Characterization of yeast cell death caused by harpin from *Pseudomonas syringae* pv. *syringae*

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

By

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CERTIFICATE

This is to certify that Ms. P. Sripriya has carried out the research work embodied in the present thesis under my supervision and guidance for a full period prescribed under the Ph.D. ordinance of this University. We recommend her thesis ***"Characterization of galactose-inducible yeast cell death caused by harpin from Pseudomonas syringae pv. syringae"*** for submission for the degree of Doctor of Philosophy of the University.

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DECLARATION

I hereby declare that the work embodied in this thesis entitled ***"Characterization of galactose-inducible yeast cell death caused by harpin from Pseudomonas syringae pv. syringae"*** has been carried out by me under the supervision of Prof. Appa Rao Podile and this has not been submitted for any degree or diploma in any other University earlier.

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Priya

Dedicated
to
Almighty
&
Beloved Parents

CONTENTS

ABBREVIATIONS

µg	:	microgram
µM	:	micro molar
^{0}C	:	degree centrigrade/degree Celsius
BCIP	:	5-bromo-4-chloro-3-indolyl phosphate
ALP	:	alkaline phosphatase
bp	:	base pair
C-terminal	:	carboxy terminal
Cyt C	:	cytochrome C
DAPI	:	4'6-diamino phenyl indole
DNA	:	deoxy ribonucleic acid
DTT	:	dithiothreitol
Ea	:	*Erwinia amylovora*
EDTA	:	ethylene diamine tetra acetic acid
FDA	:	fluoresceine diacetate
g	:	gram
h	:	hour(s)
Hrp	:	harpin
hrpZ	:	gene encoding harpin
IPTG	:	isopropyl ß-D-thiogalactoside
kb	:	kilobase pair
kAc	:	potassium acetate
kDa	:	kilodalton
l	:	litre
LB	:	Luria-Bertani
LS	:	leader sequence
mg	:	milligram
min	:	minute
ml	:	milliliter
mM	:	millimolar
NBT	:	nitroblue tetrozolium

Ni-NTA	:	Nickel-nitroacetic acid agarose
nm	:	nanometers
N-terminal	:	amino terminal
OD	:	optical density
ORF	:	open reading frame
PAGE	:	polyacrylamide gel electrophoresis
PBS	:	phosphate buffered saline
PCR	:	polymerase chain reaction
PEG	:	polyethylene glycol
PI	:	propidium iodide
pmole	:	picomole
Pss	:	*Pseudomonas syringae* pv. *syringae*
Psph	:	*Pseudomonas syringae* pv. *phaseolicola*
Pst	:	*Pseudomonas syringae* pv. *tomato*
PVDF	:	polyvinylidene difluoride
rpm	:	revolutions per minute
SD		synthetic drop out media
SDS	:	sodium dodecyl sulphate
ssDNA	:	single stranded DNA
TE	:	TRIS-EDTA
TEMED	:	N,N,N',N'-tetramethylene diamine
TTC	:	2,3,5-triphenyl tetrazolium chloride
Tris	:	tris-(Hydroxymethyl) aminoethane
UV	:	ultraviolet
V	:	volts
YCD	:	yeast cell death
YMM	:	yeast minimal media

LIST OF TABLES

LIST OF FIGURES

Introduction

Cell wall reinforcement, phytoalexin production and the accumulation of anti-microbial properties are effectively employed in plant defense reactions to stall the infection process. Their temporal and spatial regulations are the decisive factors governing the outcome of the host-pathogen interactions. In numerous incompatible interactions, these reactions are often associated with the death of a small number of cells, at and around the site of infection, often referred to as the 'hypersensitive response' (HR). Membrane damage, necrosis, and collapse of challenged cells are the common features of the HR, but the early stages of the response remain poorly defined. HR is a best-studied form of genetically regulated programmed cell death (PCD) in plants (Greenberg *et al.*, 1994) and shares some phenotypic features with apoptosis (Levin *et al.*, 1996). Cell death that occurs as a part of normal developmental processes, is referred to as PCD. Gilchrist (1998) reviewed some of the fundamental characteristics of apoptosis in animals and points to a number of connections to PCD in plants that may lead to both a better understanding of the HR and novel strategies for engineering disease resistance in plants. The HR and other examples of cell death in plants, therefore, have become the focus of intensive research.

1.1 HR limits pathogen growth and activates defense genes in plants

Plant HR is a means to eliminate the microbes that have a potential to cause plant disease. The most common expression of host resistance, and a frequent expression of non-host resistance, is the HR, a rapid death of cells at the infection site that is associated with pathogen limitation as well as with defense gene activation (Goodman and Novacky, 1994). The HR serves to inhibit the growth of the invading pathogen by killing infected and uninfected cells and producing a physical barrier composed of dead plant cells. The rapid dehydration that follows the death of plant tissue may also have deleterious effects on pathogen growth by limiting the available nutrients. In addition, during the HR, dying plant cells strengthen their cell walls and accumulate certain toxic compounds such as different phenolics and phytoalexins (Dangl *et al.*, 1996). Bacteria, fungi, and viruses induce different types of cell death with different morphological and physiological characteristics, and at a different rate. This function may be similar to the activation of apoptosis in response to infection with viruses or bacteria in animal cells (Mittler and Lam, 1996). Thus, activation of cell death as a means of preventing further infection by an invading pathogen appears to be a general theme in the biology of

multicellular organisms. Plant HR is triggered by (a) interaction of '*R-avr*' gene products or (b) pathogen-derived molecules.

1.1.1 Interaction of '*R-avr*' gene products: HR is one of the classical examples of disease resistance with a strong genetic basis, often referred as "gene-for-gene" interaction. It occurs as a result of an interaction between pathogens' avirulence (*avr*) gene and the hosts' resistance (*R*) gene (Flor, 1955) and postulated that pathogens contain a specific '*avr*' gene whose expression is recognized by a corresponding '*R*' gene in the plant. When matching *avr-R* genes are present, 'incompatible' interaction occurs (the plant is resistant and the pathogen is avirulent). A 'compatible' interaction occurs if either the plant lacks the appropriate *R* gene or the pathogen lacks the corresponding *avr* gene. The compatible or incompatible interaction is determined by the alleles of one specific plant *R* gene and a pathogen's *avr* gene. If the plant carries the correct *R* gene, and if the pathogen carries the corresponding *avr* alleles, recognition occurs and the HR follows. However, if the plant does not carry the *R* allele (but rather the non-functional allele *r*), or if the pathogen does not have the *avr* allele (recessive allele *avr*), there is no recognition and the result is disease (Ellingboe, 1976).

1.1.2 R-proteins as guards of cellular machinery: The 'guard hypothesis' provides an intriguing conceptual framework for the action of disease effectors and the R-protein complex. Nucleotide-binding leucine-rich-repeat (NB-LRR) proteins constitute the major R-protein class and specify in gene-for-gene plant resistance to animal, fungal, bacterial and viral pathogens, and collectively constitute a comprehensive pathogen-detection system (Hammond- Kosack and Jones, 1997; Milligan *et al.*, 1999; Wang *et al.*, 1999; Rossi *et al.*, 1998; Vos *et al.*, 1998). This innate, genetic recognition-response apparatus resembles the animal immune system. R-proteins might detect the association of plant pathogenicity targets with pathogen virulence factors that are then destined to become Avr products. Guard hypothesis was put forward to rationalize why Pto protein kinase requires the NB-LRR protein Prf to activate defense upon recognition of AvrPto. According to this model, Pto is a general component of host defence, perhaps in a pathway for response to nonspecific elicitors of phytopathogenic bacteria (van der Biezen and Jones, 1998). Conceivably, one particular Avr product corresponds to one specific pathogenicity target, which, in turn, could be safeguarded by one matching R protein. In order to adapt rapidly to pathogen Avr modification or loss, novel

recognition speificities in R proteins are created through the generation of sequence variation in the β–sheet (functions as a ligand-binding surface) of the LRR domains (Parniske *et al.*, 1997; Thomas *et al.*, 1997).

The guard hypothesis provides a step beyond the previous notion that R proteins are simply direct receptors for Avr proteins. This elicitor/receptor model may still be true for some systems, but for many others, the lack of direct R/Avr interaction is sufficiently convincing that it can be excluded (Dangl and Jones, 2001).

1.1.3 Pathogen-derived molecules trigger plant HR: A variety of fungal products elicit inducible defensive plant responses in both host and non-host plants, and that such responses can also be triggered by plant products released during cell-wall degradation. These 'non-specific elicitors' are the prime inducers of defense responses in non-host plant-pathogen interactions. Evidence suggests that cryptogein, one family of proteinaceous elicitors produced by *Phytophthora* species, has binding sites on cells from both plant species that do and that do not defensively respond to the elicitor raising important questions about receptor and signal pathway differences between species (Bourque *et al.*, 1999).

Non-specific elicitors from bacteria have been the cell-death-eliciting harpins, heat-stable proteins, encoded by members of the *hrp* (hypersensitive response pathogenicity) gene cluster of some Gram-negative bacteria (Collmer *et al.*, 2000). The harpin binds to the lipid bilayers and forms an ion-conducting pore permeable to the cations (Lee *et al.*, 2001).

1.2 Programmed cell death (PCD) is essential in plants' life

In plants, PCD is essential for development and survival. Available evidences suggest that plant cell death, in some cases might be mechanistically similar to apoptosis in animal cells, since the dying plant cells appear morphologically similar to apoptotic cells (Bennetzen *et al.*, 1988; Bent 1992). In addition, some types of plant cell death are accompanied by DNA cleavage, often with the characteristics of endonucleolytically processed DNA, one hallmark of apoptosis (Bennetzen *et al.*, 1988; Bent 1992; Bestwick *et al.*, 1995; Bowler *et al.*, 1989). Typical apoptotic events include the fragmentation of DNA into internucleosomal-sized fragments with 3'OH ends indicative of

endonucleolytic cleavage (DNA ladders), membrane blebbing, nuclear condensation, and fragmentation with nucleic acids found in membrane-bound vessels (apoptotic bodies), and cytoplasmic condensation (Greenberg 1997).

1.2.1 Mitochondria play a key role in inducing disease resistance and cell death in plants: Involvement of mitochondria in pathogen-induced plant defense responses has long been implicated. Salicylic acid (SA)-induced tobacco resistance to Tobacco mosaic virus (TMV) is sensitive to salicylhydroxamic acid (SHAM), an inhibitor of the terminal oxidase of the mitochondrial alternative pathway (Chivasa *et al.*, 1997). The respiratory inhibitors like antimycin A and cyanide induced both the accumulation of alternative oxidase transcript and resistance to TMV. Cyanide also restored *N* gene-mediated resistance to TMV in transgenic tobacco expressing the salicylate hydroxylase (nahG) gene (Chivasa and Carr, 1998). These findings suggest that certain functions of plant mitochondria do play an important role in SA-induced disease resistance. The mitochondrial connection to PCD in plants was suggested by Lacomme and Santa Cruz (1999). This may act via leakage of cytochrome C, which has not been demonstrated in plant PCD. Altered mitochondrial functions play an important role in harpin-induced hypersensitive cell death in tobacco (Xie and Chen, 2000).

1.2.2 The HR: An example of PCD

The HR is genetically controlled and coordinately regulated with other defense-related biochemical events typical of the resistant response (Dietrich *et al.*, 1994; Dinesh-Kumar and Baker, 2000). Plants must have active protein synthesis machinery to show an HR induced by bacteria (Croft *et al.*, 1990; Keen *et al.*, 1981). Purified bacterial elicitors of the HR require plants to have active metabolism to show cell death (He *et al.*, 1993). Over production of a component of the *R* gene *Pto* signal transduction pathway of tomato expressed in tobacco caused an amplification of the HR. Mutants exist in maize, rice, tomato, barley and also in *Arabidopsis* that mimic the effect of infection in the absence of the pathogen (Jones and Dangl, 1996). The *Arabidopsis* mutant phenotypes suggest that the genes defined represent the steps along normal disease resistance response pathways.

1.3 Phytopathogenic bacteria

Phytopathogenic bacteria generally have limited host ranges, often confined to members of a single plant species or genus. This appears to result from negative factors restricting the host range rather than from positive factors, which allow the pathogen to infect its hosts. Phytopathogenic bacteria specialize in colonizing the apoplast and from this location outside the walls of living cells they incite diseases in most cultivated plants to cause rots, spots, vascular 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*. Two features characterize bacteria-plant relationships. First, during their parasitic life, most bacteria reside within the intercellular spaces of the various plant organs or in the xylem. Second, many cause considerable plant tissue damage by secreting toxins, extracellular polysaccharides (EPSs), or cell wall-degrading enzymes at some stage during pathogenesis.

Several bacterial genes, referred to collectively as the hypersensitive response and pathogenicity cluster (*hrp* cluster), are absolutely required for bacterial pathogenesis. Many *hrp* gene sequences from plant pathogenic bacteria are very similar to the genes required for pathogenesis in bacteria that infect animals, which suggest that these distinct pathogens utilize similar virulence strategies and emphasize that during the evolution of bacterial colonization of animals and plants, certain common mechanisms may have been retained (Alfano *et al.*, 2000; Cao *et al.*, 2001; Keen *et al.*, 2000).

1.4 Type III secretion system (TTSS)

Genetic and molecular studies unraveled important mechanisms underlying bacterial pathogenicity. The molecular cross-talk between pathogens and their host is a specified protein delivery system, the type III secretion system (TTSS). TTSSs are present in many Gram-negative pathogens of both plants and animals (Hueck, 1998; Galan and Collmer, 1999; Cornelis and Gijsegem, 2000). 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 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 ORF1 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 genome of *P. syringae* pv. *tomato* DC3000 revealed 33 confirmed effectors and several effector candidates (Buell *et al.*, 2003; Collmer *et al.*, 2002; Guttman *et al.*, 2002; Petnicki-Ocwieja *et al.*, 2002; Zwiesler-Vollick *et al.*, 2002). Some effectors can block the ability of other 'masked' effectors to trigger the HR, which suggested that they may allow subversion of the HR and lead to disease development. Indeed, several *P. syringae* effectors were recently shown to suppress plant defenses (Abramovitch *et al.,* 2003; Axtell and Staskawicz 2003; Bretz *et al.*, 2003; Espinosa *et al.*, 2003; Mackey *et al.*, 2003). Several of the effectors that suppress the HR can also suppress Bax-triggered PCD in yeast and plants. $AcrPphE_{Pto}$, $AvrPpiBl_{Pto}$, HopPtoE, AvrPtoB, HopPtoF, and HopPtoG effectors possess suppressor activity (Jamir *et al.*, 2004), which provides a global picture of the capacity of this bacterium to regulate PCD pathways in plants.

1.5 Harpins are the proteinaceous elicitors of phytopathogenic bacteria

Harpins, bacterial proteinaceous elicitors of HR, have been isolated from Gram-negative phytopathogenic bacteria (Table 1). HrpN of *E. amylovora* was the first Hrp protein shown to elicit an HR in tobacco (Wei *et al.*, 1992), secreted *via* the TTSS system. The properties of HrpN define the characteristics of the class of HR elicitors known as harpins. They are hydrophilic, rich in glycine, heat stable, lack cysteine, and elicit an HR when infiltrated into the apoplast of certain plants (Bauer *et al.*, 1995). The *P. syringae* pv. *syringae* 61 *hrpZ* gene encodes $harpin_{Pss}$, a 34.7 kD extracellular protein that elicits HR in tobacco and other plants. Interestingly, four truncated harpin peptides, of different sizes, elicit HR that is indistinguishable from that of full-length harpin (Alfano *et al.*, 1996).

Table 1 Harpins of phytopathogenic bacteria.

Organism	Gene	Protein Size (kDa)
Erwinia amylovora	*hrpW*	45
Erwinia carotovora	*hrpN*	36.3
Erwinia chrysanthemi	*hrpN*	34.7
Pseudomonas syringae pv. *syringae*	*hrpZ*	34.7
Pseudomonas syringae pv. *phaseolicola*	*hrpZ*	35
Pseudomonas syringae pv. *tomato*	*hrpZ*	38.4
Ralstonia solanacearum	*popA*	34.5

As a characteristic feature of proteins secreted by TTSS, harpins lack N-terminal signal peptide (Wei *et al.*, 1992). Detailed studies revealed that harpins differ substantially in their primary structure and their contribution to Hrp phenotypes, and their actual function is unknown (Arlat *et al.*, 1994; Bauer *et al.*, 1995; Cui *et al.*, 1996; He *et al.*, 1993). $Harpin_{Pss}$, a 34.7kDa extracellular protein, is glycine-rich, dissimilar in amino acid sequence to any known protein, and is produced only in apoplastic fluid-mimicking minimal media (Alfano *et al.*, 1996).

The harpin genes of *E. amylovora* (*hrpN*) (Wei *et al.*, 1992), *E. chrysanthemi* ($hrpN_{Ech}$) (Bauer *et al.*, 1994) and *R. solanacearum* (*PopA*) (Arlat *et al.*, 1994) are located adjacent to or near their respective *hrp* clusters, whereas the *P. syringae hrpZ* gene resides within a *hrp* operon (He *et al.*, 1993). *E. chrysanthemi hrpN* mutants are reduced in infectivity at low inoculum levels and are unable to elicit the HR (Bauer *et al.*, 1994), but harpin gene mutations in *E. amylovora* CFBP1430 (a highly virulent strain) (Baray, 1995), *R. solanacearum* (Arlat *et al.*, 1994), and *P. syringae* (Alfano *et al.*, 1996) produce weak phenotype or no phenotype. The potential role of harpins in determining host specificity is uncertain. *PopA* may be a host specificity factor because the isolated protein elicits the HR selectively in those plants in which *R. solanacearum* also elicits the HR, whereas the isolated harpin from *E. amylovora* and three *P. syringae* pathovars trigger the HR in various plants in a manner that shows no relationship to bacterial host range (He *et al.*, 1994; He *et al.*, 1993; Wei and Beer, 1993). The $harpin_{Psph}$ resembles that *hrpZ* gene products identified in *P.syringae* pvs. *glycinea*, *syringae*, or *tomato* (Preston *et al.*, 1995).

$Harpin_{Ea}$-induced responses in tobacco suspension cells such as oxidative burst (Baker *et al.*, 1993), extracellular alkalinization, active oxygen species production, and membrane depolarization are blocked by lanthanum chloride, a Ca^{++} channel blocker and K252a, a protein kinase inhibitor (Baker *et al.*, 1993, He *et al.*, 1993, Popham *et al.*, 1995). Though structurally different, both cause immediate K^+ efflux and extracellular alkalinization in tobacco suspension-cultured cells (Wei *et al.*, 1992, He *et al.*, 1993, Popham *et al.*, 1995). These events suggest that $harpin_{Pss}$ triggers a signal transduction pathway that involves active oxygen species production, protein phosphorylation, and Ca^{++} influx. Alkalinization was induced immediately after addition of different concentrations of full-length $harpin_{Pss}$. The pH change caused by the same concentrations of truncated $harpin_{Pss}$ is similar in magnitude to the changes caused by the full-length protein (Alfano *et al.*, 1996).

The *hrpZ* gene product from the bean halo-blight pathogen, *Pseudomonas syringae* pv. *phaseolicola* ($HrpZ_{Psph}$), is secreted in an *hrp*-dependent manner and exported by the TTSS. $HrpZ_{Psph}$ associates stably with liposomes and synthetic bilayer membranes (Lee *et al.*, 2001). $HrpZ_{Psph}$-related proteins from *P. syringae* pv. *tomato* or *syringae* triggered ion current similar to those stimulated by $HrpZ_{Psph}$. The $HrpZ_{Psph}$-mediated ion-conducting pore was permeable for cations but did not mediate fluxes of Cl^-. Such pore-forming activity may allow nutrient release and/or delivery of virulence factors during bacterial colonization of host plants.

1.5.1 Harpin function is intriguing: The function of $harpin_{Pss}$ is particularly puzzling. Several observations suggest a simple, direct role for HrpZ in HR elicitation. HrpZ is the predominant protein secreted by the *P. syringae* Hrp system in culture (He *et al.*, 1993; Yuan and He, 1996), the *hrpZ* gene is conserved in divergent *P. syringae* pathovars (Preston *et al.*, 1995), and the isolated protein elicits an apparent PCD in plants that is indistinguishable from HR elicited by living bacteria (He *et al.*, 1993). Furthermore, *hrpZ* deletion mutations in the cosmid pHIR11 functional cluster of *P. syringae* pv. *syringae hrp* genes strongly reduce the HR elicitation activity of *E. coli* cells carrying only pHIR11. The same mutation only slightly reduces the HR in *P. syringae* pv. *syringae*, postulating that possibly due to a second harpin encoded elsewhere in the bacterial genome (Alfano *et al.*, 1996).

However, other observations show that the relationship of HrpZ to HR elicitation is more complex. Mutation of *hrmA* (Hue and Hutchenson, 1993), which is in a variable region flanking the conserved *hrp* cluster in pHIR11, abolishes HR activity in tobacco without diminishing HrpZ synthesis or secretion (Alfano *et al.*, 1996). Thus, isolated HrpZ was sufficient to elicit a HR in tobacco leaves but HrpZ produced by bacteria in plants is not. Instead, HrmA, with no apparent function in the Hrp secretion apparatus, is necessary for bacterial elicitation of the HR, and thus, HrmA appears to be the actual elicitor of the HR produced by bacteria carrying pHIR11. HrmA has several characteristics of an Avr protein (Alfano and Collmer, 1997).

Mutations in the *hrpN* gene abolish the ability of *E. amylovora* to elicit the HR in non-host tobacco to elicit disease in highly susceptible pear fruit (Wei *et al.*, 1992). In contrast, the *P. solanacearum* PopA1 protein also elicits the HR in tobacco but *PopA* mutants retain their ability to elicit the HR in this and other non-host plants and

to incite disease in tomato (Arlat *et al.*, 1994). Mutants in $hrpN_{Ech}$ have an intermediate phenotype of abolished HR elicitation and reduced frequency of infection (Bauer *et al.*, 1994). Therefore, the relative contribution of these elicitors to plant-bacterium interactions varies.

1.6 Structure-function relationship

Comparative or homology protein structure modeling builds a three-dimensional model for a protein of unknown structure (the target) based on one or more related proteins of known structure (the templates) (Blundell *et al.*, 1987; Greer, 1981; Johnson *et al.*, 1994; Sali and Blundell, 1993; Sali, 1995, Sanchez and Sali, 1997; Marti-Renom *et al.*, 2000; Fiser *et al.*, 2001, Fiser *et al.*, 2002, Sanchez and Sali, 2000). The necessary conditions for calculating a useful model are (1) detectable similarity between the target sequence and the template structures and (2) availability of a correct alignment between them. The comparative approach to protein structure prediction is possible because a small change in the protein sequence usually results in a small change in its 3D structure (Chothia and Lesk, 1986). It is also facilitated by the fact the 3D structure of proteins from the same family is more conserved than their primary sequences (Lesk and Chothia, 1980). Therefore, if the similarity between two proteins is detectable at the sequence le vel, structural similarity can usually be assumed. Moreover proteins that share low or even non-detectable sequence similarity many times also have similar structures. Ab initio structure prediction (Bonneau and Baker, 2001), another method of protein structure prediction, where the structure of the protein's native state is predicted from the protein's amino acid sequence. It is generally assumed that a protein sequence folds to a native conformation or ensemble of conformations that is at or near the global free-energy minimum. Thus, the problem of finding native-like conformations for a given sequence can be decomposed into two subproblems: a) developing an accurate potential and (b) developing an efficient protocol of searching the resultant energy landscape. Several methods have made good predictions in the ab initio category, and some ab initio methods outperformed fold recognition methods for certain proteins in the fold recognition categoty (Murzin, 1999; Orengo *et al.*, 1999; Orengo *et al.*, 1999). Despite progress in ab initio protein structure prediction, comparative modeling remains the only method that can reliably

predict the 3D structure of a protein with accuracy comparable to a low-resolution experimentally determined structure (Marti-Renom *et al.,* 2000).

He *et al.* (1993) analysed the DNA sequence and predicted its product harpin$_{Pss}$, to be a glycine-rich protein with no extensive similarity to known proteins. Harpin$_{Pss}$ has no significant sequence similarity with sequences deposited in major sequence databases accessible with the Blast search program (Altschul *et al.*, 1990), nor were motifs of known biological significance detected for harpin $_{Pss}$ using the MOTIF program. However, they found an intriguing, albeit limited, significa nce similarity was detected between harpin$_{Pss}$ and harpin $_{Ea}$ over a stretch of 22 amino acids He *et al.* (1993). Because the gene encoding harpin$_{Pss}$ showed little relationship with the *hrpN* gene of *E. amylovora* and encodes the apparent end product of the *P. s.* pv. *syringae* 61 *hrp* cluster, it was designated *hrpZ*. He *et al.* (1993) also reported that carboxy-terminal 148 amino acid portion of harpin$_{Pss}$ contains two directly repeated sequences of GGGLGTP and QTGT and is sufficient and necessary for elicitor activity. The same group showed that all four HrpZ fragments elicit an HR that is indistinguishable from HrpZ-elicited HR in tobacco (Alfano *et al.,* 1996). Since these HrpZ fragments represent non-overlapping fragments, it was concluded that the elicitor activity of HrpZ is not confined to one region on the protein.

1.7 Protein crystallization

The crystallization of proteins currently has three major applications: (1) structural biology and drug design, (2) bioseparations, and (3) controlled drug delivery. In the first application, the protein crystals are used with the techniques of protein crystallography to ascertain the three-dimensional structure of the molecule. This structure is indispensable for correctly determining the often complex biological functions of these macromolecules. The design/drugs is related to this, and involves designing a molecule that can exactly fit into a binding site of a macromolecule and block its function in the disease pathway. Producing better quality crystals will result in more accurate 3D protein structures, which in turn means its biological function can be known more precisely, also resulting in improved drug design.

Modern crystallography is intimately linked with the ability of crystals to diffract X-rays. The resulting diffraction profile can be used to determine the structure of the crystal, as well as the 3D molecular structure of the crystalline material. The ability to

know the precise molecular structure of biological macromolecules has revolutionized the study of their functions in many fields of biology. The process of determining this structure begins with the crystallization of the macromolecule.

Many plant proteins have been crystallized, to mention a couple of them; the banana lectin, 29.4kD from *Musa paradisiaca* has been isolated, purified and crystallized. The structure of the subunit was found to be similar to that of jacalin-like lectins (Singh *et al.*, 2004). Mexicain, a 23kD papain-like cystein protease from the tropical plant *Jacaratia mexicana*, was purified and crystallized (Oliver-Salvador *et al.*, 2004).

1.8 Yeast serves as a useful model to study the cell death machinery of eukaryotes

Caenorhabditis elegans has provided information from a genetic screen for genes that regulate and execute PCD in basal metazoan cell death machinery, which includes *ced*-9, *ced*-3, and *ced*-4. Yeasts, both fission and budding, have been used as tools to examine the functions of bonafide regulators/effectors of metazoan apoptosis. This approach has proved valuable in shedding light on the obscure functions of the proapoptotic Bcl-2 family homologues of the CED-9 of *C. elegans*. Expression of either of the two mammalian proapoptotic Bcl-2 family members, BAX and BAK, in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* results in cytotoxicity with similar phenotypes in each case (Greenhalf *et al.*, 1996; Ink *et al.*, 1997).

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 yeast may become an important model to investigate the conserved steps of apoptosis. Similar properties of Bax or Bak are required to kill both mammalian and yeast cells (Shaham *et al.*, 1998), and a Bax inhibitor-1 (BI-1) blocks the cell death induced by Bax-over expression (Xu and Reed, 1998). An evolutionarily conserved plant homologue of the BI-1 gene has been detected that is capable of the suppression of Bax-induced cell death in yeast (Kawai *et al.*, 1999). Mitochondria can play a central role in apoptosis (Desagher and Martonan, 2000) and also appear to be involved in harpin-induced HR (Xie and Chen, 2000). Boccara *et al.* (2001) by

adapting infra-red thermography, revealed a role for mitochondria in pre-symptomatic cooling during harpin-induced hypersensitive response. They showed the affect in complex I structure and function and over-expressing alternative oxidase, indicating that they are directly or indirectly mediated by mitochondrial function. Recently it was reported by Krause and Durner (2004) that treatment of *Arabidopsis* cells with harpin protein induced a rapid release of cytochrome C from mitochondria into the cytosol, which is regarded as a hallmark of apoptosis.

Conditional expression of $harpin_{Pss}$ caused yeast cell death (YCD) indicating that yeast might share, with plants conserved components in cell death pathway (Podile *et al.,* 2001). In the $harpin_{Pss}$-induced plant HR and YCD, oxidative burst plays a role and a protein kinase inhibitor (K252a) suppresses the cell death.

Objectives of the present study

The present study is focused on characterization of the conditional expression of $harpin_{Pss}$-mediated YCD and the structure-function relationship of $harpin_{Pss}$, an unusual peptide.

Materials and Methods

2.1 Growth media

2.1.1 Yeast media: Strains were grown with aeration at 30^{0}C in YEPD medium (1% (w/v) yeast extract, 2% (w/v) bactopeptone, and 2% (w/v) glucose) and Yeast Minimal Medium (YMM) containing a yeast minimal base (Difco), ammonium sulfate (Sigma-Aldrich), and tryptophan/uracil dropout supplement (Clontech) with glucose or galactose (2%) (Sigma-Aldrich) as the source of carbon (referred to as glucose/galactose-containing medium, respectively). GGYP (4% (v/v) glycerol, 0.2% (w/v) glucose, 1% (w/v) yeast extract, 1% (w/v) bactopeptone, and 3gm/l KH_2PO_4) and YEPG (YEPD with glucose replaced by 3% (v/v) glycerol) was used to study the 'petite' phenotype.

2.1.2 *Escherichia coli* media: LB (1% tryptone, 0.5% yeast extract and 1% sodium chloride) was used to grow *Escherichia coli* cultures with appropriate antibiotic.

2.2 Strains, plasmids, primers and constructs

Strains of *Saccharomyces cerevisiae* (Y187, DY150, W303, Sey211, BY4741, and BJ2168 (Table 2) and *E. coli* DH5α and M15 were used. pQE30 (Qiagen) and pYEUT (Prof. T-Y Feng, Taiwan) were used to express harpin$_{Pss}$ in *E. coli* and *S. cerevisiae* respectively. The details of the primers and constructs used in the present study are given in Tables 3 and 4 respectively. To use tryptophan auxotrophy as a selectable marker in *S. cerevisiae* Y187 (Clontech), the complete ORF of TRP1 gene from pSR424 was PCR amplified adding *Nsi* I (5') and *Eco* RI (3') restriction sites and cloned into pYEUra3 (Clontech) with galactose-inducible GAL1 promoter. The modified vector, designated as pYEUT (7.45kb) (Podile *et al.,* 2001) was kindly provided by Prof. T-Y Feng, Academia Sinica, Taiwan.

A 1.02kb *hrpZ* encoding full-length harpin$_{Pss}$ was obtained from p*hrpZ* as a *Bam* HI (5') and *Xho* I (3') fragment (Podile *et al.*, 2001). Twelve different truncated mutants of *hrpZ* were amplified using PCR primers (Table 3) that add *Bam* HI (5') and *Xho* I (3') restriction sites and appropriate translation initiation and termination codons, using pSYH10 (carrying complete *hrpZ* ORF) as templa te. PCR cycling conditions are described in Table 5.

The products were cloned into the *Bam* HI and *Xho* I site of pYEUT under the regulatory control of the GAL1 promoter in which the expression of *hrpZ* and the

Table 2 Details of strains of *Saccharomyces cerevisiae*.

Yeast strains	Genotypes
Y187	MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, met$^-$, gal80Δ, URA3::GAL1$_{UAS}$-GAL1$_{TATA}$-lacZ
BY4741	his3, leu2, met1, ura3
W303	trp-1, leu2-3, ura3-1, his3-11, ade2-1, can1-100
DY150	ura3, leu2, trp1, ade2, his3, can1
Sey6211	ade2-1, his3, trp1, ura3-52
BJ2168	prb1-1122, prc1-407, pep4-3, trp1, leu2, ura3-52

Table 3 Details of primers used in the present study.

Primer	Sequence (5' to 3')	Targetgene	5'/3'	Rest.Site added
Mut 1	CG*GGATCC*CGATGCAGAGTCTCAGTCTTAACA	Pss	5'	*Bam* HI
C-1-5	CG*GGATCC*CGATGACCAAGCAGGATGGCGGGA	C-1	5'	*Bam* HI
C-2-5	CG*GGATCC*CGATGGACCGTGGCCTGCAATCGG	C-2	5'	*Bam* HI
C-3-5	CG*GGATCC*CGATGAACACCCCGCAGACCGG	C-3	5'	*Bam* HI
C-4-5	CG*GGATCC*CGATGGATCTTGATCAGTTAGCTGGG	C-4	5'	*Bam* HI
C-5-5	*CGGGATCCCGATGACGCTCAAGGATGCCGG*	*C-5*	5'	Bam HI
C-6-5	AA *GGA TCC* ATG CAG TCG AGC GCT G	C-6	*5'*	*Bam HI*
C-7-5	AA *GGA TCC* ATG ACG CTG CTG CAA	C-7	*5'*	*Bam HI*
N-1-3	CCG*CTCGAG* CTGCTGTCCGGTACCCGAGGCG	N-1	3'	*Xho* I
N-2-3	CCG*CTCGAG* ATCCATGAACTGCGCGATCTTG	N-2	3'	*Xho* I
N-3-3	AA CTC GAG TTA GTT TGC CCA ATG GCG	N-3	3'	*Xho* I
N-4-3	AA *CTC GAG* TTA TCT CGG TTT CAG GAC	N-4	3'	*Xho* I
Mut 6	CCG*CTCGAG*TCAGGCTGCAGCCTGATTGCGG	Pss	3'	*Xho* I
Mut 6a	CCG*GAGCTC*TCAGGCTGCAGCCTGATTGC	Pss	3'	*Sac* I
NCLF	*GGATCC*ATGCGCAACGGTCAACTCGA	NCL	5'	*Bam* HI
NCLR	G*CTCGAG* TTACCAGCAACTGGTCAAG	NCL	3'	*Xho* I
Psph-F	AA*GGATCC*ATGAAGAGTCTCAGTCTTAAC	Psph	5'	*Bam* HI
Psph-R	AA*TCTAGA*TCAGGCAGCAGCCTGGTTTTTA	Psph	3'	*Xba* I
Pst-F	AA*GGATCC*ATGCAAGCACTTAACAGCATCA	Pst	5'	*Bam* HI
Pst-R	AA*TCTAGA*TCAGGCCACAGCCTGGTTAG	Pst	3'	*Xba* I
LSF	AA*GGATCC*CTCAAAATGAGATTTCCTTCA	LS	5'	*Bam* HI
LSR	AA*GGATCC*GAATTCAGCTTCAGCCTCTC	LS	3'	*Bam* HI

The italicized and underlined sequences shows the restriction sites added

Table 4 Details of constructs used in the present study.

Plasmid	Purpose	Comment	Ref
pYEUra3	Vector with GAL1 promoter	Inducible expression	Prof. Feng
pYEUT	pYEUra3 with TRP1 ORF	Full length harpin expression	Podile *et al*
pYEUT-*amy*	Complete *amy* ORF under GAL1 promoter of pYEUT	Conditional expression	Podile *et al*
p*hrpZ*	Source of *hrpZ* ORF	~1.0 *hrpZ* as *Bam* HI and *Xho* I fragment	Podile *et al*
pSYH10	Template to isolate *hrpZ* mutants	-	
pYEUT-*hrpZ*	Complete *hrpZ* ORF under GAL1 promoter of pYEUT	Conditional expression	Podile *et al*
pYEUT-N1	Mutant coding N-terminal 109 a.a. of HrpZ	Conditional expression	Podile *et al*
pYEUT-N2	Mutant coding N-terminal 153 a.a. of HrpZ	Conditional expression	Podile *et al*
pYEUT-N3	Mutant coding N-terminal 62 a.a. of HrpZ	Conditional expression	*Present study*
pYEUT-N4	Mutant coding N-terminal 28 a.a. of HrpZ	Conditional expression	*Present study*
pYEUT-C1	Mutant coding C-terminal 213 a.a. of HrpZ	Conditional expression	Podile *et al*
pYEUT-C2	Mutant coding C-terminal 86 a.a. of HrpZ	Conditional expression	Podile *et al*
pYEUT-C3	Mutant coding C-terminal 70 a.a. of HrpZ	Conditional expression	*Present study*
pYEUT-C4	Mutant coding C-terminal 54 a.a. of HrpZ	Conditional expression	*Present study*
pYEUT-C5	Mutant coding C-terminal 38 a.a. of HrpZ	Conditional expression	*Present study*
pYEUT-C6	Mutant coding C-terminal 27 a.a. of HrpZ	Conditional expression	*Present study*
pYEUT-C7	Mutant coding C-terminal 13 a.a. of HrpZ	Conditional expression	*Present study*
pYEUT-NCL	Mutant lacking N and C terminal153 a.a. of HrpZ	Conditional expression	*Present study*
pYEUT-$hrpZ_{Psph}$	Complete $hrpZ_{Psph}$ ORF under GAL1 promoter of pYEUT	Conditional expression	*Present study*
pYEUT-$hrpZ_{Pst}$	Complete $hrpZ_{Pst}$ ORF under GAL1 promoter of pYEUT	Conditional expression	*Present study*
pYEUT-*hrpZ*+LS	Complete *hrpZ* ORF under GAL1 promoter of pYEUT with leader sequence cloned upstream of *hrpZ*	Conditional expression	*Present study*
pQE30-*hrpZ*	Complete *hrpZ* ORF under lac promoter of pQE30	Inducible expression	*Present study*

Table 5 Cycling conditions to amplify full length and truncated $hrpZ_{Pss}$, $hrpZ_{Psph}$, $hrpZ_{Pst}$ and alpha factor leader sequence

Steps	*Full length hrpZ and other mutants*	$hrpZ_{Psph}$ *and* $hrpZ_{Pst}$	alpha factor leader sequence
Step 1	94^0C-4 min	95^0C-3 min	95^0C-3 min
Step 2	94^0C-1 min	95^0C-1min	95^0C-1min
Step 3	65^0C-1 min	60^0C-1 min	68^0C-1 min
Step 4	72^0C-1 min	72^0C-2 min	72^0C-2 min
Step 5	Go to step '2' 30 more times		
Step 6	72^0C-5min	72^0C-20 min	72^0C-20 min

mutants is inducible by galactose. The derivatives were designated as pYEUT-*hrpZ*, pYEUT-N1, pYEUT-N2, pYEUT-N3, pYEUT-N4, pYEUT-C1, pYEUT-C2, pYEUT-C3, pYEUT-C4, pYEUT-C5, pYEUT-C6, pYEUT-C7 and NCL Mutant (details in table 4). The *hrpZ* sequences in C1, C2, N1 and N2 constructs are essentially the same as pSYH10, pCPP2999, pCPP2992, pSYH5 and pSYH26 of Alfano *et al.*, (1993) respectively, except for a minor modification. Another pYEUT-derivative expressing functional α-amylase under galactose induction (obtained from Prof. T.T. Kuo) was used as positive control for heterologous protein expression in *S. cerevisiae* Y187.

2.3 Transformation

2.3.1 ***E. coli:*** Competent cells of *E. coli* DH5α were transformed (Sambrook *et al.*, 1989) with pYEUT-amylase, pYEUT and its thirteen different derivatives containing either full-length or truncated sequences of *hrpZ* (described above), pYEUT-*hrpZ*$_{Psph}$, pYEUT-*hrpZ*$_{Pst}$ and pYEUT-*hrpZ*+LS (alpha factor leader sequence upstream of *hrpZ*) and selected on LB-ampicillin plates. Competent cells of *E. coli* M15 were transformed with pQE30-*hrpZ* and selected on LB plate containing ampicillin and kanamycin.

2.3.2 Yeast: Plasmid minipreps (using Qiagen spin columns) were prepared from *E. coli* DH5α cultures containing pYEUT-amylase, pYEUT and its thirteen different derivatives containing either full-length or truncated sequences of *hrpZ*, pYEUT-*hrpZ*$_{Psph}$, pYEUT-*hrpZ*$_{Pst}$ and pYEUT-*hrpZ*+LS to transform *S. cerevisiae* Y187 (Clontech) using a one-step transformation protocol (Chen *et al.*, 1997). Actively growing cells, from 2-3 days-old plate, were scraped using a sterile toothpick. The cells were resuspended in 100 µl of one-step buffer, which comprised of 45% PEG and 0.1 M lithium acetate. To this, 5 µl each of 2 M DTT and ssDNA were added. Plasmid at the concentration of 10-100 µg was added and incubated at 45^{0}C for 1 h. The transformed yeast cells were selected on glucose-containing medium plates with tryptophan drop out-supplement.

2.4 Galactose-induction of harpin$_{Pss}$ and its truncated mutants in *S. cerevisiae*

A single colony of transformants of *S. cerevisiae* Y187 (with pYEUT or its derivatives containing *hrpZ* sequences) and different strains of *S. cerevisiae* (Table 2)

were grown in glucose-containing medium with vigorous aeration at 30^0C to an optical density of ~ 1.0 at 600nm (OD_{600}). Cells were pelleted by centrifugation at 4500 rpm for 10 min and resuspended in glucose- or galactose-containing media to achieve an OD_{600} of ~0.4-0.7. In the yeast cells cultured in glucose-containing medium, the *hrpZ* under GAL promoter is repressed and expressed in galactose-containing medium (induction medium). After culturing for various times, aliquots were removed for the trypan blue staining, counting 400 total (live or dead) cells, colony forming ability, genomic DNA isolation, propidium iodide uptake, and OD_{600} estimations.

To study the expression of $harpin_{Pss}$ in the pYEUT-*hrpZ* transformed *S. cerevisiae* Y187, the cells were first cultured in glucose-containing medium. Once the culture reached the log phase, the cells were pelleted, washed and then shifted to galactose-containing medium. Total protein extracts prepared at 0, 1 and 3 h from cells cultured in galactose-containing medium were analysed on a Western blot with 5 µg extracts (quantified by Bio-Rad protein assay) using an 1:1000 diluted polyclonal antibodies raised against $harpin_{Pss}$ in rabbit and compared to $harpin_{Pss}$ expressed in *E. coli*. The immunoblot was visualized by the alkaline phosphatase catalysed colour reaction (Boehringer Mannheim).

2.4.1 Trypan blue staining: The cells that exclude the vital dye trypan blue are viable. To 0.5 ml of a yeast cell suspension, in an eppendorf, 0.1 ml of 0.4% trypan blue (Sigma-Aldrich) was added, mixed thoroughly, and allowed to stand for 5 min at 30 $\pm$ 2^0C. Trypan blue stained cells were counted using hemocytometer, under the microscope, for stained non-viable cells and viable cells excluding the stain (Freshney, 1987).

2.5 $Harpin_{Pss}$ purification

$Harpin_{Pss}$ was expressed in *E. coli* M15 cells harbouring pQE30-*hrpZ* grown at 37^0C to midlogarithmic phase was induced with 1mM IPTG for 3 h. Bacteria were harvested by centrifugation, washed once in 10mM phosphate buffer (pH 6.5), and resuspended in 0.1 volume of the same buffer supplemented with 1mM PMSF. The bacterial suspension was immediately sonicate d (30 sec pulse on and 1 min pulse off – 7 cycles, Bandelin MS-72). The sonicate was then incubated at 100^0C for 10 min,

followed by centrifugation at 16,500×g for 20 min. The supernatant was transferred to a fresh tube for further analysis. $Harpin_{Pss}$ was purified using Ni-NT agarose (Invitrogen) column as per the manufacturers instructions.

2.5.1 SDS-PAGE analysis: The protein was quantified by Lowry's method (1951) and SDS-PAGE analysis of protein fractions was carried out on vertical slab gels (Bio-rad) according to the method of Laemmli (1970). Samples containing protein were dissolved in sample buffer containing 1% SDS (w/v) and 12% glycerol (v/v), in 0.063 M Tris-HCl, pH-6.8. The samples were boiled at 100^0C for 5 min and subjected to electrophoresis at 50 V in stacking gel and at 100 V in resolving gel. The stacking gel contained 4.5% polyacrylamide in 0.125 M Tris-HCl, pH-6.8 and the resolving gel contained 10% polyacrylamide in 0.375 M Tris-HCl, pH 8.8. Electrode buffer contained 0.025 M Tris-HCl, 0.192 M glycine and 0.1% (w/v) SDS with pH-8.5. Molecular weight markers were run simultaneously with the samples.

The gels were stained according to the procedure of Blum *et al.,* (1987) by incubating in the fixative (50% methanol, 12% acetic acid and 0.05% formaldehyde) for 1 h followed by three washings in 50% ethanol for 20 min each. Gel was soaked in sodium thiosulphate (2mg/ml) for 1 min, washed in double distilled water thrice, and stained in 0.2% silver nitrate containing 0.05% formaldehyde for 30 min. Washed the gel thrice, 20 sec each, and developed with 6% sodium carbonate containing 0.075% formaldehyde. Reaction was stopped with 1% acetic acid solution and stored in 50% methanol after thorough washing.

2.5.2 Raising of polyclonal antibodie s: Polyclonal antibodies were raised against $harpin_{Pss}$. The antibodies were raised by injecting 100µg $harpin_{Pss}$ into rabbit, subcutaneously, after mixing with 500µl of Freund's complete adjuvant and emulsification. Prior to immunization, the lateral ear vein was bled to collect pre-immune serum. After two weeks, a booster injection of 50µg $harpin_{Pss}$ emulsified with 500µl Freund's incomplete adjuvant was given. The second booster injection was given after a week of first booster injection and the blood was collected after 10 days of the second booster injection. The collected blood was left overnight at 4^0C for clotting and serum was collected by centrifuging at 7,000 rpm for 20 min. The serum was aliquoted and stored at -20^0C after adding 0.01% sodium azide.

2.5.3 Western blot analysis: The harpin$_{Pss}$ was resolved in 12% SDS-PAGE along with protein molecular weight standards and then transferred onto nitrocellulose membranes (Bio-rad). The membranes were blocked with 5% (w/v) non-fat dry milk then incubated with the primary antibodies raised against harpin $_{Pss}$ in 10 ml of antibody-diluted buffer (1x Tris buffered saline and 0.5% Tween with 5% milk) with gentle shaking at 4^{0}C for 8-12 h and then incubated with anti-rabbit IgG ALP conjugate. The immunoblot was visualized by the alkaline phosphatase-catalysed colour reaction using BCIP-NBT, substrate for alkaline phosphatase.

2.5.4 Harpin$_{Pss}$ sequencing: PVDF (Immobilon Transfer, 0.45µm pore size Millipore), membrane was rinsed with 100% methanol prior to use and stored in transfer buffer (20% methanol, 39mM Glycine, 0.03% SDS, 48mM Tris.base pH 8.3). The harpin$_{Pss}$ was resolved in a 12% SDS-PAGE and the gel, sandwiched between a sheet of PVDF membrane and several sheets of blotting paper, was assembled into a blotting apparatus (Bio-rad) and electroeluted for 3 h at 75 V in transfer buffer. The PVDF membrane was washed in deionized water for 5 min, stained with 0.1% ponceau S in 5% acetic acid, and then destained in deionized water. The region of the PVDF membrane where the band was seen was excised, washed thoroughly, dried and sequenced (Shimadzu PPSQ21A).

2.6 Extracellular effect of harpin

2.6.1 Addition of protein into the media: pYEUT–*hrpZ* transformants of *S. cerevisiae* Y187 were cultured in glucose-containing medium at 28^{0}C, diluted to an optical density at 600 nm of ~ 0.7, and then cultured in fresh medium containing glucose where different concentrations (5µM, 10µM, 20µM) of harpin$_{Pss}$ or harpin$_{Psph}$ was added and monitored OD_{600} at different time interval. Extracellular effect was also studied in semisolid media by adding harpins before pouring the plates. *S cerevisiae* Y187 (pYEUT transformants) were streaked and incubated for two days. Cells streaked on glucose- or galactose-containing medium served as controls.

2.6.2 Cloning of alpha factor leader sequence upstream of *hrpZ*: *S. cerevisiae* exports alpha-mating factor with the help of a "leader sequence" which is attached to

alpha-factor. A 270bp leader sequence was amplified from TOPOVector (primer details given in table 3) and cloned upstream of *hrpZ* in pYEUT-*hrpZ*, resulting in pYEUT-*hrpZ*+LS for targeting harpin$_{Pss}$ outside the cell. To study the effect of the harpin$_{Pss}$ when secreted into the medium, *S. cerevisiae* Y187 was transformed with pYEUT-*hrpZ*+LS.

2.7 Role of cell cycle stage on harpin$_{Pss}$-induced YCD

To study the effect of a particular stage of cell cycle on the harpin$_{Pss}$ induced cell death, *S. cerevisiae* Y187 was arrested in S- or M-phase of cell cycle as described by Wang and Kuo (2001). For S-phase arrest, pYEUT-*hrp Z* transformant of *S. cerevisiae* Y187 was cultured in glucose-containing medium, grown at 28^{0}C, diluted to an optical density of 0.2 at 600nm, and cultured for 3 h at 24^{0}C in glucose-containing medium in the presence of 0.2M hydroxyurea (Sigma-Aldrich). Hydroxyurea is an inhibitor of ribonucleotide reductase, an essential enzyme of DNA precursor metabolism, and so inhibits DNA synthesis, arresting cells in S-phase. For M-phase arrest, cultures were grown, diluted, and cultured for 3 h at 24^{0}C in the presence of 15µg/ml nocodazole (Sigma-Aldrich). Nocodazole is an inhibitor of microtubule polymerization, and so prevents mitosis. The S- and M-phase-arrested *S. cerevisiae* Y187 pYEUT-*hrpZ* cells were washed and suspended in fresh medium, and cultured at 28^{0}C in glucose and galactose-containing media.

2.8 Assessment of nuclear morphology and chromatin condensation

A single colony of pYEUT-*hrpZ* transformant of *S. cerevisiae* Y187 was cultured in glucose-containing medium with vigorous aeration at 30^{0}C to ~ 1.0 (OD_{600}). Cells were pelleted by centrifugation at 4500 rpm for 10 min and resuspended in glucose- or galactose-containing media to achieve an OD_{600} of ~0.4-0.7. A positive control with 3 mM H_2O_2 in glucose-containing media was maintained simultaneously.

2.8.1 Yeast chromosomal DNA isolation: The culture was prepared as described in section 2.8. Chromosomal DNA from *S. cerevisiae* Y187 was isolated essentially following Breeden LL's Lab protocols described in their website. After 3 h of induction, chromosomal DNA was prepared from 25 ml culture. To the cells, 25 ml 100% ethanol

(prechilled at -20^{0}C) and 1 ml 0.5M EDTA were added and stored at -20^{0}C. The cells were thawed, washed once with water and resuspended in 0.5 ml spheroplast buffer (1.2 M sorbitol; 0.1 M EDTA; 1% ß-mercaptoethanol; 0.1% zymolyase) and incubated at 37^{0}C for 30 min. To this was added 0.5 ml proteinase K buffer (50 mM EDTA; 0.3% SDS; 0.01% proteinase K) and incubated at 65^{0}C for 30 min. Finally 0.2 ml of 5 M potassium acetate was added and incubated on ice for 10 min and centrifuged at 10,000 rpm for 15 min. The supernatant was transferred to a fresh tube and ethanol precipitated. The DNA was resuspended in 500µl 1x TE, digested with RNase I for 30 min at 37^{0}C and extracted with phenol/chloroform. The DNA was again ethanol precipitated and dissolved in 1x TE.

2.8.2 4'6 diamino phenyl indole (DAPI) staining: DAPI exclusively stains the nuclei as bright blue spots. DAPI staining of yeast cells was carried out as described by Tocyski *et al.* (1997). Cells were cultured as described in section 2.8. After 3 h of induction, 1 ml of culture was fixed with absolute ethanol for 30 min, stained with 10 µg/ml of DAPI and observed under fluorescence microscope with BF filter.

2.8.3 Electron Microscopy: Cells were cultured as described in section 2.8. After 3 h of induction, yeast cells were fixed with phosphate-buffered glutaraldehyde, cell walls were removed enzymatically, and the cells were postfixed with osmium tetroxide and uranyl acetate, and then dehydrated as described by Byers and Goetsch (1991). After the 100% ethanol washes, cells were washed with 100% acetone, infiltrated with 50% acetone/50% Epon for 30 min and with 100% Epon for 20 h. Cells were transferred to fresh 100% Epon and incubated at 56^{0}C for 48 h before cutting thin sections.

2.8.4 Fluorescein diacetate (FDA) and propidium iodide (PI) staining: A combination of two fluorescent dyes, fluorescein diacetate (FDA) and propidium iodide (PI) was used for assessment of membrane damage. Cells were cultured as described in section 2.8. After 3 h of induction 1 ml of culture was washed and resuspended in same volume of phosphate buffered saline (PBS buffer pH 7.2). To this was added 10µl FDA (stock 5mg/ml) and 10µl PI (stock 10mg/ml), incubated for 15 min. Surviving and dead cells were observed under fluorescence microscope.

2.9 Generation of 'petites'

'Petites' were generated by the "margin of growth" technique, which ensures exposure to the highest concentration of the drug compatible with growth (Clark-Walker 1972). A drop of ethidium bromide solution (10mg/ml) was placed 1 to 2 cm from the perimeter of a GGYP plate and allowed to dry. A loopful of an actively growing culture was drawn over the ethidium bromide drop. After two to three days incubation at 30^0C, the colonies growing nearest to the margin of the drop were substreaked. After a further two to three days at 30^0C, respiratory deficient 'petite' colonies were distinguished by their small size from the larger 'grande' colonies on the medium.

2.9.1 Detection of 'petites' by tetrazolium overlay technique: 'Petite' colonies were detected using the standard 2,3,5-triphenyl tetrazolium chloride (TTC) method of Ogur *et al* (1957). The plate containing 'petite' and 'grande' colonies were overlaid with 20 ml of 0.1% TTC agar (at 40^0C). Red and white colonies were scored 1h after overlay. Differential staining of colonies is based on TTC reaction with the respiratory chain *via* cytochrome oxidase and reducing it to formazan. Formazan accumulates as an insoluble, red pigment in the cells. Actively respiring cells produce red colonies on high-glucose agar plates, whereas cultures incapable of respiration produce white or pink colonies.

2.9.2 Plating on YEPG plates: 'Petite' and 'grande' colonies were spotted on YEPG plates. YEPG medium is same as YEPD except that glucose is replaced with glycerol.

2.9.3 Release of cytochrome C into the cytosol in harpin$_{Pss}$-induced YCD: *S. cerevisiae* Y187 was cultured as described in section 2.8. After 3 h of induction, the cytosolic fractions were prepared using cell fractionation kit (BD Biosciences) as per the manufacturers instructions from the cultures grown in presence of glucose, galactose and 80 mM acetic acid treated yeast cells. The proteins resolved on a 15% SDS-PAGE were subjected to western blot analysis.

2.9.4 Effect of cyclosporine A on *S. cerevisiae* Y187: The effect of cyclosporin A (7µg/ml) (Jung *et al.*, 1997) on pYEUT-*hrpZ* transformants cultured in galactose-containing media was studied (culture conditions described in section 2.8). Aliquots were drawn to assess colony-forming ability and OD_{600} at regular intervals.

2.10 Effect of staurosporine and acetic acid on *S. cerevisiae* Y187

Preculture conditions were described in section 2.8. After resuspending the culture in glucose-containing medium was added staurosporine and acetic acid at the final concentration of 1µM and 80mM, respectively to induce cell death in yeast. Aliquots were drawn at regular intervals for OD_{600} estimation.

2.11 Effect of harpins from two other pathovars of *Pseudomonas syringae*

A 1.037kb and 1.112 kb fragments encoding full-length $hrpZ_{Psph}$ and $hrpZ_{Pst}$, respectively, were PCR amplified (primer details in Table 3) and cloned into pYEUT using *Bam* HI (5') and *Xba* I (3') sites, placing $hrpZ_{Psph}$ and $hrpZ_{Pst}$ under the control of the GAL1 promoter for conditional expression of these genes when cells are grown in galactose-containing media. *S. cerevisiae* Y187 transformed with pYEUT-$hrpZ_{Psph}$ and pYEUT-$hrpZ_{Pst}$ for conditional expression of the $harpin_{Psph}$ and $harpin_{Pst}$. The $hrpZ_{Psph}$ and $hrpZ_{Pst}$ encoding full-length $harpin_{Psph}$ and $harpin_{Pst}$ were kindly provided by Dr. Justin Lee, Institute of Plant Biochemistry, Halle, Germany.

2.12 Effect of N-terminal and C-terminal deletions on cell death activity of harpin $_{Pss}$: The size of the full-length *hrpZ* is of 1.02kb. Different truncated mutants were generated by PCR amplification using primers listed in Table 3 and the cycling conditions described in Table 5. The truncation was done either at N- or C-terminal end or either ends of the full-length $harpin_{Pss}$. The PCR amplified truncated mutants were cloned in *Bam* HI and *Xho* I sites of pYEUT and the pYEUT-*hrpZ* mutants constructs were then transformed in *S. cerevisiae* Y187. The effect of these truncations on cell death activity of $harpin_{Pss}$ was studied, as described in the section 2.4, using trypan blue staining.

2.13 Sequence analysis of harpin $_{Pss}$ in a bioinformatic approach

2.13.1 Detection of homology be tween harpin$_{Pss}$ sequences: To study the homology existing between harpin sequences, HrpZ sequence of *Pseudomonas syringae* pv. *syringae* was blasted against Swissprot databank using BlastP and PSI-BLAST programs; the later provides an advantage over normal blast in the detection of distantly related sequences. The PSI-BLAST program uses the information from any significant alignments returned to construct a position-specific score matrix, which replaces the query sequences for the next round of database searching. PSI-BLAST may be iterated until no new significant alignments are found.

2.13.2 Comparison of harpin sequences– Multiple Sequence Alignment: A multiple sequence alignment was carried out for the harpin sequences of *Erwinia amylovora, E. carotovora and E. chrysanthemi* and harpins of *Erwinia* sps. and *Pseudomonas syringe* was done using ClustalW algorithm.

2.13.3 Secondary structure prediction: Harpin$_{Pss}$ secondary structure was predicted using PHD algorithm, a neural network system (a sequence-to-structure level and a structure-to-structure level) to predict secondary structure of protein. A substantial increase in both the accuracy and quality of secondary-structure predictions are possible using a neural-network algorithm. The main improvements come from the use of multiple sequence alignments (better overall accuracy), from "balanced training" (better prediction of beta-strands), and from "structure context training" (better prediction of helix and strand lengths).

2.13.4 Tertiary structure prediction: The most successful protein structure prediction method is homology modeling (also known as comparative modeling). The approach is based on the structural conservation of the framework regions between the members of a protein family. Since the 3D structures are more conserved in evolution than sequence, even the best sequence alignment methods frequently fail to correctly identify the regions that possess the desired level of structural similarity, and the quality of alignment is the single most important factor determining the accuracy of 3D model.

2.14 Crystallization of harpin$_{Pss}$

2.14.1 Crystallization of macromolecules: Increasing the concentration of a precipitating agent to a point just below super saturation, and then adjusting the pH or temperature to further reduce the solubility of the protein may achieve the crystallization of macromolecules (Mc Pherson, 1999).

2.14.2 Hanging drop method: The disposable 24 wells (1.7cm diameter, 1.6 cm depth) plastic tissue culture plates (Linbro or VDX plates) were used. The wells have flat ground rims that allow sealing from the exterior by application of a light coating of silicone grease or vaseline to the circumference. The protein microdroplets were composed on glass coverslips that have been siliconized to ensure against wetting and drop spread, and subsequently suspended over wells in a plastic plate. Round glass coverslips were used. The individual wells have, prior to this operation, been prefilled with 1 ml of different combinations of precipitating solution (Table 6). The mother liquor was prepared by dissolving 3 mg of pure harpin$_{Pss}$ in 300µl sterile double distilled water. Each well was covered by a coverslip with a drop of 6 µl protein solution hanging from its underside, which equilibrates with the reservoir solution, over time, through the vapor phase, causing precipitant and protein concentrations to increase in the drop, and thus induce crystallization of the protein. Different combinations and concentrations of precipitant with varied pH were used (Table 6) to standardize the harpin$_{Pss}$ crystallization process.

Table 6 Conditions used for crystallization of harpin$_{Pss}$

Reservoir components	Different set-ups															
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
PEG (%)	20	20	20	20	20	10	40	60	75	40	60	75	50	55	60	65
Buffer	A	B	C	D	E	C	C	C	C	E	E	E	C	C	C	C
pH	4.6	5.6	6.5	7.5	8.5	6.5	6.5	6.5	6.5	8.5	8.5	8.5	6.5	6.5	6.5	6.5
SDDW (µl)	700	700	700	700	700	825	450	200	13	450	200	13	325	263	200	138
Total vol. (ml)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Five different buffers were used in standardizing harpin$_{Pss}$ crystallization viz., (A) Sodium acetate pH 4.6, (B) Sodium citrate pH 5.6, (C) Sodium cacodylate pH 6.5, (D) Sodium HEPES pH 7.5 and (E) Tris.HCl pH 8.5. The alphabets mentioned in the table correspond to the buffers described here. The precipitant used is 80% PEG stock and the PEG concentration used in the study range from 20-75%. The stock and working concentration of the buffers used are 1 M and 0.05M respectively.

Results

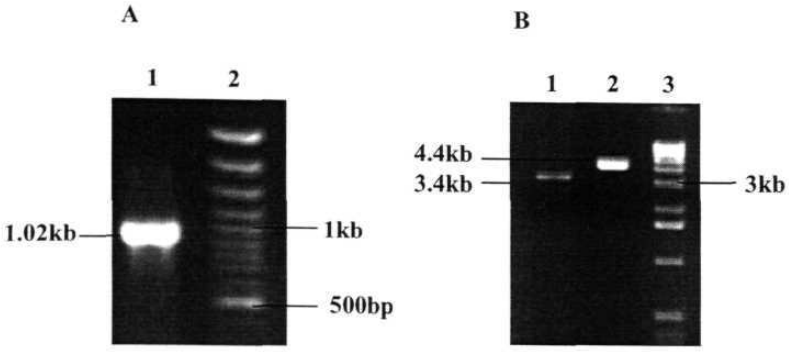
3.1 Harpin$_{Pss}$ purification

Harpin$_{Pss}$ was expressed in *E. coli*. The *hrpZ* gene (1.02kb) encoding full-length harpin$_{Pss}$ was PCR amplified as a single band (Fig.1A) and cloned in *Bam* HI and *Sac* I sites of pQE30 (Fig. 1B). *E. coli* cells were transformed with pQ E30-*hrpZ*. A single colony of pQE30-*hrpZ* transformant of *E. coli* M15 was grown in LB broth to OD_{600} of ~ 0.6, and induced with IPTG. After 3 h of induction, the protein was extracted from the culture by sonication. The harpin$_{Pss}$ was partially purified by boiling the protein extract, where only few proteins remained (Fig. 2A -left side gel). A distinctly expressed harpin$_{Pss}$ was detected as the major component of the protein extract. The partially purified harpin$_{Pss}$ (Fig. 2A) extract was passed through Ni-NT agarose, to obtain pure harpin$_{Pss}$ (Fig.2A-right side gel).

3.1.1 Raising of polyclonal antibodies: About 100µg of purified harpin$_{Pss}$ was injected into rabbit subcutaneously after mixing with Freund's complete adjuvant and emulsification to raise antibodies. The reactivity of antibody was tested in an immunoblot. The membrane was incubated with the HrpZ antibodies and then incubated with the anti-rabbit IgG ALP conjugate. The immunoblot was visualized by the alkaline phosphatase catalyzed colour reaction using BCIP -NBT. Band corresponding to harpin$_{Pss}$ was detectable, confirming the specificity of the antibody (Fig. 2B). No other cross-reaction was visible on the membrane (not shown).

3.1.2 Harpin$_{Pss}$ sequencing: Purified harpin$_{Pss}$ resolved on a SDS-PAGE was transferred to a PVDF membrane. The region of the PVDF membrane with the band was excised, washed thoroughly, dried and sequenced. On sequencing, the amino-terminus of the purified harpin$_{Pss}$ confirmed the start codon of harpin$_{Pss}$ and revealed the sequence similarity with the deduced sequence available in the database. Sequencing was repeated at least five times to confirm the sequence similarity with the sequence available in the database. The obtained sequence is seven amino acid residues of the N-terminus of harpin$_{Pss}$ and among these 7 a.a., 5 a.a., were matching with the deduced sequence available in the database.

Figure 1 PCR amplification and cloning of *hrpZ*$_{Pss}$ in pQE30.



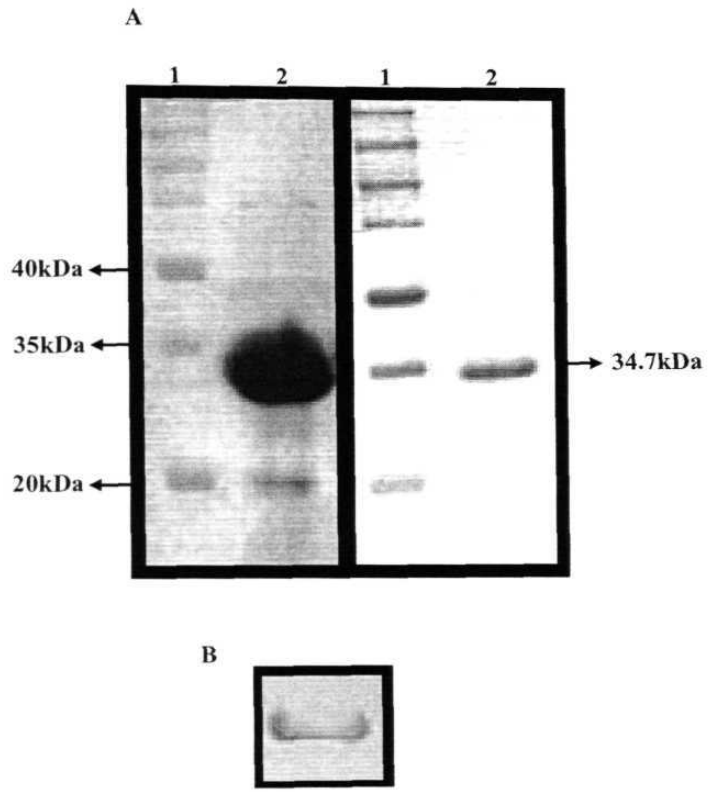
A) A 1.02kb *hrpZ* gene encoding full-length harpin$_{Pss}$ was PCR amplified using the primer sequences (Table 3) that add *Bam* HI (5') and *Sac* I (3') restriction sites. The PCR product was resolved on a 1% agarose gel electrophoresis.

Lane 1: A 1.02kb *hrpZ*$_{Pss}$ amplicon
Lane 2: 100bp Molecular weight marker

B) The PCR amplified *hrpZ* was digested with *Bam* HI and *Sac* I and cloned in *Bam* HI and *Sac* I digested pQE30 and transformed in *E. coli* DH5α. *E. coli* DH5α cells containing pQE30 and cells containing pQE30-*hrpZ* construct were cultured in LB broth and plasmid minipreps was prepared from them. The pQE30 and pQE30-*hrpZ* plasmids were linearised with *Bam* HI and resolved on a 1% agarose gel electrophoresis.

Lane 1: linearised pQE30
Lane 2: linearised pQE30-*hrpZ*
Lane 3: Molecular weight marker

Figure 2 Harpin$_{Pss}$ expression in *E. coli.*



A) SDS-PAGE of harpin$_{Pss}$ expressed in *E. coli* and B) Western blot analysis of harpin$_{Pss}$.

E. coli M15 competent cells were transformed with pQE30-*hrpZ*, grown in LB broth to OD_{600} ~0.6. After 3 h of IPTG induction, the crude protein was extracted and subjected to SDS-PAGE.
A) Both the gels (left and right) show prestained marker (Bio-rad) in lane 1. Lane 2 is the partially purified harpin$_{Pss}$ (gel on the left) and Ni-NTA purified harpin$_{Pss}$ (gel on the right).

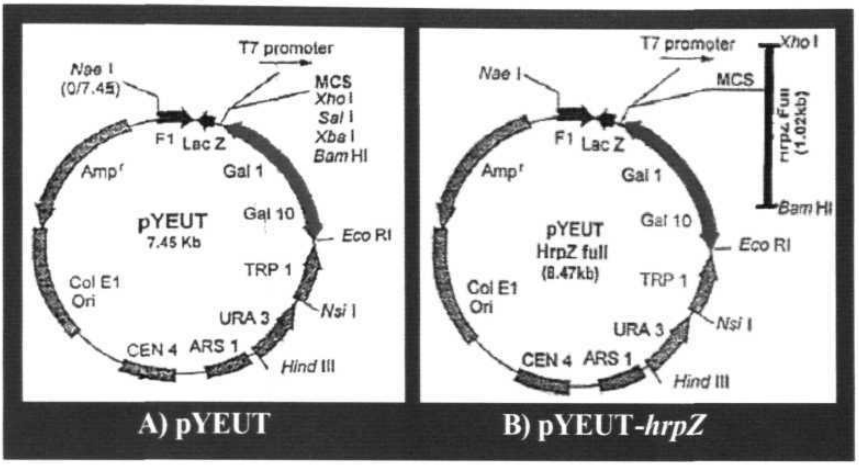
B) Purified harpin$_{Pss}$ was resolved on a 12% SDS-PAGE, transferred to a nitrocellulose membrane, blocked with 5% non-fat dry milk, incubated with the harpin$_{Pss}$ antibodies and subsequently incubated with anti-rabbit IgG ALP conjugate. The immunoblot was visualized by the alkaline phosphatase catalyzed colour reaction using BCIP-NBT.

3.2 Harpin$_{Pss}$ causes yeast cell death

A 1.02kb fragment encoding full-length *hrpZ* was cloned into pYEUT, placing *hrpZ* under the control of the GAL1 promoter for conditional expression of harpin$_{Pss}$ (Fig. 3) when cells were shifted to galactose-containing media from glucose-containing media. Plating of pYEUT-*hrpZ* transformants on semisolid medium that contained galactose resulted in essentially complete inhibition of colony formation, whereas colony formation on glucose-based medium occurred with approximately the same efficiency as observed for control transformants containing pYEUT (Fig. 4). pYEUT-amylase construct was transformed in *S. cerevisiae* Y187. When transformant of pYEUT-amylase of *S. cerevisiae* Y187 was plated onto glucose- and galactose-containing medium, the colony forming ability of the pYEUT-amylase transformant was not different from the cells grown in glucose-containing medium both on glucose- and galactose-containing media indicating that heterologous expression of proteins using pYEUT in *S. cerevisiae* Y187 did not affect the growth. The western blot analysis confirmed the expression of harpin$_{Pss}$ in yeast cells expressing the *hrpZ* in pYEUT-*hrpZ* under the GAL1 promoter in galactose-containing medium within 1 h of induction (Fig. 5). The level of expression of harpin$_{Pss}$ in pYEUT-*hrpZ* of *S. cerevisiae* increased with time.

To characterise the effects of HrpZ on yeast cells, pYEUT-*hrpZ* and pYEUT transformed cells were grown in glucose-containing medium and then switched to a fresh medium containing either glucose or galactose. After various time intervals, the cell density was determined on the basis of the ability to exclude trypan blue dye. Fig. 6A presents results from a representative experiment, showing a time-dependent decline in the percentage of trypan blue-excluding cells in cultures of pYEUT-*hrpZ* transformants when cultured on galactose-containing medium. In the yeast cultures expressing harpin$_{Pss}$, cells that failed to exclude trypan blue appeared within 3 h after shifting the cells to the galactose-containing medium. Within 3 h of induction, the percentage of cells excluding trypan blue in cells cultured in galactose-containing medium markedly reduced to 60% when compared to the cells cultured in glucose-containing medium. By 24 h, the percentage of viable cells in the cells cultured in galactose-containing medium was only 20% compared to the control. In contrast, cells grown in glucose-containing medium remained mostly dye-negative.

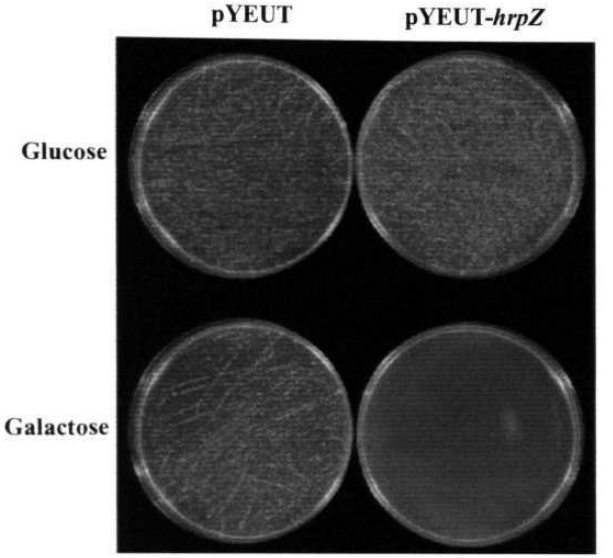
Figure 3 Construction of pYEUT-*hrpZ* for conditional expression of full length and truncated harpins in *S. cerevisiae* Y187.



A) To use tryptophan auxotrophy as a selectable marker in *S. cerevisiae* Y187 (Clontech), the complete ORF of TRP1 gene from pSR424 was PCR amplified adding *Nsi* I (5') and *Eco* RI (3') restriction sites and cloned into pYEUra3 (Clontech) with galactose inducible GAL1 promoter. The modified vector was designated as pYEUT (7.45kb) (Kindly provided by Prof. T-Y Feng, Academia Sinica, Taiwan)

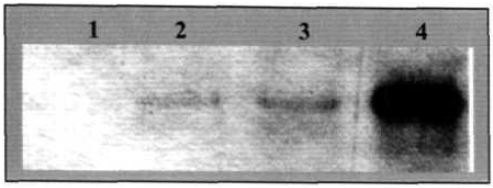
B) A 1.02kb *hrpZ* encoding full-length harpin$_{Pss}$ was obtained from p*hrpZ* as a *Bam* HI (5') and *Xho* I (3') fragment and cloned into pYEUT in the *Bam* HI and *Xho* I sites under GAL1 promoter for conditional expression of *hrpZ* and truncated mutants in *Saccharomyces cerevisiae* Y187.

Figure 4 Conditional expression of harpin$_{Pss}$ in *S. cerevisiae* Y187.



pYEUT and pYEUT-*hrpZ* was transformed in *S. cerevisiae* Y187 by one-step yeast transformation protocol as described in material and methods. A thick suspension of yeast cells transformed with either pYEUT or pYEUT-*hrpZ* was plated on glucose- or galactose-containing medium plates. Photographs were taken 2 days after incubation at 30^0C.

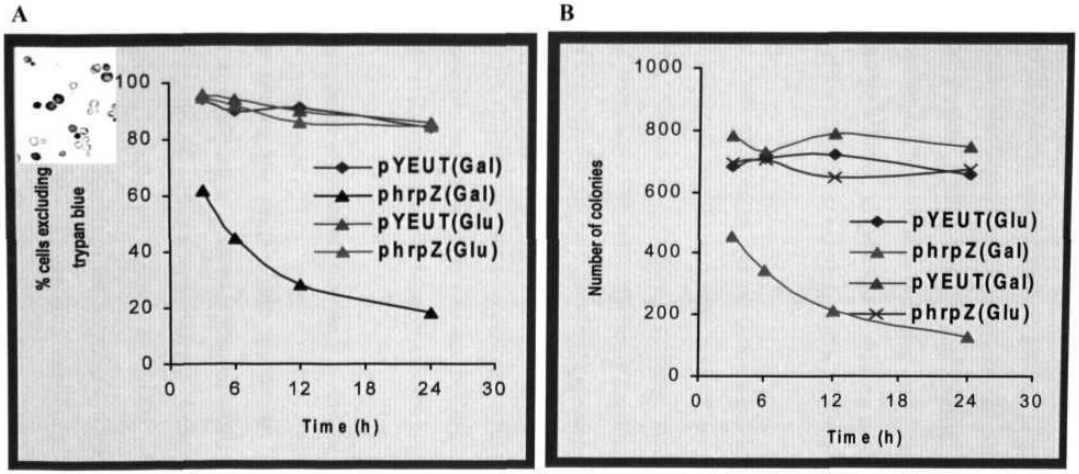
Figure 5 Immunoblot of harpin$_{Pss}$ expression in *S. cerevisiae* Y187 and *E. coli*.



Immunoblot of harpin$_{Pss}$ expressed in *S. cerevisiae* Y187 or *E. coli* under inducible conditions. *S. cerevisiae* Y187 cells were transformed with pYEUT-*hrpZ*, grown in glucose-containing medium with tryptophan drop out-supplement of OD_{600} of ~0.3. Cells were pelleted, washed and introduced into galactose-containing medium and pelleted at 0, 1 and 3 h after transfer and homogenized by glass beads. Supernatant, containing 2μg total proteins of the aliquot, was subjected to SDS-PAGE and detected using polyclonal antibodies raised against harpin$_{Pss}$ in rabbit. Expression of harpin$_{Pss}$

Lane 1: at 0 h
Lane 2: after 1 h
Lane 3: after 3 h
Lane 4: in *E. coli* (3 h after induction)

Figure 6 Yeast cell death induced by conditional expression of *hrpZ* in *S. cerevisiae* Y187 assessed in terms of cell viability.



Characterization of YCD caused by conditional expression of *hrpZ* in *S. cerevisiae* Y187. *S. cerevisiae* Y187 cells were transformed with pYEUT (control) or pYEUT-*hrpZ,* grown in glucose-containing medium with tryptophan drop out-supplement to mid-log phase (OD_{600} of ~ 1.0). Cells were then washed and introduced into glucose- or galactose-containing medium to achieve an OD_{600} of 0.4–0.7, incubated for a different time duration, as indicated on the x-axis (A) Percentage of cells excluding trypan blue was determined by counting a total of 400 cells. (B) The number of colonies formed after 48 h of culture at 30^0C was counted. The inset shows trypan blue stained *S. cerevisiae* Y187cells. The data are representative of five individual experiments.

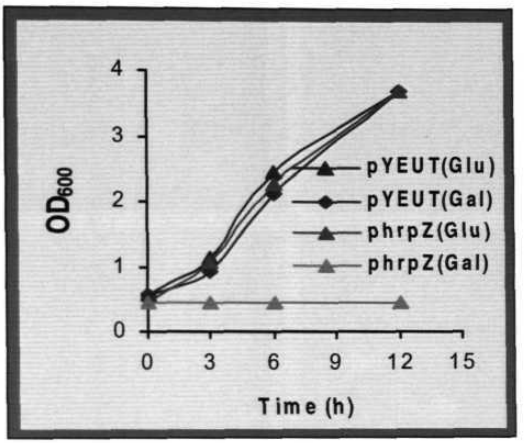
To further examine the kinetics of *hrpZ*-mediated YCD, pYEUT transformants were cultured for various times in galactose-containing medium to induce *hrpZ* expression and were plated on glucose-based medium. The number of colonies from galactose-grown cultures markedly reduced to about 50% within 6 h (Fig 6B), when compared to the colony forming ability of glucose-grown cultures. By 24 h, very few viable colony-forming cells remained in the cultures. The data in Fig. 7 on the growth of pYEUT or pYEUT-*hrpZ* transformants in glucose or galactose-containing media clearly indicated that the *hrpZ* expressing *S. cerevisiae* did not multiply in galactose-containing medium. The growth of pYEUT-*hrpZ* transformed *S. cerevisiae*, in terms of OD_{600}, in glucose-containing medium steadily increased upto 12 h. Similarly, pYEUT transformed cells also was not affected in galactose-containing medium. These observations further confirmed that the conditional expression of *hrpZ* in yeast cells inhibited proliferation.

3.2.1 Yeast cells expressing harpin$_{Pss}$-mediated lethal phenotype are small: *S. cerevisiae* Y187 cells transformed with pYEUT-*hrpZ*, grown on glucose-containing medium, were shifted to galactose-containing medium, to initiate the inducible expression of harpin$_{Pss}$. The cells expressing harpin$_{Pss}$ with the lethal phenotype were much smaller compared to the cells growing in glucose-containing medium (Fig. 8). The number of cells cultured in galactose-containing medium is very less compared to the number of cells cultured in glucose-containing medium. The yeast cells in glucose-containing medium were similar in size and healthy, in contrast to different sizes and "sick-looking" cells expressing the lethal phenotype in galactose-containing medium.

3.3 Extracellular effect of harpins on *S. cerevisiae*

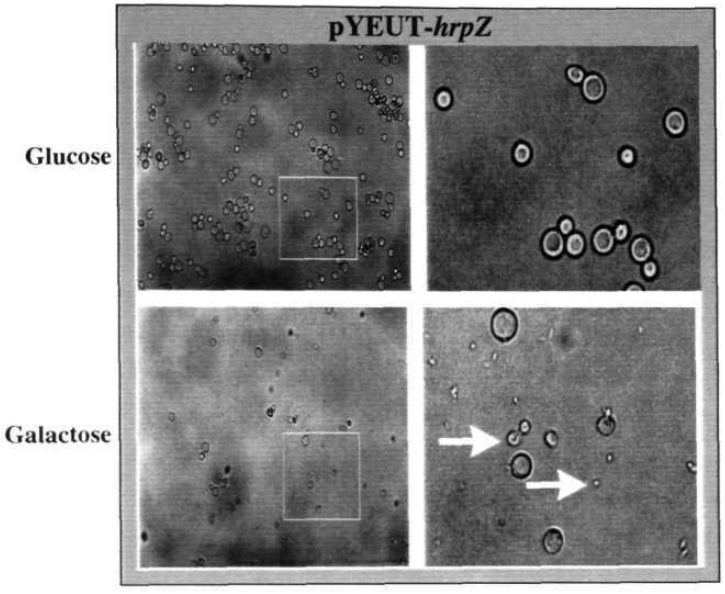
3.3.1 By adding the protein into the media: Single colony of pYEUT-*hrpZ* transformant was cultured in glucose-containing medium with vigorous aeration at 30^0C to an optical density of ~1.0 at 600nm (OD_{600}). Cells were pelleted and resuspended in glucose-containing media to achieve an OD_{600} of ~0.4-0.7, to which different concentrations (5μM to 20μM) of the harpin$_{Pss}$ and harpin$_{Psph}$ individually was added to study the extracellular effect of them on *S. cerevisiae*. The growth of *S. cerevisiae* was not affected by the presence of harpin$_{Pss}$ and harpin$_{Psph}$ in the liquid and solid media. The growth, in presence of harpins, was similar to the growth of *S.*

Figure 7 Yeast cell death induced by conditional expression of *hrpZ* in *S. cerevisiae* Y187 assessed in terms of cell density determined as optical density.



Yeast cells were transformed with pYEUT (control) of pYEUT-*hrpZ* grown in glucose-containing medium with tryptophan drop out-supplement to mid-log phase (OD_{600} of ~ 1.0). Cells were pelleted, washed and introduced into glucose- or galactose-containing medium to achieve an OD_{600} of 0.4–0.7, incubated for a different time duration, as indicated on the x-axis and OD_{600} estimation was carried out. The data are representative of five individual experiment

Figure 8 *S. cerevisiae* Y187 cells expressing harpin$_{Pss}$-mediated cell death.



S. cerevisiae Y187 transformed with pYEUT-*hrpZ* grown to OD_{600}~ 1.0 in glucose-containing medium and shifted to glucose- or galactose-containing medium. After 18 h of culturing at 30^{0}C, 10µl of culture was placed on a clean glass slide and covered with a thin glass. Photomicrographs in the left panel are of the representative population of cells (400X) – from glucose- (top) and galactose (bottom) containing medium, and in the right panel the magnified view.

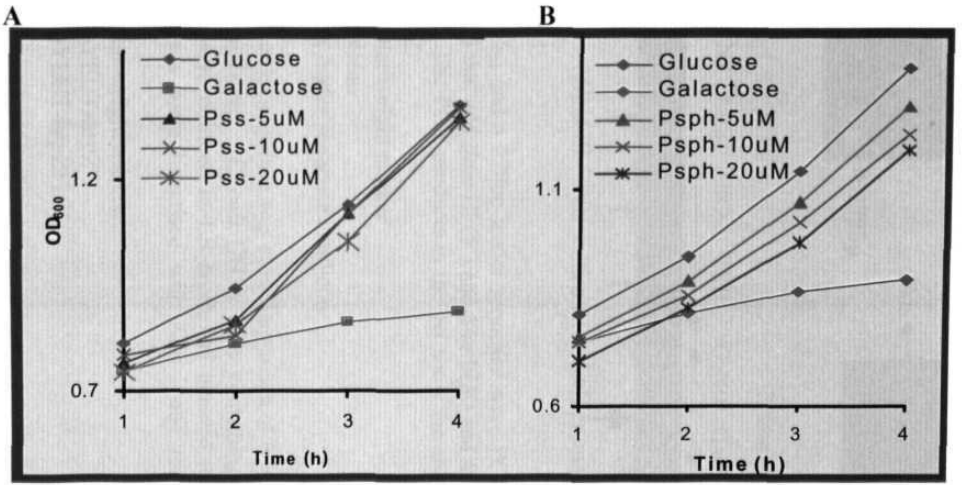
cerevisiae in glucose-containing media, while the galactose-grown cultures were unable to grow upto 4 h (Fig. 9A & B). Extracellular effect of $HrpZ_{Pss}$ and $HrpZ_{Psph}$ was studied in the semisolid medium by adding the protein in the medium just before pouring the plate. After solidification, pYEUT-*hrpZ* transformant was streaked onto the media and the plate was observed three days after incubation at 30^0C. The colonies of the *S. cerevisiae* pYEUT-*hrpZ* transformant on harpin-containing media were indistinguishable from the colonies that appeared on glucose-containing medium. These observations, confirmed that harpins, when provided in the medium did not affect the *S. cerevisiae* pYEUT-*hrpZ* transformants. Cells cultured in glucose- and galactose-containing media were maintained simultaneously as controls, where inhibition of cell proliferation was observed in cells cultured in galactose-containing medium (Fig. 9A & B).

3.3.2 Cloning of leader peptide (alpha-factor leader sequence): The leader sequence (LS) was PCR amplified as a single fragment (Fig. 10A) and cloned in *Bam* HI digested pYEUT upstream of *hrpZ* to let the yeast target the protein into the media when cultured. Before transforming *S. cerevisiae* Y187 with pYEUT-*hrpZ*+LS, the construct was subjected to restriction analysis using *Xho* I and the linearised plasmid confirmed the cloning (Fig. 10B & C) and direction of cloning of the alpha factor was confirmed by PCR using the forward primer of the alpha factor and reverse primer of *hrpZ*. *S. cerevisiae* Y187 was transformed with pYEUT-*hrpZ*+LS construct. When the transformants of pYEUT-*hrpZ*+LS were plated onto semisolid media containing glucose or galactose, the colonies on galactose-containing plate were unaffected and were similar to the colonies observed on glucose-containing medium (Fig. 11). The growth of pYEUT-*hrpZ*+LS transformant of *S. cerevisiae* Y187 was unaffected when the harpin was secreted into the medium. This observation, further confirmed that $harpin_{Pss}$ had no effect on *S. cerevisiae* Y187 cells, extracellularly.

3.4 Role of cell cycle stage on $harpin_{Pss}$-induced YCD

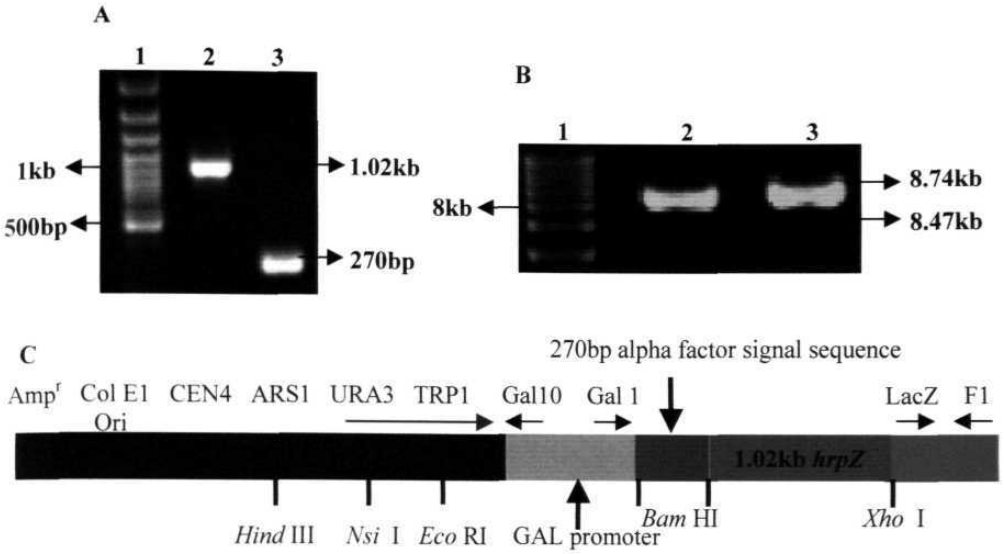
To see the role of cell cycle stage on $harpin_{Pss}$-mediated YCD, $harpin_{Pss}$-mediated YCD was studied by arresting the yeast cells in two different phases of cell cycle. *S. cerevisiae* Y187 transformed with pYEUT-hrpZ was grown in glucose-containing medium with tryptophan drop out-supplement to OD_{600}~0.6. For S- and M-phase arrest,

Figure 9 Extracellular effect of harpins on *S. cerevisiae* Y187.



S. cerevisiae Y187 transformed with pYEUT-*hrpZ*$_{Pss}$ grown in glucose-containing medium to OD_{600}~0.6. Cells were pelleted, washed and introduced into glucose-containing medium to which was added three different concentrations of A) harpin$_{Pss}$ and B) harpin$_{Psph}$, expressed in modified pET vector pJC40 (kindly provided by Dr. Justin Lee, Institute of Plant Biochemistry, Halle, Germany) and cultured further to study the effect of harpin on *S. cerevisiae*. Cells cultured in the presence of glucose- or galactose-containing medium were maintained simultaneously as controls. OD_{600} estimation was carried out at regular time intervals for 4 h as indicated in the x-axis. The data are representative of five individual experiments.

Figure 10 PCR amplification and cloning of alpha factor leader sequence upstream of *hrpZ*.

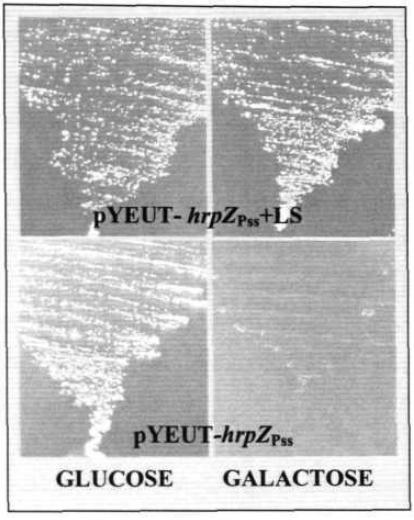


A) A 1.02kb $hrpZ_{Pss}$ and a 270bp leader sequence were PCR amplified using the primer sequences (Table 3) that add *Bam* HI (5') and *Xho* I (3') and *Bam* HI (5') and (3') restriction sites, respectively. The PCR products in the gel show the amplification of leader sequence and full-length *hrpZ*.
Lane 1: 1kb molecular weight marker
Lane 2: $hrpZ_{Pss}$ amplicon
Lane 3: alpha-factor leader sequence amplicon

B) PCR amplified leader sequence was cloned upstream of *hrpZ* for extracellular targeting of $harpin_{Pss}$ in *S. cerevisiae*. *E. coli* DH5α cells containing contructs pYEUT-*hrpZ* and pYEUT-*hrpZ*+LS were cultured in LB broth and plasmid minipreps were prepared from them. The plasmids were linearised with *Xho* I and resolved in a 1% agarose gel electrophoresis.
Lane 1: the molecular weight marker
Lane 2: linearised pYEUT-*hrpZ*
Lane 3: linearised pYEUT-*hrpZ*+LS

C) Diagramatic representation of the linearised pYEUT-*hrpZ*+LS to show the cloning of PCR amplified leader sequence upstream of *hrpZ* for letting the yeast target the protein outside the cell. As indicated in the figure, alpha factor leader sequence is of 270bp and *hrpZ* of 1.02kb in molecular weight. The blue arrow (→) indicates the direction of transcription.

Figure 11 Conditional expression of pYEUT-*hrpZ*+LS in *S. cerevisiae* Y187.



pYEUT-*hrpZ* transformed in *S. cerevisiae* Y187 by one-step transformation protocol as described in material and methods. The upper half of the figure shows the pYEUT-*hrpZ*+LS transformant and pYEUT-*hrpZ*+LS (leader sequence cloned upstream of *hrpZ*) were streaked onto the glucose- or galactose-containing medium on the left and right panels respectively. The lower half of the figure shows the pYEUT-*hrpZ* transformant streaked onto the glucose- or galactose-containing medium indicated on the left and right panels respectively. The photographs were taken after 48 h incubation at 28^0C.

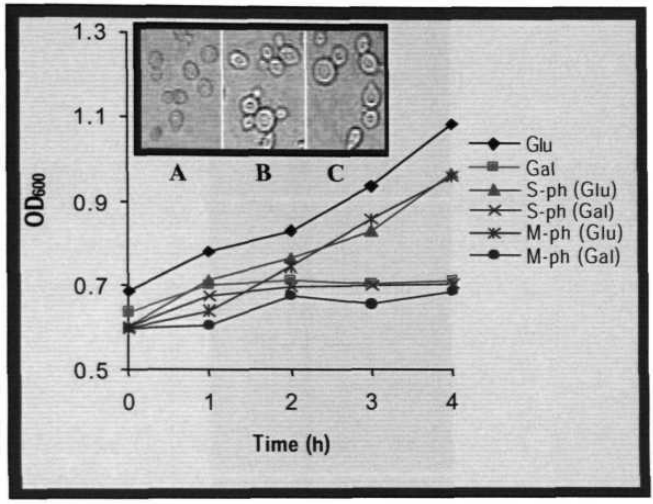
cells were grown in the presence of 0.2M hydroxyurea and of 15µg/ml nocodazole, respectively, for 3 h at 24^{0}C in glucose-containing medium. Cells in S phase are in the budding stage where the buds are much smaller in size when compared to the mother cell and in M phase the size of the buds are almost of the mother cell and are in the separating stage from the mother cell (inset in Fig.12). The S- and M-phase arrested cells were then washed and resuspended in glucose- or galactose-containing fresh medium, and cultured at 28^{0}C. After every 1 h, cells were observed under microscope and OD_{600} was recorded. It was noted in microscopic observations that, within 1 h of induction, cell death was taking place both in S- and M-phase arrested cells cultured in galactose-containing medium. The data in Fig. 12 on the growth of pYEUT-*hrpZ* transformed *S. cerevisiae* in glucose or galactose-containing media, clearly indicated that the *hrpZ* expressing S- and M-phase arrested pYEUT-*hrpZ* transformed *S. cerevisiae* did not multiply in galactose-containing medium. The growth of the pYEUT-*hrpZ* transformed *S. cerevisiae* in terms of OD_{600}, in glucose-containing medium steadily increased upto 4 h. These observations confirmed that harpin$_{Pss}$-mediated cell death was independent of the stage of cell cycle.

3.5 Assessment of nuclear morphology and chromatin condensation

The culture was grown in glucose-containing medium with tryptophan drop out-supplement to OD_{600}~0.6. Cells were pelleted, washed and introduced into glucose- or galactose-containing media; cultures in 3mM H_2O_2 added glucose-containing medium served as positive control.

3.5.1 Chromatin condensation and fragmentation in harpin$_{Pss}$-induced YCD: Harpin$_{Pss}$ expressed *S. cerevisiae* Y187 cells were studied for morphological signs of apoptosis by staining with DNA-binding fluorochrome DAPI. Cells were cultured as described in section 3.5. After 3 h of induction, 1 ml of the culture was fixed with ethanol for 30 min and then stained with DAPI On observation under fluorescence microscope, DAPI stained yeast cells from exponential and stationary phase cultures in glucose-containing medium showed a continuous DNA ring within the nucleus in the cells. DAPI staining of H_2O_2-treated cells showed a typical fragmentation (nuclear fragments) (Fig. 13), while there was no evidence of nuclear fragmentation in the cells

Figure 12 Cell cycle synchronization of pYEUT-*hrpZ* transformant of *S. cerevisiae* Y187.



S. cerevisiae Y187 transformed with pYEUT-*hrpZ*, grown in glucose-containing medium with tryptophan drop out-supplement to OD_{600}~0.6. Cells were pelleted, washed and introduced in glucose-containing medium to which was added 0.2 M hydroxyurea and 15µg/ml nocodazole for S-phase and M-phase arrest, respectively, and cultured for 3 h at 24^0C. After the arrest, the cells were observed under microscope to confirm the arrest. After the arrest, cells were washed and introduced in glucose- or galactose-containing medium. OD_{600} estimations were carried out at regular time intervals for 4 h as indicated in the x-axis. The data are representative of five independent experiments.

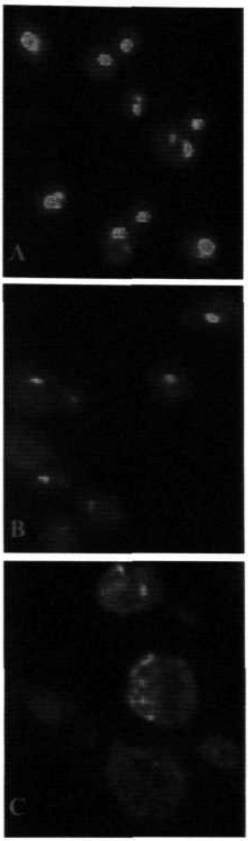
The inset shows the photomicrographs of control and arrested cells.

A) control culture without any arrest

B) cells arrested in S-phase (with buds much smaller then the mother cell)

C) cells arrested in M-phase (the buds almost the size of the mother cell and is about to separate for the mother cell)

Figure 13 DAPI staining of pYEUT-*hrpZ* transformant of *S. cerevisiae* Y187.



S. cerevisiae Y187 transformed with pYEUT-*hrpZ*, grown in glucose-containing medium with tryptophan drop out-supplement to OD_{600}~0.6. Cells were pelleted, washed and introduced in glucose- or galactose-containing medium and 3mM H_2O_2 in glucose-containing medium. After 3 h of induction, 1 ml culture was taken, pelleted, washed and fixed with ethanol for 30 min, stained with DAPI and then the cells were observed under fluorescence microscope. The photomicrographs of cells cultured in presence of

A) glucose

B) galactose and

C) 3mM H_2O_2 in glucose-containing medium

cultured in galactose-containing medium, revealing that the chromosomal DNA fragmentation does not seem to occur in the harpin$_{Pss}$-mediated YCD.

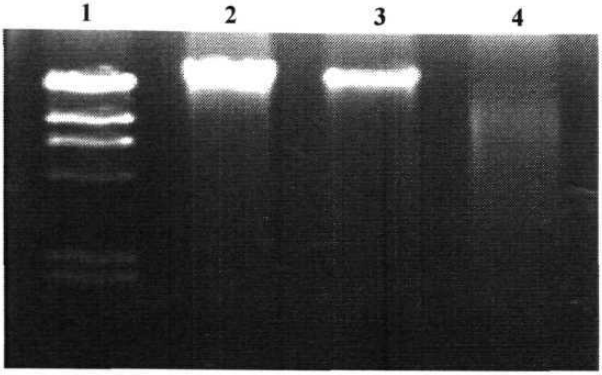
3.5.2 Alteration in genomic DNA: Cells were cultured as described in section 3.5. After 3 h of induction, genomic DNA was extracted from 25 ml cultures, dissolved in equal volume of 1xTE and 5µl of the samples were resolved on an agarose gel electrophoresis. Yeast chromosomal DNA prepared from cells cultured in galactose-containing medium did not reveal the oligonucleosomal pattern of DNA, typical of apoptotic cells, suggesting that this feature of apoptosis was absent in harpin$_{Pss}$-mediated cell death (Fig. 14). In the positive control (H_2O_2 treated cells), a diffuse smear of DNA fragments was evident. The genomic DNA was extracted from equal volume of cultures, dissolved in equal volume of TE was loaded the gel with equal volume, there was difference in the intensity of the bands clearly indicating that the number of cells in galactose-containing medium and H_2O_2 treated cells were less due to the occurrence of cell death.

3.5.3 Electron microscopy of pYEUT-*hrpZ* transformed *S. cerevisiae*: The culture was prepared as described in section 3.5. After 3 h of culturing, EM analysis of yeast cells growing in glucose-containing medium, in which harpin$_{Pss}$ expression was not induced, *S. cerevisiae* cells were normal with a central vacuole and a normal nucleus. Electron microscopic investigation of cells expressing *hrpZ* also revealed absence of chromatin condensation while, yeast cells treated with 3mM H_2O_2 revealed extensive chromatin condensation along with the nuclear envelope typical for apoptosis (Fig. 15), cells containing multiple nuclear fragments, corresponding to the damage observed in DAPI-stained cells.

The observations under section 3.5 (Fig. 13-15) clearly indicated that the harpin$_{Pss}$–mediated YCD does not seem to follow mechanisms of mammalian apoptotic pathway.

3.5.4 Possible loss of membrane integrity in pYEUT-*hrpZ* transformed *S. cerevisiae*: A combination of two fluorescent dyes, fluorescein diacetate (FDA) and propidium iodide (PI) was used for assessment of membrane damage. Cells were cultured as described in section 3.5. After 3 h of culturing, when the pYEUT-*hrpZ* transformants cultured in glucose- or galactose-containing medium and 3mM H_2O_2 in

Figure 14 Alteration of genomic DNA in pYEUT-*hrpZ* transformant of *S. cerevisiae* Y187.



S. cerevisiae Y187 transformed with pYEUT-*hrpZ*, grown in glucose-containing medium with tryptophan drop out-supplement to OD_{600}~0.6. Cells were pelleted, washed and introduced in glucose- or galactose-containing medium and 3mM H_2O_2 in glucose-containing medium. After 3 h of induction, genomic DNA was extracted from 25 ml cultures, dissolved in equal volume of 1xTE and 5µl of the samples were resolved on an 1% agarose gel electrophoresis and stained with ethidium bromide.

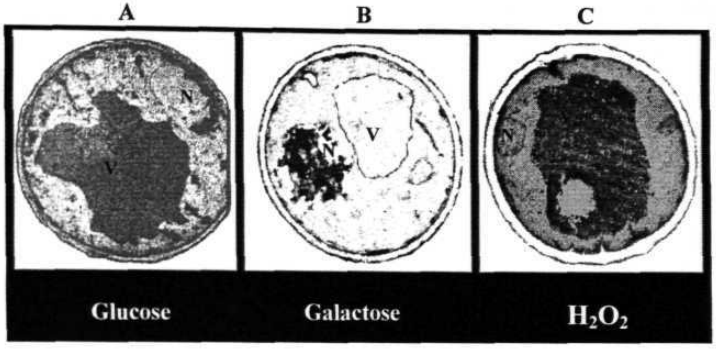
Lane 1: λ *Hind* III molecular weight markers

Lane 2: genomic DNA extracted from cells cultured in glucose-containing medium

Lane 3: genomic DNA extracted from cells cultured in galactose-containing medium

Lane 4: genomic DNA extracted from cells treated with 3mM H_2O_2 in glucose-containing medium

Figure 15 Electron micrographs of pYEUT-*hrpZ* transformant of *S. cerevisiae* Y187.



S. cerevisiae Y187 transformed with pYEUT-*hrpZ* grown in glucose-containing medium to OD_{600}~0.6. Cells were pelleted, washed and introduced into glucose- or galactose-containing medium and 3mM H_2O_2 in glucose-containing medium. After 3 h, electron microscopic analysis was undertaken for the cells grown in glucose- or galactose-containing medium and H_2O_2-treated cells. Electron micrographs of cell cultured in

A) glucose-containing medium
B) galactose-containing medium and
C) 3mM H_2O_2 in glucose-containing medium

N - Nucleus
V - Vacuole

glucose-containing media were stained with FDA and PI simultaneously to study the loss of membrane integrity, all the cells grown in presence of H_2O_2 and cells cultured in galactose-containing medium fluoresced orange/red implying loss of membrane integrity, whereas the cells grown in presence of glucose fluoresced green, taking up FDA, a membrane permeant stain implying that they had an intact membrane (Fig. 16). It could be concluded that there was loss of membrane integrity in harpin$_{Pss}$-mediated YCD.

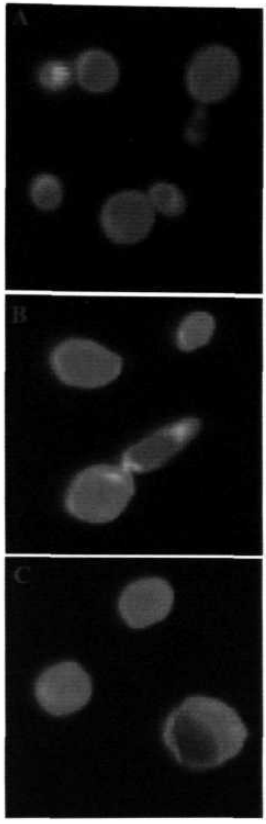
3.6 *S. cerevisiae* 'petites' were insensitive to harpin$_{Pss}$-mediated YCD

'Petites', respiratory deficient mutants, lacking functional mitochondria were generated by the "margin of growth" technique, and tested by overlaying TTC agar (at 40^0) on the plates. Red and white colonies were scored 1h after overlay (Fig. 17A). Actively respiring cells produce red colonies on high-glucose agar plates, whereas cultures incapable of respiration produce white or pink colonies.

The 'petite' mutants of *S. cerevisiae* Y187 after confirmation by TTC overlay technique were transformed with pYEUT-*hrpZ*. When pYEUT-*hrpZ* transformants of 'petite' mutants of *S. cerevisiae* Y187 were plated onto semisolid media containing glucose or galactose, growth on galactose-containing plate was unaffected and was similar to the colonies formed on glucose-containing medium. The pYEUT-*hrpZ* transformants of 'petite' mutants of *S. cerevisiae* Y187 are therefore insensitive to harpin$_{Pss}$-mediated cell death (Fig. 17B), indicating possible involvement of the mitochondrion, in this form of YCD.

3.6.2 Release of cytochrome C (Cyt C) in harpin$_{Pss}$-mediated YCD: *S. cerevisiae* Y187 transformed with pYEUT-*hrpZ* was grown in glucose-containing medium with tryptophan drop out-supplement to OD_{600}~0.6. Cells were pelleted, washed and introduced in glucose- or galactose-containing medium and 80mM acetic acid in glucose-containing medium. To check whether harpin$_{Pss}$-mediated YCD process was accompanied by release of Cyt C from mitochondria to cytosol, the levels of Cyt C if any, in cytosolic fractions from *S. cerevisiae* Y187 cells undergoing harpin$_{Pss}$-induced YCD were detected by western blot analysis. Chronic myeloid leukemia cells induced with an apoptosis inducing agent (positive control) was run in the gel along with the other samples. There was a significant release of Cyt C in the 80mM acetic acid treated

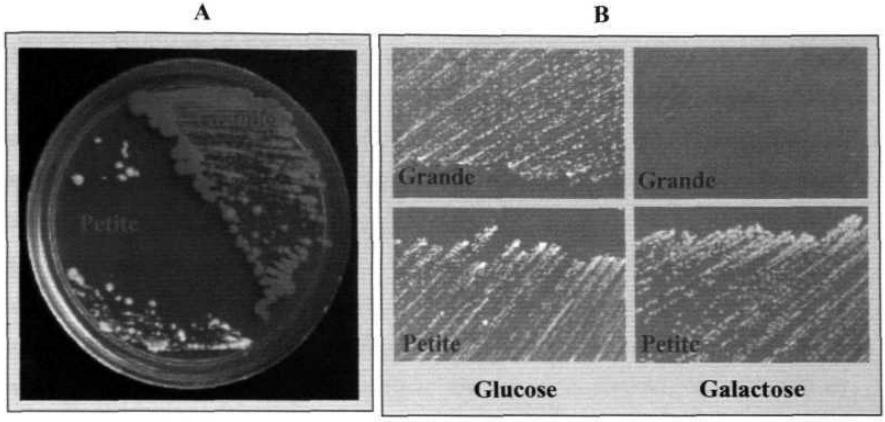
Figure 16 FDA and PI staining of pYEUT-*hrpZ* transformant of *S. cerevisiae* Y187.



S. cerevisiae Y187 transformed with pYEUT-*hrpZ*, grown in glucose-containing medium with tryptophan drop out-supplement to OD_{600}~0.6. Cells were pelleted, washed and introduced in glucose- or galactose-containing medium and 3mM H_2O_2 in glucose-containing medium. After 3 h of induction, 1 ml culture was taken, pelleted, washed and resuspended in phosphate buffered saline (PBS). This was incubated with FDA and PI for 5 min at RT and observed under fluorescence microscope. The photomicrographs of cell cultured in presence of

A) glucose
B) galactose and
C) 3mM H_2O_2 in glucose-containing medium

Figure 17 Expression of pYEUT-*hrpZ* in 'petite' mutants of *S. cerevisiae* Y187.



A) Petite mutants were generated by "margin of growth" technique using ethidium bromide as described in material and methods. The petite mutants were selected by TTC overlay method where 0.1% of 20ml of TTC agar was overlaid onto a plate containing grande and petite colonies. Red (grande) and white (petite) colonies were selected after 1h of overlay.

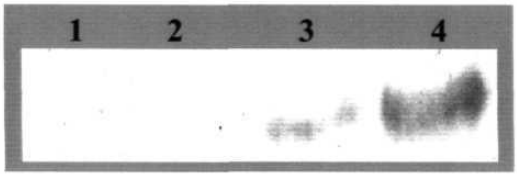
B) 'Petite' mutant of *S. cerevisiae* Y187 was generated by 'margin of growth' technique and selected by TTC overlay technique as described in materials and methods. The mutants were transformed with pYEUT-*hrpZ* by one-step transformation protocol. The pYEUT-*hrpZ* transformants of *S. cerevisiae* Y187 petite mutants were streaked onto semisolid media containing glucose or galactose. The lower half of the figure shows the pYEUT-*hrpZ* transformants of *S. cerevisiae* Y187 petite mutants streaked onto glucose-containing medium (left panel) and onto galactose-containing medium (right panel) and in the upper half of the figure indicates pYEUT-*hrpZ* transformants of *S. cerevisiae* Y187 grande streaked onto glucose- and galactose-containing medium on the left and right panels respectively.

yeast cells and the chronic myeloid leukemia cells while, there was no evidence for the leakage of Cyt C in the pYEUT-*hrpZ* transformants cultured in galactose-containing medium. This study revealed that there was no leakage of Cyt C from the mitochondrial membrane into the cytosol (Fig. 18).

3.6.3 Effect of cyclosporine A: Cyclosporine A (CsA) is a potent inhibitor of permeability transition pore formation. PTP is formed in the mitochondrial membrane during apoptosis, which results in the leakage of Cyt C into the cytosol. *S. cerevisiae* Y187 transformed with pYEUT-*hrpZ* was grown in glucose-containing medium with tryptophan drop out-supplement to OD_{600}~0.6. Cells were then washed and introduced in galactose-containing medium containing 7µg/ml CsA to study whether or not PTP formation is taking place in $harpin_{Pss}$-mediated YCD. Cells introduced in glucose- or galactose-containing medium served as controls. CsA did not affect the pYEUT-*hrpZ* transformants of *S. cerevisiae* Y187 (Fig. 19). Since there is no leakage of Cyt C into the cytosol, probably, CsA did not have any effect on $harpin_{Pss}$-mediated cell death. These observations further supported the data under section 3.6.2, to conclude that there was no release of Cyt C into the cytosol in $harpin_{Pss}$-mediated YCD.

3.7 Effect of cell death inducers on *S. cerevisiae* Y187: To compare the effect of the cell death inducers like staurosporine and acetic acid on yeast cells, pYEUT-*hrpZ* transformants of *S. cerevisiae* Y187 was cultured in glucose-containing medium with tryptophan drop out-supplement to OD_{600}~0.6. Cells were pelleted, washed and introduced in glucose-containing medium containing staurosporine and acetic acid at the final concentrations of 1µM and 80mM, respectively, to induce cell death in *S. cerevisiae*. The data in Fig. 20 on the growth of pYEUT-*hrpZ* transformed *S. cerevisiae* in glucose-containing media in presence of staurosporine and acetic acid clearly indicated that the pYEUT-*hrpZ* transformed *S. cerevisiae* did not multiply in presence of acetic acid while, staurosporine did not affect the growth of the pYEUT-*hrpZ* transformant of *S. cerevisiae* Y187. The growth of the pYEUT-*hrpZ* transformed *S. cerevisiae* in terms of OD_{600}, in glucose-containing medium steadily increased upto 4 h. These observations confirmed that, only acetic acid caused cell death in *S. cerevisiae*, and staurosporine had no effect (Fig. 20).

Figure 18 Release of cytochrome C in harpin$_{Pss}$-mediated YCD.



S. cerevisiae Y187 transformed with pYEUT-*hrpZ*, grown in glucose-containing medium with tryptophan drop out-supplement to OD_{600}~0.6. Cells were pelleted, washed and introduced in glucose- or galactose-containing medium and 80mM acetic acid in glucose-containing medium. After 3 h of induction, cytosolic fractions of the cultures were prepared, resolved in a 15% SDS-PAGE and subjected to a western blot analysis. The membrane was incubated with the Cyt C antibodies and subsequently with the anti-rabbit IgG ALP conjugate. The immunoblot was visualized by the alkaline phosphatase catalyzed colour reaction using BCIP-NBT. Cytosolic fractions of cells cultured in presence of

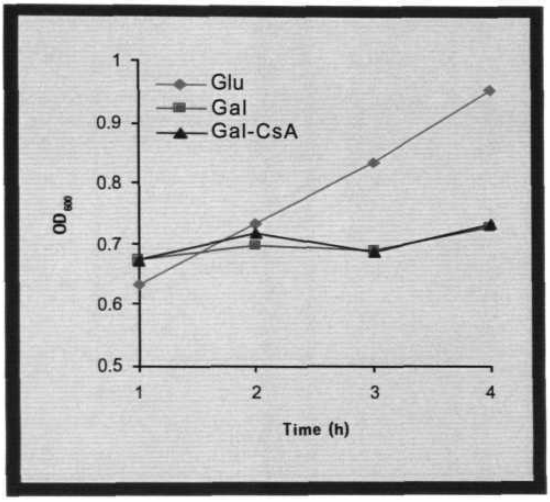
Lane 1: glucose

Lane 2: galactose

Lane 3: 80mM acetic acid in glucose-containing medium

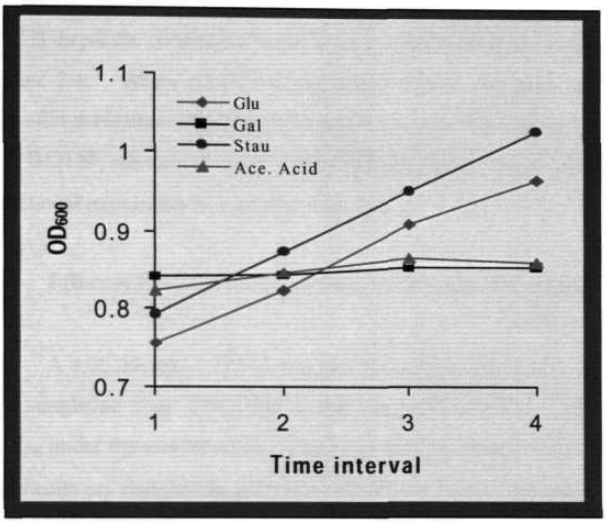
Lane 4: A positive control for Cyt C from chronic myeloid leukemia cells induced for apoptosis with a cell death inducer.

Figure 19 Effect of cyclosporin A on pYEUT-*hrpZ* transformant of *S. cerevisiae* Y187.



S. cerevisiae Y187 transformed with pYEUT-*hrpZ*, grown in glucose-containing medium with tryptophan drop out-supplement to OD_{600}~0.6. Cells were pelleted, washed and introduced in galactose-containing medium containing 7μg/ml CsA to study the effect of CsA on pYEUT-*hrpZ* transformants of *S. cerevisiae* Y187 cells. Cells introduced in glucose- or galactose-containing medium served as controls. Observations were recorded at regular time intervals for 4 h as indicated in the x-axis. The data are representative of five individual experiments.

Figure 20 Effect of cell death inducers on *S. cerevisiae* Y187 cells.



S. cerevisiae Y187 transformed with pYEUT-*hrpZ*, grown in glucose-containing medium with tryptophan drop out-supplement and diluted to OD_{600}~0.2. Cells were then washed and introduced in glucose-containing medium containing two different cell death inducers namely staurosporine and acetic acid at the concentrations of 1μM and 80mM respectively. Cells introduced into glucose- or galactose-containing medium served as controls. OD_{600} estimation was recorded at regular time intervals as indicated in the x-axis. The data are representative of five individual experiments.

3.8 Effect of harpin$_{Pss}$ on different strains of *S. cerevisiae*

Conditional expression of harpin$_{Pss}$ causing yeast cell death was studied in *S. cerevisiae* Y187. The other strains of *S. cerevisiae* used in this study are DY150, W303, Sey6211, BY4741 and BJ2168 (Table 2). All these strains were transformed with pYEUT-*hrpZ* for conditional expression in galactose-containing medium as described in section 2.4. When pYEUT-*hrpZ* transformants of these strains were plated onto semisolid medium containing galactose, YCD was observed in transformants of Sey6211 and BJ2168 similar to Y187 whereas, the other three strains were insensitive to conditional expression of harpin $_{Pss}$ (Fig. 21).

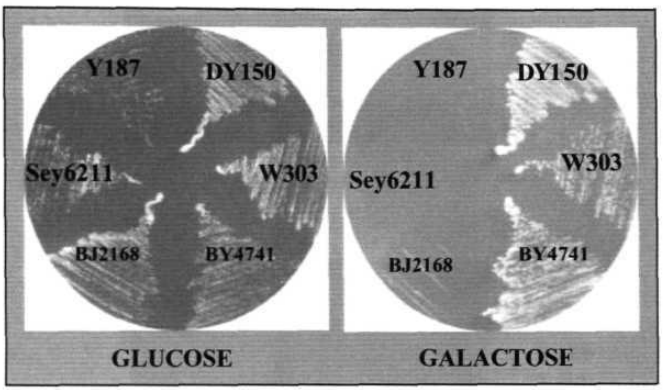
3.9 Effect of harpins from two other pathovars of *Pseudomonas syringae*

A 1.037kb and 1.112 kb fragments encoding full-length *hrpZ*$_{Psph}$ and *hrpZ*$_{Pst}$ were PCR amplified (Fig. 22A) and cloned into pYEUT (Fig. 22B), placing *hrpZ*$_{Psph}$ and *hrpZ*$_{Pst}$ under the control of the GAL1 promoter for conditional expression of these genes when cells are cultured in galactose-containing media. Plating of pYEUT-*hrpZ*$_{Psph}$ and pYEUT-*hrpZ*$_{Pst}$ transformants on semisolid media containing galactose resulted in complete inhibition of colony formation, whereas growth on the glucose-containing medium was unaffected (Fig. 23). Both pYEUT-*hrpZ*$_{Psph}$ and pYEUT-*hrpZ*$_{Pst}$ transformed *S. cerevisiae* Y187 showed YCD similar to that pYEUT-*hrpZ*$_{Pss}$.

3.10 Effect of N-terminal and C-terminal deletions on cell death activity of harpin $_{Pss}$

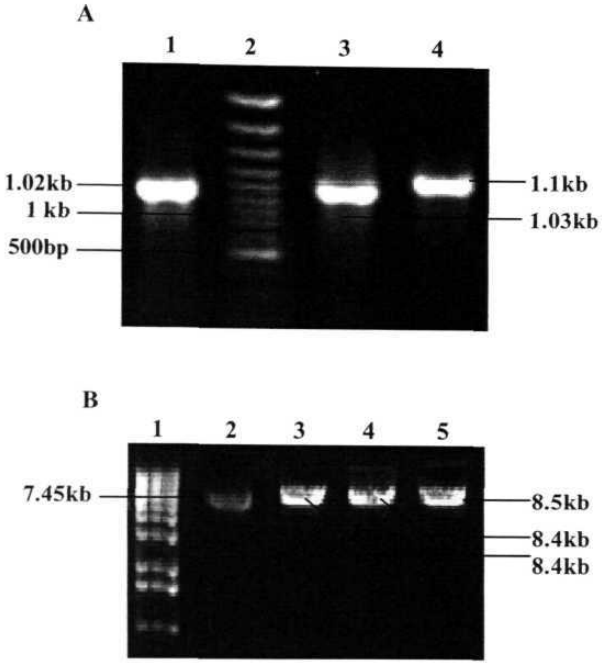
Twelve different truncations were made in the *hrpZ*$_{Pss}$ in a PCR-based approach to the minimum of 13 a.a. retaining the C-terminal end and minimum of 28 a.a. towards the N-terminal end. The truncation was done either at N- or C-terminal end or either ends of the full-length harpin$_{Pss}$ (Fig.24). The truncated mutants generated by PCR method, when analysed on the agarose gel electrophoresis showed distinct single band of the respective sizes of the fragments (Fig. 25). Twelve truncated harpin$_{Pss}$ peptides, of

Figure 21 Conditional expression of *hrpZ* in different strains of *S. cerevisiae*.



Strains of *S. cerevisiae* listed in Table 2 were transformed with pYEUT-*hrpZ* by one-step transformation protocol to study conditional expression of harpin$_{Pss}$ as described under section 2.4. pYEUT-*hrpZ* transformants of different strains of *S. cerevisiae* were streaked onto semisolid medium containing either glucose (left) or galactose (right).

Figure 22 PCR amplification and cloning of *hrpZ*$_{Pss}$, *hrpZ*$_{Psph}$ and *hrpZ*$_{Pst}$ in pYEUT.



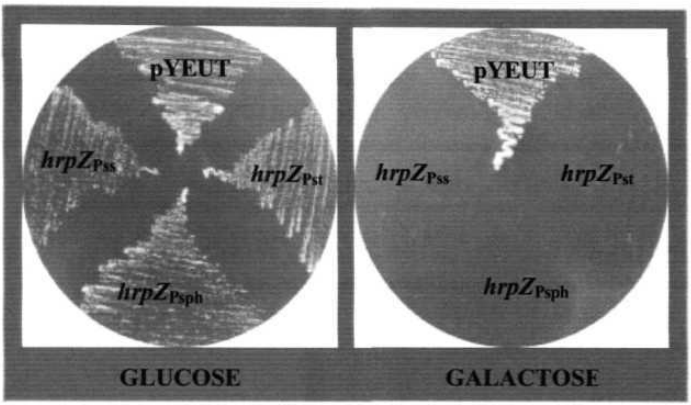
A) A 1.02kb *hrpZ*$_{Pss}$, 1.03kb *hrpZ*$_{Psph}$ and 1.13kb *hrpZ*$_{Pst}$ were PCR-amplified using the primer sequences (Table 3) that add *Bam* HI (5') and *Xho* I (3') restriction sites to *hrpZ*$_{Pss}$ and *Bam* HI (5') and *Xba* I (3') restriction sites *hrpZ*$_{Psph}$ and *hrpZ*$_{Pst}$, resolved on a 1% agarose gel electrophoresis.

Lane 1: *hrpZ*$_{Pss}$ amplicon
Lane 2: 100bp molecular weight marker
Lane 3: *hrpZ*$_{Psph}$ amplicon
Lane 4: *hrpZ*$_{Pst}$ amplicon

B) The PCR-amplified 1.03kb *hrpZ*$_{Psph}$ and 1.13kb *hrpZ*$_{Pst}$ were cloned in *Bam* HI and *Xba* I digested pYEUT and transformed in *E. coli* DH5α. *E. coli* DH5α cells containing pYEUT and cells containing pYEUT-*hrpZ*$_{Pss}$, pYEUT-*hrpZ*$_{Psph}$ and pYEUT-*hrpZ*$_{Pst}$ constructs were cultured in LB broth and plasmid minipreps, linearised with *Bam* HI and resolved on a 1% agarose gel electrophoresis.

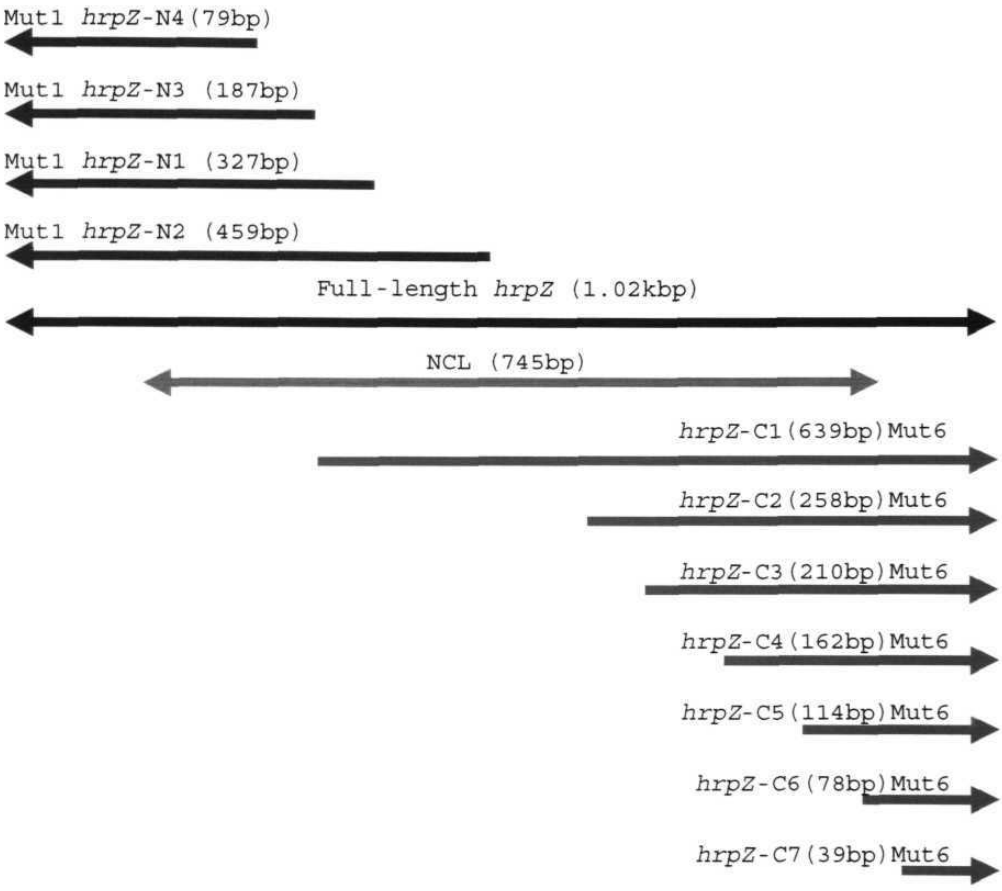
Lane 1: 1kb ladder molecular weight marker
Lane 2: linearised pYEUT
Lane 3: linearised pYEUT- *hrpZ*$_{Pss}$
Lane 4: linearised pYEUT-*hrpZ*$_{Psph}$
Lane 5: linearised pYEUT-*hrpZ*$_{Pst}$

Figure 23 Conditional expression of *hrpZ*$_{Psph}$ and *hrpZ*$_{Pst}$ in *S. cerevisiae* Y187.



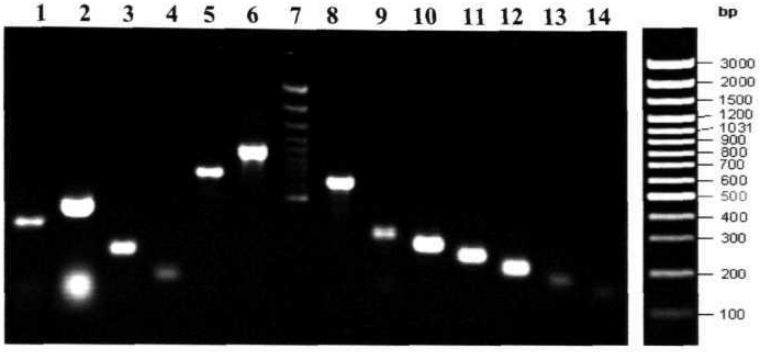
pYEUT-*hrpZ*$_{Psph}$ and pYEUT-*hrpZ*$_{Pst}$ were transformed in *S. cerevisiae* Y187 by one –step transformation protocol as described in materials and methods. A single colony of the above mentioned transformants were streaked onto semi-solid media containing glucose (left) and galactose (right). Controls (pYEUT and pYEUT-*hrpZ*$_{Pss}$), were streaked simultaneously on the glucose- and galactose-containing media.

Figure 24 Digramatic presentation of the details of *hrpZ*$_{Pss}$ and twelve different truncated mutants.



The size of the full-length *hrpZ* is of 1.02kb. Truncated mutants of *hrpZ* fragments were generated to study the structural signature of harpin$_{Pss}$. Different truncated mutants were generated by PCR-amplification using the primers listed in Table 3 and the cycling conditions described in Table 5. The truncation was done either at N- or C-terminal end or either ends of the full-length harpin$_{Pss}$. The smallest mutant generated was of 13 a.a. towards the C-terminal end of the protein.

Figure 25 PCR amplification of *hrpZ* truncated mutants.



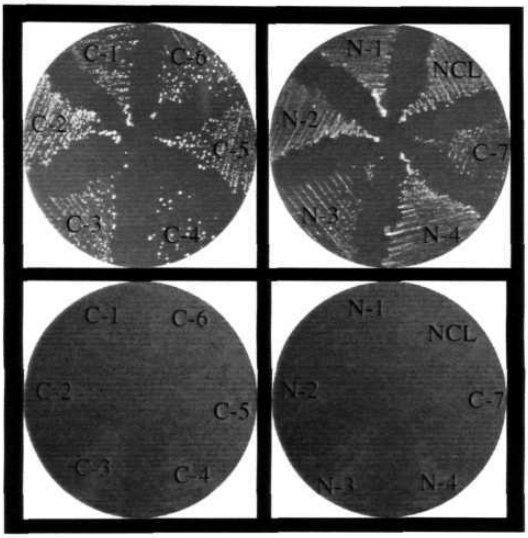
Different truncated mutants of *hrpZ* were PCR-amplified using the primer sequences (Table 3) that add *Bam* HI (5') and *Xho* I (3') restriction sites, resolved on a 1.5% agarose gel. Molecular weights of the bands in the marker used, adjacent to the gel for reference.

Lane 1: N-1 mutant
Lane 2: N-2 "
Lane 3: N-3 "
Lane 4: N-4 "
Lane 5: NCL "
Lane 6: Full-length $hrpZ_{Pss}$
Lane 7: 100bp molecular weight marker
Lane 8: C-1 mutant
Lane 9: C-2 "
Lane 10: C-3 "
Lane 11: C-4 "
Lane 12: C-5 "
Lane 13: C-6 "
Lane 14: C-7 "

different sizes caused YCD indistinguishable from that of a full-length harpin$_{Pss}$. The full-length and truncated mutants, on conditional expression under the GAL1 promoter caused cell death in *Saccharomyces cerevisiae* Y187. Plating of pYEUT-*hrpZ* transformants on a medium containing galactose resulted in complete inhibition of colony formation, whereas their growth on a glucose-based medium was unaffected. All of these twelve different mutants of *hrpZ* when cloned separately in pYEUT and transformed *S. cerevisiae* Y187, all of them retained biological activity in terms of yeast cell death. The smallest fragment, generated in our study, which retained the biological activity of the harpin$_{Pss}$, is 13 a.a. towards the C-terminal end of the protein. The mutants which either lacks the C- and N-terminal end of the full-length harpin$_{Pss}$ indicates that C- or N-terminal end of the protein is not having any specific amino acid residue to retain the activity and the mutant which lacks either ends of the full-length harpin$_{Pss}$ further confirms that even with the absence of either of terminal, the protein is retaining the biological activity. All these mutants, causing cell death in *S. cerevisiae* Y187, reveals that harpin$_{Pss}$ is a unique protein and thus retains the biological acitivity in any part of the protein in causing cell death in *S. cerevisiae*.

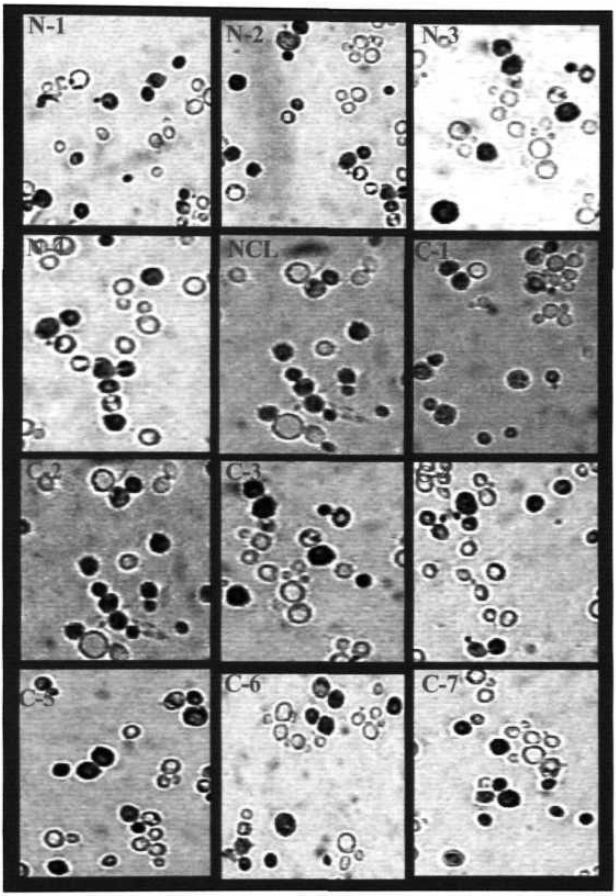
3.10.1 Harpin$_{Pss}$ mutants also cause YCD: Since the yeast cells expressing full-length *hrpZ* undergo YCD in galactose-containing media, we hypothesized that the conditional expression of truncated versions of harpin$_{Pss}$ also might cause YCD. Twelve different truncated mutants of *hrpZ* were cloned into pYEUT under the control of the GAL1 promoter (Table 5) for conditional expression of these mutants when cells are cultured in galactose-containing medium. Plating the transformants on galactose-containing medium resulted in complete inhibition of colony formation, whereas colony formation on the glucose-based medium was not affected (Fig. 26). To study the effect of mutants on yeast cells, pYEUT-N1, pYEUT-N2, pYEUT-N3, pYEUT-N4, pYEUT-C1, pYEUT-C2 pYEUT-C3, pYEUT-C4, pYEUT-C5, pYEUT-C6, pYEUT-C7 and NCL transformed cells were grown in glucose-containing medium with vigorous aeration at 30^{0}C to an optical density of ~1.0 at 600nm (OD_{600}). Cells were pelleted by centrifugation at 4500 rpm for 10 min and resuspended in either glucose- or galactose-containing media to achieve an OD_{600} of ~0.4-0.7. After various time intervals, the cell density was determined on the basis of the ability of the cells to exclude trypan blue (Fig. 27). In the yeast cultures expressing truncated harpin$_{Pss}$, cells that failed to exclude trypan blue appeared within 3 h after shifting the cells to the galactose-containing medium. Within 3

Figure 26 Conditional expression of pYEUT-*hrpZ* truncated mutants in *S. cerevisiae* Y187.



Constructs of pYEUT-*hrpZ* mutants were transformed in *S. cerevisiae* Y187 for conditional expression of truncated mutants of $harpin_{Pss}$ in galactose-containing medium. A single colony of the transformants were streaked onto semisolid medium containing either glucose (upper half of the figure) or galactose (lower half of the figure). The labels indicate designation of mutants.

Figure 27 Trypan blue staining of pYEUT-*hrpZ* truncated mutants of *S. cerevisiae*.



A single colony of the pYEUT-*hrpZ* mutants were cultured in glucose-containing medium to achieve an OD_{600} ~ 1.0. Cells were pelleted, washed and introduced in fresh medium containing either glucose or galactose. After 3 h of culturing in galactose- containing medium, 0.5 ml of culture was stained with 0.1 ml of 0.4% trypan blue. The suspension was left at room temperature for 5 min and then observed under light microscope. Photomicrographs of the designated mutants can be seen.

h of induction, the percentage of cells excluding trypan blue in cells cultured in galactose-containing medium markedly reduced to 60% when compared to the cells cultured in glucose-containing medium. Figs. 28 and 29A & B present results from a representative experiment showing a time dependent decline in the percentage of cells excluding trypan blue in pYEUT-*hrpZ* truncated mutants when cultured in galactose-containing medium. By 24 h, the percentage of viable cells in the cells cultured in galactose-containing medium was only 20% compared to the control. The colony forming ability of cells in glucose-containing media, and the change in OD_{600} all suggest that the YCD caused by full-length and truncated harpins was identical.

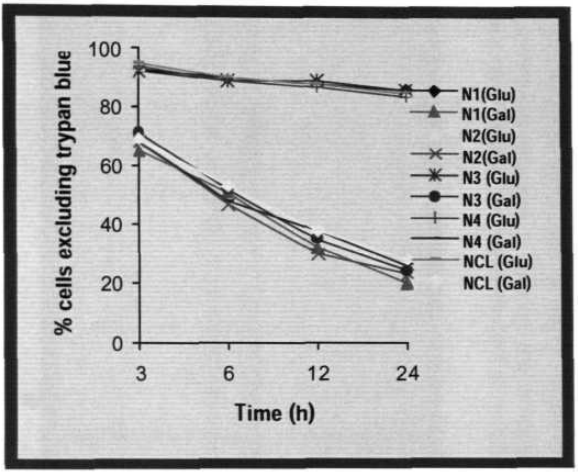
3.11 Sequence analysis

3.11.1 Detection of homology between harpin sequences: To study the homology existing between harpin sequences, HrpZ sequence of *Pseudomonas syringae* pv. *syringae* was blasted against Swissprot databank (Protein sequence database) using BlastP program. On performing this blast, none of the harpins appeared in the result list indicating that they are distantly related and there was less sequence similarity existing between them. When BlastP program could not successfully detect homology with any of the available sequences in the database, harpin was blasted against Swissprot using PSI-BLAST to distantly related sequences. After four iterations, homology was detected between the harpins from *Pseudomonas syringae* and *Erwinia chrysanthemi, E. coratovora* and *E. amylovora* (Fig. 30).

3.11.2 Multiple Sequence Alignment (MSA): A MSA with ClustalW algorithm of harpin homologues *Erwinia* sps. revealed well-conserved individual amino acids and extended regions of high similarity, mostly at the C- terminal end (Fig. 31). When MSA of harpins of *Erwinia* sps. revealed well-conserved individual amino acids, MSA was carried out for harpins belonging to *Erwinia* sp. and *P. syringae*, which showed less similarity between them and most of the conserved residues were present at the C-terminal end. Notable feature observed while carrying out MSA was, most of the glycines were conserved among these harpins (Fig. 30).

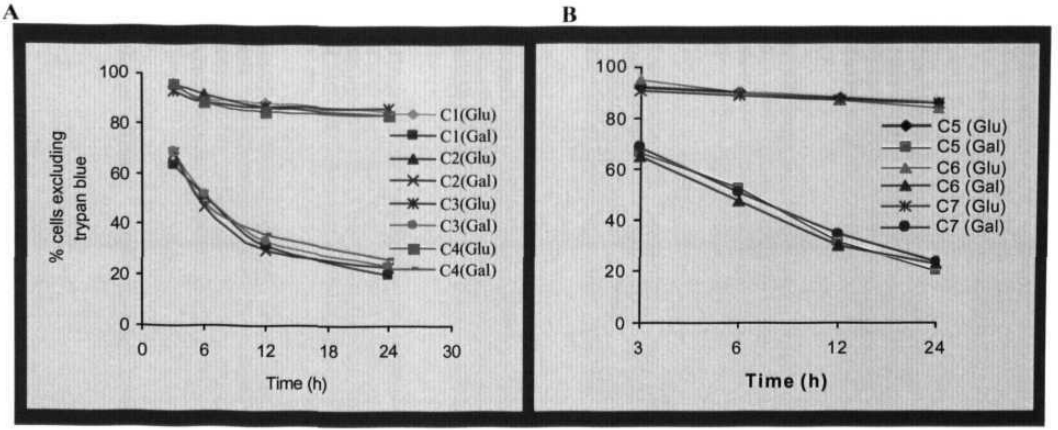
3.11.3 Secondary and tertiary structure prediction: $Harpin_{Pss}$ secondary structure, predicted using PHD algorithm, showed that among 341 residues, 48.97% had the

Figure 28 Characterization of pYEUT-*hrpZ* N1 to N4 and NCL mutants of *S. cerevisiae* Y187.



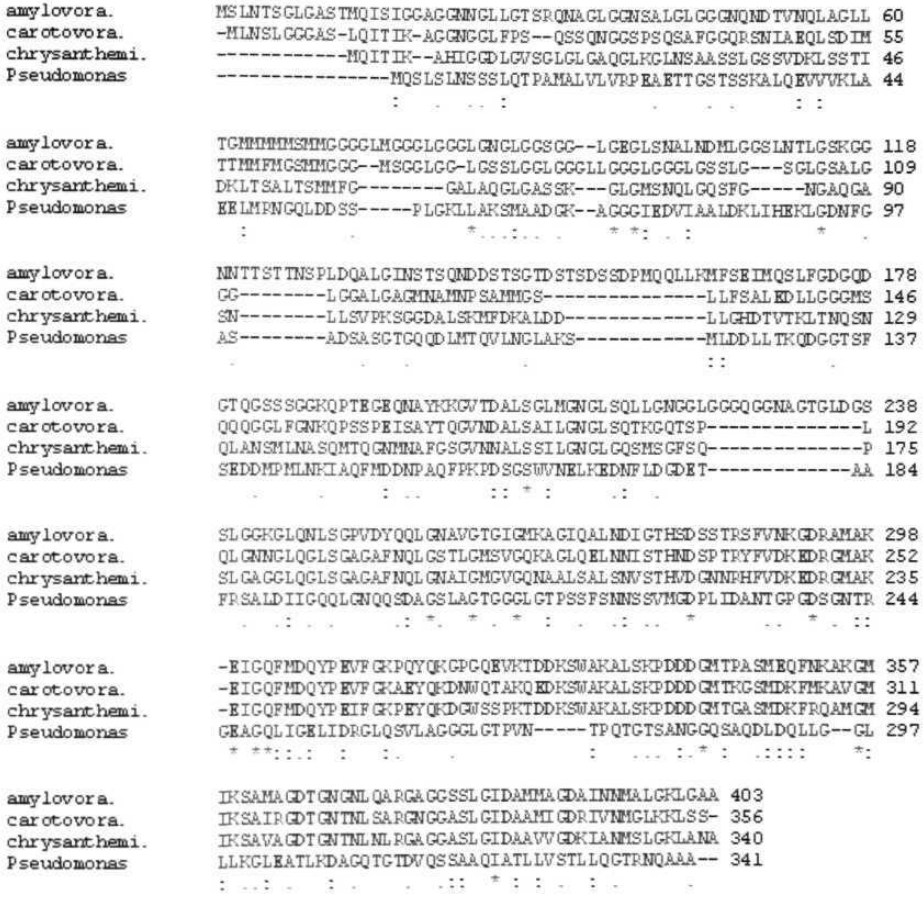
S. cerevisiae Y187 cells were transformed with pYEUT-*hrpZ* mutants grown in glucose-containing medium with tryptophan drop out-supplement to mid-log phase (OD_{600} of ~ 1.0). Cells were then washed and introduced into glucose- or galactose-containing medium to achieve an OD_{600} of 0.4–0.7, incubated for a different time duration, as indicated on the x-axis. Percentage of cells excluding trypan blue was determined by counting a total of 400 cells. The data is representative of five independent experiment.

Figure 29 Characterization of pYEUT-*hrpZ* C1 to C7 mutants of *S. cerevisiae* Y187.



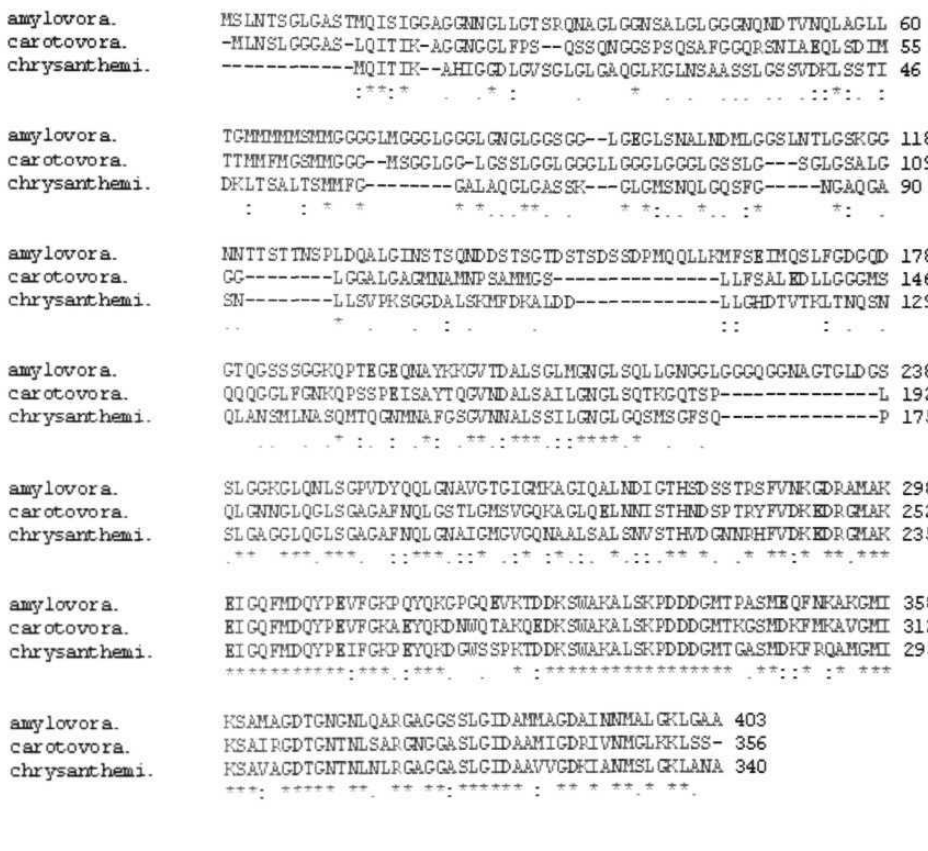
S. cerevisiae Y187 was transformed with pYEUT-*hrpZ* mutants, grown in glucose-containing medium with tryptophan drop out-supplement to mid-log phase (OD_{600} of ~ 1.0). Cells were then washed and introduced into glucose- or galactose-containing medium to achieve an OD_{600} of 0.4–0.7, incubated for a different time duration, as indicated on the x-axis. A and B indicate the percentage of cells excluding trypan blue was determined by counting a total of 400 cells for the mutants C-1 to C-4 and C-5 to C-7 respectively. The data are representative of five individual experiments.

Figure 30 Multiple sequence alignment of harpins from *Erwinia* and *Pseudomonas syringae*



In the alignment '*' indicates identity ':' indicates high similarity and '.' indicates weak similarity.

Figure 31 Multiple sequence alignment of harpins from *Erwinia* sp.



In the alignment '*' indicates identity ':' indicates high similarity and '.' indicates weak similarity.

tendency to form alpha helices, 46.92% and 4.11% had the tendency to form random coils and extended sheets (Fig. 32), respectively. Since, majority of the residues have the tendency to form alpha helices, it was predicted that the harpins have a helical structure. No significant homologues of harpin$_{Pss}$ were found in the PDB databank for homology modeling, therefore, three-dimensional model of HrpZ of *P. syringae* could not be predicted.

3.12 Crystallization of harpin$_{Pss}$

Since bioinformatics approaches to predict the structures of the harpin$_{Pss}$ were not successful, an attempt was made to crystallize harpin$_{Pss}$. The mother liquor prepared by dissolving 3mg of pure harpin $_{Pss}$ in 300µl sterile double distilled water was used for crystallization study. A 24-well disposable plastic tissue culture plates were used (Fig. 33). Out of the different combinations used (Table 6), 60% PEG and Tris.Cl pH 8.5 and 60% PEG and 0.05M sodium cacodylate pH 6.5 were found to be inducing crystals and among these two conditions, 60% PEG and Tris.Cl pH 8.5 was ideal to create the better supersaturated state leading to successful growth of harpin$_{Pss}$ crystals (Fig. 34). The crystals obtained in 60% PEG and 0.05M Tris.Cl pH 8.5 was rectangular in shape and was about 0.9mm in size. The crystal obtained in 60% PEG and 0.05M Sodium cacodylate pH 6.5 was hexagon in shape and was about 0.6mm in size. Needle-shaped crystals were commonly obtained in most of the set-ups.

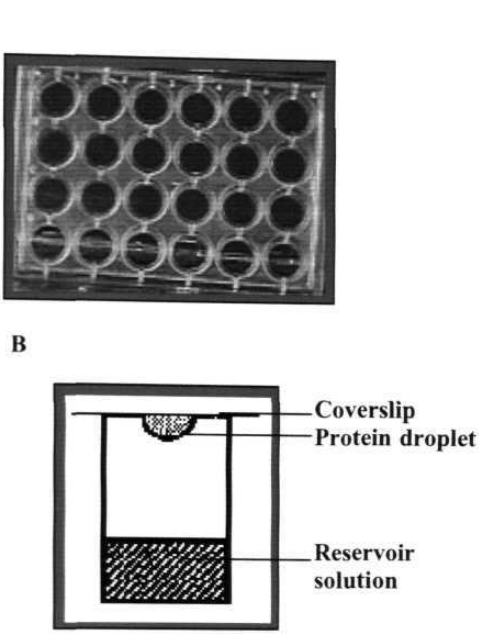
Figure 32 Secondary structure prediction for harpin belonging to *Pseudomonas syringae* (*hrpZ*).

```
          10        20        30        40        50        60        70
          |         |         |         |         |         |         |
MQSLSLNSSSLQTPAMALVLVRPEAETTGSTSSKALQEVVVKLAEELMRNGQLDDSSPLGKLLAKSMAAD
CCcEeeCCCCCCcccceEEEEEcccCCCCCCCcHHHHHHHHHHHHHHHHHHHHcCCCCCChhHHHHHHHHHHh
GKAGGGIEDVIAALDKLIHEKLGDNFGASADSASGTGQQDLMTQVLNGLAKSMLDDLLTKQDGGTSFSED
ccCCCCHHHHHHHHHHHHHHHHhCCCcccecCCcCCcchHHHHHHHHHHHHHHHHHHHHHHHhcCCCceccCC
DMPMLNKIAQFMDDNPAQFPKPDSGSWVNELKEDNFLDGDETAAFRSALDIIGQQLGNQQSDAGSLAGTG
ChHHHHHHHHHHhCCccCCCCCCCChHHHhhccCCCCCCchHHHHHHHHHHHHHHHHcCccccCCcccCCCC
GGLGTPSSFSNNSSVMGDPLIDANTGPGDSGNTRGEAGQLIGELIDRGLQSVLAGGGLGTPVNTPQTGTS
CCccCcCCCcCCCccCCCcccccCCCCCCCCCCCChHHHHHHHHHHHHHhchhheeecCCCCCCCCCCCCcC
ANGGQSAQDLDQLLGGLLLKGLEATLKDAGQTGTDVQSSAAQIATLLVSTLLQGTRNQAAA
CCCCCccHHHHHHHhHHHHHHHHHHHHHHHHcCCChHHHHHHHHHHHHHHHHHHhhhhhcCC
```

Sequence length : 341
PHD :

Alpha helix	**(Hh)**	**:**	**167 is 48.97%**
3_{10} helix	(Gg)	:	0 is 0.00%
Pi helix	(Ii)	:	0 is 0.00%
Beta bridge	(Bb)	:	0 is 0.00%
Extended strand	(Ee)	:	14 is 4.11%
Beta turn	(Tt)	:	0 is 0.00%
Bend region	(Ss)	:	0 is 0.00%
Random coil	(Cc)	:	160 is 46.92%
Ambigous states	(?)	:	0 is 0.00%
Other states		:	0 is 0.00%

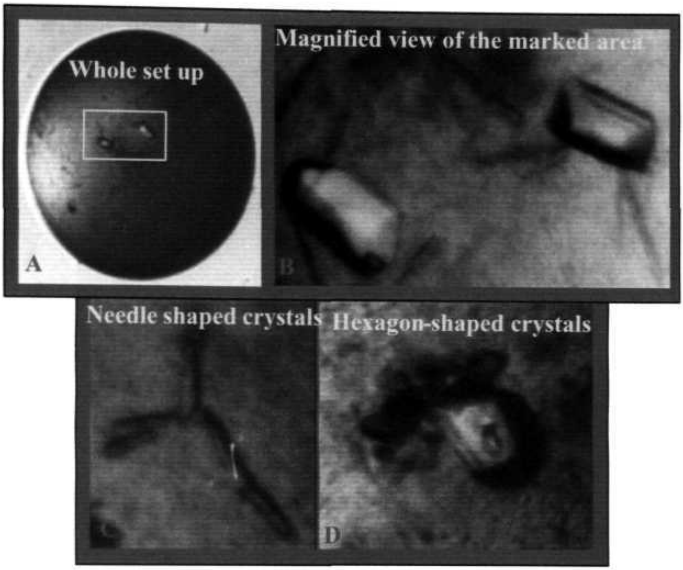
Figure 33 Protein crystallization set-up.



A) The disposable plastic tissue culture plate (Linbro or VDX plates) that have 24 wells (1.7cm diameter, 1.6 cm depth) was used. The wells have flat ground rims that allow sealing from the exterior by application of a light coating of silicone grease or vaseline to the circumference.

B) The protein microdroplet is composed on glass coverslip that have been siliconized to ensure against wetting and drop spread, and subsequently suspended over wells in a plastic plate. A protein microdroplet on the underside of the coverslip equilibrates with the reservoir solution over time through the vapor phase, causing precipitant and protein concentrations to increase in the drop, and thus induce crystallization of the protein.

Figure 34 Harpin$_{Pss}$ crystals.



The mother liquor was prepared by dissolving 3mg of pure harpin$_{Pss}$ in 300µl sterile double distilled water. The coverslips used were round glass. The individual wells have, prior to this operation, been filled with 1ml of different combinations of precipitating solution (Table 7). Each well was covered by a coverslip with a drop of 6 µl protein solution hanging from its underside, which equilibrated with the reservoir solution [(a) 60% PEG and 0.05M Tris.Cl pH 8.5 and (b) 60% PEG and 0.05M Sodium cacodylate pH 6.5] over time through the vapor phase, causing precipitant and protein concentrations to increase in the drop, and thus induced crystallization of harpin$_{Pss}$.

A) mother liquor drop on the coverslip with two well formed crystals (condition a)
B) magnified view of the marked area in picture a (condition a)
C) needle-shaped crystals (condition b)
D) hexagon-shaped crystal (condition b)

Discussion

Model organisms including bakers yeast (*Saccharomyces cerevisiae*), the fruitfly (*Drosophila melanogaster*), plant (*Arabidopsis thaliana*) and the nematode worm (*Caenorhabditis elegans*) were used to study developmental processes, disease development and gene function. *C. elegans* has provided information from a genetic screen for genes that regulate and execute PCD in basal metazoan cell death machinery, which includes *ced*-9, *ced*-3, and *ced*- 4. This approach has proved valuable in shedding light on the obscure functions of the Bcl-2 family homologues of the CED-9 of *C. elegans*. Yeasts, both fission and budding, have been used as tools to examine the functions of bonafide regulators/effectors of metazoan apoptosis by Madeo *et al.* (1997). The budding yeast *S. cerevisiae* and the fission yeast *S. pombe* have been employed extensively as models for genetic analysis of a variety of complex pathways and processes, including cell division, secretion, transcription, and receptor-mediated signal transduction. The great facility with which it is possible to genetically manipulate these single cell organisms makes yeast ideal for applying genetic approaches to many biological questions. The expression of Bax was lethal in yeast (Greenhalf *et al.,* 1996), which could be prevented by the co-expression of anti-apoptotic genes such as *Bcl-2* or *Bcl-X_L* (Ink *et al.,* 1997). Expression of CED-4 from *C. elegans* in *Scizosaccharomyces pombe* also leads to an apoptotic phenotype. Coexpression of CED-9, a bcl-2 homologue, prevents chromatin condensation. Obviously, Bax, Bak, CED-4 and caspases do not simply act as cytotoxic substances in yeast but seem to activate the same or a similar mechanism as in metazoan organisms. According to Fraser and James (1998), yeast will provide a powerful complementary system for the analysis of fundamental mechanism of metazoan cell death. An evolutionally conserved plant homologue of the *BI-1* gene, capable of suppressing Bax-induced cell death in yeast, was isolated from rice and *Arabidopsis* (Kawai *et al.,* 1999), indicating the components of the cell death pathway controlled by Bax and other Bcl-2 family proteins may be conserved from simple unicellular eukaryotes to multicellular organisms. In many instance, discoveries made originally in yeast have been experimentally validated in higher eukaryotic cells, suggesting the yeast-based strategies for studying apoptosis genes will continue to provide novel insights into conserved cell death mechanisms.

4.1 Characterization of harpin$_{Pss}$-mediated YCD

We have directly cloned the full-length or truncated sequences of *hrpZ* under the GAL1 promoter to provide expression only in galactose-containing medium and not in glucose-containing medium to study the effect of *hrpZ* expression *in vivo*. In harpin$_{Pss}$-mediated YCD, a characteristic morphological feature of the cells expressing lethal phenotype was their small size. Shrinkage is one of the notable features of cells undergoing PCD (Pennel and Lamb, 1997 and Wang *et al.*, 1996). Glucocorticoids are well known to stimulate apoptosis in immature rat thymocytes leading to cell shrinkage and DNA fragmentation (Hughes and Cidlowski, 1998).

The HR elicited by different harpins involves similar biochemical processes. The use of inhibitors to study the events of cell death pathways in general and plant HR in particular has revealed a role of protein phosphorylation and an oxidative burst. In our attempt to compare the biochemical events of plant HR and YCD, the use of protein kinase inhibitor K252a completely suppressed the YCD, as observed in some forms of the plant HR (Adam *et al*, 1997; Baker *et al*, 1993). The amount of cell death greatly increased when the H_2O_2 production was enhanced by inhibiting catalase (He *et al*, 1994). Similarly harpin $_{Pss}$-induced cell death decreased significantly in the presence of catalase (Desikan *et al*, 1996; Podile *et al*, 2001). The protective effect of catalase in the yeast cells expressing the lethal phenotype in galactose-containing medium suggested the involvement of oxidative burst in YCD, another feature similar to the plant HR. The difference between the protective effect of K252a and catalase in harpin$_{Pss}$-induced YCD, also observed by Desikan *et al.* (1996) in *Arabidopsis* suspension cultures, suggests the importance of protein phosphorylation as the major event in harpin$_{Pss}$-induced cell death.

Harpin$_{Psph}$ and harpin$_{Pst}$ belong to the same family of proteins produced by *P. syringae*. Recombinant harpins from *P.s. syringae* (harpin$_{Pss}$) and *P. s. tomato* (harpin$_{Pst}$) are related to the harpin$_{Psph}$ (77% and 53% identical at the amino acid level) and exhibited competitor activity similar to that of harpin$_{Psph}$ indicating that they targeted the same binding site in tobacco (Lee *et al.*, 2001). In this study conditional expression of the gene encoding the full-length harpin$_{Psph}$ and harpin$_{Pst}$ under galactose-inducible promoter caused cell death of *S. cerevisiae* Y187 similar to that harpin$_{Pss}$.

Hoyos *et al.* (1996) indicated that harpin$_{Pss}$ is localized in outer portion of plant cell, probably in the cell wall, and that the cell wall is crucial for the harpin$_{Pss}$-HR

induction of tobacco suspension cells. HrpZ protein encoded by the *hrpZ* genes of *P. sryingae* pv. *tomato* ($HrpZ_{Pst}$) or *syringae* ($HrpZ_{Pss}$) and *P. s phaseolicola* ($HrpZ_{Psph}$) evoked ion currents of very similar unitary conductance (Lee *et al.*, 2001). Since, $harpin_{Pss}$ causes YCD when expressed inside the cell under GAL1 promoter (Podile *et al.*, 2001), in the present study three different concentrations of both $harpin_{Pss}$ and $harpin_{Psph}$ were checked for the extracellular effect of harpins on yeast cells both in liquid and semisolid media. There was no cell death in both the cases even with 20μM of harpin. Extracellular expression of *hrpZ*, with the help of an alpha factor leader sequence also confirmed that $harpin_{Pss}$ does not cause YCD outside the yeast cells. We have reported that the use of protein kinase inhibitor K252a completely suppressed the YCD (Podile *et al.*, 2001) as observed in some forms of the plant HR. It is possible that the harpin triggers a Wak1 type of kinase located in plasma membrane that transforms the signal via second messengers as proposed by Pickard (1994).

Uchiyama *et al.* (1998) reported that the specific secretion rate of rice α-amylase fluctuated during the cell cycle and reached a maximum during the M phase, although the basis of the cell cycle dependency was unknown. They also developed a mathematical model describing the cell cycle dependency of rice α-amylase production in yeast cultured in a fed-batch fermentation (Uchiyama and Shioya, 1999). Wang and Kuo (2001) checked the effects of the synthesis of foreign protein on the mechanism of the cell cycle perturbation and checkpoint response in yeast. When it was studied, whether or not $harpin_{Pss}$-mediated cell death was cell cycle dependent by arresting the cells in two stages namely S- and M-phase and then introducing into galactose-containing medium, cell death occurred irrespective of the of stage of cell cycle and revealed that $harpin_{Pss}$ expression takes place irrespective of the cell cycle stage and $harpin_{Pss}$-mediated cell death was cell cycle independent.

In YCD, the cell death process induced by $harpin_{Pss}$ did not resemble apoptosis, inasmuch as no evidence of nuclear fragmentation and chromatin condensation was evident by EM and DAPI and did not involve the oligonucleosomal degradation pattern which classically occurs in mammalian cells when induced to undergo apoptosis. Though DNA ladders that can be detected by conventional agarose gel electrophoresis are not necessarily seen in apoptotic cells (Reed *et al.*, 1991; Ucker, 1991; Wyllie *et al.*, 1980), the morphological changes in nuclear shape and chromatin condensation are essentially universal features of apoptosis in mammalian cells but were not found in

$harpin_{Pss}$-expressing *S. cerevisiae* Y187. H_2O_2 induces apoptotic cell death in yeast, chromatin condensation and DNA smearing (Madeo *et al.*, 1999). pYEUT-*hrpZ* transformant of *S. cerevisiae* Y187 treated with 3mM H_2O_2 was, therefore, used as a control in the present study, and analysis of these cells revealed DNA fragmentation, ofcourse not a typical ladder formation but a smear. The smear instead of typical ladder is probably due to the presence of very little linker DNA between the nucleosomes (Lowary and Widom 1989). In addition, apoptosis without the occurrence of a DNA ladder has been described for several metazoan cell types (Oberhammer *et al.*, 1993). FDA and PI staining of the cells cultured in galactose-induced YCD and H_2O_2-induced apoptosis confirmed the loss of active membrane, a feature commonly observed both in apoptosis and necrosis.

All PCDs need not occur with the classical features of apoptosis in animal and plant species (Schwartz *et al.*, 1993). For example, the death of intersegmental muscle cells that occurs near the end of metamorphosis in the moth *Manduca sexta* involves the cell shrinkage typically seen during apoptosis, and nuclear fragmentation do not occur (Schwartz *et al.*, 1993). PCD also occurs in the absence of DNA degradation that can be detected by either routine agarose gel electrophoresis or pulsed-field gel electrophoresis (Cornillon *et al.*, 1994). Likewise, the PCD response induced by pathogens in tobacco (HR) was associated with induction of endonuclease activities and genomic DNA digestion, but the DNA fragmentation does not involve the oligonucleosomal pattern of DNA cleavage or nuclear morphological changes often seen in apoptosis (Mittler and Lam, 1995).

Bax induces apoptosis in mammalian cells by the activation of ICE (Interleukin-1beta-Converting Enzyme) proteases (Chinnayan *et al.*, 1996), which mediate the leakage of several proteins including those of the nuclear matrix and nuclear envelope, finally leading to DNA fragmentation. Bax-induced cell death in *S. pombe* was not accompanied by any classical morphological feature of apoptosis. Neither evidence of nuclear fragmentation nor of chromatin margination against the nuclear envelope, not even internucleosomal DNA fragmentation was observed. Jurgensmeier *et al.* (1997) therefore labelled the Bax-induced effect as "cytotoxicity", to distinguish it from apoptosis.

Herker *et al.* (2004) demonstrated that chronologically aged yeast cultures die exhibiting typical markers of apoptosis, and proposed that yeast cells commit altruistic

suicide to provide nutrients for other, probably younger and fitter cells. This provided for the first time an explanation on how apoptosis can be advantage even for unicellular organisms. Madeo *et al.* (2002) showed that oxygen stress induces yeast apoptosis *via* activation of the yeast caspase Yca1p (Disruption of YCA1 also increased the survival rate of chronologically aged cultures). It was demonstrated *in vivo* that aging cells show caspase activity using a fluorogenic caspase substrate (Herker *et al.*, 2004). The amount of cell death greatly increased when the H_2O_2 production was enhanced by inhibiting catalase (Desikan *et al.*, 1996). $Harpin_{Pss}$-induced YCD decreased significantly in the presence of catalase. The protective effect of catalase in the yeast cells expressing the lethal phenotype in galactose-containing medium suggested the involvement of oxidative burst in YCD (Podile *et al.*, 2001).

No systematic comparison between PCD and $harpin_{Pss}$-mediated plant HR is known except that Desikan *et al.* (1998) argued that both harpin and H_2O_2 initiate PCD in *Arabidopsis* suspension cultures. In harpin-mediated YCD a characteristic morphological feature of the cells expressing lethal phenotype was their decreasing size. The yeast cells expressing YCD and H_2O_2-mediated cell death were apparently similar. The genomic DNA in cells expressing $harpin_{Pss}$ showed no signs of fragmentation, and it remains to be seen whether there is DNA fragmentation in plant cells undergoing $harpin_{Pss}$-mediated HR.

Unlike multicellular organisms, the yeast *S. cerevisiae* has the unique ability to survive without functional mitochondria and to live by glycolysis alone. Bcl-2 family members are localized, though not exclusively, in the outer membrane of mitochondria. That mitochondria play some role in apoptosis is widely accepted, although the precise nature of that role is unclear. The release of Cyt C from the intermembranal space seems to be an early event in apoptosis, and Bcl-2 is able to inhibit the release of Cyt C from mammalian mitochondria (Kluck *et al.*, 1997). BAX-mediated cytotoxicity in yeast is also associated with release of Cyt C from the mitochondrion (Manon *et al.*, 1997). Greenhalf *et al.* (1996) observed that Bax expression does not lead to death in 'petite' mutants, arguing that fully active mitochondria are essential for Bax-induced cytotoxicity in yeast. It was reported by Xie and Chen (2000) that plant PCD induced during harpin-induced HR is also associated with altered mitochondrial functions, inducing inhibition of ATP synthesis in tobacco cell cultures. Boccara *et al.* (2001) by adapting infra-red thermography, revealed a role for mitochondria in pre-symptomatic cooling during

harpin-induced HR. The petite mutants being insensitive to $harpin_{Pss}$-mediated yeast cell death suggested the role of mitochondria in $harpin_{Pss}$-mediated YCD.

One of the major apoptotic pathways is activated by the release of apoptogenic protein, Cyt C, from mitochondria into the cytosol. The release of Cyt C, one of the most important respiratory-chain proteins, from the mitochondria into the cytosol is the hallmark of cells undergoing apoptosis. In mitochondria, Cyt C is required as an electron carrier in oxidative phosphorylation, a process, which generates the majority of intracellular ATP (Hatefi, 1985). Cyt C resides in the space between the outer and inner membrane of mitochondria, where it snuggles up to the Cyt C oxidase complex located in the inner membrane. Several apoptosis inducing agents are known to trigger mitochondrial uncoupling leading to the rupture of outer membrane. Insensitivity of 'Petite' mutant to $harpin_{Pss}$-mediated YCD indicated that mitochondria were playing a role in $harpin_{Pss}$-induced YCD, while there was no evidence of Cyt C in $harpin_{Pss}$-mediated YCD. The same was confirmed using CsA on yeast cells. CsA, a potent inhibitor of mitochondrial permeability transitition inhibits apoptosis in yeast (Severin and Hyman, 2002), did not protect *S. cerevisiae* Y187, probably because of the non-involvement of Cyt C leakage in $harpin_{Pss}$-mediated YCD. In *Arabidopsis* cells, harpin induced a rapid release of Cyt C from mitochondria into the cytosol (Krause and Durner, 2004), which is regarded as a hallmark of PCD or apoptosis. Bax expression induces the release of Cyt C from mitochondria and decrease Cyt C oxidase (Manon *et al.*, 1997), similar to its effects in mammals. However, a yeast strain containing only a Cyt C-green fluorescent protein fusion which is not released upon the expression of Bax in yeast cell also dies, indicating that mitochondrial release of Cyt C is not essential for killing (Roucou *et al.*, 2000).

In yeast cells undergoing a PCD process induced by acetic acid, translocation of Cyt C to the cytosol and reactive oxygen species production, two events known to be proapoptotic in mammals, were observed (Ludovico *et al.*, 2002). Staurosporine (a non-specific protein kinase inhibitor inducing apoptosis in cells of higher organisms) stimulated reactive oxygen species (ROS) production to kill *Tetrahymena* (Christensen *et al.*, 1998). Effect of acetic acid and staurosporine on yeast cells was compared with $harpin_{Pss}$-mediated YCD. Acetic acid caused cell death in yeast whereas staurosporine had no effect on yeast cells but $harpin_{Pss}$-mediated YCD was not similar to acetic acid induced cell death. We have reported that $harpin_{Pss}$-mediated YCD is similar to H_2O_2 induced cell death by carrying out a flow cytometric analysis (Podile *et al.*, 2001), but

other features like DNA fragmentation and chromosomal condensation are not seen in harpin$_{Pss}$-mediated YCD. Release of Cyt C is not reported till date in H_2O_2-induced yeast cell death, a feature, which is also observed in harpin$_{Pss}$-mediated YCD.

Strains of *S. cerevisiae* DY150, W303, Sey211, BY4741, and BJ2168 cells when transformed with pYEUT-*hrpZ* grow normally on glucose/galactose-containing media except for strains Sey211 and BJ2168, which were showing conditional expression similar to that Y187. It is not uncommon that certain strains are insensitive to conditional cell death induced with the expression of cell death-associated protein from heterologous systems, a phenomenon observed in 'petite' mutants.

4.2 Structure -function of harpin $_{Pss}$

It was reported by He *et al.* (1993) that carboxy-terminal 148 amino acid portion of harpin$_{Pss}$ contains two directly repeated sequences of GGGLGTP and QTGT and is sufficient and necessary for elicitor activity. Later, the same group showed that all four HrpZ fragments elicit an HR that is indistinguishable from HrpZ-elicited HR in tobacco (Alfano *et al.*, 1996). Since these HrpZ fragments represent non-overlapping fragments, it was concluded that the elicitor activity of HrpZ is not confined to one region on the protein. Initially four truncated *hrpZ* mutants were generated similar to that of the *hrpZ* fragments studied by Alfano *et al.* (1996) and these caused YCD similar to the full-length harpin. This demonstrated that the harpin$_{Pss}$-induced plant HR and YCD show obvious similarities (Podile *et al.*, 2001). We further constructed pYEUT that would express smaller fragments to search for a structural signature for regions with elicitor activity. Studies to gain more insight into the structural features of HrpZ to identify the smallest harpin sequence that can cause YCD, different truncations were made, cloned and when expressed in yeast, a small harpin peptide with 13 amino acids retained the elicitor activity in terms of YCD. It was reported that N-terminal (NT) end of Bax a, containing first a helix (Ha1), is a functional mitochondrial-addressing signal both in mammals and in yeast. It was suggested that Bax is targeted to mitochondria by its NT and thus through a pathway that is unique for a member of BCL-2 family. Mutations at the N-terminal end confirmed the inhibitory function of the zone known as ART (Apoptotic Regulation of Targeting). Mutations at the C-terminal end of the protein support the hypothesis that the hydrophobic helix a9 is not required for the insertion of Bax, which suggested that the conformation of a9-helix plays a significant role in

Bax/Bcl-xl interaction (Arokium *et al.,* 2004). To study whether such a specific role is played by N-terminal or C-terminal sequence in YCD, a mutant which lacked either ends of $harpin_{Pss}$ was generated and cloned in pYEUT to study the conditional expression of *hrpZ*, which also caused YCD.

Our study confirmed that $harpin_{Pss}$ retains the activity even in the 13 a.a. towards the C-terminal end. Attempts to search for the homologues of harpin, in a bioinformatic approach, indicated that the harpins might share homology with chaperones. Type III chaperones have been identified in several animal pathogens (Menard *et al.,* 1994, Day and Plano, 1998; Fu and Galan 1998; Wainwright and Kaper, 1998; Page *et al.*, 2001) and, other than between homologues, they share little amino acid sequence similarity with each other. However, they do share several general structural characteristics such as a small size, an acidic isoelectric point and predicted a-helical secondary structure (Bennett and Hughes, 2000; Plano *et al.*, 2001). The harpin fragment retaining the activity was a small peptide, acid isoelectric point and predicted helical secondary structure, similar to Type III chaperone.

TTSS effectors are commonly associated with mobile genetic elements, and many appear to have been acquired by horizontal gene transfer (Arnold *et al.,* 2003; Kim and Alfano, 2002). For example, the *P. syringae* effector genes are associated with regions missing in the related bacteria *P. aeruginosa* and *P. putida*, and some are carried on plasmids or in hypervariable regions of the genome, such as the exchangeable effector locus (Alfano *et al*, 2000; Bretz *et al.*, 2003; Buell *et al.,* 2003; Deng *et al.*, 2003). In all the genomes that have been sequenced to date, the *hrp/hrc* genes are found clustered in a single region of the chromosome, or on a 0.1-Mb megaplasmid in the case of *Ralstonia solanacearum* or a 150-kb plasmid in *Pantoea agglomerans* (*Erwinia herbicola*) pv. *gypsophilae* (Buttner *et al.,* 2003; Manulis and Barrash, 2003). The exchangeable effector locus in *P. syringae* is one such hypervariable region, and another apparent hot spot for effector gene recombination has been identified elsewhere in the *P. syringae* genome (Alfano *et al.,* 2002; Arnold *et al.,* 2001). Duplications within genomes of effector genes and associated mobile genetic elements may support ongoing insertions and deletions of effector genes. Allelic variations in effectors that are present in many (or all) pathovars are important determinants of host specificity (Stevens *et al.,* 1998). Importantly, an example of host range limitation by a single TTSS effector has recently been reported: PthG in *Pantoea agglomerans* pv. *gypsophilae* appears to be the sole

factor preventing this gypsophila pathogen from expanding its host range to beet (Feldman and Cornelis, 2003).

Research on the evolution and function of TTSS in *Salmonella* and *Yersinia* spp. has yielded two revolutionary insights. First, genes associated with pathogenicity, such as those encoding TTSS, are often clustered in horizontally acquired pathogenicity islands (Pais) that may enable the evolution of virulence in "quantum leaps" (Groisman and Ochman, 1996). Second, TTSS have the remarkable ability to inject bacterial proteins into the cytoplasm of eukaryote host cells (Rosqvist *et al.*, 1994; Sory and Cornelis, 1994).

The likely universality of harpins among plant pathogenic bacteria that elicit the HR in nonhosts finds further experimental support in the report of Genin *et al.* (1993) that *P. solanacearum* produces one or more heat-stable, protease-sensitive factors that are secreted by Hrp^+ cells and elicit HR-like necrosis in tobacco. The *hrp/hrc* genes are probably universal among necrosis-causing Gram-negative plant pathogens, and they have been sequenced in *P. syringae* pv. *syringae* 61, *E. amylovora* Ea321, *X. campestris* pv. *vesicatoria* 85-10, *and R. solanacearum* GM11000 (Alfano and Collmer, 1997). The discrepancy between the distribution between these bacteria and the phylogeny of the bacteria provides some evidence that *hrp/hrc* gene clusters have been horizontally acquired and therefore may represent Pais (Alfano and Collmer, 1997).

Loss of gene function also may be important in the evolution of virulence (Parkhill, 2002). Several candidate effector genes in DC3000 are disrupted by mobile genetic elements (Buell *et al.*, 2003; Greenberg and Vinatzer, 2003); the truncated product of two such genes can be translocated into plant cells by the *P. syringae* TTSS (Schechter *et al.*, 2004), frameshifted effector pseudogenes have been found in the exchangeable effector loci of several *P. syringae* pathovars (Charity *et al.*, 2003; Deng *et al.*, 2003); and many effector genes have limited distribution among pathovars and races of *P. syringae* and *X. campestris* and affiliated species.

$Harpin_{Pss}$ was reported by He *et al.* (1993) to have no significant similarity with sequences deposited in major sequence databases accessible with the Blast search program homology (Altschul *et al.*, 1990), nor were motifs of known biological significance detected for $harpin_{Pss}$ using the MOTIF program in the Genetics Computer Group Sequence Analysis Software Package (Devereaux *et al.*, 1984). However, limited sequence similarity was detected in their study between $harpin_{Pss}$ and $harpin_{Ea}$ over a stretch of 22 amino acids. We used existing databases to search for homologs of harpin.

On using the increased power algorithms such as BLAST (i.e., position specific iterated BLAST) (Altschul and Koonin, 1998) affords the opportunity for identification of distance homologies that can be the basis of understanding the structure-function relationship. This search resulted in finding three homologs, harpins of *Erwinia amylovora*, *E. chrysanthemi* and *E. coratovora*. MSA of harpins from *Erwinia* sps. revealed well-conserved residues and high similarity among them. When MSA of harpins from *Erwinia* sps. and *P. seudomonas* was carried out, there was less similarity between them and one notable feature in both the cases was most of the glycines were conserved and the homology was mostly towards the C-terminal end.

Secondary prediction using PHD algorithm revealed that harpins have tendency to form alpha helices and based on this result, we conclude that harpin has helical structure. Comparative or homology protein structure modeling builds a three-dimensional model for a protein of unknown structure (the target) based on one or more related proteins of known structure (the templates). Therefore, if the similarity between two proteins is detectable at the sequence level, structural similarity can usually be assumed. When an attempt was made to predict the 3D structure of harpin, we were not successful since no significant homologs were found in the PDB databank.

Since, 3D structure prediction was not successful by bio-informatic approaches, crystallization study was carried out and was successful in crystallizing harpin$_{Pss}$, the first step in studying the 3-Dimensional structure of the protein. 3D structure of the protein helps in understanding the structure-function relationship of the protein. Crystallization and X-ray analysis of a complex between the *Plasmodium vivax* sexual stage 25kDa protein Pvs25 and a malaria transmission-blocking antibody fab fragment was successful (Saxena *et al.*, 2004) which will provide an understanding of the interaction between Pvs25 and 2A8 antibody that inhibits ookinete development in the mosquito and should aid in the development of transmission-blocking vaccines against *P. vivax* malaria. We have crystallized harpin$_{Pss}$, for the first time and the X-ray analysis and 3-D structure of the same would reveal the functional domain(s) of the protein.

Expression of harpin encoded by *hrpZ* from *Pseudomonas syringae* pv. *syringae* 61 under control of GAL1 promoter in *Saccharomyces cerevisiae* Y187 resulted in galactose-inducible cell death sharing features of plant HR with a notable feature of cell shrinkage which is observed in many apoptotic cells. 'Petite' mutant being insensitive to harpin$_{Pss}$-induced cell death suggested that the YCD caused by harpin$_{Pss}$ is principally associated with mitochondria in yeast cells indicating a feature of apoptosis while there

is absence of the other hallmarks of apoptosis like leakage of cytochrome C, nuclear fragmentation and chromosomal condensation in $harpin_{Pss}$-mediated YCD. Overall, characterization of $harpin_{Pss}$-mediated cell death implies that this kind of cell death is neither similar to that of mammalian apoptosis nor necrosis but certainly shares to some extent, some features of both the kinds of cell death.

Summary and Conclusions

5.1 Background and Objective: *Pseudomonas syringae* pv. *syringae* 61 *hrpZ* encodes harpin$_{Pss}$, a 34.7kD extracellular protein that elicits hypersensitive response (HR) in plants. We showed that conditional expression of harpin$_{Pss}$ causes yeast cell death hypothesizing that yeast might share, with plants, conserved components in cell death pathway (Podile *et al.*, 2001). We have also reported that harpin-induced plant HR and YCD are similar because: conditional expression of harpin$_{Pss}$-causes YCD, oxidative burst plays a role in harpin $_{Pss}$-mediated YCD and a protein kinase inhibitor (K252a) that suppresses plant HR also suppresses YCD.

With the above background, the present study was focused on: a) characterization of the conditional expression of harpin$_{Pss}$-mediated YCD and b) the structure-function relationship of harpin$_{Pss}$, an unusual peptide, causing cell death in both plants and *Saccharomyces cerevisiae* Y187.

5.2 Characterization of conditional expression of harpin$_{Pss}$-mediated YCD: Harpin$_{Pss}$ was expressed in *E. coli*, purified, polyclonal antibodies were raised in rabbit and also the protein was sequenced. Amino-terminal sequencing of the purified harpin$_{Pss}$ confirmed the start codon of harpin$_{Pss}$ and revealed the sequence similarity with the deduced amino acid sequence for the gene sequence available in the database.

A 1.02kb full-length *hrpZ* from *P.s.* pv. *syringae* was cloned into pYEUT under the control of the GAL1 promoter for conditional expression of harpin and cells were shifted to galactose-containing media from glucose-containing media. Plating of pYEUT-*hrpZ* transformants on semisolid medium containing galactose resulted in complete inhibition of colony formation, whereas growth on the glucose-based medium was unaffected. The western blot analysis confirmed the expression of harpin$_{Pss}$ in yeast cells expressing the *hrpZ* gene in pYEUT-*hrpZ* under the GAL1 promoter in galactose-containing medium within 1 h of induction. A time-dependent decline in the percentage of trypan blue-excluding cells in cultures of pYEUT-*hrpZ* transformants when cultured on galactose-containing medium was observed. In contrast, cells grown in glucose-containing medium remained mostly dye-negative. The number of colonies markedly reduced to about 50% within 6 h. By 24 h, very few viable colony-forming cells remained in the cultures. Thus, conditional expression of *hrpZ* resulted in irreversible inhibition of colony formation, consistent with a lethal phenotype.

Extracellular effect of harpin when studied, by adding different concentrations of harpins extracellularly ranging from 5µM to 20µM on pYEUT-*hrpZ* transformant of *S.*

cerevisiae Y187 cultured in glucose-containing medium, revealed that harpins had no effect on yeast cells cultured both in liquid and semisolid media and thus cells were growing normally. Extracellular expression of *hrpZ*, with the help of an alpha factor leader sequence also confirmed that harpin does not cause YCD from outside the yeast cells.

S- and M-phase arrested yeast cells after 3 h of induction indicated that harpin$_{Pss}$-mediated cell death occurred in both S- and M-phases, independent of the stage of cell cycle.

To assess chromatin condensation and nuclear morphology of the cells expressing harpin$_{Pss}$, DAPI staining, genomic DNA and EM analyses were carried out. DAPI stained cells grown in the presence of galactose did not show chromosomal condensation, which reveals that chromosomal DNA fragmentation does not seem to occur in the harpin$_{Pss}$-induced YCD. Analysis of genomic DNA on a conventional agarose gel electrophoresis confirmed that there was no genomic DNA fragmentation or ladder formation in cells cultured in galactose-containing medium suggesting that this feature of apoptosis was not seen in harpin$_{Pss}$-mediated cell death, while there was genomic DNA fragmentation, a typical marker of apoptosis in H_2O_2-treated cells. Electron microscopic observations further confirmed that the yeast cells expressing *hrpZ* revealed no evidence of chromatin condensation. Overall this study confirmed that there was no chromatin condensation and nuclear fragmentation in harpin$_{Pss}$-mediated YCD.

Possible loss of membrane integrity in harpin$_{Pss}$-mediated YCD when studied by staining the cells with FDA and PI simultaneously, cells cultured in galactose-containing medium fluoresced orange/red taking up PI, implying loss of membrane integrity, whereas the cells grown in presence of glucose were green, taking up FDA, implying that they had intact membrane, indicating that there could be loss of membrane integrity in harpin$_{Pss}$-mediated YCD.

Mitochondria play a central role in programmed cell death. To study the role of mitochondria in harpin$_{Pss}$-mediated YCD, 'petite' mutants (respiratory deficient mutant) of *S. cerevisiae* Y187 were generated by "margin of growth" technique and transformed with pYEUT-*hrpZ*. The transformants of 'petite' mutants of *S. cerevisiae* being insensitive to harpin$_{Pss}$-mediated cell death suggested the role of mitochondria in this form of YCD.

One of the major apoptotic pathways is accompanied by the release of cytochrome C (Cyt C) from mitochondria into the cytosol. Western blot analysis of the

cytosolic fractions of pYEUT-*hrpZ* transformant of *S. cerevisiae* Y187 cells cultured in galactose-containing medium revealed that there was no evidence for the leakage of Cyt C from the mitochondria into the cytosol. Cyclosporine A, a potent inhibitor of permeability transition pore formation, did not have an affect on harpin$_{Pss}$-mediated YCD.

The observed cell death in *S. cerevisiae* Y187 when studied in other strains of yeasts *viz.*, *S. cerevisiae* DY150, W303, Sey6211, BY4741 and BJ2168, YCD was observed in pYEUT-*hrpZ* transformants of Sey6211 and BJ2168 similar to Y187, whereas, the other three strains were insensitive to harpin$_{Pss}$-mediated YCD.

A comparative account of described characteristics of plant HR and harpin-induced YCD is presented as Table 7.

5.3 Structure-function relationship of harpin: Harpin$_{Psph}$ and harpin$_{Pst}$ belong to the same family of proteins produced by *P. syringae*. Conditional expression of harpin$_{Psph}$ and harpin$_{Pst}$ under galactose-inducible promoter caused cell death of *S. cerevisiae* Y187 similar to harpin$_{Pss}$.

To study the effect of N-terminal and C-terminal deletions on cell death activity of harpin$_{Pss}$, twelve different mutants were generated by PCR-based approach by truncating either at N- or C-terminal end or either ends of the full-length harpin$_{Pss}$. When these mutants were cloned and expressed in *S. cerevisiae* Y187, all the mutants retained the biological activity, similar to the full-length *hrpZ* in terms of YCD.

An attempt was made to study the structure-function relationship of harpin using bio-informatic approaches. Homology existing between harpins when studied using Blast P program revealed that harpins are distantly related and there is less sequence similarity existing between them. So, harpin was blasted against Swissprot using PSI-BLAST and then homology was detected between the harpins of *Erwinia amylovora, E. chrysanthemi* and *E. carotovora*. A multiple sequence alignment (MSA) of these homologues revealed well-conserved individual amino acids and extended regions of high similarity, mostly at the C- terminal end. MSA of harpins from *Erwinia* sp. and *Pseudomonas syringae* showed very less similarity between them. Most of the conserved residues were present at the C-terminal end and notable feature observed in performing the MSA was, most of the glycines are conserved in harpins.

When harpin secondary structure was predicted using PHD algorithm, the results showed that all harpins have tendency to form alpha helices. There is a report showing that Type III chaperone is known to have helical secondary structure. Attempt to predict the three-dimensional model of HrpZ of *P. syringae* was not successful since no significant homologues were found in the PDB databank.

Table 7 Comparison of the characteristic features of plant HR and YCD.

Features of HR/YCD	**Plant HR**	**YCD**
Triggered by interaction of '*R-avr*' gene products	Yes	Not known
Triggered by pathogen-derived molecules like harpins	Yes	Yes
Reduction in cell size and localized cell death	Yes	Yes
Truncated harpins cause cell death similar to full-length *hrpZ*	Yes	Yes
Involves a serial signal transduction and *de novo* synthesis of transcripts and proteins	Yes	Yes
Triggers oxidative burst	Yes	Yes
Kinase activity blocked by K252a, a protein kinase inhibitor	Yes	Yes
Features of apoptosis like membrane blebbing, formation of apoptotic bodies, etc.,	Yes	No
Maintenance of plasmalemmal integrity	Yes	No
Chromatic condensation and DNA	Yes	No
Involvement of mitochondria	Yes	Yes
Leakage of cytochrome C from mitochondria into the cytosol	Yes	No

Since bio-informatic approaches to predict the structure of the harpin were unsuccessful, an attempt was made to cystallize harpin$_{Pss}$. Out of the different combinations used, 60% PEG and Tris.Cl pH 8.5 was ideal to create the supersaturated state for successful growth of harpin crystals.

5.4 MAJOR FINDINDS OF THE PRESENT WORK

- Harpin$_{Pss}$-mediated yeast cell death was characterized:
  - Conditional expression of harpin$_{Pss}$ caused YCD
  - Harpins have no extracellular effect on *S. cerevisiae* Y187 cells
  - Harpin$_{Pss}$-mediated YCD was independent of the stage of cell cycle
  - Chromosomal condensation and nuclear fragmentation does not seem to occur in harpin$_{Pss}$-induced YCD
  - Possible loss of membrane integrity in harpin$_{Pss}$-induced YCD was observed
  - 'Petite' mutants being insensitive to harpin$_{Pss}$-induced YCD indicate the possible role of mitochondria in harpin$_{Pss}$-induced YCD
  - No evidence for the leakage of Cyt C in harpin$_{Pss}$-induced YCD
  - The observed cell death in *S. cerevisiae* Y187 was observed in Sey6211 and BJ2168 and the remaining three strains DY150, W303 and BY4741 were insensitive to harpin$_{Pss}$-induced YCD
- Structure-function relationship
  - Full-length *hrpZ*$_{Psph}$ and *hrpZ*$_{Pst}$ caused YCD similar to *hrpZ*$_{Pss}$
  - Deletion mutation revelaed that harpin $_{Pss}$ is a unique protein that retains the biological activity even in the 13 a.a. peptide (towards the C-terminal end), and any portion of the *hrpZ* cause YCD
  - Sequence analysis revealed that harpin shares no homology with the known proteins whose structure was elucidated
  - The predicted secondary structure of harpin is helical in nature
  - Bio-informatic study revealed harpin$_{Pss}$ to have features of Type III chaperones
  - Harpin$_{Pss}$ crystallization was successful.

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