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Resistance through inhibition: Ectopic expression of serine protease inhibitor offers stress tolerance via delayed senescence in yeast cell

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ABSTRACT

Protease inhibitors have been known to confer multiple stress tolerance in transgenic plants. We have assessed growth of yeast (*Pichia pastoris* GS115) strains expressing inhibitory repeat domains (PpIRD$^+$) of previously characterized *Capsicum annuum* protease inhibitors under high salt, heavy metal and oxidative stress. PpIRD$^+$ strains exhibited multiple stress tolerance and showed differential molecular responses at transcriptional and translational level on exposure to stress inducing agents like heavy metal, high salt and H_2O_2. PpIRD$^+$ strains display significant reduction in metacaspase (Yca1) activity, the key enzyme in apoptosis, indicates the possibility of cross reactivity of IRDs (serine protease inhibitor) with cysteine proteases. PpIRD$^+$ and *Saccharomyces cerevisiae* knockout with Yca1 (ΔYca1) strain showed similar growth characteristics under stress, which indicated the delayed senescence due to cellular metacaspase inhibition. Molecular docking study showed a close proximity of IRDs reactive site and the active site of metacaspase in the complex that signified their strong interactions. Maintenance of GAPDH activity, primary target of metacaspase, in PpIRD$^+$ strain evidenced the inhibition of metacaspase activity and survival of these cells under stress. This report demonstrates a potential molecular mechanism of protease inhibitor-based multiple stress tolerance in yeast strains.

1. Introduction

Plant precept both abiotic and biotic stress signals and subsequently transduced to switch on the adaptive response, which is part of the important survival tactics [1]. Stress signal induces several signaling molecules and results in activation of a battery of stress-inducible genes [2]. It has further been noted that an exposure to one kind of stress makes the plant tolerant to others, showing that there is a cross-talk between different pathways [1,3]. Amongst the unique stress-inducible genes, plant protease inhibitor (PIs) is one of the most important gene family. For example, potato inhibitor-II (Pin-II) gene expression is regulated by an alternative pathway (other than classical herbivory induced octadecanoid pathway), mediating the electrical/mechanical wound response and thus primed the stress tolerance in plants [4,5]. Furthermore, the constitutive expression of the *Oryza sativa* chymotrypsin inhibitor in transgenic rice enhanced drought tolerance [4–6].

PIs are widely distributed throughout the plant kingdom and most importantly play an important role in the defense against herbivores and pathogens [7–9]. They form complexes with proteases, thereby inhibiting their proteolytic activity, while serve as storage proteins in plant storage organs [7]. PIs are divided into four major classes, i.e. serine, cysteine, aspartic and metallo PIs [7,10]. Of these, the most abundant are serine PIs are well known for the role in environmental response and also in development [6]. Numerous independent studies have demonstrated enhanced expression of serine PIs in response to abiotic stresses such as drought, abscisic acid (ABA), sodium chloride (NaCl) and hydrogen peroxide treatments (H_2O_2) in various plants [11–13]. A chymotrypsin inhibitor from rice was also strongly induced under dehydration and ABA treatments [6]. Furthermore, Shitan et al. showed that expression Bowman Birk inhibitors award heavy metal and multiple drug tolerance in yeast [14]. Recently, transgenic plants with constitutive protease inhibitor expression showed tolerance to pH variations in the culture medium along with additional stresses [15]. All these reports suggest that the PIs are involved in abiotic stress, though their exact role and mechanism are yet to be determined.

In the present study, we report the response of yeast strains expressing *C. annuum* protease inhibitors (CanPIs) single inhibitory repeat units (IRD-7, IRD-9 and IRD-12) to various abiotic stresses. It was observed that ectopic expression of IRDs in *Pichia pastoris* conferred tolerance towards different stresses such as NaCl, heavy

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metal and oxidative stress (H_2O_2). We have investigated the impact of unusual stress on the activity of serine and cysteine proteases in yeast cells. We attempted to find effect of IRDs expression on metacaspase activity. We have studied growth characteristics of yeast metacaspase knockout to confirm the role of metacaspase in survival of the cells. All these observations highlight the underlining reason in tolerance against various abiotic stresses.

2. Materials and methods

2.1. Materials

Enzymes, fine chemicals, synthetic inhibitors and substrates were available from Sigma Chemical Co., St. Louis, MO, USA.

2.2. Viability and growth studies of EV and PpIRD$^+$ strains

Yeast cells were transformed with *C. annuum* PIs genes i.e. IRD-7, -9 and -12 in the pPIC9 vector [16]. Four different *in vitro* phenotypes, containing empty and IRD containing vector, were assessed on various condition by spotting 10 μl aliquots of diluted stationary phase cultures on plates containing high salt (1 M NaCl), metal (50 μM $CdCl_2$ and 50 μM $HgCl_2$) and 1 mM H_2O_2. These phenotypic tests were performed by serial dilutions of cultures onto solid agar YEPD-based plates. Yeast cultures were grown overnight in liquid YEPD and diluted to a density of 1.5×10^5 cells/ml. Four serial 10-fold dilutions were performed at a final dilution containing 1.5×10^1 cells/ml. Four microlitre of each dilution were spotted onto YEPD-based plates and incubated at 28 °C for 48 h.

To assay the viability of yeast containing empty and inhibitor containing vector, the overnight cultures were adjusted to OD_{600} of 0.1, and the cultures were grown for 14 h at 28 °C with shaking (220 rpm), and the OD_{600} was monitored after every 2 h. Time course growth was plotted and further analyzed [17].

2.3. Trypsin PI (TPI) activity assay

Different yeast strains under control and stress conditions were grown and pellet down. Cell mass was suspended in lysis buffer without any protease inhibitor. Protein lysate of yeast was incubated with trypsin for 15 min at 37 °C and residual protease activity was estimated by BA*p*NA assay. The details of the assay were described previously [18].

2.4. Protease assay

Enzymatic assays using azocasein and BA*p*NA as substrates were performed in order to estimate total protease-like and trypsin-like activities, respectively. The details of the assay were described previously [18]. Protease inhibitors, TLCK (1 mM) and NEM (1 mM), were first pre-incubated for 15 min at ambient temperature with enzyme extracts prior to azocasein in addition, and activities were measured as described above. Control assays were performed with the corresponding solvents.

2.5. Metacaspase activity assay

Metacaspase activities in yeast cells were estimated using a commercially available fluorogenic system that uses the peptide Ac-Val-Arg-Pro-Arg-7-amido-4-methylcoumarin (Ac-VRPR-amc) as substrates and procedure described by Vercammen et al. [19]. Detailed procedure is given in Supplementary data 2.

2.6. Quantitative real-time PCR

Relative transcript abundance of subtilisin-like protease 3, calpain-like protease 1, metacaspases and IRDs was determined by quantitative Real-Time PCR (qRT-PCR). Details procedures about RNA extraction, cDNA preparation and qRT-PCR reaction setup were described in Chikate et al. [20]. Actin (ACT1; Accession No.: CAA24598) was used as a reference gene for normalization and list of gene specific primer is given in Supplementary data 1.

2.7. Molecular docking study

In order to analyse the interaction between yeast metacaspase and IRDs, protein–protein docking was carried out. Structure of yeast metacaspase Yca1 was retrieved from PDB (PDB ID: 4F6O) and structures of IRDs were predicted using homology modeling [17,21]. The catalytic residues of Yca1 (CYS220, CYS276) were allowed to interact with the reactive loop of the IRDs (37CPxNC41). Details of docking procedure using ZDOCK and best complex selection criteria were discussed in Joshi et al. [16,22]. Binding energy was estimated using PDBePISA server (http://www.ebi.ac.uk/msd-srv/prot_int/cgi-bin/piserver) [23].

2.8. Viability and growth studies of metacaspase knockout *S. cerevisiae* strain (*ΔYca1*)

Viability and growth curve assay of homozygous diploid *S. cerevisiae* metacaspase knockout (ΔYca1) was performed to confirm the role of metacaspase in apoptosis and its correlation with delayed senescence in the presence of PIs. Detailed procedure of growth curve analysis is given in Section 2.2.

2.9. GAPDH activity assay

Silva et al. had showed that yeast metacaspase specific substrate and primary target in the cell is GAPDH and hence its relevance to yeast apoptosis [24]. Detailed procedure of GAPDH activity assay is given in Supplementary data 2.

2.10. Statistical analysis

All data were statistically examined by independent sample *t*-test. Asterisks indicate significant differences ($^*p < 0.05$; $^{**}p < 0.01$).

3. Result and discussion

3.1. Ectopic expression of IRDs conferred tolerance towards different stresses

Stress tolerance of PpIRD$^+$ strains was evaluated in serial dilution tests, as showed in Fig. 1. Yeast cell with an empty vector (EV) showed susceptibility to elevated salt, heavy metal and oxidative stress as significant growth retardation was marked (Fig. 1A). PpIRD$^+$ strains appeared to be highly tolerant to various stress inducing agents. Our previous study have showed that IRD-9 have higher protease inhibition potential as compared to IRD-7 and -12 [16], similarly in present study the PpIRD-9$^+$ strain (Fig. 1B) showed higher tolerance to multiple stress than that of IRD-7 (Fig. 1C) and -12 (Fig. 1D).

In growth curve analysis (14 h), IRD-9 showed faster rescue of the phenotype under the stress conditions analogous to that of the control condition. In case of PpIRD-7$^+$ and -12$^+$ strains, initially growth was retarded up to 4 h, indicating as extension of the lag phase under stress condition; later the cells were adapted to stress condition. Stress adaptation results in the normal growth of yeast

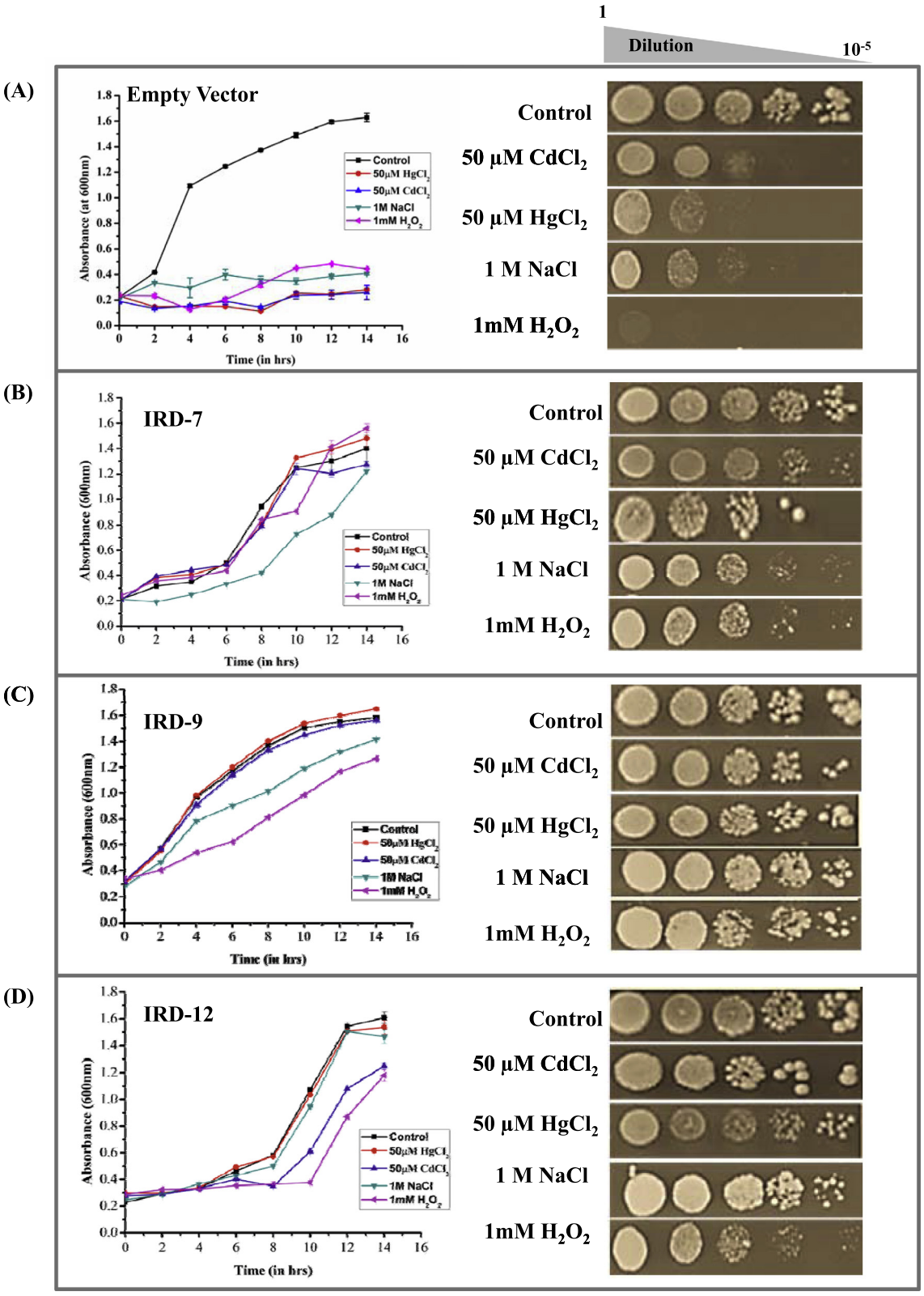

Fig. 1. Growth of yeast cells expressing (A) empty pPIC9 vector (EV) and IRDs namely (B) IRD-7, (C) -9, (D) -12 in the yeast extract peptone dextrose (YPD) broth and plate supplemented with 50 µM $CdCl_2$, 50 µM $HgCl_2$, 1 M NaCl and 1 mM H_2O_2 stress. PpIRD$^+$ cells showed tolerance against all the stresses, while cells with empty vector showed significant retardation of growth. Furthermore, within the different IRDs, yeast cells expressing IRD-9 showed highest tolerance against multiple stresses.

cells in the exponential phase. These results demonstrated that expression of PIs provides endurance against various abiotic stresses. There is a definite correlation in growth rate and inhibition potential of IRDs indicates the significance of protease inhibition on cell survival under stress.

3.2. Enhanced expression and activity of TPI in PpIRD$^+$ stains

Gene expression analysis of IRDs showed that PIs genes namely IRD-7, -9 and -12 were found to be overexpressed in all PpIRD$^+$ as compared to EV strain under stress inducing condition (Fig. 2A). In response to this transcriptional activation, TPI activity was higher in PpIRD$^+$ strains after stress inducing agent's treatment as compared to EV strain (Fig. 2B). Upon stress treatment, there was a sig-

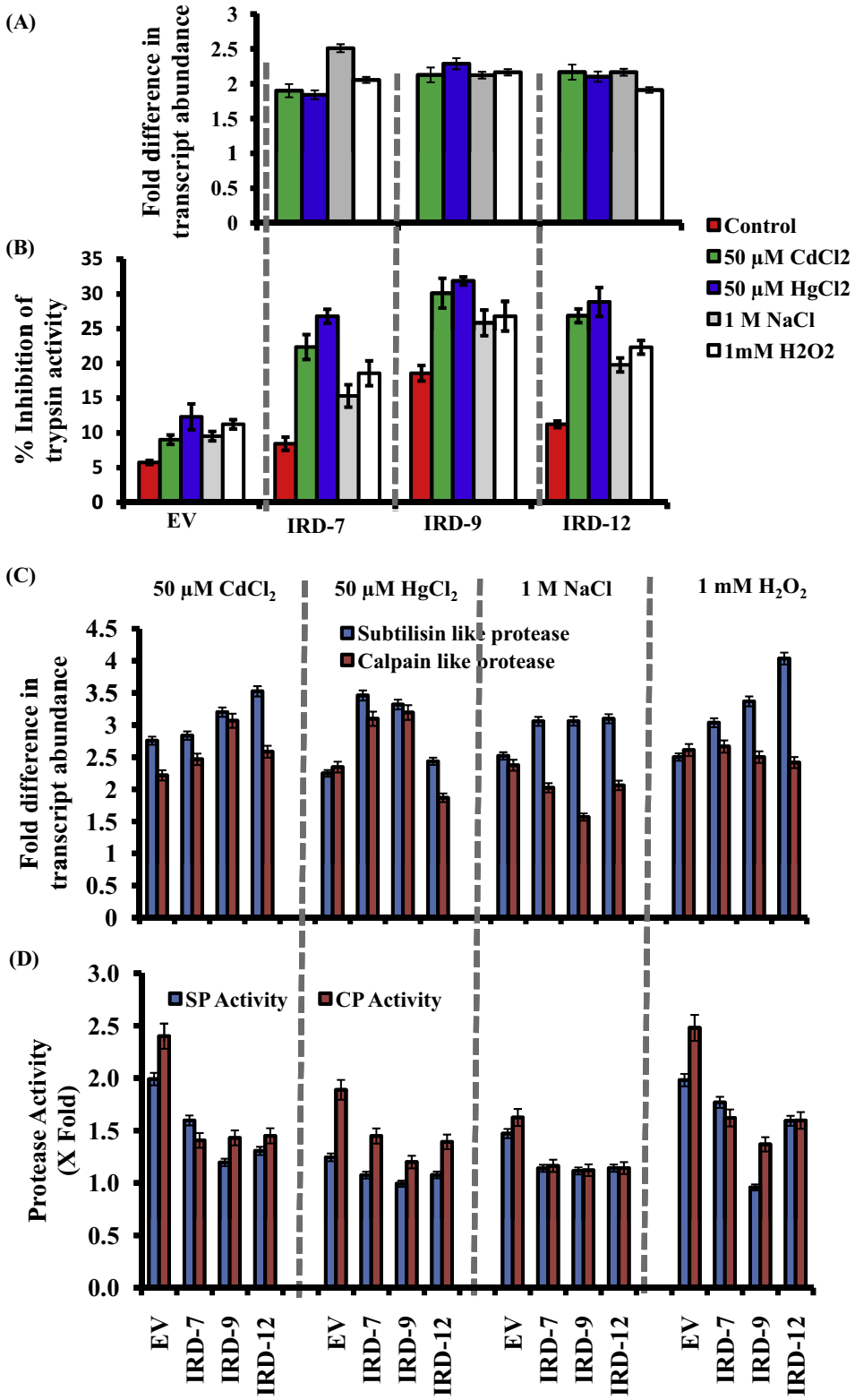

Fig. 2. Protease inhibitor and protease gene expression and activity of CanPI expressing yeast cells under stress. (A) Fold difference in transcript abundance of IRDs; (B) trypsin inhibitory activity; (C) fold difference in transcript abundance of subtilisin like and calpain like protease and (D) serine and cysteine protease activities in of yeast cell expressing IRD-7, -9 and -12 under stress inducing agents like 50 µM $CdCl_2$, 50 µM $HgCl_2$, 1 M NaCl and 1 mM H_2O_2. Values are the means of three measurements with standard error.

nificant increase in intracellular TPI activity in PpIRD$^+$ strain as compared to control (empty vector; EV). As pPIC9 have alpha secretion tag before IRD sequence thus, TPI activity was higher in extracellular as compared to the intracellular environment (Supplementary Fig. 1). Cell lysate of IRD-9 expressing strain showed maximum protease inhibitor activity as compared to IRD-7 and -12 expressing *P. pastoris* strains. Domash et al. has discussed about regulation of proteases expression through the modulation of protease inhibitors expression under stress conditions [25]. Similarly, PpIRD$^+$ strains showed enhancement of PIs activity on stress stimuli, which might inhibit the cellular proteases which are involved in protein degradation under stress.

3.3. Inhibition of serine (SP) and cysteine protease (CP) activities in PpIRD$^+$ stains

In stress conditions, the gene expression and hence, the activities of SP and CP increased rapidly in EV cells (Fig. 2C and D). Overexpression of subtilisin-like protease 3 and calpain-like protease transcripts was observed in PpIRD$^+$ strains (Fig. 2C). Induction of protease under stress condition could help the cells in clearance of misfolded protein load and thus to overcome the lethal effect of stress. However, at activity there is no significant difference in SP and CP activities was observed in stress inducing agent treated cells as compared to control cells. EV strains showed elevated level of SP and CP activity, while PpIRD$^+$ cells showed optimal level of protease. The inhibition of SP and CP activity might be due to elevated expression and activity level of PIs i.e. IRDs under stress (Fig. 2D). Although IRDs are SP inhibitor but, the cross reactivity of IRDs toward inhibition of CP might have influence on regulation of CP expression and activity in PpIRD$^+$ strains.

According to various reports, increase in protease activity under stress conditions could be related to physiological processes such as senescence and programmed cell death [26]. An increase in proteolytic activity under stress conditions can lead to a disturbance in the balance between protein synthesis and decay, which might cause premature senescence and cell death [27]. To prevent this, valid mechanisms are required to control the proteolytic activity at the transcriptional, translational, and post-translational levels. Solomon et al. has showed that expression of cystatin, an endogenous cysteine protease inhibitor gene, can cause inhibition of the programmed cell death related cysteine proteases, which are induced by biotic or oxidative stress [28]. Quian et al. evidenced that expression of oryzacystatin-I inhibited cysteine proteases participate in the control of growth and stress tolerance through effects on strigolactones [29]. Thus, these observations suggest that ectopic expression of PIs might lead to inhibition of proteases participating in the growth control and thus provides multiple stress tolerance in yeast cells.

3.4. PpIRD$^+$ exhibited reduced metacaspase activity under stress conditions

Level of metacaspase activity is an indicator of survival status of the cell. Enhanced transcriptional and translation level of metacaspase was observed in the apoptosis or senescence cells [30,31]. The metacaspase mRNA level was markedly elevated in EV and PpIRD$^+$ strains. The metacaspase expression level appeared to stimulate by stress-inducing agents thus conferred to have major role in senescence and cell death (Fig. 3A). We used metacaspase specific substrate to access the level of activity in EV and PpIRD$^+$ under various stress conditions. In EV cells, higher metacaspase activity leads to activation of apoptosis pathway and that may result in reduced growth under stress condition. While, PpIRD$^+$ stains showed 50–60% reduction in metacaspase activity as compared to EV strain (Fig. 3B). This indicated that under stress condition the metacaspase activity was inhibited by the induced expression of IRDs and thus results in delayed senescence in PpIRD$^+$ strains.

It is known that expression of yeast metacaspase is regulated by apoptosis stimuli like oxidative stress, low pH, and high salinity stress. Numerous reports highlight the interlinking in ectopic expression of PI, its effect on cysteine proteases and abiotic stress tolerance [28,29]. Yeast metacaspase have arginine/lysine-specific endo-peptidase activity thus, various synthetic substrates containing an arginine/lysine residue at the P1 position could be cleaved by the metacaspase examined in this study [19]. The reactive loop of expressed IRD contains arginine/lysine residue at the P1 position, thus enhancing the specificity of IRDs toward Yca1. These data, in turn, suggest that Yca1 interact with IRDs in substrate specific manner, and this result in competitive inhibition of its activity.

3.5. Reactive loop of IRDs forms multiple contacts with the active site of Yca1

Comprehensive view of IRD-metacaspase complex showed that IRD binds at the active site region of metacaspase and block the access of the substrate to the active site (Fig. 3C–E). Close view of this interaction depicted the formation of a hydrogen bond in the reactive loop of IRDs (CPXXC) and the active site of metacaspase (His220 and Cys276). In IRD-7 and -9, carbonyl oxygen atoms of Asn40 of IRD reactive loop forms hydrogen bond with metacaspase active site residue i.e. His220 (Fig. 3C and D). In case of IRD-12 and metacaspase complex, there is absence of polar contact in interacting regions (Fig. 3E). Binding energy of these complexes was estimated using PDBePISA server [24]. IRD-9 showed stronger binding with metacaspase with energy of −32 kcal/mol as compared to IRD-7 (−18 kcal/mol) and IRD-12 (−19 kcal/mol). Results derived from energy calculations are well accordance with our earlier reports. This indicates that interaction of IRD with metacaspase is similar to IRD-trypsin interaction.

Substrates with arginine/lysine residue at the P1 position can act as superior and specific substrates for metacaspase [31]. The S1–S3 position of binding pocket of Yca1 constitutes negative environment and showed enhanced binding affinity toward positively charged amino acids. The reactive loop of IRDs contains arginine/lysine (positively charged) residue at the P1 position, thus increasing the reactivity and specificity of IRDs toward Yca1. Yca1-IRDs complex showed enzyme-substrate like binding with relatively higher binding energy. Docking studies have provided supportive indication of the interaction between two heterogeneous partners, Yca1 and IRDs. From these results, we can suggest that strong interaction between the reactive loop of IRD with the active site of metacaspase might be the key reason behind delayed apoptosis and multiple tolerance in yeast cells.

3.6. Metacaspase knockout S. cerevisiae strain showed PpIRD$^+$ like growth characteristic

To examine the effect of various stress on the growth of ΔYca1 cells, we measured the time-dependent changes in optical density (OD_{600}) of culture subjected to various stresses. As showed in Fig. 4A, growth of ΔYca1 strain was delayed during the first 2 h of exposure to stress inducing agents, but it starts to increase after 3 h, the density of the ΔYca1 culture was higher and almost alike of those observed in the absence of stress. Growth pattern of the ΔYca1 strain was comparable like PpIRD$^+$ strains, specifically more corroborative with growth of IRD-9 expressing yeast cell under stress. Assessment of various protease activities showed that SP activity was increased after stress treatment, while CP activity was marginally increased. Metacaspase activity was almost absent in all different conditions (Fig. 4B).

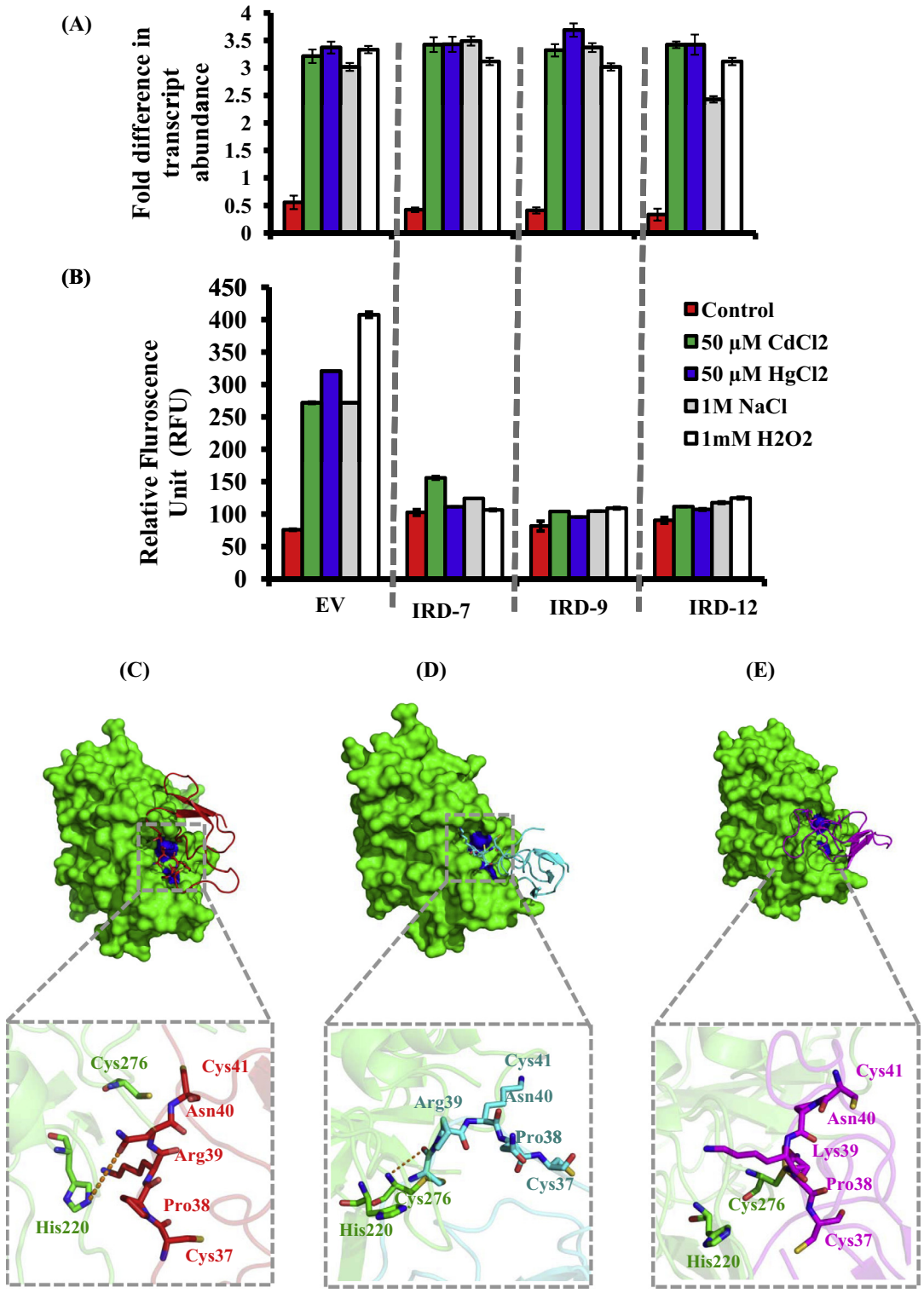

Fig. 3. (A) Metacaspase gene expression and (B) intracellular metacaspase activity of CanPI expressing yeast cells under stress inducing agent like 50 µM $CdCl_2$, 50 µM $HgCl_2$, 1 M NaCl and 1 mM H_2O_2. Significant inhibition of metacaspase activity was observed in PpIRD$^+$, which was indicative of delayed apoptosis and senescence. Values are the means of three measurements with standard error. Interaction between the active site of yeast metacaspase (Yca1) and reactive loop of IRD was evaluated by docking analysis. Reactive loop of (C) IRD-7, (D) IRD-9 and (E) IRD-12, showed close interaction with metacaspase active site residues (His220 and Cys276). In case of IRD-7 and IRD-9, Arg39 of reactive loop forms hydrogen bond with His220 and Cys276, respectively.

Growth and metacaspase activity analysis of this knockout indicate that ΔYca1 strain exhibit similar growth characteristics like PpIRD$^+$ strains and suggest that metacaspase activity of yeast cell under stress might be inhibited by IRD expression and thus exhibited phenotype like ΔYca1 strain [30].

3.7. Inhibition of metacaspase leads to maintenance of GAPDH activity in PpIRD$^+$ strains

Assessment of metacaspase inhibition was performed indirectly by estimation of *in vivo* GAPDH activity, as it is the primary target

of metacaspase during apoptosis process. It was observed that ΔYca1 and PpIRD$^+$ strains showed maintenance of GAPDH activity under various stresses, while there is considerable reduction in this activity in the case of EV strains. On stress response, in case of EV strain 50% of GAPDH activity was reduced, while it is kept at a normal level in PpIRD$^+$ strains (Fig. 4C). Silva et al. showed that in yeast cells GAPDH is the primary target of metacaspase [24]. Increased activity of metacaspase under stress enhances GAPDH degradation and thus it will inhibit the basic energy metabolism of the cell [16]. Inhibition of metacaspase activity by PIs expression causes maintenance of GAPDH level; this in turn helps in survival and growth maintenance of the yeast cells under stress.

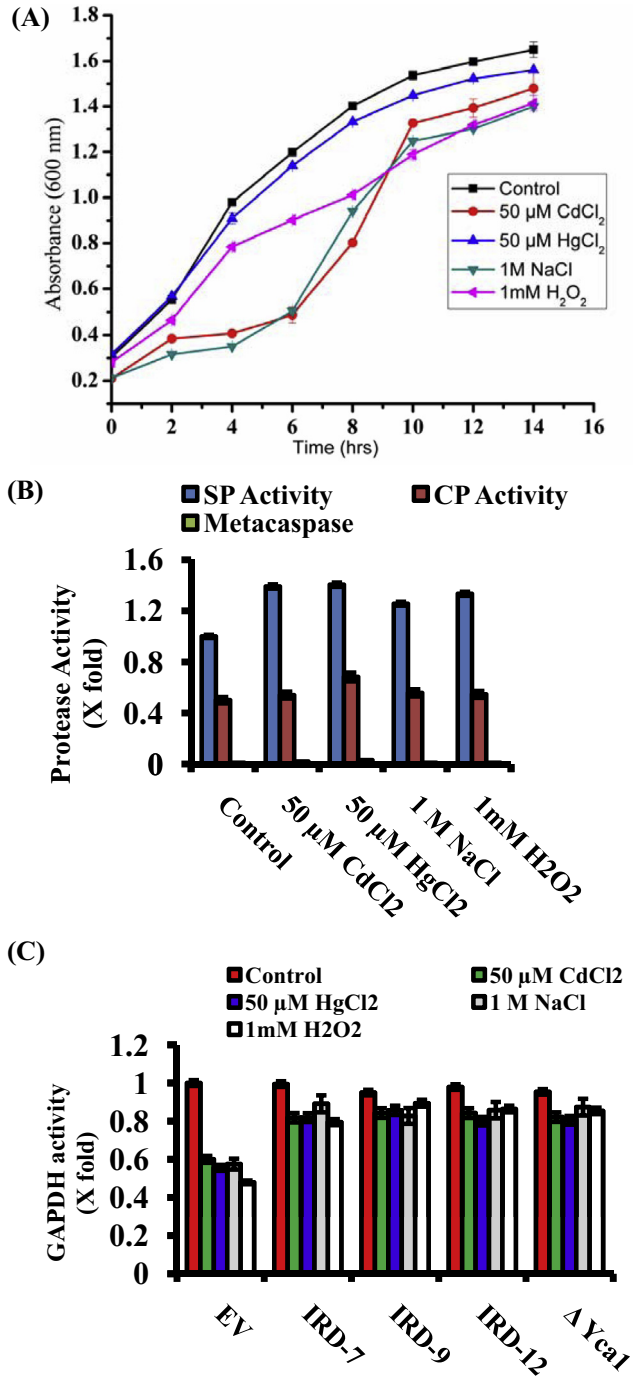

Fig. 4. (A) Growth of metacaspase knockout yeast cell (ΔYca1) in the YPD medium supplemented with 50 µM $CdCl_2$, 50 µM $HgCl_2$, 1 M NaCl and 1 mM H_2O_2. (B) Intracellular serine protease and metacaspase activity in ΔYca1 cell under stress. (C) GAPDH activity of EV, PpIRD$^+$ (IRD-7, -9 and -12) and ΔYca1 strain under multiple stresses. Values are the means of three measurements with standard error.

4. Conclusion

Expression of IRDs in yeast cells exhibited multiple stress tolerance against various abiotic stresses. This investigation provided insight into the role of inhibitor expression on the viability and survival of yeast cells under multiple stresses. Exposure of PpIRD$^+$ to stress inducing agents exhibited differential molecular response of TPIs and intracellular protease at transcriptional and translational level. Inhibition assay, docking and knockout study has showed cross-reactivity of expressed IRDs (serine PIs) towards yeast metacaspase. Inhibition of metacaspase might be the key reason behind the stress tolerance in PpIRD$^+$ strains. Assessment of intracellular activity showed that inhibition of metacaspase activity causes the maintenance of cell's energy metabolism and thus its survival. This phenomenon of multiple stress tolerance via delayed senescence is mediated through maintenance of cells protein turnover and energy metabolism due to inhibition of cellular metacaspase and proteases.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.08.075.

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