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Characterization of the maize b-32 ribosome inactivating protein and its interaction with fungal pathogen development

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Abstract

Plants respond to attack by pathogenic fungi with a complex network of responses, including the production and accumulation of proteins, such as the Ribosome Inactivating Proteins (RIPs) that are toxic or inhibitory to pathogens. In maize endosperm, a cytosolic albumin termed b-32 (RIP1) is synthesized in temporal and quantitative coordination with the deposition of storage proteins. Research has shown that b-32 is able to i) enzymatically inactive ribosomes modifying rRNA inhibiting protein synthesis *in vitro*, ii) inhibit the growth of *Rhizoctonia solani* mycelia in an in vitro and in planta assays, iii) reduce *Fusarium culmorum* head blight in wheat transgenic plants expressing b-32, and iv) diminish *Fusarium verticillioides* attack symptoms in leaf tissues assays of maize transgenic expressing ectopically b-32 protein. Similarly to other RIPs, maize b-32 is accumulated in the seed as an inactive precursor, which is converted into an active form by proteolytic processing which removes peptide segments from the N (residues 1-16 of pro-RIP) and C (residues 295-301) termini and also from the center (linker domain) of the polypeptide. In this review we will summarize evidence and advances related to the ability of the b-32 protein in contrasting pathogen attacks by considering and describing i) *in vivo* b-32 antifungal activity and ii) *in vitro* fungal development inhibition. These data provide information for assessing b-32 in developing plants with a higher capacity to contrast damages induced by pathogens.

Keywords: Ribosome-Inactivating Proteins, b-32, antifungal protein, Fusarium

Introduction

Plants are exposed to a vast number of pathogenic fungi and have evolved a variety of potent defense mechanisms. Although, plants do not have an immune system, they possess a network of defense against pathogens, that include a large array of proteins and other defensive molecules produced before infection or during pathogen attack. Not all pathogens can attack all plants and a single plant is not susceptible to the whole plethora of plant pathogenic fungi. A major area of research in this field has been devoted to identify, and characterize genes involved in disease resistance. The identification of these genes has suggested to plant scientists to evaluate their specific roles and importance in disease response pathways via the use of transgenic plants (Punja, 2001). In fact, recombinant DNA technologies may permit to exploit inherent plant responses against pathogen by either using single dominant resistance genes not normally present in susceptible plants (Keen et al, 1999; Melchers and Stuiver, 2000) or by choosing genes that intensify or trigger the expression of existing defense mechanisms (Rommens and Kishore, 2000). In this context, the genes encoding many antifungal proteins were frequently used to develop genetically modified plants possessing an increased fungal resistance in field trials (Selitrennikoff, 2001; Hernández et al, 2005). Thus, the identification of such proteins, which are generally not race or species-specific and possess a broad spectrum of activity, may allow the use of the corresponding genes to develop transgenic plants with an increased disease resistance (Ferreira et al 2007).

Ribosome Inactivating Proteins

Plants constitutively accumulate proteins that are either toxic or inhibitory against pathogens, including the Ribosome Inactivating Proteins (RIPs). These RIPs are N-glycosidases that depurinate the universally conserved α-sarcin loop of large rRNAs (Barbieri et al, 1993; Metha and Boston, 1998). This depurination inactivates ribosomes, by an irreversible modification that blocks elongation factor EF-1 and EF-2 dependent GTPases activities and renders the ribosome unable to bind EF-2 with consequent arrest of protein synthesis (reviewed in van Damme et al, 2001). Although RIPs were first identified more than 25 years ago, their biological function(s) still remains open to speculation. A working model to summarize and rationalize their biological function in plants was reported by Park et al (2004). These workers have shown that plants possess multiple RIPs and were found in different organs (seed, root, and leaf) in concentrations ranging from few micrograms to several hundred

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milligrams per 100 g of tissue. Moreover, it was found that plant RIPs inactivate foreign ribosomes, *in vitro* and *in vivo*, of distantly related species and of other eukaryotes, including fungi (Iglesias et al, 1993).

Classification and properties of RIPs

RIPs are currently classified into three groups based on their physical properties (Metha and Boston, 1998; Mundy et al, 1994).

Type 1 RIPs. They include Pokeweed Antiviral Protein (PAP), saporin (from soap-wort, *Saponaria officinalis* L.), trichosantin, gelonin, and barley seed RIP (RIP30). They have basic isoelectric point, and are monomeric enzymes of approximately 30 kDa with a single polypeptide chain that contains the ribosome-inactivating activity (Irvin, 1975; Yeung et al, 1998). Type 1 RIPs are not toxic to intact cells, although their enzymatic activity may be several folds higher than that of Type-2 RIPs.

Type 2 RIPs. These RIPs are mainly represented by ricin and abrin. They are highly toxic heterodimeric proteins with enzymatic and lectin activities in separate polypeptide subunits, each of approximate molecular weight of 30 kDa: a polypeptide chain (A chain) that contains the ribosome-inactivating activity is linked by a disulphide bridge to a second chain galactose-binding lectin (B chain) that promotes uptake by the cell (Olsnes and Pihl, 1973; Stirpe et al, 1978). Thereby, once it reaches the cytosol, the A-chain of the RIP has access to the translational machinery and inactivates ribosomes interrupting protein synthesis. Only some Type 2 RIPs, namely ricin, abrin, modeccin, volkensin, and viscumin, are higly toxic to cells and animals; while others, namely ebulin, nigrin, cinnamomin, iris lectin are not toxic; however, the reason(s) for the difference is still unknown.

Type 3 RIPs. These RIPs include maize b-32 or RIP1 and barley JIP60 (Walsh et al, 1991; Chaudhry et al, 1994) They are synthesized as single-chain proenzymes, inactive precursors (proRIPs) that require proteolytic processing events to produce two noncovalently linked chains equivalent to a Type 1 RIP.

In maize, b-32 or RIP1 has been described as a holo-RIP, two-chain type-1 RIP, whereas JIP60 as a chimero-RIP, true Type-3 RIP (Nielsen and Boston, 2001; Van Damme et al, 2001). These RIPs are less abundant than Type 1 or Type 2 RIPs. The function of the extra domains in the Type 3 RIP is not known. However, once they are removed, the processed active protein is similar in charge and enzymatic activity to Type 1 RIPs (Walsh et al, 1991; Hey et al, 1995; Krawetz and Boston, 2000). For the maize RIP1, the extra domains are unlikely to be protective features to prevent self-inactivation of maize ribosomes because ribosomes from seed and other plant parts are resistant to maize proRIP and active RIP (Hey et al, 1995; Bass et al, 1992). The mode of uptake of Types 1 and 3 RIPs by cells is unknown.

Although the enzymatic activities of RIPs have been shown *in vitro*, their role in plant defence is less clearly defined. Historically, RIPs have been linked to plant protection, since crude extracts of pokeweed (*Phytolacca americana*) leaves were first shown to have inhibitory activity against viral infections in plants (Irvin, 1975). Subsequent purification of the inhibitory proteins led to their identification as RIPs and the development of transgenic plants have promoted the antimicrobial activity of RIPs for practical application in plant defense (Lam et al, 1996; Zoubenko et al, 1997).

Cereal RIPs

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. (reviewed by Balconi et al, 2010).

Maize RIP1 protein

In maize, RIPs are present in at least two forms of non-allelic genes, one in the endosperm (Di Fonzo et al, 1986, 1988; Walsh et al, 1991) and the other in leaf tissues (Bass et al, 1995). The maize endosperm RIP1 (b-32) has been largely studied (reviewed by Motto and Lupotto, 2004). The main finding emerging from these studies indicated that this RIP is a cytosolic albumin with a molecular weight of 32 kDa (termed b-32) synthesized in temporal and quantitative coordination with the deposition of storage proteins. It is present in the endosperm as inactive zymogen (pro-RIP), representing up to 1% of the total seed proteins. Moreover, it was shown that its N-terminal, C-terminal, and internal domains can be enzymatically removed from pro-RIP to yield two chains α-β that interact non-covalently to form a much more active enzyme (Walsh et al, 1991; Bass et al, 1992). The process involves removal of a 16 amino acid residue of 1763 D from the N-terminus (residues 1 to 16), a 25 amino acid residue of 2708 D from the acidic central region of polypeptide (residues 162 to 186), and 14 amino acids of 1336 D from the C-terminus (residues 289 to 301). The two final peptides of 16.5 and 8.5 kDa generated, tightly linked in a non-covalent manner, represent the activated form of RIP, termed $\alpha\beta$-RIP (Walsh et al, 1991). The activated form inhibits translation in a cell free rabbit reticulocyte system with an IC50 (concentration causing 50% inhibition) of 28-66 pM, at least 10,000 times more active than the pro-RIP (Walsh et al, 1991). Further support for a proteolytic activation of pro-RIP was found in the

demonstration of increases in RIP activity in coincidence with the onset of protease synthesis and protein degradation during germination (Bass et al, 1992; Hay et al, 1991). The proteolytic cleavage that occurs *in vivo* during germination, can also be performed *in vitro* by a variety of non-specific proteases such as papain and subtilisin Carlsberg (Walsh et al, 1991), thus demonstrating that the RIP activation is due to a proteolytic processing of central acidic domain.

The synthesis of inactive precursor forms of enzymes, the zymogens, appears to be a specific way to regulate their activity by suppressing the enzymatic capacity until conversion of the zymogens to the active form, when needed, occurs by proteolytic cleavage (Neurath, 1989). There is no evidence from literature that maize endosperm RIP1 has a specific subcellular targeting (Walsh et al, 1991); the maize pro-RIP is in all cases described as a cytosolic protein not secreted via the endoplasmic reticulum.

Gene expression studies have demonstrated that the *b-32* (*RIP1*) gene, as well as genes encoding the 22 kDa zeins, are co-ordinately controlled by the endosperm regulatory locus *Opaque-2* (*O2*) (Olsnes and Pihl, 1982; Soave et al, 1981). O2 protein belongs to the b-ZIP family of transcriptional regulatory proteins, and affects expression of the major seed storage protein genes, in particular those encoding the 22 kDa α-zeins (Hartings et al, 1989; Schmidt et al, 1990). Levels of b-32 and 22 kDa zeins are greatly decreased in *o2* mutants. The role of b-32 in defence against pathogens was so suggested by an increased susceptibility of opaque-2 (o2) mutant kernels (in which the level of b-32 is greatly decreased), to fungal attack (Loesch et al, 1976; Warren, 1978) and insect feeding (Gupta et al, 1970). In addition, the results about pure inbred lines, and their isogenic *o2* mutants, tested in field experiments with Silk Channel Inoculation Assay (SCIA) and Kernel Inoculation Assay (KIA) on adult plants, showed that the *o2* mutants resulted significantly more susceptible to the *F. verticillioides* attack than the normal version (Balconi et al 2005; Torri 2011). The increased susceptibility in the absence of the pro-RIP1 is consistent with a defence function, although the experimental results cannot be attributed to the maize pro-RIP1 *a priori* because the *O2* locus regulates transcription of several genes that may contribute to a complex mutant phenotype. These observations suggest that will be interesting to verify if the expression of RIP1 in an *o2* mutant might increase tolerance to fungal pathogen attack in kernels.

In vivo maize b-32 antifungal activity

The use of a purified protein creates an artificial situation because the amount of RIP that might actually be released from cells during an endogenous insect-plant or fungal-plant interaction is very likely approximated. The effectiveness of an anti-fungal protein in planta may be determined, at least in part, by studying i) its expression levels in the host tissues and ii) by the timing of its expression, such as suitable levels accumulate before the host becomes most vulnerable to infection. The expression of antimicrobial proteins in plants or plant tissues, in which they are not normally produced, may have a greater potential to limit pathogen infection or growth. In this perspective, a useful strategy is to deploy an antifungal protein (normally expressed in the kernel) in a non-seed tissue that is critical for infection (e.g. stover).

Ectopic expression of RIPs in transgenic plants can solve this problem by allowing the exposure of the pest or pathogen to the RIP only during interactions with the plant. In several studies, transgenic plants expressing cereal RIPs have been used to test defence properties attributed to these proteins (Punja, 2001; Hartley et al,1996). The Type 1 barley RIP, expressed under a 35S-CaMV promoter or a wound-inducible promoter in tobacco, conferred some reduction to disease symptoms caused by the fungus *R. solani*. Moreover, the addition of a signal sequence to target the RIP to endomembrane system improved resistance in transgenic plants producing detectable levels of RIP (Logemann et al,1992; Jach et al,1995). On the other hand, expression of Type 1 barley RIP30, expressed under the control of a strong constitutive promoter 35S-CaMV, had little effect against infection by the fungal pathogen *Erysiphe graminis* in transgenic wheat as reported by Bieri et al (2000). In this study, the RIP30 was targeted through the endoplasmic reticulum (ER) to the apoplastic space to ensure the presence of RIP at the place where initial interactions with the fungus occur. It was found that RIP30 was effectively localized to the intracellular space and the intercellular wash fluids of transgenic wheat lines, and strongly inhibited a rabbit reticulocyte lysate transcription/translation system. However, the anti-fungal efficiency of RIP30, as assayed by infection of detached leaves with *E. graminis*, were small. In addition, further studies have demonstrated that the combined expression of chitinase and RIP in transgenic tobacco had a more inhibitory effect on *R. solani* development than the individual proteins (Jach et al,1995). Therefore, dissolution of the fungal cell wall by hydrolytic enzymes appears useful to enhance the efficacy of antifungal proteins and peptides in transgenic plants.

Transgenic rice plants expressing the maize *b-32* gene were produced by Kim et al (1999). These authors found that the level of the b-32 expression was ca. 0.5-1% of total soluble protein in leaf tissues, a value comparable to the expression of barley RIP detected in fungus-resistant transgenic tobacco plants (Logemann et al, 1992). Furthermore, data reported by Kim et al (1999) indicate that the b-32 was proteolytically processed in germinating rice seeds and

young leaves of transgenic plants, in a similar manner to that found in germinating maize kernels; however, no processing was detected in mature leaf tissues. The same authors reported also that disease severity caused by infection with the fungal pathogens *Rhizoctonia solani* and *Magnaporthe grisea*, was not significantly reduced in the transgenic rice plants expressing the b-32 as compared to control plants, suggesting that a processing of b-32 protein may be required to have antifungal activity *in planta*. However, in the mentioned research, whether transgenic plant fungal resistance requires proteolytic processing of the maize pro-RIP1 is not clearly defined. In fact, transgenic tobacco plants, in which the expression of maize *b-32/RIP1* gene is driven by the wun 1 promoter, had increased protection against infection of the soil-borne fungal pathogen *R. solani* (Maddaloni et al, 1997).

Figure 1 - Visual scale to estimate severity of Fusarium head blight following *F. culmorum* artificial inoculation. C: control plants inoculated with sterile distilled water; from right to left: 20-50-90-100 % infected spikelets/spike. Adapted from Balconi et al (2007).

Transgenic wheat plants expressing RIP1- b-32 protein

Research in our laboratory indicated that maize b-32 protein is effective in wheat transgenic lines as anti-fungal protein by reducing Fusarium head blight (FHB) symptoms (Balconi et al, 2007). Transgenic wheat plants were obtained via biolistic transformation, in which the b-32 gene is driven by the 35S-CaMV promoter in association with the bar gene as a selectable marker. b-32 wheat transgenic lines were characterised; all plants had a normal phenotype not distinguishable from control plants from the cv. Veery, except a slightly smaller plant size. A differential b-32 expression in the various progenies was also recorded. To assure that the b-32 expression was maintained during plant development, immunoblot assays against b-32 were performed at three different stages of plant development: a) fourth leaf stage, b) tillering, and c) ten days after anthesis. The results showed a stable expression level of *b-32* in green tissues of the transgenic lines throughout plant development.

Pathogenicity tests for Fusarium head blight (FHB) were performed on the b-32 transgenic wheat lines in comparison to the control plants via the "single floret injection inoculation method" on immature spikes with spores of *Fusarium culmorum*. The plants were analyzed for FBH via visual inspection of the injected heads by counting the number of spikelets showing premature bleaching on emerging heads. In Figure 1 the disease severity in a range from 20 to 100% infected spikelets is shown. Moreover, no changes in colour or other morphological alterations were observed in glumes of control water-inoculated plants (Figure 1C).

The severity of FHB reported as percent of infected spikelets/head, scored at 7 and 14 days after inoculation, were significantly lower in transgenic plants than in control plants. Another parameter used to attest scab disease severity in the tested genotypes was the percentage of "tombstones" (shriveled, light weight, dull greyish or pinkish in colour kernels) with respect to total seeds, recorded at maturity in control plants and in the transgenic lines. Independently from the differential b-32 level of the transgenic lines, the disease severity and disease control, were equally reduced in all cases, in comparison with controls. Moreover, it was found that the protection due to the presence of b-32 was not dependent by the increasing levels of the RIP protein in the tissues, because the lowest level of b-32 was effective. Therefore, it was concluded that transgenic wheat plants constitutively expressing the antifungal maize RIP1-b-32 protein showed a higher level of resistance to FHB, reflected in reduced fungal colonization after artificial single spikelet inoculation. This confirmed that maize RIP b-32 was effective as in vivo antifungal protein in wheat, which normally does not produce this protein (Balconi et al, 2007).

Transgenic maize plants expressing b-32 protein

To further explore the antifungal activity of the maize b-32 protein, transgenic maize plants, containing the b-32 coding sequence under a constitutive 35S-CaMV promoter, were obtained in our laboratory through genetic transformation (Lanzanova et al, 2009). Specifically, in this study four homozygous transgenic lines with differential ectopic expression of b-32 were challenged, in comparison with plants expressing b-32 only in the endosperm. More in detail, four Basta resistant progenies (SM 3.4; SM 16.1; SM 19.4; SM 20.2), one Basta sensitive progeny (SM 20.4), were analyzed in the research. All plants of

these progenies were fertile and had a normal phenotype, when compared to the negative control, thus confirming that the ectopic expression of the b-32 RIP did not interfere with normal plant development, as previously observed for wheat by Balconi et al (2007).

The evaluation, in the negative control, of b-32 expression at the protein level, in endosperm and leaf tissues, confirmed the endosperm-tissue specificity of this gene; on the other hand, transgenic progenies showed detectable b-32 protein in leaf and endosperm tissues. At flowering, a comparison of b-32 protein amounts in leaf extracts, was performed by immunoblot imagine scanning showing a differential b-32 expression among the various progenies. The identification of progenies with a differential b-32 expression in the leaves was useful for setting up pathogenicity experiments, aimed at evaluating a possible differential response to a Fusarium attack in leaf tissue colonization bioassays. Non-inoculated and sterile water inoculated leaves (controls), did not show any mycelial growth, or any changes in colour or other morphological alterations; hence, the protocol adopted for leaf tissue surface sterilization, was effective in eliminating all external contaminations and appeared useful for the pathogenicity bioassays. Different spore concentrations were tested on the control progeny (SM 20.4, Figure 2A). The following bioassay parameters, i.e. i) spore suspension containing 10^6 spores/ml, and ii) 3-4-7 DAI (Days After Inoculation) as detection time were adopted for pathogenicity experiments including together with the negative control SM 20.4 also individuals of the four transgenic progenies. Results indicated that fungal colony diameters measured around the inoculated leaves of SM 20.4 (negative control), were, at all detection times, significantly larger than that observed in all four progenies expressing b-32. In addition, the fungal colony diameters measured around the inoculated leaves of SM 19.4, the progeny with the lowest level of b-32, were, at all detection times, significantly larger than that observed in the other three progenies expressing

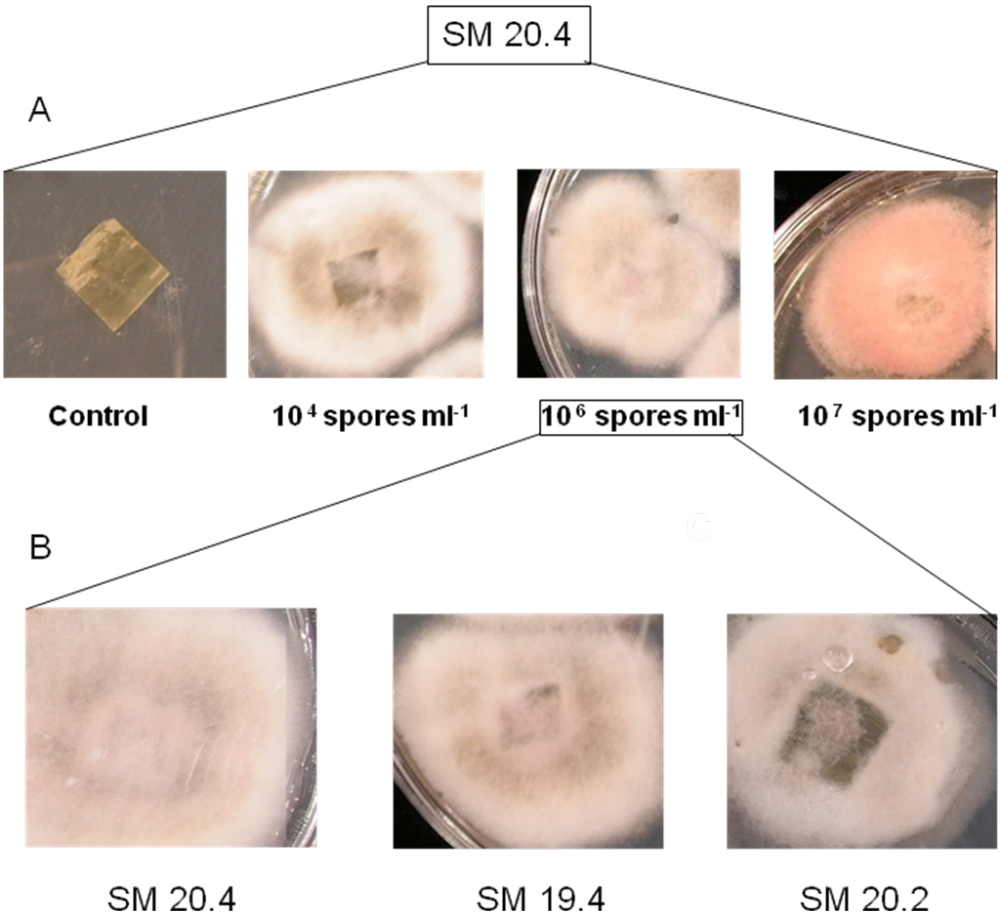

Figure 2 - Progression of *F. verticillioides* infection
(A) SM20.4 (negative control) at 7 days after inoculation (DAI) with different spore concentrations (from left to right: 10^4, 10^6 and 10^7 spores/ml) in SM. Control: leaf tissue inoculated with sterile water. (B) SM 20.4, SM 19.4 and SM 20.2 progenies at 10 DAI after inoculation with 10^6 spores/ml. Adapted from Lanzanova et al (2009).

b-32. Therefore, the suppression of Fusarium leaf colonization (growth inhibition, % relative to the control) in the SM 19.4 progeny was significantly lower than that observed in the other three transgenic progenies, at all detection times. For each genotype, observations about the Fusarium attack and the progression of the infection on the leaf segments, were recorded.

From the previous experiments a correlation between the b-32 content in the leaves and the level of resistance to Fusarium attack was observed. In the case of progenies with high b-32 content in the leaves (SM 20.2), in addition to a reduced mycelial growth around the cut edges of the leaves, a very weak growth on leaf surfaces was observed in comparison to progeny (SM 19.4) exhibiting the lowest b-32 content in leaves (Figure 2B). Collectively, these data suggested that b-32 protein does not prevent the Fusarium attack, but rather promotes the reduction of mycelial growth on the colonized tissue. As previously reported in wheat for FHB, b-32 crop protection appeared due to the avoiding the spread of infection in all directions from the point of inoculation (PI) (Balconi et al, 2007).

The expression of antifungal proteins in plants or plant tissues, in which they are not normally expressed, is very appropriate to reduce pathogen colonization and growth. In this perspective, a reduction of *F. verticillioides* infection in maize leaves and stalk, may be useful to arrest the fungal infection from spreading to the exposed silks and consequently to reduce fumonisin contamination in grains (Lauren and Di Menna, 1999).

In vitro b-32 activity against fungal development

Fungal bioassays offer the advantage of testing a single protein for its effect. Additionally, tests can be done with concentrations of the protein under study equivalent to those found in particular plant organs (Dowd et al, 1998).

An *in vitro* bioassay to test the b-32 antifungal activity was previously performed in our laboratory (Maddaloni et al, 1997); the results of the microtiter dish method experiment showed that b-32 protein, extracted and purified from maize endosperm, has an inhibitory effect on the growth of *R. solani*, the causal agent of root rot, stem canker and damping off observed in a wide range of crops, including tobacco (Ogoshi, 1987; Leah et al, 1991).

The toxicity of maize RIP1 toward fungi has been tested by Nielsen et al (2001); the authors developed a microculture assay useful to monitor the cellular growth and morphology of fungi upon addition of purified RIP. In this study it was found that the activated maize RIP (MOD1, created as a deletion of the proRIP1) altered the growth and morphology of *Aspergillus flavus*, a maize fungal pathogen, and *Aspergillus nidulans*, a non-pathogen. Moreover, the data reported from the enzymatically inactive MOD1 mutant (mutation that abolish RIP activity) treatment, showed that the effect of RIP on *A. flavus* and *A. nidulans* requires the catalytic ribosome-inactivating activity of the protein (Nielsen et al, 2001). In this study, pro-RIP did not show any antifungal activity against tested fungi, suggesting that the protein must be activated to have antifungal activity. However, this did not rule out activation occurring in a number of ways *in vivo*. For example, *A. flavus* was shown to lyse and degrade cells at fungal invasion front, presumably by the action of proteases and other degradative enzymes secreted by the fungus (Smart et al, 1990). Proteases stored intracellularly might also be released from the plant cells that were lysed by the invading fungus. The inhibitory activity of activated maize RIP1 against normal fungal growth is consistent with a biological function to protect seeds from fungal invasion.

Further research in our laboratory was developed in order to: i) deepen our knowledge about relationships between structure and substrate specificity of b-32 protein; ii) clarify the role of the processed segments of b-32 gene on the ability of maize RIP to inhibit fungal growth. Thereby, a series of *in vitro* gene constructions was made by selectively deleting the N-terminal, or C-terminal or internal linker domain. Genetic deletions of the b-32 gene, that is apparently responsible for suppressing enzymatic activity in the precursor, were primarily expressed in *Escherichia coli* to produce sufficient quantities of modified proteins (Lanzanova et al, unpublished results).

Fusarium verticilliodes growth conditions set up

This first step of the research was carried out using two *F. verticillioides* strains (#289 and #294) maintained on Potato Dextrose Agar (PDA) medium in Petri dishes, at 26°C with a photoperiod 16 h light/8 h dark. At regular intervals of time, subcultures were performed, taking a portion of mycelium from the front of fungal colony and plating it on fresh PDA medium. Spore suspensions were prepared washing the plate surface with sterile distilled water (SDW) and the conidial suspension adjusted to the final desired concentration using a Bürker haemocytometer. In order to find the best experimental conditions to observe a regular radial growth, useful for the antifungal *in vitro* bioassays, the center of 5 ml PDA medium plates was inoculated with 10 µl of spores suspensions at different concentration.

The two *F. verticillioides* strains were considered separately and inoculated at three different spore concentrations: 5,000 spores/ml PDA, 500 spores/ml PDA and 50 spores/ml PDA (10^5 spores/plate, 10^4 spores/plate, 10^3 spores/plate). Five replicates were made for each concentration tested. Radial growth was determined from 3 Days After Inoculation (DAI), until 7 DAI, by measuring the diameter of the fungal colony. As reported in Figure 3, at all tested spore concentrations, the colony diameter was comparable for both strains, excepted for a lower growth rate of

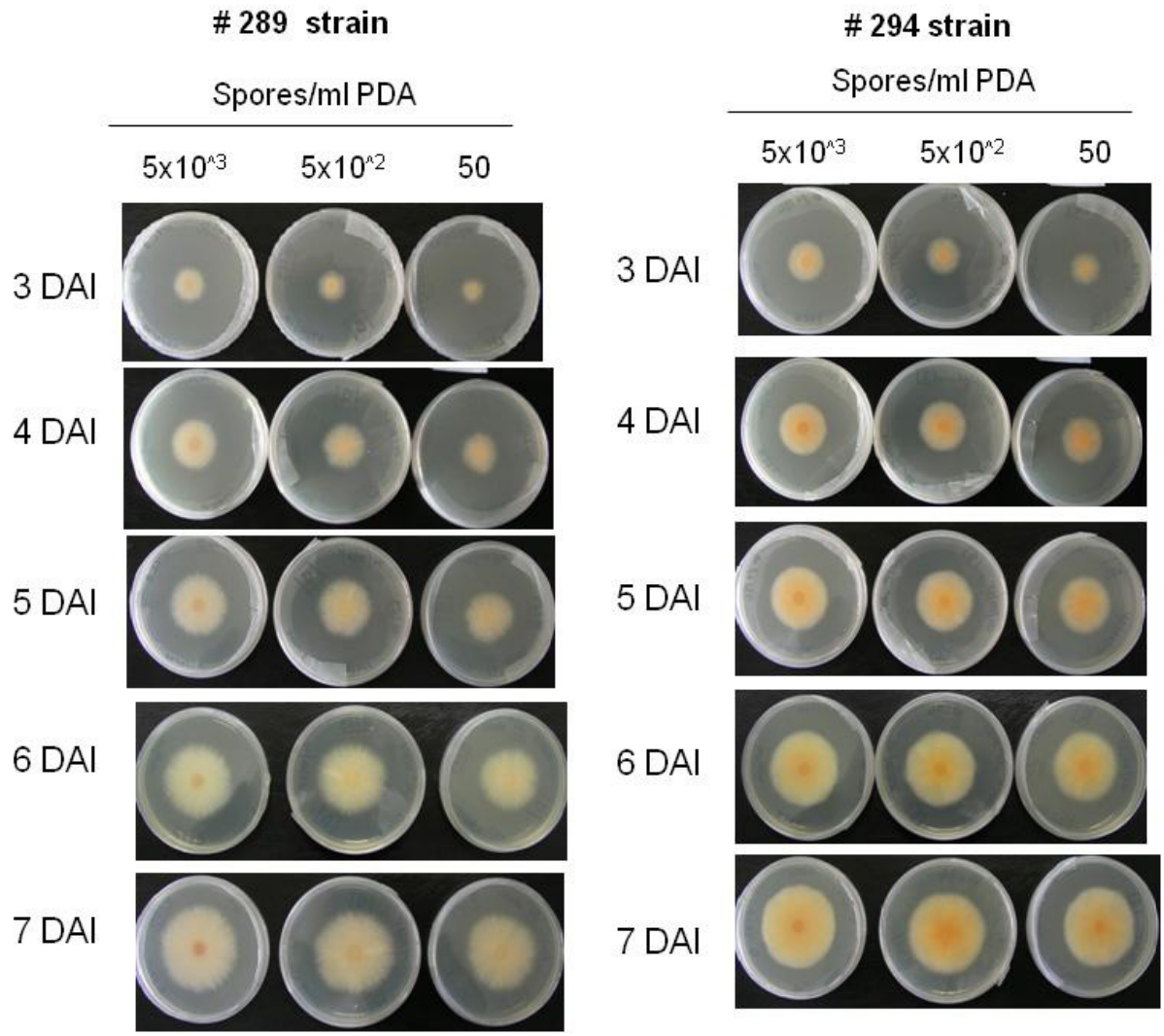

Figure 3 - Growth pattern of #289 and #294 strains of *F. verticillioides* from 3 to 7 DAI.

#289 strain, at the 10^3 spore/ml concentration. More in detail, the colony diameter ranged around 20-50 mm from 3 to 7 DAI for strain #289 at 10^5 and 10^4 spores/plate, and around 18- 45 mm at 10^3 spores/plate. For strain #294 the average colony diameter ranged 25-52 mm at all the three tested spore concentrations.

However, from a qualitative point of view, a substantial difference between the two strains was noticed: in fact, #294 showed a more compact and regular mycelium growth than that observed for #289 (Figure 3). Following these observations the strain #294 and the lowest amount of spores, i.e. 10^3 spores/plate (50 spores/ml PDA) were chosen, respectively, as material and experimental conditions, for setting up antifungal bioassays to test the bioactive property of maize b-32.

Antifungal bioassays

Two different bioassays of radial growth inhibition, i.e. a) bioactive protein spread on the plate and b) bioactive protein pipetted on paper discs), were performed, to evaluate the inhibitory effect of RIP1 maize b-32 on *F. verticillioides* growth and development.

As above mentioned, the strain #294 and the lowest amount of spores 10^3spores/plate (50spores/ml PDA) were chosen, respectively, as material and inoculum, for antifungal bioassays. Experiments were devoted to test the antifungal activity of the commercial RIP (Saporin - from *Saponaria officinalis* seeds, SIGMA S9896.) against *F. verticillioides*. The Saporin commercial product, supplied as a lyophilized powder containing approximately 30% protein (Lowry) in buffer salts (50% Glucose, 25% Sodium Phosphate buffer pH6), was considered as control (Buffer SAP),

in our antifungal bioassays.

A) Radial growth inhibition - (Saporin spread on the plate)

Experiments were conducted following directions reported by Krishnamurthy et al (2001), spreading two different amounts of Saporin (75 µg and 150 µg/plate) on 5 ml PDA plates and inoculating the plates with 10^3 spores of *F. verticillioides* (#294 strain). As controls, the growth of both fungal strain alone and in the presence of the Buffer SAP, was followed to observe whether this buffer could interfere with the growth of the fungus. Five replicates for each treatment were considered. Fungal toxicity was recorded in terms of percentage of colony diameter inhibition and calculated according to Amadioha (2000). Radial growth of the pathogen was measured, as colony diameter, from 3 to 7 DAI (Figure 4).

As reported in Figure 4 an inhibition of fungal growth after treatment with saporin was observed, in comparison with fungal growth in the presence of Buffer SAP. The percentage of Fusarium growth inhibition, at 2 DAI, is around 30%-35 %. This inhibition decreases in the following days, reaching 25% with 75 µg of SAPORIN and around 18% with 150 µg of commercial RIP.

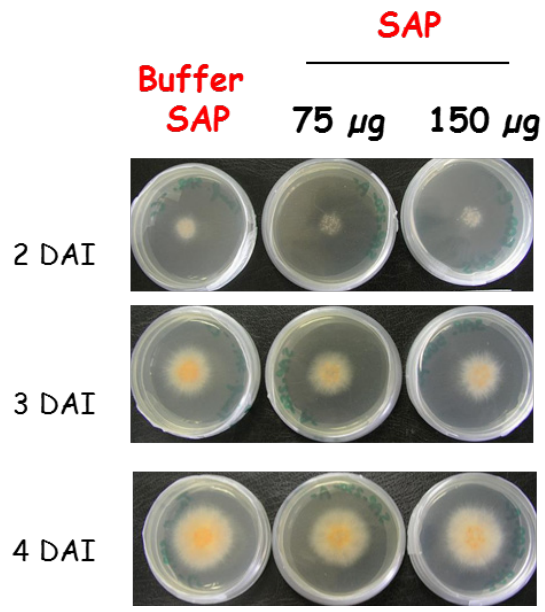

Figure 4 - Test of *F. verticillioides* radial growth inhibition with 75 and 150 µg Saporin spread on the plate from 2 to 4 DAI.

B) Radial growth inhibition- (Saporin pipetted on paper discs).

The growth inhibition of *F. verticillioides* was also tested by pipetting 20 µg of Saporin on paper discs, to assess the effect of a certain amount of protein concentrated in a specific point at the mycelia front, as described in a study reported by Park et al (2002).

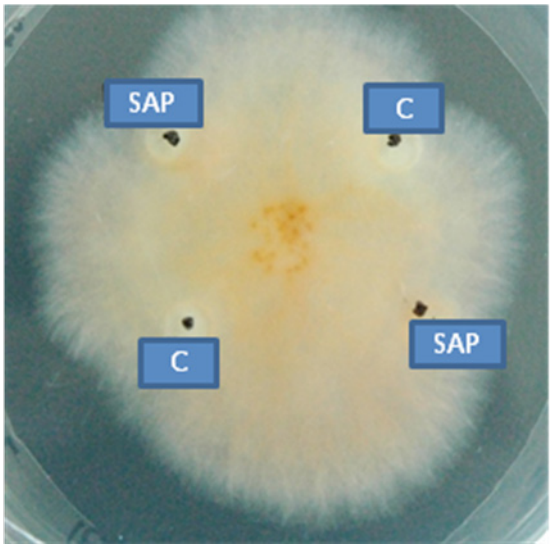

Figure 5 - Inhibition Assay of *F. verticillioides* radial growth (20 µg SAP pipetted on paper discs), 4 days after the assay was set up.

PDA plates were previously inoculated with 10^2 spores of *F. verticillioides* (#294 strain). As control, on the same plate, Buffer SAP (C) was pipetted on paper discs. As shown in Figure 5, Saporin antifungal activity was observed as a crescent-shaped zone of inhibition at the mycelia front.

The effect on fungal growth was expressed qualitatively, according to the procedure of Schlumbaum et al (1986). Specifically, the percentage of inhibition was around 23% at 4 days after the paper discs assay was set up and decreased slowly during the following days.

Antifungal assays performed with commercial RIP-Saporin were useful to set up the experimental conditions to be adopted for testing other bioactive proteins. Experiments are in progress in our laboratory to dissect the antifungal activity of maize b-32-RIP as plant defence protein against *F. verticillioides* and other fungal pathogens.

Conclusions and future perspectives

Plant diseases have major effects on agricultural production and food supply. Although application of fungicides has helped control plant diseases, chemical control is economically costly as well as environmentally underisable. The development of new strategies based on plant's own defense mechanisms for disease control is therefore critical for sustaining agricultural production and improving our environment and health. Basic research on the genetic bases of disease resistance in plants and of host-pathogen interactions has greatly improved the efficiency of manipulating disease resistance genes in practical breeding programs. These researches resulted in the deployment of high-yielding genetically resistant crop cultivars that in some cases have been grown over

vast areas. However, much remains to be learnt at the interface of the genetics of resistance and crop physiology. The cloning of resistance genes and corresponding avirulence genes has indicated considerable complexity not only in structure but also in the way in which gene products interact and trigger resistance. Hence, our overall understanding of the process is still fragmentary. Furthermore, many gaps remain in our models of the defense signal transduction network and these must be bridged before we can design truly rational strategies to activate the network.

Because most mycotoxin contaminants are due to pathogenic fungi infecting plants directly in the field, strategies are needed to prevent infection by growing plants tolerant to toxigenic fungi; efforts to control infection or mycotoxin development in maize, through conventional breeding and genetic engineering were reported by several authors (e.g. Munkvold, 2003).

The effectiveness of an anti-fungal protein in planta may be predicted, in part, by its expression levels in the crucial host tissues and by the timing of expression, because suitable levels should accumulate before the host becomes most vulnerable to infection. In this context, the maize endosperm albumin b-32, as a RIP has been the subject of extensive studies aimed at investigating and at exploiting its action as a defence protein against fungi (Balconi et al, 2010).

The results from our research, carried out in diverse crop plants, such as tobacco, wheat and maize, confirm that the incorporation of maize b-32 gene and the ectopical expression of b-32 protein, appears an effective and reliable tool in crop disease management programs (Maddaloni et al, 1997; Balconi et al, 2007; Lanzanova et al, 2009). An important issue in fungal protection against maize fusariosis is to verify the influence of the anti-fungal b-32 protein in the containment of mycotoxins, mainly fumonisins (Duvick, 2001). This finding will help to develop plants able to contrast infection of fungi producing toxins.

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