Genetics of Ribosome-Inactivating Proteins

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Abstract: Ribosome-inactivating proteins (RIPs) are a heterogeneous group of enzymes found mainly in plants and a few bacteria that possess N-glycosidase activity on ribosomes and a related polynucleotide adenosine glycosidase activity on naked nucleic acids. They encompass single enzymatic chains, heterodimeric toxic lectins and related agglutinins. Plants commonly produce several RIP isoforms encoded by multi-gene families. The toxic lectins possess adaptations related to their cytotoxic role.

Keywords: Ribosome-inactivating protein, Ricin, Pokeweed antiviral protein, RNA N-glycosidase.

INTRODUCTION AND CLASSIFICATION OF RIBOSOME-INACTIVATING PROTEINS

The term Ribosome-inactivating Protein (RIP) was introduced before structural details and the nature of the enzymatic activity that inactivated ribosomes were known [1]. The discovery of the RNA N-glycosidase activity towards ribosomes is used as the diagnostic feature of the extended family of RIPs. This activity requires for its substrate an exposed GAGA-containing tetraloop structure in the context of the ribosome, although it can also act with similar sequence specificity on naked RNA, but with an approx. 10^5-fold lower catalytic efficiency [2]. The majority of RIPs also contain a related polynucleotide adenosine glycosidase activity, which acts on a variety of naked RNA and DNA substrates to release multiple adenines [3]. Again, the catalytic efficiency of this activity is considerably lower than the RNA N-glycosidase activity towards ribosomes and for this reason is probably not the primary cause of the cytotoxic action of RIPs. Several other activities have also been ascribed to individual RIPs .

RIPs have been classified into two [4] or three [5] groups in attempts to encompass their heterogeneity. Type 1 RIPs, such as Pokeweed Antiviral Protein (PAP) tritin (from wheat endosperm) and trichosanthin (from tubers of the Chinese cucumber) are basic, single chain proteins or glycoproteins of ~ 30 KDa. They share a number of invariant amino acid residues, including Y21, F24, R26, Y80, Y123, R134, L144, E177, R180 and W211 (ricin A-chain numbering) [6]. Of these, Y80, Y123, E177, R180 and W211 are located in the active site [6]. Although they are distinctly different in overall sequence homology and post-translational modifications, they share a highly conserved tertiary structure [12]. Type 1 RIPs are only weakly cytotoxic because they lack a cell binding moiety; however, they can enter cells by fluid phase endocytosis and are thus toxic to macrophages and trophoblasts. With the exception of the RIPs produced in the endosperm of certain cereal seeds, the majority of type 1 RIPs are highly active on conspecific ribosomes and are targeted to the apoplast (cell wall plus intercellular space) and/or vacuoles [7]. They are synthesised as precursors with N-terminal signal sequences and C-terminal extensions [7]. It can thus be assumed that they enter the secretory pathway and are either secreted from the cell by the default pathway, or are targeted to the vacuole [8]. In contrast, the type 1 RIPs produced in the endosperm of Poaceae seeds (wheat and barley) are not made as precursors and accumulate in the cytosol of endosperm cells [9,10]. Although the ribosomes of these species are susceptible to high concentrations of the homologous RIPs *in vitro* [10,11], the finding that endosperm ribosomes of wheat are not modified *in situ* during the developmental stage of tritin accumulation argues against a cytotoxic role in the programmed senescence of this tissue [10].

Type 2 RIPs of plant origin, which include the well known toxic lectins ricin and abrin, consist of a heterodimer of an N-glycosidase domain (the A-chain), which is structurally and functionally equivalent to a type 1 RIP, linked through a disulphide bond to an unrelated B-chain with lectin activity [12]. Both chains are derived from a single precursor. Van Damme *et al.* [13] include the classical type 2 RIPs as one category of chimero RIPs which additionally includes both covalently and non-covalently linked dimers (e.g. the agglutinins from *Ricinus communis* and *Abrus precatorius* seeds) and the tetrameric RIPs from *Polygonatum multiflorum*, in which the monomers are non-covalently associated, and *Sambucus nigra* (SNA I) in which the monomers are linked through disulphide bonds between the B-chains[14]. A few plants produce both type 1 and type 2 RIPs, including *S. nigra, Iris hollandica* and *Cinnamomum camphora* [17]. For example, *I. hollandica* bulbs produce three type 1 and two type 2 RIPs [17,18]. On the basis of multiple amino acid sequence alignments, it is thought that the type 1 RIPs in *I. hollandica* have arisen through the deletion of the B-chains of the type 2 RIP [18]. The RIPs of bacterial origin are also usually classified as type 2. These include Shiga toxin produced by *Shigella dysenteriae* and Shiga-like Toxins (SLTs) produced by enterohaemorrhagic strains of *Escherichia coli* [19]. Infection in humans causes diarrhoea leading to colitis and may progress to Haemolytic Uremic Syndrome (HUS) as a direct result of SLT-induced kidney damage. They consist of an A-chain analogous to the A-chains of plant type 2 RIPs with a C-terminal extension, which is non-covalently associated with a doughnut-shaped pentamer of B-chains [20]. The

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toxins specifically recognise the globo series of glycolipids [19].

Most of our knowledge about the synthesis and processing of plant type 2 RIPs has been derived from studies on ricin. Ricin is synthesised as a single precursor molecule (preproricin) in which the A- and B-chains of the mature toxin are joined by a 12 amino acid linker peptide, and in which the N-terminus of the mature A-chain is preceded by a 35 residue pre-sequence, the first 26 residues of which represent the ER signal peptide [21, 22]. Within the ER proricin is glycosylated and disulphide bonded within the B-chain and between the A- and B-chains. In castor bean endosperm cells, proricin is then transported via the Golgi complex, where it acquires complex glycans, to precusor-accumulating vesicles and eventually to protein storage vacuoles in which the linker and N-terminal propeptides are proteolytically cleaved, generating the mature toxin [23]. The vacuolar sorting determinant of proricin has recently been shown to reside in the 12 residue internal linker which contains the motif LLIRP, resembling the N-terminal NPIRL sequence-specific vacuolar sorting signal necessary for targeting sporamin to the vacuole [24]. Mutation of the Ile residue in the LLIRP motif of proricin causes its secretion in tobacco protoplasts [24].

The more recently discovered type 3 RIPs are synthesised as inactive proRIPs that contain an internal propeptide in the N-glycosidase domain. Proteolytic processing results in an active $\alpha\beta$ dimer in which the chains are tightly bound by non-covalent interactions. To date, only two type 3 RIPs (also know as two chain type 1 RIPs) have been described - in maize endosperm and barley leaves [25, 26]. The maize proRIP (also known as b32) accumulates in the cytosol of developing endosperm cells and is proteolytically activated during germination by the removal of a 25 residue acidic propeptide from the centre of the proRIP [25]. Barley leaves synthesise a type 3 RIP, JIP 60 (jasmonate-induced protein of 60 kDa) in response to the volatile plant signalling molecule methyl jasmonate, which causes an accelerated senescence response in this tissue [26]. The N-terminal half of JIP 60 resembles the maize endosperm proRIP and contains a putative internal propeptide at a similar position [26]. The C-terminal half of JIP 60 (~ 25 kDa) is unrelated to any other RIP and shows weak homology to eukaryotic initiation factor eIF4G. *In vitro* studies have shown that several processing events, including the removal of the C-terminal domain and the internal propeptide, are required for activation [26]. The precise polypeptide composition of the mature RIP is unknown, and it appears that a large proportion of JIP 60 remains unprocessed *in vivo* [27].

RIP GENE STRUCTURE AND ORGANISATION

Most plants that contain RIPs produce several isoforms that may be present together in the same organ, or restricted to particular organs. These isoforms can arise either from the expression of different members of a gene family, or from differences in the processing and/or glycosylation of a primary gene product.

Type 1 RIP Genes

The majority of type 1 RIPs (but excluding those of endosperm of the Poaceae) are encoded by intron-less genes that specify proRIPs with N- and C-terminal extensions with respect to the mature forms [28-30]. For example, four isoforms of Pokeweed Antiviral Protein (PAP), a RIP from *Phytolacca americana* notable for its antiviral properties and its high enzymatic activity on ribosomes from diverse phyla have been described (Table 1). They are encoded by a gene family comprising approximately nine members [31]. PAP and PAPII are leaf isoforms that appear in spring and summer respectively, whereas PAP-S is a seed isoform that has the highest activity *in vitro* of all of the isoforms [31-34]. PAP and PAP-S share 76% sequence identity, whereas PAP and PAP II are only 33% identical. A further isoform, α-PAP, which is similar in sequence to PAP-S, is constitutively expressed in all organs [34]. An analysis of genomic clones for PAP, PAP II and PAP-S revealed that PAP and PAP-S lack introns, whereas one copy of two otherwise identical PAP II genes contained a 734 bp intron at its centre (lying within the central domain of the native protein), separating PAP II into two equal sized exons corresponding to amino acid residues 1 to 134 and 135 to 285 [35]. The only other RIP gene shown to contain an intron is Mirabilis Antiviral Protein (MAP) in which the 162 bp intron is inserted between Lys178 and Ile179 in the C-terminal domain of native MAP [36]. Poyet and Hoeveler [35] propose that the intron-containing PAP II represents an ancestral RIP gene, and that PAP and PAP-S lost their introns during evolution. However, in view of the finding that the positions of the introns in the PAP II and MAP genes differ, and that the sequences of the PAP II intron-containing and intron-less genes are identical, it could be argued that the acquisition of the introns is a recent event.

Table 1. Isoforms of Pokeweed Antiviral Protein. (ND = not determined)

Isoform	Number of aminoacyl residues			Intron	% identity to PAP	Ref.
	Mature protein	N-terminal extension	C-terminal extension			
PAP	262	22	29	No	-	31
PAP II	285	25	ND	Yes	33	32
PAP-S	262	24	28	No	76	33
PAP	261	24	9	No	74	34

It is known that some type 1 RIP isoforms represent glycosylation variants of the same primary sequence. For example, the leaves of *Phytolacca dioica* contain four RIP isoforms termed PD1, PD2, PD3 and PD4; PD3 was shown to be a glycosylated form of PD4 [37].

Type 2 RIP Genes

Ricin, and its dimeric isoform *Ricinus communis* agglutinin (RCA), are the best characterised type 2 RIPs in terms of their gene structure and expression. Several isoforms of ricin and RCA are known to exist among different varieties of *R. communis* and within single varieties. A combination of direct protein sequencing and cDNA sequencing of prominent ricin isoforms, termed ricin D and RCA I revealed that their A-chains contain 267 and 266 amino acid residues respectively and share 93% sequence identity, differing at only 18 positions [38]. Significantly, Gly156 in ricin A-chain is replaced by Cys in RCA A-chain. This is located on hydrophilic loop that protrudes from the surface between two major helices and in RCA, and forms a disulphide bond with an adjacent molecule [39]. The B-chains of ricin and RCA contain 262 residues and share 84% identity [38]. Their most significant difference is that the sugar platform aromatic residue Tyr248 in ricin B-chain is replaced by His in RCA, which causes a loss of sugar binding activity of domain 2 of RCA [40]. The B-chain of a variant form of ricin, termed ricin E appears to be a hybrid ricin/RCA sequence that has arisen through a recombination event [41]. Hedge and Podder [42] have described two isoforms of ricin, designated ricin II and III on the basis of their differing isoelectric point (Pi) values. The Pi values of RCA variants fell between those of ricin II and III, but the Pi values of urea-denatured RCA monomers were identical to those of ricin II and III, leading to the conclusion that RCA is heterogeneous and is composed of one ricin II and one ricin III-like monomer, and that each RCA monomer has evolved from ricin II and III-like genes. Unfortunately, these conclusions are not supported by sequence information. Molecular hybridisation studies using a ricin cDNA probe have shown that the ricin/RCA gene family is composed of 6-8 members [43, 44], although at least two of these are pseudogenes [44]. It has been proposed that the abrin/*A. precatorius* agglutinin gene family comprises at least 30 genes in three independent groups to account for all of the subunit variants [45].

Recently, extensive studies on the genus *Sambucus* have revealed the presence of complex mixtures of RIPs and related lectins that differ in subunit composition, activity and carbohydrate-binding properties (reviewed in [13]). For example, *S. nigra* (elderberry) produces five type 2 RIPs in the bark. Two of these (SNAI and SNAI') are NeuAcα(2-6)Gal/GalNac-specific lectins that differ in structure. SNAI B-chain contains an additional Cys residue (Cys327) with respect to ricin A-chain, present in a surface-exposed loop, and participating in interchain disulphide bond formation to form a $[A\text{-s-s-B-s-s-B-s-s-A}]_2$ tetramer. SNAI' is similar to SNAI but lacks this additional Cys residue and exists as a non-covalently associated dimer [46, 47]. *S.nigra* agglutinin V (SNAV) has a GalNac-binding specificity and *S.nigra* lectin-related proteins (SNALRP1 and SNALRP2) are type 2

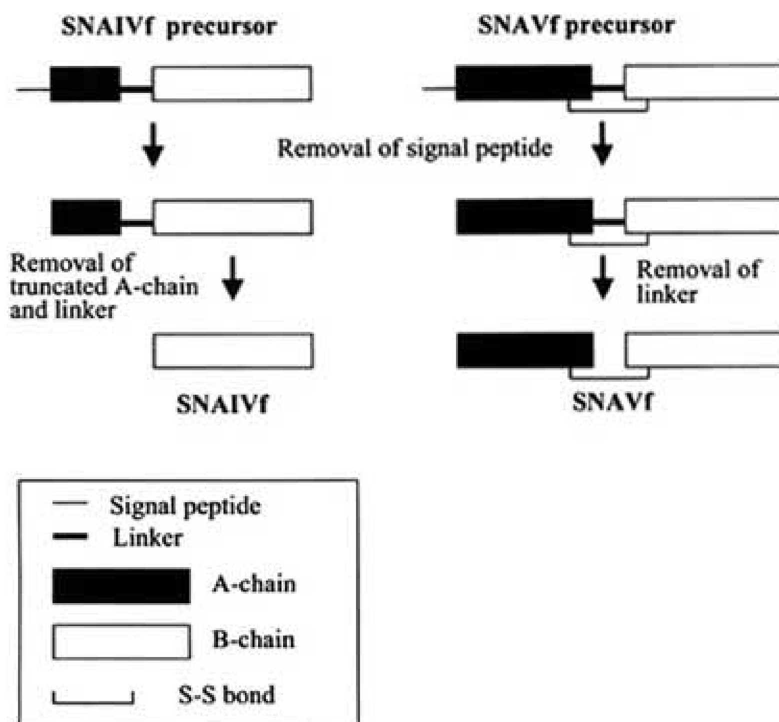

Fig. (1). Schematic representation of the processing of the precursors of SNAIVf and SNAVf in the fruit of *Sambucus nigra.* For details, see text. From [50].

RIPs with inactive B-chains [48]. In addition to these chimero RIPs, *S. nigra* bark also produces a lectin that is a homodimer of a subunit identical to SNAV B-chain, but lacking the first eight N-terminal residues [49]. Similarly, the major elderberry fruit protein is a GalNac-specific lectin that is a dimer of 32 KDa subunits that strongly resemble the B-chain of a less prevalent type 2 RIP (SNAVf) that is also expressed in the fruit [50]. An analysis of cDNA clones and PCR-amplified genomic sequences revealed unambiguously that the SNAIVf sequence is almost identical to that of SNAVf, except that the A-chain is truncated through an internal deletion corresponding to amino acid residues 22-315 of the mature SNAVf A-chain and the first four residues of the 16 residue linker connecting the A- and B-chains[50] (Fig. **1**). The N-terminal signal peptide sequences of SNAIVf and SNAIV are identical. The authors suggest that novel type 1 RIPs could be generated by the successful integration of the excised A-chain fragment.

REGULATION OF RIP GENE EXPRESSION

The majority of RIP genes show organ-specific expression patterns, and in the case of seed RIPs, also show temporal control. For example, ricin transcripts and protein accumulate in endosperm cells during the later stages of seed maturation when the testa has formed [51], and tritin and barley RIP transcripts accumulate during the latter third of seed development [9,10]. The details of the molecular basis for such regulation are largely unknown, although the presence of putative promoter elements that confer organ-specific expression for other genes have been described. For example, a functional ricin gene contains two copies of a motif closely resembling the CATGCATY legumin box motif at -113 and -145 bp [44]. The legume box is found in most legume seed storage proteins and is known to be involved in their expression [51]. A genomic clone for tritin contains three sequences in its 5' flanking region that are homologous to maize opaque-2 binding sequences (see below) suggesting that the tritin gene is controlled by a similar transcription factor [52]. The maize RIP is the only RIP gene for which a detailed mechanism of transcriptional regulation is known. The maize RIP (b-32) was first identified in the albumin fraction of developing kernels, but was absent in kernels homozygous for the recessive allele *opaque*-2 (O_2) in which the accumulation of zeins is greatly reduced [53]. The O_2 gene encodes a 48 kDa transcription factor of the b ZIP class. Transactivation of the b-32 promoter was shown by the transient expression in tobacco leaf protoplasts of a plasmid containing a constitutively expressed O_2 cDNA and a β-glucuronidase reporter gene fused to a genomic sequence extending from -1283 to +4 positions of the b-32 gene [54]. Five binding sites (GATCAPuPuTGPu) for the factor were mapped by footprinting and two of these are homologous to the endosperm box, which comprises a 20 bp motif present in most cereal prolamin storage protein promoters [55].

The accumulation of type 1 RIPs in several species is known to be enhanced by biotic and abiotic stress and signalling molecules. The activity of RIPs in *Hura crepitans* and *P. americana* leaves increased up to 15-fold following heat treatment and osmotic stress [56]. Beetins 27 and 29 in the leaves of *Beta vulgaris* accumulate in response to infection with beet mild yellowing virus, and in virus-free plants following treatment with salicylic acid and hydrogen peroxide, which are considered to be mediators of virally-induced acquired resistance [57]. In the halophytic ice plant (*Mesembranthamum crystallinum*), which shows a switch from C3 photosynthesis to the water-conserving crassulacean acid metabolism during salinity tolerance, salinity also induces the expression of a type 1 RIP. In this species, the level of RIP transcripts also shows a diurnal fluctuation [58]. The plant signalling molecule methyl jasmonate induces the synthesis of JIP 60 (a type 3 RIP) in young detached barley leaves, and acts synergistically with the inhibitor abscisic acid [26]. In older leaves, on the point of senescence, JIP 60 accumulates without the need for exogenous signalling molecules [26]. The finding that ribosomes isolated from jasmonate-treated barley leaves show characteristic depurination of their 25S rRNA has been interpreted to show that JIP 60 brings about senescence by inhibiting protein synthesis *in planta* [59]. However rRNA extracted directly from jasmonate-treated leaves in the presence of guanidine hydrochloride produced non-depurinated 25S rRNA, indicating that the depurination observed had occurred during the extraction of the ribosomes [27].

It is interesting to note that RIP-free callus and suspension cultures of *P.americana* have been obtained [60]. As RIP-isoforms are ubiquitously expressed in all organs of the plant, a gene-silencing event must have occurred during the establishment of the cultures.

ORIGIN AND MOLECULAR EVOLUTION

RIPs have been identified in two species of bacteria, approximately 100 angiosperm species and recently in an alga [61] and a Basidiomycete [62]. In angiosperms, they have been described in 20 families, including 17 dicot and 3 monocot families [13]. On the basis of their distribution in angiosperms, Van Damm *et al* [13] propose that RIPs are the exception rather than the rule, and the fact that the complete genome of *Arabidopsis thaliana* does not contain an obvious RIP-like sequence challenges the notion that all angiosperms produce RIPs, although this remains contentious. Of the plant families known to produce RIPs, some have only a few RIP-producing members. For example the only members of the extended legume family known to produce RIPs are the garden pea and the jequirity bean (*Abrus precatorius)* whereas other families have many, for example the Poaceae, Euphorbiaceae, Cucurbitaceae, Caryophyllaceae and Nyctaginaceae. Within the Iridaceae, Euphorbiaceae and Cucurbitaceae species produce both type 1 and type 2 RIPs and within the Poaceae barley produces a type 1 and a type 3 RIP (reviewed in [13]).

It is generally believed that type 2 RIPs have arisen through the fusion of an ancestral type I RIP and a lectin, giving rise to the A- and B-chains respectively. Although elements of the three domain structure of type 1 RIPs are found in other RNA-binding proteins such as RNase H from *E.coli* and the retroviral reverse transcriptases [63], no close homologue of the N-glycosidase domain has been found in other proteins. The fact that this domain is found in

organisms as diverse as bacteria and angiosperms begs the question as to where it arose. This is not a straightforward question to answer because sequence comparisons between the A-chain of the bacterial toxins and higher plants reveal a maximum identity of only 14%, and it is proposed that horizontal gene transfer has occurred between pro- and eukaryotes and v*ice versa* [64].

Van Damme *et al* [13] have constructed dendrograms from a distance matrix from the complete amino acid sequences of all of the 31 type 1 RIPs, the A-chains of 17 type 2 RIPs and the two type 3 RIPs published by 2001. Unfortunately, sequences are not available for the algal [61] and fungal [62] RIPs. The phylogenetic tree consists of four branches; branch I contains the type 1 and A-chains of the type 2 RIPs from *Iris hollandica.* It is postulated that in these species the type 1 RIPs evolved recently from an ancestral type 2 RIP through the deletion of the B-domain [65]. The second branch (P) of the dendrogram groups all the Poaceae type 1 and type 3 RIPs, including tritin, barley seed RIP, maize RIP and JIP 60. This suggests that the C-terminal region of JIP 60, which lacks homology to any other RIP, arose by a recent fusion between a maize-like type 3 RIP and an unrelated domain. The third branch (C) includes all the type 1 RIPs of species in the order Caryophyllales, comprising the families Aizoaceae, Nyctaginaceae, Phytolaccaceae, Chenopodiaceae, Amaranthaceae, Basellaceae and Caryophyllaceae. The fourth branch (E) comprises the A-chains of all currently known plant type 2 RIPs, with the exception of those from *Iris*. and includes the type 1 RIPs from the families Euphorbiaceae, Cucurbitaceae and Araliaceae and Lamiaceae. The fact that the type 1 RIPs in the above families more closely resemble the A-chains of the type 2 RIPs in the same families than those of group C type 1 RIPs suggests that they arose from type 2 RIPs through deletion of the B-chain. A major discrepancy in the dendrogram is that the A-chain of the type 2 RIP from *Polygonatum multiflorum* (a monocot) is placed in the same cluster as that from *Sambucus nigra* (a dicot). This may be the result of a recent horizontal gene transfer.

The B-chain of type 2 RIPs and related lectins consists of duplicate copies of a tripartite galactose-binding peptide, termed the α, β and γ subdomains, in which each subdomain comprises ~40 amino acid residues. The sugar binding pockets are created by a sharp bend in the backbone formed by the sequence Asp, Val and Arg, plus a variable aromatic residue, which provides the binding platform for the sugar [66]. It is believed to be derived from an ancestral lectin of ~40 amino acid residues by two duplication/in tandem insertion events, which occurred before its fusion with the ancestral N-glycosidase domain [67]. Unlike the N-glycosidase domain of the A-chain, the β-trefoil structure of the individual domains of the B-chain is widely distributed and extremely ancient. It is found in discoidin II, from the slime mold *Dictyostelium discoideum*, in human interleukins, human fibroblast growth factor, β-xylanase of *Streptomyces olivacoviridis* and in the murine mannose receptor, mCys-MR (reviewed in [13]). All of these are now classified as members of the ricin B family. A dendrogram of the B-chains of 18 type 2 RIPs constructed by Van Damm *et al* [13] shows a general resemblance to that of the respective A-chains.

EVOLUTIONARY ADAPTATIONS OF TYPE 2 RIPS

The fact that the N-glycosidase domain of RIPs can occur in the single, non-toxic polypeptide of type 1 RIPs and as the cytotoxic moiety of type 2 RIPs makes it very difficult to envisage an all-embracing physiological role for RIPs. However, it can be stated with certainty that several properties of the "classical" type 2 RIPs have evolved to enable them to perform their cytotoxic role. These include cell-surface binding and endocytosis, the retrograde translocation of a small proportion of the endocytosed toxin to the endoplasmic reticulum, and the translocation of the A-chain into the cytosol by a mechanism that hijacks the ER quality control system to target the A-chain to Sec 61p channels [68]. The reverse translocation of misfolded/ unfolded proteins into the cytosol is normally closely coupled to their ubiquitination and proteasomal degradation in the process of ER-associated degradation (ERAD [69]. The first step in proteasome-mediated degradation involves polyubiquitination of target protein lysines [70]. It has been noted that the A-chains of toxins that are translocated from the ER have an unusually low lysine content, possibly enabling such toxins to escape the fate of ubiquitin mediated proteolysis [71]. For example, Shiga-like toxin and ricin A-chain contain only two lysyl residues, and abrin A-chain contains three [68]. In contrast, the A-chains of the non-toxic type 2 RIPs, including nigrin and ebulin contain an average of 7.4 lysyl residues, while type 1 RIPs contain an average of 18.6 [68]. The introduction of four additional lysyl residues into the A-chains of ricin and abrin at positions that did not compromise the structure or N-glycosidase activity of the toxins resulted in ~100-fold lower LD_{50} values on Vero cells when compared to the wild type toxins. Furthermore, in the presence of the specific proteasome inhibitor, *claso*-lactacystin β-lactone, the potency of the high lysine mutant A-chains was comparable to those of the wild type toxins [68].

CONCLUDING REMARKS

RIPs have been investigated for many years for their cytotoxic effects, as agents to study structure/function relationships within the ribosome and have been exploited for clinical and agricultural applications. Although suggestions about their physiological roles in plants have continued to increase, their effects on the phenotype and fitness of plants are unknown. The evolution of great structural and functional diversity within the RIP family almost certainly reflects a diversity of physiological roles. Transgenic plant technology has made it possible to express RIPs ectopically in plants, and to target RIPs to cellular compartments where they are not found in Nature. The discoveries being made from these approaches should provide valuable new insights into the roles of RIPs in plants.

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