2S Albumin Gene Expression in Castor Plant (*Ricinus communis* L.)

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ABSTRACT: In the castor plant (*Ricinus communis* L.), 2S albumins are highly allergenic seed proteins and are detrimental to the reintroduction of castor as a U.S. crop. Others have shown that the two 2S albumins are encoded by a single gene and processed from a precursor protein in castor. Little is known about the expression pattern of the 2S albumin gene. As part of a genetic approach to eliminating 2S albumin from castor, we investigated the developmental expression of this gene in castor seed. To assess seed developmental age quickly and accurately, we established a set of simple criteria, which included two visual markers, seed coat color and endosperm volume, and defined three phases that encompass the course of castor seed development. Northern analysis indicated that the 2S albumin mRNA is highly abundant in the mid-phase of seed development. A comparison of 2S albumin genomic DNA and cDNA clones from two castor cultivars indicated that only a single gene is expressed. Protein domain sequence analysis revealed that castor 2S albumin contains the trypsin/α-amylase inhibitor family domain, suggesting a role for the albumin in insect resistance.

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KEY WORDS: Biobased products, castor, oilseed, ricinoleate, 2S storage protein.

The castor plant (*Ricinus communis* L.) produces seed containing a unique oil that has numerous nonfood applications. Up to 90% of the FA content of castor oil is ricinoleate (12-hydroxy oleate). Castor oil and ricinoleate have many important industrial uses, serving as a source of lubricants, coatings, plastics, and fungicides (1). However, castor cultivation and processing can expose workers to potent allergens that evoke an immunoglobulin E (IgE) response and lead to serious medical consequences (2–4). Based on fractionation studies, 2S albumin proteins were eventually identified as the primary allergenic components of castor seed meal (5,6). The amino acid sequence of one of these castor 2S albumins (7) allowed isolation of a full-length cDNA and a genomic clone (8,9). Sequence analysis of the clones indicates a castor 2S albumin gene with a 776-bp open reading frame encoding a precursor protein of 258 amino acids. The precursor produces two heterodimeric 2S albumin proteins, RicC1 (7) and RicC3 (10,11). We report here the pattern of the seed morphological changes and the expression of the 2S albumin gene during various stages of seed development. This initial information is critical to developing and implementing a genetic silencing approach to eliminate 2S albumin from castor seed.

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EXPERIMENTAL PROCEDURES

Plant material. The castor (*R. communis* L.) seeds, PI215769, were obtained from USDA-Germplasm Resources Information Network, Southern Regional Plant Introduction Station (Griffin, GA). Plants were germinated and grown in a greenhouse at temperatures ranging between 28 (day) and 18°C (night), with supplemental metal halide lighting to provide a 15-h-day length (1000 to 1250 μeinstein/m^{-2}/s^{-1}). Plants used for physiological studies of seed development were grown from seeds germinated in June to ensure the most favorable day-length condition for flowering. The initial flowering occurred 55 to 65 d after planting. Fully opened new female flowers were individually pollinated and tagged, and the tagging dates were recorded as 0 d after pollination (0 DAP). Capsules were harvested at 7-d intervals from 12 to 61 DAP. Qualitative characteristics of the capsules and seeds were recorded immediately upon collection. Measurements of at least 18 capsules and at least 50 seeds were taken at each time. For Northern analysis, separate sets of seeds were frozen immediately in liquid nitrogen after dissection and stored at –80°C.

Isolation of total RNA and Northern analysis. Total RNA was extracted from the fresh frozen seeds using TRIzol reagent (Life Techologies, Rockville, MD). RNA pellets were dissolved in diethyl pyrocarbonate-treated water, and the RNA was quantified by using a spectrophotometer (A_{260}/A_{280}) and checked for quality by gel electrophoresis, using ethidium bromide as a stain (12). Northern blotting was carried out as described by Sambrook *et al.* (12). The DNA probes (730 to 1207 bp according to the Genbank gene sequence, X54158) were generated using digoxigenin (DIG)-labeled nucleotides incorporated by polymerase chain reaction (PCR) and, after immunolabeling, detected by chemiluminescence (Roche, Indianapolis, IN).

First-strand cDNA synthesis and amplification of target DNA. Total RNA from a mixed sample of 26 to 40 DAP seeds was first treated with DNase I, and then reverse-transcribed to first-strand cDNA by using Oligo(dT) primer and SUPERSCRIPT II (SUPERSCRIPT™ First-Strand Synthesis System for reverse transcription-PCR (RT-PCR; Life Technologies). Target DNA was amplified using Pwo DNA Polymerase

(Roche). Gene-specific PCR primers, based on the castor 2S albumin gene sequence published in NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov; Genbank accession No. X54158), were used to amplify the 2S albumin cDNA and genomic fragments.

Cloning of PCR-amplified DNA, endosperm cDNA library construction, and DNA sequencing. PCR products were ligated into a pCR®4Blunt-TOPO® vector and transformed into TOP 10 *Escherichia coli* cells using the Zero Blunt TOPO PCR Cloning Kit for Sequencing (Life Technologies). For construction of a castor endosperm cDNA library, poly (A^+) RNA was extracted from the total RNA of seeds between 26 to 35 DAP by using the PolyATtract mRNA Isolation SystemII (Promega, Madison, WI). The cDNA synthesis was performed with a ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA) using an oligo(dT) primer with *Xho*I linker. The cDNA was inserted into λ ZAP arms following addition of an *Eco*RI adaptor. DNA was sequenced by the USDA Sequencing Facility (Albany, CA).

RESULTS AND DISCUSSION

Morphological criteria for assessing seed developmental ages. The basic structures of a mature castor seed include testa, caruncle, endosperm, and embryo. The seed consists of a mass of endosperm and an embryo with two thin papery cotyledons lying in the center of the endosperm. The endosperm is not absorbed by the embryo until seed germination. Castor plants produce a racemic, monoecious inflorescence, with male and female flowers blooming asynchronously, so pollination must be used to provide a common starting point for determining seed developmental age. We manually pollinated and tagged female flowers and investigated the external and internal morphological features of developing seeds as a function of age. Among all features we examined, the testa color and the endosperm volume were most distinctive in determining the developmental age of castor seed. Figure 1 illustrates the morphology of a developing seed. Fresh seed weight, seed size, and capsule size are plotted in Figure 2.

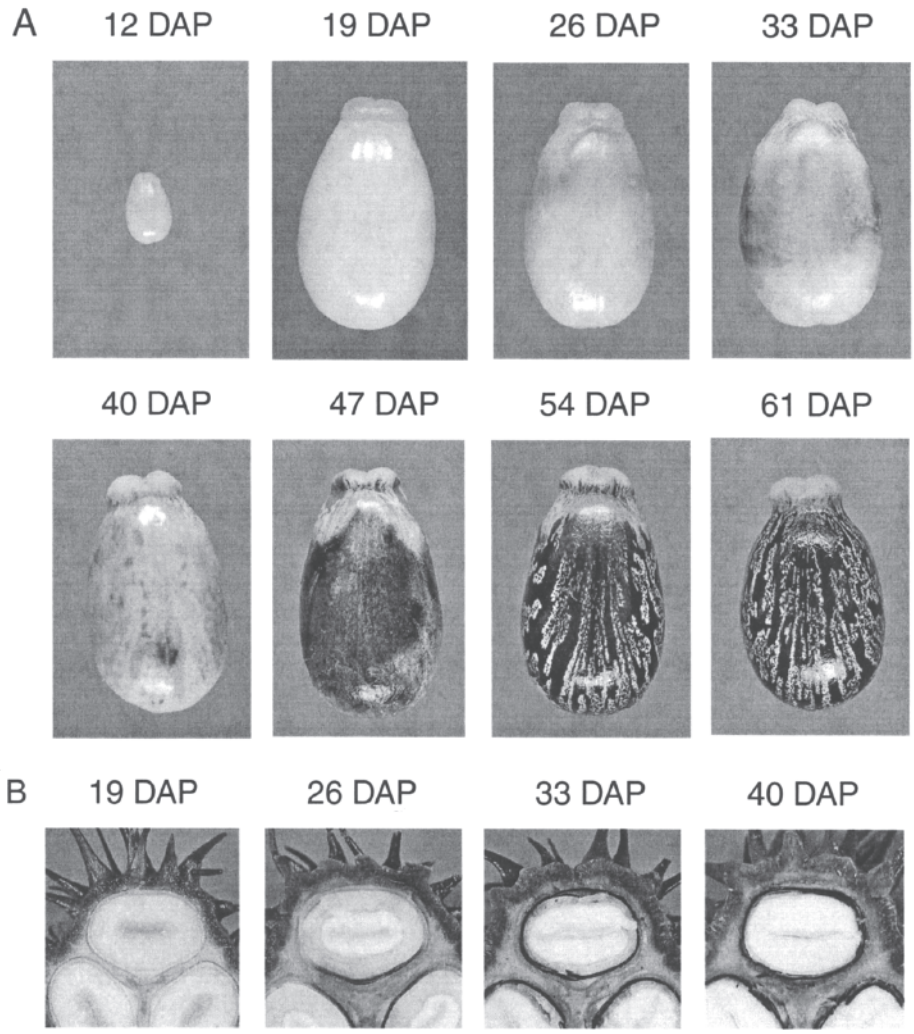

FIG. 1. The morphological changes of PI215769 castor seeds during development. (A) Whole seed; (B) transverse cross section. Endosperm is shown in opaque color. DAP, days after pollination.

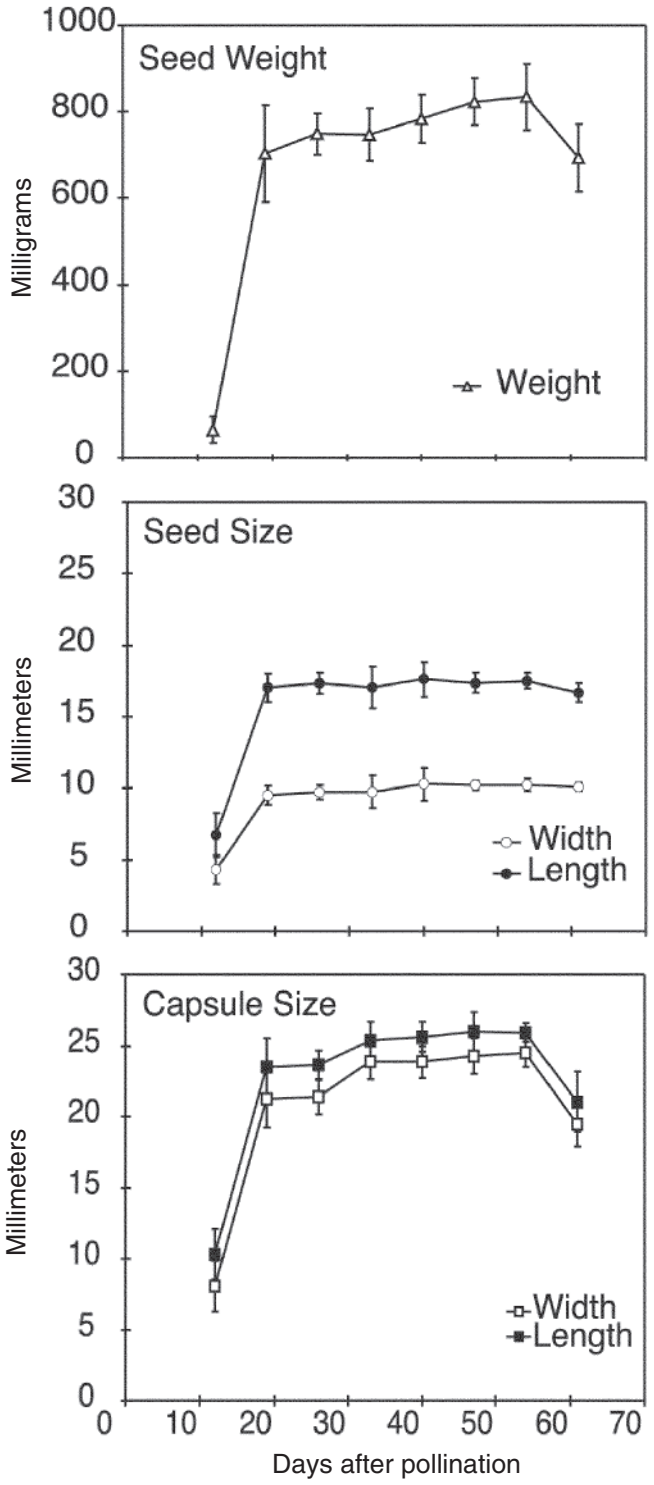

FIG. 2. The growth curves of seed development. Each data point represents the mean ± SD of at least 18 capsules and at least 50 seeds.

Based on changes of these two features, we divided seed developmental stages into three phases (I to III), each phase spanning approximately 20 d. During phase I of seed development, the capsule and seed develop their basic tissues and grow rapidly to almost full size at 19 DAP (Fig. 1). Seeds have an ivory-white testa color and the endosperm tissue is not yet expanded. The major volume of seed is filled with ground tissue of inner integument. In phase II, the volume of endosperm increases dramatically (Fig. 1B, 26 to 40 DAP). The growing endosperm ultimately displaces the inner integument and accounts for about 80% of the seed mass. The expansion of endosperm volume coincides with the changes of testa color, which start from the caruncle end and spread to the opposite chalazal end. By the end of this phase (about 40 DAP), the endosperm tissue has reached full expansion. The testa is covered with uneven shades of purple and brown color and partially sclerified. Seeds in phase III (47 to 61 DAP) can be distinguished by filled endosperm and sclerified, pigmented testa. After 54 DAP, seed testa are mature with a shiny mosaic color of chocolate and silver in the case of PI215769. At about 61 DAP, capsules senesce and desiccate, showing reduced seed weight and capsule size (Fig. 2).

The method of determining seed developmental stage is critical for drawing accurate comparisons between experiments. The use of morphological markers for determining seed age reduces variability and increases reliability of comparisons among experiments and among cultivars, where maturation times may differ. Morphological markers for seed development in dicotyledonous plants have been well established for *Phaseolus coccineus* (13) and *P. vulgaris* (14). They have also been used to describe castor seed development for the cultivar Hale (15). However, such criteria require a number of time-consuming measurements, including capsule and seed length, embryo appearance and size, specific endosperm features, and testa color. Since plant materials usually need to be frozen or used immediately upon collection for many molecular and biochemistry studies, it is necessary to establish a simple set of criteria for a quick and accurate assessment of seed development. The color of testa and the endosperm volume are two reliable morphological markers and easy to identify. We are able to accurately designate seed age ±3 d over the entire developmental period. It is clearly advantageous in comparing results from different experiments. In addition, by using our morphological criteria, we can divide castor seed development into three distinct phases, which are comparable to the three major physiological periods assigned for the seed development in Hale castor based on more elaborate criteria (15).

The expression of 2S albumin gene exhibits a temporal pattern coinciding with endosperm expansion. To determine the 2S albumin gene expression pattern during seed development, Northern blot analysis was performed on total RNA from developing seeds. Figure 3 shows the 2S albumin messenger RNA levels in seeds collected at 12, 19, 26, 33, 40, and 47 DAP. A sample of total RNA from young leaves was included in the same blot to test for 2S albumin gene expression in vegetative tissue of castor. During the course of castor seed development, the expression of the 2S albumin was very low in phase I (12 and 19 DAP). The mRNA levels rose sharply during phase II (26 to 33 DAP) when the endosperm underwent rapid expansion (Fig. 3A). Once the endosperm tissue had fully expanded (40 DAP), the level of 2S albumin mRNA decreased (Fig. 3A). In phase III, the mRNA level

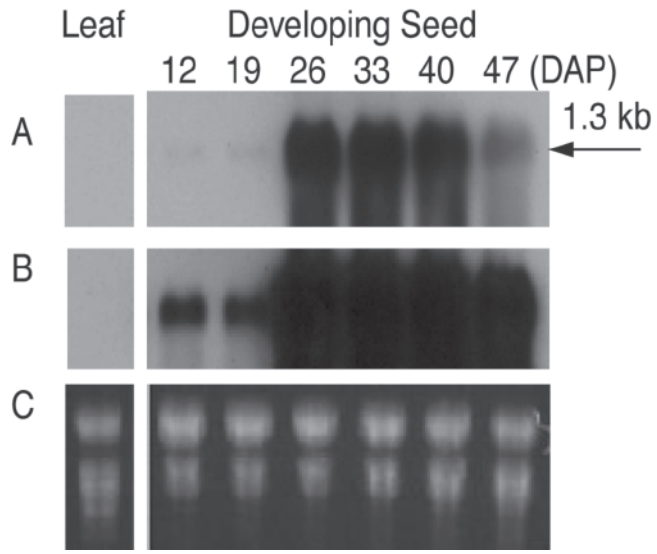

FIG. 3. Northern analysis of 2S albumin expression in PI215769 castor. Approximately 5 µg of total RNA was loaded on each lane. The same blot was exposed to film for 10 s (A) or 10 min (B). (C) Ethidium bromide staining of the gel as a control for equal RNA loading. RNA size (kb) is indicated on the right.

continued to decrease as shown for mRNA from 47 DAP (Fig. 3A). After 47 DAP, the mRNA degraded and did not show signal integrity (data not shown). No 2S albumin mRNA was detected in leaf tissue even after prolonged exposure of the Northern blot (Fig. 3B).

The observed temporal expression pattern of 2S albumin gene is similar to the expression profiles of 2S albumins from oilseed rape (16) and *Arabidopsis* (17). The castor 2S albumin mRNA level reaches its peak at phase II (26 to 33 DAP) of seed development. During the same period, the endosperm dimension increases significantly. It is known that castor endosperm is the storage compartment for various seed proteins including 2S albumins (18,19). The timing suggests that endosperm development and the 2S albumin gene expression are spatially and temporally coregulated. Irwin *et al.* (8) examined castor 2S albumin mRNA levels during seed development, but in their study, the developing seeds were divided into six stages based on the size and testa formation, and they did not provide information on seed developmental stages in days. They did not detect any signal in the early three stages and detected weak RNA signals in seeds from the later three stages with a relatively stronger signal shown in the desiccating seeds from the last stage. In contrast, we observed low expression of 2S albumin in early stages (phase I), maximum expression in middle stages (phase II), and decreasing expression in late the phase (phase III). As the developing seeds used in our study are associated with seed developmental ages, we present a defined developmental profile for the 2S albumin gene expression during seed development.

The 2S albumin gene is conserved among different castor cultivars and abundantly expressed in the endosperm. Using primers derived from the known 2S albumin gene sequence (Genbank X54158, Sudanese origin), we isolated additional genomic DNA fragments covering 2S albumin genes from PI215769 (Peru) and Maui (Hawaii) castor cultivars. Despite their different origins, sequence comparison indicates that the 2S albumin gene is almost completely conserved among castor cultivars. There is only 1 bp difference between the X54158 and PI215769 cultivars. Position 1507 of X54158 was reported as cytosine, whereas the corresponding base in PI215769 is an adenine. The difference is located at the 3′-end of the noncoding region of the genes, and we excluded the possibility of PCR error because multiple genomic PI215769 clones were sequenced. Indeed, sequences of two full-length cDNA clones, one from the

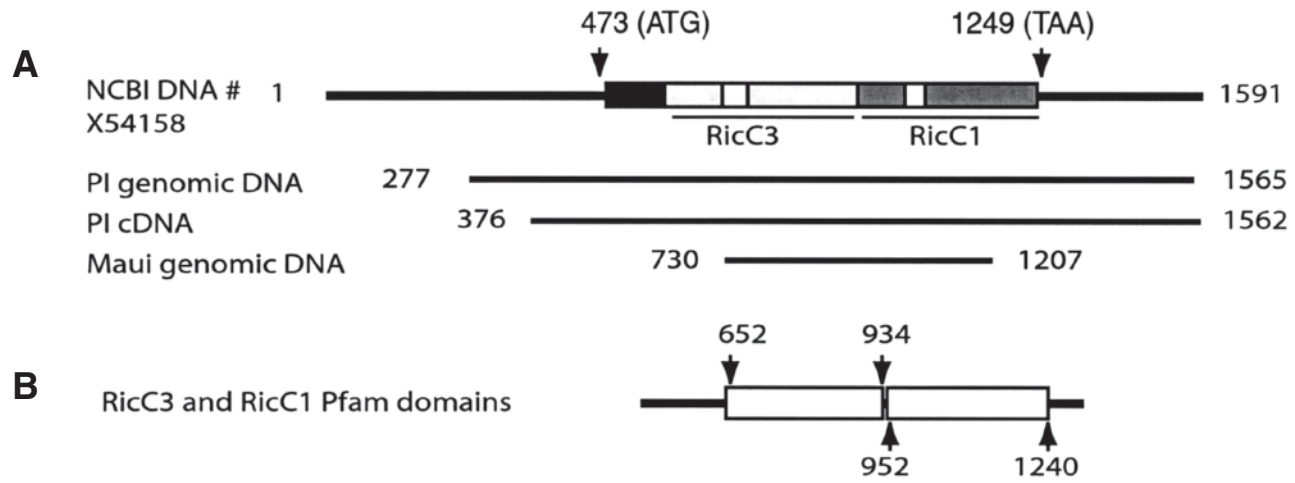

FIG. 4. Schematic representation of the castor 2S albumin gene structure. (A) Reverse transcriptase-polymerase chain reaction isolated clones and sequenced DNA fragments of PI (PI215769) and Maui are aligned with the known gene (Genbank accession no. X54158) from 1 to 1591 bp. The solid box indicates the signal peptide, the light- and dark-shaded boxes refer to the small and large subunits of RicC3 and RicC1, respectively, and the open boxes indicate linking sequences between the small and large subunits. The arrows at 473- and 1249-bp indicate the start codons (ATG) and stop codons (TAA), respectively. (B) Pfam search results: the open boxes indicate the RicC3 and RicC1 Pfam domains (Pfam ID: PF00234). Numbers indicate DNA sequence positions corresponding to the Genbank sequence (X54158).

same laboratory of X54158 genomic clone (8) and one from our laboratory (Fig. 4A), also confirmed that it is an adenine at the position of 1507 bp. It has been suggested that the 2S albumin gene comprises a family of genes (8). We have found a similar Southern blot result in PI215769 cultivar (data not shown) but do not yet know whether the expressed castor 2S albumins are encoded by a single gene or by the entire gene family.

The 2S albumin gene is abundantly expressed in the endosperm of castor seeds. We sequenced 96 clones randomly selected from a castor endosperm cDNA library and found that 11 of them are 2S albumin clones and their sequences are identical to the known 2S albumin gene. The expression of castor 2S albumin is not detectable in the leaf tissue (Fig. 3), indicating seed-specific regulatory elements associated with the 2S albumin gene. There are 472 nucleotides of upstream regulatory sequence available in the corresponding genomic clone (NCBI DNA No. X54158). A comparison of the region immediately upstream of the putative TATA box of the castor 2S albumin gene (bases 343 to 414) with the corresponding regions upstream of the TATA box of the oilseed rape and *Arabidopsis* 2S albumin shows a common motif, CATGCA, from base 388 to 393 (8). This motif is two bases shorter than the CATGCATG box, or RY element, which is conserved among various seed-protein genes including lectin, legumin-type, vicilin-type, and albumin (20). Searches of other well-characterized seed-specific regulatory cis-elements in the upstream region of the TATA box also revealed an abscisic acid-responsive core element (ACGT, bases 292 to 295) and soybean embryo factor binding motif (AGCCCA, bases 415 to 420). RY elements in combination with ACGT or AGCCCA have been identified as essential for seed-specific expression of 2S albumin from rapeseed (21) and 7S β-conglycinin from soybean (22,23), respectively. These putative cis-elements found in the castor 2S albumin gene may confer its temporal and seed-specific expression.

Castor 2S albumins are members of the trypsin/α-amylase inhibitor family, and this family includes a number of seed allergenic proteins. The major *in vivo* role of 2S albumins is clearly as storage proteins (18). However, the conserved nature of castor 2S albumins suggests they may have some important intrinsic features that could serve other biological functions beyond that of food reserve for seed germination. To answer this question, we performed a protein domain analysis on 2S albumin. A Hidden-Markov-Models search of the Pfam database (http://pfam.wustl.edu) identified a trypsin/α-amylase inhibitor family domain (Tryp_alpha_amyl, PF00234) on the 2S albumin protein sequence (Fig. 4B). Castor 2S albumins are homologous to amylase inhibitor and trypsin inhibitor genes in plants (8,11). A number of *in vitro* biochemical activities have been reported for Brassicaceae 2S albumins, including antifungal capacity, trypsin inhibitory activity, and calmodulin antagonist activity (24). Recently, trypsin inhibitory activity was demonstrated *in vivo* for the 2S albumin from *Brassica juncea* (25), which, interestingly, is active only in the precursor form, and its inhibitory activity disappears upon processing. As the 2S albumin precursor is expressed at high levels during mid-phase seed development, it should provide an additional protection to young seeds when insect damage is most likely to occur. Although RicC1 and RicC3 2S albumin share limited sequence identity with the 2S albumin from *B. juncea*, 32 and 24%, respectively (pairwise comparison results of 2S albumin mature peptides), they do contain a trypsin/α-amylase domain. It is possible that castor 2S albumins serve as both trypsin inhibitors, which are thought to confer some insect resistance, and as storage proteins during seed development.

The trypsin/α-amylase inhibitor Pfam family includes cereal seed trypsin/α-amylase inhibitor, nonspecific plant lipid transfer protein, and 2S albumin storage proteins. According to the Food Allergy Research Resource Program (FARRP) database (http://www.allergenonline.com/), about 33% of well-known food allergens have protein sequences that fall into the trypsin/α-amylase inhibitor family (26). These proteins are evolutionarily related and have a conserved "skeleton" of eight cysteine residues (C…C…CC…CXC…C…C) (27). Moreover, all these genes lack introns (27). The molecular basis of the allergenicity of some proteins from this family has been analyzed by determining the allergen-specific IgE-binding sequence or epitopes, as well as their 3-D structures (28). An epitope core sequence, RGEE, identified in the hypervariable region of Jug r1 2S albumin from English walnut (29), is comparable to HGEE, which is also located in the hypervariable region of castor RicC3 (30). It is possible that the HGEE may function as a castor epitope involved in IgE-RicC3 interaction. The 3-D structure of castor RicC3 has been determined by NMR (30). The RicC3 belongs to a singlefold class described in the SCOP (Structural Classification of Protein) database (31) as "4 helices; folded leaf; right-handed superhelix; disulfide-rich." Available structures from the same family members show that they have a similar fold and fall into the same SCOP class (26). There is not yet sufficient information to conclude common structural characteristics of 2S albumin allergen, but this could change as more epitope mapping and crystallographic or NMR studies are completed. The ability to suppress castor 2S albumin will help the development of castor as a domestic crop. Moreover, it can serve as a model for allergen suppression in other seeds.

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