Ribosome-Inactivating Proteins in Cereals

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Abstract Plants constitutively accumulate proteins that are either toxic or inhibitory against pathogens, including ribosome-inactivating proteins (RIPs) and *N*-glycosidases that depurinate the universally conserved α-sarcin loop of large rRNAs. Cereal RIPs share a high similarity with all the other RIPs; however, they retain characteristic features forming a distinct class which diversified significantly during evolution**.** They appear involved in several different physiological roles, such as defense against pathogens and/or involved in regulatory and developmental processes. RIPs from cereals generally have low activity against plant ribosomes. In this chapter are reported recent advances in research related to cereal RIPs, with particular emphasis to the maize RIP (b-32) expressed in transgenic plants as an antifungal protein and reliable tool in crop disease management programs.

1 Introduction

Ribosome-inactivating proteins (RIPs) are a widely distributed family of toxic plant proteins that catalytically inactivate eukaryotic ribosomes (Barbieri et al. 1993; Metha and Boston 1998). RIPs function as *N*-glycosidases to depurinate the universally conserved α-sarcin loop of large rRNAs, selectively cleaving an adenine residue at a conserved site of the 28S rRNA (26S rRNA in yeast), such as the adenine$_{4324}$ of rat liver 28S rRNA (Endo and Tsurugi 1988). This depurination inactivates the ribosome, thereby blocking its further participation in protein synthesis; in particular, this irreversible modification 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 (Barbieri et al. 1993; Metha

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and Boston 1998; Endo and Tsurugi 1988; Nielsen and Boston 2001; Peumans et al. 2001; van Damme et al. 2001).

Although, RIPs were first identified more than 25 years ago, their biological function(s) still remains open to speculation. In addition, to their known activity of depurinating ribosomes at the sarcin/ricin (S/R) loop in vitro, there is no unequivocal answer to the question of why plants synthesize and accumulate RIPs. To summarize and rationalize their biological function in plants, progress on RIPs and a working model have been recently presented and discussed (Park et al. 2004).

It was originally believed that RIPs do not occur universally among plants, pointed out by the failure to identify RIPs in model plant systems such as *Arabidopsis thaliana* or *Nicotiana tabacum*. However, Sharma et al. (2004) have isolated and characterized from *N. tabacum*, a 26-kDa RIP-like protein termed TRIP, possessing *N*-glycosidase activity. In this context, it was found that TRIP is expressed in the leaf at very low levels (≈0.01% fresh weight), a finding that suggests the difficulty in identifying an RIP from tobacco. This discovery raised the possibility that other plant species also possess one or more *N*-glycosidases. Accordingly, it has been shown that plants possess multiple RIPs and their activity has been found in different organs (seed, root, leaf) in concentrations ranging from few micrograms to several hundred milligrams per 100 g of tissue (Stirpe et al. 1992; Hartley and Lord 1993).

2 Classification of RIPs

On the basis of their physical properties, RIPs are classified into three classes (Metha and Boston 1998; Mundy et al. 1994). Type 1 RIPs such as Pokeweed Antiviral Protein (PAP), saporin (from soap-wort, *Saponaria officinalis* L.), trichosanthin, gelonin, and barley seed RIP (RIP30) 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; Stirpe et al. 1980; Asano et al. 1984; Maraganore et al. 1987; Yeung et al. 1988). Type 1 RIPs are only weakly toxic to intact cells, although their enzymatic activity appears to be at least greater than that of type-2 RIPs (Barbieri et al. 1993).

Type 2 RIPs, such as ricin and abrin, are highly toxic heterodimeric proteins with enzymatic and lectin properties in separate polypeptide subunits, each of an approximate MW 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 1973a; Olsnes and Pihl 1973b; Stirpe et al. 1978). The B-lectin chain can bind to galactosyl moieties of glycoproteins and/or glycolipids that are found on the surface of eukaryotic cells and mediates retrograde transport of the A-chain through the secretory pathways into the cytosol (Beaumelle et al. 1993; Sandvig and van Deurs 1994). Therefore, when it reaches the cytosol, the A-chain of the RIP has access to the translational machinery and inactivates ribosomes interrupting protein

synthesis. Type 2 RIPs were valuable for investigating endocytosis and intracellular transport in mammalian cells (Lord and Roberts 1996; Sandvig and van Deurs 1999; Hazes and Read 1997). Only some type 2 RIPs, namely ricin, abrin, modeccin, volkensin, and viscumin, are highly toxic to cells and animals; on the contrary, others type 2 RIPs, namely ebulin, nigrin, cinnamomin and iris lectin are not toxic, the reason(s) for the difference being still unknown.

Type 3 RIPs, such as maize b-32 and barley JI60 (Walsh et al. 1991; Chaudhry et al. 1994), are synthesized as single-chain proenzymes, inactive precursors (pro-RIPs) that require proteolytic processing events to produce two noncovalently linked chains equivalent to a type 1 RIP. Recently, b-32 has been described as a holo-RIP, two-chain type-1 RIP, whereas JIP60 as a chimero-RIP, true type-3 RIP (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: once they are removed, the processed active protein is similar in charge and enzymatic activity to type 1 RIPs (Hey et al. 1995; Krawetz and Boston 2000; Walsh et al. 1991). For maize, the extra domains are unlikely 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; Krawetz and Boston 2000; Bass et al. 1992). For barley, however, induction of JIP60 was reported to coincide with a decrease in protein synthesis followed by a decrease in polysome size (Reinbothe et al. 1994b). Thus, the possibility of JIP60 accumulating as a proenzyme to protect barley ribosomes cannot be ruled out. The mode of uptake of types 1 and 3 RIPs by cells is still unknown.

3 Applied Research on RIPs

Because of their peculiar biological activities towards animals and human cells as cell-killing agents, RIPs have received remarkable attention in biological and biomedical applied research. In fact, due to their selective toxicity, RIPs have been primary candidates for the toxic moiety of immunotherapeutics (Spooner and Lord 1990; Pastan and Fitzgerald 1991; Olsnes and Pihl 1982). Therefore, a great deal of the literature reflects attempts to isolate and characterize RIPs from new plant sources and to exploit these RIPs as anticancer agents (Barbieri et al. 1993; Gasperi-Campani et al. 1985; Stirpe et al. 1992). Studies were also focused on enzymology, uptake of lectin-associated RIPs into target cells, and subsequent transport to ribosomal targets in the cytosol (Lord and Roberts 1998; Olsnes and Sandvig 1988; Sandvig and van Deurs 1999). This research has provided a broad knowledge base for understanding biochemical and therapeutic properties of RIPs. However, less frequent are studies into the biological function of RIPs in plants, even if in recent years, investigations of RIP activities have increased, especially as tools for gene isolation and transgenic expression became available.

RIPs are widely distributed throughout the plant kingdom and are active against ribosomes from different species, though the level of activity depends on the source

of RIP and of the ribosome (Bass et al. 1992; Battelli et al. 1984; Harley and Beevers 1982; Stirpe et al. 1992).

Although, the enzymatic activities of RIPs have been shown in vitro, their role in plant defense is less clearly defined. Historically, RIPs have been linked to plant protection, because crude extracts of pokeweed (*Phytolacca americana*) leaves were first shown to have inhibitory activity against viral infections in plants (Irvin 1975). Work performed to assay extracts from over 50 plant species pointed out that most had translational inhibitory activity in vitro (Barbieri et al. 1993; Gasperi-Campani et al. 1985; Stirpe et al. 1992), while purification of the inhibitory proteins have led to their identification as RIPs. Collectively, this information has promoted several biotechnological approaches to generate transgenic plants to exploit the antimicrobial activity of RIPs. For example, in tobacco, increased virus resistance was achieved with the expression of trichosanthin (Lam et al. 1996), PAP (Lodge et al. 1993), PAP II (Wang et al. 1998), virus-induced dianthin (Hong et al. 1996) and C-terminally deleted, inactive PAP (Tumer et al. 1997); altogether these data indicate that the resistance may not necessarily be linked to *N*-glycosidase activity on "self" ribosomes. Increased fungal resistance against *Rhizoctonia solani* was obtained with PAP II (Wang et al. 1998), and a truncated PAP version (Zoubenko et al. 1997).

4 Properties of Cereal RIPs

RIPs from cereals share a high similarity to all other RIPs, retaining, however, characteristic features which group them into a distinct class which diversified significantly during evolution (Jensen et al. 1999). These proteins appear to be involved in quite different physiological roles, such as defense against pathogens and/or in regulatory and developmental processes (Motto and Lupotto 2004). While some RIPs, such as PAP, are very active against both animal and plant ribosomes, on the other side, RIPs from cereals generally have low activity against plant ribosomes (Madin et al. 2000); an exception to this rule is the RIP of wheat leaves, which can modify plant ribosomes at concentrations where the seed RIP does not (Massiah and Hartley 1995).

4.1 Rice RIPs

A genome-wide identification of the RIP family in rice, based on the complete genome sequence analysis, was recently reported by Jiang et al. (2008), who identified at least 31 members of the RIP family all belonging to the type 1 RIP genes. It was also found that some members of this family were expressed in various tissues with differentiated expression abundances, whereas several members showed no expression under normal growth conditions or various environmental stresses.

On the other hand, the expression of many RIP members appears regulated by various abiotic and biotic stresses. Therefore, the previous authors suggested that specific members of the RIP family in rice might play important roles in biotic and abiotic stress-related biological processes and function as regulators of various environmental cues and hormone signaling. Consequently, they can potentially be useful in improving plant tolerance to various abiotic and biotic stresses by over-expressing or suppressing their genes.

4.2 Wheat RIPs

Tritin, a single-chain RIP in wheat seeds, was first identified as a component of a wheat-germ protein-synthesizing system that caused inhibition of protein synthesis in an ascites cell-free system (Stewart et al. 1977); this protein represents approximately 2% of the total soluble protein in mature wheat seeds (Coleman and Roberts 1982). Furthermore, two distinct forms of RIPs were purified from wheat germ and leaves, termed tritin-S and tritin-L, respectively; these forms differ in size and charge and are antigenically unrelated (Massiah and Hartley 1995). Tritin-S and tritin-L differ in both their ribosome substrate specificities and cofactor requirements: tritin-S shows only barely detectable activity on ribosomes from the endosperm, whereas tritin-L is highly active on leaf ribosomes; tritin-S, unlike tritin-L shows a marked requirement for ATP in its action (Massiah and Hartley 1995). The finding that tritin-S is inactive on wheat-germ ribosomes is consistent with the observation that the genomic sequence does not encode an *N*-terminal signal sequence (Habuka et al. 1993); from this it can be inferred that the RIP accumulates in the cytosol in contact with ribosomes.

Sawasaki et al. (2008) recently reported that: (1) tritin RIP activity could be one of the key steps in the development of senescence in wheat coleoptiles (2) transgenic tobacco plants expressing glucocorticoid-induced tritin developed senescence-like phenotype. The above mentioned data confirm previous indications about the induction of RIP activity in stressed leaves, including senescent leaves, of several plant species (Stirpe et al. 1996; Rippmann et al. 1997).

4.3 Barley RIPs

In the barley endosperm, three similar type 1 RIP isoforms – I, II (RIP30), and III – have been identified and described. The RIP30 isozyme fraction showed 50% inhibition of RNA translation (reticulocyte lysate) at concentration of 3–30 nM (Asano et al. 1986; Leah et al. 1991). Barley RIP30 inactivates rat liver ribosomes in the same manner as ricin A-chain by hydrolysing a single *N*-glycoside bond at A_{4324} of 28S rRNA to release adenine (Endo and Tsurugi 1988). Additionally, this barley toxin has been shown to be especially active on isolated fungal ribosomes of

Neurospora crassa (Roberts and Selitrennikoff 1986). RIP30 is a cytosolic protein lacking a signal peptide extension and it is probably only weakly active or completely inactive on ribosomes of the producing cells (Leah et al. 1991). The starchy endosperm-specific deposition of RIP30 suggests that it may also function as an albumin in storage polypeptide. Starchy endosperm cells differentiate terminally during development and are metabolically senescent at maturity. Therefore, it is likely that RIP30, despite its inhibitory specificity towards "foreign" ribosomes (Stirpe and Hughes 1989), is only mildly cyotototoxic to barley cells. If this is the case, starchy endosperm cells apparently form one of the tissues where high levels of ribosome-inactivating proteins accumulate in cereal plants. These proteins are likely potential determinants of the terminally differentiated fate of this cell type. However, addition of barley RIP30 was inhibitory to in vitro translation and to fungal growth on solid media when tested against *Trichoderma reesei* (Leah et al. 1991), suggesting a protective role. On the other hand, the same authors reported that inhibition of growth in liquid media was minimal with barley RIP alone, but increased dramatically when β-1,3-glucanase or a chitinase, or both were included (Leah et al. 1991).

Studies with the type 3 barley jasmonate-induced RIP, termed JIP60, suggested that ribosome susceptibility to RIPs is a dynamic process than an innate property. Incubation of polysomes with JIP60 resulted in a shift to monosomes only if the polysomes had been prepared from stressed leaf tissue that had undergone 36 h of desiccation or 24 h of methyl jasmonate treatment. On the other hand, water-treated controls had no change in polysome size (Reinbothe et al. 1994a,b). However, these indications should be carefully interpreted, because ribosomes were not assayed for depurination at the α-sarcin loop; moreover, it was reported that JIP60 is not competent for translational inhibition unless proteolytic processing with at least two cleavage events has occurred (Chaudhry et al. 1994).

4.4 Maize RIPs

In maize, proRIP – classified as type 3 RIPs (Nielsen and Boston 2001) or, as two-chain type 1 RIPs (van Damme et al. 2001), are present in at least two forms encoded by nonallelic genes, one expressed in the endosperm (Walsh et al. 1991; Di Fonzo et al. 1986, 1988) and the other in leaf tissues (Bass et al. 1995). The maize endosperm RIP (b-32) has been widely investigated (Motto and Lupotto 2004).

This protein is a cytosolic albumin with a molecular weight of 32 kDa synthesized in temporal and quantitative coordination with the deposition of storage proteins (Soave et al. 1981a; Barbieri et al. 1997); it is present in the endosperm as inactive zymogen (proRIP), representing up to 1% of the total seed proteins (Soave et al. 1981b).

N-terminal, C-terminal and internal domains can be enzymatically removed from proRIP to yield two chains α–β that interact noncovalently 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 1–16), a 25 amino acid residue of 2708 D from the acidic central region of polypeptide (residues 162–186), and 14 amino acids of 1336 D from the C-terminus (residues 289–301) (Hey et al. 1995). The two final peptides of 16.5 and 8.5 kDa generated, tightly linked in a noncovalent manner, represent the activated form of RIP, termed αβ-RIP (Walsh et al. 1991). The activated form inhibits translation in a cell-free rabbit reticulocyte system with an IC_{50} (concentration causing 50% inhibition) of 28–66 pM, at least 10,000 times more active than the proRIP (Walsh et al. 1991). Further support for a proteolytic activation of proRIP 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 nonspecific proteases such as papain and subtilisin Carlsberg (Chaudhry et al. 1994), thus demonstrating that the RIP activation is due to a proteolytic processing of central acidic domain. Bass et al. (1992) have identified a second maize RIP that appears to require both N-terminal and internal processing events for enhancing its enzymatic activity. This second RIP shows no enhanced expression in the kernel, but it appears expressed in all maize tissues.

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 and Walsh 1976; Neurath 1989). There is no evidence that maize endosperm RIP b-32 is housed in intracellular organelles or secreted (Walsh et al. 1991; Di Fonzo et al. 1986); the maize proRIP is, in all cases described, a cytosolic protein not secreted via the endoplasmic reticulum (ER). This feature distinguishes the maize RIP from many other plant RIPs such as ricin (Lamb et al. 1985), trichosanthin (Chow et al. 1990) or momorcharin (Ho et al. 1991).

Gene expression studies have demonstrated that the *b-32* gene, as well as genes encoding the 22 kDa zeins, are coordinately controlled by the endosperm regulatory locus *Opaque-2* (*O2*) (Soave et al. 1981a, b). O2 protein belongs to the b-ZIP family of transcriptional regulators (Hartings et al. 1989), 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 defense against pathogens is therefore suggested by an increased susceptibility of *o2* mutant kernels, where the level of b-32 is greatly decreased, to fungal attack (Loesch et al. 1976; Warren 1978) and insect feeding (Gupta et al. 1970). Moreover, the results obtained by testing a set of pure inbred lines, and their isogenic *o2*-mutants, in field experiments with Silk Channel Inoculation Assay (SCIA) on adult plants, showed that the *o2* mutants resulted significantly more susceptible to the *Fusarium verticillioides* attack than the normal version (Balconi et al. 2005). The increased susceptibility in the absence of the proRIP b-32 is consistent with a defense function, although the experimental results cannot be attributed to the maize proRIP b-32 a priori because O2 regulates transcription of a large number of genes that may contribute to

a complex mutant phenotype (Hartings et al. 2009). These observations suggest that it would be interesting to verify if the expression of b-32 in an *o2* mutant could increase the tolerance to fungal pathogen attack in the kernel.

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

5 Transgenic Plants Expressing RIPs

The ectopic expression of antimicrobial proteins in plants or plant tissues has the potential to limit pathogen infection or growth. In this perspective, a successful strategy would be to deploy an antifungal protein (normally expressed in the kernel) in a nonseed tissue that is a site of infection (e.g., silk, husk, leaves). Ectopic expression of RIPs in transgenic plants may solve this infection 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 were used to test defense properties attributed to this group of proteins (Hartley et al. 1996; Punja 2001). The type 1 barley RIP expressed under a 35S-CaMV promoter or a wound-inducible promoter in tobacco conferred a reduction of disease symptoms caused by the fungus *R. solani*; 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). In contrast, 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 (Bieri et al. 2000). In the last cited study, the RIP30 was targeted through the ER to the apoplastic space in order to ensure the presence of RIP at the place where initial interactions with the fungus occur; RIP30 was effectively localized to the intracellular space, and the intercellular wash fluids (IWF) of expressing transgenic wheat lines, strongly inhibited a rabbit reticulocyte

lysate transcription/translation system, but the antifungal effects of RIP30, as assayed by infection of detached leaves with *E. graminis*, were small (Bieri et al. 2000). Likewise, RIP30 driven by the strong maize constitutive *ubiquitin-1* promoter was introduced in wheat and transgenic plants engineered with three single antifungal genes – *RIP30*, *Ag-AFP* and *chitinase-II* – then challenged for response to powdery mildew and rust; the results obtained showed a significant reduction of infection in Ag-AFP and Chi-II expressing plants, but not in RIP30-expressing wheat lines compared with the control (Oldach et al. 2001).

Further studies have demonstrated that 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 should enhance the efficacy of antifungal proteins and peptides in transgenic plants.

Transgenic rice plants expressing the maize RIP b-32 have been produced by Kim et al. (1999). These workers noted that the level of the b-32 expression was approximately 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). The data reported in this research indicated 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 protein processing was detected in mature leaf tissues (Kim et al. 1999). The authors also reported that disease severity caused by infection with the fungal pathogens *R. solani* and *Magnaporthe grisea*, was not significantly reduced in the transgenic plants expressing the RIP b-32 as compared to control plants, suggesting that processing of b-32 protein may be required to exhibit antifungal activity *in planta*. Whether transgenic plant fungal resistance requires proteolytic processing of the maize proRIP (b-32) is not clearly defined. In fact, a research developed in our laboratory showed that transgenic tobacco plants, expressing the maize RIP b-32 gene driven by the *wun 1* promoter, had increased protection against infection of the soil-borne fungal pathogen *R. solani* (Maddaloni et al. 1997).

Similarly, further research carried out in our laboratory with wheat transgenic lines, indicated that maize RIP b-32 protein was effective, as an antifungal protein, in reducing Fusarium head blight (FHB) symptoms (Balconi et al. 2007). Transgenic approaches to combat FHB in wheat and barley were recently reviewed by Dahleen et al. (2001). They pointed out that various degrees of protection against FHB may be achieved by introducing, *in planta*, heterologous genes encoding for proteins with anti*Fusarium* activity.

A variety of antifungal genes have been isolated: some of their products have been shown to inhibit *Fusarium* growth in vitro and *in planta* (McKeehen et al. 1999). Our experiments to evaluate the action of the protein RIP b-32 in transgenic wheat plants, confirmed an increase of FHB resistance. 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. The six homozygous transgenic lines used for the investigation were all phenotypically

normal when compared to the parental nontransgenic cv. Veery, except for a slightly smaller size, were fully fertile and set seeds, confirming that expression of the exogenous RIP did not interfere with normal plant development. B-32 expression was maintained at comparable levels during various developmental stages of the transgenic wheat plants which include the seedling stage, tillering, and up to 10 days after anthesis. It was also interesting to note that no endogenous b-32 expression was observed in control wheat plants, while the typical pattern (a double banding) was observed in the transgenic line at the three stages of development at comparable levels, and in the control maize endosperm W64A, as expected (Balconi et al. 2007).

A similar pattern of b-32 expression was also detected in immature spikelets and rachis. Collectively, these results confirmed a stable expression level of b-32 in green tissues of transgenic lines throughout their development. The comparison of b-32 amounts in protein leaf extracts of transgenic lines at the heading stage allowed the identification of lines with high, intermediate, and low b-32 content in leaves. This finding is a useful range of expression for pathogenicity experiments, in order to evaluate an eventual differential response to fungal pathogens attack. Resistance to FHB was evaluated by the "single floret injection inoculation method" on immature spikes of the b-32 transgenic wheat lines in comparison to the parental cv. Veery, with spores of *Fusarium culmorum*. The plants were analyzed for FBH by visual inspection and the data suggest that protection due to the presence of b-32 was not dependent on increasing levels of the RIP protein in the tissues, but also that the lowest level of b-32 was effective.

In this study, it was also observed, by applying another index associated with tolerance, such as the percentage of "tombstones" (shriveled, light weight, dull grayish or pinkish in color kernels)/total seeds, recorded at maturity, that independently from the differential b-32 content of the transgenic lines, the percentage of "tombstones" was equally reduced in all cases, in comparison with control cv. Veery plants (Balconi et al. 2007). Therefore, disease control by b-32 protein was observed as a reduction of visible FHB symptoms, early after inoculation, reflected in reduced fungal colonization after artificial single spikelet inoculation and also at maturity, as a reduction in damaged seeds percentage.

Collectively, these results indicate that RIP b-32 was effective as an in vivo antifungal protein in wheat, which normally does not produce this protein. In fact, one of the most devastating fungal diseases of wheat was therefore controlled at significant level.

To further explore the antifungal activity of the maize RIP b-32, Lanzanova et al. (2009) developed transgenic maize plants containing the *b-32* coding sequence driven by a constitutive *35S-CaMV* promoter were obtained through genetic transformation. In this study four homozygous independent maize transgenic lines, with differential ectopic expression of b-32 (SM 3.4; SM 16.1; SM 19.4; SM 20.2), were evaluated, in comparison with plants expressing b-32 only in the endosperm (SM 20.4), for response to *F. verticillioides* colonization by leaf tissue bioassays. All transgenic plants were phenotypically normal, when compared to the negative control and fertile, confirming that the ectopic expression of the b-32 RIP did not

interfere with normal plant development, as previously underlined (Maddaloni et al. 1997; Balconi et al. 2007).

The evaluation, in the B73 inbred line and in the negative control (SM 20.4), of b-32 expression at the protein level, in endosperm and leaf tissues, confirmed the endosperm-tissue specificity of this gene (Fig. 1, lanes 1 and 3); it is also evident in the same figure that those materials did not exhibit any expression of cross-reacting proteins in leaf tissues (lanes 2 and 4, respectively). On the other hand, SM 20.2 transgenic progeny showed detectable b-32 cross-reacting bands both in leaf and endosperm tissues (Fig. 1, lanes 5 and 6).

Additionally, proteomic analyzes on leaf extracts showed that the presence of both the b-32 and herbicide resistance enzyme, were the only significant variations detected between the transgenic and the Basta-sensitive progeny protein profiles. The identification of progeny with a differential b-32 expression in the leaves was useful for setting up pathogenicity experiments. These were aimed at evaluating a possible differential response to a *Fusarium* attack in leaf tissue colonization bioassays.

A comparison of b-32 protein amounts in leaf extracts at flowering stage, performed by immunoblot image scanning, manifested a differential b-32 expression among the various progeny. Progeny SM 3.4, SM 16.1 and SM 20.2 showed b-32 contents significantly higher than observed in progeny SM 19.4; as expected, SM 20.4, i.e. negative control, showed nondetectable b-32 content (n.d.) in leaf tissues.

The transgenic progenies expressing b-32 in leaf tissues were less susceptible than the negative control, when evaluated for response to the *F. verticillioides* attack, showing significantly reduced colony diameter around the inoculated leaves (Table 1). Similar results were reported concerning blast inoculation assays conducted on detached leaves of transgenic rice plants expressing the antifungal AFP protein (Coca et al. 2004). A good correlation between the b-32 content in the leaves and the level of resistance to *Fusarium* attack was observed (Table 1). In particular, data in this table indicated that 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. The expression of herbicide resistance protein (the enzyme, phosphinothricin acetyltransferase) apparently does not interfere with mechanism(s)

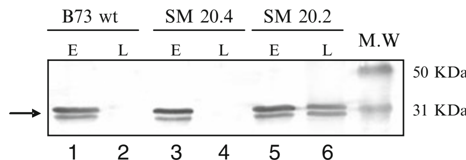

Fig. 1 Western-blot analysis of b-32 expression at protein level. Endosperm and leaf tissue respectively of inbred line B73 (*lanes 1 and 2*), negative control line SM 20.4 (*lanes 3 and 4*), and transgenic line SM 20.2 (*lanes 5 and 6*), were tested for the presence of b-32 protein (modified from Lanzanova et al. 2009)

Table 1 Severity of *Fusarium* leaf tissue colonization and suppression of *Fusarium* leaf colonization in the b-32-expressing transgenic progenies (modified from Lanzanova et al. 2009)

Progenies	Days after inoculation (DAI)			
	3 DAI		7 DAI	
	Colony diameter (mm)[a]	Growth inhibition (%)[b]	Colony diameter (mm)[a]	Growth inhibition (%)[b]
SM 20.4	7.6 ± 0.5	–	25.0 ± 0.0	–
SM 3.4	2.7 ± 0.9	64.0 ± 11.3	12.3 ± 0.9	50.8 ± 3.5
SM 16.1	2.9 ± 0.6	61.9 ± 7.7	12.8 ± 1.3	47.3 ± 3.7
SM 19.4	5.3 ± 0.7	30.6 ± 7.8	18.9 ± 0.9	23.0 ± 3.2
SM 20.2	2.7 ± 0.5	64.2 ± 7.9	12.6 ± 0.5	49.8 ± 2.0
LSD (0.05)	0.8	12.7	0.9	3.3

[a]Triplicate samples measured as mycelial radial growth 3–7 days after *F. verticillioides* leaf tissues inoculation (10^6 spores/ml) in the negative control (SM 20.4 progeny) and in the transgenic progenies. All values were analyzed by MSTAT-C-Program (Michigan State University, Version 1991)

[b]Percent of radial growth inhibition compared to negative control, SM 20.4 progeny, calculated individually for each replicate before statistical analysis

involved in *F. verticillioides* maize leaf colonization; the increased resistance to *Fusarium* colonization, observed in the transgenic progenies is attributable to the expression of b-32, excluding potential additional transgenic effects (Lanzanova et al. 2009).

Even though the two low molecular weight peptides, reported to be the activated form of RIP (Walsh et al. 1991), were not detected in the transgenic leaf tissues, the expression of b-32 protein (reported to be the 32 kDa proenzyme, proRIP) supported the increase of *Fusarium* leaf colonization resistance. This result is in accordance with previous studies on tobacco, showing that transformed plants were more tolerant to *R. solani* infection than the negative control even if no low molecular weight immunoreactants were detectable with b-32 antiserum (Maddaloni et al. 1997).

In all tested transgenic progenies and in the negative control, 100% of inoculated leaf squares showed *Fusarium* colonization (data not shown) suggesting, as expected, 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 for FHB, b-32 crop protection may be due to the disease being limited in its spread in all directions from the point of inoculation (PI) (Balconi et al. 2007).

An important issue in view of fungal protection against maize fusariosis is to verify the influence of the engineered antifungal b-32 protein in the containment of mycotoxins (fumonisins, FB) in the plants infected by *F. verticillioides* (Duvick 2001). Since most mycotoxin problems develop in the field, strategies are needed to prevent infection of growing plants by toxigenic fungi (Munkvold 2003). 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, could be very useful to limit the fungal infection from spreading

to the exposed silks and consequently to reduce the grain fumonisin contamination (Lauren and Di Menna 1999). In this respect, various benchmarks for the success of this approach to FB reduction will all determine the extent of disease tolerance in a transgene. These include: (1) protein localization in the plant tissue in relation to fungal substrate accessibility; (2) kinetic parameters of the enzyme(s) in the context of its interaction with the plant substrate (substrate Km, pH optimum, substrate range, potential inhibitors); (3) stability and activity of the enzyme during plant growth and development conductive to fungal growth. Equally important will be experiments that verify the nutritional properties of the transgenic grain under various environmental conditions.

6 Conclusions

In spite of RIPs having been thoroughly investigated as potentially useful toxins, their role and function in plants and their distribution in nature remain enigmatic. Use of the recently identified RIP from *N. tabacum* (Sharma et al. 2004), in conjunction with genomic and biotechnological approaches, such as mutagenesis and gene silencing, appear as an effective strategy to bridge the existing gaps in our knowledge. In the future, it is likely that progress in this field will accelerate, leading to elucidation of the biological function of RIPs and establishing their fundamental role in the plant cell and their potential significance in several therapeutic applications, including the preparation of immunotoxins targeting tumors and hematological malignancies.

The maize b-32 RIP is one of the most thoroughly investigated cereal RIPs because of its peculiar presence, action, processing, and effects in the maize kernel physiology. In addition, its presence in connection to regulatory processes involved in the zein storage protein deposition, as well as direct evidence of a protective role of the seed, render the maize RIP an interesting matter of investigation as a multifunctional system of the plant biology. Despite of the body of work performed, several topics correlated to RIP b-32 role in the maize kernel, remain to be clarified. RIP b-32 is expressed under the control of the seed-specific transcriptional activator *O2* and is synthesized as zymogen that increases its catalytic activity after proteolytic activation. B-32 accumulates in the kernel as a proenzyme (proRIP) accounting for 1–3% of the soluble protein; therefore its role is also consistent with a storage albumin function. This RIP clearly shows unusual regulatory properties at both the gene and protein level. The differences in maize RIP and other RIPs might also be useful in understanding the mechanisms involved in physiology and biochemistry of endosperm development and reserve mobilization.

Attempts to use RIPs as biological pesticides or cell-killing agents can benefit from information about the mechanism of RIP uptake by pathogens and about their protective role in transgenic plants. The effectiveness of an antifungal 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 defense protein against fungi.

Recent results from our laboratory (Lanzanova et al. 2009), in agreement with other findings reported for tobacco and wheat (Maddaloni et al. 1997; Balconi et al. 2007), confirm that the incorporation of maize *b-32* gene and the ectopical expression of b-32 RIP protein, may represent an effective and reliable tool in crop disease management programs.

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