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A Simple and Sensitive Assay for Distinguishing the Expression of Ricin and *Ricinus communis* Agglutinin Genes in Developing Castor Seed (*R. communis* L.)

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Castor oil is the only commercial source of ricinoleic acid and has numerous industrial applications. Among the factors limiting domestic production of castor oil is the presence of the toxin ricin and its less toxic homologue *Ricinus communis* agglutinin (RCA) in seeds. Although the sequences of ricin and RCA genes are known, their transcriptional expression patterns have not been distinguished due to their high degree of sequence similarity. As the information is critical for assessing success in developing a ricin-free castor crop using genetic silencing, we have designed a gene specific reverse transcription-polymerase chain reaction (RT-PCR) assay to examine the expression of the ricin and RCA genes in developing seeds. The results show that the ricin and RCA mRNA are highly abundant in seeds during the development of endosperm, and the expression pattern is similar to that observed in the Northern analysis. The RT-PCR results can be confirmed by a simple RT-PCR-based restriction fragment analysis.

KEYWORDS: ***Ricinus communis*; oilseed; ricin gene; agglutinin gene**

INTRODUCTION

Castor (*Ricinus communis* L.) is a nonedible oil crop having much economic significance because of its unique oil composition. The fatty acid component of castor oil contains up to 90% ricinoleate (12-hydroxy oleate). Ricinoleate and its derivatives have many important industrial applications, such as lubricants, coatings, plastics, and fungicides (*1*). However, castor cultivation and processing result in exposure to the highly toxic seed protein ricin and its less toxic homologue *R. communis* agglutinin (RCA) (*2*−*4*). Ricin is a dimeric glycoprotein composed of a toxic A chain and a lectin B chain linked by disulfide bonds. The A chain is a ribosome-inactivating enzyme, which depurinates a specific adenine residue of 28S ribosomal RNA, thereby inactivating eucaryotic protein synthesis (*5*). The B chain, which contains two galactose binding sites, binds specifically to cell surface glycoproteins or glycolipids and facilitates the movement of the A chain into cells (*6*). RCA is a tetramer consisting of two ricinlike dimers held together by noncovalent bonds. Mature RCA is less toxic than ricin and causes agglutination of red blood cells in mammals (*2*, *3*).

The complete primary sequences of ricin and RCA proteins have been determined chemically (*7*, *8*) and deduced from the nucleotide sequence of cloned cDNAs and genomic clones (*9*−*11*). Both ricin and RCA genes encode a precursor protein containing an N-terminal signal sequence, an A chain, a 12 amino acid linking region, and a B chain (*9*, *10*, *12*). The A chains of ricin and RCA differ in 18 of their 267 residues and are thus 93% identical at the amino acid level, whereas the B chains differ in 41 of 262 residues giving 84% identity (*9*). A Northern analysis has shown that the expression of ricin and RCA genes is seed specific and not detectable in either leaf or root tissue or in the endosperm of germinating seeds (*12*). However, because ricin and RCA gene sequences share a high similarity, their transcripts are not distinguishable in Northern analysis. Here, we report a method for separate identification of ricin and RCA transcripts using a reverse transcription−polymerase chain reaction (RT-PCR) and the characterization of their temporal expression patterns during various stages of seed development. The information is critical in developing and implementing a genetic silencing approach to eliminate ricin from castor seed.

MATERIALS AND METHODS

Plant Material and Growth Conditions. The castor (*R. communis* L.) seeds, PI215769, were obtained from USDA-GRIN, Southern Regional Plant Introduction Station (Griffin, GA). Seeds were germinated, and plants were 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 (1000−1250 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 55−65 days after planting. Fully opened new female flowers were individually pollinated, and the date of pollination was recorded. Capsules were harvested at 7 day intervals from 12 days after pollination (DAP) to 61 DAP. Qualitative characteristics of the capsules and seeds were

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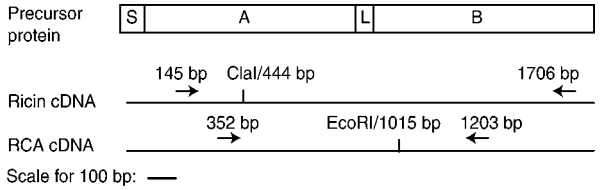

Figure 1. Scale diagram showing a precursor protein with positions of the signal peptide (S), A chain (A), linker (L), and B chain (B), and the cDNAs of ricin and RCA with the primer sites (arrows) and unique restriction enzyme sites indicated.

recorded immediately upon collection. For Northern analysis, separate sets of seeds were frozen immediately in liquid nitrogen after dissection and stored at −80 °C.

Total RNA Isolation and Northern Analysis. Total RNA was extracted from fresh frozen seeds using TRIzol Reagent (Invitrogen/Life Techologies, Carlsbad, CA). RNA pellets were dissolved in DEPC-treated water, and the RNA was quantified using a spectrophotometer (A_{260}/A_{280}) and checked for quality by gel electrophoresis using ethidium bromide as a stain. Northern blot was analyzed using a digoxigenin (DIG) nucleic acid labeling and detection kit (Roche, Indianapolis, IN). The DIG-labeled ricin DNA probes were generated by PCR using primers derived from the ricin gene sequence (Genbank X52908), and detected using immunolabeling and chemiluminescence (Roche).

RT-PCR. The total RNA samples were first treated with DNase I and then reverse transcribed to first-strand cDNA by using an Oligo-(dT) primer and the SUPERSCRIPT First-Strand Synthesis System (Life Technologies, Rockville, MD). Target cDNA was amplified using Taq Polymerase (puRe Taq, Amersham Biosciences, Piscataway, NJ). Gene specific PCR primers derived from the castor ricin gene (5′-gcgggtgccactgtgcaaagcta-3′ and 5′-tttgggtcaccatggagagggtaaaga-3′, GenBank X52908) and RCA gene (5′-tgccgcgctggaaatagcgcctatt-3′ and 5′-acgggtggcaccaactgtagcggta-3′, GenBank M12089) were used to amplify the cDNA fragments. Primers derived from the castor actin gene (5′-aggggataaccaccccatgaatcca-3′ and 5′-tgcatggtctcctgatacggccaag-3′, GenBank T14887) were used as the constitutive control. In a standard 25 μL RT-PCR reaction, amounts of first strand cDNA templates corresponding to 3 ng of total RNA were used for ricin and RCA genes and 15 ng total RNA for actin gene. The PCR program was set as follows: a 3 min initial denaturation at 95 °C was followed by 28 cycles of denaturation (95 °C for 30 s), annealing (64 °C for 30 s), and extension (72 °C for 3 min); and finishing with an additional 5 min extension at 72 °C, at the end of the 28th cycle. The amplified cDNA fragments were examined by electrophoresis of 15 μL of RT-PCR products in a 1% agarose gel stained with ethidium bromide.

RT-PCR-Based Restriction Fragment Analysis. To obtain enough RT-PCR-amplified DNA for enzyme digestions, the RT-PCR reaction volume was scaled up to 100 μL and the amplified DNA fragments were then purified using QIAquick PCR purification kit (Qiagen, Valencia, California). About 10 units of restriction enzymes was used per μg of DNA incubating at 37 °C for 1 h, followed by heat inactivation at 75 °C for 15 min. The amplified DNA fragments were examined by electrophoresis using 1% agarose gel stained with ethidium bromide.

RESULTS

The DNA sequences of ricin and RCA genes are 93% identical [pairwise comparison of National Center for Biotechnology Information (NCBI) ricin gene X52908 and RCA gene M12089], with the mismatches scattered along the aligned sequences. To identify ricin and RCA specific transcripts, two sets of gene specific primers (see Materials and Methods) were designed from castor ricin and RCA sequences. **Figure 1** illustrates a scaled diagram showing a precursor protein, cDNAs of ricin and RCA genes with the primers and unique restriction enzyme sites indicated. Using the selected primers, RT-PCR should amplify a 1562 bp ricin cDNA, which would generate

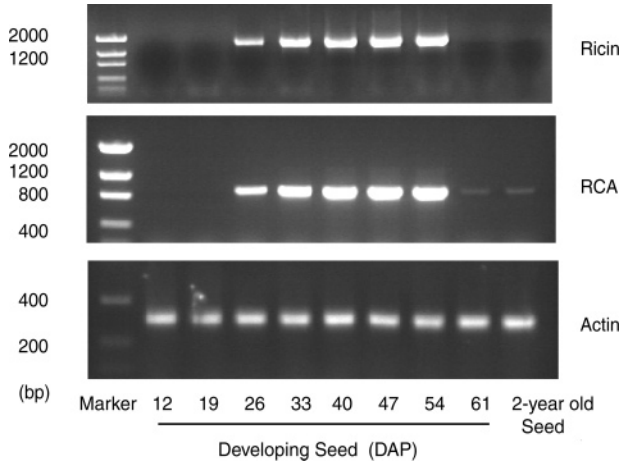

Figure 2. RT-PCR analysis showing the expression of the ricin (**A**) and RCA (**B**) genes and, as a constitutive control, the actin gene (**C**).

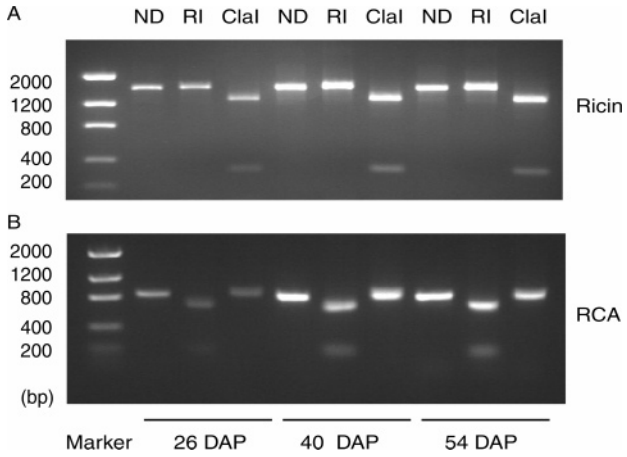

Figure 3. RT-PCR-based restriction fragment analysis. The RT-PCR products of the ricin gene (**A**) and RCA gene (**B**) were loaded for undigested samples (ND) and samples digested with *Eco*RI (RI) and *Cla*I restriction enzymes.

1263 and 299 bp fragments after the unique *Cla*I digestion. In the case of RCA, the cDNA is 852 bp and has a different unique restriction site, *Eco*RI, which would cut the product into two fragments, 663 bp and 189 bp. With this design, we examined the expression of ricin and RCA genes during the seed development. As shown in **Figure 2**, no ricin and RCA cDNA was detected in young seeds of 12 and 19 DAP, but the expression of each increased significantly in 26 DAP seeds, and the upward trend continued into later stages until 54 DAP. A trace amount of RCA transcripts could still be detected in desiccating 61 DAP seeds and in 2 year old dormant seeds. The identity of the RT-PCR products was further validated by sequencing (data not shown). However, the different unique restriction enzyme sites allow simple verification for the authenticity of RT-PCR products by restriction fragment analysis. The RT-PCR products from 26, 40, and 54 DAP developing seeds were digested with *Cla*I and *Eco*RI. The resulting restriction fragments are shown in **Figure 3**. As predicted, *Cla*I digestion produced 1263 and 299 bp fragments for ricin cDNA, while *Eco*RI digestion produced 663 and 189 bp fragments for RCA cDNA. To confirm the expression patterns of ricin and RCA genes revealed by the RT-PCR method, a Northern analysis was performed on total RNA from the same set of seeds. The result showed a pattern similar to that obtained using RT-PCRs (**Figure 4**).

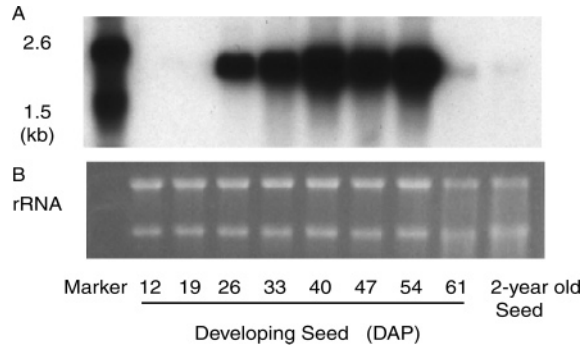

Figure 4. Northern analysis of ricin and RCA gene expression. Approximately 5 μg of total RNA was loaded on each lane. The blot was exposed to film for 10 s (**A**). Bottom, an ethidium bromide stained gel as a control for equal RNA loading (**B**).

DISCUSSION

The developmental expression of ricin and RCA genes was studied in castor seed using RT-PCR assay and Northern analysis. To make accurate comparisons between experiments, we first carefully determined seed development age using a set of simple criteria established at our laboratory (*13*). The criteria include two visual markers, seed coat color, endosperm volume, and three phases for the whole course of castor seed development. In brief, seeds do not develop endosperm tissue until about 26 DAP and then the endosperm undergoes development until 54 DAP. At 61 DAP, seeds mature and desiccate. The results from this study indicate that the expression of ricin and RCA genes corresponds to the developmental profile of endosperm. The mRNA signal is not detectable before the endosperm begins to develop (12 and 19 DAP) but becomes significant between 26 and 54 DAP when the endosperm expands to occupy most of the seed volume. When seeds enter the desiccation stage (61 DAP), the expression of ricin and RCA genes drops to a trace level. These observations are similar to those of lectin and ribosome inactivating proteins from various species including soybean, common bean, wheat, and barley (*14*). In soybean, the expression of a lectin gene is transcriptionally activated and spatially regulated during the midmaturation phase of embryogenesis and repressed in late maturation when seeds enter the desiccation phase and begin dormancy (*15*, *16*). In castor seed, the ricin and RCA mRNA levels are correlated with the dimension of endosperm, suggesting that they are also spatially and temporally regulated. It has been shown that castor endosperm is the site for synthesis and storage of various seed proteins including ricin and RCA (*17*). A previous study examined ricin and RCA mRNA levels during castor seed development (*12*), but the authors noted that the Northern blot did not distinguish ricin or RCA transcripts due to their high degree of sequence identity. In that study, the developing seeds were divided into six stages based on seed size and seed coat formation and did not take into account the stages of endosperm development. Thus, our RT-PCR results supported by our Northern analysis not only present the specific temporal expression patterns for ricin or RCA genes but also define their developmental profiles based on ages and factors more directly related to distinct chemical stages in castor seed development.

We have demonstrated that an RT-PCR assay and restriction fragment analysis can be used to determine the transcriptional expression patterns of ricin and RCA genes. Although ricin and RCA share substantially similar biochemical characteristics, they are distinctive genes and may have different functions. To develop safe castor cultivars using genetic engineering, we are generating a series of plants with different expression levels of ricin and RCA genes. Selection of plants with diminished hazard but normal biological properties is important to all users including grower, processor, and consumer. Therefore, it is critical to establish sensitive methods for detecting expression of ricin and RCA genes, respectively. Sensitive immunoassay methods have been developed for detecting ricin protein at 0.1 ng/mL levels (*18*, *19*); however, the possibility of cross-reaction with RCA remains. The RT-PCR assay/restriction fragment analysis that we report here is definitive and a highly sensitive method to detect the expression of ricin or RCA genes at the transcriptional level. Therefore, this technique provides us with a method to screen the effectiveness of different silencing constructs in individual transformants for the level of success in suppressing expression of ricin and RCA genes in developing castor seed.

ABBREVIATIONS USED

DAP, days after pollination; NCBI, National Center for Biotechnology Information; RCA, *Ricinus communis* agglutinin; RT-PCR, reverse transcription polymerase chain reaction.

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