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 pQE30-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$_2$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 \( E. coli \) DH5\( \alpha \). \( E. coli \) DH5\( \alpha \) 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<sub>pss</sub> expression in *E. coli*.

A) SDS-PAGE of harpin<sub>pss</sub> expressed in *E. coli* and B) Western blot analysis of harpin<sub>pss</sub>.

*E. coli* M15 competent cells were transformed with pQE30-*hrpZ*, grown in LB broth to OD<sub>600</sub> ~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<sub>pss</sub> (gel on the left) and Ni-NTA purified harpin<sub>pss</sub> (gel on the right).

B) Purified harpin<sub>pss</sub> was resolved on a 12% SDS-PAGE, transferred to a nitrocellulose membrane, blocked with 5% non-fat dry milk, incubated with the harpin<sub>pss</sub> 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 HarpinPss 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 harpinPss (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 harpinPss 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 harpinPss 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 harpinPss, 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 harbip 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$_{ps}$ 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°C.
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)
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°C was counted. The inset shows trypan blue stained *S. cerevisiae* Y187 cells. 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<sub>600</sub>, in glucose-containing medium steadily increased up to 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<sub>Pss</sub>-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<sub>Pss</sub>. The cells expressing harpin<sub>Pss</sub> 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°C to an optical density of ~1.0 at 600nm (OD<sub>600</sub>). Cells were pelleted and resuspended in glucose-containing media to achieve an OD<sub>600</sub> of ~0.4-0.7, to which different concentrations (5μM to 20μM) of the harpin<sub>Pss</sub> and harpin<sub>Psp</sub> 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<sub>Pss</sub> and harpin<sub>Psp</sub> 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 pYEUJ-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$_{PS}$-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°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 up to 4 h (Fig. 9A & B). Extracellular effect of HrpZ_{pss} and HrpZ_{pssph} 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°C. 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-/ir/?Z+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-/wpZ+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-hrpZpss grown in glucose-containing medium to OD\textsubscript{600}=0.6. Cells were pelleted, washed and introduced into glucose-containing medium to which was added three different concentrations of A) harpin\textsubscript{pss} and B) harpin\textsubscript{psh}, 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\textsubscript{600} estimation was carried out at regular time intervals for 4 h as indicated in the xaxis. The data are representative of five individual experiments.
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 \textit{S. cerevisiae}. \textit{E. coli} DH5α cells containing constructs 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-\textit{hrpZ+LS} in \textit{S. cerevisiae} Y187.

pYEUT-\textit{hrpZ} transformed in \textit{S. cerevisiae} Y187 by one-step transformation protocol as described in material and methods. The upper half of the figure shows the pYEUT-\textit{hrpZ+LS} transformant and pYEUT-\textit{hrpZ+LS} (leader sequence cloned upstream of \textit{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-\textit{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°C.
cells were grown in the presence of 0.2M hydroxyurea and of 15µg/ml nocodazole, respectively, for 3 h at 24°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°C. After every 1 h, cells were observed under microscope and OD600 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 OD600, in glucose-containing medium steadily increased up to 4 h. These observations confirmed that harpinPsp-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 OD600~0.6. Cells were pelleted, washed and introduced into glucose- or galactose-containing media; cultures in 3mM H2O2 added glucose-containing medium served as positive control.

3.5.1 Chromatin condensation and fragmentation in harpinPsp-induced YCD:
HarpinPsp 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 H2O2-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<sub>600</sub>~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°C. 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<sub>600</sub> 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-\textit{hrpZ} transformant of \textit{S. cerevisiae} Y187.

\textit{S. cerevisiae} Y187 transformed with pYEUT-\textit{hrpZ}, grown in glucose-containing medium with tryptophan drop out-supplement to OD\textsubscript{600}~0.6. Cells were pelleted, washed and introduced in glucose- or galactose-containing medium and 3mM H\textsubscript{2}O\textsubscript{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\textsubscript{2}O\textsubscript{2} in glucose-containing medium
cultured in galactose-containing medium, revealing that the chromosomal DNA fragmentation does not seem to occur in the harpin\textsubscript{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\textsubscript{Pss}-mediated cell death (Fig. 14). In the positive control (H\textsubscript{2}O\textsubscript{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\textsubscript{2}O\textsubscript{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\textsubscript{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\textsubscript{2}O\textsubscript{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\textsubscript{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\textsubscript{2}O\textsubscript{2} in
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$_2$O$_2$ 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$_2$O$_2$ in glucose-containing medium
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$_2$O$_2$ in glucose-containing medium. After 3 h, electron microscopic analysis was undertaken for the cells grown in glucose- or galactose-containing medium and H$_2$O$_2$-treated cells. Electron micrographs of cell cultured in

A) glucose-containing medium
B) galactose-containing medium and
C) 3mM H$_2$O$_2$ 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$_2$O$_2$ 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\(^\circ\)) 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$_2$O$_2$ 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$_2$O$_2$ 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 1μg/ml CsA to study whether or not PTP formation is taking place in harpin_{ps}-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_{ps}-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_{ps}-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 harpinPSST-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 7 μg/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<sub>Pss</sub> on different strains of <i>S. cerevisiae</i>

Conditional expression of harpin<sub>Pss</sub> causing yeast cell death was studied in <i>S. cerevisiae</i> Y187. The other strains of <i>S. cerevisiae</i> used in this study are DY150, W303, Sey6211, BY4741 and BJ2168 (Table 2). All these strains were transformed with pYEUT-<i>hrpP</i> for conditional expression in galactose-containing medium as described in section 2.4. When pYEUT-<i>hrpP</i> 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<sub>Pss</sub> (Fig. 21).

3.9 Effect of harpins from two other pathovars of <i>Pseudomonas syringae</i>

A 1.037kb and 1.112 kb fragments encoding full-length <i>hrpP<sub>Psp</sub></i> and <i>hrpP<sub>Pst</sub></i> were PCR amplified (Fig. 22A) and cloned into pYEUT (Fig. 22B), placing <i>hrpP<sub>Psp</sub></i> and <i>hrpP<sub>Pst</sub></i> under the control of the GAL1 promoter for conditional expression of these genes when cells are cultured in galactose-containing media. Plating of pYEUT-<i>hrpP<sub>Psp</sub></i> and pYEUT-<i>hrpP<sub>Pst</sub></i> 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-<i>hrpP<sub>Psp</sub></i> and pYEUT-<i>hrpP<sub>Pst</sub></i> transformed <i>S. cerevisiae</i> Y187 showed YCD similar to that pYEUT-<i>hrpP<sub>Pss</sub></i>.

3.10 Effect of N-terminal and C-terminal deletions on cell death activity of harpin<sub>Pss</sub>

Twelve different truncations were made in the <i>hrpP<sub>Pss</sub></i> 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<sub>Pss</sub> (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<sub>Pss</sub> 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 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*<sub>pss</sub>, *hrpZ*<sub>psph</sub> and *hrpZ*<sub>pst</sub> in pYEUT.

A) A 1.02kb *hrpZ*<sub>pss</sub>, 1.03kb *hrpZ*<sub>psph</sub> and 1.13kb *hrpZ*<sub>pst</sub> were PCR-amplified using the primer sequences (Table 3) that add *Bam* HI (5') and *Xho* I (3') restriction sites to *hrpZ*<sub>pss</sub> and *Bam* HI (5') and *Xba* I (3') restriction sites *hrpZ*<sub>psph</sub> and *hrpZ*<sub>pa</sub>, resolved on a 1% agarose gel electrophoresis.

- Lane 1: *hrpZ*<sub>pss</sub> amplicon
- Lane 2: 100bp molecular weight marker
- Lane 3: *hrpZ*<sub>psph</sub> amplicon
- Lane 4: *hrpZ*<sub>pst</sub> amplicon

B) The PCR-amplified 1.03kb *hrpZ*<sub>psph</sub> and 1.13kb *hrpZ*<sub>pst</sub> 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*<sub>pss</sub>, pYEUT-*hrpZ*<sub>psph</sub> and pYEUT-*hrpZ*<sub>pst</sub> 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*<sub>pss</sub>
- Lane 4: linearised pYEUT-*hrpZ*<sub>psph</sub>
- Lane 5: linearised pYEUT-*hrpZ*<sub>pst</sub>
Figure 23  Conditional expression of \( hrpZ_{Psph} \) and \( hrpZ_{Pst} \) in \textit{S. cerevisiae} Y187.

pYEUT-\( hrpZ_{Psph} \) and pYEUT-\( hrpZ_{Pst} \) were transformed in \textit{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-\( hrpZ_{Psph} \)), were streaked simultaneously on the glucose- and galactose-containing media.
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.
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
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\textsubscript{Pss}. The full-length and truncated mutants, on conditional expression under the GAL1 promoter, caused cell death in \textit{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 \textit{hrpZ} when cloned separately in pYEUT and transformed \textit{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\textsubscript{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\textsubscript{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\textsubscript{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 \textit{S. cerevisiae} Y187, reveals that harpin\textsubscript{Pss} is a unique protein and thus retains the biological acitivity in any part of the protein in causing cell death in \textit{S. cerevisiae}.

3.10.1 Harpin\textsubscript{Pss} mutants also cause YCD: Since the yeast cells expressing full-length \textit{hrpZ} undergo YCD in galactose-containing media, we hypothesized that the conditional expression of truncated versions of harpin\textsubscript{Pss} also might cause YCD. Twelve different truncated mutants of \textit{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\degree C to an optical density of ~1.0 at 600nm (OD\textsubscript{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\textsubscript{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\textsubscript{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-\textit{hrpZ} truncated mutants in \textit{S. cerevisiae} Y187.

Constructs of pYEUT-\textit{hrpZ} mutants were transformed in \textit{S. cerevisiae} Y187 for conditional expression of truncated mutants of harpin$_{PN}$ 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-<i>hrpZ</i> truncated mutants of <i>S. cerevisiae</i>.

A single colony of the pYEUT-<i>hrpZ</i> mutants were cultured in glucose-containing medium to achieve an OD<sub>600</sub> ~ 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 OD₆₀₀ 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: Harpinₚₛ secondary structure, predicted using PHD algorithm, showed that among 341 residues, 48.97% had the
S. cerevisiae Y187 cells were transformed with pYEUT-hrpZ mutants grown in glucose-containing medium with tryptophan drop out-supplement to mid-log phase (OD₆₀₀ of ~ 1.0). Cells were then washed and introduced into glucose- or galactose-containing medium to achieve an OD₆₀₀ 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<sub>Pss</sub> were found in the PDB databank for homology modeling, therefore, three-dimensional model of HrpZ of <i>P. syringae</i> could not be predicted.

### 3.12 Crystallization of harpin<sub>Pss</sub>

Since bioinformatics approaches to predict the structures of the harpin<sub>Pss</sub> were not successful, an attempt was made to crystallize harpin<sub>Pss</sub>. The mother liquor prepared by dissolving 3mg of pure harpin<sub>Pss</sub> 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<sub>Pss</sub> 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*).

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Sequence length : 341

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Figure 33  Protein crystallization set-up.

A) The disposable plastic tissue culture plate (Linbro or VDX plates) that have 24 wells (1.7 cm 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.
The mother liquor was prepared by dissolving 3mg of pure Harpin_{ps} 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_{ps}. 

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)