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Research Article

Analysis of castor bean ribosome-inactivating proteins and their gene expression during seed development

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Abstract

Ribosome-inactivating proteins (RIPs) are enzymes that inhibit protein synthesis after depurination of a specific adenine in rRNA. The RIP family members are classified as type I RIPs that contain an RNA-N-glycosidase domain and type II RIPs that contain a lectin domain (B chain) in addition to the glycosidase domain (A chain). In this work, we identified 30 new plant RIPs and characterized 18 *Ricinus communis* RIPs. Phylogenetic and functional divergence analyses indicated that the emergence of type I and II RIPs probably occurred before the monocot/eudicot split. We also report the expression profiles of 18 castor bean genes, including those for ricin and agglutinin, in five seed stages as assessed by quantitative PCR. Ricin and agglutinin were the most expressed RIPs in developing seeds although eight other RIPs were also expressed. All of the RIP genes were most highly expressed in the stages in which the endosperm was fully expanded. Although the reason for the large expansion of RIP genes in castor beans remains to be established, the differential expression patterns of the type I and type II members reinforce the existence of biological functions other than defense against predators and herbivory.

Keywords: agglutinin, evolution, lipase, *Ricinus communis*, ricin, RIPs.

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Introduction

Ribosome-inactivating proteins (RIPs) are enzymes that irreversibly inhibit protein synthesis by cleaving the glycosidic bond between adenine and ribose in the exposed loop of 28S rRNA that is involved in the interaction between rRNA and elongation factors 1 and 2, *i.e.*, RIPs act as specific RNA-*N*-glycosidases (Endo and Tsurugi, 1987). This specific depurination of rRNA blocks the interaction between rRNA and elongation factors and interrupts protein synthesis (Endo *et al.*, 1991).

RIPs are classified as type I RIPs that consist of a single polypeptide chain (~30 kDa) with RNA-*N*-glycosidase activity and correspond to the A chain, and type II RIPs that consist of a protein dimer containing an A chain and a B chain (~35 kDa) linked by disulfide bonds (Peumans *et al.*, 2001; Stirpe, 2004). The B chain has lectin activity, recognizes terminal galactose residues and facilitates the interaction between type II RIPs and the cell membrane (Lord *et al.*, 1994).

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RIPs have been identified primarily in plants but have also been found in bacteria, algae and fungi (Girbes *et al.*, 2004). The castor bean (*Ricinus communis*), an oil-rich crop (Scholz and da Silva, 2008), contains the proteins ricin and agglutinin, both of which are type II RIPs. Ricin is the best-characterized RIP of the group (Lord *et al.*, 1994). The interest in ricin is related to its cytotoxicity towards mammalian cells (Spivak and Hendrickson, 2005), its potential use in biological weapons (Franz *et al.*, 1997), its use as a research tool to study intracellular transport (Roberts and Smith, 2004) and its therapeutic uses against tumors (Schnell *et al.*, 1996; Wang *et al.*, 1998) and AIDS (Scadden *et al.*, 1998; Donayre Torres *et al.*, 2009).

Genomic and transcriptomic analyses of ricin-encoding genes (Halling *et al.*, 1985; Lamb *et al.*, 1985) have shown that this protein is synthesized from a single mRNA that encodes a preproprotein known as preproricin, which contains the A and B chains. Preproricin contains 576 amino acids, the first 35 of which correspond to the signal peptide required for translocation of the protein to the endoplasmic reticulum (ER) lumen. The 267 residues beyond the signal peptide form the A chain which is linked to the 262 residues of the B chain by a linker peptide of 12 residues (Lamb *et al.*, 1985; Frigerio and Roberts, 1998). The

linker peptide is a signal for vacuolar sorting (Frigerio *et al.*, 2001). Once the protein is sorted into a vacuole the linker peptide is proteolytically removed to promote ricin activation (Frigerio *et al.*, 1998).

Agglutinin shares high structural similarity with ricin (Roberts *et al.*, 1985), which suggests that agglutinin may have evolved from duplication of the ricin gene (Ready *et al.*, 1984). Agglutinin has strong agglutinating activity but low RIP activity (Hegde and Podder, 1998). Like ricin, agglutinin is expressed as a preproprotein, with the formation of a self-assembled tetramer determining its RIP and agglutinating activities (Roberts *et al.*, 1985).

X-ray crystallography (Monzingo and Robertus, 1992) and site-directed mutagenesis (Kim *et al.*, 1992) studies have identified the ricin residues that are critical for catalysis. The adenine of rRNA binds between two conserved tyrosine residues and the core active site is formed by two residues, a glutamic acid and an arginine (Day *et al.*, 1996). More recently, a tryptophan residue located near the active site is apparently important for preservation of the active site structure (Ding *et al.*, 2003). Site-directed mutagenesis, protein structural analyses and protein kinetics experiments have shown that, in addition to its RIP activity, ricin also has lipolytic activity that is targeted to glycerophospholipids and especially triglycerides (Morlon-Guyot *et al.*, 2003). In this same study, the active site of ricin, which contains histidine, serine (A chain) and glutamic acid (B chain), was also characterized.

The paralogous ricin genes were first characterized by Southern blot analysis and six genes were proposed to be members of the ricin gene family (Halling *et al.*, 1985). Later, the ricin agglutinin family was found to contain eight members (Tregear and Roberts, 1992). The recent publication of the castor bean draft genome revealed the presence of 28 putative RIP genes, including those for ricin, agglutinin and possibly pseudogenes (Chan *et al.*, 2010). To date, six ricin-like castor bean proteins have been shown to contain RIP activity (Leshin *et al.*, 2010).

To gain an overall picture of the *R. communis* RIP gene family, we have re-examined the 28 candidate castor bean RIP genes and the genome draft. Additional plant type I and type II RIPs were identified in addition to the foregoing ricin and agglutinin genes. The expression patterns of the castor bean RIP genes were analyzed by quantitative PCR at five stages of seed development. The phylogenetic relationship of these proteins with other plant RIPs and the functional divergence among type I and II castor bean RIPs are discussed.

Materials and Methods

Plant material

The inflorescences of mature AL-Guarany 2002 castor bean plants cultivated at EMBRAPA Clima Temperado (Pelotas, RS, Brazil) were collected for seed dissection. Five seed developmental stages (S1 to S5) were selected based on morphological differences described in previous work (Chen *et al.*, 2004) and in a recent publication by our group (Cagliari *et al.*, 2010). Briefly, the first stage (S1) contains seeds ~0.5 cm wide that are green-yellow and lack a testa (seed coat) and caruncle, stage S2 contains seeds ~1 cm wide that are light yellow and lack a testa and early caruncle, stage S3 contains seeds ~1 cm wide that are yellow with dark spots and show early formation of the testa and the caruncle during maturation, stage S4 contains seeds with ~1 cm wide that are dark brown and contain a formed testa and caruncle and stage S5 contains mature seeds.

Identification of RIPs

The castor bean ricin sequence (60629.m00002) was used as a query for BLAST searches against plants with sequenced genomes in the Phytozome database and against *Jatropha curcas* in the NCBI database. The 28 putative *R. communis* RIPs (Chan *et al.*, 2010) and rice RIPs (Jiang *et al.*, 2008) were retrieved from the Phytozome database by a keyword search. All of the sequences were screened using the InterProScan (Quevillon *et al.*, 2005) tool and were inspected manually for the presence of RIP motifs. The FancyGene (Rambaldi and Ciccarelli, 2009) and WebLogo (Crooks *et al.*, 2004) programs were used to illustrate the castor bean gene composition and amino acid conservation, respectively. The crystallographic model of ricin (pdb code 2AAI) was used with the software Swiss-Pdb-Viewer (version 4.0.1) (Guex and Peitsch, 1997) to illustrate the active site of the RIPs and their association with lipase activity.

Phylogenetic analysis and functional divergence

The retrieved RIP sequences were aligned using MUSCLE (Edgar, 2004) implemented in MEGA 5.0 (Tamura *et al.*, 2011) and contained customized adjustments. The protein sequence alignments were used for the phylogenetic analyses that were done using the Bayesian and maximum-likelihood (ML) methods. The Bayesian analysis was done with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) using the mixed amino acid substitution model plus gamma and invariant sites. Two independent runs of 5,000,000 generations each with a parallel Metropolis-coupled Markov chain Monte Carlo (MCMCMC) were run in parallel (each started from a random tree). The Markov chains were sampled every 100 generations and the first 25% of the trees were discarded as burn-in. The remaining generations were used to compute the majority rule consensus tree (Allcompat command in MrBayes), the posterior probability of the clades and the branch lengths. The convergence of the two runs was assessed by checking the average standard deviation of the split frequencies (< 0.01) and the potential scale reduction factor (PSRF, which was very close to 1.0 for all of the parameters). The maximum-likelihood (ML) method was run in PhyML 3.0 (Guindon *et al.*, 2010) and the branch points were tested for

their significance by bootstrapping with 100 replicates. The sequences that covered only the A chain domain were used to construct RIP trees. The phylogenetic trees were rooted with a medium point and visualized using FigTree software (http://tree.bio.ed.ac.uk/software/figtree/).

The functional divergence was estimated with DIVERGE 2.0 software (Gu and Vander Velden, 2002) which detects evolutionary functional divergence. This software used a phylogenetic tree to assess site-specific changes in the evolutionary rates within the amino acid alignments between the subgroups (type I and type II RIPs). The coefficient of evolutionary functional divergence (θ) was used to measure changes in the site-specific evolutionary rate. A value of $\theta = 0$ indicated a lack of functional divergence, while increasing values of θ indicated increasing functional divergence, with $\theta = 1$ being the maximum.

RNA extraction, primer design and expression analysis by RT-qPCR

Total RNA from the dissected seeds was extracted with NucleoSpin columns (Macherey-Nagel) and purified with an PolyATract mRNA isolation system III (Promega) according to the manufacturer's specifications for optimal mRNA isolation. Reverse transcription of the first strand of cDNA was done using 2 μg of purified mRNA, the primer T25V (1 μg/μL) and 200 units of M-MLV reverse transcriptase (Promega) in a final volume of 50 μL. The cDNA products were diluted 1:10 and stored at -80 °C. The reverse transcriptase reaction was done as follows: a denaturation step of 5 min at 70 °C was followed by a rapid thaw on ice and elongation at 42 °C for 1 h. Genomic DNA from the S5 seed stage was extracted with a PureLink Total DNA purification kit (Invitrogen) according to the manufacturer's instructions.

Primer3 (Rozen and Skaletsky, 2000) was used to design the *R. commnuis* RIP-specific primer pairs (Table 1). All of the PCR amplification products were sequenced using an automatic sequencer (ABIPRISM 3100 Genetic Analyzer, Applied Biosystems) to confirm the identity of the *R. commnuis* RIP cDNAs and the specificity of the primers.

The gene expression patterns in the five stages of seed development were assessed by quantitative real-time PCR (RT-qPCR) that was done in a StepOnePlus real-time PCR system (Applied Biosystems) using SYBR green dye. Briefly, the cDNAs were diluted up to 100-fold and mixed with primers (0.2 μM), dNTPs (25 μM), 1X PCR reaction buffer (20 mM Tris-HCl and 50 mM KCl), 3 mM $MgCl_2$, 0.1X SYBR green, Platinum *Taq* polymerase (0.25 U/μL) and DNAse-free water in a total volume of 20 μL per reaction tube. The RT-qPCR conditions were as follows: an initial hot-start step at 94 °C for 5 min was followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at

Table 1 - *Ricinus communis* ribosome-inactivating protein (RIP) access codes in the Phytozome database and the acronyms and primer sequences that were designed for RT-PCR and RT-qPCR.

Access code	Acronym	Primer sequences		Amplicon size (bp)
		Forward primer	Reverse primer	
29638.m000512	Rco_I1	TTAAGTATGCTTCCTCTCCTTCAACT	TGTCTGAATGGCAATGGAAA	197
29638.m000513	Rco_I2	AACCTATGATGGGACTCTTGCTT	TTGACAGCAGCTGGATGAAG	93
60638.m00022	Rco_I3	AATATATCGAGGAAAAATTGGTTGAG	TTGCAGATGTCTGAATTGCTTC	117
28842.m000952	Rco_I4	AGAGAATACCACATTGGGAACCTA	GCAGCCTCTGAAACCATTTG	129
29852.m001982	Rco_I5	GAGAGTACAACACTGGGAAGTGG	CAATTCTTGCTCGATATATGTGAATC	156
29942.m000748	Rco_I6	CCAAAACGAGGGGATTTGA	TGTGGCCAAGTACAGCAAGA	183
30113.m001449	Rco_I7	AATAGGATTGCAAGATGAAAACTACC	GCATTGGCAAGAACTTTTTGAT	146
60629.m00002	Rco_Ric	GCGTATCGTAGGTCGAAATGG	GCGTTTCCGTTGTGAATC	62
60637.m00004	Rco_Aggl	GAGCCCATAGTGCGTATCGTA	CTGTCCTGGACTGGACTTGG	198
60638.m00019	Rco_II3	TCACGGATGTTCCAAATCC	TAAAGCGCTGAGATAGCATCC	130
60638.m00018	Rco_II4	GTGCACGTCCACCATCAA	CATTTCGACCCGCGATAC	114
29988.m000128	Rco_II5	TTGTTTGCATCCAAATGGC	GCAGATCTTCGATTGTGTCG	118
29988.m000125	Rco_II6	TTCAACCACTTACGGGTACAA	TCTGGGATTTATGATGGTTCC	92
60638.m00023	Rco_II7	GCTCAAACAATACCCAACGG	AAAGCTCTGCCTGATTTGTGA	200
60637.m00006	Rco_II8	GGTCATTCACATTGCAGGATAGT	ACGTCATCTCCAGCTAATAATTGA	145
29791.m000533	Rco_II9	TCAACCAGCTTGGCGG	CTGTCACGCAGCACTGGTAT	173
29942.m000749	Rco_II10	GGATATGCTTTGGCAGCATC	TGCAGGACTGCAAGATGAAA	312*
60638.m00025	Rco_II11	GATACCATGCTTAAGCTCGAGAAG	TCCACTTTTTCTCCGCTCAC	138

*183 bp in genomic DNA.

60 °C for 10 s, extension at 72 °C for 15 s and an additional step for the data recording at 60 °C for 35 s; after the cycling stage, an additional melting curve was obtained with an initial step at 50 °C for 120 s that was followed by a stepwise increase in temperature (0.3 °C/step) up to 99 °C.

The previously described reference genes, *i.e.*, elongation factor-B (29785.m000934), ubiquitin (30169.m006323) and the ubiquitin conjugation protein rad6 (29736.m002026) (Cagliari *et al.*, 2010), were used to normalize the castor bean RIP expression levels by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). All experiments were done in quadruplicate.

Results

Identification of RIP genes

To identify new RIP-encoding genes in plants, BLAST and keyword searches were done in the Phytozome and NCBI databases. Eighty-four putative RIP sequences were retrieved (Supplementary material Table S1). The numbers of genes varied from one copy in *Cucumis sativus* (Csa) to 20 members in *Oryza sativa* (Osa). The *R. communis* (Rco) genome contained 18 RIP-encoding genes. Of the 84 RIPs, 30 have not previously been published, including genes from *Manihot esculenta*, *Prunus persica*, *Cucumis sativus*, *Populus trichocarpa*, *Sorghum bicolor*, *Setaria italica*, *Brachypodium distachyon* and *Zea mays.* Although *Z. mays* and *S. bicolor* also contained type II RIPs in their genomes, the canonical amino acids in the active site that were responsible for the toxic proprieties were replaced in these species (Table S1).

Alignment of the 18 castor bean sequences revealed the presence of 7 type I and 11 type II RIPs. In the present work, ricin and agglutinin were annotated as Rco_Ric and Rco_Aggl, respectively. The remaining *R. communis* RIPs were annotated and numbered according to their relative type. Figure 1 shows the detailed structure of the genes for the 18 *R. communis* RIPs, including the coding sequences, introns and linker peptide positions. Rco_II3, Rco_II5 and Rco_I4 contained a single intron each whereas Rco_II10 contained seven introns; all of the other members lacked introns. We used the linker peptide position to estimate the boundary of the A and B chains and to determine their relative lengths. The size of the A chains was more uniform when compared to that of the B chain counterparts. Rco_II3 and Rco_II10 contained B chains that were half the size of the other *R. communis* type II RIPs.

One striking finding was that Rco_II5 and Rco_II11 showed no linker peptide while their B chain counterparts were classified as type II RIPs (Figure 1). Sequence analysis revealed that following the gene duplication that gave rise to Rco_II5, a guanine base was introduced into the sequence and resulted in a chain termination mutation that introduced a premature stop codon (Figure 2). The sequence alignment of Rco_II4 and Rco_II5 showed that the introduced stop codon was present in a different frame in Rco_II4 (Figure 2). Indeed, the downstream Rco_II5 sequence, which is out of the translational frame, may be a portion of a non-functional A chain. In this case, the upstream 29988.t000011 gene (ricin-agglutinin family protein) was most likely a part of the Rco_II5 B chain and the current Rco_II5 sequence is a portion of a truncated A chain. The downstream sequence of Rco_II5 shared high nucleotide identity with the equivalent region of Rco_II4, which supports the finding that the Rco_II5 downstream region is a non-functional portion of Rco_II5 (Figure 2). However, Rco_II11 did not contain a linker peptide region and the 5-kbp downstream sequence of the Rco_II11 gene showed no similarities to or signatures of the B chain (data not shown).

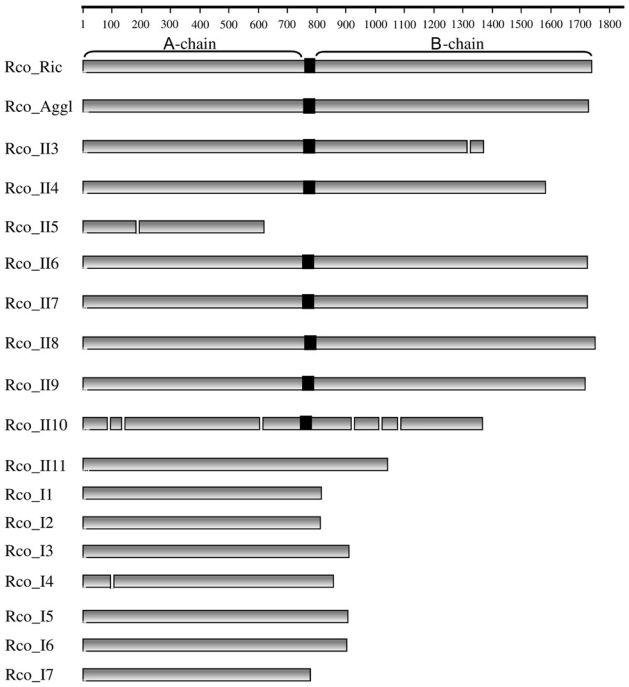

Figure 1 - Structure of the 18 *R. communis* RIP (Rco_RIP)-encoding genes. The exons are shown in grey boxes and the linker peptide regions that delimit the A and B chains are indicated by black boxes. The introns that were detected are shown out of scale by interruption of the grey boxes.

The conservation of the amino acid sequences of *R. communis* RIPs was analyzed with the WebLogo tool. The residues in the active site cleft, which is responsible for the toxic properties of RIPs (Figures 3A,B), and the catalytic triad, which is responsible for the lipolytic activity (Figures 3A,C,D), showed high conservation. This conservation was maintained even among the A chains of type I and type II Rco RIPs (Figure 3A). These findings indicate that other plant species in addition to *R. communis* contain RIP genes. These findings indicate that despite the high diversity among *R. communis* RIP gene structures there is strong amino acid conservation among the proteins encoded by these genes.

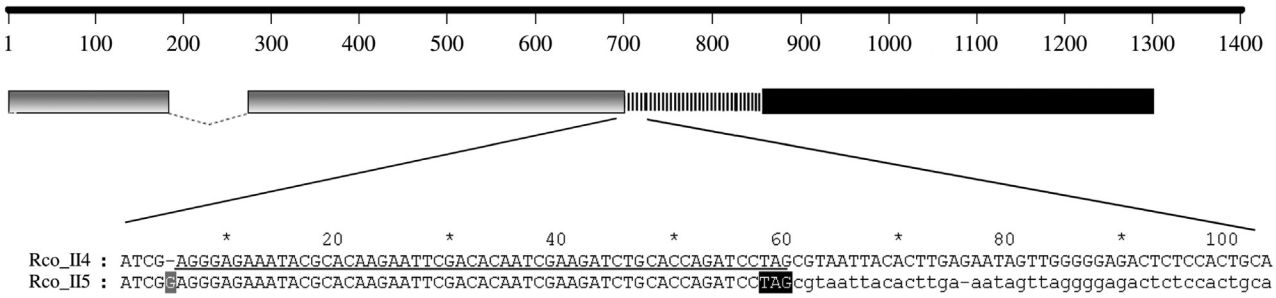

Figure 2 - Structures of the 29852.t001982 (Rco_II5) and 29988.t000011 (ricin-agglutinin family protein) genes and the sequence alignment of Rco_II5 and Rco_II4. The Rco_II5 exons are shown in grey boxes and the introns are indicated by dashed lines. The intergenic regions are identified by the striped box and the 29988.t000011 gene is shown as a black box. The sequence alignment of Rco_II5 and Rco_II4 showed that a chain termination event caused by the addition of a guanine base (amino acid identified by grey shading) introduced a translational frame shift that resulted in a premature stop codon (amino acid identified by black shading) and occurred after the Rco_II5 and Rco_II4 duplication event. The intergenic region, indicated by lower-case letters, also demonstrates the high sequence identity of the Rco_II4-equivalent region.

Type I and II plant RIPs show independent parallel evolution

Bayesian and maximum likelihood (ML) trees were constructed based on the 84 RIP sequences that were retrieved (Figure 4 and Supplementary material Figure S1, respectively). The Bayesian and ML trees showed similar topologies and revealed evolutionary partitioning of the type I and II RIPs, which indicated that RIP clustering was based mostly on the protein type rather than the species of origin. The *R. communis* RIPs showed a clustering pattern in which type I RIPs were more closely related to other Euphorbiaceae type I RIPs than to type II RIPs. A similar patern of clustering was observed for Zma_II1, Sbi_II1 and type II *R. communis* RIPs (Figure 4 and Figure S1). The phylogenetic tree also showed that duplication events occurred independently in both of the *R. communis* type clades and that the internal duplication events were recent. The exceptions were Rco_II10 and Rco_II11, neither of which grouped with their corresponding clades (Figure 4 and Figure S1).

To complement the phylogenetic studies, the extent of functional divergence between the RIP genes was analyzed using the DIVERGE software. The coefficient of functional divergence theta (θ) represents the extent of divergence of a specific amino acid site between two protein groups, where θ = 1 represents the maximum divergence. The mean θ between *R. communis* type I and II RIPs and the *J. curcas*, *M. esculenta* and monocot type I RIPs was calculated (Figure 5) and the results confirmed the clustering pattern for the RIP types shown in Figure 4. The value of θ was always smaller when RIPs of the same type were compared, indicating that RIPs are more functionally divergent between types than within the same type (Figure 5). The phylogenetic and functional analyses indicated that RIPs of the same type were more evolutionarily related to each other and less functionally divergent than were RIPs of different types or clades.

Rco_Ric and Rco_Aggl are the most highly expressed RIPs in seeds

PCR using the genomic DNA of seeds was done to confirm the presence of the *R. communis* RIPs identified *in silico* in the castor bean genome. All 18 of the *R. communis* RIP amplicons produced PCR bands of the predicted size (Table 1 and Figure S2), thus confirming the existence of the *R. communis* RIP genes that had been retrieved from the Phytozome database. The identities of the PCR products from the 18 *R. communis* RIPs were also confirmed by sequencing (data not shown).

The expression of these RIPs during seed development was initially assessed by RT-PCR using cDNA from mature seeds. In this seed stage, only Rco_Ric (ricin), Rco_Aggl (agglutinin) and Rco_II5 showed detectable amplicons, indicating that these RIPs were highly expressed in mature seeds (Figure S2). Because RT-PCR is not a sensitive method for the detection of low levels of gene expression, we also undertook accurate gene expression quantification analysis by RT-qPCR in five seed developmental stages.

These expression analyses revealed that 10 *R. communis* RIP genes were expressed in developing seeds (Figure 6). Among the type I *R. communis* RIPs, Rco_I3, Rco_I4, Rco_I5 and Rco_I7 showed a similar pattern of expression, with the highest expression occurring in stages S3 and S4 (Figure 6A). The type II *R. communis* RIPs Rco_Ric, Rco_Aggl, Rco_II4, Rco_II5, Rco_II6 and Rco_II11 also showed the highest expression in stages S3 and S4 (Figure 6B). Together these results indicate that the 18 *R. communis* RIPs that were identified *in silico* are present in the *R. communis* genome and that four type I and six type II RIP genes are expressed during seed development.

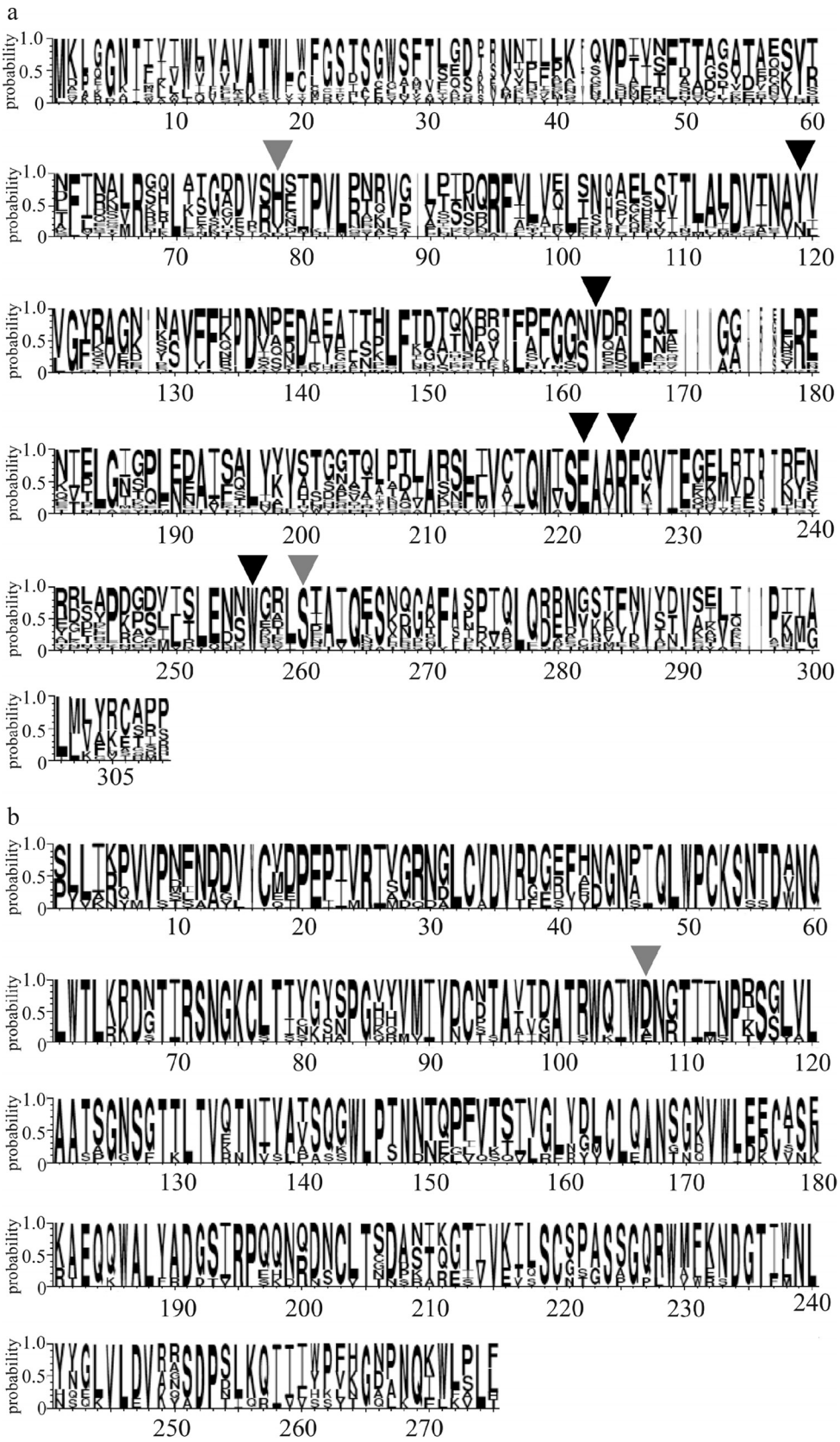

Figure 3 - Logo and crystallographic visualization of the A and B chains of *R. communis* RIPs. (a) Logo representation of all the *R. communis* A chains. (b) Logo representation of all the *R. communis* B chains. The logo heights in these graphs represent the amino acid probability for each position. The residues of the active site of Rco_Ric that are responsible for toxicity are indicated by the black arrowheads, and the active site residues that are responsible for the lipolytic activity are indicated by the grey arrowheads.

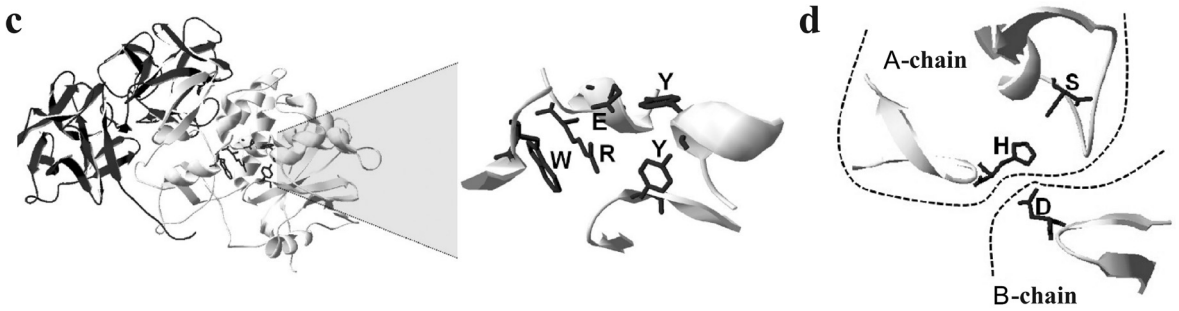

Figure 3 (cont.) - (c) A crystallographic model of ricin (pdb 2AAi) with the A chain in gray and the B chain in black. The spatial conformation of the active site responsible for toxicity is indicated. (d) Model representing the spatial conformation of the lipolytic active site of ricin.

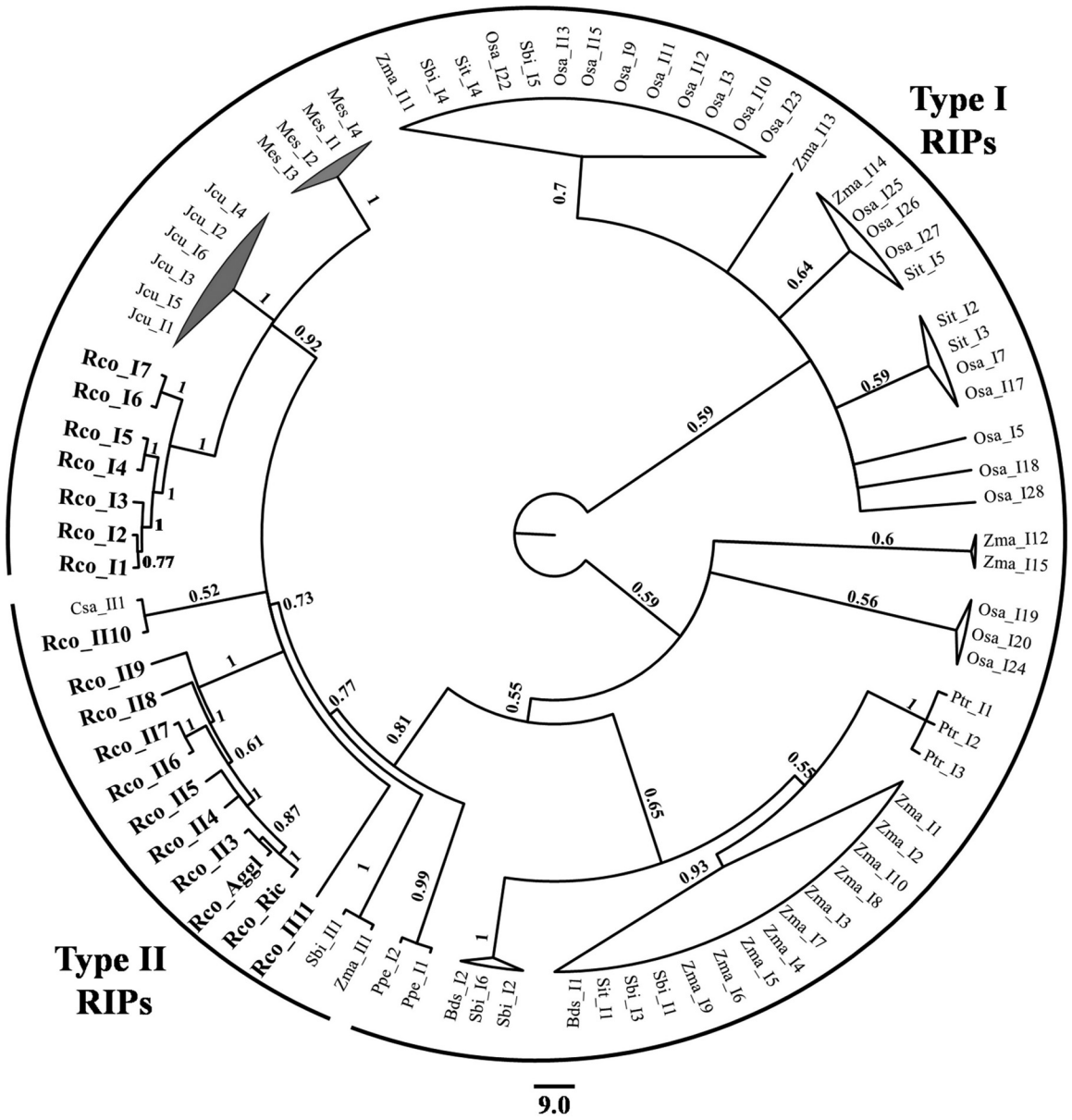

Figure 4 - Bayesian phylogenetic tree showing the evolutionary relationships among the *R. communis* (Rco), *Manihot esculenta* (Mes), *Jatropha curcas* (Jcu), *Prunus persica* (Ppe), *Cucumis sativus* (Csa), *Populus trichocarpa* (Ptr), *Zea mays* (Zma), *Sorghum bicolor* (Sbi), *Setaria italic* (Sit), *Brachypodium distachyo* (Bdi) and *Oryza sativa* (Osa) RIPs. The primary clustered monocot RIPs are shown by white sectors and Euphorbiaceae plants (with the exception of *R. communis*) are shown by gray sectors. The midpoint rooting and *a priori* posterior probability values are also shown.

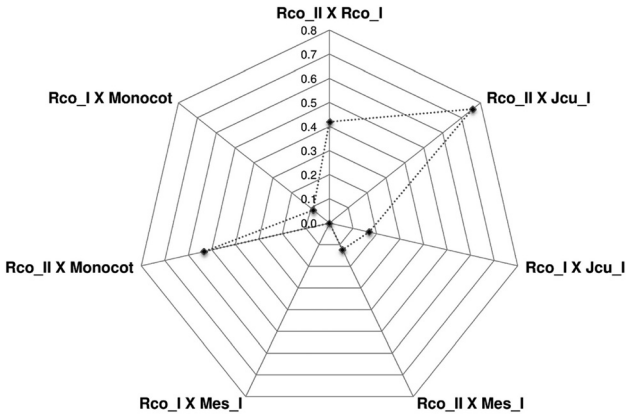

Figure 5 - Evolutionary functional divergence analysis of RIP genes showing the mean values of the divergence coefficient (θ) between the type I and II *R. communis* (Rco), *M. esculenta* (Mes), *J. curcas* (Jcu) or monocot RIPs. The divergence coefficient measures the site-specific evolutionary rate, where θ = 0 indicates no functional divergence and increasing values indicate increasing functional divergence, with θ = 1 being the maximum.

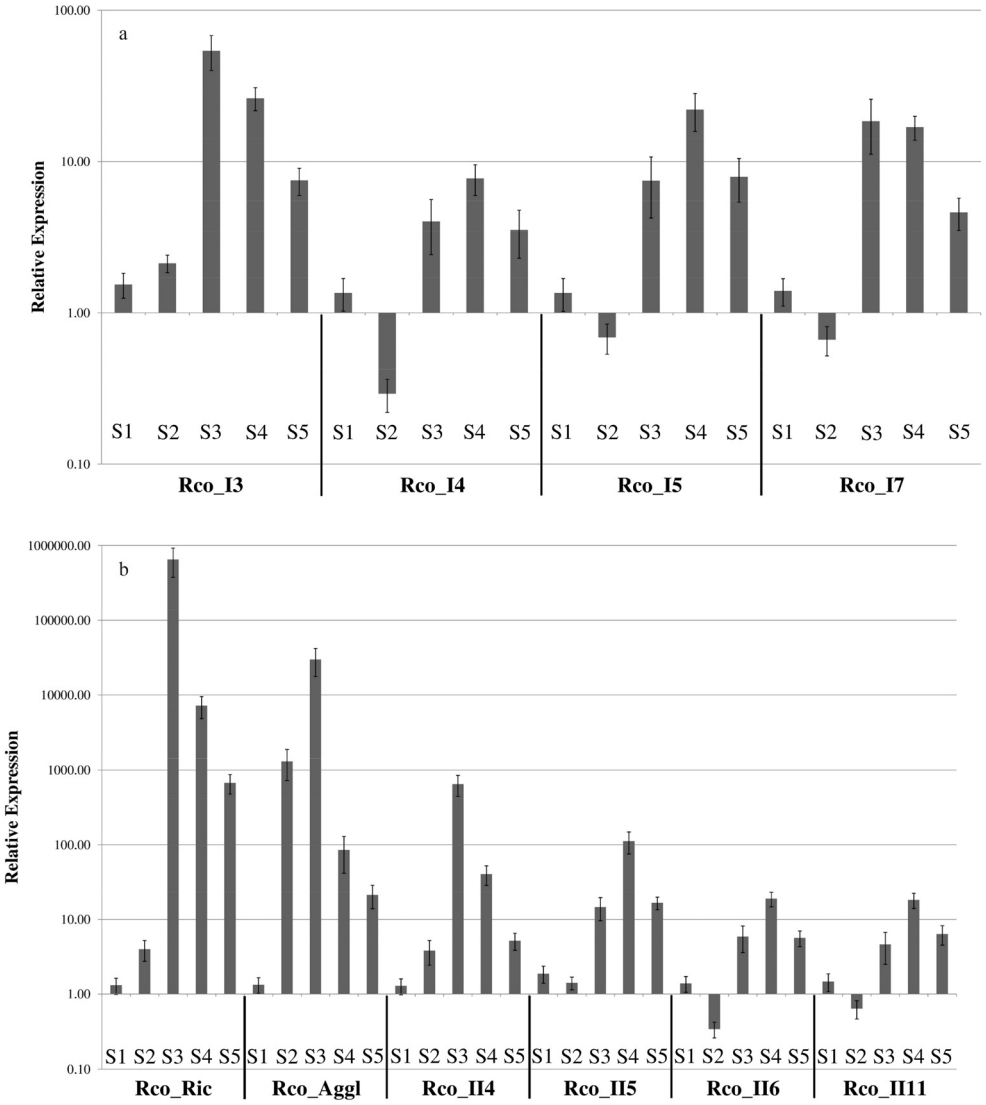

Figure 6 - Relative gene expression of *R. communis* RIPs as measured by RT-qPCR in the five stages of seed development. (A) The relative expression levels of Rco_I3, 4, 5 and 7. (B) The relative expression levels of Rco_Ric, Rco_Aggl and Rco_II 4, 5, 6 and 11. The expression data are shown on a logarithmic scale. The RIP gene expression levels were normalized at the S1 stage by the $2^{-\Delta\Delta Ct}$ method using the elongation factor B (29785.m000934), ubiquitin (30169.m006323) and ubiquitin conjugation protein rad6 (29736.m002026) genes. The bars represent the mean ± standard deviations (n = 4).

Discussion

Identification of plant RIPs

A systematic search in the public Phytozome and NCBI databases resulted in the identification of 84 putative ribosome-inactivation proteins (RIPs), 18 of which occurred in castor bean. Of the 26 plant species deposited in the Phytozome database, only 10 contained RIPs and five of them were monocots. Although pectin methyl esterase has RIP activity in *Arabidopsis* (De-la-Pena *et al.*, 2008) no RIP coding sequences were identified in the *Arabidopsis* genome.

Basal organisms, such as moss and chlorophytes, do not contain RIP genes. However, RIP activity has been identified in the fruiting bodies of the mushrooms *Hypsizygus marmoreus* (Wang *et al.*, 2006), *Volvariella volvacea* (Yao *et al.*, 1998), *Pleurotus tuberregium* (Wang and Ng, 2001), *Lyophyllum shimeji* (Lam and Ng, 2001) and *Flammulina velutipes* (Wang and Ng, 2001) and in the brown algae *Laminaria japonica* (Liu *et al.*, 2002). Unfortunately, genome sequences are presently unavailable for these organisms. Although *Escherichia coli* and *Shigella dysenteriae* contain RIPs, they were not included in our analysis because bacterial RIPs are evolutionarily distant from those of plants and their inclusion in this study could have led to a bias in the phylogenetic analysis.

At least 36 plant RIP genes have been characterized and their protein sequences are available (Girbes *et al.*, 2004), although there is little information on the genome structure and organization for most of these species. Consequently, the abundance of type I and II RIPs cannot be assessed. For this reason, we focused on plant species for which a full genome was available. We identified 30 unpublished RIPs belonging to eight plant species; three of these genes were for type II RIPs, which are far less abundant than type I RIPs (Girbes *et al.*, 2004). Moreover, the three new type II RIPs rom *C. sativus*, *Z. mays* and *S. bicolor* differed in their core active sites that are responsible for toxicity. Changes in the core active site residues of ricin are related to a decrease in toxicity (Day *et al.*, 1996; Leshin *et al.*, 2010). These three species have been subjected to genetic breeding (Li *et al.*, 2011; Tenaillon and Charcosset, 2011) that may have selected for plants that contain nontoxic RIPs. However, this hypothesis needs to be confirmed by examining the RIP genomic sequences from wild-type relatives of *C. sativus*, *Z. mays* and *S. bicolor*.

The recently published draft of the *R. communis* genome sequence revealed the existence of 28 members that were associated with the ricin/agglutinin gene family. These members included seven putative type II RIPs, nine putative type I RIPs, four putative pseudogenes and eight fragments of the B chain (Chan *et al.*, 2010). In contrast, in the *R. communis* genome, our analysis identified seven type I and nine type II RIP genes, in addition to ricin and agglutinin. Our analysis also identified a previously characterized pseudogene (29988.m000128), referred to here as Rco_II5. Rco_II5 and Rco_II11 (60638.m00025) had gene lengths similar to type I RIPs but phylogenetic analyses showed that they were grouped with Rco_ric (ricin), Rco_Aggl (agglutinin) and other putative type II RIPs.

Chan *et al.* (2010) identified two cases in which adjacent ricin family members accumulated frame-shift mutations and could correspond to pseudogenes. Our phylogenetic analysis suggested that the recent duplication event that generated Rco_II4 and Rco_II5 introduced a guanine into the coding sequence and that this introduced a chain termination mutation by incorporating a stop codon; this mutation resulted in the formation of a truncated protein. The sequence downstream of the stop codon, which is characterized as intergenic, is actually a fragment of the A and B chains, and the upstream gene (29988.t000011) is a portion of the Rco_II5 B chain. However, we observed no B chain counterpart upstream of the Rco_II11 gene. This gene may have originated from an early duplication event that occurred within the type II RIP group, with the Rco_II11 B chain counterpart being lost during subsequent evolution.

The ricin B chain counterparts are lectins that recognize carbohydrate cellular receptors and mediate intracellular glycoprotein trafficking; this contributes to the greater toxicity of type II RIPs compared to type I RIPs (Dodd and Drickamer, 2001). The B chains of ricin and agglutinin contain four domains and amino acid substitutions could account for differences in toxicity between ricin and agglutinin (Roberts *et al.*, 1985). Rco_II3 and Rco_II10 contain a shorter B chain than ricin and agglutinin because of the absence of 1-2 lectin domains, although the influence of this lack of B chain domains on the function of Rco_II3 and Rco_II10 remains unclear. Neither gene was expressed in any of the five seed stages analyzed, perhaps because Rco_II3 and Rco_II10 are pseudogenes. Rco_II10 contains a greater number of introns than other castor bean RIPs. This finding supports the pseudogene hypothesis but requires confirmation.

The evolution of plant RIPs

General phylogenetic and evolutionary studies of RIPs have shown that the distribution of these proteins in plants and bacteria is the product of independent parallel duplications (Girbes *et al.*, 2004; Jiang *et al.*, 2008). In the present work, a robust phylogenetic analysis of plants for which the genomes have been sequenced confirmed that RIPs evolved in an independent, parallel manner.

Our phylogenetic analyses showed that the amino acid sequences of genes from members of the same RIP type are related. This finding suggests that the evolution of these proteins was based on the RIP type and not on the taxon, in contrast to previous proposals. For example, Girbes *et al.* (2004) proposed that all of the monocot type 1

RIPs were related, a conclusion that resulted in the family Poaceae appearing as a homogeneous group. Jiang *et al.* (2008) subsequently showed that the *O. sativa* sequences were separated into subclades, a conclusion that supports our findings for *O. sativa* and other members of the Poaceae. Our phylogenetic analyses also showed that all type II monocot RIPs were clustered with type II eudicot RIPs, indicating that that Poaceae is indeed a heterogeneous group.

The A chains of all type II RIPs are related, which suggests that they have a common origin (Girbes *et al.*, 2004). Our phylogenetic analyses showed that the separation between type I and II occurred before the monocot/eudicot split in the late Jurassic-early Cretaceous periods (140-150 million years ago) (Chaw *et al.*, 2004).

With the exception of Rco_II10 and Rco_II11, the *R. communis* RIPs clustered into two main groups that corresponded to type I and II RIPs. Most of the *R. communis* RIPs are products of recent duplication events; however, Rco_Ric and Rco_Aggl have been suggested to be the products of an ancestral RIP gene duplication (Roberts *et al.*, 1985). The phylogenetic tree shown in Figure 4 confirms that Rco_II3 is a product of an Rco_Aggl duplication. Rco_Aggl, or agglutinin, is a dimeric protein and this conformational structure is related to its lower toxicity when compared to ricin (Olsnes, 2004). Rco_Aggl exists as a heterodimer in which the non-identical protein monomers differ in their surface charge and molecular mass (Hegde and Podder, 1998). Rco_II3 may also exist as a heterodimer but this remains to be confirmed experimentally.

Girbes *et al.* (2004) demonstrated that *Iris hollandica* type I RIP was phylogenetically related to *I. hollandica* type II RIPs, possibly because of a B chain deletion. A similar B chain deletion event clearly occurred with Rco_II5 (Figures 1, 2 and 4) and may also have generated Rco_II11.

The functional divergence analysis between the Euphorbiaceae and monocots strongly corroborated our phylogenetic analyses, which showed that RIPs of the same type or class were less functionally divergent even when derived from different taxa. The lack of strong functional divergence between type I and type II *R. communis* RIPs may be indicative of their overlapping functions.

Expression of *R. communis* RIPs during seed development

PCR and DNA sequencing confirmed the presence of the 18 RIPs in the castor bean genome and the specificity of each primer. Expression of the Rco RIP gene was assessed qualitatively by RT-PCR based on the absence/presence of PCR amplicons in mature seed cDNA. Our findings agreed with previous studies showing that Rco_Ric and Rco_Aggl were expressed in *R. communis* seeds (Chen *et al.*, 2005). Since Rco_Ric and Rco_Aggl show seed-specific expression in castor beans (Barnes *et al.*, 2009) the current study focused primarily on RIP expression during five stages of seed developmental as assessed by RT-qPCR. Our results corroborated previous findings obtained by northern blotting (Tregear and Roberts, 1992), RT-PCR (Chen *et al.*, 2005; Barnes *et al.*, 2009), expressed sequence tags (Lu *et al.*, 2007) and seed proteomics (Houston *et al.*, 2009), all of which demonstrated that Rco_Ric and Rco_Aggl were expressed at higher levels in seeds that contain a fully expanded endosperm (S3-S5). Indeed, Rco_Ric and Rco_Aggl were the RIPs with the highest levels of expression in developing seeds, perhaps because these RIPs were the first to be discovered (Olsnes and Kozlov, 2001). Our results also provide the first evidence that four other type I and II *R. communis* RIPs are expressed in seeds, including Rco_II5, which was previously characterized as a pseudogene (Chan *et al.*, 2010).

Jiang *et al.* (2008) analyzed the expression of *O. sativa* RIPs in seedlings and suggested that RIPs that were not expressed may have been pseudogenes. As shown here, eight castor bean RIPs showed no detectable expression during seed development. However, broader expression analyses that include different tissues, developmental stages and responses to biotic and abiotic stress, are required to determine whether these eight RIPs actually correspond to pseudogenes.

The functions of castor bean RIPs

Plant RIP genes were reported to be regulated by abiotic stress and senescence (Stirpe *et al.*, 1996), salinity (Rippmann *et al.*, 1997), drought (Bass *et al.*, 2004) and oxidative stress (Iglesias *et al.*, 2005, 2008), and by biotic stress, such as viral infections (Iglesias *et al.*, 2005), insect herbivory (Zhou *et al.*, 2000; Bertholdo-Vargas *et al.*, 2009) and fungal infections (Xu *et al.*, 2007). RIP expression patterns can be modulated by plant hormones such as jasmonic acid (Reinbothe *et al.*, 1994; Xu *et al.*, 2007), abscisic acid (Xu *et al.*, 2007), gibberellic acid (Ishizaki *et al.*, 2002) and ethylene (Park *et al.*, 2002).

Recently, Rco_Ric (ricin) was associated with the inhibition of seed predation (Barnes *et al.*, 2009) and some RIPs have dual activities on superoxide dismutase (Sharma *et al.*, 2004) and lipase (Morlon-Guyot *et al.*, 2003). RIPs may initially have been related to pathogen defenses (Stirpe, 2004; Stirpe and Battelli, 2006). During the evolution of RIPs, one or more gene fusion events probably occurred among type I RIPs and the lectin genes that correspond to the B chain (Figure 7). These events may have resulted in the appearance of type II RIPs, which have greater toxicity and show greater inhibition of herbivory. Morlon-Guyot *et al.* (2003) demonstrated that in addition to its well-demonstrated toxicity, ricin also has lipolytic activity that could facilitate its translocation from the membrane to the cytosol. Recently, Leshin *et al.* (2010) analyzed the RIP activity of six type II *R. communis* RIPs that displayed toxicity but the lipolytic activity was not analyzed. The *R. communis* RIP genes have an expression pattern similar to

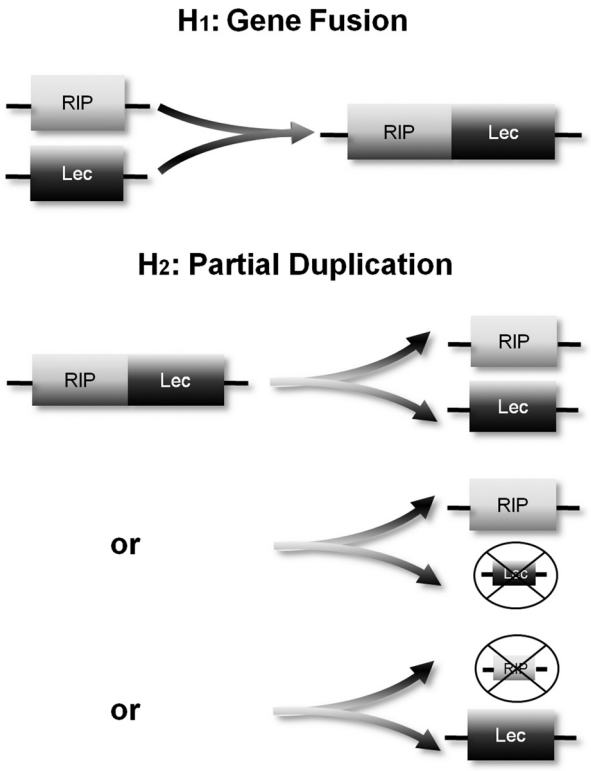

Figure 7 - Hypotheses to explain the appearance of type II RIPs and their putative return to the independent, separate domains of type I RIPs and lectins. (H1) Gene fusion: the result of gene duplication of an RIP gene and lectin gene is fusion leading to a type II RIP. (H2) Partial duplication: a type II RIP may be partially duplicated, with separation of the RIP and lectin genes or loss of the A or B chain.

that of genes involved in the castor bean triacylglycerol (TAG) biosynthesis pathway (Cagliari *et al.*, 2010). We speculate that the lipolytic activity of ricin may be associated with TAG metabolism. Ricin gene knockdown or knockout protocols (Sujatha and Sailaja, 2005; Sailaja *et al.*, 2008) may help to elucidate the role of RIPs in TAG metabolism.

In conclusion, we have identified 30 new RIP-encoding genes from eight plant species, including three new type II RIPs in the castor bean RIP gene family. Expression analyses revealed that, in addition to ricin and agglutinin, eight RIPs are expressed during seed development. Phylogenetic analyses also showed that the type I and type II RIP gene divergence event most likely occurred before the separation of the monocots and eudicots.

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Internet Resources

Phytozome database, http://www.phytozme.org.

Supplementary Material

The following material concerning this article is available online:

Table S1 - Description of Ribosome Inactivating Proteins (RIPs) data used for phylogenetics analysis.

Figure S1 - Maximum likelihood phylogenetic tree for plant RIPs.

Figure S2 - Agarose gel electrophoresis of the *R. communis* RIP PCR products.

This material is available as part of the online article at http://www.scielo.br/gmb.

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