



Short communication

Convergent evolution led ribosome inactivating proteins to interact with ribosomal stalk

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ABSTRACT

Ribosome-inactivating proteins (RIPs) inhibit protein synthesis by depurinating an adenine on the sarcin–ricin loop (SRL) of the large subunit ribosomal RNA. Several RIPs interact with the C-terminal end of ribosomal stalk P proteins, and this interaction is required for their full activity. In contrast, the activity of Pokeweed Antiviral Protein is not affected by blocking this stalk component. Here, we provide evidence from phylogenetic analyses and sequence alignments suggesting that the interaction with the C-terminal end of P proteins evolved independently in different RIPs by convergent evolution.

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The large subunit of the eukaryotic ribosome has a long and protruding stalk formed by ribosomal P proteins. These proteins share a conserved, highly acidic motif at their C-terminal end. This motif is essential for the interaction of the ribosome with Elongation Factor 2 (EF-2); a GTPase protein which catalyzes the translocation of peptidyl-tRNA from the A to the P site, during the protein synthesis process (Lavergne et al., 1987).

Ribosome inactivating proteins (RIPs; EC 3.2.2.22) are toxins present in plants and bacteria (Stirpe, 2004). Early studies reported RIP activity in several fungi, such as in *Flammulina velutipes* (Ng and Wang, 2004; Wang and Ng, 2001), *Hypsizygus marmoreus* (Lam and Ng, 2001a), *Lyophyllum shimeji* (Lam and Ng, 2001b) and *Pleurotus tuberregium* (Wang and Ng, 2001). However, even when N-terminal sequencing of purified polypeptides was performed, these sequences are too short for alignment construction and phylogenetic analysis. Classically, RIPs are

classified as type 1 and 2, according to the absence or the presence, respectively, of a lectin chain which mediates toxin cell entry. RIPs irreversibly modify ribosomes through its RNA N-glycosidase activity that depurinates an adenine residue in the conserved α -sarcin/ricin loop (SRL) of the 28S rRNA (Endo et al., 1987; Endo and Tsurugi, 1987, 1988; Hudak et al., 1999; Rajamohan et al., 2001). This modification prevents the interaction of the ribosome with EF-2. Although RIPs are able to cleave both prokaryotic and eukaryotic naked rRNA, its k_{cat} is 10^5 -fold lower than that for rRNA within an intact ribosome (Endo and Tsurugi, 1988). Some RIPs (e.g. ricin) are only active against eukaryotic ribosomes (Endo and Tsurugi, 1988). In contrast, other RIPs (e.g. Shiga toxin) inactivate both prokaryotic and eukaryotic ribosomes (Suh et al., 1998). These findings strongly suggest that ribosomal proteins are involved in rendering the rRNA susceptible to inactivation by RIPs, and that different RIPs would interact with different proteins. It has also been shown that some RIPs remove adenine residues from polynucleotides (Girbes et al., 2004).

Several RIPs, namely ricin (Chiou et al., 2008), trichosanthin (TCS) (Chan et al., 2007; Juri Ayub et al., 2008), shiga-like toxins 1 and 2 (SLT-1 and SLT-2) (Chiou et al., 2008; McCluskey et al., 2008), and maize RIP (MOD)

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(Yang et al., 2010), interact with the acidic conserved C-terminal end of ribosomal P proteins. The RIP's residues responsible for the interaction with the stalk have been mapped in TCS (Chan et al., 2007; Too et al., 2009) and MOD (Yang et al., 2010). This interaction is required for full activity. Based on these observations, it was initially proposed that the stalk structure would be a generic binding site for RIP toxins required to gain access to the SRL of the 28S rRNA (McCluskey et al., 2008). However, studies from other researchers and ourselves have recently demonstrated that Pokeweed Antiviral Protein (PAP) does not interact with this motif (Chiou et al., 2008; Juri Ayub et al., 2008).

These data suggest two alternative hypotheses:

- i) the ability to interact with the stalk was a feature of an ancestral RIP, which has been conserved in many of them (at least ricin, shiga like toxins, TCS and MOD), and has been lost in other RIPs (at least in PAP);
- ii) the ability to interact with the stalk evolved later independently in different RIPs, as a result of convergent evolution or evolutionary parallelism.

To test these hypotheses, we did an exhaustive database search of RIP sequences, selected 54 representative sequences and performed sound phylogenetic analyses using Bayesian inference and Maximum Likelihood. For

this, a multiple amino acids sequence alignment was constructed using a conserved region of the RIP domain (residues Y14 to S196 according to TCS). Based on this alignment (Fig. 1), we performed Bayesian (MB) and maximum likelihood (ML) analyses using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) and PhyML 3.0 (Guindon et al., 2010), respectively. MrBayes was run for 10⁶ generations and the average standard deviation of split frequencies obtained was <0.01. PhyML was run using the algorithm Subtree Pruning and Regrafting (SPR) (Hordijk and Gascuel, 2005) with 5 initial starting trees. To estimate the robustness of the phylogenetic inference, we ran 500 bootstrap replicates. The WAG substitution matrix (Whelan and Goldman, 2001) and gamma distribution model with invariable sites was selected as the model that best fits our dataset using ProtTest (Abascal et al., 2005).

The phylogenetic tree (Fig. 2) was, in general, consistent with the main conclusions obtained by Peumans and Van Damme (Peumans and Van Damme, 2010):

- i) many of the current RIP genes have suffered numerous duplication events and are paralogous;
- ii) type 1 (thick branches) and type 2 (thin branches) RIPs are not monophyletic.

The phylogenetic analysis presented here allowed us to reach additional conclusions. Fig. 2 shows that the recently

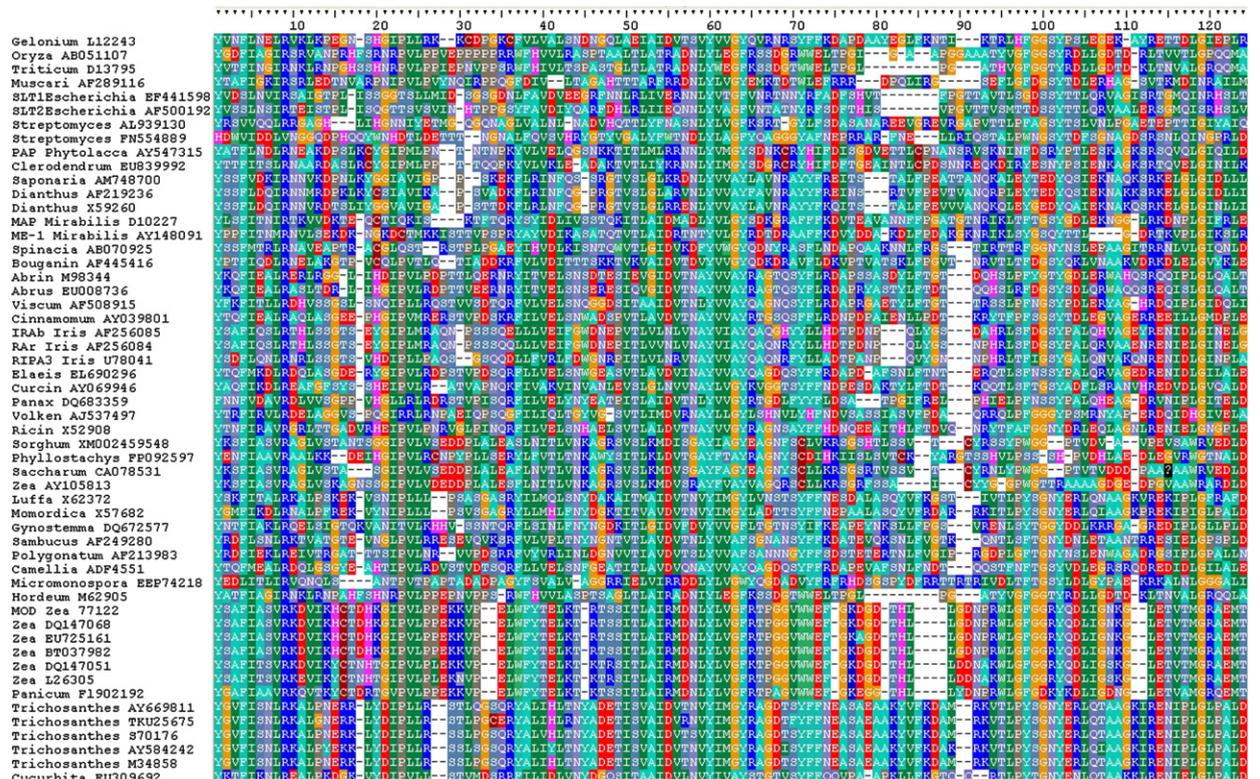


Fig. 1. Sequence alignment of the RIP domain used for phylogeny reconstruction. Sequences were aligned using TCOFFEE under default parameters and edited visually. Residues forming the active site are shown by arrows. The regions corresponding to the stalk interacting motifs from MOD and TCS are indicated by blue and red bars, respectively (figure continued in the next page). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

described type 2 RIPs from *Poaceae*; *Sorghum* (XM_002459548), *Saccharum* (CA255160), *Zea* (AY105813) and *Phyllostachys* (FP092597), are phylogenetically closer to ricin (X52908.1), which is a RIP from the dicot plant *Ricinus communis*, than to other monocot RIPs (BS = 78, BPP = 0.99). Also, the phylogenetic tree suggests a close relationship between bacterial RIPs and *Poaceae* type 1 RIPs, although with low bootstrap support. This contrasts with the hypothesis that some bacterial RIPs (e.g. Shiga-like toxins) are more closely related to type 2 RIPs than to type 1 RIPs (Peumans and Van Damme, 2010).

Most importantly, Fig. 2 shows that those RIPs interacting with the ribosomal stalk (circles) are widely and patchy distributed across the phylogenic tree. Next, we analyzed whether the residues involved in the interaction with the stalk were conserved in different RIPs. Fig. 1 clearly shows that amino acids interacting with P proteins from TCS (K¹⁷³, R¹⁷⁴ and K¹⁷⁷) (Chan et al., 2007; Too et al., 2009) and MOD (K¹⁴³, K¹⁴⁴, K¹⁴⁵, K¹⁴⁶) (Yang et al., 2010) are located on different regions of the peptide chain. Moreover, it is worth noting that these residues are not conserved in other RIPs that also interact with the stalk and for which the interacting residues have not been determined, such as ricin and SLT-1 and 2. These findings suggest that the ability to interact with

the ribosomal stalk arose independently and it represents a case of convergent or parallel evolution. Future studies mapping those residues that interact with the stalk in other RIPs would allow further testing of this model. We predict that unrelated RIPs will show different interacting residues.

In order to further test the hypothesis of convergent evolution, we analyzed stalk-interacting motifs in sequences closely related to MOD from the plant genera *Hordeum*, *Oryza*, *Triticum*, and *Zea*, and in sequences closely related to TCS from the plant genera *Cucurbita*, *Luffa*, *Momordica*, and *Trichosanthes* (Fig. 3). For instance, the stalk-interacting motif of TCS (**KRADK**) is conserved only in *Trichosanthes* species, but not in the homologous sequences from *Momordica*, *Luffa* and *Cucurbita*. A similar situation is observed in MOD-related sequences, where the motif **KIKKK** is only present in some of the sequences from *Zea*. These observations strongly suggest that these stalk interacting motifs are located in regions highly variable and have evolved rather recently during evolution.

In conclusion, we have performed for the first time, Bayesian and Maximum Likelihood phylogenetic analyses of bacterial and plant RIP domains. All the evidence taken together (phylogenetic trees and sequence alignments), support the hypothesis that the ability of different RIPs to

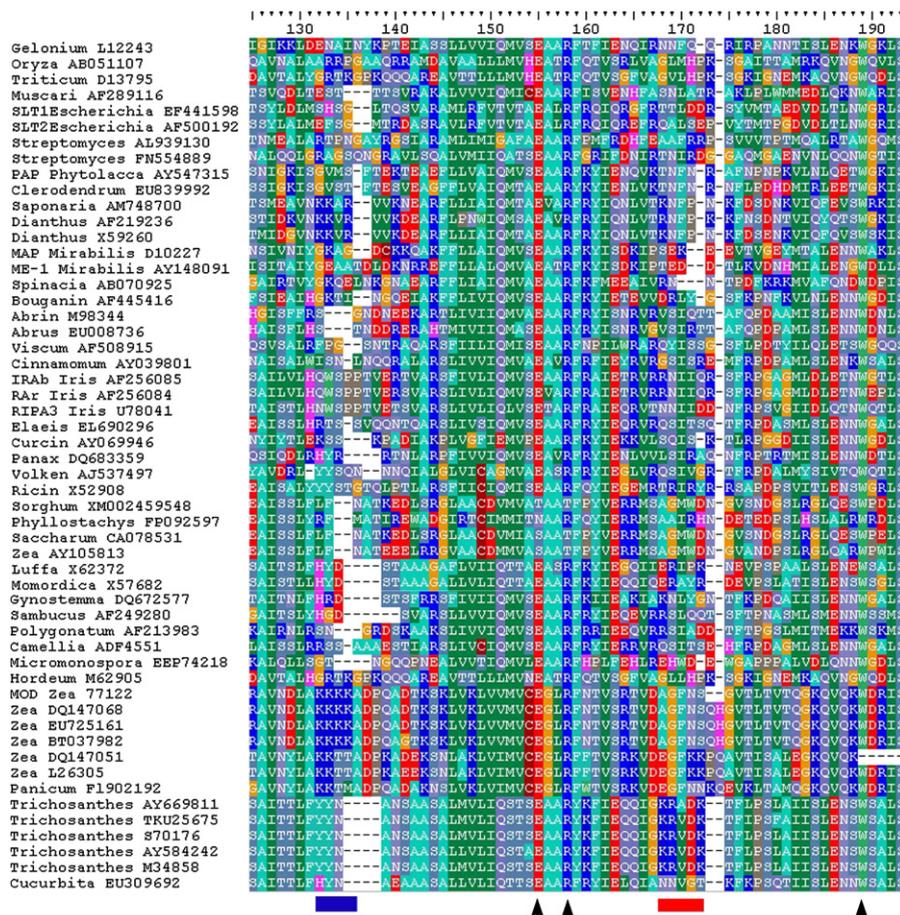


Fig. 1. Continued

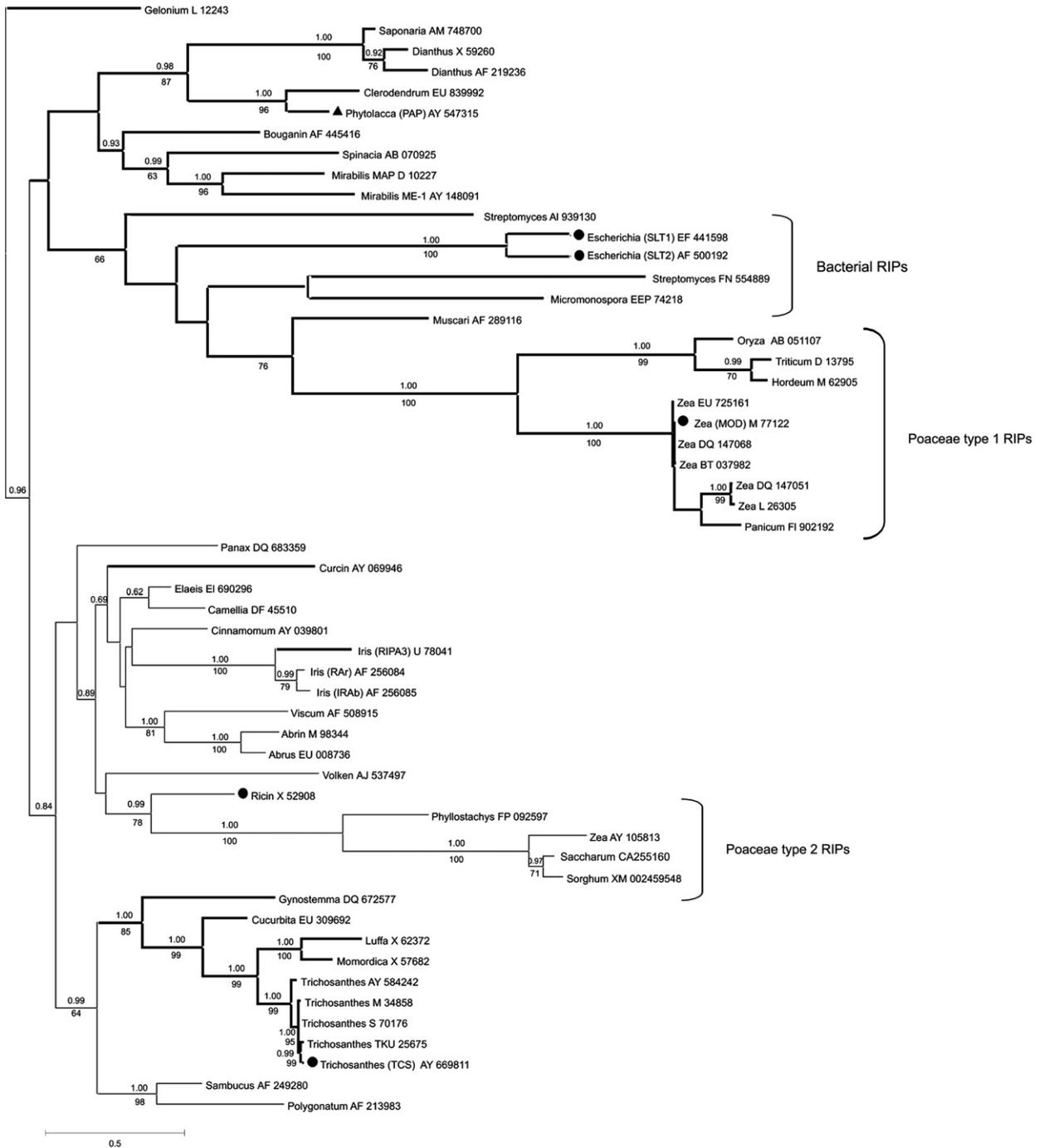


Fig. 2. Most likely phylogenetic tree of RIPs. Numbers above branches indicate Bayesian Posterior Probabilities (BPP) and numbers below branches are Bootstrap (BS) support values from the ML analysis. Type 1 and type 2 plant RIPs are indicated by thick and thin lines, respectively. RIPs interacting and non-interacting with ribosomal stalk are indicated with circles and triangles, respectively. GenBank accession numbers are shown for each sequence. Shiga toxin is not included because it only has one amino acid difference comparing to Shiga-like toxin 1.

interact with the ribosomal stalk evolved independently, as a result of convergent or parallel evolution.

Since bacterial ribosomes lack P proteins (their L7/L12 orthologous proteins have no acidic C-terminal ends), it is reasonable to postulate that the ability to interact with the P protein motif originated during evolution of eukaryotic

EF-2. Our conclusion about the parallel evolution of this ability in different RIPs, suggests that interaction with the stalk gives an adaptive advantage and does not have strong sequence constraints, which makes it easy for different proteins to acquire this feature. Our results suggest that the ability to interact with the stalk, and

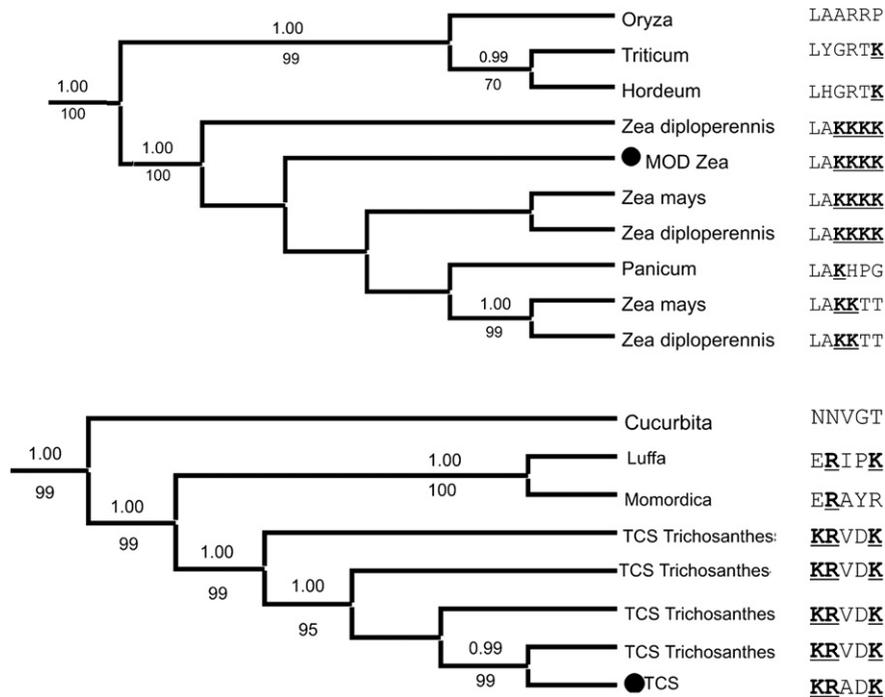


Fig. 3. Inferred phylogenetic relationships amongst RIPs closer to MOD and TCS, along with the amino acid sequences from the stalk interacting motif. Residues responsible for the interaction are shown in bold and underlined.

probably with other ribosomal proteins, has developed independently, during the evolution of different RIPs, leading to enhanced activity.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Ethical statement

The authors have no ethical statement to declare.

References

- Abascal, F., Zardoya, R., Posada, D., 2005. ProtTest: selection of best-fit models of protein evolution. *Bioinformatics* 21, 2104–2105.
- Chan, D.S., Chu, L.O., Lee, K.M., Too, P.H., Ma, K.W., Sze, K.H., Zhu, G., Shaw, P.C., Wong, K.B., 2007. Interaction between trichosanthin, a ribosome-inactivating protein, and the ribosomal stalk protein P2 by chemical shift perturbation and mutagenesis analyses. *Nucleic Acids Res.* 35, 1660–1672.
- Chiou, J.C., Li, X.P., Remacha, M., Ballesta, J.P., Tumer, N.E., 2008. The ribosomal stalk is required for ribosome binding, depurination of the rRNA and cytotoxicity of ricin A chain in *Saccharomyces cerevisiae*. *Mol. Microbiol.* 70, 1441–1452.
- Endo, Y., Mitsui, K., Motizuki, M., Tsurugi, K., 1987. The mechanism of action of ricin and related toxic lectins on eukaryotic ribosomes. The site and the characteristics of the modification in 28 S ribosomal RNA caused by the toxins. *J. Biol. Chem.* 262, 5908–5912.
- Endo, Y., Tsurugi, K., 1987. RNA N-glycosidase activity of ricin A-chain. Mechanism of action of the toxic lectin ricin on eukaryotic ribosomes. *J. Biol. Chem.* 262, 8128–8130.
- Endo, Y., Tsurugi, K., 1988. The RNA N-glycosidase activity of ricin A-chain. The characteristics of the enzymatic activity of ricin A-chain with ribosomes and with rRNA. *J. Biol. Chem.* 263, 8735–8739.
- Girbes, T., Ferreras, J.M., Arias, F.J., Stirpe, F., 2004. Description, distribution, activity and phylogenetic relationship of ribosome-inactivating proteins in plants, fungi and bacteria. *Mini Rev. Med. Chem.* 4, 461–476.
- Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O., 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst. Biol.* 59, 307–321.
- Hordijk, W., Gascuel, O., 2005. Improving the efficiency of SPR moves in phylogenetic tree search methods based on maximum likelihood. *Bioinformatics* 21, 4338–4347.
- Hudak, K.A., Dinman, J.D., Tumer, N.E., 1999. Pokeweed antiviral protein accesses ribosomes by binding to L3. *J. Biol. Chem.* 274, 3859–3864.
- Juri Ayub, M., Smulski, C.R., Ma, K.W., Levin, M.J., Shaw, P.C., Wong, K.B., 2008. The C-terminal end of P proteins mediates ribosome inactivation by trichosanthin but does not affect the pokeweed antiviral protein activity. *Biochem. Biophys. Res. Commun.* 369, 314–319.
- Lam, S.K., Ng, T.B., 2001a. Hypsin, a novel thermostable ribosome-inactivating protein with antifungal and antiproliferative activities from fruiting bodies of the edible mushroom *Hypsizygus marmoreus*. *Biochem. Biophys. Res. Commun.* 285, 1071–1075.
- Lam, S.K., Ng, T.B., 2001b. First simultaneous isolation of a ribosome inactivating protein and an antifungal protein from a mushroom (*Lyophyllum shimeji*) together with evidence for synergism of their antifungal effects. *Arch. Biochem. Biophys.* 393, 271–280.
- Lavergne, J.P., Conquet, F., Reboud, J.P., Reboud, A.M., 1987. Role of acidic phosphoproteins in the partial reconstitution of the active 60 S ribosomal subunit. *FEBS Lett.* 216, 83–88.
- McCluskey, A.J., Poon, G.M., Bolewska-Pedyczak, E., Srikumar, T., Jeram, S.M., Raught, B., Gariepy, J., 2008. The catalytic subunit of

- shiga-like toxin 1 interacts with ribosomal stalk proteins and is inhibited by their conserved C-terminal domain. *J. Mol. Biol.* 378, 375–386.
- Ng, T.B., Wang, H.X., 2004. Flammin and velin: new ribosome inactivating polypeptides from the mushroom *Flammulina velutipes*. *Peptides* 25, 929–933.
- Peumans, W.J., Van Damme, E.J.M., 2010. Evolution of plant ribosome-inactivating proteins. In: Lord, J.M., Hartley, M.R. (Eds.), *Toxic Plant Proteins*. Springer, Berlin, pp. 1–26.
- Rajamohan, F., Ozer, Z., Mao, C., Uckun, F.M., 2001. Active center cleft residues of pokeweed antiviral protein mediate its high-affinity binding to the ribosomal protein L3. *Biochemistry* 40, 9104–9114.
- Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19, 1572–1574.
- Stirpe, F., 2004. Ribosome-inactivating proteins. *Toxicon* 44, 371–383.
- Suh, J.K., Hovde, C.J., Robertus, J.D., 1998. Shiga toxin attacks bacterial ribosomes as effectively as eucaryotic ribosomes. *Biochemistry* 37, 9394–9398.
- Too, P.H., Ma, M.K., Mak, A.N., Wong, Y.T., Tung, C.K., Zhu, G., Au, S.W., Wong, K.B., Shaw, P.C., 2009. The C-terminal fragment of the ribosomal P protein complexed to trichosanthin reveals the interaction between the ribosome-inactivating protein and the ribosome. *Nucleic Acids Res.* 37, 602–610.
- Wang, H.X., Ng, T.B., 2001. Isolation of pleuteregins, a novel ribosome-inactivating protein from fresh sclerotia of the edible mushroom *Pleurotus tuber-regium*. *Biochem. Biophys. Res. Commun.* 288, 718–721.
- Whelan, S., Goldman, N., 2001. A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. *Mol. Biol. Evol.* 18, 691–699.
- Yang, Y., Mak, A.N., Shaw, P.C., Sze, K.H., 2010. Solution structure of an active mutant of maize ribosome-inactivating protein (MOD) and its interaction with the ribosomal stalk protein P2. *J. Mol. Biol.* 395, 897–907.