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Involvement of Mitochondria and Metacaspase Elevation in Harpin$_{Pss}$-Induced Cell Death of *Saccharomyces cerevisiae*

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ABSTRACT

Expression of a proteinaceous elicitor harpin$_{Pss}$, encoded by *hrpZ* of *Pseudomonas syringae* pv. *syringae* 61, under GAL1 promoter in *Saccharomyces cerevisiae* Y187 resulted in galactose-inducible yeast cell death (YCD). Extracellular treatment of harpin did not affect the growth of yeast. The observed YCD was independent of the stage of cell cycle. "Petite" mutant of *S. cerevisiae* Y187 pYEUT-*hrpZ* was insensitive to cell death indicating the involvement of mitochondria in this YCD. Loss in mitochondrial potential, but no leakage of Cytochrome *c* from mitochondria into the cytosol, were notable features in harpin$_{Pss}$-induced YCD. Cyclosporin A had no effect on *hrpZ* expressing yeast cells, further confirmed that there was no release of Cytochrome *c*. Elevation of caspase activity has been reported for the first time in this form of cell death induced by harpin expression. Release of reactive oxygen species and clear loss of membrane integrity were evident with the absence of nuclear fragmentation and chromosomal condensation, while annexin V and propidium iodide staining showed features typical of necrosis. J. Cell. Biochem. 107: 1150–1159, 2009.

KEY WORDS: HARPIN; *Saccharomyces cerevisiae*; GAL1; PLANT HYPERSENSITIVE RESPONSE; MITOCHONDRIA; REACTIVE OXYGEN SPECIES; METACASPASE

The plant hypersensitive response (HR), a defense mechanism, involves interaction between products of an "*avr*" gene of the pathogen and a matching "*R*" gene of the plant [Dodds et al., 2006]. The HR is one kind of programmed cell death (PCD) associated with the death of a small number of cells at and around the site of infection. An HR also occurs when unique proteinaceous bacterial elicitors like "harpin" interact with non-host plants. Membrane damage, necrosis, and collapse of challenged cells are the common features in a highly orchestrated form of genetically regulated PCD in plants [Greenberg et al., 1994].

Pseudomonas syringae pv. *syringae* 61 *hrpZ* encodes harpin$_{Pss}$, a 34.7 kDa extracellular protein, that elicits HR in plants [He et al., 1993]. Harpin encoded by the *hrpZ* genes of *P.s.* pv. *tomato* (HrpZ$_{Pst}$) or *P.s.* pv. *syringae* (HrpZ$_{Pss}$) and *P.s.* pv. *phaseolicola* (HrpZ$_{Psph}$) evoked ion currents of very similar unitary conductance [Lee et al., 2001]. We have earlier reported that galactose-inducible intracellular expression of harpin$_{Pss}$ caused cell death in *Saccharomyces cerevisiae* suggesting that the yeasts might share with plants, conserved components in cell death pathways [Podile et al., 2001], although the harpin receptor in plants was supposed to be localized in outer portion of plant cell membrane, probably in the cell wall [Hoyos et al., 1996] as simple infiltration of the protein into leaf intercellular spaces would cause hypersensitive necrosis. Recently a few of the type III effectors from *Pseudomonas syringae* pv. *tomato* were also shown to inhibit growth in yeast and cause cell death during ectopic expression [Munkvold et al., 2008].

A characteristic feature of apoptosis in mammalian cells is the nuclear condensation and fragmentation of DNA, typically involves cleavage of nuclear DNA, first into large 50–300 kb fragments [Oberhammer et al., 1993] and eventually into oligonucleosomal fragments through the action of undefined nucleases. Many, but not all, cases of cell deaths that occur under normal physiological circumstances, involve characteristic morphological changes, which include nuclear fragmentation, chromatin condensation, cell shrinkages, plasma membrane blebbing, and budding-off of cellular fragments [Wyllie et al., 1984]. Bax-induced cell death in *Schizosaccharomyces pombe* was not accompanied by morphological features of apoptosis [Jurgensmeier et al., 1997].

Paranthaman Sripriya and Vedantam Lakshmi Vasudev contributed equally to this work.

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Mitochondria play a central role in apoptosis, and in harpin-induced HR [Xie and Chen, 2000]. Bax does not lead to death in "petite" mutants, suggesting that fully active mitochondria are essential for Bax-induced cytotoxicity in yeast [Greenhalf et al., 1996]. Harpin induces rapid release of Cytochrome *c* from mitochondria into the cytosol in *Arabidopsis* cells, a hallmark feature of PCD [Krause and Durner, 2004].

S. cerevisiae genome sequence have revealed a few orthologues of mammalian apoptotic regulators such as the metacaspase (YCA1/MCA1) [Madeo et al., 2002]. YCA1 also enhances the responsiveness of mitochondrial-associated factors, proteins functioning in oxidative stress response and nucleotide excision repair processes [Khan et al., 2005; Lee et al., 2008]. YCA1 was reported to be involved in yeast cell death triggered by different stimuli [Herker et al., 2004; Silva et al., 2005].

In the present article we report the various features of cell death during harpin expression in yeast and emphasize that harpin$_{Pss}$-induced cell death of *S. cerevisiae* involves metacaspase activation and definitely involved reactive oxygen species (ROS) and mitochondria as evident with the testing on "petite mutants," similar to Bax-induced YCD, while there was no release of cytochrome *c*.

MATERIALS AND METHODS

STRAINS, PLASMIDS, AND GROWTH CONDITIONS

S. cerevisiae Y187 (Clontech) strain was used in the present study. The plasmid constructs pYEUT, pYEUT-*hrpZ* were generated by Podile et al. [2001] and the "petite" mutant of *S. cerevisiae* Y187 was generated as described by [Greenhalf et al., 1996]. *S. cerevisiae* Y187 was grown with aeration at 28°C in YEPD medium and *S. cerevisiae* Y187-pYEUT-*hrpZ* was grown in yeast minimal medium (YMM) containing yeast nitrogen base and tryptophan/uracil dropout supplement (Difco) with raffinose or galactose (2%) (Sigma) as the carbon source. GGYP (4% (v/v) glycerol, 0.2% (w/v) glucose, 1% (w/v) yeast extract, 1% (w/v) bactopeptone, and 3 gm/L KH_2PO_4) and YEPG (YEPD with glucose replaced by 3% (v/v) glycerol) was used to test strains for the "petite" phenotype. To induce *hrpZ* expression, the transformed cells grown to an early exponential phase in 2% raffinose medium were washed and resuspended in tryptophan drop-out supplement medium containing 2% galactose. Either H_2O_2 or acetic acid in raffinose-containing medium was used as a positive control to induce cell death in *S. cerevisiae*. Luria-Bertani medium with kanamycin was used to culture *E. coli (BL-21)* harboring pET28a-*hrpZ*, induced with 1 mM IPTG for harpin expression and purified it using Ni-NTA agarose column (Qiagen).

EXTRACELLULAR AFFECT OF HARPIN

pYEUT-*hrpZ* transformant of *S. cerevisiae* Y187 was grown at 28°C, to an $OD_{600} \sim 0.5$, and then cultured in fresh medium containing raffinose where different concentrations (5, 10, 20 μM) of harpin$_{pss}$ and harpin$_{psph}$ (from *Psuedomonas syringae* pv. *phaseolicola*) was added. The culture OD was recorded at different time intervals. Extracellular affect was also observed on semisolid media with both the harpins. Cells cultured in raffinose or galactose-supplemented medium for harpin expression served as controls.

CELL CYCLE SYNCHRONIZATION

pYEUT-*hrpZ* transformant of *S. cerevisiae* Y187 was cultured in raffinose-containing medium, grown at 28°C to 0.5 (OD_{600}), washed, resuspended and cultured for 3 h at 24°C in the presence of 0.2 M hydroxyurea and 15 μg/ml nocodazole (Sigma–Aldrich) for S- and M-phase arrest, respectively [Wang and Kuo, 2001]. The S- and M-phase-arrested *S. cerevisiae* Y187, pYEUT-*hrpZ* cells were then washed and suspended in fresh medium, and cultured at 28°C in raffinose and galactose-containing medium with timely recordings at OD_{600}.

ASSESSMENT OF NUCLEAR MORPHOLOGY, CHROMATIN CONDENSATION, AND DNA LADDERING

S. cerevisiae Y187 pYEUT-*hrpZ* was cultured in raffinose-supplemented medium with vigorous aeration at 28°C to ~0.5 (OD_{600}). Cells were pelleted and again resuspended in raffinose or galactose-containing media for harpin induction. A positive control with 3 mM H_2O_2 treatment for yeast cells was also maintained simultaneously. After 3 h of induction, DAPI staining, chromosomal DNA isolation and transmission electron microscopy were carried out.

DAPI (4′,6-DIAMINO PHENYL INDOLE) STAINING

DAPI staining of yeast cells was carried out as described by Tocyski et al. [1997]. After 3 h of induction, 1 ml (~1×10^6 cells) of culture was fixed with 3.7% formaldehyde for 30 min, stained with 2 μg/ml of DAPI and observed under Leika epifluorescence microscope with BF/UV filter.

YEAST CHROMOSOMAL DNA ISOLATION

Chromosomal DNA from *S. cerevisiae* Y187 was isolated essentially following Breeden LL Lab's yeast protocols (http://www.fhcrc.org/labs/breeden/Methods/chromosomal-DNA.html). After 3 h of induction in galactose and H_2O_2, chromosomal DNA was prepared from 25 ml culture each. The DNA was resuspended in 500 μl 1× TE, digested with RNase I for 30 min at 37°C and extracted with phenol/chloroform. The DNA was again ethanol precipitated and dissolved in 1× TE and analyzed on a 1.5% agarose gel.

ELECTRON MICROSCOPY

Yeast cells were fixed with phosphate-buffered 2.5% glutaraldehyde overnight at 4°C. Cell walls were removed enzymatically, and the cells were postfixed with osmium tetroxide and uranyl acetate, and then dehydrated as described by Byers and Goetsch [1991]. After 100% ethanol washes, cells were washed with 100% acetone, infiltrated with 50% acetone/50% Epon for 30 min and with 100% Epon for 20 h. Cells were transferred to fresh 100% Epon and incubated at 56°C for 48 h before cutting thin sections. Micrographs were taken with a Zeiss EM electron microscope.

H_2DCFDA STAINING FOR ROS

Release of reactive oxygen species (ROS) in harpin$_{pss}$-induced YCD was detected by staining with H_2DCFDA (2′7′-dichloro dihydro fluorescein diacetate) [Ludovico et al., 2002]. After 90 min of induction, cell culture (~1×10^6 cells ml^{-1}) was pelleted, resuspended in phosphate buffered saline and incubated with

15 μM H_2DCFDA for 30 min in dark at 28°C. The cells were then acquired in the BD FACS Calibur flow cytometer at 488–529 nm using FL1-H filter for. A total of 20,000 cells were analyzed per sample.

FDA AND PI STAINING (FLUORESCEIN DIACETATE AND PROPIDIUM IODIDE)

Possible loss of membrane integrity in harpin$_{Pss}$-mediated yeast cell death was studied using a combination of two fluorescent dyes FDA and PI. After 3 h of induction 1 ml of culture was taken, washed and resuspended in phosphate buffered saline (PBS buffer pH 7.2). To this were added 50 μg/ml of FDA and 100 μg/ml of PI and incubated for 15 min in dark at 28°C. Cells were then observed under Leika epifluorescence microscope equipped with a 50 W mercury lamp and appropriate filter settings.

RH-123 STAINING FOR MITOCHONDRIAL POTENTIAL

S. cerevisiae cells cultured for 200 min and for 12 h were harvested ($\sim 1 \times 10^6$ cells ml^{-1}), washed and resuspended in 1 ml phosphate buffered saline (PBS, pH 7.0) and incubated with 100 nM Rhodamine-123 [Ludovico et al., 2001] for 30 min in dark at 28°C. 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 529 nm using FL1-H filter. A total of 20,000 cells were analyzed per sample.

RELEASE OF CYTOCHROME *c* IN HARPIN$_{PSS}$-INDUCED YCD

Cytosolic fractions were prepared using cell fractionation kit (BD Biosciences) from *S. cerevisiae* cells grown in presence of raffinose, galactose and 80 mM acetic acid for 3 h. The proteins resolved on a 15% SDS–PAGE were subjected to western blot analysis and the membrane was incubated with Cyt C antibodies and subsequently with the anti-rabbit IgG ALP conjugate. The immunoblot was visualized by alkaline phosphatase catalyzed color reaction using BCIP-NBT.

EFFECT OF CYCLOSPORINE A

To study the effect of cyclosporin A (CsA) on yeast cells, CsA (7 μg/ml) [Jung et al., 1997] was added to pYEUT-*hrpZ* transformants cultured in galactose-containing media and continued to grow at 28°C. Aliquots were drawn to carryout OD_{600} at regular intervals.

DETECTION OF METACASPASE ACTIVATION

Caspase activation was assessed using the fluorescent caspase inhibitor "CaspACE, FITC-VAD-fmk In Situ Marker" (Promega) [Madeo et al., 2002]. Briefly, 1×10^6 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 ($\lambda_{exc} = 488$ nm and $\lambda_{em} = 529$ nm). A total of 20,000 cells were analyzed per sample. Cells were simultaneously counter stained with 2 μg ml^{-1} of PI for 10 min at room temperature and observed under epifluorescence microscope to ensure any non-specific staining of FITC-VAD-fmk to dead cells (not shown).

ANNEXIN V AND PI STAINING

S. cerevisiae pYEUT-*hrpZ* cells cultured for 200 min were harvested ($\sim 1 \times 10^6$ ml^{-1}), washed with PBS (pH 7.0) and treated with 15 U lyticase (Sigma) 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) and then incubated for 20 min in dark at 30°C. After incubation, the cells were washed with binding buffer and then resuspended in 1 ml of PBS. The samples were then acquired in the BD FACS calibur flow cytometer using FL 1-H filter ($\lambda_{exc} = 488$ nm and $\lambda_{em} = 529$ nm) on *X*-axis and FL 2-H filter ($\lambda_{exc} = 605$ nm and $\lambda_{em} = 640$ nm) on *Y*-axis. A total of 20,000 cells were analyzed per sample.

RESULTS

EXTRACELLULAR AFFECT OF HARPIN

S. cerevisiae Y187, exposed extracellularly to different concentrations (5–20 μM) of harpin$_{Pss}$ and harpin$_{Psph}$, individually, was not affected in both liquid and solid media. The growth, in presence of either of the harpin proteins was similar to the growth of *S. cerevisiae* in raffinose supplemented media (Fig. 1), while it was inhibited in galactose-supplemented cultures.

ROLE OF CELL CYCLE STAGE ON HARPIN$_{PSS}$-INDUCED YCD

S. cerevisiae Y187 pYEUT-*hrpz* cells, grown in raffinose medium and synchronized to S- and M-phase were shifted to galactose-containing medium to induce harpin$_{Pss}$ expression. Cell death occurred both in S- and M-phase arrested cells within 1 h (Fig. 2), similar to unsynchronized cell population indicating that harpin-mediated YCD was independent of the stage of cell cycle.

ASSESSMENT OF NUCLEAR MORPHOLOGY AND CHROMATIN CONDENSATION

Staining of harpin$_{Pss}$-expressing yeast cells with DAPI revealed absence of nuclear fragmentation (Fig. 3A). Cells grown in raffinose medium were normal with a central vacuole and homogenous nuclear chromatin, where as cells grown in galactose-supplemented medium also showed a central vacuole and homogenous nuclear chromatin but watery cytoplasm, a feature of necrosis, indicating that the cytoplasmic contents have leaked out (Fig. 3B). In contrast, cells treated with H_2O_2, showed extensive chromatin condensation towards the nuclear envelope, a typical marker of apoptosis. Galactose-grown *S. cerevisiae* Y187 cells had no oligonucleosomal pattern of DNA bands typical of apoptotic cells, while H_2O_2-treated cells showed nuclear fragmentation. The chromosomal DNA from H_2O_2-treated cells showed a diffused smear of DNA fragments (Fig. 3C).

RELEASE OF ROS AND LOSS OF MEMBRANE INTEGRITY

Less than 2% cells cultured in raffinose-supplemented medium exhibit ROS, where as 45% of acetic acid-treated cells, and 39% of

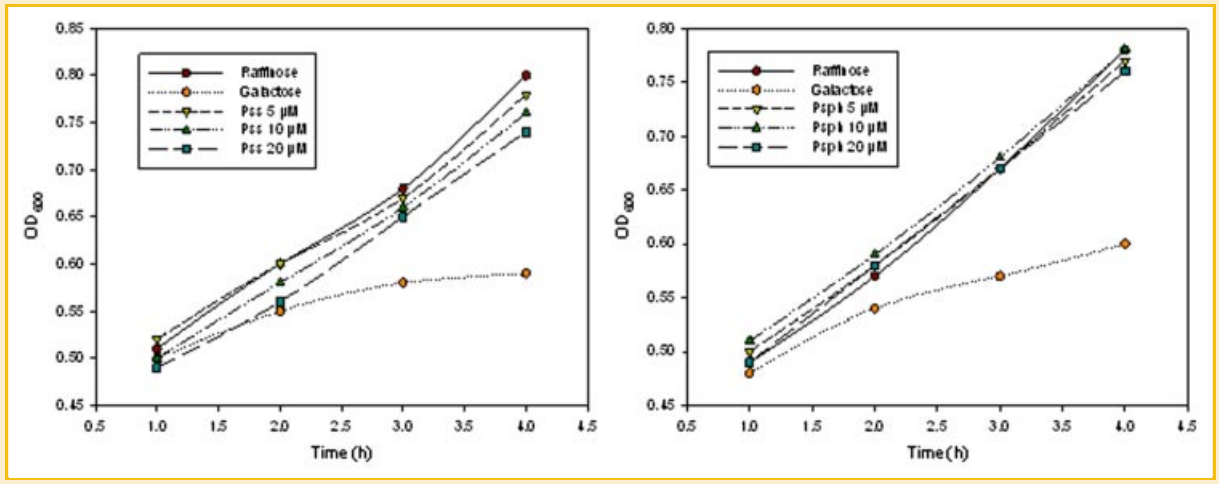

Fig. 1. Extracellular affect of harpin on yeast cells. *S. cerevisiae* Y187 pYEUT-*hrpZ* cells were cultured in raffinose medium with extracellular treatment (5–20 µM) of harpin$_{Pss}$ and harpin$_{Psph}$ (expressed in modified pET vector pJC40). Untreated cells served as controls. Growth at 28°C for 4 h of incubation was recorded at OD_{600}. Data represent one of three independent experiments.

yeast cells cultured in galactose-supplemented medium (Fig. 4A) resulted in formation of ROS indicating its involvement in harpin$_{Pss}$-mediated YCD. Staining with FDA and PI, showed loss of membrane integrity in the cells cultured in galactose-supplemented medium (Fig. 4B) similar to H_2O_2 treated cells, whereas the cells cultured in raffinose-containing medium, did not stain PI and fluoresced green taking up FDA indicating intact membrane integrity.

TESTING ON "PETITE" MUTANTS AND ROLE OF MITOCHONDRIA

The "petite" mutants of *S. cerevisiae* Y187 pYEUT-*hrpZ* were plated onto semisolid media containing glucose or galactose. Growth of "petite" mutants on galactose-containing plate was unaffected and was similar to the colonies formed on glucose-containing medium (Fig. 5). The "petite" mutants of *S. cerevisiae* Y187, therefore, were insensitive to harpin$_{Pss}$-mediated cell death, indicating the involvement of mitochondria, in YCD, similar to Bax-induced

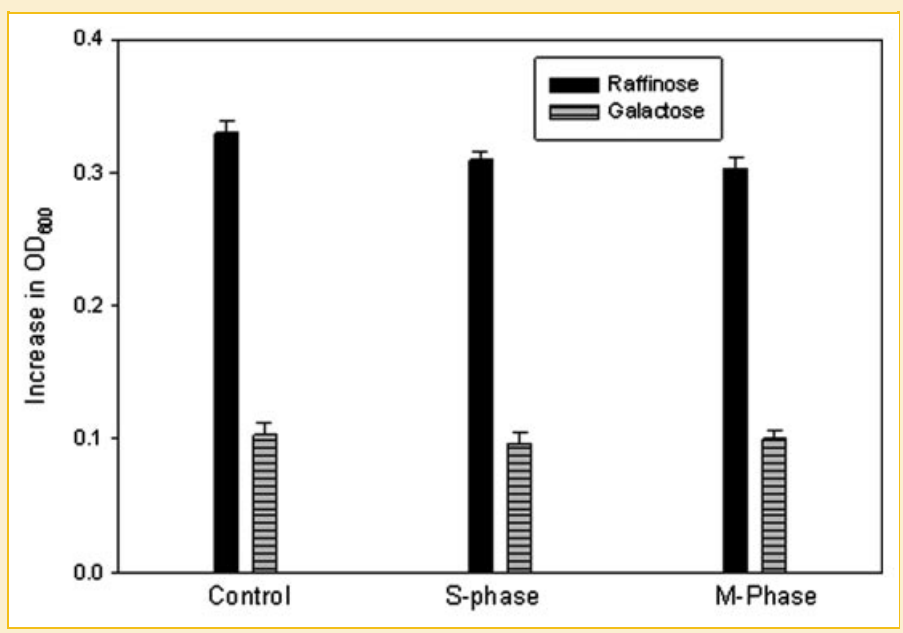

Fig. 2. Role of cell cycle stage on harpin$_{Pss}$-induced YCD. *S. cerevisiae* Y187 pYEUT-*hrpZ* cells arrested in S- and M-phase were cultured in raffinose or galactose-containing medium to monitor the influence of cell cycle phase. Unarrested cells were used as controls. Growth at 28°C after 4 h incubation was recorded on *Y*-axis as increase in OD_{600}, representing average of three independent observations.

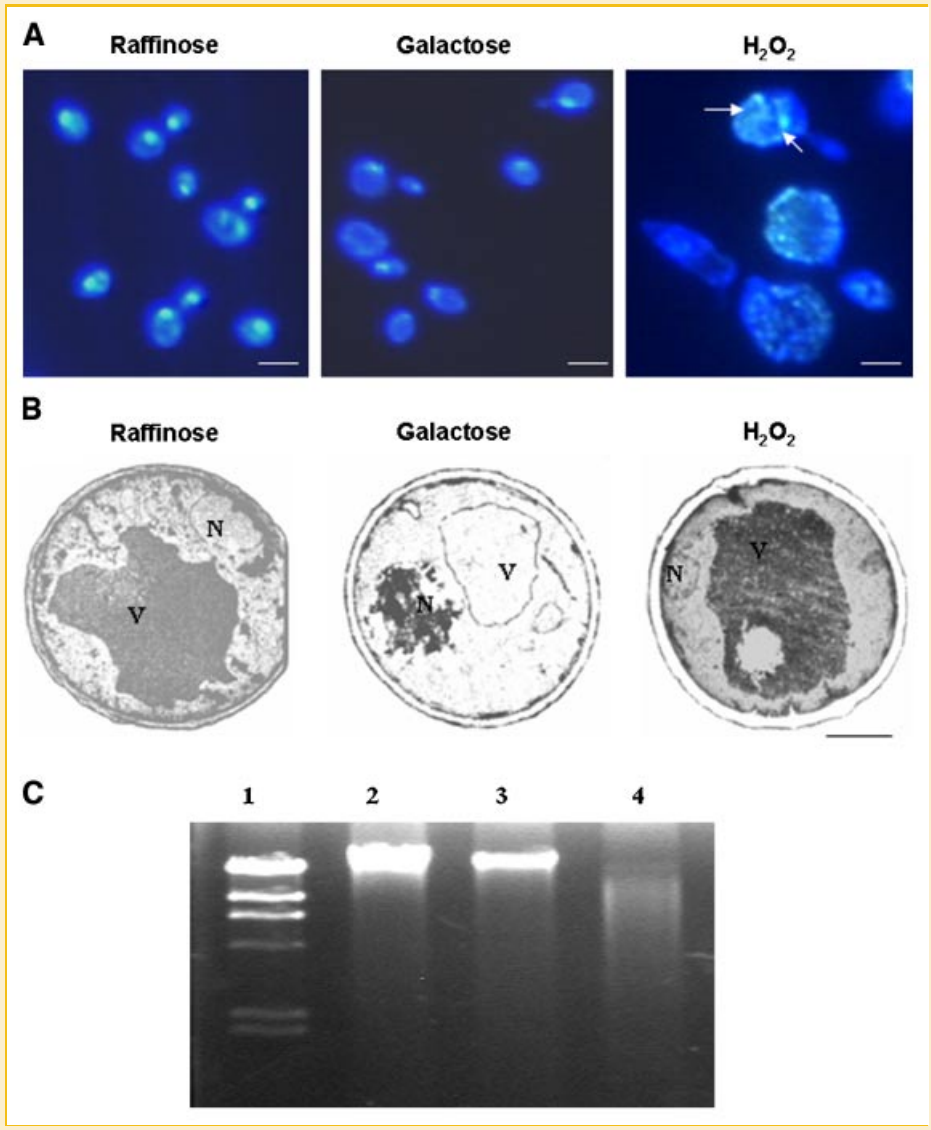

Fig. 3. Assessment of nuclear morphology, chromatin condensation and DNA laddering. A: *S. cerevisiae* Y187-*hrpZ* cells stained with DAPI to identify nuclear fragmentation. Cells cultured in presence of raffinose (control), galactose (harpin expressed) and H_2O_2 treated where the fragmentation is shown with arrows (Bar, 10 μm). B: Electron micrographs of yeast cells cultured in raffinose, galactose, and H_2O_2 treatment. N, nucleus; V, vacuole. Bar, 1 μm. C: Genomic DNA isolated from yeast cells and analyzed on an agarose gel for laddering. λ *Hin*d III molecular weight markers (lane I), genomic DNA extracted from cells cultured in raffinose medium (lane 2), in galactose medium (lane 3), and cells treated with H_2O_2 (lane 4). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cytotoxicity in yeast [Greenhalf et al., 1996]. Yeast cells expressing harpin or cells treated with acetic acid exhibit hyperpolarization during early hours and depolarization of mitochondrial membrane during late hours of induction, respectively (Fig. 6) indicating changes in mitochondrial potential during harpin expression.

ROLE OF CYTOCHROME *c* AND CYCLOSPORINE A

Cytochrome *c* was released into the cytosol in acetic acid-treated yeast cells and in chronic myeloid leukemia cells (Fig. 7A), while, there was no evidence of leakage of Cyt C from the pYEUT-*hrpZ* cells cultured in galactose-containing medium. Cyclosporine A (PTP blocker) also did not alter the fate of *S. cerevisiae* Y187 cells expressing $harpin_{Pss}$ (Fig. 7B), further suggested that there was no release of Cytochrome *c* from the mitochondria into cytosol in $harpin_{Pss}$-mediated YCD.

ASSESSMENT OF METACASPASE ACTIVITY

In order to confirm caspase activation in harpin-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 [Madeo et al., 2002]. Yeast cells cultured for 4 h in raffinose, galactose and acetic acid treatment were analyzed for caspase activity. In comparison, galactose cultured yeast cells (harpin expressed) and acetic acid treated cells exhibit higher metacaspase activity than the control cells (Fig. 8) indicating the elevation of metacaspases in harpin-induced yeast cell death.

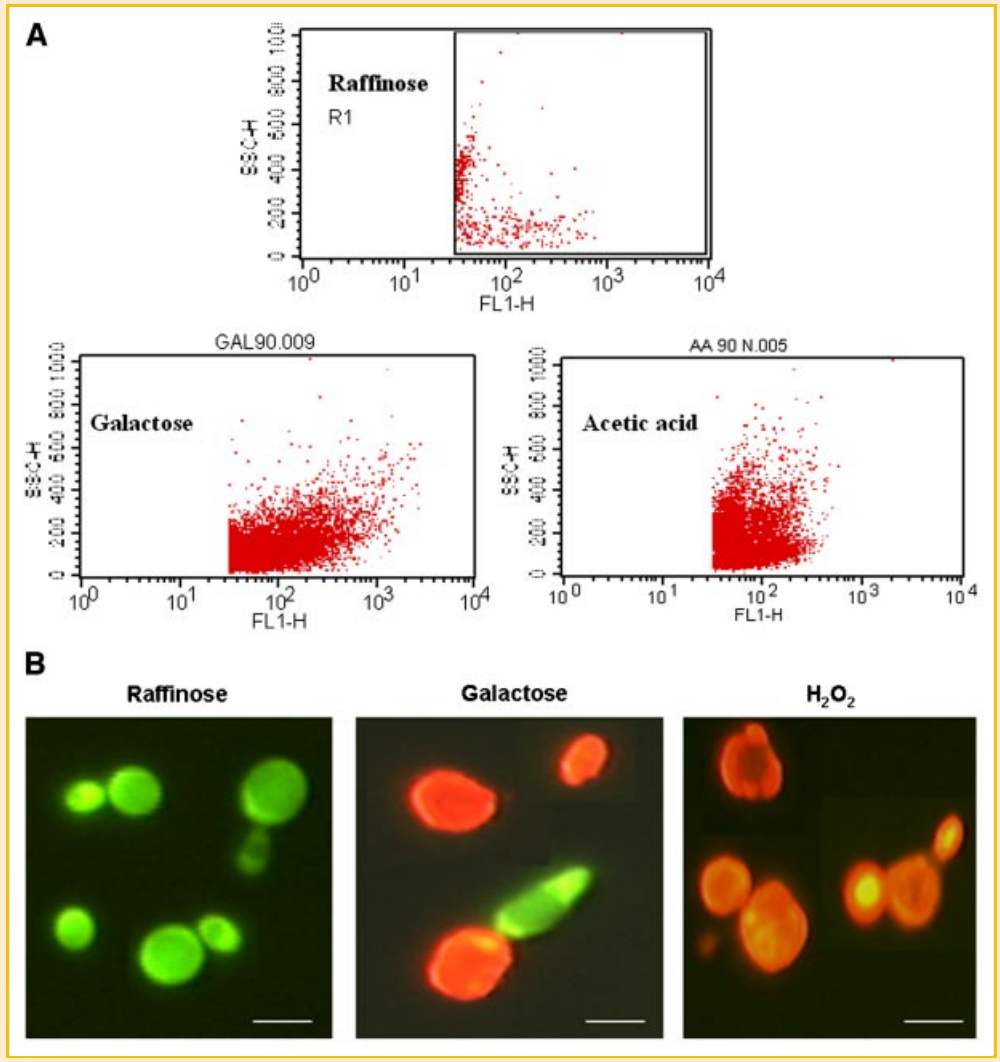

Fig. 4. Detection of ROS and loss of membrane integrity in harpin$_{pss}$-mediated yeast cell death. A: Yeast cells were stained with H_2DCFDA to monitor ROS levels. Cells cultured in raffinose served as control, galactose supplementation for harpin-induction, and acetic acid treated cells as positive control. B: Yeast cells were incubated with FDA and PI to visualize the membrane integrity. Cells cultured in presence of raffinose (control), galactose (harpin-induced), and H_2O_2 treated cells as positive control (Bar 10 μm). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

ASSESSMENT OF YCD BY ANNEXIN V AND PI

In harpin$_{Pss}$-induced YCD 29% of galactose-supplemented cells (Fig. 9) and 53% of acetic acid treated cells stained PI, as compared with 3% of control cells cultured in raffinose-supplemented medium. Nearly 2% of acetic acid-treated cells and 0.2% of harpin$_{Pss}$-induced cells tested positive for annexin V staining indicating that harpin$_{Pss}$-expressed yeast cells exhibit more necrosis than apoptosis.

DISCUSSION

Harpin$_{Pss}$ was supposed to be localized in outer portion of cell membrane, probably in the cell wall. The cell wall, therefore, is crucial for the harpin-induced HR in tobacco suspension cells [Hoyos et al., 1996]. Harpin could differentially interact with a putative host specific protein in the plant cell membrane [Li et al., 2005] which could not be facilitated in the yeast cell membrane or cell wall. Induction of YCD by harpin did not occur when added extracellularly, both in liquid and semisolid media, suggesting the possibility that harpin probably triggers a cell wall associated kinase (Wak1) in the plant cell [He et al., 1996]. Extracellular secretion of *hrpZ* with the help of alpha factor leader sequence also confirmed that harpin does not cause YCD outside the yeast cells (our unpublished data).

Differential expression of proteins during the cell cycle was studied in synchronized cell fractions using a mathematical model [Uzbekov, 2004]. The expression profiles of several proteins cyclically changes during the cell cycle or the effectiveness of several proteins varies differentially depending on the cell cycle phase but in contrast, there are proteins, whose activity does not depend on the stage of the cell cycle. Harpin$_{Pss}$ expressing S- and

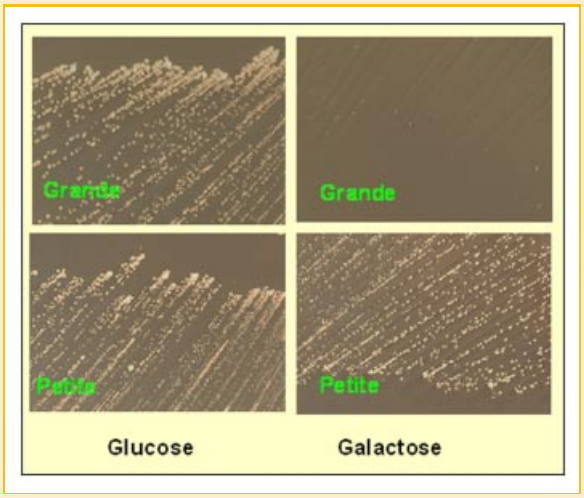

Fig. 5. Effect of harpin on petite mutants of *S. cerevisiae* Y187. pYEUT-*hrpZ* transformants of *S. cerevisiae* Y187 "petite" mutants grow on both glucose-supplemented medium (left panel) and galactose-containing medium (right panel) and pYEUT-*hrpZ* transformants of *S. cerevisiae* Y187 "grande" grow only on glucose-containing medium (upper left). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

M-phase arrested *S. cerevisiae* cells did not multiply in galactose-containing medium suggesting that harpin$_{Pss}$-mediated YCD was independent of the stage of cell cycle.

DNA ladders that can be detected by conventional agarose gel electrophoresis are not necessarily seen in apoptotic cells [Reed et al., 1991], but the morphological changes in nuclear shape and chromatin condensation are essentially universal features of apoptosis in mammalian cells, not observed in harpin$_{Pss}$-expressing *S. cerevisiae* Y187. In H_2O_2-treated cells, DNA fragmentation appeared as a smear, though not a typical ladder, probably due to the presence of very little linker DNA between the nucleosomes [Lowary and Widom, 1989]. Apoptosis without DNA laddering has been described for several metazoan cell types [Oberhammer et al., 1993].

Formation of ROS was common to both apoptotic and necrotic forms of cell death, and plays a central role in the induction and execution of PCD in yeast [Frohlich and Madeo, 2000]. In harpin$_{Pss}$-induced YCD, we observed extensive ROS generation which likely results from the changes in the electron transport chain events (complex I and III) of the mitochondria. Diphenylene iodonium (DPI), an inhibitor of complex I, completely suppressed ROS generation from the mitochondria during harpin treatment in tobacco cell cultures [Xie and Chen, 2000; Amirsadeghi et al., 2007]. The protective effect of catalase in yeast cells expressing the lethal phenotype in galactose-supplemented medium indicated the involvement of oxidative burst in YCD [Podile et al., 2001], a feature similar to plant HR. FDA and PI staining of the cells cultured in galactose-supplemented medium as well as cells induced for apoptosis with H_2O_2 confirmed the loss of membrane integrity, a feature commonly observed both in apoptosis and necrosis. In our attempt to compare the biochemical events of plant HR and YCD, the use of protein kinase inhibitor K252a completely suppressed the YCD [Podile et al., 2001], as observed in some forms of plant HR [Adam et al., 1997]. The amount of cell death greatly increased when the H_2O_2 production was enhanced by inhibiting catalase, similarly harpin$_{Pss}$-induced cell death decreased significantly in the presence of catalase. The difference between the protective effect of K252a and catalase in harpin$_{Pss}$-induced YCD, also observed by Desikan et al. [1996] in *Arabidopsis* suspension cultures, suggests the importance of protein phosphorylation and oxidative burst as the major events in harpin$_{Pss}$-induced cell death.

Harpin$_{Pss}$ expressed yeast cells exhibited transient hyperpolarization during initial hours of induction and depolarization during late hours of induction similar to acetic acid-treated cells indicating that there was loss of mitochondrial membrane potential ($\Delta\Psi_m$) in harpin$_{Pss}$-induced YCD. Since "petite" mutants of *S. cerevisiae* were insensitive to expression of harpin$_{Pss}$,

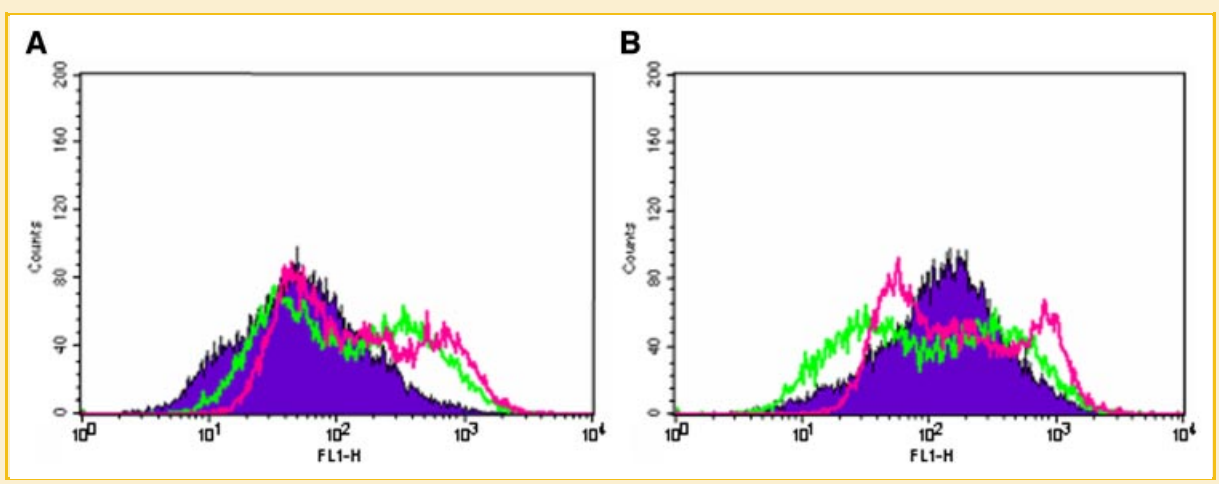

Fig. 6. Detection of mitochondrial membrane potential (ψ) in harpin$_{pss}$-induced YCD. Histogram representation of Rhodamine-123 stained yeast cells. The area in blue represents the cells cultured in raffinose-supplemented medium, green line for cells cultured in galactose-supplemented medium (harpin-induced), and pink for acetic acid treated cells. Cells were cultured for (A) 200 min, and (B) 12 h. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

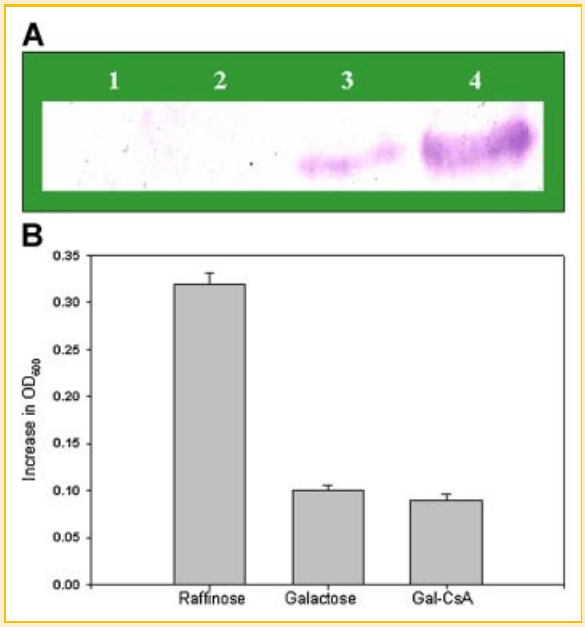

Fig. 7. A: Western blot analysis to detect the release of Cytochrome c. Cytosolic fractions resolved on SDS–PAGE were transferred to the membrane and incubated with Cyt C antibodies (Clontech) and subsequently with the anti-rabbit IgG ALP conjugate. The blot was visualized by alkaline phosphatase catalyzed color reaction using BCIP-NBT. Cells cultured in presence of raffinose (lane 1), galactose for harpin induction (lane 2), acetic acid treated yeast cells as a positive control (lane 3), and a second positive control for Cyt C from chronic myeloid leukemia cells (lane 4). B: Effect of Cyclosporine A in harpin-induced YCD. *S. cerevisiae* Y187 pYEUT-*hrpZ* cells were cultured in galactose-supplemented medium with Cyclosporine A. Cells introduced in raffinose or galactose-containing medium served as controls. Growth at 28°C for 4 h was recorded on *Y*-axis as increase in OD_{600} representing average of three independent observations. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

mitochondria were playing a major role in $harpin_{Pss}$-induced YCD similar to Bax-induced YCD [Greenhalf et al., 1996], while there was no evidence of Cyt C release. Cyclosporine A (PTP blocker) inhibits apoptosis in yeast [Severin and Hyman, 2002], did not protect *S. cerevisiae* Y187 pYEUT-*hrpZ* cells, probably because of the non-involvement of Cyt C leakage. A central feature of mitochondrial dysfunction during animal apoptosis is the opening of permeability transition pore (PTP), releasing Cyt C and intermembrane space proteins into the cytosol, but in $harpin_{Pss}$-induced YCD, PTP seems to have no prominent role as evidenced by the affect of CsA and no release of Cyt C. In *Arabidopsis* cells, $harpin_{Pss}$ treatment resulted in a decline of $\Delta\Psi_m$ and cellular ATP levels with the appearance of cytosol-localized Cyt C from the mitochondria [Krause and Durner, 2004]. Bax expression induced the release of Cyt C from mitochondria and decreased Cyt C oxidase in yeast [Manon et al., 1997], similar to its effects in mammals. Roucou et al. [2000] showed that the Cyt C-green fluorescent protein fusion was not released from mitochondria into cytosol upon expression of Bax in the yeast cell, indicating that mitochondrial release of Cytochrome *c* was not mandatory for YCD.

S. cerevisiae contains a few orthologues of mammalian cell death regulators such us the YCA1/MCA1 coding for metacaspases which are supposed to be involved in yeast cell death triggered by different stimuli [Silva et al., 2005]. In mammalian cells, caspase activity also stimulates several non-death functions like cell cycle, proliferation, inflammation and differentiation [Li et al., 2007]. Yeast metacaspase (MCA1) was also shown to accelerate G1/S phase of cell cycle and antagonize G2/M transitions [Lee et al., 2008]. The role of YCA1 in yeast cell death remains elusive, YCA1 independent cell death [Buttner et al., 2007] and YCA1 independent caspase like activities [Vachova and Palkova, 2005] have also been reported, yet the involvement of metacaspases remains debatable in yeast cell death. YCA1 seems to have a prominent role in acetic acid induced yeast cell death, in a manner unrelated to its caspase activity [Guaragnella

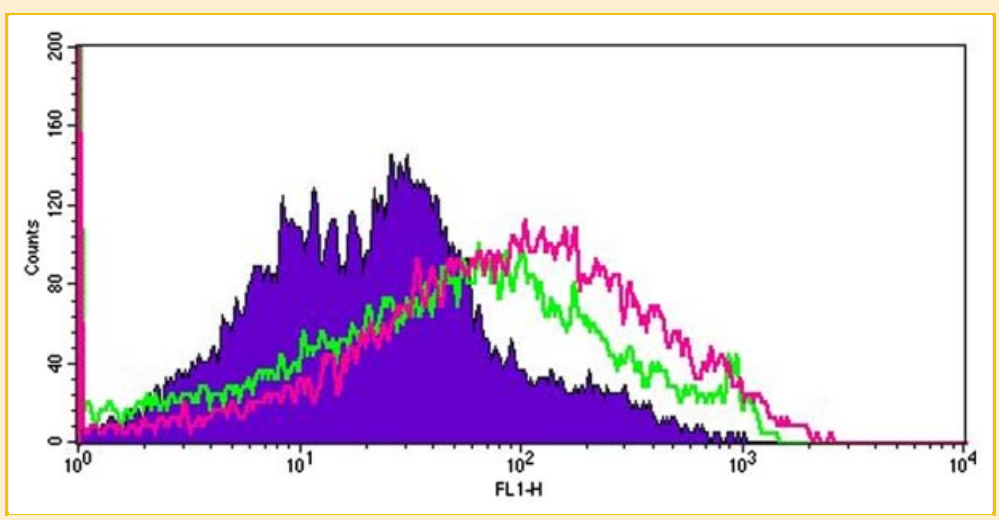

Fig. 8. Detection of Metacaspase activity in harpin-induced yeast cell death. Histogram representation of FITC-VAD-fmk stained yeast cells. Area in blue represents cells cultured in raffinose-supplemented medium (control), green line for cells cultured in galactose (harpin expressed) and pink for acetic acid treated cells. Data represents one of three independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

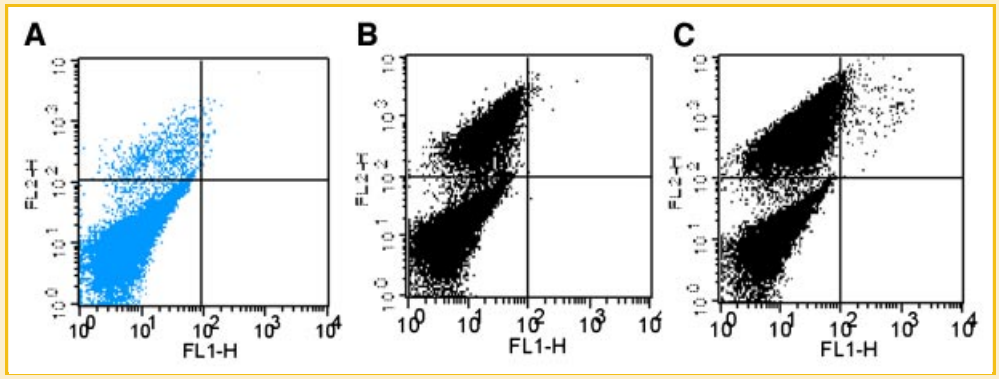

Fig. 9. Assessment of cell death by annexin V and PI staining. Cell death during harpin-induced YCD was assessed using Annexin V and PI. Cells cultured in raffinose served as control (A), galactose-supplementation where harpin expression is induced in yeast cells (B) and cells treated with acetic acid (C). Data represents one of three independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

et al., 2006] indicating that it could be associated with other cellular functions.

Cytochrome *c* and caspases are the key factors for induction of apoptosis in animal cells but in yeast the absence of cytochrome *c* or metacaspase may only reduce but not abolish the cell death processes. Unlike in animal cells, the release of cytochrome *c* upstream of metacaspase activation is not very much essential in yeast [Silva et al., 2005] or caspase activation may not be always associated with the release of cytochrome *c* and yeast might possess alternative death pathways which do not involve the role of cytochrome *c* [Pereira et al., 2007].

In the present study, FITC-labeled caspase in-situ marker VAD-fmk was used, which binds specifically to the active site of metazoan caspases and detected by flow cytometry. Harpin expressing yeast cells showed elevated metacaspase activity (similar to acetic acid treated cells) compared with the control cells. This is the first report on the induction of metacaspase activity during harpin-induced YCD or even in plant cells where caspase activation during harpin expression has not been reported earlier, however the role of metacaspase (YCA1) in this form of YCD needs to be further investigated.

In yeast, as in mammalian cells, phosphatidylserine (PS) has an asymmetric distribution in the lipid bilayer of the cytoplasmic membrane. The exposure of PS residues at the outer surface of the cytoplasmic membrane occurs at the early stages of apoptosis [Martin et al., 1995] when membrane integrity is still retained. In the present study more percentage of harpin$_{Pss}$-expressed cells stained positive for PI than for annexin V similar to acetic acid-treated cells (induced for a necrotic response) suggesting that the harpin$_{Pss}$-induced YCD was more necrotic rather than apoptotic.

As a whole our results presented here reveal the basic physiological changes in harpin-induced yeast cell death giving us an indication that this is a necrosis like PCD, although caspase activation has been observed [Vachova and Palkova, 2007]. Harpin has a rapid and dramatic impact on mitochondria, in functioning, that it binds and triggers ion currents in lipid bilayers [Lee et al., 2001] and forms ion conducting pores and probably disrupting the function of mitochondria in the cell. The necrosis seen in this YCD could be due to the consequence of severe oxidative-stress, involving mitochondria and related enzymes which are currently under investigation. This necrosis like PCD seems to be comparable to the cellular events during HR response in plants. Thus the possibility of using yeast as a model organism throws light in elucidating the mechanism of action of several molecules in higher eukaryotic systems however there might be little variations from one system to another due to its evolutionary genetic make up and environmental influences.

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