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The b-32 ribosome inactivating protein from maize influences fumonisin accumulation in *in-vitro* bioassays

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Abstract

Fungi of the genus Fusarium are common plant pathogens mainly associated with cereal crops. In particular, *Fusarium verticillioides* Sacc. is the most common toxigenic fungus in maize worldwide, causing root, stalk, and ear rot. Fusarium spp. can produce a wide range of secondary metabolites, some of which can unfavourably affect human and animal health. Owing to the potential risks of fumonisins *F. verticillioides* secondary metabolites, new regulations for the allowable mycotoxin limit in food and feed have been put in place by most agencies worldwide. Plants act on the attack of pathogenic fungi through a complex network of active responses such as the production of proteins toxic or inhibitory to pathogens such as RIP (Ribosome-Inactivating-Protein). The RIP present in maize endosperm (termed b-32) has been widely investigated. Similarly to other RIPs, is accumulated in the seed as an inactive pro-RIP precursor, which is converted into an active form by proteolytic processing. To understand the relationships between structure and substrate specificity of the maize b-32 RIP protein, a series of recombinant b-32 sequences, by selective deleting of different domains (RIP b-32, RIP ΔN, RIP ΔC and RIP-ΔC (Ala), were prepared. Recombinant sequences were expressed in *Escherichia coli* to obtain high levels of recombinant proteins, which were subsequently tested for their potential ability to reduce both the colonization of *F. verticillioides* and fumonisin accumulation.

KeyWords *Antifungal protein, Fusarium verticillioides, crop protection, Zea mays, mycotoxins*

Introduction

Plants are exposed to a large number of pathogenic fungi, against which they have developed a variety of defence mechanisms, including the synthesis of proteins and peptides with antifungal activity (Theis and Stahl., 2004; Lucca et al., 2005). These proteins appear to be involved in either constitutive or induced resistance to fungal attack. The identification of such proteins could lead to the isolation of genes which could be employed in the development of transgenic plants with increased disease resistance as discussed by Ferreira et al., 2007. An example of plant potential antifungal compounds with a possible use in agriculture and food processing are Ribosome Inactivating Proteins (RIPs). These proteins act as N-glycosidases and irreversibly block protein synthesis by modifying the large rRNA unit so that it is not longer recognized by translational elongation factors (Hartley et al., 1996).

RIPs are widely distributed throughout the plant kingdom and are active against ribosomes from different species. RIPs from cereals have high similarity to all other RIPs, retaining however characteristic features which group them into a distinct class which diversified significantly during evolution. Three classes of RIPs have been identified (Balconi et al., 2010) and, although the enzymatic activities of RIPs have been shown *in vitro*, their role in plant defence is less clearly defined. In maize, pro-RIP, classified as a type3 RIP (Nielsen and Boston, 2001), is present in at least two forms encoded by non allelic genes, one expressed in the endosperm (Di Fonzo et al., 1988) and the other in leaf tissue (Bass et al., 1995).

The RIP present in maize endosperm has been widely investigated (Motto and Lupotto, 2004; Balconi et al., 2010); this protein is a cytosolic albumin with a molecular weight of 32 KDa (termed b-32) and is synthesized in temporal and quantitative coordination with the deposition of storage proteins. Similarly to other RIPs, maize b-32 is accumulated in the seed as an inactive pro-RIP precursor, which is converted into an active form by proteolytic processing. This involves the removal of 16 amino acids from the N-terminus (residues 1-16 of pro-RIP), several amino acids from the C-terminus (residues 295-301), and 25 amino acids from the centre of the RIP polypeptide (residues 162-186) (Hey et al., 1995). In past years, maize b-32 was shown i) to enzymatically

inactive ribosomes and inhibit protein synthesis *in vitro* (Maddaloni et al., 1991); ii) to inhibit the growth of *Rhizoctonia solani* mycelia in an *in vitro* bioassay and plant assays (Maddaloni et al., 1997). We have also shown that maize b-32 was effective in wheat transgenic lines as an antifungal protein by reducing *Fusarium culmorum* head blight (FHB) (Balconi et al., 2007) and in maize transgenic lines reducing *Fusarium verticillioides* attack symptoms in leaf tissues assays (Lanzanova et al., 2009). The role of maize b-32 in defence against pathogens was also suggested by an increased susceptibility of *opaque-2 (o2)* mutant kernels (in which the level of b-32 is decreased), to fungal attack (Loesch et al., 1976) and insect feeding. In addition, results obtained with inbred lines and their isogenic *o2*-mutants, tested in field experiments with artificial inoculation assays (KIA Kernel Inoculation Assay; SCIA Silk Channel Inoculation Assay) on adult plants, showed that *o2 mutants* were significantly more susceptible to *F. verticillioides* than the wild-type inbred lines (Balconi et al., 2005; Torri et al., 2011).

To understand the relationships between structure and substrate specificity of the maize b-32 RIP protein, a series of recombinant b-32 sequences were prepared by selective deletion of different domains. Recombinant sequences were intracellularly expressed in *Escherichia coli* to obtain high levels of recombinant proteins, which were subsequently tested for their potential ability to reduce both the colonization of *F. verticillioides* and fumonisin accumulation.

Materials and Methods

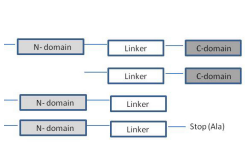
Engineered plasmids

A plasmid carrying the entire cDNA sequence of the maize *b-32* gene was used as a source of recombinant sequences obtained with the of the Flexi Vector System (C8640 Promega). A tool for primer design (Figure 1a)

5' Primer	
RIP ATG	5'-AAGAGCGATCGCCATGGCCGAGACAAATCCAGAGTTGAGT-3'
RIP ΔN	5'-CCCGGCGATCGCCAAAAAAATAGTACCAAAGTTCACTG-3'
RIP ATG-2	5'-GTCGGCGATCGCCATGGCCGAGACAAATCCAGAGTTGAGTGATCTT-3'
RIP ATG-3	5'- AGGAGCGATCGCCATGGCCGAGACAAATCCAGAG-3'
3' Primers	
PIR STOP	5'-TGGTGTTTAAACGGCCTCGTCATCGTCGTTGTCAG-3'
PIR STOP ΔN	5'-TTGTGTTTAAACGGCCTCGTCATCGTCGTTGT-3'
PIR Linker	5'- GGGGGTTTAAACCGCCGCCGCCAGTTCAGCGG-3'
PIR ΔC (Ala)	5'-CGCGGTTTAAACAGTAGTTTGATTCTTAACGAGCGCA-3'

Figure 1a-PCR primers used for engineering the maize b-32 sequence for bacterial expression. Sequences in red indicate the SgfI restriction site while the blue sequences indicate the PmeI restriction site.

is available on the Promega web site. The description of different b-32 sequences obtained is reported in Figure 1b



Plasmids	Primer combination	PCR (bp)	Protein (KDa)
RIP b-32	RIP ATG-PIR STOP	937	33
RIP ΔN	RIP ΔN-PIR STOPΔN	889	32
RIP ΔC	RIP ATG 2-PIR Linker	580	20
RIP-ΔC (Ala)	RIP ATG 3- PIR ΔC (Ala)	883	31

Figure 1b-Description of different b-32 plasmids. RIP b-32 contains the entire sequence of the b-32 gene (pro-RIP). RIP ΔN is deleted for the N-terminal leader sequence (residues 1-16 of pro-RIP). RIP ΔC contains a deletion of the sequence downstream the linker region (186-305 residues of pro-RIP). RIP-ΔC (Ala) has a partial deletion of the C-terminal domain (residues 286-305 of pro-RIP) resulting in polypeptide stopped before the (Ala) sequences. Primer combinations, length of fragment obtained in PCR (bp) and size of recombinant protein (KDa) are indicated.

PCR

Gene sequences were amplified by PCR utilizing DNA (20 ng) as template, 10X Taq polymerase buffer, dNTP (200 μM), primers (0.1 μM) and Pfu DNA Polymerase (1. 25U) in a final volume of 50 μL. Amplification was carried out over 35 cycles, after an initial denaturation step at 95°C for 2 min. Each cycle consisted of a denaturation step at 95°C for 50 sec, annealing at 60°C for 30 sec and extension at 72°C for 2 min. Final elongation was carried out at 72°C for 5 min. Amplified products were electrophoresed on a 1.3 % agarose gel, purified using the High pure PCR purification kit (Roche), digested with PmeI and SgfI (Promega) and ligated into pF1A T7 vector in order to obtain different b-32 carrying plasmids. *E. coli* competent cells were transformed with recombinant plasmids. The correct sequences of the different clones were verified by DNA sequencing.

Recombinant and native protein purifications

Single step (KRX) competent cells (L3002 Promega) were used for b-32 protein expression. These cells incorporate a copy of the T7 RNA polymerase gene under control of the rhamnose promoter, which controls expression of recombinant proteins. Recombinant KRX *E.coli* colonies were cultivated overnight at 37°C in LB medium with ampicillin and 0.4% glucose. Overnight cultures were diluted (1:100) in fresh LB medium containing ampicillin and grown at 37°C with shaking at 275 rpm, until they reached an optical density (OD600) of 0.4-0.5. Thereafter cultures were incubated at 20°C at 275 rpm. When cultures reached an OD600 of 0.5-0.6, inducers (0.1% L-Rhamnose monohydrate and 1 mmol L-1 IPTG) were added and induction was performed over night at 20°C and 200 rpm. Cells were harvested by centrifugation at 5,000 rpm for 10 min.

B-PER™ (Pierce) extraction buffer was used for extraction of recombinant proteins from bacterial cells. Protocols for mini and midi-scale bacterial protein extraction

(see instructions B-PER™) were used for both soluble, insoluble and inclusion body purification from total bacterial cell lysates. Total protein extracts, from mature endosperm of the B73 wt inbred line, were obtained as described by Lanzanova et al., (2009).

Fusarium isolates and antifungal bioassays

F. verticillioides strain No. 294 was maintained on Potato Dextrose Agar (PDA) medium in Petri dishes, at 26°C with a photoperiod of 16h light / 9h dark. An *in vitro* radial growth inhibition bioassay was performed to evaluate the inhibitory effect of recombinant RIP-b32 proteins on *F. verticillioides* growth as described in Lanzanova et al., 2011. Five replicates for each treatment were considered for each experiment, and the experiment was performed twice.

Fumonisin detection

After blocking at 7 DAI (Days After Inoculation) the fungal growth by placing the plates containing the Fusarium mycelium at 4°C, fumonisin content was determined according to Ridascreen fumonisin test kit (r-Biopharm), as reported by Berardo et al. (2011). Two replicates for each treatment were considered for each experiment, and the experiment was performed three times.

Statistical analysis

Results of experiments are summarized by means accompanied by standard errors (SEs). Statistical significance was given by using Student's t-test with $P < 0.05$.

Results

Recombinant RIP expression in E.coli

The highest recovery of b-32 RIP protein was obtained using overnight induction, B-PER™ Reagent (Pierce) and midi-scale bacterial protein extraction for both soluble, insoluble and inclusion bodies purification from total bacterial cell lysates. The recombinant b-32 RIP protein was expressed and clearly visible on the SDS-PAGE gel in all three induced (soluble, insoluble, inclusion bodies) fractions (Figure 2, lanes 4, 5, and 6, respectively) in comparison to uninduced samples (Figure 2, lanes 1, 2, and 3, respectively). As a positive control, a total protein extract from B73wt endosperm was used and allowed to visualize a band of about 32 KDa, which cross-reacted with b-32 specific antibodies (Figure 2, lane 8).

In order to verify the expression of the recombinant b-32 proteins, soluble fractions of induced cells transformed with RIP b-32, RIP ΔN, RIP ΔC, and RIP-ΔC (Ala) recombinant b-32 plasmids, respectively were analysed by Western blot (Figure 3, lanes 1, 2, 3, and 4) All recombinant proteins were expressed, although

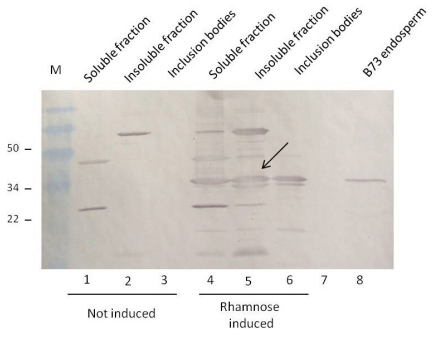


Figure 2- SDS PAGE of recombinant RIP b-32 protein. M: standard protein marker; lanes 1-2-3: soluble, insoluble and inclusion bodies of uninduced fraction, respectively; lanes 4-5-6: soluble, insoluble and inclusion bodies of induced fraction, respectively; lane 7: blank; lane 8: B73 wt endosperm protein extract .

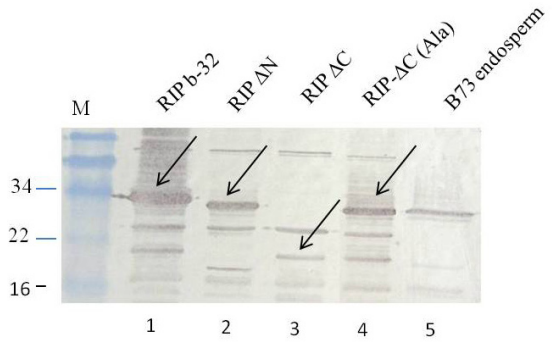


Figure 3-Western blot of the soluble fractions of engineered b-32 plasmids. M: standard protein marker; lanes 1 to 4: RIP b-32, RIP ΔN, RIP ΔC, RIP-ΔC (Ala), respectively; lane 5: extract from B73 endosperm. Bands corresponding to recombinant b-32 proteins are indicated with arrows.

to a lesser extend than the wild-type b-32 protein (see black arrows in Figure 3).

Effect of recombinant proteins on F. verticillioides growth and fumonisin accumulation

In order to evaluate the inhibitory effect on *F. verticillioides* growth of the recombinant b-32 proteins obtained, radial growth inhibition bioassays were performed. Petri plates containing 5 ml of PDA (Potato Dextrose Agar) were inoculated with 500 µg of the different recombinant proteins (RIP b-32, RIP ΔN, RIP ΔC and RIP-ΔC (Ala) and 100 spores of Fusarium per plate. As a control, the growth of Fusarium in the presence of B-PER buffer, was measured. The radial growth of the pathogen, measured as colony diameter from 3 to 5 DAI (Days After Inoculation), in the presence

Table 1-Effect of recombinant b-32 proteins on *F. verticillioides*. Radial growth and fumonisin content at 5 Days After Infection are indicated. Values are averaged over five replicates. Means and standard Errors (SEs) are indicated. Radial growth inhibition and fumonisin content were compared to B-PER buffer treated Fusarium. Statistical significance was given by using Student's t-test with P <0.05.

	Radial growth		Fumonisin	
Proteins	Colony diameter (mm)	Growth inhibition (%)	FUM content (µg Kg -1)	Inhibition (%) u
B-PER	31.1 ± 3.5		19150	
RIP b-32	32.8 ± 3.1	0%*	15850	16.60% **
RIP ΔN	31.3 ± 1.5	0%*	7633	60.10%**
RIP ΔC	31.9 ± 1.4	0%*	10516	45%**
RIP-ΔC (Ala)	33.5 ± 0.2	0%*	10700	44.10%**

or absence of recombinant b-32 protein allowed to measure the relative percentage of inhibition of each modified b-32 protein. In Table 1, both the growth inhibition of Fusarium mycelium relative to the negative control and the reduction in fumonisin content relative to the negative control at 5 DAI are reported. The average colony diameter of the treatments ranged from 31.1±3.5 (B-PER) to 33.5±0.2 mm RIP ΔC (Ala). Therefore, no significant difference in growth of *F. verticillioides* in the presence of RIP b-32, RIP ΔN, RIP ΔC and RIP-ΔC (Ala) proteins with respect to the control (B-PER) was observed. Conversely, fumonisin accumulation was reduced by 16.6% in the presence of b-32 protein (Table 1). A more significant reduction in fumonisin production was observed with the b-32 variants carrying a deletion of the N or C terminal ends of the mature protein. The highest reduction of mycotoxin production (60,1%) was observed in the presence of RIP ΔN protein, which carries a deletion of the N-terminal leader sequence (residues 1-16). Reductions of 45% and 44,1% were observed with proteins RIP ΔC and RIP-ΔC(Ala), respectively, both exhibiting a deletion of the C-terminal b-32 sequence.

Discussion

Fumonisins (FBs) are mycotoxins produced in *F. verticillioides* infected maize grain (Berardo et al., 2011). Attention has focused on FBs because of their widespread occurrence, acute toxicity to certain livestock, and their potential carcinogenicity. Since mycotoxin development occurs mainly in the field, strategies are needed to prevent infection of growing plants by toxigenic fungi (Duvick et al., 2001).

Research has been focused on the ability of plant RIP proteins to inhibit fungal growth (Nielsen et al., 2001); these authors developed a microculture assay useful to monitor, during very preliminary stages (18 hours), the cellular growth and morphology of fungi upon addition of purified RIP proteins. In their study alterations in growth and morphology of *Aspergillus flavus*, a corn fungal pathogen, and *Aspergillus nidulans*, a non-pathogen strain, suggest that the effect of RIP on *A. flavus and A. nidulans* requires the catalytic ribosome-inactivating activity of the protein. The inhibitory activity of RIPs against normal fungal growth is consistent with the biological function to protect the seed from fungal invasion during seed germination (data not shown).

Although, in literature, the effect of maize RIP b-32 in contrasting Aspergillus development was observed, no studies on the effect of this protein in contrasting *F. verticillioides* growth and fumonisin accumulation were reported.

To assess the role of bioactive b-32 recombinant proteins in protection against *F. verticillioides*, a series of preliminary *in vitro* bioassays with recombinant RIP b-32 proteins [RIP ΔN, RIP ΔC, and RIP-ΔC (Ala)] were performed in order to analyze their effect on fungal growth and fumonisin accumulation. We have observed that all different recombinant b-32 proteins did not affect *F. verticillioides* growth and development in an *in vitro* bioassay, as there were no significant differences between the treatments and the control (B-PER) from 3 to 5 DAI. An important aspect of maize fungal protection regards the capacity of antifungal proteins to prevent or reduce mycotoxin production. In this respect, it is interesting to note that, although no reduction of *F. verticillioides* mycelium growth was observed during our experiments, a clear decrease in the accumulation of fumonisins in presence of different b-32 recombinant proteins could be shown. Evidence for a possible role of b-32 RIP in plant defence was attained from the observation that the native protein was able to reduce fumonisin accumulation. Moreover, the deletion of N or C terminal domains from the mature peptide resulted in a greater containment of toxin. These observations are in good agreement with earlier results reported by Hey et al., 1995. They observed that during the activation of maize RIP, the removal of the N and C terminal segments increased the activity 6 and 5 fold, respectively.

The protective role of the b-32 protein in favour of the safety of the maize kernel was also observed by comparing fumonisin accumulation in corn kernels of a A69Y *opaque-2* line (in which the level of b-32 is decreased), with respect to A69Ywt when subjected to an artificial inoculation with *F. verticillioides* (Torri 2011). The opaque mutant was not only more susceptible to the fungal attack, but also accumulated more fumonisin than the wild-type line.

The preliminary observations reported in this paper are encouraging in prospecting the use of maize b-32 RIP protein in the containment of fumonisin and possibly Fusarium with the aim to improve the quality and safety of the maize grain.

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