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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

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