JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Temporal and Spatial Expression of 2S Albumin in Castor (*Ricinus communis* L.)

YEH-JIN AHN† AND GRACE Q. CHEN*

Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 800 Buchanan Street, Albany, California 94710

We studied the temporal and spatial expression of the 2S albumin in castor (*Ricinus communis* L.) during seed development, germination, post-germination, and plant development. Quantitative polymerase chain reaction analysis showed that the 2S albumin transcript accumulated to a maximum level at the middle of seed development, showing a bell-shaped temporal pattern. Residual levels of the transcript were present in the mature seed and degraded rapidly upon germination. Immunodetection analysis was performed using an anti-2S albumin antibody under reducing conditions. During seed development, the 2S albumin precursor pro-protein began to be synthesized at 26 days after pollination (DAP); the pro-protein was thereafter processed to mature proteins at 40 DAP, suggesting that the post-translation modification of 2S albumin takes place during this time period. Both the 2S albumin precursor pro-protein and the mature proteins accumulated throughout seed maturation and desiccation stages. During seed germination, both forms of the 2S albumin proteins were present in endosperm and cotyledon until the completion of germination and degraded rapidly afterwards. However, the antibody also detected a group of proteins/peptides in endosperm and cotyledon when the seeds progressed to germination and post-germination stages. A 14 kDa protein in the leaves of fully developed seedlings and mature plants also reacted to the anti-2S albumin antibody. The identity of the proteins accumulated in germinating seed and leaf remains unknown.

KEYWORDS: 2S albumin; oilseed; *Ricinus communis* L.; seed development; seed germination; storage protein; temporal/spatial expression; transcript/protein accumulation

INTRODUCTION

Plant seeds contain a large amount of starch, proteins, and/or lipids as storage reserves. Storage proteins consist of 10 to 40% of the seed dry weight (*1*). During seed germination, they are mobilized to support the growth of a young seedling. 2S albumins are low molecular weight storage proteins that are widely distributed in dicotyledonous seeds (*1*). Youle and Huang (*2*) showed the presence of the proteins in a specialized storage compartment, a protein storage vacuole (PSV), in the seed endosperm of castor (*Ricinus communis* L.). In an oilseed crop castor, the 2S albumin gene is present as a precursor form [776 base pair (bp) in size; (*3*)]. During seed development, it is expressed to yield a single 2S albumin precursor pro-protein (prepro2S albumin; 29.3 kDa) containing a signal peptide at the N-terminal (*3, 4*). The signal peptide is cleaved off in the lumen of the endoplasmic reticulum, and the resulting protein (pro2S albumin) is transferred to the PSV. Then, it is processed into two hetero-dimeric mature proteins, RicC1 and RicC3. Each mature protein is composed of a small and a large subunit linked by disulphide bonds (*5–7*). The schematic diagram of the prepro2S albumin and the two mature 2S albumins is shown in **Figure 1**. During castor seed germination, storage proteins, including 2S albumins, are converted into amino acids by proteases, such as cystein endopeptidase (*8, 9*). When the mobilization of storage reserves is complete, castor endosperm undergoes nuclear DNA fragmentation and programmed cell death (*8, 10*).

In addition to its primary role as a storage protein, 2S albumin is also the major allergen in castor seeds. Most (96%) castor seed-sensitive patients developed the IgE antibody specific to the 2S albumin protein (*11*). Accordingly, it poses serious health concerns to the castor growers and castor oil factory workers (*12*). The presence of 2S albumins is one of the reasons that the American farmers have lost interest in growing castor commercially, although the lucrative market for the oil still exists. 2S albumins in other plant species, such as mustard seeds (*13*), sesame seeds (*14*), Brazil nuts (*15*), English walnuts (*16*), and cashew nuts (*17*) are also known to have allergenic properties, suggesting that the 2S albumin protein family is intrinsically allergenic.

We are developing a safe castor crop by eliminating 2S albumin from castor seed through a gene silencing approach. As part of the approach, we previously characterized the 2S albumin gene

* To whom correspondence should be addressed. Tel: 510-559-5627. Fax: 510-559-5768. E-mail: qhgc@pw.usda.gov.

† Current address. Major in Life Science, College of Natural Sciences, Sangmyung University, 7 Hongji-dong, Jongno-gu, Seoul 110-743, Korea.

10.1021/jf071272p
Published on Web 10/26/2007

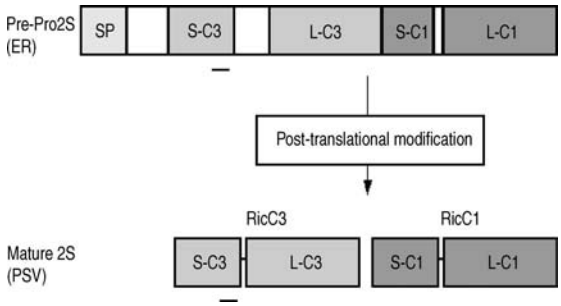

Figure 1. Schematic representation of the prepro2S albumin and the mature 2S albumins in castor. The peptide region, against which an anti-2S albumin antibody was generated, is marked with bars. The schematic diagram is proportional to the actual size of the proteins. ER, endoplasmic reticulum; L-C1, large subunit of RicC1; L-C3, large subunit of RicC3; S-C1, small subunit of RicC1; S-C3, small subunit of RicC3; and SP, signal peptide.

and its transcript in developing seeds using RNA hybridization analysis (*18*). We report here a comprehensive survey on the temporal and spatial expression patterns of the 2S albumin transcript and proteins in various tissues and at different developmental stages. In addition, our analysis revealed a group of unknown proteins whose expressions were detected by an anti-2S albumin antibody in germinating seed, true leaf of seedling, and leaf of mature plants. The results raise interesting questions for future investigation on the identity and function of these proteins.

MATERIALS AND METHODS

Plant Materials. Castor seeds (*R. communis* L. cultivar "Hale") were obtained from the U.S. Dept. of Agriculture, Germplasm Resources Information Network, Southern Regional Plant Introduction Station (Griffin, GA). Seeds were planted in a commercial peat-vermiculite growth mixture (Scotts, Marysville, OH), and plants were grown in a greenhouse at temperatures ranging from 18 to 28 °C (night/day) under a 15/9 h (day/night) photoperiod with light supplemented by metal halide lighting at an intensity of 1000 to 1250 μeinstein m^{-2} s^{-1}. Developing seed samples were obtained as described previously (*18*). Briefly, mature female flower buds just before opening were hand pollinated and labeled as 0 days after pollination (DAP). Developing seeds harvested at 7 day intervals from 19 to 61 DAP were dissected out from seed coat, frozen in liquid nitrogen, and stored at −80 °C until use. For seed germination, mature seeds were surface sterilized in a 0.25% (v/v) sodium hydrochloride solution for 15 min followed by five rinses in sterile deionized water. The seeds were then placed on the germination medium [half-strength MS medium (*19*), pH 5.7, without sucrose] and incubated at 30 °C in the dark. The endosperm, cotyledon, and embryo axis were separated by using a dissecting knife and pooled from at least five germinating seeds. The tissues were collected daily and frozen at −80 °C until use. In mature and germinating seeds until 6 days after imbibition (DAI), the cotyledon was tightly attached to the endosperm; thus, the cotyledon samples obtained during this period contained some of the surrounding endosperm tissues.

Quantitative Polymerase Chain Reaction (qPCR) Analysis. Total RNA was isolated from various castor tissues using RNeasy plant mini kit (Qiagen, Valencia, CA). The buffer RLC, which contains guanidine hydrochloride, was used to lyse cells. The concentrations of RNA samples were estimated by using a spectrophotometer and gel electrophoresis. Purified RNA (1 μg) was reverse transcribed using the QuantiTect reverse transcription kit (Qiagen, Valencia, CA), which includes a genomic DNA removal step. Primers for the 2S albumin gene (GenBank accession X54158) were designed using the Beacon designer software (PREMIER Biosoft International, Palo Alto, CA) as follows: forward (5′-ACGAGTCAAAGGGTGAAAGG) and reverse (5′-CTGCTGAATCTGATCCTCTGC). This primer set would amplify a 267 bp fragment. A set of primers (forward 5′-GAATCCACGAGACTACATACAAC and reverse 5′-TTATGAAGGTTATGCTCTC, amplicon size: 176 bp) was also designed for the actin gene (GenBank accession AY360221) to be used as an internal control for the normalization of the 2S albumin gene expression.

Real-time polymerase chain reaction (PCR) analysis was performed using the SYBR Green I supermix (Bio-Rad, Hercules, CA) and the iCycler system (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. The PCR mix (25 μL) contained 10 μL of cDNA template (0.05 μg per reaction), 1X SYBR Green I supermix, and 300 nM forward and reverse primers. The PCR thermal-cycling parameters were as follows: initial 95 °C for 3 min, followed by 40 cycles each of 95 °C for 10 s and 55 °C for 30 s. The melting curve was generated to confirm the specificity of the amplified products, using thermal cycles of 95 °C for 1 min and 50 °C for 1 min, and followed by 80 cycles of a temperature increase by 0.5 °C (for 10 s) from 50 °C.

To confirm the amplification efficiency of the primers, standard curves were generated using six serial dilutions (10-fold) of the template, which was a mixture of cDNAs from 19 to 54 DAP samples with the starting concentration at 2.5 ng/μL. Correlation coefficients of 0.99 or higher and PCR amplification efficiencies between 85 and 105% were accepted. PCR products were separated by gel electrophoresis to ensure a single product with the expected size.

For the statistical analysis, the threshold cycle (Ct) of each sample was obtained using the iCycler software. Relative gene expression was calculated according to the Pfaffl model (*20*). The ratio of the 2S albumin cDNA in comparison to the actin cDNA at 33 DAP was given an arbitrary value of 10. Expression levels of triplicates were averaged for each sample, and the reproducibility of PCR results was confirmed in at least two independent experiments.

Protein Extraction and Electrophoresis. Total proteins from various castor tissues were extracted by first homogenizing samples in the extraction buffer [0.3% sodium dodecyl sulfate (SDS), 200 mM dithiothreitol, and protease inhibitor cocktail (Complete Mini, Roche, Indianapolis, IN) in 1X phosphate buffered saline (Invitrogen, Carlsbad, CA)]. Lysate was incubated at 70 °C for 5 min and, then, on ice for 10 min. After centrifuging for 5 min at 12000 × *g* (4 °C), clear supernatant was taken, avoiding the precipitants and the upper oil layer. The centrifugation was repeated until a clear protein solution was obtained. Protein quantification was performed according to the Bradford assay (*21*). An equal quantity of protein (20 μg) was resolved on an 18% SDS–polyacrylamide gel electrophoresis (SDS-PAGE; Invitrogen, Carlsbad, CA). To examine the total protein profile, gels were stained with the Coomassie Blue G-250 (Bio-Rad, Hercules, CA) and dried using the DryEase Mini-Gel Drying System (Invitrogen, Carlsbad, CA), according to the manufacturers' instructions.

Protein Immunodetection. Proteins resolved on an 18% SDS-PAGE gel were electroblotted to a polyvinylidene difluoride (PVDF) membrane (Roche, Indianapolis, IN). Immunodetection analyses were performed using a polyclonal antibody raised against a peptide sequence from the small subunit of RicC3 (RQEVQRKDLSSCERYLRQS; Custom antibody, Invitrogen, Carlsbad, CA). This peptide region (**Figure 1**) was recommended by the manufacturer because of its high antigenicity when the whole prepro2S albumin sequence was analyzed using the manufacturer's algorithms. Accordingly, the anti-2S albumin antibody was expected to react to both the prepro2S albumin (29.3 kDa) and the small subunit of the mature 2S albumin RicC3 (4.2 kDa) when proteins were extracted under a reducing condition. The ECL Plus system (Amersham Biosciences, Pittsburgh, PA) was used to visualize the proteins. Chemiluminescent signals were detected on Hyperfilm (Amersham Biosciences, Pittsburgh, PA). The protein immunodetection analysis was performed three times independently. To quantify the accumulation of the 14 kDa protein in leaf, a densitometer (Bio-Rad Gel Doc 2000, Hercules, CA) was used. The level of the protein in the young leaf was given an arbitrary value of 2, and it was used to normalize that level in the mature leaf. The densitometry was performed twice using two independent immunodetection data sets, and the bar graph represents the average of the trials.

RESULTS

Morphological Changes during Seed Development, Seed Germination, and Post-Germination. A mature castor seed consists of a mass of cellular endosperm and an embryo that

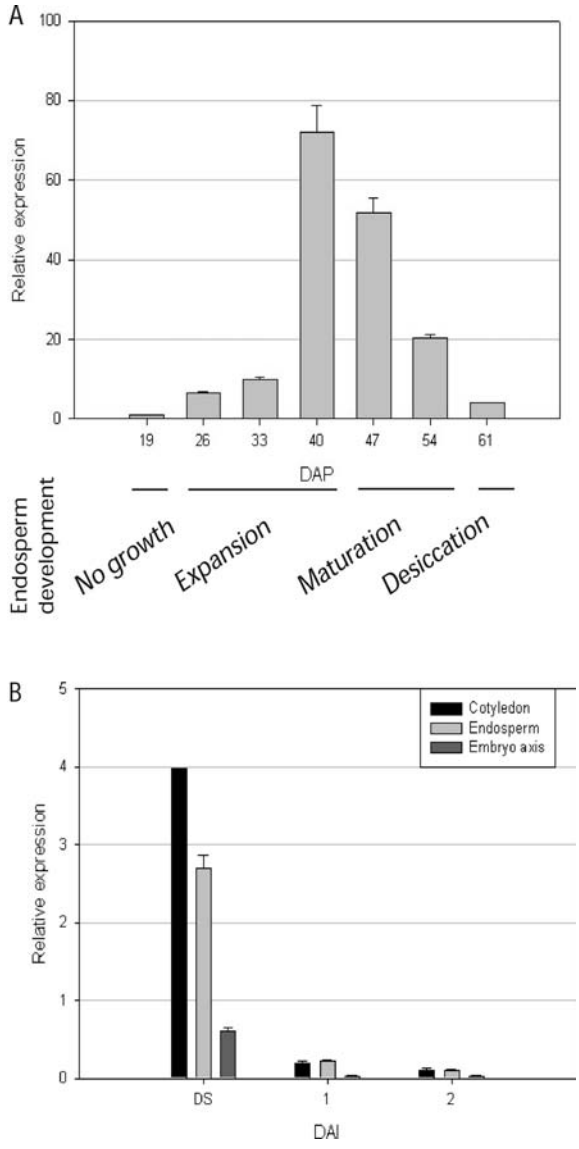

Figure 2. Expression profile of the 2S albumin gene in castor seeds. The transcript levels of the 2S albumin in (**A**) developing seeds and in (**B**) three different tissues (cotyledon, endosperm, and embryo axis) of germinating seeds were analyzed by using quantitative real-time PCR analysis. The levels of the 2S albumin transcript were normalized to that of the actin gene. The error bars show the standard error of the means. DS, dry seed.

has two papery thin cotyledons attached to an embryo axis lying in the center of the endosperm. We have previously characterized the morphological changes of developing castor seeds (germplasm line PI215769) and established a staging system of castor seed development (*18*). In this study, we collected developing seeds from a cultivar "Hale" based on the staging system and observed similar morphological changes during seed development (data not shown). Briefly, after pollination, seeds grew rapidly and reached their full sizes at 19 DAP. The majority of the volume in seeds was filled with inner integument tissue that contained mostly water. At 26 DAP, the seed endosperm started to expand, replacing the inner integument, and eventually occupied more than 90% of the seed volume by 40 DAP. The endosperm then underwent maturation up to 54 DAP. At 61 DAP and after, the seeds were completely mature and began desiccation.

Upon imbibition, castor seeds started to swell, taking up water (data not shown). At 4 DAI, the embryo axis emerged out of the seed coat, completing germination. During the post-germination stage, after 5 DAI, the cotyledons and endosperm continued to expand, and the hypocotyl and root system started to develop. At 7 DAI, the endosperm was consumed and detached from the cotyledons of the young seedling. Because the embryo axis develops into the hypocotyl and the root of a young seedling after germination is complete at 4 DAI, mixed samples from both the hypocotyl and the root tissues were used for total protein and inmmunodetection analyses for the **Figure 3G**,**H**, 5 to 8 DAI lanes.

Transcript Level of the 2S Albumin Gene in Castor. The transcript level of the 2S albumin gene in a cultivar "Hale" was determined using qPCR. During seed development, the 2S albumin transcript was at the lowest level in seeds at 19 DAP (**Figure 2A**). The transcript increased slowly to higher levels at 26 and 33 DAP and then rose quickly to a maximum level at 40 DAP when the cellular endosperm reached its full expansion (*18*), resulting in a seven-fold induction of the transcript between 33 and 40 DAP. After, the amount of the transcript decreased gradually. Residuals of the 2S albumin transcript remained in dry seeds (**Figure 2B**). When examined closely, the cotyledon exhibited a slightly higher level of the transcript than the endosperm. The amount of the transcript was the lowest in the embryo axis, showing 15% of the level in the cotyledon. When the seed started imbibing and germinating, the residual transcripts degraded very rapidly in all the tissues within 2 DAI. After 3 DAI, the 2S albumin transcript was no longer detectable. In addition, the 2S albumin transcript was not found in any nonseed tissues, such as flower (female and male), leaf, stem, and root from 4-month-old adult plants (data not shown).

Profile of Total Proteins and 2S Albumin in Developing Castor Seeds. The accumulation of the total protein in developing castor seeds is shown in **Figure 3A**. In the seeds at early developmental stages between 19 and 33 DAP, proteins with high molecular weight from 40 to 60 kDa were dominantly present. These proteins degraded rapidly after 40 DAP. However, in 26 DAP seed, when the endosperm starts to develop (*18*), three groups of proteins with molecular masses of 30–37, 19–22, and 4 kDa started to be synthesized. The proteins became predominant at the time of maturation (47–61 DAP), which suggested that they were storage proteins. There were minor proteins detected at 14 and 10 kDa in developing seeds after 26 DAP. Our results showed that the developing castor seed undergoes coordinated changes in protein expression from high to low molecular mass ranges. The synthesis of the seed storage proteins coincided with the endosperm development.

As stated in the Introduction, a single prepro2S albumin undergoes post-translational modification to produce two mature 2S albumin proteins, RicC1 and RicC3, consisting of hetero-dimeric subunits linked by disulfide bonds (**Figure 1**). A polyclonal antibody was raised against a peptide region, whose sequence was derived from the small subunit of RicC3. In this study, total proteins were extracted under reducing conditions, separating the small and large subunits of the mature proteins. Accordingly, our anti-2S albumin antibody was expected to react to the prepro2S albumin (29.3 kDa) and the small subunit of the mature protein RicC3 (4.2 kDa) from the seed protein extract. This antibody allowed us to examine not only the accumulation of the two different forms of 2S albumins but also the timing of the post-translational modification. As shown in **Figure 3B**, the anti-2S albumin antibody detected only the prepro2S albumin and the small subunit of the mature RicC3 in developing seed, showing a high specificity. The prepro2S albumin started to be synthe-

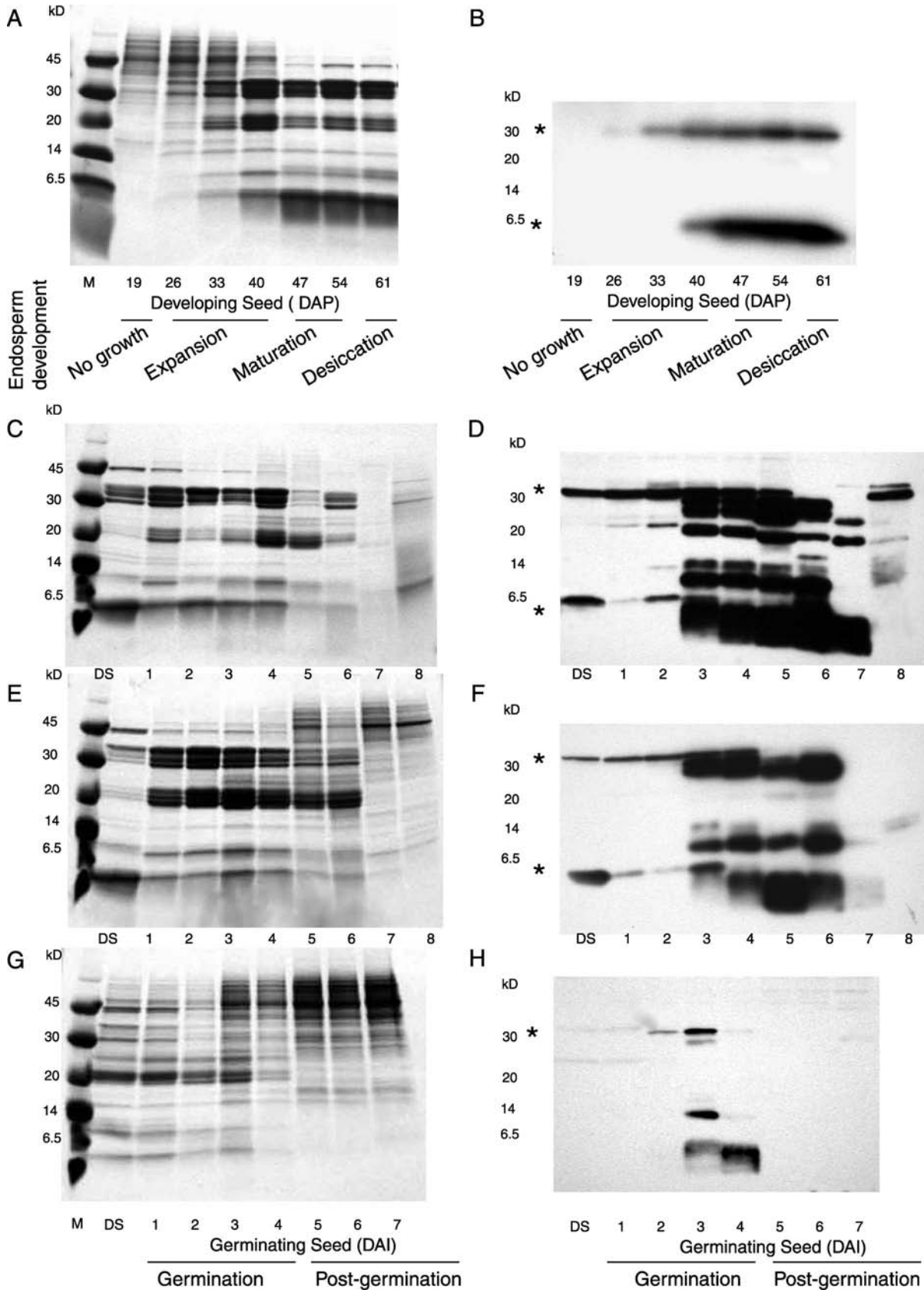

Figure 3. Expression profiles of the total proteins and the 2S albumins during castor seed development and germination. Total proteins were extracted from (**A**, **B**) developing seeds and from (**C** and **D**, endosperm; **E** and **F**, cotyledon; **G** and **H**, embryo axis) different tissues of germinating seeds and resolved on an 18% SDS-PAGE gel. (**A**, **C**, **E**, and **G**) Visualization of total proteins by using Coomassie staining. (**B**, **D**, **F**, and **H**) Immunodetection of the 2S albumins by using an anti-2S albumin antibody, which detects both the prepro2S albumin and small subunit of RicC3. Asterisks denote the prepro2S albumim (29.3 kDa) and the small subunit of RicC3 (4.2 kDa). DS, dry seed; M, molecular marker.

sized at 26 DAP. The levels of the protein increased to a plateau at about 40–47 DAP when the endosperm reached its maximum volume (*18*) and remained at the same high level thereafter. Whereas, the small subunit of the mature

RicC3 appeared at 40 DAP, 2 weeks after the synthesis of the prepro2S albumin, and its level reached a plateau after 47 DAP.

Profile of Total Proteins in Germinating Castor Seeds. To closely examine the expression pattern of total proteins and the degradation of the 2S albumins, we investigated its accumulation during seed germination in three different tissues: endosperm, cotyledon, and embryo axis. The endosperm in the germinating castor seed mobilizes storage lipids and proteins for the growth of the embryo (cotyledon and embryo axis). When the mobilization is complete, the endosperm undergoes programmed cell death while the embryo initiates active cell division to develop into a young seedling. In the endosperm, two dominant groups of proteins with molecular masses of 30–37 kDa and 4 kDa were present until germination was complete at 4 DAI (**Figure 3C**). There were less abundant proteins detected, including a 45 kDa protein at dry stage and 1 DAI and two groups of proteins with molecular masses of 19–22 kDa and 10–12 kDa in seeds at 1–6 DAI. From 7 DAI, after the endosperm was completely detached from the cotyledon, rapid protein degradation occurred, resulting in no or little protein present in the entire molecular mass range.

In the cotyledon of dry seeds, there were dominant protein bands detected at 45, 37, and 4 kDa and faint bands at 30–35, 19–22, and 10 kDa (**Figure 3E**). Among the dominant protein bands, the 45 and 4 kDa proteins degraded gradually and were absent after 7 DAI. However, two groups of proteins with sizes of 30–37 and 19–22 kDa started accumulating predominantly from 1 to 6 DAI and then disappeared after 7 DAI. During postgermination (5–8 DAI), following the radicle emergence and completion of germination, high molecular mass proteins from 40 to 60 kDa started to be synthesized. With the disappearance of other proteins after 7 DAI, these high molecular mass proteins became abundant, when the cotyledon completely separated from the endosperm.

In the embryo axis of dry seeds and germinating seeds by 2 DAI, we observed a wide range of protein bands, including dominant ones at 45, 37, 30, 22, 10, and 4 kDa (**Figure 3G**). After 3 DAI, proteins with molecular mass higher than 40 kDa increased dramatically, while most of the proteins smaller than 25 kDa degraded.

Immunodetection of 2S Albumin in Germinating Castor Seeds. Immunodetection analysis showed that, in the endosperm of germinating seeds, the prepro2S albumin and the mature albumin were present until 5 and 3 DAI, respectively (**Figure 3D**). Between 3 and 6 DAI, additional protein bands with sizes of 25–28, 22, 10–14, and <3 kDa reacted to the anti-2S albumin antibody. Most of these proteins degraded at 7 DAI, with the exception of the bands at 25, 22, and <3 kDa. At 8 DAI, protein bands at 29 and 30 kDa were detected. Although these proteins migrated to the size close to that of the prepro2S albumin, it does not appear to be that the prepro2S albumin is being resynthesized because the 2S albumin transcript was not detected after 2 DAI (**Figure 2B**). In the germinating cotyledon, the prepro2S albumin and the mature 2S albumin were present until 4 and 3 DAI, respectively, and degraded rather rapidly afterward (**Figure 3F**). However, additional groups of proteins at 25–29, 10–14, and <3 kDa reacted to the anti-2S albumin antibody from 3 to 6 DAI. After 7 DAI, when the cotyledons were completely detached from the endosperm, no proteins were detected. In the embryo axis of dry seed and germinating seed at 1 DAI, trace amounts of proteins at 30 and 20 kDa were detected which degraded completely after 2 DAI (**Figure 3H**). The antibody also reacted transiently to a number of other proteins: a 29 kDa protein at 2 and 3 DAI, 27 and 12 kDa proteins at 3 DAI, and a <3 kDa protein(s) from 3 to 4 DAI. During post germination after 5 DAI, the embryo axis developed into young hypocotyl and roots where no 2S albumin was detected using the antibody.

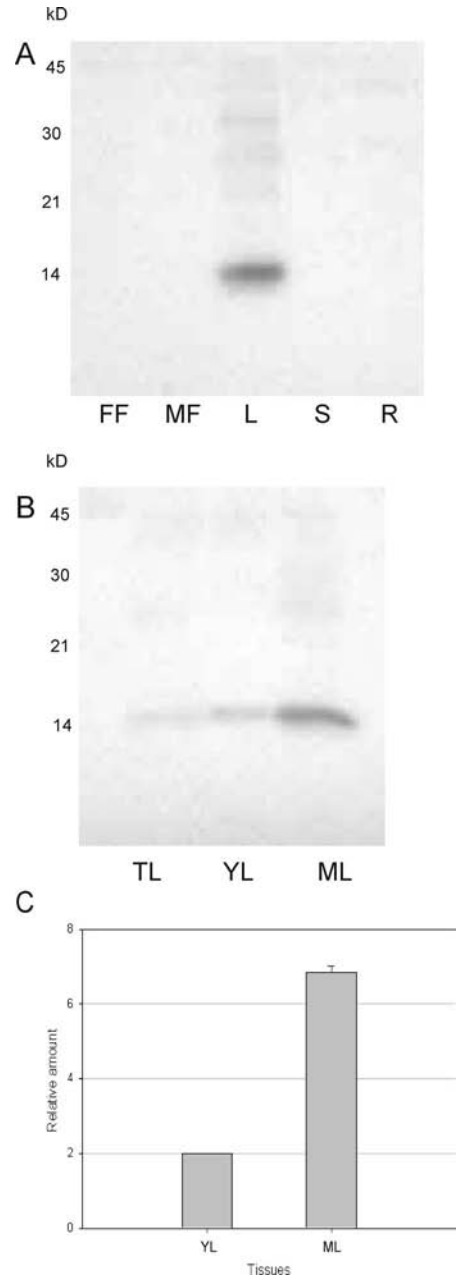

Figure 4. Detection of a 14 kDa protein in castor leaves by using the anti-2S albumin antibody. (**A**, **B**) Total proteins were extracted from nonseed tissues of 4-month-old castor plants and 14-day-old seedlings and resolved on an 18% SDS-PAGE gel. Chemiluminescent signals were detected and (**C**) quantified using a densitometer. Immunodetection analysis was performed twice, and the value of the protein in the mature leaf was normalized to that in the young leaf. FF, female flower; MF, male flower; ML, mature leaf (>20 cm in length); L, leaf; R, root; S, stem; TL, true leaf; and YL, young leaves (<10 cm in length).

Immunodetection of a 14 kDa Protein in Leaf. Nonseed tissues of mature castor plants (4-month-old), such as the flower (female and male), leaf, stem, and root, were also subjected to immunodetection using the anti-2S albumin antibody. Both forms of the 2S albumin proteins were not present in any of the tissues analyzed (**Figure 4A**). However, a 14 kDa protein

was detected in leaves at various developmental stages, including the true leaves from 14-day-old seedlings, young leaves at shoot tips, and mature leaves at the bottom of 4-month-old plants (**Figure 4B**). Densitometry showed that mature leaves, which were longer than 20 cm in length, accumulated the protein at an approximately 3 times higher rate than young leaves shorter than 10 cm (**Figure 4C**).

DISCUSSION

We have characterized the gene expression and protein accumulation of castor 2S albumin to understand this important seed storage protein and potent allergen. We chose the cultivar "Hale" because it is the most commonly used genotype for molecular and genomic studies. Besides, "Hale" has superior properties such as high oil contents and dwarfism well-suited for commercial cultivation and oil processing (*22*). Using qPCR, we examined the transcript accumulation of the castor 2S albumin in various tissues, such as in the developing and germinating seeds, female and male flower, leaf, stem, and root, at different developmental stages. Our results indicated that the 2S albumin gene was expressed exclusively in the developing seed. In a previous study, we compared the sequences of 2S albumin genomic DNA and cDNA clones from the developing seeds of two different castor genotypes, a germplasm line PI215769 and a cultivar "Maui", and found that a single gene was predominately expressed during seed development (*18*). In addition, the predominant 2S albumin gene showed nucleotide sequences identical to the known gene (GenBank accession X54158, Sudanese origin), which indicates the conserved nature of the 2S albumin gene among castor genotypes. As the qPCR analysis is highly sequence specific, the 2S albumin transcript we describe here is likely to have an identical sequence to the known gene.

Our temporal expression analysis on the 2S albumin transcript in the cultivar "Hale" revealed a pattern similar to that in the germplasm line PI215769 (*18*). First, in both "Hale" and PI215769, we observed the initial increase of the 2S albumin transcript, which occurred at 26 DAP, coinciding with the beginning of the endosperm expansion. In earlier studies, we observed the increased expression of ricin (*23*) and lipid genes (*24*) that also started accumulating at 26 DAP. These results all suggest that the time between 19 and 26 DAP is a critical switchover stage for the synthesis of seed proteins and storage lipids during castor seed development. Second, we observed the similar bell-shaped temporal patterns of the 2S albumin transcript accumulation during seed development in both PI215769 and "Hale" but with a slight difference on the timing of their peak expressions. The transcripts accumulated to a peak at 30 DAP in PI215769 (*18*) but at 40 DAP in "Hale" in this study. The discrepancy could be due to different transcriptional regulations involved in the 2S albumin gene expression in different genotypes. Despite the difference in the timing of the transcript accumulation, both studies showed a decrease in the transcript level at the last stage of seed development, leaving only a residual amount of the transcript in the mature seed (61 DAP). A similar expression profile was also observed when the 2S albumin genes were examined in other plant species, such as *Arabidopsis* (*25*) and coniferous Douglas-fir (*Pseudotsuga menziesii*) (*26*). Overall, our analyses on the temporal and spatial expression of the 2S albumin transcript in castor revealed a single 2S albumin gene, whose expression was highly regulated throughout seed development, that contributes to the synthesis of 2S albumins in castor seed. This gene is responsible for the 2S albumin allergenicity in castor seed (*27, 28*). Such information is critical to developing a tightly regulated silencing construct for genetic engineering of a safe castor crop.

To profile the 2S albumin protein expression pattern in castor, we designed an anti-2S albumin antibody with a peptide of 19 amino acids from the 2S albumin protein sequences. Antibodies raised by using such a length of a peptide should recognize proteins in a sequence-specific manner under a reducing condition (*29*). As we expected, the 2S albumin antibody reacted to the prepro2S albumin and the mature 2S albumin in developing seeds with high specificity (**Figure 3B**). The prepro2S albumin started to accumulate at the onset of endosperm development at 26 DAP and subsequently was processed to mature 2S albumins. We observed that both forms of the 2S albumin proteins continued to accumulate during the rest of the stages of seed development. The steady levels of the prepro2S albumin and the mature protein during the last stage of seed development are due to the stability of the proteins, not the constant protein synthesis, considering the decrease in the amount of the 2S albumin transcript after 40 DAP (**Figure 2A**). The accumulation of castor 2S albumins took place from the middle stage of the seed development, which is consistent with the temporal patterns of seed storage protein synthesis in many plants (*30*).

Both the prepro2S albumin and the mature 2S albumin were present in the dry and germinating seed at early stages (**Figure 3D**,**F**). They degraded after the seed germination was complete, which is a typical pattern for storage proteins. In Brazil nut (*Bertholletia excelsa* H.B.K.) (*31*) and Indian mustard (*Brassica juncea*) (*32*), the levels of the prepro2S albumins decreased upon the post-translational modification and the subsequent accumulation of the mature 2S albumins during seed development. The prepro2S albumins were not present in the mature seed of these plant species. The biological significance of the presence of the prepro2S albumin in mature castor seeds remains to be investigated. In *B. juncea*, the prepro2S albumin possessed biological activity as a trypsin inhibitor, and transgenic tobacco and tomato plants expressing the prepro2S albumin conferred insect resistance (*32*). On the basis of the bioinformatics data on castor 2S albumins having trypsin/α-amylase inhibitor family domains (*18*), it is possible that castor prepro2S albumins also serve as both trypsin inhibitors, which are thought to confer insect resistance, and storage proteins in seeds.

When profiling the castor 2S albumin during seed germination, we detected a group of bands that reacted to the anti-2S albumin antibody from 3 to 6 DAI (**Figure 3D**,**F**). It is possible that the group proteins are proteolytic peptides of the prepro2S albumin, and the mature 2S albumin accumulated during seed germination. However, on the basis of the temporal pattern, the group of proteins/peptides accumulated when the level of the prepro2S albumin was still at its maximum (**Figure 3D**,**F**). It suggests that they may not be all the proteolytic peptide fragments of the prepro2S albumin but some newly synthesized proteins that also reacted to the antibody. In addition, the antibody reacted to a 14 kDa protein in the true leaves from seedlings and the leaves of mature plants (**Figure 4**). It is evident that these proteins share sequence homology to the known 2S albumin but are unlikely to serve a storage function on the basis of their spatial and temporal expression patterns. In fact, the castor 2S albumin gene may consist of a family with three or four members (*3*). Whether these proteins are 2S albumin gene family members remains a subject for future investigations. Nonetheless, our results should facilitate future studies, such as immuno-precipitation using the anti-2S albumin antibody, that examine the identity of these proteins. Such information is

critical to developing and implementing a genetic approach to effectively silence the expression of the specific 2S albumin gene in castor seed.

ABBREVIATIONS USED

DAI, day after imbibition; DAP, day after pollination; qPCR, quantitative polymerase chain reaction.

LITERATURE CITED

(1) Shewry, P. R.; Napier, J. A.; Tatham, A. S. Seed storage proteins: Structures and biosynthesis. *Plant Cell* **1995**, *7*, 945–956.

(2) Youle, R. J.; Huang, A. H. C. Albumin storage proteins in the protein bodies of castor bean. *Plant Physiol.* **1978**, *61*, 13–16.

(3) Irwin, S. D.; Keen, J. N.; Findlay, J. B. C.; Lord, J. M. The *Ricinus communis* 2S albumin precursor: A single preproprotein may be processed into two different heterodimeric storage proteins. *Mol. Gen. Genet.* **1990**, *222*, 400–408.

(4) Sharief, F. S.; Li, S. S. L. Amino acid sequence of small and large subunits of seed storage protein from *Ricinus communis*. *J. Biol. Chem.* **1982**, *257*, 14753–14759.

(5) Hara-Nishimura, I.; Shimada, T.; Hatano, K.; Takeuchi, Y.; Nishimura, M. Transport of storage proteins to protein storage vacuoles is mediated by large precursor-accumulating vesicles. *Plant Cell* **1998**, *10*, 825–836.

(6) Brown, J. C.; Jolliffe, N. A.; Frigerio, L.; Roberts, L. M. Sequence-specific, golgi-dependent vacuolar targeting of castor bean 2S albumin. *Plant J.* **2003**, *36*, 711–719.

(7) Jolliffe, N. A.; Brown, J. C.; Neumann, U.; Vicre, M.; Bachi, A.; Hawes, C.; Ceriotti, A.; Roberts, L. M.; Frigerio, L. Transport of ricin and 2S albumin precursors to the storage vacuoles of *Ricinus communis* endosperm involves the golgi and VSR-like receptors. *Plant J.* **2004**, *39*, 821–833.

(8) Gietl, C.; Schmid, M. Ricinosomes: an organelle for developmentally regulated programmed cell death in senescing plant tissues. *Naturwissenschaften* **2001**, *88*, 49–58.

(9) Greenwood, J. S.; Helm, M.; Gietl, C. Ricinosomes and endosperm transfer cell structure in programmed cell death of the nucellus during *Ricinus seed* development. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 2238–2243.

(10) Gietl, C.; Wimmer, B.; Adamec, J.; Kalousek, F. A. Cysteine endopeptidase isolated from castor bean endosperm microbodies processes the glyoxysomal malate dehydrogenase precursor protein. *Plant Physiol.* **1997**, *113*, 863–871.

(11) Thorpe, S. C.; Kemeny, D. M.; Panzani, R. C.; McGurl, B.; Lord, M. Allergy to castor bean. II. Identification of the major allergens in castor bean seeds. *J. Allergy Clin. Immunol.* **1988**, *82*, 67–72.

(12) Davison, A. G.; Britton, M. G.; Forrester, J. A.; Davies, R. J.; Hughes, D. T. Asthma in merchant seamen and laboratory workers caused by allergy to castor beans: analysis of allergens. *Clin. Allergy* **1983**, *13*, 553–561.

(13) Gonzalez de la Pena, M. A.; Villalba, M.; Garcia-Lopez, J. L.; Rodriguez, R. Cloning and expression of the major allergen from yellow mustard seeds, Sin a 1. *Biochem. Biophys. Res. Commun.* **1993**, *190*, 648–653.

(14) Pastorello, E. A.; Varin, E.; Farioli, L.; Pravettoni, V.; Ortolani, C.; Trambaioli, C.; Fortunato, D.; Giuffrida, M. G.; Rivolta, F.; Robino, A.; Calamari, A. M.; Lacava, L.; Conti, A. The major allergen of sesame seeds (*Sesamum indicum*) is a 2S albumin. *J. Chromatogr., B: Biomed. Sci. Appl.* **2001**, *756*, 85–93.

(15) Pastorello, E. A.; Farioli, L.; Pravettoni, V.; Ispano, M.; Conti, A.; Ansaloni, R.; Rotondo, F.; Incorvaia, C.; Bengtsson, A.; Rivolta, F.; Trambaioli, C.; Previdi, M.; Ortolani, C. Sensitization to the major allergen of Brazil nut is correlated with the clinical expression of allergy. *J. Allergy Clin. Immunol.* **1998**, *102*, 1021–1027.

(16) Teuber, S. S.; Dandekar, A. M.; Peterson, W. R.; Sellers, C. L. Cloning and sequencing of a gene encoding a 2S albumin seed storage protein precursor from English walnut (*Juglans regia*), a major food allergen. *J. Allergy Clin. Immunol.* **1998**, *101*, 807–814.

(17) Robotham, J. M.; Wang, F.; Seamon, V.; Teuber, S. S.; Sathe, S. K.; Sampson, H. A.; Beyer, K.; Seavy, M.; Roux, K. H. Ana o 3, an important cashew nut (*Anacardium occidentale* L.) allergen of the 2S albumin family. *J. Allergy Clin. Immunol.* **2005**, *115*, 1284–1290.

(18) Chen, G. Q.; He, X.; Liao, L. P.; McKeon, T. A. 2S albumin gene expression in castor plant (*Ricinus communis* L.). *J. Am. Oil Chem. Soc.* **2004**, *81*, 867–872.

(19) Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **1962**, *15*, 473–497.

(20) Pfaffl, M. W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **2001**, *29*, 2002–2007.

(21) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.

(22) Brigham, R. D. Registration of castor variety Hale. *Crop Sci.* **1970**, *10*, 457.

(23) Chen, G. Q.; He, X.; McKeon, T. A. A simple and sensitive assay for distinguishing the expression of ricin and Ricinus communis Agglutin genes in developing castor seed (*R. communis* L.). *J. Agric. Food Chem.* **2005**, *53*, 2358–2361.

(24) Chen, G. Q.; Turner, C.; He, X.; Nguyen, T.; McKeon, T. A.; Laudencia-Chingcuanco, D. Expression profiles of genes involved in fatty acid and triacylglycerol synthesis in castor bean (*Ricinus communis* L.). *Lipids* **2007**, *42*, 263–274.

(25) Guerche, P.; Tire, C.; Grossi de Sa, F.; De Clercq, A.; Van Montagu, M.; Krebbers, E. Differential expression of the Arabidopsis 2S albumin genes and the effects of increasing gene family size. *Plant Cell* **1990**, *2*, 469–478.

(26) Chatthai, M.; Misra, S. Sequence and expression of embryogenesis-specific cDNAs encoding 2S seed storage proteins in *Pseudotsuga menziesii* [Mirb.] Franco. *Planta* **1998**, *206*, 138–145.

(27) Youle, R. J.; Huang, A. H. C. Evidence that the castor bean allergens are the albumin storage proteins in the protein bodies of castor bean. *Plant Physiol.* **1978**, *61*, 1040–1042.

(28) Bashir, M. E. H.; Hubatsch, I.; Leinenback, H. P.; Zeppezauer, M.; Panzani, R. C.; Hussein, I. H. Ric c1 and Ric c 3, the allergenic 2S albumin storage proteins of *Ricinus communis*: Complete primary structures and phylogenetic relationships. *Int. Arch. Allergy Immunol.* **1998**, *115*, 73–82.

(29) Van Regenmortel, M. H. V.; Choulier, L. Recognition of peptides by antibodies and investigations of affinity using biosensor technology. *Comb. Chem. High Throughput Screening* **2001**, *4*, 385–395.

(30) Hills, M. J. Control of storage-product synthesis in seeds. *Curr. Opin. Plant Biol.* **2004**, *7*, 302–308.

(31) Sun, S. S. M.; Altenbach, S. B.; Leung, F. W. Properties, biosynthesis and processing of a sulfur-rich protein in Brazil nut (*Bertholletia excelsa* H.B.K.). *Eur. J. Biochem.* **1987**, *162*, 477–483.

(32) Mandal, S.; Kundu, P.; Roy, B.; Mandal, R. K. Precursor of the inactive 2S seed storage protein from the Indian mustard Brassica juncea is a novel trypsin inhibitor. *J. Biol. Chem.* **2002**, *277*, 37161–37168.

Received for review April 30, 2007. Revised manuscript received August 2, 2007. Accepted August 16, 2007.

JF071272P