3.1 Harpin_{pss}-induced cell death events in yeast

3.1.1 Detection of ROS in \textit{S. cerevisiae} cells expressing harpin_{pss}

\textit{S. cerevisiae} Y187 cells harboring pYEUT-hrpZ were cultured in raffinose-supplemented medium, pelleted and resuspended in YMM containing raffinose or galactose or treated with acetic acid. ROS is detected using 2’7’-dichloro dihydro fluorescein diacetate, a non-fluorescent cell-permeable compound, which is changed to fluorescent compound 2’7’-dichlorofluorescein upon oxidation by ROS. Cells with ROS release exhibit greenish yellow fluorescence which could be detected in the flow cytometer using FL1-H filter at $\lambda_{\text{exc}} = 488$ nm and $\lambda_{\text{em}} = 520$ nm. Only 1.7% cells cultured in raffinose-supplemented medium exhibit ROS levels where as 45% of acetic acid treated cells exhibit ROS levels (Fig.3.1) and 39% of yeast cells cultured in galactose-supplemented medium shows ROS levels indicating that reactive oxygen species (ROS) are involved in harpin_{pss}-mediated yeast cell death.

![Fig 3.1 Detection of ROS in harpin_{pss}-mediated yeast cell death](image)

\textit{S. cerevisiae} Y187 cells harboring pYEUT-hrpZ were cultured in raffinose-supplemented medium with tryptophan drop out to an OD\textsubscript{600} of 0.6. Cells were then pelleted, washed and resuspended in minimal medium containing raffinose or galactose or treated with 80 mM acetic acid. After 90 min of induction, cell culture (~ 1x10\textsuperscript{6} cells ml\textsuperscript{-1}) was pelleted, resuspended in PBS and incubated with 15 µM H\textsubscript{2}DCFDA for 30 min in dark at 28\textdegree C. The flow cytometry pictures of cells cultured in media supplemented with A) raffinose B) galactose C) 80 mM acetic acid in raffinose. Data represent the result from one of three similar experiments.

---

\textit{Results}
3.1.2 Determination of $O_2$ consumption during harpin$_pss$-induced YCD

*S. cerevisiae* Y187 pYEUT-hrpZ cells cultured in raffinose-containing medium were shifted to raffinose or galactose-supplemented medium to induce harpin$_{pss}$ expression. Oxygen consumption of yeast cells was monitored using an ‘Oxygraph’ consisting of a ‘Clark $O_2$ electrode’ that measures the amount of dissolved oxygen in the medium. After 4h incubation, $O_2$ consumption by yeast cells expressing harpin$_{pss}$ was determined to be $0.9 \pm 0.2$ nmoles/min/5 million cells (Fig 3.2), where as the $O_2$ consumption by control cells cultured in raffinose-supplemented medium was determined to be $3.4 \pm 0.3$ nmoles/min/5 million cells. Harpin$_{pss}$-expressed yeast cells exhibit a four-fold reduction in the rate of oxygen consumption which indicates the impairment of mitochondrial function, thus leading to respiratory deficiency in harpin$_{pss}$-induced yeast cell death.

![Fig 3.2 Estimation of $O_2$ consumption during harpin$_{pss}$-induced yeast cell death](image)

*S. cerevisiae* Y187 transformed with pYEUT-hrpz was grown in raffinose-supplemented medium with tryptophan drop out to an OD$_{600}$ of 0.6 at 30°C. The cells were then centrifuged and suspended in raffinose or galactose containing medium and incubated for 4h. Cells (~$5\times10^6$) from both the samples were pelleted and resuspended in 1ml of YMM containing 2% raffinose or galactose. The 1ml cell suspension was loaded into the oxygraph chamber and monitored for $O_2$ uptake for equal period of time. Vertical bars on the histogram indicate standard error of three independent experiments.

---

**Results**
3.1.3 Assessment of mitochondrial membrane potential ($\psi$) in yeast cells expressing harpin$_{Pss}$

*S. cerevisiae* Y187 pYEUT-hrpZ cells cultured in raffinose or galactose or acetic acid treated cells were monitored for changes in mitochondrial potential using rhodamine-123, a lipophilic cationic dye that is accumulated by intact mitochondria possessing negative charge. The rate of accumulation of the dye depends on the mitochondrial membrane potential. Rh-123 could be detected in the flow cytometer using FL1-H filter at $\lambda_{ex}=488$ nm and $\lambda_{em}=529$ nm. Yeast cells expressing harpin$_{Pss}$ and acetic acid treated cells exhibit hyperpolarization (Fig 3.3A) during the initial hours of culturing as compared to that of control cells grown in raffinose-containing medium. During the late hours of induction (i.e. 12 h), harpin$_{Pss}$-expressed yeast cells and acetic acid-treated cells showed depolarization (Fig 3.3B) of mitochondrial membrane as compared to that of control cells.

![Fig 3.3 Detection of mitochondrial membrane potential ($\psi$) in harpin$_{Pss}$-induced YCD](image)

*S. cerevisiae* Y187 pYEUT-hrpZ cells cultured in raffinose-supplemented medium were pelleted and resuspended in minimal medium containing raffinose or galactose or treated with 80 mM acetic acid. After 3 h of induction, $\sim1 \times 10^6$ cells ml$^{-1}$ was pelleted, resuspended in PBS and incubated with 100 nM Rh-123 for 30 min at 30$^\circ$C and then acquired in the flow cytometer. The area in violet color represents the cells cultured in raffinose-supplemented medium, the green colored line represents the cells cultured in galactose-supplemented medium where the expression of harpin$_{Pss}$ is induced and the pink colored line represents the cells cultured in raffinose containing medium with acetic acid induced cell death. Cells cultured for A) 3 h and B) 12 h after induction. Data represent the result from one of three similar experiments.
3.1.4 Assessment of yeast cell death induced by harpin$_{\text{Pss}}$

*\textit{S. cerevisiae* Y187 pYEUT-hrpZ*} cells cultured in raffinose-supplemented medium were pelleted and resuspended in minimal medium containing raffinose or galactose or treated with acetic acid. Nature of cell death (whether apoptotic or necrotic) was assessed using annexin V, a protein of 36 kDa that binds to the phosphatidyl serine residues and detected by the conjugated fluorescent molecule FITC in the flow cytometer or fluorescent microscope. Cells which show the binding of annexin V are said to be in the phase of apoptotic cell death. Annexin V-FITC is detected using FL 1-H filter at an excitation of 488 nm and an emission of 529 nm. Cells were simultaneously counter stained with another fluorescent dye propidium iodide which is taken up by cells in the phase of necrotic cell death. PI is detected in flow cytometer using FL 2-H filter at an excitation of 605 nm and an emission of 640 nm. In harpin$_{\text{Pss}}$- induced YCD 29% of galactose-supplemented cells (fig 3.4) and 53% of acetic acid treated cells have taken up PI showing a shift towards the upper left quadrant as compared with 3% of control cells cultured in raffinose-supplemented medium. Only 1.6% of acetic acid treated cells and 0.2% of harpin$_{\text{Pss}}$- induced cells tested positive for annexin V staining (upper right quadrant). Hence the yeast cell death induced by harpin$_{\text{Pss}}$ expression and acetic acid (130mM) treatment has more orientation towards necrosis rather than apoptosis.

---

**Fig 3.4 Assessment of yeast cell death induced by harpin$_{\text{Pss}}$**

*S. cerevisiae* Y187 transformed with pYEUT-hrpz was grown in raffinose medium, centrifuged and resuspended in raffinose or galactose or treated with acetic acid. After 4h culturing cells were pelleted and treated with lyticase for 30min and stained with annexin V- FITC (20µg/ml) and PI (50µg/ml). The flow cytometry pictures of cells (A) cultured in raffinose served as control, (B) galactose-supplementation where harpin expression was induced and (C) cells treated with acetic acid. Data represent the result from one of three similar experiments.

---

_results_
3.1.5 Yeast cell cycle analysis

*S. cerevisiae* Y187 pYEUT-hrpZ cells cultured in raffinose-containing medium were shifted to raffinose or galactose-supplemented medium or treated with hydroxy urea. HarpinPss-induced YCD was verified by flow cytometric analysis of cellular DNA content stained with propidium iodide which directly represents different phases of the cell cycle. PI could be detected in the flow cytometer using FL2- A filter at $\lambda_{\text{exc}} = 605$ nm and $\lambda_{\text{em}} = 640$ nm. The cell cycle histograms of *S. cerevisiae* Y187 cultured in different conditions and time points are illustrated in Fig 3.5 and the percentage of cells in different phases of the cell cycle at three different time points are represented in table 3.1. The FACS analysis of control cells (cultured in raffinose) showed prominent G1, followed by S and G2/M phases except in late hour (24h) sample where more number of cells are in stationary phase showing increased accumulation in the G1 phase. HarpinPss-expressing yeast cells (galactose cultured) showed increased accumulation in G0/G1 sub phase (resembling positive control) with increase in culturing time as compared to the control cells. Yeast cells cultured in presence of hydroxy urea (positive control) exhibit blockage of cell cycle at the S-phase showing more accumulation of cells at the G1-phase. Thus harpinPss- expression in yeast leads to DNA damage resulting in cell cycle blockage ultimately leading to yeast cell death.

<table>
<thead>
<tr>
<th>Cell cycle phase</th>
<th>Cells cultured in Raffinose (%)</th>
<th>Cells cultured in Galactose (%)</th>
<th>Cells cultured in Hydroxy urea (‘S’ block) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4h</td>
<td>12h</td>
<td>24h</td>
</tr>
<tr>
<td>G0 (M4)</td>
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</tr>
<tr>
<td>G1 (M1)</td>
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</tr>
<tr>
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<td>16.1</td>
<td>13.4</td>
</tr>
<tr>
<td>G2M (M3)</td>
<td>31.1</td>
<td>22.3</td>
<td>21.3</td>
</tr>
</tbody>
</table>

Table 3.1 Percentage of yeast cells in different phases of the cell cycle at three different time points
Fig 3.5 Cell cycle analysis in harpinₚₛₚ-mediated yeast cell death.

*S. cerevisiae* Y187 pYEUT-hrpZ cells were grown in raffinose-containing medium with tryptophan drop out to an OD ~0.6 at 30°C. Cells were then pelleted and resuspended in YMM containing raffinose or galactose or raffinose with 0.2M Hydroxy urea. After incubating for a period of 4h, 12h and 24h, cells were collected from each sample, fixed with 70 % ethanol and stained with PI (4µg/ml) and then acquired in flow cytometer. Violet colored histograms represents cells grown in raffinose-containing medium, green and brown histograms represent cells grown in galactose (harpinₚₛₚ-expressing) and in presence of S-phase blocker. Data represent the result from one of three similar experiments.

3.1.6 Determination of GSH and GSSG content

*S. cerevisiae* Y187 harboring pYEUT-hrpZ was grown in raffinose-supplemented medium, pelleted and resuspended in YMM containing raffinose or galactose. After incubation for 12 h, cell extracts were prepared and used for the estimation of glutathione. Glutathione is present in the oxidized form (GSSG) which is readily converted to the reduced (GSH) form by the enzyme glutathione reductase. Reduced

Results
glutathione is predominant in biological tissues than oxidized glutathione. During oxidative stress, GSH gets oxidized and is converted to GSSG. The relative comparison of GSH/GSSG ratio between the control and stress samples indicates the extent of oxidative stress. The ratio of GSH/GSSG for raffinose grown cells was 0.77, while it was 0.43 for cells expressing harpin_{Pss}. S. cerevisiae Y187 expressing harpin_{Pss} exhibit 37% reduction in GSH levels (Fig 3.6) and significant decrease in the GSH/GSSG ratio compared to the control cells indicating the extent of oxidative stress in cells expressing harpin_{Pss}.

![Fig 3.6 GSH and GSSG estimation in harpin_{Pss}-induced YCD](image)

**S. cerevisiae** Y187 transformed with pYEUT-hrpZ was grown in raffinose-containing medium with tryptophan drop out to an OD ~0.6 at 30°C. Cells were then pelleted and resuspended in YMM containing raffinose or galactose. After incubation for a period of 12h, the cell extracts were prepared by homogenization and ultracentrifuged followed by the estimation of reduced and oxidized glutathione. Vertical bars on the histogram indicate standard error of three independent experiments.

### 3.1.7 Assessment of metacaspase activity

*S. cerevisiae* Y187 pYEUT-hrpZ cells cultured in raffinose-supplemented medium were transferred to minimal medium containing raffinose or galactose or treated with acetic acid for 4 h. In order to confirm caspase activation in harpin_{Pss}-induced YCD, cells were incubated with CaspACE, FITC-VAD-fmk *in situ* marker that binds to the active site of caspases and detected using flow cytometry. In comparison, galactose cultured yeast cells (harpin_{Pss}-expressed) and acetic acid treated cells exhibit higher metacaspase activity than
the control cells (Fig 3.7) indicating the elevation of metacaspases in harpinPss-induced yeast cell death.

![Graph showing metacaspase activity in yeast cell death](image)

**Fig 3.7 Detection of metacaspase activity in harpinPss-induced yeast cell death.**

*S. cerevisiae* Y187 cells harboring pYEUT-hrpZ were cultured in raffinose-supplemented medium with tryptophan drop out to an OD_{600} of 0.6. Cells were then pelleted, and resuspended in minimal medium containing raffinose or galactose or treated with 80 mM acetic acid. After 4h of induction, ~1x10^6 cells ml^-1 were pelleted, resuspended in PBS and incubated with FITC-VAD-fmk for 30 min at 30°C and then acquired in flow cytometer. Area in blue represents cells cultured in raffinose-supplemented medium (control), green line for cells cultured in galactose (harpinPss expressed) and pink for acetic acid treated cells. Data represent the result from one of three similar experiments.

### 3.1.8 Mitochondrial enzyme assays

**A) Isolation of mitochondria**

The mitochondria were isolated (Daum *et al*, 1982) as described in materials and methods and suspended in 100 μls of 0.6 M Mannitol and 10 mM Tris-Cl, buffer pH 7.4.

**B) Visualization of mitochondria by confocal microscopy**

The yeast mitochondria isolated were stained with a fluorescent dye, JC-1, which enters the mitochondria exhibiting a fluorescence shift from red to green, observed at λ_{exc}= 605
nm and $\lambda_{em} = 640$ nm for red filter (Fig 3.8B) and $\lambda_{exc} = 514$ nm and $\lambda_{em} = 529$ nm for green filter (Fig 3.8A), along with the transmission image (Fig 3.8C) taken under the confocal microscope.

Fig 3.8 Confocal image of yeast mitochondria stained with JC-1 showing green (A) and red (B) fluorescence respectively, shown along with the transmission image (C).

C) Assay for NADH dehydrogenase (Complex I) activity

Assay for NADH Dehydrogenase activity was done by recording the change in absorbance (OD measured at 420 nm) plotted on Y-axis against the time (in sec) taken on
X-axis (Fig 3.9). The specific activity of the NADH dehydrogenase enzyme from yeast grown in raffinose-supplemented medium is 150.48 μ moles of NADH oxidized or reduced / min/mg protein and for yeast grown in galactose-supplemented medium, it was 101.76 μ moles of NADH oxidized or reduced / min/ mg protein. *S. cerevisiae* Y187 expressing harpin$_{Ps}$ exhibited 32% reduction in the NADH dehydrogenase levels compared to the control cells.

![Graph](image)

**Fig 3.9 Assay for NADH dehydrogenase activity**

NADH dehydrogenase activity of the mitochondria isolated from cells cultured in galactose-supplemented medium or raffinose-supplemented was estimated spectrophotometrically at 420 nm on a time based scale for 200 sec.

**D) Assay for Cytochrome c oxidase (Complex IV) activity**

Assay for Cytochrome c oxidase activity was done by recording the change in absorbance (OD measured at 550 nm) plotted on Y-axis against the time (in sec) taken on X-axis (Fig 3.10). The specific activity of Cytochrome c oxidase from the control cells cultured in raffinose-supplemented medium was 43.4 μ moles of Fe Cytochrome c oxidized/min/mg protein, while it was 24.2 μ moles of Fe Cytochrome c oxidized/min/mg protein for the cells induced with harpin$_{Ps}$ expression i.e. in galactose-supplemented medium.
S. cerevisiae Y187 expressing harpin\textsubscript{Pss} exhibited 44% reduction in complex IV activity compared to the control cells.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Fig3.10.png}
\caption{Assay for Cytochrome c oxidase activity}
\end{figure}

The Cytochrome C oxidase activity of mitochondria isolated from cells cultured in galactose-supplemented medium or raffinose-supplemented was estimated spectrophotometrically at 550 nm on a time based scale for 300 sec.

E) Assay for mitochondrial ATPase (Complex V) activity

Assay for mitochondrial ATPase activity was done by recording the change in absorbance (OD measured at 360 nm) plotted on Y-axis against the time (in sec) taken on X-axis (Fig 3.11). The specific activity of mitochondrial ATPase from the control cells cultured in raffinose-supplemented medium was 253 \( \mu \) moles of NADH oxidized / min/mg protein, while the cells cultured in galactose-supplemented medium had 176.8 \( \mu \) moles of NADH oxidized /min/mg protein. S. cerevisiae Y187 expressing harpin\textsubscript{Pss} exhibit 30% reduction in complex V activity compared to the control cells.
Fig 3.11 Assay for mitochondrial ATPase activity

The mitochondrial ATPase activity of mitochondria isolated from cells cultured in galactose-supplemented medium or raffinose-supplemented was estimated spectrophotometrically at 360 nm on a time based scale for 300 sec.

3.2 HarpinPss-induced cell death events in Jurkat cell lines

3.2.1 Effectiveness of HarpinPss on cancer cell lines

The effectiveness of harpinPss has been tested on animal cell lines (Jurkat cells-T-cell lymphoma cell line). Cell proliferation, on harpinPss treatment was evaluated using MTT assay which is based on the mitochondrial reduction of the MTT salt to formazan crystals by living cells. Jurkat cells were cultured (serum free) with or without harpinPss (10 –80 µM) for 24h. A dose dependent decrease in Jurkat cell proliferation was observed at 24h of harpinPss treatment with maximum decrease in cell proliferation at a concentration of 40 µM (1.5 mg/ml) where the percent inhibition (IC₅₀) was 50% (Fig. 3.12). Thus harpinPss treatment appears to be efficient causing cell death in Jurkat cells.

Results
Fig. 3.12 MTT assay for determination of cell viability

Jurkat cells were cultured in presence (10 – 80 µM) or absence (control) of harpin$_{Psa}$ for 24h. After treatment, cell viability was determined by the addition of MTT followed by recording the absorbance values at 570 nm on an ELISA reader. Vertical bars on the histogram indicate standard error of three independent experiments.

3.2.2 Morphological changes

Harpin$_{Psa}$ treated Jurkat cells showed typical features of cell death compared to the untreated control cells. Phase contrast microscopic observations revealed changes in cell shape, organelle distribution and membrane blebbing ultimately resulting in cell burst and release of small cell fragments (Fig.3.13) called as the apoptotic bodies.

Fig. 3.13 Photomicrographs of Jurkat cells (A) untreated control cells and (B) cells treated with harpin$_{Psa}$ showing features of cell death. White arrow points to membrane blebbing and red arrow shows the apoptotic bodies.

Results
3.2.3 DNA fragmentation induced by harpin<sub>Pss</sub> in Jurkat cells

Induction of apoptosis in Jurkat cells treated with harpin<sub>Pss</sub> was further evaluated by DNA fragmentation, a hallmark feature of apoptosis. Jurkat cells treated with 40 µM harpin<sub>Pss</sub> showed DNA fragmentation (Fig 3.14) evidenced by a ladder of 180-200 base pairs which corresponds to internucleosomal cleavage, a characteristic feature of apoptotic cells. Untreated Jurkat cells (lane 1) did not show any such fragmentation pattern.

![Fig. 3.14 Analysis of DNA fragmentation in Jurkat cells treated with harpin<sub>Pss</sub>](image)

Jurkat cells were treated with 40 µM harpin<sub>Pss</sub> for 24 h and 12 h. DNA was isolated from the treated cells and separated on 1.5% agarose gels. DNA was stained and visualized under UV light. Lane 1: Untreated control cells; Lane 2: Cells treated with harpin for 24h; Lane 3: Cells treated with harpin for 12h; Lane 4: 100 bp marker.

3.2.4 Effect of harpin<sub>Pss</sub> on cell cycle profile of Jurkat cells

The induction of apoptosis in cells treated with harpin<sub>Pss</sub> was further evaluated by flow cytometric analysis of DNA content. Jurkat cells treated with or without 40 µM harpin<sub>Pss</sub> were stained with propidium iodide (50µg/ml) for 30 min and subjected to FACS analysis. The FACS analysis of control cells, showed prominent G1, followed by S and G2/M phases. Only 3 % of untreated cells showed hypodiploid DNA (sub G0/G1 peak). The percentage of hypodiploid DNA increased gradually to 12 % (Fig. 3.15) in the cells treated for 12 h with harpin<sub>Pss</sub> which further increased to 27 % at 24 h.

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Results
3.2.5 Effect of harpinPss treatment on Cytochrome c release, Bcl-2 and Bax proteins in Jurkat cells

The process of apoptosis is associated with the disruption of mitochondrial membrane potential, which results from the opening of permeability transition pores in the mitochondrial membrane, leading to the release of cytochrome c. The expression levels of Bcl-2 and Bax proteins was associated with mitochondrial membrane integrity and play a crucial role in the regulation of apoptosis. No appreciable change in the levels of Bax protein was observed at all the time periods (Fig. 3.16A) but a time dependent decrease of Bcl-2 protein levels was observed after treatment with harpinPss (Fig 3.16B). These results suggest an altered Bcl-2/Bax ratio (Fig. 3.16C) in the cells treated with harpinPss. Furthermore, to determine whether there is any release of cytochrome c from the mitochondria into the cytosol, cytosolic fractions from the cells treated with 40 µM harpinPss were analyzed.
harpin_{Pss} for 3, 6, 12 or 18 h were subjected to western blot analysis. A time dependent elevation in the levels of cytochrome c with maximum increase at 12 and 18 h was observed (Fig. 3.17).

**Fig. 3.16 Western blot analysis of Jurkat cells treated with harpin_{Pss}**

The cell extracts of Jurkat cells treated with 40 µM harpin_{Pss} at 0, 6, 12 and 24 h were resolved on 15% SDS-PAGE and probed against Bax (A) and Bcl-2 (B) antibodies followed by incubation with anti-mouse IgG secondary antibody and developed using BCIP-NBT substrate. β actin was used as loading control. The histograms (C) represent the altered Bcl2/Bax ratio analyzed with Scion imaging.

**Results**
Fig. 3.17 Cytochrome c release from Jurkat cells

Jurkat cells were treated with 40 µM harpin_{Pss} for 0, 3, 6, 12 and 18 h. Equal quantities of cytosolic protein (30 µg) was resolved on a 15% SDS-PAGE and immunoblotted with cytochrome c antibody. β actin was used as loading control.

3.3 Bio-physical studies on harpin_{Pss}

3.3.1 Harpin_{Pss} expression and purification

Harpin_{Pss} was expressed in *E. coli*. The *hrpZ* gene (1.02 kb) encoding full length harpin_{Pss} was PCR amplified as a single band (Fig. 3.18A) and cloned under *NdeI* and *XhoI* sites of pET 28a vector. Cloning was confirmed by release of 1 kb insert upon double digestion of the cloned plasmid on a 1% agarose gel (Fig.3.18B). *E. coli* BL 21 competent cells were prepared and transformed with pET 28a-*hrpZ* and selected on an LB kanamycin (50µg/ml) plate. A single colony of pET 28a-*hrpZ* transformant of *E. coli* BL 21 (Rosettae) was grown in LB broth to OD_{600} ~ 0.5 and induced with 1mM IPTG, with hourly sample collection for confirmation of expression on a 12% SDS PAGE (Fig.3.18C). After 3h of induction, the cells were pelleted and sonicated for protein extraction. Antibodies were raised against harpin_{Pss} and the reactivity was tested on an immunoblot. The membrane was incubated with harpin_{Pss} antibody and then with the secondary antibody, anti-rabbit IgG- ALP conjugate. The blot was visualized with alkaline phosphatase color reaction using BCIP-NBT substrate. Band corresponding to harpin_{Pss} was detectable, confirming the specificity of the antibody (Fig.3.18D). Harpin_{Pss} was purified from the protein extract by passing through Ni-NTA column to obtain pure protein shown on a 12% SDS gel (Fig. 3.18E). Thepolydispersed, oligomeric nature of harpin_{Pss} resolved on an 8% native PAGE is also shown in Fig. 3.18F.
Fig. 3.18 PCR amplification, cloning, expression and purification of harpin<sub>Pss</sub>.

A) 1.02 kb <i>hrpZ</i> gene encoding full length harpin<sub>Pss</sub> was PCR amplified using gene specific primers and resolved on a 1% agarose gel. Lane 1: 1 kb DNA ladder. Lane 2: A 1.02 kb <i>hrpZ</i> amplicon.

B) Confirmation of <i>hrpZ</i> cloning in pET 28a vector by double digestion shown on a 1% agarose gel. Lane 1: 1 kb DNA ladder. Lane 2: Double digestion of pET 28a-<i>hrpZ</i> showing the release of 1.02 kb harpin<sub>Pss</sub> fragment along with the 5.3 kb pET 28a vector.

C) 12% SDS PAGE showing hourly expression of harpin<sub>Pss</sub> (shown with arrow) from <i>E. coli</i> BL 21 cells along with the un-induced control (ctrl) and protein molecular weight marker (M).

D) Western blot analysis carried out with alkaline phosphatase color reaction showing the specific detection of harpin<sub>Pss</sub>, i.e. induced expression (I), uninduced control (ctrl) and protein marker (M).

E) Ni-NTA purified protein (PP), harpin<sub>Pss</sub> (38 kDa) showed on a 12% SDS gel along with the protein molecular weight marker (M).

F) 8% native PAGE showing the polydispersed, multimeric (mono, di, trimeric etc) nature of harpin<sub>Pss</sub>.

Results
3.3.2 MALDI-TOF analysis of harpin$_{Ps}$

The Gauss filter-smoothed mass spectrum (TOF/TOF) of harpin$_{Ps}$ shows a singly charged ($[M_1+H]^+$) fundamental peak corresponding to a mass of 38,138 Da (Fig 3.19) and the other two major peaks represent a double charge ($[M_1+2H]^{2+}$) at 19,070 Da, as well as the triple charge ($[M_1+3H]^{3+}$) corresponding to a peak of 12,715 Da. The very low intensity tiny peaks could be due to minute impurities. Of the 367 amino acid residues in the expressed recombinant protein, 26 residues, including the twelve histidines [one His(6) tag at N-terminus and other at C-terminus) are due to the pET 28a Vector, where as the calculated molecular weight of the actual 341 amino acid harpin$_{Ps}$ is 34,700 Da at pH 7.0.

![MALDI-TOF spectrum of harpin$_{Ps}$ indicating the molecular mass at 38,138 Da and the other peaks showing the double and triple charged status of the protein](image)

3.3.3 Secondary structure and CD spectroscopy

CD spectrum of native harpin$_{Ps}$ in the far UV region (240-190 nm) is shown in Fig. 3.20. Two minima centered around 206 nm and 223 nm are clearly discernible in the spectrum, suggesting the presence of helical structure along with other secondary structural elements. To estimate the content of different types of secondary structures in harpin$_{Ps}$, the CD spectrum shown in Fig. 3.20 has been analyzed by three different programs, namely CDSSTR, CONTINLL, SELCON3 using the routines available at

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*Results*
DICHROWEB. Reference set 4 containing 43 proteins was used for fitting the experimental spectra. The calculated fit obtained using CDSSTR program is in excellent agreement with the experimentally obtained spectrum of harpin_{Pss}, indicating high accuracy in the estimates obtained from this analysis. The values obtained for the different types of secondary structures are: \(\alpha\)-helix (51.5\%), \(\beta\)-sheet (8.6\%), \(\beta\)-turns (15.6\%) and unordered structures (25.8\%) (Table 3.2).

![CD spectrum of harpin_{Pss}](image)

**Fig. 3.20** CD spectrum of harpin_{Pss}

Circular dichroic spectrum of harpin_{Pss} at far UV region. The spectrum was recorded at 25 °C and validated with the calculated fit obtained from the CDSSTR program which indicates that \(\alpha\)-helix is more predominant in the protein. MRE indicates mean radial ellipticity.

<table>
<thead>
<tr>
<th>Programme</th>
<th>(\alpha)-Helix (%)</th>
<th>(\beta)-sheet (%)</th>
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<th>Coil(%)</th>
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</tbody>
</table>

**Table 3.2** Results of CD spectral analysis of Harpin_{Pss}.
3.3.4 Fluorescence quenching of harpin_{Pss}

Fluorescence quenching study was carried out with harpin_{Pss} using a neutral quencher, acrylamide or an anionic quencher, iodide (I). The fluorescence emission spectra of native harpin_{Pss}, recorded in the absence or in the presence of increasing concentrations of acrylamide or KI are shown in Fig 3.21A and 3.21B respectively. In both cases, spectrum 1 is that of the native harpin_{Pss} in the absence of quencher and spectra 2-20 correspond to harpin_{Pss} in the presence of increasing concentrations of acrylamide or iodide. The significantly lower quenching observed with iodide in the native harpin_{Pss} indicates that the tryptophan residue (W167) in this protein is buried in the hydrophobic core of the protein, while as the higher quenching observed with the neutral quencher, acrylamide also confirms the non-polar environment around the tryptophan residue in the protein.

Fig. 3.21 Fluorescence quenching of harpin_{Pss}

Fluorescence spectra of native harpin_{Pss} in the absence and presence of acrylamide (A) or potassium iodide (B). Spectral line 1 corresponds to harpin_{Pss} alone and spectral lines from 2-20 corresponds to harpin_{Pss} with increasing concentrations of acrylamide or iodide.
3.3.5 Differential scanning calorimetry

In order to characterize the thermal unfolding mechanism and to rationalize the high thermal stability of HarpinPss we have performed differential scanning calorimetric studies. A heating thermogram of the protein in PBS (pH 7.5) is given in Fig. 3.22. The thermogram indicates the presence of three distinct transitions centred at 50.0 °C, 59.9 °C and 93.6 °C, respectively. Changing the protein concentration between 0.1 mg/ml and 1.5 mg/ml did not alter the overall features of the thermogram. Deconvolution of the thermogram of harpinPss is also shown (dotted lines) in the same figure. Thermodynamic parameters, viz, change in calorimetric enthalpy (ΔHc), change in van't Hoff enthalpy (ΔHv) and transition temperature (Tm) for individual transitions obtained from the deconvolution analysis, are listed in Table 3.3. Deconvolution analysis shows that the first, second and third transitions start at 26 °C, 44 °C, 76 °C and end at 72 °C, 76 °C and 108 °C, respectively.

To check the reversibility of heat induced harpinPss unfolding, sample was heated to 90 °C in the DSC cell, and then cooled rapidly to room temperature. After an equilibration period of 10 minutes the sample was subjected to a second heating scan. The resulting thermogram contained all the three endothermic transitions, which establishes the reversibility of transitions 1 and 2 in harpinPss. The calorimetric enthalpy (ΔHc) corresponding to transition 1 was found to vary considerably when repeated experiments were performed (involving protein obtained in different batches of purification) whereas enthalpy corresponding to transitions 2 and 3 were reproducible. These thermodynamic data strongly suggest that transition 1 most likely corresponds to dissociation of a polydisperse, oligomeric aggregate of harpinPss and transitions 2 and 3 correspond to temperature induced structural changes in the protein. A hypothetical diagram representing the probable mechanistic pathway for thermal dissociation of harpinPss is shown in Fig. 3.23.
Fig. 3.22 Differential scanning calorimetry of harpin$_{Pss}$

DSC scan of a 0.545 ml sample of 0.028 mM (monomer)/0.014 mM (dimer) of harpin$_{Pss}$ protein in 0.5 M sodium phosphate, pH 7.5 at a scan rate of 60 K h$^{-1}$. Deconvolution analysis of DSC data is also shown.

<table>
<thead>
<tr>
<th>Transition</th>
<th>$T_m$ (°C)</th>
<th>$\Delta H_c$ (kcal.mol$^{-1}$)</th>
<th>$\Delta H_v$ (kcal.mol$^{-1}$)</th>
<th>$\Delta H_c/\Delta H_v$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transition 1</td>
<td>50.0</td>
<td>217.3±69.3</td>
<td>224.1±7.2</td>
<td>-</td>
</tr>
<tr>
<td>Transition 2</td>
<td>59.9</td>
<td>154.5±10.0</td>
<td>330.2±42.5</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td></td>
<td>309.1±20.1*</td>
<td></td>
<td>0.94*</td>
</tr>
<tr>
<td>Transition 3</td>
<td>93.6</td>
<td>59.1±4.4</td>
<td>258.1±11.0</td>
<td>-</td>
</tr>
</tbody>
</table>

*In analysis protein was considered as dimeric and dimer concentration was used.

Table 3.3 Thermodynamic parameters of thermal unfolding of Harpin$_{Pss}$.
Fig. 3.23 Probable mechanistic pathway for thermal unfolding of harpinPss.

An oligomeric protein (A) unfolding to a dimer (B), then to a semi/partially unfolded dimer (C) and finally to a fully unfolded monomer (D). Cylinders represent folded helices, whereas curved lines represent unfolded random coils.

3.3.6 Temperature dependent CD

To understand the structural basis of the complex nature of the thermal unfolding process of harpinPss as revealed by the DSC thermograms, CD spectra of the protein were recorded at various temperatures. Each spectrum was analysed using the CDSSTR program to get the average $\alpha$-helical content and $\beta$-sheet content at different temperatures. Fig. 3.24 presents the dependence of $\alpha$-helical content and $\beta$-sheet structure on temperature. Up to 45 °C, before the onset of transition 2, there is no obvious change in helical and sheet content, suggesting that no change in secondary structure occurs below this temperature. The changes observed in the helical content at higher temperatures suggest the temperature-induced unfolding of the protein. Temperature-dependent CD shows that at high temperature the $\alpha$-helical content is reduced, whereas $\beta$-sheet structure increases with temperature.
Temperature dependence CD of harpin\textsubscript{Pss} shows no significant change in $\alpha$-helical or $\beta$-sheet content up to 45-50 °C. Increase in temperature results in (A) decrease of $\alpha$-helix and (B) increase in $\beta$-sheet content.

3.3.7 Reversible aggregation of harpin\textsubscript{Pss}

Oligomeric structures of protein can be easily detected via native polyacrylamide gel electrophoresis (PAGE). Generally proteins with oligomeric form give a smear instead of distinct bands. To demonstrate the reversibility of aggregate formation in thermal unfolding, gel electrophoresis was performed by heating harpin\textsubscript{Pss} at 90 °C for 5 min, cooled to RT and again loaded on to the gel. Fig. 3.25 shows the reversibility of the protein in aggregate formation. The aggregate formation is relatively a fast process as the gel electrophoresis was carried out immediately after cooling the heated sample. Therefore the aggregate formation is an inherent property in harpin\textsubscript{Pss} and the phenomena may play an important role for its thermal stability and biological activity.

Fig. 3.25 Electrophoretic profile of harpin\textsubscript{Pss} resolved on an 8 % native-PAGE showing reversible aggregation of the protein. Lane 1, Harpin\textsubscript{Pss}, lane 2, Harpin\textsubscript{Pss} heated at 90 °C for 10 min, cooled and loaded on the gel.
3.3.8 Dynamic light scattering

The hydrodynamic radius of HarpinPss as a function of temperature (up to 61 °C, which is the highest temperature where the DLS experiments could be carried out) is shown in Fig. 3.26. The data presented here indicates that the hydrodynamic radius (R_h) decreases with increase in temperature which is quite interesting, because for a monomeric protein the R_h is expected to increase with increase in temperature. The average R_h of the native protein is 20.54 ± 6.19 nm, which is significantly higher than the predicted value of 2.58 nm (according to Equation 1). The predicted R_h of native protein and unfolded protein comes from two empirical equations. Equation (1) is for native protein, equation (2) is for fully unfolded protein and N is the number of residues in the protein.

\[
R_h = 4.75N^{0.29} \text{Å} \tag{1}
\]

\[
R_h = 2.21N^{0.57} \text{Å} \tag{2}
\]

Here, large R_h of HarpinPss at room temperature may be attributed to the aggregation of monomeric protein. The decrease in R_h with temperature suggests the dissociation of oligomeric structure, which is consistent with the DSC and temperature dependent CD data. For a thermostable protein like harpin it is obvious that total thermal unfolding can’t be accomplished at 60-65 °C. At 61 °C the R_h is 9.35 ± 1.04 nm which is also much higher than R_h value of 6.14 nm predicted by Eqn. 2 for the denatured protein (monomer). It may be possible that higher R_h at 61 °C is due to partially unfolded dimeric structure as estimated R_h for fully unfolded dimeric harpinPss falls in the range of 12-13 nm. The partially unfolded dimeric protein may convert to fully unfolded monomeric protein at higher temperatures.

![Fig. 3.26 DLS study of harpinPss](image)

Temperature dependence of the hydrodynamic radius (R_h) of harpinPss showing the decrease in R_h of the protein with increase in temperature. Vertical lines represent standard error in R_h measurement, taken out of three independent observations.

---

**Results**
3.3.9 Congo red binding

The β-sheet fibrils referred to as amyloids typically bind azo dye congo red (CR). Binding of CR to amyloids leads to a characteristic red shift of the absorption spectrum. At room temperature a blue shift of the absorption maxima ($\lambda_{\text{max}}$) from 499 to 485 nm was observed with harpin$_{\text{Pss}}$ while at 55 °C there was no shift in $\lambda_{\text{max}}$ (Fig. 3.27). The blue-shift of the absorption spectrum indicates aggregation of the dye.

![Congo red binding of harpin$_{\text{Pss}}$ oligomer.](image)

3.3.10 Atomic force microscopy

To visualise the morphology of aggregates and corroborate the above DLS data, the harpin$_{\text{Pss}}$ samples were imaged using atomic force microscopy (AFM). Fig. 3.28 gives four different AFM images of harpin$_{\text{Pss}}$ and all the images show the heterogeneity in size and shape among aggregates of this protein. Small rod-like structures (Fig 3.28 B) may correspond to protofibrils or rod like aggregates. However, the sizes of the aggregates

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*Results*
shown in the AFM picture appears to be slightly bigger than the $R_h$ values obtained from DLS measurements. This could be due to the fact that the solutions for DLS studies were filtered through a 0.22 μm filter, resulting in the removal of larger aggregates that might have been present in the samples before filtration. Also it should be kept in mind that DLS measures average size of the aggregates present in solution.

![AFM image of harpinPss showing heterogeneity in size and shape (shown with arrows) among the aggregates of the protein.](image_url)

**Fig. 3.28** AFM image of harpinPss showing heterogeneity in size and shape (shown with arrows) among the aggregates of the protein.

### 3.3.11 Crystallization of harpinPss

HarpinPss was set up for crystallization by ‘Sitting drop’ method as described in materials and methods and the plates were observed for crystal growth for 2 months. Most of the set ups had precipitation of the protein and some contained micro crystalline precipitates

---

**Results**
which apparently resulted from the polydispersed, oligomeric and non-specific aggregation of the protein.

3.3.12 Oligomeric form and leucine-zipper

Examination of the primary structure of harpin\textsubscript{Pss} revealed the presence of two leucine zipper-like motifs (amino acids 71-90 and 302–323) in harpin\textsubscript{Pss} (Fig 3.29) Sequence alignment, shown in Fig. 3.30 A and B clearly describes that these particular segments consists of heptad repeat of leucine or other hydrophobic amino acids such as isoleucine, phenylalanine, alanine, methionine and valine. Analysis of primary structure of other harpins also shows the existence of one or more leucine-zipper like motifs (Fig. 3.30 A and B). Some of the harpins have coiled-coil motif where the first (a) and fourth (d) positions of heptad repeats were occupied by hydrophobic amino acids (Fig. 3.30 C). The presence of at least two leucine-zipper like motif in harpins can in principle nucleate a variety of different oligomerization states (Fig. 3.31).

\begin{verbatim}
MQSLSLNSSSLQTPAMALVLVRPEAETTGSTSSKALQEVV
VKLAELMRNGQLDDSSLPGKLLAKSMAADGKAGGGIED
VIAALDKLHEKLGDNFASADSASGTTQDLQMTQVLNGL
AKSMLDLLTQKDGSFSEDMPMLNKIAQFMDDNPAQ
FKPDSGWSVNLKEDNFGLDEATAAFRSALDDIIQQLGN
QQSDAGSLAGTGGGLGTPSSFNSSVMGDPLIDANTGP
GDSGNTRGEAGQLIGELIRGLQSVLAGGGLGTPVNTPTQ
GTSANGQSAQDLQLLGGGQLLKGLEATLKDAGQGTGTDV
QSSAQQIATLLVSTLLQGTRNQAAA
\end{verbatim}

Fig 3.29 Primary structure of harpin\textsubscript{Pss} (341 aa) with leucine zippers.

Amino acid sequence analysis of harpin\textsubscript{Pss} reveals the presence of two leucine zipper like motifs in the protein.

\underline{Results}
Panel A

71 GKA
gG G
ED VIAALDK
LIH

P.s pv syringae

12 S
tMQIISGG
AGGNLLGGT
SRGNAGLGGNAS
LG

-- Erwinia amylovora

31 NGA
S
SPSQSGFGGR
ORSNIAEQLSDI
MTTMMFMGS

-- Erwinia carotovora

23 QGL
KLGLNSA
ASLGSSVDKLST
IDKLTSAL
TS

-- Erwinia chrysanthemi

11 S

STMQISGGAGGGN
LLGGTSRQNA
LGDDHSL

-- Erwinia pyrifolia

106 S

ALCGGLG
ALAGMN
PSAMMGSL

FSALE

-- Pectobacterium

58 S

SPL
GLSLKGK
MAASGKAG
GGLEDIK
AALDTLI
H

-- P. s pv phaseolicola

41 Q

QLLTQLIMAL
LGGSSNNAE

Q

-- Xanthomonas axonopodis

Panel B

380 SSLGIDAMMAGDAINNMAL

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P.s pv syringae

300 KG

GLEATLKDAGQT
GDVQSSAQQIA

AT

Erwinia amylovora

191 A

ALCNQLAGQCN
SPGLCLGN
LG

-- Erwinia carotovora

209 SALSNVSTHVDGN

RNFVD

-- Erwinia chrysanthemi

312 SF

VF

NKGDRA
AKEEGQFM

FDQYPEF

G

-- Erwinia pyrifolia

304 SG

MDKFMAVG

GMIKAEVAG

------------

Pectobacterium

91 QALMNIVDI

LAQNGGOF

-- Xanthomonas axonopodis

322 TN

LNIKNSSA
KAGAD

DVQ

-- Pseudomonas syringae pv. tomato

Panel C

adadadada

23 QG

LKLGL

NSAASSLGSSV

DKLSSTIDKLTSAL

-- Erwinia chrysanthemi

27 TTG

STSSKALQEG

VVKL

-- P.s pv syringae

300 GSDMKPMAVG

GMIKAVAG

------------

Pectobacterium

91 QALMNIV

DII

LAQNGG

-- Xanthomonas axonopodis

58 S

SPLG

KLGLG
KAMAASGKAG

GLEDIKAALDL

-- P. s pv phaseolicola

301 VHAQ

NVE

DILTV

KEGGA

AVTNLNIK

NSSAKGAD

DVQ

-- P.s pv tomato

adadadadad

Fig. 3.30 Occurrence of leucine zipper like motifs and heptad repeats in harpins.

Panel A and B, showing amino acid sequence alignment of one or more leucine-zipper like motifs in several harpins including harpinPss. Panel C, some harpins are also have coiled-coil motifs, where each (a) and (d) position of heptad repeat are occupied by hydrophobic amino acids. Heptadic amino acids are marked in bold letters.

Results
Harpins have at least two leucine-zipper like motifs and hence can nucleate variety of different oligomerization states. Cylinders represent folded helices and two small distorted ellipsoids attached to cylinder are the leucine-zipper like motifs.