 2005). Necrosis is associated with acute injury to cells, leading to loss of membrane integrity, swelling and disruption of the cells. During necrosis, cellular contents are released uncontrolled into the cell’s environment which results in damage of surrounding cells and a strong inflammatory response in the corresponding tissue (Leist and Jaatela, 2001).
Since, other types of PCD have been proposed, for which cell death was found to occur in a programmed fashion, but in complete absence and independent of caspase activation. PCD has been classified into three main types according to Clarke’s classification based on lysosomal involvement (Clarke, 1990; Chipuk and Green, 2004; Kim, 2005) - apoptosis (or type I cell death), autophagic cell death (also known as cytoplasmic, or type II cell death) and necrosis-like cell death (also known as type III or non-lysosomal cell death).

Type I cell death, apoptosis, is a form of PCD morphologically defined by condensation of the nucleus and cytoplasm, association of chromatin with the nuclear periphery, DNA fragmentation, membrane blebbing, and engulfment and lysosomal degradation of the dying cell by a phagocyte (Kerr et al., 1972). Biochemical evidence has indicated the caspase family of cysteine protease as well as certain proteins of the mitochondria to be mediators of type I PCD. Type II, autophagic cell death, is characterized by sequestration of bulk cytoplasm and organelles in double or multi-membrane autophagic vesicles and their delivery to and subsequent degradation by the cell’s own lysosomal system before the nucleus is destroyed (Bursch et al., 2000; Levine and Klionsky, 2004). The sequestered cytoplasmic components may be degraded prior to heterophagocytosis of cellular remains. Type III, non-lysosomal or necrosis-like cell death, is characterized by breakdown of the plasma membrane, swelling of organelles, lysosome-independent formation of ‘empty spaces’ in the cytoplasm and disintegration of the cytoplasm (Gozuacik and Kimchi, 2004).

Types I and II cell death have been observed in many animal species during development, whereas type III cell death is common in pathological conditions. Types II and III cell death are genetically regulated and often have morphological features resembling necrosis, yet their underlying molecular mechanisms are unclear. The various types of PCD have in common that they are executed by active cellular processes that can be intercepted by interfering with intracellular signaling. Those types cannot be categorized because they might overlap since they share the same activation intermediaries or they can be activating each other (Lockshin and Zakeri, 2004). The

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cellular components are safely isolated by membranes, and then consumed by adjacent cells and/or resident phagocytes without inflammation. The elimination of PCD debris may remain virtually unnoticed by the body which distinguishes them from “accidental” necrosis. The caspase-independent cell death pathways - type II and type III cell death - are important safeguard mechanisms to protect the organism against unwanted and potential harmful cells when caspase-mediated routes fail but can also be triggered in response to cytotoxic agents or other death stimuli. In the case of accidental necrosis, cytosolic constituents spill into extracellular space through damaged plasma membrane and provoke an inflammatory response. The necrotic cell removal induces and amplifies pathological processes.

1.7 Key elements involved in regulation of cell death
Mitochondria play not only a key role in cellular metabolism and in signal transduction cascades but also an important role in the regulation of PCD (Ferri and Kroemer, 2001). Mitochondrial alterations – following release of sequestered apoptogenic proteins, loss of transmembrane potential, production of ROS, disruption of electron transport chain, and decreases in ATP synthesis - have been shown to be responsible for the different types of cell death (Bras et al., 2005). Thus, the mitochondria can be viewed as a central regulator of the decision between cellular survival and cell death.

During cell death, the ATP levels are determinant in directing toward PCD or necrosis (Leist et al., 1997; Nicotera et al., 1998; Formigli et al., 2000). The disruption of the mitochondrial electron transport chain would result in diminished ATP production and consequently in a striking perturbation of the bioenergetic state of the cell. The inhibition of ATP production has been observed in both type I and type III cell death. However, this phenomenon occurs relatively late in type I cell death, as the complete apoptotic program involves the energy-dependent formation of the apoptosome (cytochrome c/ Apaf-1 / dATP complex) and hydrolysis of macromolecules. By contrast, type III cell death is characterized by an early loss of ATP synthesis and seems to proceed in conditions of low cytosolic ATP levels (Kim et al., 2003). ATP dependency has been observed for the autophagic type II pathway, apparently at the lysosomal level (Plomp et al., 1989).
Moreover, intracellular nucleotides can regulate apoptosis, they can directly block the cytochrome c initiated apoptosome formation and the caspase activation by interfering with Apaf-1 (Chandra et al., 2006).

Calcium is a key regulator of mitochondrial function and acts at several levels within the organelle to stimulate ATP synthesis. Mitochondrial matrix Ca$^{2+}$ overload can lead to enhanced generation of ROS. The ROS triggers the opening of the mitochondrial permeability transition pore (MPTP). If the pore remains open, cells do not maintain their ATP levels and this leads to cell death by necrosis. When cells experience a less severe insult, the MPTP may open transiently. The resulting mitochondrial swelling may be sufficient to cause release of cytochrome c and activation of the apoptotic pathway rather than necrosis (Green and Kroemer 2004).

The efflux of cytochrome c from mitochondria is also a pivotal event in apoptosis, as it drives the assembly of the apoptosome in the cytoplasm, the activation of a proteolytic cascade involving caspase proteases is an irreversible step (Abraham and Shaham, 2004). Caspases cleave a variety of proteins after specific aspartate residues, ultimately leading to cell death. The contents of dead cells are packaged into apoptotic bodies, which are recognized by neighboring cells or macrophages and cleared by phagocytes. De-regulation of apoptosis may lead to pathological disorders such as developmental defects, autoimmune diseases, neurodegeneration or cancer.

1.8 Apoptotic pathways
The apoptotic cascade can be initiated via two major pathways (Fig 1.4). These pathways involve either the activation of death receptors in response to ligand binding (death receptor pathway), or the release of cytochrome c from the mitochondria (mitochondrial pathway) (Ashkenazi and Dixit 1998; Hengartner, 2000). Both pathways involve a specific family of cysteine proteases, the caspases, that are activated to execute PCD. The execution of PCD results in the typical morphologic changes (Degterev et al., 2003). In mammals, the cell surface death receptor-mediated pathway involves cell surface death receptors such as Fas, Tumor Necrosis Factor (TNF), or TRAIL receptors (Ashkenazi and
Dixit 1998). Death ligand stimulation, via a series of protein-protein interactions, results in oligomerization of the receptors and recruitment of an adaptor protein and caspase-8 or -10, forming a death-inducing signalling complex (DISC). Autoactivation of caspase-8 at the DISC is followed by activation of other caspases, including caspase 3, 6 and 7. These activated caspases function as downstream effectors of the cell death program.

Fig. 1.4 Apoptotic pathways in mammalian cell death.

The extrinsic apoptotic pathway (left) is induced by a death receptor ligand (TNF, TRAIL, FasL etc) which results in the recruitment and formation of a multiprotein complex DISC that includes the death receptor, intracellular adaptor proteins (TRADD, FADD, RAIDD) and initiator caspases (procaspase-8 or -10). The complex leads to autocatalytic processing and activation of the initiator caspase. The intrinsic pathway (right) is initiated by the majority of apoptotic stimuli, including irradiation, cytotoxic drugs, DNA damage etc. Loss of mitochondrial membrane potential and release of pro-apoptotic cell death proteins results in the formation of another multiprotein complex, the apoptosome, that includes Apaf-1, cytochrome c, ATP/dATP and the initiator caspase, procaspase 9. That complex leads to autocatalytic activation of caspase-9 and subsequent effector caspases. Pro- and anti-apoptotic bcl-2 homologues regulate the release of pro-cell death mitochondrial proteins (adapted from Gaussand, 2007).
The other caspase activation pathway in mammals is the mitochondria pathway which is characterized by a depolarization of the mitochondrial membrane and a release of mitochondrial proteins (Danial and Korsmeyer, 2004) including pro-apoptotic proteins, such as cytochrome c, into the cytosol. A cytosolic complex, the apoptosome, is formed which consists of oligomerised Apaf-1 (apoptotic protease-activating factor 1), ATP/dATP, cytochrome c and the initiator caspase, pro-caspase-9 (Riedl and Shi, 2004). Oligomerisation of Apaf-1 allows the recruitment and autocatalytic activation of caspase-9, and consequently the propagation of a death signal by proteolytic processing and activation of effector caspases (Li et al., 1997).

Apart from the pathways now described, there is another cell death pathway. That pathway involves the endoplasmic reticulum (ER), and it is known as the apoptotic pathway of ER stress-mediated cell death (Momoi, 2004). The ER is the site of assembly of polypeptide chains that are destined for secretion or routing into various subcellular compartments. The ER-initiated PCD pathway comprises the activation of caspase-12 and/or the cytochrome c-dependent apoptotic pathway.

1.9 **Yeast serves as a useful model organism to study the phenomenon of cell death**

*Saccharomyces cerevisiae* has been successfully used as model system to solve complex biological questions related to the mechanism of action of several molecules or genes or proteins. Fundamental results unraveling the deadly function of mitochondria during apoptosis execution in yeast have been achieved (Skulachev, 2006). Yeasts, both fission and budding, have been used as tools to examine the functions of bonafide regulators/effectors of metazoan apoptosis and this approach has proved valuable in shedding light on the obscure functions of the proapoptotic Bcl-2 family homologues of the CED-9 of *Caenorhabditis elegans*. Like metazoan cells, yeast cells undergo cell death showing characteristic apoptotic markers such as externalization of phosphatidylserine to the outer leaflet of the plasma membrane, DNA fragmentation and chromatin condensation (Madeo et al., 1997). Expression of either of the two mammalian proapoptotic Bcl-2 family members, BAX and BAK, in *S. cerevisiae* and
Schizosaccharomyces pombe results in cytotoxicity with similar phenotypes. Moreover, the yeast genome codes for many proteins of the basic molecular machinery executing cell death, including orthologues of caspases (Madeo et al., 2002), apoptosis inducing factor (Wissing et al., 2004), HtrA2/Omi (Fahrenkrog et al., 2004), and inhibitor of apoptosis (IAP) proteins (Walter et al., 2006). Notably, histone phosphorylation, which is considered to be a universal prerequisite for apoptosis execution (Cheung et al., 2005), was shown to be necessary for cell death induction upon oxidative stress in yeast. Ahn et al. (2006) suggested that a regulated cross-talk between deacetylation and phosphorylation within Histone H2B tails was required for induction of apoptosis in yeast. Physiological scenarios of yeast apoptosis have been demonstrated during ageing (Fabrizio et al., 2004; Herker et al., 2004), the mating process (Severin and Hyman, 2002), and also the development of yeast multicellular colonies (Vachova and Palkova, 2005).

Some features of PCD appear to be conserved from bacteria to fungi to plants and animals (Ameisen, 1996). The accumulated evidence strongly suggests that the cytotoxic effects of the expression in the yeast of mammalian BAX or BAK are relevant to the mechanism of their proapoptotic action in mammalian cells (Fraser and James, 1998) and thus yeast has become an important model to investigate the conserved steps of apoptosis. The programmed death of yeast has been linked to complex mitochondrial processes, such as cytochrome c and AIF release, channel opening upon human Bax expression, depolarization of mitochondrial membrane potential, and mitochondrial fragmentation. These mitochondrial events are more or less common to various scenarios of yeast apoptosis, namely chronological and replicative ageing, decreased actin dynamics, as well as apoptosis induced by acetic acid (Ludovico et al., 2001), H₂O₂ (Madeo et al., 1997), amiodarone, or α-factor. Especially in the fields of ageing and neurodegeneration, intricate experimental and genetic problems present in higher organisms were addressed in yeast. The discovery of an apoptotic yeast strain carrying a CDC48 mutation (Madeo et al., 1997) shed light on its mammalian orthologue VCP (Shirogane et al., 1999), which is involved in several polyglutamine triggered neurodegenerative disorders and the crucial involvement of mitochondria in the CDC48 connected yeast cell death pathway.
was also observed (Braun et al., 2006; Zischka et al., 2006). Galactose-induced expression of harpin\textsubscript{Pss} caused yeast cell death indicating that the yeast might share, with plants, conserved components in cell death pathway (Podile et al., 2001) and in both, harpin\textsubscript{Pss}-induced YCD as well as plant HR, oxidative burst seems to play a prominent role. Partial characterization of harpin\textsubscript{Pss}-induced YCD has been carried out by Sripriya (2004), and reported the following:

- Galactose-induced expression of harpin\textsubscript{Pss} caused cell death in \textit{S. cerevisiae} Y187.
- Harpin\textsubscript{Pss} has no extracellular effect on the yeast cells.
- Chromosomal condensation or genomic DNA fragmentation was not observed during harpin\textsubscript{Pss}-induced YCD.
- Loss of membrane integrity was observed in yeast cells during harpin\textsubscript{Pss} expression.
- Harpin\textsubscript{Pss}-induced YCD is independent of the stage of cell cycle.
- Yeast ‘petite’ mutants (lacking functional mitochondria) were insensitive to harpin\textsubscript{Pss}-induced YCD, indicating the possible involvement of mitochondria in this form of cell death.
- Cytochrome c release from mitochondria was not observed in harpin\textsubscript{Pss}-induced YCD.

Thus many discoveries made originally in yeast have been experimentally validated in higher eukaryotic cells, suggesting that yeast-based strategies for studying apoptotic genes will continue to provide more insights into conserved cell death mechanisms.

1.10 Bio-physical features of harpin may provide clues on the structure-function relationship of this enigmatic protein

Harpins are hydrophilic in nature, rich in leucine & glycine and highly thermal stable proteins (He et al., 1993) which retain their activity even after boiling to 90\textdegree C for 10 min. Report from Alfano et al. (1996) demonstrated that four different truncations of harpin\textsubscript{Pss} elicit similar HR activity that is indistinguishable with that of the HR elicited by full length harpin\textsubscript{Pss} in tobacco. Similarly, Sripriya (2004), also showed that, truncation of the protein either towards the \textit{N}-terminal or \textit{C}-terminal region caused cell death during in
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Harpins exhibit the property of forming amyloid like fibrils and possess helical structure (Oh et al., 2007) and the predicted α-helix in the N-terminal region of HpaG (harpin from Xanthomonas axonopodis) was supposed to be responsible for the HR elicitor activity and mutations in the α-helix region of HpaG abolished the HR elicitation activity (Kim et al., 2004 and Wang et al., 2008). Harpin\textsubscript{Pss} exists as a polydispersed, multimeric protein in nature, the forms include, dimer, trimer, tetramer and octamer (Chen et al., 1998) and the functional significance for the existence of the protein in many oligomeric forms, still remains unclear. Interestingly harpin\textsubscript{Pss} has a single ‘Tryptophan’ (W167) residue which makes it a protein of special interest for fluorescence quenching studies. Harpin\textsubscript{Pss} sequence analysis reveals the dissimilarity in amino acid sequence (He et al., 1993) or the absence of homology with any known protein in the protein data base, which makes it a peculiar biological molecule for functional or structural studies. Above all, the crystal structure of harpin (any of the known harpins) has not been elucidated till date and which really makes it a challenging biological molecule and one of the top proteins on the earth. Biophysical techniques like circular dichroism (CD), differential scanning calorimetry (DSC) and dynamic light scattering (DLS) have been extremely useful in revealing the structural and functional aspects of many biological macromolecules with special reference to proteins. CD was extensively used in studying the secondary structural confirmations of proteins like Cytochrome c and its interactions with Cytochrome c oxidase (Michel et al., 1989). DSC and DLS have been used for studying the thermal denaturation and light scattering or protein-ligand interactions of hexokinase (Barone et al., 1995) and calmodulin (Andriyka et al., 2002). Although harpin\textsubscript{Pss} has been extensively characterized biochemically and via mutational analysis, speculation of HR elicitation and high thermal stability of the protein have not been well addressed from a structural point of view. It would be quite interesting to look at the reasons for oligomerization from a structural point of view. CD analysis reveals that the protein is predominantly α-helical (51.5%) and the amino acid sequence shows high percentage (13.5%) of leucine (He et al., 1993). These two clues suggested that a leucine-zipper like
motif might be present in harpin\textsubscript{Pss} as leucine-zipper is a motif, responsible for the dimeric or oligomeric form in many \(\alpha\)-helical proteins (Landschulz \textit{et al.}, 1988). A leucine zipper is a super secondary structural motif found in proteins that creates adhesion forces in parallel alpha helices (Landschulz \textit{et al.}, 1988). Each half of a leucine zipper consists of a short alpha-helix with a leucine residue at every seventh position, known also as the heptad repeat. The standard 3.6 residues per turn alpha-helix structure changes slightly to become 3.5 residues per turn alpha-helix. Leucine zipper motifs are protein-protein dimerization or oligomerization motifs consisting of heptad repeats of leucine or hydrophobic amino acid residues that form a coiled-coil structure (O'Shea \textit{et al.}, 1989; Gonzalez \textit{et al.}, 1996). Classical coiled-coil proteins share a characteristic seven-amino acid repeat containing hydrophobic side chains at the first (a) and fourth (d) positions (Lupas \textit{et al.}, 1991, Conway and Parry, 1990). The lack of structure-activity relationship prompted us to take up the bio-physical studies of the protein and to look for common structural motifs among harpins which might be responsible for the functional nature of the molecule.

\textbf{1.1 Objectives of the present study}

Against this background, we have set two major objectives to understand more about the harpin\textsubscript{Pss}-mediated cell death and the structure-function relationship of this protein viz.

1) Study of harpin-mediated cell death events:

   a) in yeast as a model system to decipher the physiological mechanism of action of harpin\textsubscript{Pss}.

   b) on Jurkat cell lines (human T-cell lymphoma cell line) to test the effectiveness of harpin\textsubscript{Pss}.

2) Investigation on the bio-physical features of harpin\textsubscript{Pss} for a better understanding of the structure- function relationship of the protein.