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The maize (*Zea mays*) b-32 protein shows RIP activity in yeast cells

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Abstract

Ribosome-inactivating proteins (RIPs) are either single-chain (type 1) or two-chain (type 2) toxins. They are toxic to eukaryotic cells by cleaving a N-glycosidic bond in an extremely conserved loop located in the 28S RNA. This releases a specific adenine and inactivates the ribosome, ultimately inhibiting protein synthesis. Plant RIPs have been intensely investigated because of their projected antiviral, antifungal and insecticidal activity. RIPs also have biomedical applications as the toxic mojety of immunotoxins. Given their biotechnological potentials, it is strategic to develop platforms to rapidly evaluate the activity of recombinant RIPs. This investigation fills this need in that it reports that the yeast *Saccharomyces cerevisiae* is a model system to assess the impact of genetic manipulations on the functionality of a recombinant *Zea mays* RIP named b-32.

Keywords: b-32, Ribosome Inactivating Protein, *Saccharomyces cerevisiae*, maize endosperm

Introduction

The network of defence mechanisms deployed by plants in response to pathogens and herbivores is a multi-layered and highly integrated innate immune system (reviewed in Gomez-Casati et al, 2015; Pechanova and Pechan, 2015 and references therein). Besides strengthening the cell wall, plants respond to attack through the activation of several pathways, ultimately leading to the production of hormones, macromolecules and a plethora of secondary metabolites, many of which are grouped under the umbrella name of phytoalexins (Pusztahelyi et al, 2015). Proteins with activities against pathogens and herbivores are an integral part of the plant defence mechanisms (Carbonell and Carrington, 2015; Dang and Van Damme, 2015; Schuman and Baldwin, 2015). Not differently from mammalian innate immune system, plants sense their encounters with microorganisms upon perception of conserved microbial structures and damage-associated patterns using cell-surface and intracellular immune receptors. Accordingly, hydrolytic enzymes such as chitinases and 1-3 β-glucanases play a pivotal role because, besides degrading pathogens' cell wall, also cause the release of fragments which boost the defence response (Lannoo and Van Damme, 2014; Miedes et al, 2014). Another class of proteins, collectively known as ribosome inactivating proteins (RIP) has been shown to be involved in the inhibition of pathogen infection. RIPs are severe inhibitors of eukaryotic protein synthesis by cleaving a specific N-glycosidic bond in a highly-conserved loop of the 28S rRNA so that the elongation factor-2 cannot properly interact with the ribosome thus leading to disruption of protein synthesis (reviewed in Stirpe, 2013). RIPs are produced by a number of plants (Schrot et al, 2015) and are usually grouped in two major classes. Type 1 RIPs consist of a single catalytic domain with an approximate MW of 30-34 KDa. Type 2 RIPs are heterodimers composed by two subunits called A and B, thus paralleling the A/B model of bacterial toxins (Olsnes et al, 1990), which are joined by a disulphide bond. The A chain is homologous in aminoacid composition and enzymatic activity to type 1 RIPs, whereas the B chain is responsible for the docking and internalization of the holoprotein into the target cells. The presence of the B chain increases type 2 RIP toxicity to eukaryotic cells up to 10^6 times compared to type 1 RIP. Cereal grains have been reported to contain large amounts of RIPs (Coleman and Roberts, 1982; Motto and Lupotto, 2004; Balconi et al, 2010). In agreement with the proposed role in plant defence a RIP isolated from dehusked barley grain was shown to inhibit the growth of fungi (Roberts and Selitrennikoff, 1986) and its ectopic expression in transgenic tobacco proved effective in protection against fungal pathogens (Logemann et al, 1992). A subsequent elegant investigation showed that barley RIP anti-fungal activity is enhanced by 1-3 β-glucanase and chitinase either alone or in combination (Jach et al, 1995). A novel RIP protein was discovered in maize endosperm which is a storage tissue whose main function is to accumulate and store nitrogen and carbon utilized by the seedling during the first stages of germination. The synthesis and deposition of storage proteins is under genetic

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and physiological control (Balconi et al, 1993; Motto et al, 1996). The transcriptional activator *Opaque-2* (*O2*), which belongs to the leucine-zipper family, plays a pivotal role in orchestrating the carbon/nitrogen metabolism in maize endosperm (Soave et al, 1981; Hartings et al, 1989; Lohmer et al, 1991). *O2*, besides regulating its own transcription and that of 22 KDa storage protein zeins, also controls a very abundant cytosolic albumin with a molecular weight of approximately 32 KDa, hence named b-32. The gene encoding for b-32 has been cloned (Di Fonzo et al, 1988). The primary aminoacid sequence of the b-32 protein bore resemblance to the barley RIP and, accordingly, inhibited eukaryotic protein synthesis in vitro by using a cell-free rabbit reticulocytes system (Maddaloni et al, 1991). Molecular studies clarified that b-32 actually is a small gene family (Hartings et al, 1990) and that is synthesized and stored as a proenzyme. During germination the proenzyme is processed to produce two tightly-associated peptides with a MW of 16.5 and 8.5 KDa. This post-translational modification lead to a dramatic shift in the isoelectric point, from 6.5 to >9, and more importantly to a 10^4-fold increase in the inhibition of rabbit ribosomes (Walsh et al, 1991). Transgenic tobacco, expressing maize b-32, is remarkably tolerant to infection by *Rhizoctonia solani* (Maddaloni et al, 1997). Further research showed that maize b-32 protein conferred protection against fungal infection, reducing Fusarium head blight (FHB) symptoms (Balconi et al, 2007). The antifungal activity of maize RIP b-32, was additionally evaluated in transgenic maize expressing the b-32 coding sequence under the control of a constitutive 35S-CaMV promoter (Lanzanova et al, 2009). In the latter case, maize leaves were challenged with *Fusarium verticilliodes*, whose progression of infection was reduced in the presence of b-32. Interestingly, the inihibition of Fusarium growth was inversely related to b-32 concentration in leaf tissues (Lanzanova et al, 2009). Taken together these data confirm that maize b-32 is an effective antifungal protein naturally produced in maize endosperm. Here it is shown that b-32 acts as a true RIP as determined by the specific glycosidic activity on eukaryotic ribosomes, using the yeast *Saccharomyces cerevisiae* as a model system. Considering the wide array of potential applications that RIPs have in crop protection as well as in the biomedical field (Stirpe, 2013; Gilabert-Oriol et al, 2014) this investigation also provides a simple framework to test the activity of recombinant RIPs i*n vivo*, before they are deployed for more complex and expensive experimental designs.

Materials and Methods

Yeast strains and growth conditions

The *Saccharomyces cerevisiae* strain X4004-3A (*MATa*, *lys5*, *ura3*, *met2*, *trp1*) was used in this study. Cells were grown in batch by shaking at 30°C in synthetic minimal medium containing 0.67% Yeast Nitrogen Base (YNB) (Difco) without aminoacids. Required micronutrients and the appropriate carbon source were added at 50 mg l^{-1} and 2%, respectively. YEPD complete medium (yeast extract 1%, peptone 2% and glucose 2%) was used to grow cell before transformation. Induction was achieved by adding 2% galactose, from a filter-sterilized 50% stock solution, to yeast cells exponentially growing in YNB-raffinose. Growth was monitored by counting the number of cells with a Coulter Counter ZBI (Coulter Electronics) as previously described (Martegani et al, 1984). The fraction of budded cells was determined by microscope counting as described (Martegani et al, 1984). Yeast cells were transformed using the lithium chloride method (Ito et al,1983). DNA manipulations were done in *Escherichia coli* HB101 grown in LB medium. When required ampicillin was added at 50μg ml^{-1}.

Plasmid construction and recombinant DNA techniques

A 1 Kb EcoRI DNA fragment containing the entire b-32 cDNA was blunt-ended by Klenow DNA polymerase and cloned into the SmaI site of the yeast expression vector pEMBLyex4 (Baldari and Cesareni, 1985). The resulting construct, named pEMBLy-b32, expressed maize b-32 under the control of galactose-inducible, hybrid promoter UASgal/CYC1 (Guarente et al, 1982). DNA manipulation followed standard procedures (Sambrook et al, 1989). Enzymed were purchased form Boehringer (Mannheim, Germany) and used according to manufacturer's instructions.

Western blot analysis

Crude extracts were obtained by vortexing the yeast cells with glass beads in sodium dodecyl sulfate (SDS) electrophoresis sample buffer (62.5 mM Tris, 5% sodium dodecyl sulfate, 5% β-mercaptoethanol, 5% glycerol). Protein extracts were separated by 10% SDS-polyacrylammide gel electrophoresis and electroblotted onto nitrocellulose (Towbin, 1987). Nitrocellulose filter were then probed with anti b-32 rabbit polyclonal sera (Maddaloni et al, 1997) followed by goat anti rabbit antibodies coupled to horseradish peroxidase. Reactive bands were detected by adding a chemiluminescent substrate (Amersham).

Protein synthesis inhibition assay

The rate of protein synthesis was determined by pulse labelling a 2 ml aliquot of yeast culture with (carboxy-^{14}C) leucine at a final concentration of 0.15 mM (specific activity 5 Ci Mol^{-1}). After 5 and 10 min of pulse, 0.5 ml aliquots were withdrawn in duplicate and the radioactivity incorporated in the hot trichloroacetic precipitable material was determined (Martegani et al,1984). In parallel 0.5 ml aliquots from the culture were quickly filtered and washed with ice to determine the total leucine uptake. The rate of leucine incorporation was calculated as Δcpm between 5 and 10 min and correlated with the number of cells (cpm $cell^{-1}$).

Aniline reaction

RIP proteins exert their activity on whole ribosomes however, given our experimental configuration, purification of ribosomes was not necessary. Total yeast RNA was prepared as described (Elion and Warner, 1984). Aliquots of total RNA (10 μg) were dissolved in 20 μl of 1 M aniline/acetate buffer pH 4.5 and incubated in the dark at 60°C for 2 min (Endo et al, 1988). The reactions were stopped by precipitation with 0.3 M potassium acetate and 70% ethanol. The RNA samples were then resuspended in denaturing running buffer and separated on formaldehyde-agarose gel electrophoresis. Gels were stained with ethidium bromide (0.5 μg ml^{-1} in water) and photographed under an UV transilluminator.

Results and Discussion

Expression of maize b-32 protein in S. cerevisiae

The constitutive expression of the b-32 protein is likely to be toxic to yeast however the mere impossibility to retrieve viable yeast cells expressing a functional *b-32* is only a circumstantial evidence of its inhibitory activity. In fact, a strongly diminished or absent number of transformed cells can be ascribed to a number of reasons. Moreover, the eventual retrieval of transformants could be caused by loss of the *b-32* gene or its rearrangement. Therefore it was decided to express the b-32 cDNA under an inducible promoter. The cDNA encoding for the b-32 (Di Fonzo et al, 1988) was cloned into the pEMByex4 expression vector under the control of the strong hybrid promoter UASgal/CYC1 which is inducible by galactose. The resulting construct named pMBLy-b32 was introduced into the *S. cerevisiae* X4004-3A selecting for uracil. The putatively transformed cells grow normally in minimal YNB medium containing alternatively glucose or raffinose as a carbon source but fail to grow when inoculated in minimal YNB medium containing galactose (not shown), suggesting that b-32 may have toxic effects as expected. Cells were inoculated in YNB-raffinose and then induced with galactose. The use of YNB-raffinose instead of YNB-glucose was necessary to bypass the repressing effects of glucose and its catabolites on the galactose-inducible promoter, thus leading to a cleaner and faster induction of *b-32* expression. Upon induction with galactose a strong inhibition of cell growth was detected in yeast transformed with pEMBLy-b32. Inhibition of growth was evident already at 2 hours after induction and it became complete after 6 hrs (Figure 1). Conversely, yeast cells, transformed with pEMBLyex4 control plasmid, continued to grow exponentially. Western blot analysis showed that, upon induction with galactose, pEMBLy-b32 yeast cells produced a protein that was strongly immunoreactive to a rabbit anti b-32 polyclonal serum (Figure 2C). The same protein was absent in yeast cells, transformed with pEMBLyex4 control plasmid both in

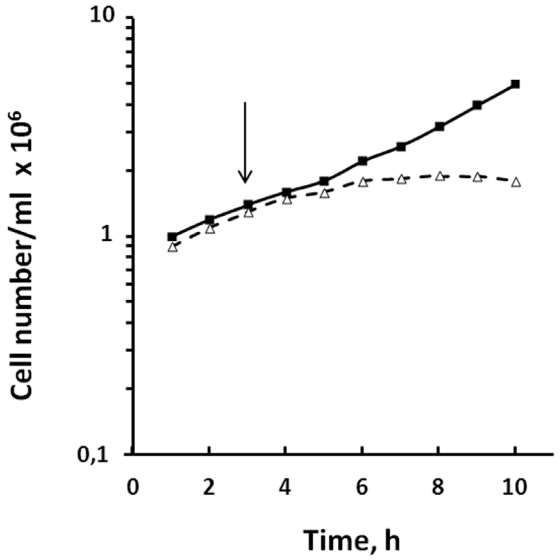

Figure 1 - Growth kinetics of *S. cerevisiae* expressing b-32. Yeast cells in exponential growth phase cultivated in YNB-raffinose were induced by adding galactose to final concentration 1%. Induction time is marked with an arrow.
(■) X4004[pEMBLyex4] control; (Δ) X4004[pEMBLy-b32]

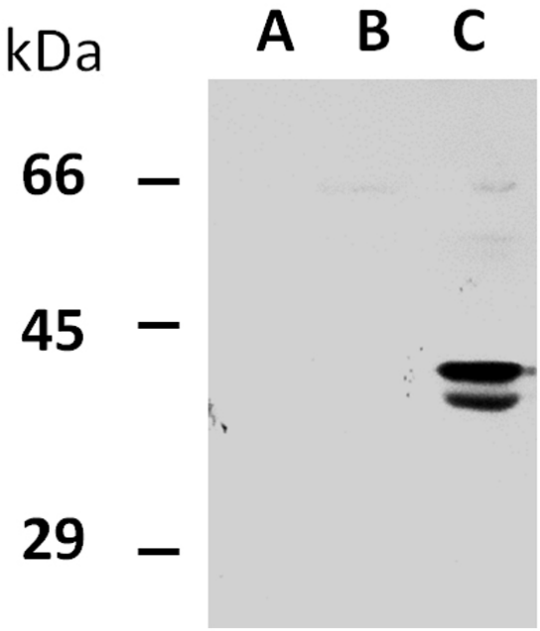

Figure 2 - Detection of maize b-32 protein in transformed *S. cerevisiae*. Total yeast protein (50mg) were loaded and separated on a10% SDS-polyacrylamide gel, blotted and decorated with in-house produced rabbit anti b-32 polyclonal serum, hybridized with HRP-conjugated goat-to-rabbit anti IgG and finally reacted with a chemiluminescence substrate as detailed in Materials and Methods. Lane A: X4004[pEMBLyex4] control grown in YNB-raffinose at 3 hrs after induction with galactose. Lane B and lane C : X4004[pEMBLy-b32] in exponential growth phase in YNB-raffinose at the time of induction and 3 hrs after induction with galactose, respectively.

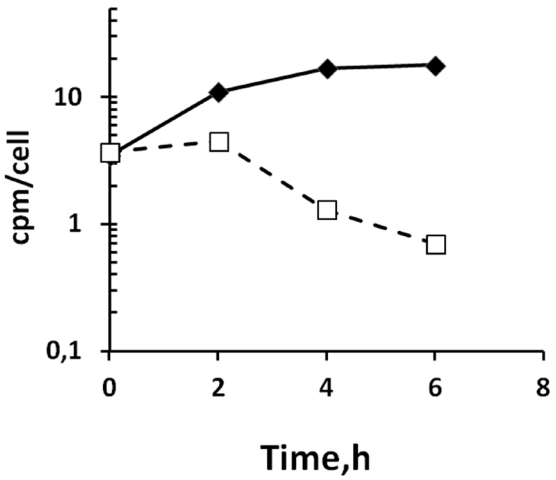

Figure 3 - Rate of ^{14}C-leucine incorporation. Yeast cells in exponential growth in YNB-raffinose were induced by addition of 1% galactose at time zero. Aliquots of cultures were pulse-labelled with ^{14}C-leucine and the hot-TCA precipitable radioactivity was measured after 5 minutes and again after 10 minutes. The rate of incorporation was calculated as counts per minute (cpm) per cell.
(♦) X4004[pEMBLyex4] control; (□) X4004[pEMBLy-b32]

YNB-raffinose (Figure 2A) and after 3 hours of induction in YNB-galactose (Figure 2B). Upon induction with YNB-galactose of pEMBLy-b32 yeast cells two bands are clearly detected at 32 and 34 KDa (Figure 2C). This feature has been consistently observed by our group and others (Di Fonzo et al, 1988; Maddaloni et al, 1991; Walsh et al, 1991). At the present it is not known if this is due to the co-existence of two molecular species caused by biochemical modifications of a fraction of molecules, or partial cleavage of the holoprotein. The growth inhibition related with the expression of maize b-32 protein is also associated to a strong inhibition of protein synthesis as monitored by incorporation of ^{14}C-labelled leucine in the hot-TCA precipitable material (Figure 3). The inhibition is not associated to a restriction of leucine uptake (data not shown) and therefore it likely reflects a damage to the protein synthesis machinery. Despite being detected shortly after induction, the inhibition is not complete after 6 hours of induction and this may be explained by *de-novo* synthesis of the damaged components or to some mechanisms of sheltering inside the cytoplasm.

The maize b-32 expressed in yeast is a true RIP

Since the maize b-32 protein showed RIP activity *in vitro* (Maddaloni et al, 1991) it was hypothesized that the inhibition of the protein synthesis observed in induced pEMBLy-b32 yeast cells was caused by the b-32 RIP activity *in vivo*. In order to ascertain this hypothesis yeast ribosomes were analysed. The specific catalytic activity of RIP proteins causes a covalent modification of the large 28S ribosomal RNA. Specifically, a single N-glycosidic bond located in an RNA loop essential for translation (A4324 in rat liver ribosomes) is cleaved. The modification of the yeast rRNA in the presence of the recombinant b- was detected by reacting the purified ribosomal RNA with aniline as described in **Materials and Methods**. Results are shown in (Figure 4). The rRNA of the host yeast strain X4004, containing the naked vector pEMBLyex4, was not modified in the presence of raffinose or galactose as carbon source (Figure 4 lanes A, B). As expected the presence of the pEMBL-b32 alone, in absence of the inducer galactose, did not cause any detectable modification of the rRNA (Figure 4 lanes C, E). Also rRNA from X4004 pEMBL-b32 induced with galactose but unreacted with aniline appears normal (Figure 4 lane D). Conversely, the rRNA of X4004 pEMBL-b32, induced in the presence of galactose and reacted with aniline, showed the expected modification caused by a RIP protein (Figure 4 lane F). The aniline test yielded two fragments of approximately 3,000 and 400 nucleotides which originated from the cleavage of the 25S RNA as expected. In fact yeast 25S RNA is 3,393 nucleotides long and the Adenine3024 corresponds to the Adenine4324 of the rat 28S rRNA (Gutell and Fox, 1988) both being the specific target for canonical RIP proteins. Data also show that a fraction of 25S RNA in yeast cells is not cleaved, suggesting that b-32 in the single chain form has a reduced activity, which is in agreement with data available in the literature (Walsh et al, 1991).

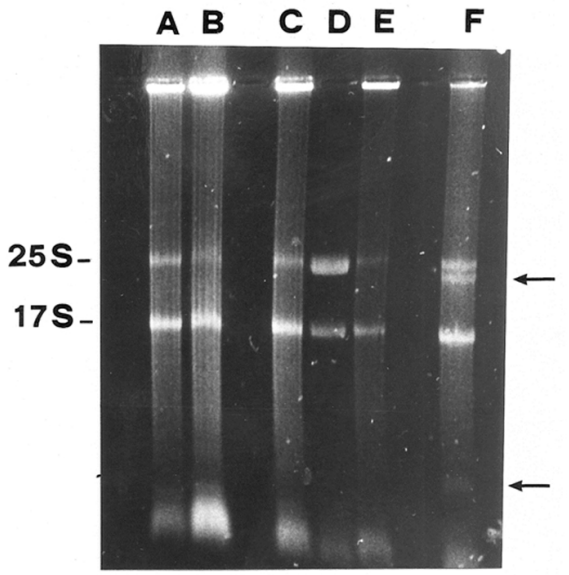

Figure 4 - RIP activity of maize b-32 expressed in S. cerevisiae. 10 μg of total yeast RNA were reacted with aniline acetate and visualized as detailed in **Materials and Methods**. Lane A: X4004[pEMBLyex4] growing in YNB-raffinose. Lane B: X4004[pEMBLyex4] at 4 hrs induction with galactose. Lane C: X4004[pEMBLy-b32] in YNB raffinose. Lane D: unreacted total yeast RNA. Lane E: X4004[pEMBLy-b32] growing in YNB-glucose. Lane F: X4004[pEMBLy-b32] at 4 hrs induction with galactose. Arrows indicate the cleavage products.

Plants accumulate proteins that are either toxic or inhibitory against pathogens, including Ribosome Inactivating Proteins (RIPs), N-glycosidases that depurinate the universally conserved α-sarcin loop of large rRNAs. Cereal RIPs share a high similarity with all the other RIPs, retaining, however, characteristic features forming a distinct class which diversified significantly during evolution. They appear to be involved in several different physiological roles, such as defence against pathogens and/or involved in regulatory and developmental processes. RIPs from cereals generally have low activity against plant ribosomes. As detailed in the introduction, several lines of evidence point at the role of b-32 in the protection of maize endosperm against fungal attacks. Intriguingly, the maize endosperm mutants *opaque-2* and *opaque-6*, lacking b-32, are more susceptible than the wild type to ear rotting diseases (summarized in Lanzanova et al, 2011). Because of their peculiar biological activities towards animals and human cells, RIPs have received substantial attention in biological and biomedical applied research. In fact, RIPs have been primary candidates for the toxic moiety of immunotherapeutics (Spooner and Lord, 1990; Pastan and Fitzgerald, 1991; Olsnes and Pihl, 1982; Pincus, 1996; Stirpe, 3013). RIPs have also received much attention as vaccines because some of them can have a dual-use in biowarfare. (Maddaloni et al, 2004; Pincus et al, 2014 and references therein). In this respect, however it is of paramount relevance to remark that maize b-32 has been ingested in massive amount, in raw and cooked form, by humans and lifestock for centuries without any negative effects. In this respects it makes an interesting candidate as an agent for unconventional crop protection (Maddaloni et al, 1997; Balconi et al, 2007). This investigation provides a convenient platform to assess the impact of genetic manipulations on the functionality of recombinant RIPs before committing to more complex and expensive experiments.

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