2.1 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 nitrogen base (Difco), ammonium sulfate (Sigma), and tryptophan/uracil dropout supplement (Clontech) with raffinose or galactose (2\%) (Sigma-aldrich) as the source of carbon (referred to as raffinose/galactose-containing medium, respectively) were used.

2.1.1 E. coli medium: Luria-Bertani medium (1\% tryptone, 0.5\% yeast extract and 1\% sodium chloride) was used to grow E. coli cultures with appropriate antibiotic.

2.2 Strains, plasmids and growth conditions Saccharomyces cerevisiae Y187 (Clontech) strain was used in the present study. The plasmid constructs pYEUT and pYEUT-hrpZ were generated by Podile et al, (2001). S. cerevisiae Y187-pYEUT-hrpZ was grown in yeast minimal medium (YMM) with raffinose or galactose (2\%) as the carbon source with aeration at 28\(^{0}\) C. To induce hrpZ expression, the transformed cells grown to an early exponential phase (OD\(_{600}\) ~0.5) in 2\% raffinose medium were washed and resuspended in tryptophan drop-out supplement medium containing 2\% galactose (induction of GAL 1 promoter). Either H\(_2\)O\(_2\) or acetic acid in raffinose medium was used as a positive control to induce cell death in S. cerevisiae.

LB medium with kanamycin was used to culture E. coli BL 21 (Rosettae) cells harboring pET28a-hrpZ at 37\(^{0}\) C and induced with 1mM IPTG for harpin\textsubscript{Pss} expression.

2.3 hrpZ cloning, expression and purification

The hrpZ gene (1.02 kb) encoding full length harpin\textsubscript{Pss} was cloned under Nde\(_1\) and Xho\(_1\) sites of pET 28a vector (Novagen) (primers used and PCR conditions are listed in table 2.1 and table 2.2). E. coli BL 21 (Rosettae) cells transformed with pET 28a-hrpZ was grown in Luria Bertani (LB) broth with Kanamycin (50 \(\mu\)g/ml) to OD\(_{600}\) ~ 0.5 and induced with 1mM IPTG. After 3 h of induction, bacterial cells were pelleted, washed and resuspended in 10 mM sodium phosphate buffer (pH 7.5) and immediately sonicated (1min pulse on and 30 sec pulse off, 7 cycles, Bandelin MS-72 probe). The sonicate was
boiled for 10 min, then centrifuged at 14,000 rpm for 20 min to remove cell debris and the supernatant was loaded on to a Ni-NTA column (sigma-aldrich). Protein was eluted with 200 mM imidazole in phosphate buffer after washing the column with 20 mM imidazole of the same buffer and then perfectly dialyzed against 10mM sodium phosphate buffer (pH 7.5). Purity of both the proteins was checked on a 12 % SDS-PAGE. The dialyzed protein was concentrated using amicon filter (10 kDa cut off, Millipore) and used after estimation by Bradford’s method (1976).

Table 2.1

Primers used in the present study

<table>
<thead>
<tr>
<th>Primer sequence (5’ to 3’ )</th>
<th>Target gene</th>
<th>5’/3’</th>
<th>Restriction site</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGAATCCCATATGCAGAGTCTCAGTCTTTAAC</td>
<td>full length harpin_Pss</td>
<td>5’</td>
<td>Nde 1</td>
</tr>
<tr>
<td>CGGGATCCCTCGAGGGCTGCAGCCTGATTGC</td>
<td>full length harpin_Pss</td>
<td>3’</td>
<td>Xho I</td>
</tr>
</tbody>
</table>

Table 2.2

PCR conditions to amplify full length harpin_Pss sequence

<table>
<thead>
<tr>
<th>Steps</th>
<th>Full length harpin_Pss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>94ºC- 5 min</td>
</tr>
<tr>
<td>Step 2</td>
<td>94ºC- 1 min</td>
</tr>
<tr>
<td>Step 3</td>
<td>65ºC- 1 min</td>
</tr>
<tr>
<td>Step 4</td>
<td>72ºC- 1 min</td>
</tr>
<tr>
<td>Step 5</td>
<td>Go to step ‘2’ for 30 times</td>
</tr>
<tr>
<td>Step 6</td>
<td>72ºC- 10 min</td>
</tr>
<tr>
<td>Step 7</td>
<td>Hold at 16ºC</td>
</tr>
</tbody>
</table>

2.3.1 Raising of polyclonal antibodies

Polyclonal antibodies were raised against harpin_Pss. The antibodies were raised by injecting harpin_Pss into rabbit subcutaneously after mixing with 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 protein emulsified

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with Freund’s incomplete adjuvant was given. The second booster injection was given after a week of first booster injection and finally the blood was collected after 10 days of the second booster injection. The collected blood was left overnight at 4°C for clotting and serum was collected for centrifuging at 7,000 rpm for 20 min. The serum was aliquoted and stored at –20°C after adding 0.01% sodium azide.

2.3.2 Western blot analysis
The protein 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, incubated with harpin primary antibody in 10 ml of antibody-diluted buffer (1x Tris buffered saline and 0.5% Tween with 5% milk) with gentle shaking at 4°C for 8-12 h and then incubated with anti-rabbit IgG-ALP conjugate secondary antibody. The immunoblot was visualized using BCIP-NBT substrate.

2.3.3 Mass spectrometry of harpinPss
Two microliters of the purified (Ni-NTA) protein solution (~ 3 µg) was mixed with 2 µl of 2% TFA (trifluoroacetic acid) and 2 µl of the matrix solution (2,5 dihydroxyacetophenone with 10 mM di-ammonium citrate). Mass measurements were performed on a Autoflex III TOF/TOF spectrometer (Bruker) in the positive linear mode of operation. About 1000 single spectra were added. Spectra were processed using 10 Da Gauss filter smoothing and baseline subtraction.

2.4 HarpinPss-mediated yeast cell death
2.4.1 H2DCFDA staining for ROS detection in yeast cells expressing pYEUT-hrpZ
S. cerevisiae cells, transformed with pYEUT-hrpZ were cultured in raffinose-containing medium with tryptophan drop out to an OD₆₀₀ of 0.5 at 30°C. The cells were pelleted, washed and resuspended in minimal medium supplemented with raffinose or galactose. A positive control with yeast cells treated with 80 mM acetic acid in raffinose containing medium was also set up. After 90 min of induction, cell culture (~ 1x10⁶ cells ml⁻¹) from
each sample was pelleted, resuspended in phosphate buffered saline (PBS) and incubated with 15 µM H$_2$DCFDA (2’7’-Dichloro dihydro fluorescein diacetate) for 30 min in dark at 28°C (Ludovico et al., 2002). The cells were then acquired in the FACS Calibur flow cytometer at 488-520 nm using FL1-H filter for measuring the reactive oxygen species (ROS). A total of 20,000 cells were analyzed per sample.

2.4.2 Measurement of O$_2$ consumption in an ‘Oxygraph’

*S. cerevisiae* pYEUT-hrpZ cells grown to an OD$_{600}$ of 0.5 were pelleted and resuspended in raffinose or galactose containing medium and cultured for 4h. Cells (~5x10$^6$) from both the samples were resuspended in 1ml of YMM, loaded into the oxygraph chamber and monitored for O$_2$ uptake for a period of 10 min. Prior to loading of cells in the oxygraph chamber, water at a constant temperature of 25°C was circulated through the outer jacket of the reaction chamber. Calibration of oxygen content in the electrode chamber was done with air saturated water, assumed to contain 253 nmol O$_2$ ml$^{-1}$ at 25°C. O$_2$ consumption by yeast cells was monitored using a Clark type O$_2$ electrode (DW2, Hansatech Ltd, King’s Lynn, UK).

2.4.3 Rhodamine-123 (Rh-123) staining for detection of mitochondrial potential

*S. cerevisiae* pYEUT-hrpZ cells cultured for 200 min and 12h were centrifuged (~ 1x10$^6$ cells ml$^{-1}$), washed and resuspended in 1ml PBS and incubated with 100 nM Rhodamine-123 (Ludovico et al., 2001) for 30 min in dark at 28°C. A positive control was also set up by adding 80 mM acetic acid in raffinose- containing medium. After incubation, the cells were washed with PBS to remove the unwanted dye, suspended in 1 ml of PBS and acquired in the BD FACS calibur flow cytometer equipped with an argon ion laser at an excitation of 488 nm and an emission of 529nm using FL1-H filter. A total of 20,000 cells were analyzed per sample.

2.4.4 Annexin V and Propidium Iodide (PI) staining

*S. cerevisiae* pYEUT-hrpZ cells cultured for 200 min were harvested (~1x10$^6$ ml$^{-1}$), washed with PBS and treated with 15 U lyticase (Sigma-aldrich) ml$^{-1}$ in sorbitol buffer
(pH 6.8) for 30 min at 28°C (Madeo et al., 1997). Cells were then washed with binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl$_2$, pH 7.4), suspended in 200 µl of binding buffer and stained with 5 µl of annexin V- FITC (20 µg/ml) (BD Biosciences) and 10 µl of PI (50 µg/ml) for 20 min in dark at 30°C. After incubation, the cells were washed with binding buffer and resuspended in 1 ml of PBS. The samples were then acquired in the BD FACS calibur flow cytometer using FL 1-H filter ($\lambda_{\text{exc}} = 488$ nm and $\lambda_{\text{em}} = 529$ nm) on X- axis and FL 2-H filter ($\lambda_{\text{exc}} = 605$ nm and $\lambda_{\text{em}} = 640$ nm) on Y-axis. A total of 20,000 cells were analyzed per sample. Acetic acid treated cell sample was used as a positive control.

2.4.5 Yeast cell cycle analysis by flow cytometry

*S. cerevisiae* Y187 pYEUT-hrpZ cells grown to an OD$_{600}$ ~0.5 were centrifuged, resuspended in YMM and synchronized in the presence of 0.2 M hydroxy urea (sigma-aldrich) for 2 h. Cells were then pelleted, washed and resuspended in YMM containing raffinose and cultured for 3 h. Cells were then recentrifuged and suspended in YMM supplemented with raffinose or galactose or raffinose with 0.2 M hydroxy urea (S-phase blocker) as a positive control. After incubating for a period of 4h, 12h and 24h, cells (~1x $10^6$) were collected from each sample, fixed with 70% ethanol for at least 30 min and resuspended in 0.5 ml of 50 mM sodium citrate containing 0.1 mg/ml RNase A and propidium iodide (Sigma-Aldrich) at a final concentration of 4 µg/ml, following the incubation of the sample at 30°C for 30 min. Then the cells were spin down and suspended in 1 ml of PBS and acquired in BD FACS calibur flow cytometer using FL 2-A filter ($\lambda_{\text{exc}} = 605$ nm and $\lambda_{\text{em}} = 640$ nm). A total of 20,000 cells were analyzed per sample.

2.4.6 Estimation of oxidized and reduced Glutathione

*S. cerevisiae* Y187 pYEUT-hrpZ cells cultured in the presence of raffinose or galactose for 12 h were pelleted and homogenized in a buffer consisting of 0.1 M sodium phosphate and 0.005 M EDTA (pH 8.0) with 25% phosphoric acid (HPO$_3$) which is used as protein precipitant. The total homogenate was ultracentrifuged at 36,000 rpm for 30
min at 4°C to obtain the supernatant for the assay of oxidized glutathione (GSSG) and reduced glutathione (GSH) (Hissin and Hilf, 1976). Protein estimation (Bradford, 1976) was carried out to take equal amounts of protein from both the samples for the assay.

**GSH assay:** To 0.5 ml of the above supernatant, 4.5 ml of the phosphate-EDTA buffer, pH 8.0 was added. The final assay mixture (2.0 ml) contained 100 µl of the diluted supernatant, 1.8 ml of phosphate-EDTA buffer and 100 µl of the O-Phthalaldehyde (OPT) solution, containing 100 µg of OPT. After thorough mixing and incubation at room temperature for 15 min, OD was recorded in a fluorescence spectrophotometer with absorbance at 420 nm and excitation at 350 nm.

**GSSG assay:** A 0.5 ml portion of the original supernatant was incubated at room temperature with 200 µl of 0.04 M N-ethylmaleimide (NEM) for 30 min to interact with GSH present in the sample. To this mixture 4.3 ml of 0.1 N NaOH was added. A 100 µl portion of this mixture was taken for measurement of GSSG, using the procedure outlined for GSH assay, except that 0.1 N NaOH was employed as diluent rather than phosphate-EDTA buffer. Standard curve for GSH and GSSG was prepared according to the method of Hissin and Hilf (1976).

### 2.4.7 Detection of metacaspase activation

*S. cerevisiae* Y187 pYEUT-hrpZ cells cultured in the presence of raffinose or galactose were assessed for caspase activation using the fluorescent caspase inhibitor ‘CaspACE, FITC-VAD-fmk In Situ Marker’ (Promega) (Madeo *et al*., 2002). Briefly, 1x 10^6 cells were washed in PBS, suspended in 200 µl staining solution containing 10 µM of FITC-VAD-FMK and incubated for 30 min at 30°C in dark. Cells were then washed once and suspended in PBS. Sample analysis was performed in a BD FACS Calibur flow cytometer equipped with FL 1-H filter (λ<sub>exc</sub> = 488 nm and λ<sub>em</sub> = 529 nm). A total of 20,000 cells were analyzed per sample. Acetic acid treated cell sample was used as a positive control.
2.4.8 Mitochondrial enzyme assays

Isolation of mitochondria

Mitochondria were isolated following the protocol of Daum et al., (1982) from both the raffinose grown (control) yeast cells, and galactose grown (harpin_pss expressed) yeast cells. *S. cerevisiae* Y187 cells cultured in raffinose or galactose-supplemented medium for 15 h at 28°C were harvested by centrifugation (5 min, 5000 rpm), washed with distilled water, suspended to 0.5 g, wet weight / ml in 0.1 M Tris.SO₄, pH 9.4, 10 mM dithiothreitol (DTT), and incubated for 10 min at 30°C. They were then washed once with 1.2 M sorbitol, 20mM potassium phosphate buffer, pH 7.4, to give 0.15 g of cell, wet weight/ml. Lyticase (Sigma-aldrich) (5mg/g of wet weight) was added and the suspension was incubated at 30°C with gentle shaking. After 50 – 60 min, all the cells had usually been converted to spheroplasts. Spheroplasts were harvested by centrifugation for 5 min at 3000 rpm, washed twice with 1.2 M Sorbitol. For homogenization, spheroplasts were suspended in 0.6 M Mannitol, 10 mM Tris Cl, pH 7.4, 0.1% bovine serum albumin (BSA), 1 mM phenylmethyisulfonylfluoride (PMSF), to a concentration of 0.15 g of spheroplasts (wet weight/ml). After chilling on ice, spheroplasts were homogenized by 10-15 strokes in a tight fitting Dounce homogenizer. From this point, all operations including centrifugations were carried out at 0 - 4°C. The homogenate was diluted with one volume of the homogenization buffer and centrifuged for 5 min at 3500 rpm. The supernatant was saved, and the pellet was rehomogenized as before and recentrifuged at 3500 rpm. The supernatants were combined and crude mitochondria were sedimented at 9000 rpm for 10 min. The mitochondrial pellet was washed twice by resuspension and recentrifugation at 9000 rpm for 10 min; for the last wash, BSA and PMSF were omitted from the washing buffer. Mitochondria were suspended in 0.6 M Mannitol, 10mM Tris-Cl, pH 7.4, to give an approximate final concentration of 10 mg of protein/ ml.

Visualization of mitochondria by confocal microscopy

Isolated mitochondria were suspended in 10mM Tris-Cl buffer, pH 7.4 and 10μl of JC-1 (1.5mg/ml) fluorescent dye was added and incubated for 20 min in dark at 4°C. A drop of the suspension was placed on a glass slide, covered with a coverslip and observed under...
the confocal microscope (Leica) at $\lambda_{\text{exc}}= 605$ nm and $\lambda_{\text{em}} = 640$ nm for red filter, and $\lambda_{\text{exc}} = 514$ nm and $\lambda_{\text{em}} = 529$ nm for green filter.

**Mitochondrial protein estimation**

The protein content of the mitochondria, isolated from the raffinose or galactose grown yeast cells was estimated by Bradford’s method (1976) against BSA standard.

**2.4.8a Assay for NADH dehydrogenase (Complex I) activity**

NADH dehydrogenase assay was carried out following the protocol of Sottocasa et al, (1967). Mitochondrial suspension (i.e. 0.1 mg mitochondrial protein) from yeast cells cultured in raffinose-supplemented medium or the mitochondrial protein (i.e. 0.1 mg) from the yeast cells cultured in galactose-supplemented medium was taken into 1 ml quartz cuvettes and the volume was made up to 0.98 ml with potassium phosphate buffer, pH 7.4 (50 mM monobasic potassium phosphate and 50 mM dibasic potassium phosphate). To this 10 $\mu$l of potassium ferricyanide (100 mM) was added and the reaction was initiated by adding 10$\mu$l of NADH (100 mM) to each of the cuvettes. Absorbance changes were measured at 420 nm at regular intervals of 50 sec for 3 min. The molar extinction coefficient of NADH is $6.22 \times 10^{-3}$. The activity of NADH dehydrogenase is expressed as $\mu$ moles of NADH oxidized or reduced per min per mg protein. Activity of NADH dehydrogenase = Slope ($\Delta$Abs/min) x 1 mg / $6.22 \times 10^{-3}$ x amount of mitochondrial protein taken in mg for assay. Percentage difference in activity = [(activity of glu-mit) – (activity of gal-mit)] x 100 / activity of glu-mit.

**2.4.8b Assay for Cytochrome c oxidase activity**

Cytochrome C oxidase activity assay from yeast mitochondria was performed following the standard instructions given in the Sigma-aldrich kit. To a final volume of 1ml, 0.85 ml of 1x assay buffer, 50 $\mu$l of Ferrocytochrome c substrate solution (reduced with 0.5 mM DTT, sigma kit) along with 20 $\mu$g of yeast mitochondrial protein was added followed by the addition of 1x enzyme dilution buffer to make up the remaining volume. Assay reaction was monitored by the oxidation of ferrocytochrome c to ferricytochrome c
at 550 nm at regular intervals of 50 sec for 5 min in an UV-Visible spectrophotometer. Reaction mixture containing all the above components, except mitochondrial protein, was used as a blank. The difference in molar extinction coefficient between reduced and oxidized cytochrome c is 21.84 at 550 nm. The activity of Cytochrome c oxidase is expressed as μ moles of Ferrocytochrome c oxidized per min per mg protein. Activity of Cytochrome c oxidase = Slope (ΔAbs/min) x 1 mg / 21.84 x amount of mitochondrial protein taken in mg for assay. Percentage difference in activity = [(activity of Raf-mit) – (activity of gal-mit)] x 100 / activity of Raf-mit.

2.4.8c Assay for yeast mitochondrial ATPase activity

Mitochondrial ATPase activity was measured following the protocol of Pullman et al., (1960). To a final volume of 1ml, 50 mM Tris Cl (pH 8.0), 1 mM ATP, 0.3 mM NADH, 3.3 mM MgCl₂, 2 μg/ml antimycin A, 1mM Phosphoenol pyruvate, 5 U/ml Lactate dehydrogenase and 2.5 U/ml Pyruvate kinase were added along with 20 μg of yeast mitochondrial protein (measured by Lowry method) at 28⁰ C. Assay reaction was monitored by the oxidation of NADH at 360 nm at regular intervals of 50 sec for 5 min in an UV-Visible spectrophotometer. Reaction mixture containing all the above components except mitochondrial protein was used as a blank. The molar extinction coefficient of NADH is 6.22 x 10⁻³. The activity of Mitochondrial ATPase is expressed as μ moles of NADH oxidized or reduced per min per mg protein. Activity of ATPase = Slope (ΔAbs/min) x 1 mg / 6.22 x 10⁻³ x amount of mitochondrial protein taken in mg for assay. Percentage difference in activity = [(activity of Raf-mit) – (activity of gal-mit)] x 100 / activity of Raf-mit.

2.5 Animal cell culture

2.5.1 Chemicals: RPMI 1640 cell culture medium (sigma-aldrich), Fetal bovine serum (FBS) (sigma-aldrich), Phosphate buffered saline (PBS), MTT (3-(4,5-dimethyl thiazol-2yl)-2,5-diphenyl tetrazolium bromide) (Himedia) were used.
2.5.2 Jurkat cell culture

Jurkat cells (human T-cell lymphoma cell line) were grown in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, 100 µg/ml Streptomycin and 2 mM L-glutamine. Cultures were maintained in a humified atmosphere with 5% CO₂ at 37°C. The cultured cells were passed twice a week seeding at a density of about 2x10⁵ cells/ml. Cell viability was determined by the trypan blue dye exclusion test.

2.5.3 Harpin₃₃ treatment/cell proliferation assay

Cell proliferation was determined using the MTT assay. Jurkat cells (2.5x10³ cells/well) were incubated in 96 well plates in the presence (10 – 80 µM) or absence of harpin₃₃ (control) for 24h in a final volume of 200 µl. After treatment, 20 µl of MTT (5 mg/ml in PBS) was added to each well and incubated for an additional 4h at 37°C. The purple blue MTT formazan precipitate was dissolved in 100 µl of dimethyl sulfoxide (DMSO) and the absorbance values were recorded at 570 nm on a multiwell plate ELISA reader (Lab systems, Multiskan, 355).

2.5.4 DNA fragmentation assay

Jurkat cells treated with 40 µM harpin₃₃ for 12 and 24 h were used for the isolation of the DNA. DNA laddering was detected by isolating fragmented DNA using the SDS/Proteinase K/ RNase A extraction method (Sambrook et al., 1989), which allows the isolation of only fragmented DNA without contaminating genomic DNA. Five million cells were pelleted, washed in cold PBS and lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.2 % Triton X-100 for 20 min at 4°C. After centrifugation at 14,000 g for 15 min, the supernatant was treated for 1 h at 37°C with RNase A (0.5 mg/ml) and then with proteinase K (0.5 mg/ml) for 1 h at 50°C. DNA was extracted with buffered phenol and precipitated with ethanol and DNA was resolved on 1 % agarose gel in TBE (44.6 mM Tris, 44.5 mM boric acid and 1 mM EDTA). DNA fragmentation was visualized upon staining gel with ethidium bromide (0.5 mg/ml) and exposed to UV light.
2.5.5 Quantification of apoptosis by flow cytometry

To quantitate apoptosis (sub G0/G1 cells), flow cytometric analysis using propidium iodide (PI) was performed. Jurkat cells treated with 40 μM harpinPss for 12 and 24 h, were prepared as single cell suspension in 200 µl PBS, fixed with 2 ml of ice-cold 70% ethanol, and maintained at 4 °C overnight. The cells were harvested by centrifugation at 500 x g for 10 min, resuspended in 500 µl PBS supplemented with 0.1 % Triton X-100 and RNase A (50 µg/ml), incubated at 37 °C for 30 min, and stained with 50 µg/ml propidium iodide (PI) in dark at 4 °C for 30 min. The red fluorescence of individual cells was measured with a FACS Calibur flow cytometer using FL 2-H filter (λexc = 605 nm and λem = 640 nm) (BD Biosciences). A minimum of 20,000 events were counted per sample.

2.5.6 Preparation of whole cell extracts and immunoblot analysis

The cell lysis was carried out as described by Sambrook et al., (1989). To prepare the whole cell extract, cells after treatment were washed with PBS and suspended in a lysis buffer (20 mM Tris, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxy cholate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml leupeptin, 20 μg/ml aprotinin). After 30 min of shaking at 4°C, the mixtures were centrifuged (9,000 rpm) for 10 min, and the supernatants were collected as the whole-cell extracts. The protein content was determined according to Bradford, (1976). An equal amount of total cell lysate was resolved on 8-12 % SDS-PAGE gels along with protein molecular weight standards, and then transferred onto nitrocellulose membranes. Membranes were stained with 0.5% Ponceau S in 1% acetic acid to check the transfer. The membranes were blocked with 5% w/v nonfat dry milk and then incubated with the primary antibodies (Bax and Bcl 2) in 10 ml of antibody-diluted buffer (1X Tris-buffered saline and 0.05% Tween-20 with 5% milk) with gentle shaking at 4 °C for 8-12 h and then incubated with ALP conjugated secondary antibodies. Signals were detected by using substrate BCIP-NBT. The blots were probed with β-actin antibodies to confirm equal loading.
2.5.7 Detection of cytochrome c release using Western blot analysis

After exposure to 40 μM harpinPss for various time periods, cells were collected and washed with PBS and subsequently with buffer A (0.25 M sucrose, 30 mM Tris-HCl, pH 7.9, 1 mM EDTA). Cells were then resuspended in buffer A containing 1 mM PMSF, 1 mg/ml leupeptin, 1 mg/ml pepstatin, 1 mg/ml aprotinin and homogenized with a glass dounce homogeniser. After centrifugation for 10 min at 16,000 rpm, protein concentration of the cytosolic extract was determined using Bradford method. Approximately, 30 μg of cytosolic protein was used for Western blot analysis as described above. Cytochrome c was detected using the mouse monoclonal antibody directed against human cytochrome c.

2.6 Bio-physical studies

2.6.1 Circular dichroism spectroscopy (CD)

Circular dichroic (CD) spectra were recorded on a Jasco J-810 spectropolarimeter (Jasco International Co., Ltd., Tokyo, Japan, website: [http://www.jasoint.co.jp](http://www.jasoint.co.jp)) equipped with a Peltier thermostat supplied by the manufacturer. Samples were placed in a 2-mm path length rectangular quartz cell. Spectra were recorded at a scan rate of 20 nm/min with a response time of 0.5 sec and a band width of 2 nm. Protein concentration was 2.25 μM for measurements in the far UV region (250–190 nm). Each spectrum was the average of 4 accumulations. In order to study the effect of temperature on the secondary structure of the protein, CD spectra were recorded in the near UV region at different temperatures.

2.6.2 Fluorescence spectroscopy

Emission spectra were recorded on a Spex Fluoromax-3 fluorescence spectrometer from Jobin-Yvon (Edison, JJ. USA, website: [http://www.jobinyvon.com](http://www.jobinyvon.com)). Slit width of 3 nm and 6 nm were used on the excitation and emission monochromators respectively. Measurements were performed by irradiating protein (harpinPss) samples (OD280 ≤ 0.1) with light of 295 nm wavelength, in order to selectively excite tryptophan residues of the protein and emission spectra were recorded above 300 nm. In fluorescence quenching experiments, small aliquots of 5 M quencher stocks (acrylamide or potassium iodide)
were added to protein samples and fluorescence spectra were recorded after each addition. The final quencher concentration attained in each case was 0.52 M. The iodide stock solution contained 0.2 M sodium thiosulphate to prevent the formation of triiodide. All the measurements were performed in duplicate at 25°C and yielded reproducible results. The average values have been reported.

2.6.3 Differential scanning calorimetry (DSC)
DSC experiments were performed on a MicroCal VP-DSC differential scanning microcalorimeter (MicroCal Inc., Northampton, MA, USA) equipped with two fixed cells, a reference cell and a sample cell. Sample and reference solutions were properly degassed with stirring in an evacuated chamber for 5 min at room temperature and then carefully loaded into the calorimeter cells. A background scan collected with buffer in both cells was subtracted from each scan. The temperature dependence of the molar heat capacity of the proteins was further analyzed by using the Origin DSC software package supplied by the manufacturer.

2.6.4 Dynamic light scattering (DLS)
DLS measurements were performed at a protein concentration ranges from 0.5-1.0 mg/ml at pH 7.5. Before the DLS measurements, the solutions were passed through a 0.22 μm filter and centrifuged at 14,000 rpm for 10 min. All experiments were performed at a 90° scattering angle on a PDExpert (Precison Detectors, Inc. Bellingham, MA, USA) DLS instrument at a wavelength of 685 nm with a power of 30 mW. The measurements were carried out at various temperatures ranges between 25-61°C using a built-in temperature controller. The diffusion coefficient (D) was calculated from the autocorrelation function, using the accessory ‘PrecisonAcquire’ software provided with the instrument. The experimentally measured hydrodynamic radius, (R_h) of the protein was determined from the Stokes–Einstein relationship.

2.6.5 Congo red binding
To measure congo red binding, 100 μg of protein was added to a 10 μM congo red (Sigma-aldrich) solution in 5 mM sodium phosphate buffer at pH 7.5. The absorbance
spectrum was measured from 400 to 600 nm using a Cary 100 UV-Vis spectrophotometer (VARIAN) equipped with a Peltier thermostat supplied by the manufacturer.

### 2.6.6 Atomic Force Microscopy (AFM)

A drop of 0.1 mg/ml protein solution was placed on a freshly cleaved mica sheet and dried immediately under nitrogen gas. The salt deposits were washed extensively by washing with miliQ water. The samples were once again dried with nitrogen gas. All the images were recorded in air under ambient conditions in semi-contact mode with a scan rate of 80 Hz using a NTMDT (Moscow) AFM instrument. The force was kept at the lowest possible value by continuously adjusting the set-point and feed-back gain during imaging. Image analysis was performed using NOVA software, supplied by NTMDT along with the instrument.

### 2.6.7 Crystallization of harpinPss

Protein (5-6 mg/ml) exchanged in 20 mM Tris-Hcl, pH 7.0 was used to set up for initial screening. ‘Crystal screen high throughput’ and ‘Index screen high throughput’ (Hampton research) were the most commonly used commercial screens for screening. Classic screens of ‘Jena Bioscience’ (JBS) were also used for screening. Crystallization was done by ‘Sitting-drop vapor diffusion’ for all the commercial screens. Sitting drop screens were set up in 96 well Greiner plates with three sub wells for holding the sample. 100 µl of screening solution (various combinations) was used per well. Drops containing 2 µl of protein and reservoir solution was placed in each sub well in a ratio of 1:1 using ‘Hydra I-e Drop’. Three different concentrations of the protein (1.5, 3, 5 mg/ml) were used in the sub wells. Plates were sealed after being set up and placed in a 277 K and 293 K ‘Rigaku robo’ incubator and imaged using a ‘Minstrel’ plate reader. Plates were observed for nucleation or crystal growth over a period of 2 months. Conditions giving micro crystalline precipitates were further expanded using ‘Crystal track’ software and dispensed using ‘Alchemist’. 96 screens were thus prepared and drops were set up and imaged. A total of 2016 set ups were screened for crystallization.