The Genetics and Properties of Cereal Ribosome-Inactivating Proteins

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Abstract: Plants contain proteins that are capable of inactivating ribosomes, commonly referred to as Ribosome Inactivating Proteins (RIPs). These particular plant proteins have received attention in biological and biomedical research because of their unique biological activities towards animals and human cells as cell-killing agents. Some of the best-characterised RIPs have been isolated from exotic plants, but they have also been found in cereals and other food crops. Cereals contain, in general, RIPs in the endosperm protein pool: they 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 quite different physiological roles, such as defence against pathogens and/or involved in regulatory and developmental processes. This review aims to provide a critical assessment to work related to cereal RIP with particular emphasis to the maize RIPs.

Keywords: Cereal species, Barley, Maize, Ribosome-inactivating protein.

INTRODUCTION

Plants contain proteins that are capable of inactivating ribosomes, commonly referred to as ribosome inactivating proteins (RIPs) [1-4]. These particular plant proteins have received attention in biological and biomedical research because of their unique biological activities towards animals and human cells as cell-killing agents [5-7]. A single RIP molecule is able to depurinate 1000-2000 mammalian cell ribosomes per minute under physiological conditions [8, 9]. Although RIPs display a myriad of properties, comprising anti-viral [10], anti-tumoral, immunosuppressive, embryotoxigenic [11], and enzymatic activities [1, 4]. It is evident, however, that plants do not produce RIPs just to fulfil the requirements of modern mankind for antitumor and antiviral drugs. Despite our detailed knowledge on the structure, activity, and action mechanisms of RIPs, there is no unequivocal answer to the question of why plant synthesize and accumulate RIPs. RIPs are present in many, probably not all, plant species. Evidence is accumulating that some RIPs play a role in plant defence and therefore can be exploited in plant protection but their biological role in plants is at present unclear [2]. Evidence for the lack of RIPs has been obtained only for *Arabidopsis thaliana*, as this plant apparently does not express detectable amounts of RIPs nor contains a sequence encoding a putative RIP in its genome [12]. This implies that RIPs are not ubiquitous and do not play a universal role in the growth, development, or protection of plants [4]. However, the question remains as to why some plants produce RIPs. This review aims to provide a critical assessment to work related to cereal RIP activities with particular emphasis to the maize RIPs.

PROPERTIES OF RIBOSOME INACTIVATING PROTEINS

RIPs are widely distributed cytotoxic enzymes that are found in over 100 different plant species and catalytically inactivate eukaryotic, and in some cases prokaryotic, ribosomes (reviewed in [1] and [13]). RIPs inhibit protein synthesis by virtue of their N-glycosidase activity, 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 [9]. 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 (reviewed in [2]).

It has been shown that plants possess multiple RIPs and their activity has been found in different organs (seed, root, leaf, lattices, and tubers depending on the species) in concentrations ranging from a few micrograms to several hundred milligrams per 100 gr of tissue [6, 14]. RIPs are stable proteins that are subdivided into three classes [13]. Type 1 RIPs such as Pokeweed Antiviral Protein (PAP), trichosanthin, gelonin, and barley seed RIP (RIP30) have basic isoelectric point and are monomeric enzymes of approximately 30 kDa [15-19]. In addition, 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, are heterodimeric proteins each with an approximate molecular weight of 60 kDa in which one polypeptide with RIP activity (A-chain) is linked by a disulphide bridge to a galactose-binding lectin (B-chain) [20-22]. The B-chain can bind to cell surfaces and mediates retrograde transport of the A-chain through the secretory pathways into the cytosol, where the A-chain inactivates ribosomes and readily disrupts protein synthesis [23]. Only some type 2 RIPs, namely ricin, abrin, modeccin, volkensin, and viscumin, are highly toxic to cells and animals, whereas other, namely ebulin, nigrin, cinnamomin, iris lectin are not toxic, the reason(s) for the difference being still unknown. Type-3 RIPs such as maize b-32 and barley JIP60 [24, 25] are formed from a larger inactive precursor by proteolytic cleavage. Their final structure resembles type-1 RIPs. (Fig. **1**). More 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 [4].

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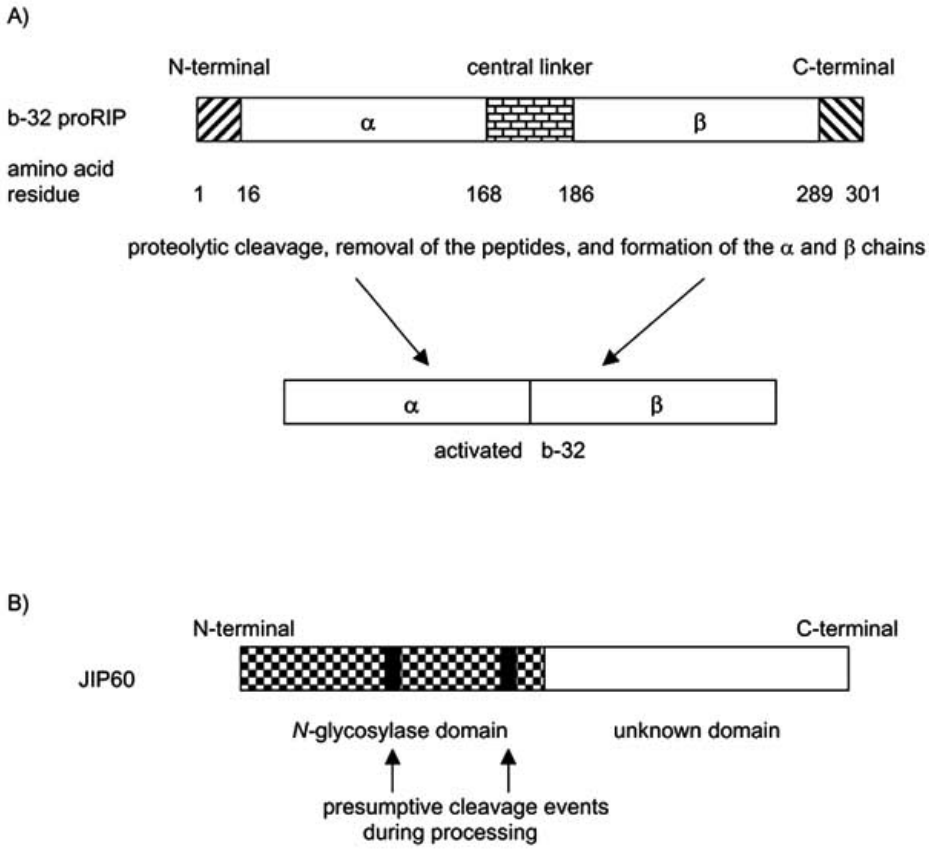

Fig. (1). Schematic representation of the molecular structure of two cereal RIPs, the maize b-32 and the barley JIP60. A) Structure of the b-32 pro-RIP form and the proposed mechanism by which the pro-RIP becomes the activated αβ-RIP, upon cleavage of the internal, and N- and C-terminal aminoacid sequences; B) General structure of the barley RIP JIP60, consisting of two distinct domains, the N-glycosidase domain, and the unknown domain. In black are marked the positions of two small polypeptides likely removed during processing (25).

CEREAL RIBOSOME INACTIVATING PROTEINS

Some of the best-characterised RIPs have been isolated from exotic plants, but they have also been found in cereals and other food crops [6, 26]. 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 [27]. Interestingly, in general, cereal crops contain RIPs in the endosperm protein pool. They appear to be involved in quite different physiological roles, such as defence against pathogens and/or in regulatory and developmental processes.

In the barley endosperm, three similar RIP isoforms I, II (RIP30), and III have been identified and described [28, 29]. The RIP30 isozyme fraction showed 50% inhibition of RNA translation (reticulocyte lysate) at concentration of 3-30 nM [28, 29]. This barley toxin 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 [9]. In particular, RIP30 has been shown to be especially active on isolated fungal ribosomes of *Neurospora crassa* [30].

The nucleotide sequence of the barley RIP30 cDNAs and the deduced amino acid sequence of the encoded protein, indicate a GC-rich open reading frame encoding a polypeptide of 29,976 Da [28, 29]. In addition, Leah *et al.* [29] found that the open reading frame starts with a ATG just before the first amino acid of the mature RIP30 protein sequence, indicating that the protein encoded by cDNA RIP30 is the native form. This suggests that RIP30 is a cytosolic protein lacking a signal peptide extension and that it is probably only weakly active or completely inactive on ribosomes of the producing cells. Furthermore, the starchy endosperm-specific deposition of RIP30 suggests that it may also function as an albumin storage polypeptide. Starchy endosperm cells differentiate terminally during development and are metabolically senescent at maturity. It is possible that RIP30, despite its inhibitory specificity toward "foreign" ribosomes [31], is mildly cytotoxic to barley cells. In this case, starchy endosperm cells would form one of the tissues where high levels of ribosome inactivating proteins could accumulate in cereal plants. These proteins might even be determinants of the terminally differentiated fate of this cell type. However, its inhibitory activity, measured by *in vitro* translation and fungal growth assays, suggests that it may play a protective role [29]. In particular, in combination with either a β-1, 3-glucanase or a chitinase, or both, it synergistically increased anti-fungal effects.

In maize, pro-RIPs are acidic proteins classified as type 3 RIPs [2] or, more recently, as two-chain type 1 RIPs [4], present in at least two forms of non-allelic genes, one in the endosperm [24, 32, 33] and the other in leaf tissues [34]. The maize endosperm RIP (b-32) is the most well-studied. It is present in the endosperm as inactive zymogen (pro-RIP1), representing up to 1% of the total seed proteins [35, 36]. Activation of the N-glycosidase activity occurs during seed germination because of the proteolytic cleavage [37]. It has been hypothesized that also the maize RIP b-32 has various roles in the seed protein synthesis machinery as well as involvement in other regulatory and defence processes. Because RNA-binding proteins participating in pathways that regulate development at the post-transcriptional level have been described [38], it is also attractive to speculate that b-32 may be involved with the translation machinery of the maize endosperm cells, for example enhancing zein synthesis.

IDENTIFICATION OF MAIZE RIBOSOME-INACTIVATING PROTEINS

A major progress in our knowledge of RIP gene expression is related to the finding that the maize pro-RIP was identical to a previously characterised maize protein, b-32 [32, 35, 36]. The b-32 protein of maize endosperm is a monomeric albumin with an apparent molecular weight of approximately 32 kDa, existing in different genotypes in two isoelectric forms: one with pI 5.8 and the second with pI 6.0. The two variants show similar amino acid composition, but minor differences are exhibited by their tryptic peptide maps. The protein is localised in the soluble part of the cytoplasm and does not bind to any particulate structure [35, 36].

The cDNA coding for the b-32 protein has been cloned and the complete amino acid sequence of the protein derived [33]. The nucleotide sequence shows that several internal repeats are present. The protein has a length of 303 amino acid residues and its sequence shows characteristic features (Fig. **2**). It contains seven tryptophan residues, an amino acid absent in maize storage proteins; polar and hydrophobic residues are spread along the sequence, and several pairs of basic residues are present in the N-terminal region. The secondary structure allows the prediction of two structural domains for the b-32 protein that would fold up giving rise to a globular shape. In addition, the maize b-32, as the very homologous barley RIP30, does not appear to possess the typical leader peptides indicative of organellar targeting.

The b-32 protein is likely the product of a single gene or a small gene family as reported by Hartings *et al.* [39]. These authors, by comparing sequences of the b-32 genomic clones, showed that although the deduced N- and C-terminal sequences were identical, the central domains were very different. When the sequence of the cDNA clone coding for b-32 was analysed in the database, it was found that it was in part homologous to the barley RIP30 [40]. According to these indications, endosperm-derived native b-32 was tested *in vitro* in a cell free reticulocyte lysate system. The results demonstrated that b-32 was able to inhibit protein synthesis, at values similar to those reported for the barley grain inhibitor RIP30 [41]. In addition, further characterisation of b-32 as an active RIP was produced in the work of Bass *et al.* [42], where purified b-32 was shown to enzymatically inactivate ribosomes both for its capacity to specifically modify rRNA and to inhibit protein synthesis *in vitro*. In this study, RNA modification assays were performed using purified endosperm-b-32 and target ribosomes from a rabbit reticulocyte cell-free translation lysate. The aniline reaction clearly demonstrated the specificity of the b-32 RIP activity with the detection of a 425 nucleotide band; the result was also indicative that b-32 itself was not acting as an endo-ribonuclease.

NOVEL FEATURES OF MAIZE RIBOSOME-INACTIVATING PROTEINS

In maize the unusual RIP is synthesized specifically in kernel endosperm as a relative native 32 kDa proenzyme (pro-RIP) [24, 42]. 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 [24, 42]. 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 the polypeptide (residues 162 to 186), and 14 amino acids of 1336 D from the C-terminus (residues 289 to 301) [43]. 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 αβ-RIP, a stable, basic protein with pI > 9.0 (Fig. **1A**). 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 pro-RIP [24]. Further support for a proteolytic activation of pro-RIP was found in the demonstration of increases in RIP activity coincident with the onset of protease synthesis and protein degradation during germination [42, 44]. 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 [24], thus demonstrating that the activation is due to a proteolytic processing of the central acidic domain. It is interesting to note that the sequence of the excised 25 residues oligopeptide is not homologous to any other published RIP sequence [24]. Immunological analysis of seed extracts from a variety of species related to maize showed that pro/αβ forms of RIP are not exclusive to maize but are also found in other members of the *Panicoideae*, including *Tripsacum* [43]. The synthesis of inactive precursor forms of enzymes, the zymogens, appears 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 [45, 46]. The extensive excision of internal amin oacid residues described by Walsh *et al.* [24], represents a novel mechanism of enzyme activation in plants and resembles processing of certain hormones, such as insulin [47].

To date, processing of type-1 and type-2 RIPs is limited to N- and/or C-terminal cleavages of pre-proteins or internal cleavages outside of active site domains [48-50]. This unusual internal processing of the type-3 maize seed RIP was found for two additional cereal RIPs. Thus, the maize protein is unique among the RIPs with respect to its

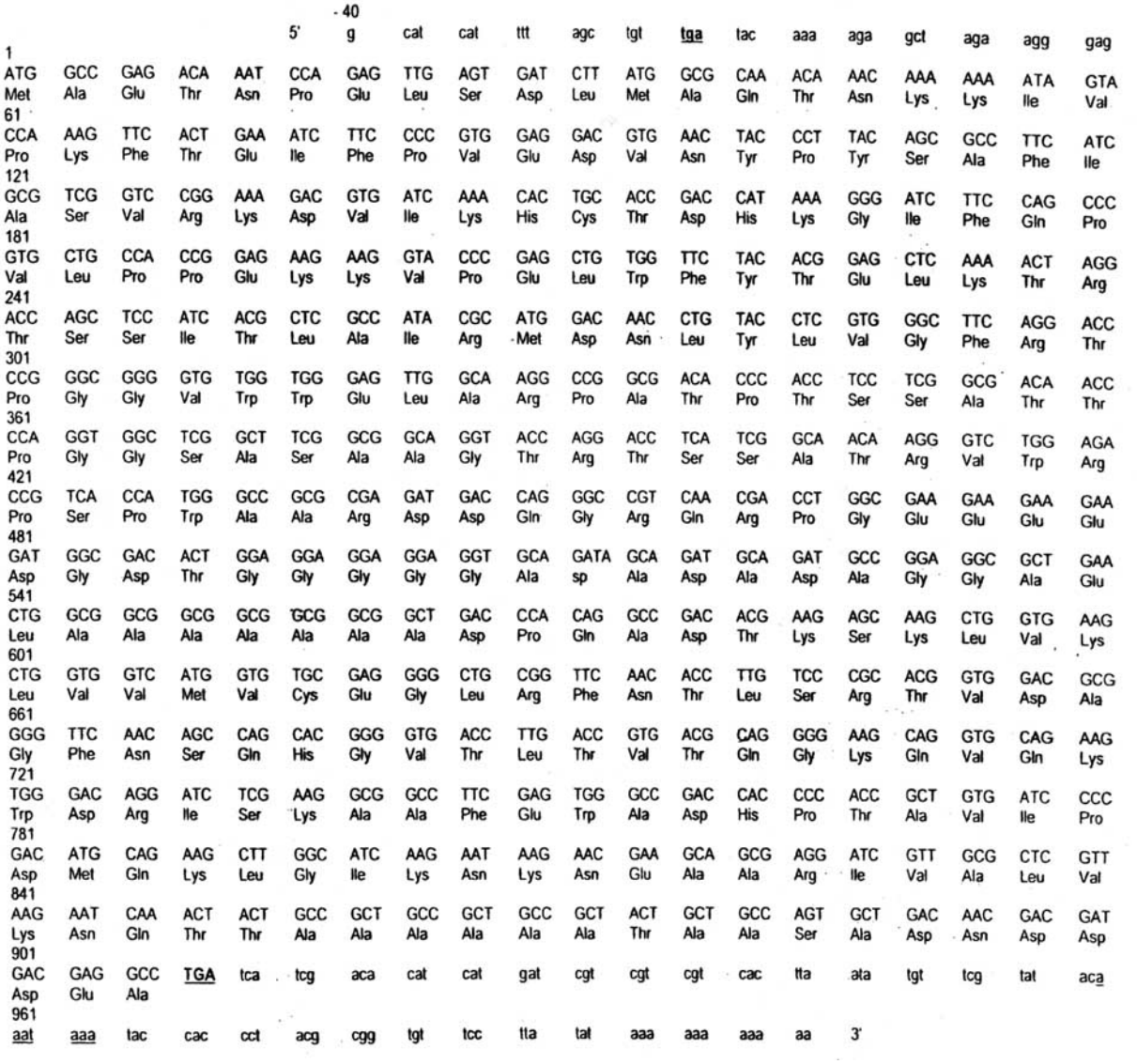

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Fig. (2). Nucleotide and deduced amino acid sequences of the b32.66 cDNA clone. Polyadenylation signal is underlined; the stop codons are in bold underlined. Poly(A) has about 11 residues (Di Fonzo *et al.*, 1988).

activation. Bass *et al.* [42] have identified a second maize RIP that appears to require both N-terminal and internal processing events for maximal enzymatic activity. This second RIP shows no enhanced expression in the kernel, but it appears expressed in all maize tissues. Similarly, Chaudry *et al.* [25] showed that removal of internal and C-terminal domains appeared to confer ribosome-inactivating activity to a 60 kDa barley protein. Synthesis of type-3 RIPs as zymogens is intriguing but the biological significance of this feature is not yet clear. Besides the RNPs (RNA-binding protein motifs) for internal interval processing motifs, the maize RIPs have additional homology with the KH RNA binding motif as well as with the RGG motif [51]. The relevance of these RNA binding motifs to RIP activity is not yet known. Even so, the difference in putative RNA binding domains is consistent with RIPs from different plants being functionally dissimilar and perhaps using slightly different recognition mechanisms.

Relating to the physical localisation of b-32 in the cell, there is no evidence in any of the analysed articles that b-32 has a specific subcellular targeting [24, 32]. The maize pro-RIP is in all cases described as a cytosolic protein not secreted *via* the endoplasmic reticulum; this peculiar feature

distinguishes the maize RIP from other plant RIPs such as ricin [52], trichosanthin [49], or momorcharin [53].

CONTROL OF MAIZE RIBOSOME-INACTIVATING PROTEIN GENE EXPRESSION

A major advance in the knowledge of RIP gene expression accompanied the finding that the maize pro-RIP1 was identical to the previously characterised maize protein b-32 [35, 36]. Both cDNA and genomic clones have been isolated [33, 39, 42]. Additionally, gene expression studies have demonstrated that the b-32 (pro-RIP), as well as genes encoding the 22 kDa zeins, are co-ordinately controlled by the endosperm regulatory locus *Opaque-2 (O2)* [35, 36] (Fig. **3**). *O2* has been identified as a DNA binding protein that affects expression of the major seed storage protein genes, particularly those encoding the 22 kDa α-zeins [54, 55], belonging to the b-ZIP family of transcriptional regulatory proteins [56]. Levels of b-32 and 22 kDa zeins are greatly decreased in *o2* mutants and transcription from the *b-32* (pro-RIP) promoter can be successfully activated by the O2 protein in transient expression assay [40]. In particular, Lohmer *et al.* [40] demonstrated that the O2 protein binds to five sites within 200 bp of the *b-32* promoter, 60 bp upstream of the TATA box consensus sequence. Interestingly, expression of the kernel RIP gene also occurs independently of the *O2* control. Bass *et al.* [57] have reported that both temporal and spatial control of RIP1 gene expression are also detectable in *o2 null* mutants. The level of *o2* independent expression is low, but varies with the inbred background at the *o2* allele. Thus, regulation of the pro-RIP1 gene by *O2* appears as a mechanism for enhancing level of the corresponding protein in the endosperm.

Even with this knowledge of pro-RIP gene regulation, however, characterisation of the protein, its activation, and its biological activity are still incomplete. By taking advantage of the availability of *o2* mutants, phenotypically normal plants except for a soft, chalky endosperm, it may be possible to make further gains in understanding the biological functions of the maize RIP.

BIOLOGICAL ROLE AND TOXICITY OF THE MAIZE RIBOSOME-INACTIVATING PROTEIN

Although RIPs have been implicated as part of the plant natural defence machinery acting against viral or fungal pathogens since the beginning of their study, the biological function and role of plant RIPs is to date not yet completely understood (for review see [2]). A potential role of RIPs as plant defence proteins has been deduced from their enzymatic activity and extracellular localisation [58]. It has been proposed that RIPs are synthesized as inactive proteins

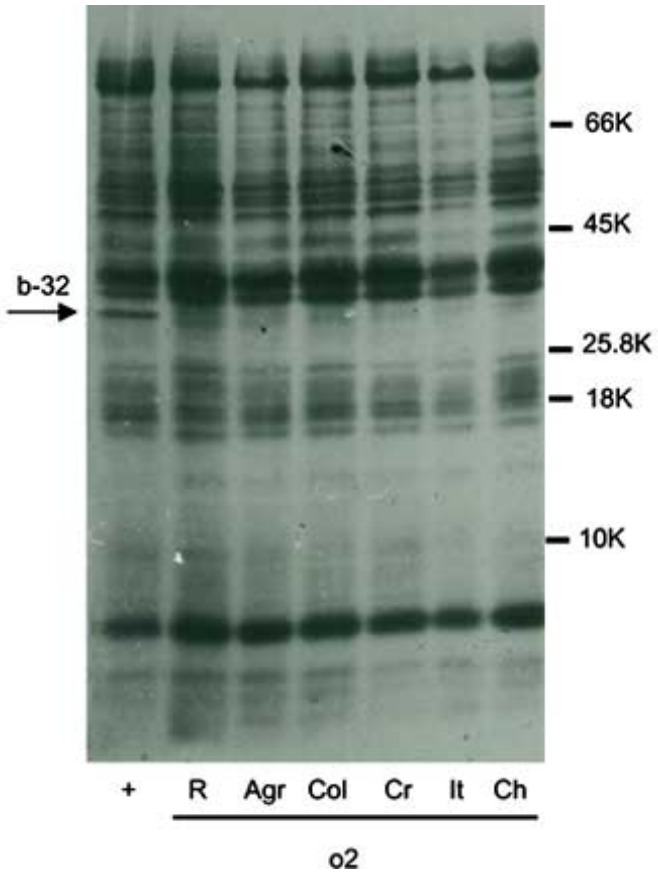

Fig. (3). SDS-polyacrylamide gel electrophoresis of S-30 proteins from normal (+) and *opaque-2* (*o2*) endosperms. Sample endosperms were collected at 30 days after pollination from normal (inbred line W64A9 and opaque-2 alleles: *o2* (R); *o2-Agr* (Agr), *o2-Col* (Col), *02-Cr* (Cr); *o2-lt* (lt), and *o2-Ch* (Ch). Endosperms were homogenised in 60 mM Tris-HCl pH 6.8, 1 mM phenylmethylsulfonyl fluoride. 50-100 ug are loaded per lane and run in a 18% SDS-polyacrylamide gel slab. Molecular marker posistions are marked at the right side [36].

sequestered in the cell wall matrix, which re-enter into the cytoplasm along with the pathogen at infection sites. Thus, RIPs are believed to arrest pathogen multiplication by inactivating the host ribosomes [58]. However, recent studies suggest that RIPs can directly inhibit pathogens by inactivating their ribosomes and causing cell death after internalisation into fungal cells via a yet-unknown mechanism [59-62].

The enzymatic activities of RIPs can produce a variety of phenotypic effects on both the homologous plant and on heterologous organisms. It is thought that in maize, RIP provides the seed with both nutritional benefits and protection against pathogen invasion of the endosperm. In addition, one or both the endogenous RIPs may provide yet-undiscovered effects on basic cellular processes. The developing kernel accumulates large quantities of proteins that are unique to the endosperm for use as nutrient sources during germination. The maize pro-RIP is high in lysine and methionine, amino acids absent and underrepresented, respectively, in the major storage proteins, zeins [63]. Consequently, it may serve as one of a number of storage albumins that partially eliminate the need for lysine and methionine biosynthesis in the germinating seedling [64].

In addition to nutritional contributions, circumstantial evidence implicates RIPs in plant defence. RIPs have features of defence agents in that they are preferentially toxic to non-plant cells and can accumulate to high levels in seeds. Maize kernels with the *o2* mutation (and thus deficient in RIP) had increased susceptibility to fungal attack [65] and insect feeding [66]. The assessment of the relative toxicity of the maize pro-RIP and its protease-activated form to insects and fungi is of particular interest, since development of pests and pathogens in plants and in stored seeds is a major concern for food safety. The maize pro-RIP and its papain-activated form were tested for their toxicity on a number of pathogenic insects [67]. The authors found that only the activated form was active against cabbage loopers (*Trichoplusia ni*) and fall army-worms (*Spodoptera frugiperda*). In addition, these authors noted no apparent effect on mortality for European corn borer (*Ostrinia nubilalis*), corn earworms (*Helicoverpa zea*), and for Indian meal moths (*Plodia interpunctella*), although a significant effect in the survivors' weight was detected in the first two experiments. In contrast, neither the pro-RIP nor the activated RIP had any significant effect on both mortality and weight of the survivors of beetles. However, the presence of both pro-RIP and activated RIP had a deterrent effect on feeding in choice assays for freeman sap beetles (*Carpophilus freemani*), dusky sap beetles (*Carpophilus lugubris*), strawberry sap beetles (*Stelidota geminata*), and maize weevils (*Sitophilus zeamais).* Collectively, these studies showed that the maize RIP is active, although to various degrees of intensity, against several pest insects for various cultures and can be ascribed to the class of toxic plant RIPs such as ricin and saporin [68], but different from most plant RIPs which are generally not toxic to insect cell cultures and insects [1].

The potential of the maize RIP against fungal ribosomes was clearly demonstrated by Maddaloni *et al.* [41] and Hey *et al.* [43]. Additionally, Nielsen *et al.* [69], with an *in vivo* bioassay with two *Aspergillus* strains, demonstrated that the activated maize RIP is capable of altering growth and morphology of the maize pathogen *Aspergillus flavus* and of the non-pathogen *Aspergillus nidulans.* In particular, the effect of the maize RIP on the pathogen *Aspergillus flavus* has a practical value, because this mold is responsible of producing mycotoxin in the grains. In this study, conidia of both fungi, mixed with the activated RIP and observed at various stages during germination and hyphal elongation, showed that the activated form proportionally decreased growth at increasing protein concentrations. Although the mechanism by which the activated RIP contrasts fungal growth remains to be clarified, these findings, taken together with its relative abundance in the maize kernel [37], and sensitivity of *o2* mutants to ear rotting fungi [65], lead to recognise a role for maize RIP in defence mechanisms against fungal pathogens.

RIBOSOMES RESISTANT TO RIBOSOME-INACTIVATING PROTEINS

Several studies have demonstrated the specificity of RIPs toward different ribosomal substrates from sources including animal, plant, and bacterial species (reviewed in [2, 6]). Most RIPs are active on a variety of ribosomes, including conspecific ones. Self-protection of RIP-producing plant cells is achieved by compartmentalisation to the extra-cellular space of the vacuole. In this case, RIPs are not able to reach the ribosome target in their own cytoplasm [70]. In general, non-plant eukaryotic ribosomes seem to be susceptible to RIP inactivation, whereas plant ribosomes are more resistant. However, susceptibility of plant ribosomes depends on the source of both ribosomes and RIP, with homologous ribosomes having at least some resistance to inactivation [42, 71-74]. Inhibition of translation from homologous ribosomes or modification of naked RNA requires at least 1000-fold more RIP than does inhibition of mammalian ribosomes [72, 75]. In a direct comparison between maize and rabbit ribosomes, it has been found that maize ribosomes are 360-fold less sensitive than rabbit ribosomes to RIP inactivation [42]. Furthermore, it was demonstrated that the b-32 displays RIP activity against heterologous ribosomes whilst maize (conspecific) and wheat (relative) ribosomes are resistant [42]. These results were later confirmed by testing the activity of the pro-RIP as well as of the activated αβ-RIP against maize ribosomes [43]. By means of the diagnostic use of the "aniline fragment", it was demonstrated that the pro-RIP as well as the αβ-RIP had no significant effect on isolated maize ribosomes at a ratio of 8:1 and 4.8:1, respectively. Conversely, treatment of yeast ribosomes with the activated αβ-RIP resulted in the release of the aniline fragment [43]. Maize ribosomes were, on the other hand, very sensitive to treatment with the heterologous RIP pokeweed antiviral protein [76] at an extremely lower ratio of RIP: ribosomes of 0.044:1. More recently, Krawetz and Boston [67] in order to determine the specific toxicity of the maize RIP against ribosomes of various origin, developed an *in vitro* quantitative assay relying on intact ribosomes as RIP substrates. In this study intact ribosomes were purified from a prokaryotic *E. coli* cells, and from eukaryotic cells derived from rabbit reticulocyte lysate, *Aspergillus flavus*, tobacco leaves, and maize kernels. The results showed, in agreement with previous findings, that maize ribosomes were resistant to the native pro-RIP as well

as to the papain-activated form, while rabbit and *Aspergillus flavus* ribosomes were more sensitive. Maize and tobacco ribosomes, although distantly related plant species, had the same sensitivity. This enzymatic specificity suggests that RIP-ribosome interaction depends on the ribosomal conformation of the affected organism [67]. The mechanism of this resistance does not appear to lie within the 28S rRNA primary sequences as the residues immediately surrounding the modification site are universally conserved in both prokaryotic and eukaryotic organisms [77]. Endo and Tsurugi [9] showed that ricin A-chain depurinates synthetic oligonucleotides corresponding to the RIP target loop but not purified *E. coli* ribosomes. Thus, it is likely that structural features and/or ribosomal proteins or other RNA-binding proteins are important in determining RIP toxicity.

The effect of RNA sequence and structure on RIP activity at the conserved loop has been determined through mutagenesis studies [75]. In contrast, little attention has been given to the formation of an RNA-protein complex. Hedblom *et al.* [78] reported binding of ricin to rat but not *E. coli* ribosomes, but Olsnes [79] failed to detect such binding in a separate study. Therefore, there is still an open area of research to unlock the mechanisms of the RIP activity.

DIFFERENCES IN RIBOSOME-INACTIVATING PROTEIN ACTIVITIES

While it is true that all plant RIPs catalyze a common biochemical reaction, physical properties of individual RIPs are quite diverse. RIPs typically exhibit amino acid sequence similarities of less than 50% and antibodies raised against RIPs seldom cross-react with RIPs from distantly related species [73, 80]. Mammalian cell lines resistant to one RIP remain sensitive to inactivation by other RIPs [6]. Likewise, only a subset of RIPs is able to act on *E. coli* ribosomes [81, 82]. Differences in RIPs have also been observed during attempts to prepare immunotoxins by coupling RIPs to antibodies. Coupling strategies that are successful for one RIP often fail with other RIPs and result in proteins that have undergone a dramatic loss of activity [83, 84]. The pokeweed RIP, PAP, resulted in increased resistance to virus when synthesized at low levels in transgenic tobacco but high levels of expression caused severe deformities [85]. Trichosanthin has been shown to have inhibitory activity *in vitro* against Human Immunodeficiency Virus (HIV) [86]. In addition, several RIPs have been reported to have nucleolytic activity on supercoiled DNA [87, 88]. Whether these features are shared with other RIPs is not known but the mechanism of viral resistance does not appear to be related to a decrease in protein synthesis. Barbieri *et al.* [89] have reported that a RIP from *Saponaria officinalis* (saporin-L) is capable to remove adenine from a variety of polynucleotides, at different rate. This substrate specificity was further confirmed in a study by Barbieri *et al.* [90], where the most known RIPs were examined for adenine release on various substrates including RNAs from different sources, DNA and poly(A). The results showed that all cell RIPs depurinate DNA, and some released adenine, from all adenine-containing polynucleotides tested.

In addition to ribosome or RNA structure, temporal or spatial separation of the RIP from ribosomes may contribute to self-protection. Many RIPs, like ricin, abrin, and α-trichosanthin, are targeted to endomembrane systems whose spatial separation from ribosomes could provide a mechanism for resistance. Synthesis of large amounts of ricin and α-trichosanthin has also been observed in transgenic and virally transfected tobacco, respectively, when endomembrane signal sequences were included in the constructs [91, 92]. Unlike these RIPs, maize proRIP1, RIP30 in barley seed, and tritin in wheat are cytoplasmic proteins. In barley seed, RIP30 is not synthesized in endosperm at early stages of development but does accumulate later [93]. Because the endosperm becomes terminally differentiated and transitionally inactive after seed maturation, accumulation of translational inhibitors late in seed development poses no problem of viability. In contrast, synthesis of maize pro-RIP is initiated in kernels as early as 10 days after pollination (DAP) and levels at maturity account for 1-3% of the soluble seed protein [35]. Perhaps the early onset of pro-RIP synthesis results in such locally high cytosolic quantities of the protein that it necessitates additional self-protection provided by accumulation as a zymogen.

Until recently, RIPs had been shown to have organ specific localisation but had not been tied to inducible defence responses. RIPs were generally thought to act as constitutive defence proteins in particular tissues. An exception to this generalisation is a 60 kDa protein from barley leaves [25, 94, 95]. This protein is encoded by a RNA induced endogenously in senescing leaves and by treatment of leaves with jasmonic acid. The N-terminal portion of this protein resembles a RIP but the C-terminal portion has a strong homology with the eukaryotic initiation factor, eIF4γ. Western blotting of leaf extracts and *in vitro* reconstitution experiments indicate that JIP60 is synthesised as a precursor, which is processed *in vivo*. This is in keeping with *in vitro* translation experiments indicating that a deletion derivative of the N-terminal region, but not the putative precursor, strongly inhibits protein synthesis on reticulocyte ribosomes. The inhibition of ribosome function is associated with depurination of 26S rRNA, characteristic of plant RIPs. This indicates that JIP60 is a novel ribosome-inactivating protein requiring at least two processing events for full activation. JIP60 derivatives do not significantly inhibit *in vitro* protein synthesis on wheat germ ribosomes. These and other results suggest that JIP60 may be involved in plant defence. Reinbothe *et al.* [94] have also reported that the protein has ribosome-inactivating activity against mammalian ribosomes as well as leaf ribosomes from stressed barley plants but not those from unstressed plants. These findings are potentially interesting; however, they should be interpreted with caution because the experimental design would not have allowed the authors to distinguish between inactivation of ribosomes by RIP and modification by RIP after inactivation by other means. Additionally, in contrast to Reinbothe *et al.* [94, 95], Chaudry *et al.* [25] have found RIP activity against mammalian ribosomes only after proteolytic processing of this gene product. Regardless of whether or not this protein is made as a zymogen, the prospect of a physiological control of self-immunity provides exciting possibilities for a basic role of this RIP in programmed cell death [96].

USE OF RIBOSOME-INACTIVATING PROTEINS AS A DEFENCE STRATEGY IN TRANSGENIC PLANTS

Plants have evolved complex, integrated defence mechanisms against diseases that include preformed physical and chemical barriers, as well as inducible defences such as antimicrobial compounds, enhanced strenghtening of cell walls, and the production of various pathogenesis related proteins [97]. It is generally assumed that a large variety of proteins play different roles in the defence of plants against bacterial and fungal pathogens, and it has been proposed that some proteins enhance the strength of extracellular matrix for passive resistance; others are enzymes involved in the biosynthesis of toxic compounds, such as phytoalexins, and a third group include those that have direct anti-microbial activity. Transgenic plants expressing various defence genes have been recently described. In these studies chitinases, glucanases, RIPs, puroindolines, and other defence proteins are expressed at significant levels conferring various degrees of pathogen resistance in bioassays and greenhouse/phytotron tests [98, 99]. However, broad and durable resistance against agronomically important pathogens have been not yet reported.

In recent years, transgenic plants expressing RIPs have been used to test defence properties attributed to this group of proteins. For example, in tobacco, increased virus resistance was achieved with the expression of trichosanthin [100], PAP [85], PAPII [101], virus-induced dianthin [102] and C-terminally deleted, inactive PAP [103], which infers 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 [101], and a truncated PAP version [104].

Studies in this field performed with cereal RIPs, have shown that transgenic tobacco plants expressing the barley RIP30 under a wound-inducible promoter led to an increased level of protection against *R. solani* [105]. Other experiments with transgenic tobacco plants have confirmed anti-fungal activity of the barley RIP30 under a constitutive promoter [106]. The latter authors suggested that this anti-fungal activity would be increased by the addition of an N-terminal signal peptide for protein export, and that a synergistic effect with barley seed chitinase could be achieved. The barley RIP30 has also been expressed by Bieri *et al.* [107] under the control of a strong constitutive promoter 35S CaMV in transgenic wheat. Plants expressing high levels of RIP were protected only moderately or not at all against infection caused by the fungal pathogen *Erysiphe graminis.*

In analogy to the cited studies, the maize RIP b-32 was introduced and expressed in tobacco in order to test its effectiveness in defence against the important soil-born fungal pathogen, *R. solani* AG4 by Maddaloni *et al.* [108]. In this study, tobacco cv. Petite Havana SR1 was transformed with *A. tumefaciens* LBA4404 carrying a wun-b32-nos3' cassette. Sixteen independent transgenic events were subjected to genetic analysis for gene segregation assessment of the kanamycin resistant marker gene, indicating that either one or two functional loci were present. Eight lines were chosen for further characterisation: sampled young leaves from T1 selfed plants and from an untransformed SR1 control plant were wounded to trigger the synthesis of b-32. Immunoblot analyses of the leaf tissue protein pool revealed that an immunoreactive band, migrating at the same position as the affinity purified maize b-32, appears specifically in protein extracts from transgenic lines. The concentration of b-32 in transgenic lines was estimated to range between 130 and 520 ng per mg of total leaf protein and the protein was stably expressed in subsequent generations. Phytopathological tests were performed on T1 plants from eight chosen lines following artificial inoculation of *R. solani* AG4. Each plant was assigned to a class ranging from 0 to 4 according to the following damage parameters: (i) the peculiar root rotting damage caused by *R. solani* and (ii) the general vigour of the above-ground organs. For both traits, transgenic plants were definitely more tolerant to *R. solani* infection than control

Fig. (4). General view of tobacco plants from transgenic lines Tr1 (left) and Tr7 (middle) and untransformed control tobacco cv. Petit Havana SR1 (right) as they appear at the time of screening after artificial inoculation with *Rhizoctonia solani* [108].

plants. Root rotting scores for transgenic lines were on average 43% lower than for control plants (0.69 vs 1.20). A similar trend of response to infection, although less pronounced, was recorded for the above-ground plant damage. Collectively, these results showed that transgenic tobacco plants, in which the expression of b-32 gene is driven by the *wun1* promoter, had increased protection against infection of the soil-borne fungal pathogen *R. solani* (Fig. **4**).

More recently, work in our laboratory utilised the same b-32 cDNA clone placed under the CaMV 35S constitutive promoter, for transformation of hexaploid wheat (*Triticum aestivum* L.). The results obtained confirmed b-32 expression in the leaf tissue of six transgenic lines at various stages of plant growth from seedling to heading (Figure. **5**). In addition, testing of wheat seedlings with artificial inoculations of *Blumeria graminis* and *Puccinia recondita,* showed enhanced resistance against both pathogens, recorded as inferior number of colonies developed and lack of fungal spread and development. b-32 expression in wheat kernels was also detected and immunoblot analysis on mono and bi-dimensional seed protein gels confirmed a high similarity with b-32 of the maize kernel [109]. Altogether the above-mentioned studies confirm the defence action of the maize b-32 against various fungal pathogens in two different host systems. Kim *et al.* [110, 111] have used a b-32-encoding cDNA clone very similar to the one described by Bass *et al.* [42], *Zmcrip3a*, to transform rice cv. Nipponbare. These authors demonstrated by immunoblot analysis that the maize RIP was expressed into leaf as well as seed tissues of transgenic lines at various levels, ranging between 0.5 and 1% of the total soluble protein. However, no challenge against plant pathogens is reported. Furthermore, the same authors found that the transgene is preferentially processed in germinating seeds and young leaves in a similar way as in maize kernels, suggesting that the processing mechanism might be highly conserved in transgenic systems.

FUTURE PERSPECTIVES

In this review we attempted to cover the recent research relating to cereal RIPs. In particular, we focused our attention to maize b-32 RIP. This is probably the most deeply investigated cereal seed RIP, because of its peculiar presence, action, processing, and effects in the maize kernel physiology. Moreover, its presence bound to regulatory processes involved in the zein storage protein deposition, as well as direct and circumstantial evidence of a protective role of the seed, render the maize RIP an intriguing matter of investigation as a multifacets system of the plant biology.

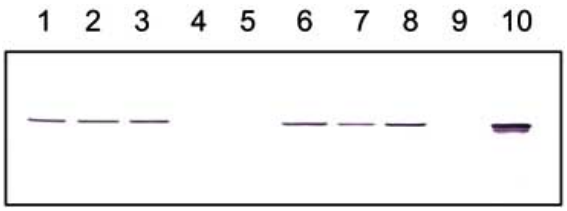

Fig. (5). Immunoblot analysis of leaf protein extracts from transgenic wheat cv.Veery (*Triticum aestivum* L.) homozygous lines, engineered with the *b32* maize gene driven by CaMV 35S constittive promoter. Polyclonal antibodies anti-GSTb32 were raised in rabbits injected with purified GST-b32 expressed in *E.coli*. Immunoblotting identifies a specific band at about 32 kDa corresponding to the b32 expressed in maize kernel. Lanes: 1 to 3 and 6 to 8 are six independent 35Sb32-wheat transgenic lines, showing expression b32; 4 and 5 are control cv.Veery plants; 9. W64A inbred maize leaf extract; 10. W64A inbred maize kernel extract.

Despite of the body of work performed and data gathered, the maize RIP b-32 still maintains its ambiguous, incompletely understood role inside the maize kernel. As highlighted in the preceding discussion this plant has two different RIPs. Both are synthesized as zymogens that gain catalytic competence only after proteolytic activation. One of these RIPs (b-32) is expressed under the control of the seed-specific transcriptional activator, *O2*. The second RIP shows no expression in the kernel. Both RIPs are apparently processed at multiple sites to yield two highly associated polypeptide chains that exhibit potent toxicity in translational inhibition assays.

The toxicity of maize b-32 *in vitro* is also suggestive of a role for RIPs in plant defence, but accumulation of the kernel proenzyme (pro-RIP) to 1-3% of the soluble protein is also consistent with a storage albumin function. Moreover, the maize RIP clearly has unusual regulatory properties at both the gene and protein level. The differences in maize RIP and other RIPs may also be important in understanding the basic physiology and biochemistry of endosperm development and mobilisation. The similarities among RIPs provide a means of exploring properties important for RIP activity. Certainly, attempts to use RIPs as biological pesticides or cell-killing agents can benefit from information about the mechanism of RIP uptake by potential pathogens and the means by which RIP can be produced in transgenic plants. Key areas of future research should include studies to define the protein domains required for pro-RIP processing and RIP activity and to identify interactions between the RIP and the RNA target site. This research activity will contribute to our understanding of the endosperm both as a source of nutrients and in protecting the kernel against plant pests. Discovery of a maize RIP synthesized co-ordinately with the major nutrition reserves of the grain provides a favourable genetic system; in addition, a number of maize *opaque* mutants deficient in RIPs are available [63]. Thus, we have a favourable opportunity to study both basic cellular processes of seed development and practical applications of the maize RIP in controlling plant pathogens.

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