# Immunolocalization of ricin accumulation during castor bean (*Ricinus communis L.*) seed development

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## Abstract

Ricin is a dimeric glycoprotein that accumulates in protein storage vacuoles of endosperm cells of *Ricinus communis* L. (castor bean). The proricin travels through the Golgi apparatus and co-localizes throughout its route to the storage vacuoles of developing castor bean endosperm. We report here the pattern of seed morphological and ultrastructural changes during various stages of seed development, associated with ricin accumulation. ELISA was used to compare the ricin content in mature seeds of four Brazilian commercial cultivars. ELISA and immunoelectron microscopy analysis were used to study ricin accumulation during seed development from 10 to 60 days after pollination (DAP). Results have shown that no ricin could be localized in the endosperm cells in the early development stages (before 20 DAP) and only a few localization points could be observed at 30 DAP. However, a significant ricin localization signal was observed at 40 DAP in the matrix of the protein storage vacuoles. The signal increased significantly from 50 to 60 DAP, when ricin was observed in both the matrix and crystalloids of the protein storage vacuoles. Understanding ricin expression at the cellular level is fundamental for the development of strategies for gene suppression using molecular breeding approaches.

## Introduction

Castor bean (*Ricinus communis* L.) seeds contain approximately 50% of an oil with unique characteristics such as high viscosity, heat and pressure stability, low freezing point, and the ability to form waxy substances after chemical treatments. Consequently, this oil has many important industrial applications, such as in the production of lubricants, biodiesel, and plastics.[1] Developing castor bean seeds accumulate lipids and storage proteins in their endosperm. In developing endosperm cells, the major storage proteins 7S lectins [ricin and its homologue *R. communis* agglutinin (RCA), which share a high similarity], 2S albumins, and 11S globulins are accumulated in protein storage vacuoles.[2,3] Castor bean cultivation and processing result in exposure to the potent toxin ricin, which is a dimeric glycoprotein composed of a toxic A-chain (RTA) and a lectin B-chain (RTB) that are linked via disulfide bonds.[4] The A-chain is a ribosome-inactivating enzyme that depurinates a specific adenine residue on the 28S ribosomal RNA from animal cells. This activity prevents the formation of a critical stem-loop configuration, to which elongation factor 2 is known to bind during the translocation step of translation. It provokes cell death by inhibiting protein synthesis.[5,6] The β-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.[7] Extremely low levels of ricin are able to inhibit protein synthesis. According to Olsnes *et al.*,[8] only one A-chain molecule of ricin is able to irreversibly inactivate 2000 ribosomes/min. In addition, Eiklid *et al.*[9] concluded that penetration of a single ricin molecule into the cytosol is enough to kill a mammalian (HeLa) cell. The estimated lethal ricin dose in humans is 1-10 μg/kg.[6,10]

Ricin is synthesized as preproprotein (64.1 kDa) in developing castor bean seeds.[4] It is inserted into the lumen of the endoplasmic reticulum (ER), when the signal peptide is removed forming the proricin (61.6 kDa). In the ER an intramolecular disulfide bond between the A and B subunits is formed, holding together the mature heterodimer. After a subsequent removal of the propeptides by a vacuolar protease, the mature dimer of 58.8 kDa is generated.[4] Ricin is assumed to serve as a seed storage protein providing nutrients for seed germination and also may act as a defense protein.[2,11,12] However, the environmental role and evolutionary aspects of ricin accumulation in castor bean seeds still are not completely clarified.

Immunoelectron microscopy and cell fractionation reveal that proricin travels through the Golgi apparatus and co-localizes throughout its route to the storage vacuoles of developing castor bean endosperm.[3] The ricin gene (coding for the ricin preproprotein) expression studied by RT-PCR and Northern analysis revealed that its expression increased significantly in seeds at 26-30 DAP, and the upward trend continued into later stages until 54-60 DAP.[13,14] Using a radial immunodiffusion assay based on ricin-specific antibodies, ricin amount was quantified in accessions from the USDA castor collections, showing an extremely wide range and differing significantly in ricin plus *Ricinus communis* agglutinin (RCA) concentrations.[15]

Ricin has been studied extensively in both medical and basic research since its discovery in the 1880s. It has been used to investigate molecular mechanisms involved in intracellular trafficking and studied for its potential for therapeutic use in cancer chemotherapy.[6,16] However, there is still limited information on ricin accumulation and genetic diversity in castor beans. There are studies on cell ultrastructure of mature and germinating seeds but detailed analysis during endosperm cell development is rare. In order to characterize ricin further, immunological techniques were used to localize protein expression in endosperm cells from developing castor bean seeds. Sections of developing castor bean seeds from six stages of development were used to characterize early ricin expression. We report here the pattern of seed morphological and ultrastructural changes during various stages of seed development, associated with ricin accu-

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Key words: electron microscopy, endosperm, immunolocalization, protein bodies, seed development.

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mulation. This information could contribute to the genetic manipulation of ricin gene expression in castor bean seeds.

## Materials and Methods

### Plant material

Seeds from castor bean plant cvs. Energia, Nordestina, Pioneira, and Paraguaçu were used for ricin content determination. Plants from cv. Energia were grown from seed in a greenhouse at Embrapa Recursos Genéticos e Biotecnologia (Brasília, DF, Brazil) under ambient conditions. Initial flowering occurred approximately 60 days after sowing. Fully opened female flowers were tagged individually and hand pollinated, and the tagging dates recorded as days after pollination (DAP). Capsules were harvested at 10-day intervals to a maximum of 60 DAP. All collected capsules were analyzed anatomically. Seed coat, caruncle, nucellus, and endosperm were separated using a scalpel and razor blade.

### Quantification of ricin content

Quantification of ricin content was carried out in the cvs. Energia, Nordestina, Pioneira, and Paraguaçu using ELISA. The cv. Energia was chosen for the analyses of ricin accumulation during seed development. Protein extraction was performed by mixing the seed powder (0.2 g) and 600 µL of sample buffer (50 mM $NaH_2PO_4$, 20 mM NaCl, 2 mM PMSF, 10 mM DTT, pH 7.0) for 30 min at 4ºC. The mixture was centrifuged (18,500 *g*) for 40 min at 4°C and the supernatant collected. Total protein was quantified using the Quick Start Bradford Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Polystyrene 96-well plates were coated with 3 µg total protein (diluted in PBS) and incubated for 4 hr at 37°C. The plates were washed three times with PBS, blocked with 200 µL/well of block solution (PBS, Tween (0.05%), and 2% defatted powdered milk) for 16 hr at 4°C. The plates were washed three times with block solution and incubated with specific goat anti-RTA antiserum (rcG-20; Santa Cruz Biotechnology Inc.) diluted in block solution (1:2,000) for 2 hr at 37°C. The rcG-20 is an affinity purified goat polyclonal antibody raised against a peptide mapping at the N terminus of the ricin precursor of *R. communis* origin. Although Western blot analysis showed that proricin precursor and glycoforms of ricin A-chain were recognized specifically, we could not exclude the possibility that the antibody would also detect the homologous RCA A-chain, GenBank accession number P06750. The plates were washed seven times with block solution and incubated with 50 µL/well of diluted secondary antibody (1:3000, rabbit anti-goat IgG conjugated with alkaline phosphatase, Santa Cruz Biotechnology Inc.) for 2 hr at 37°C. The plates were washed five times with PBS and the reaction was performed using the Alkaline Phosphatase Substrate Kit (Bio-Rad) according to the manufacturer's instructions. A standard curve was prepared using the purified ricin A (Sigma). Absorbance was measured in a microplate reader (Bio-Rad) at 405 nm. Experiments were repeated with three biological and three technical replicates.

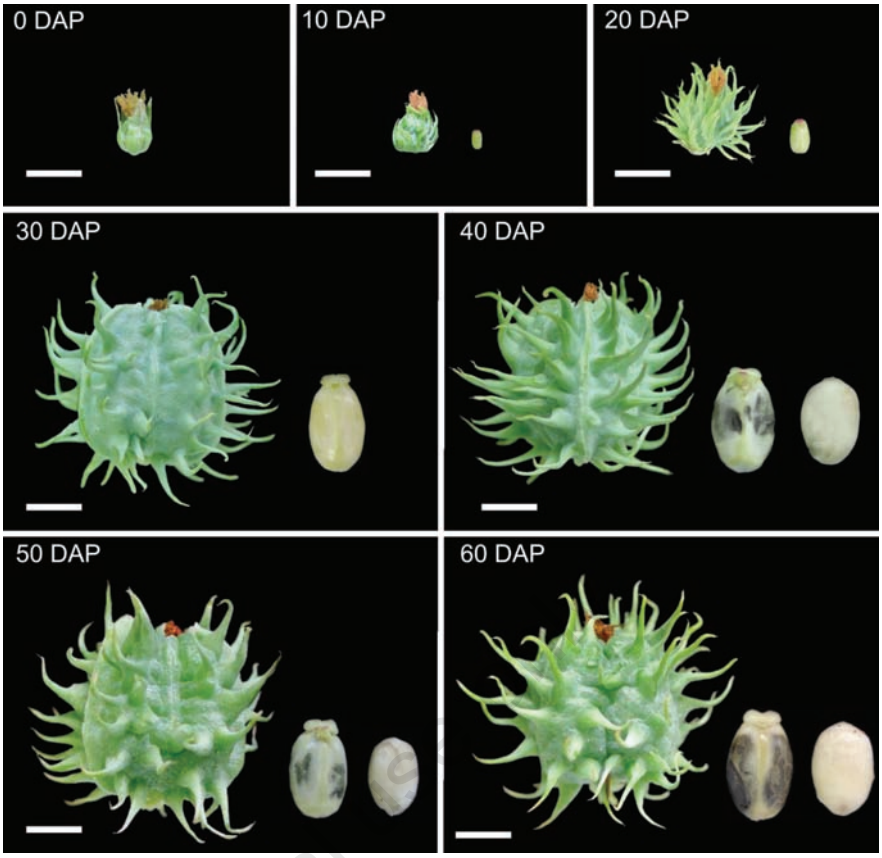


**Figure 1. The morphological changes in castor bean seeds cv. Energia during development from: flower (0 DAP), fruits (on the left, 10 to 60 DAP), coated seeds (on the right, 10 to 30 DAP; in the middle, 40 to 60 DAP), and seeds without a coat (on the right, 40 to 60 DAP). DAP, days after pollination. Scale bars represent 1 cm.**

### Morphological and ultrastructural immunocytochemistry analyses

Flowers were collected at each stage of seed development (10, 20, 30, 40, 50, and 60 DAP; Figure 1) and images of developing fruits were obtained. Samples were dissected and sectioned transversally and longitudinally. The fragments were immersed in the fixative solution of cacodilate buffer 0.05M, pH 7.0 containing 2% paraformaldehyde and 0.2% glutaraldehyde for 24 hr at 4°C, and subsequently dehydrated in an increasing series of ethanol solutions (30%, 50%, 70%, 90%, 100%), for 15 min in each solution. Samples were displaced in transparent microtubes containing the acrylic resin LR White (London Resin) for 6 days at 4°C and then cured at 4°C under UV light for 3 days. Ultra-thin sections (60 nm thick) were obtained using the ultramicrotome Leica Ultracut UCT and collected on 300 mesh copper grids recovered with Formvar film. Grids were incubated with a solution 1% of uranyl acetate and examined in a Jeol 1011 transmission electron microscope, operated at 60 kV.

For ultrastructural immunocytochemistry, grids containing ultra-thin sections were incubated with blocking solution (PBS 1X, 0.5% Tween 20, 2.5% bovine serum albumin) and primary antibody (Ricin A, rcG-20; Santa Cruz Biotechnology Inc.) diluted 1:200 for 1 hr. After incubation with primary antibody, sections were washed with PBS 1X solution and then incubated with Protein A coupled with 20 nm gold particles (SPI Supplies) diluted 1:50. Sections, with no counterstaining, were examined in the transmission electron microscope. As a control, the incubation with primary antibody was omitted. Only representative images that were consistently observed in sections of three or more individual seeds from each developmental stage were selected for presentation.

## Results

An enzyme-linked immunosorbent assay was carried out to compare the ricin content in mature seeds of four Brazilian commercial varieties and to study ricin accumulation during seed development from 10 to 60 days after pollination (DAP). Figure 1 illustrates the morphology of a developing seed. Our results showed that the cvs. Paraguaçu and Pioneira exhibit about 24% of the ricin content of the cvs. Nordestina and Energia (Figure 2A). Thus, the cv. Energia was chosen for further studies of ricin localization during seed development. ELISA analysis revealed that ricin could be detected in the seeds (cv. Energia) only at 30 DAP, and a significant increase was observed from 30 to 40 DAP (4.2 fold), from 40 to 50 DAP (5.8 fold), and from 50-60 (1.4 fold) (Figure 2B).

Endosperm cells at the early developing stage (30 DAP) presented large vacuoles and other organelles such as ER, nuclei, and mitochondria (Figure 3A). At 40 DAP cells were still vacuolated (Figure 3B) and the development of other storage organelles, such as protein storage vacuoles and lipid bodies, was first observed in some cells (Figure 3C). Phytin globoid cavities were also observed (Figure 3B-C). At 50 to 60 DAP the cytosol was not evident and the cellular space was filled by protein storage vacuoles and lipid bodies (Figure 3D-F). At 50 DAP protein crystalloids could be observed inside the protein storage vacuoles, while in some cells the structure of the protein storage vacuoles still was not mature (Figure 3D). At 60 DAP the protein crystalloids were more evident (Figure 3E and F).

No ricin could be localized in the endosperm cells in the early developing stages (before 20 DAP) and only a few points could be observed at 30 DAP (*data not shown*). A significant ricin localization signal was observed at 40 DAP in the matrix of the protein storage vacuoles (Figure 4A). A significant increase in the ricin localization signal was observed from 50 to 60 DAP, and ricin could be observed both in the matrix and crystalloids of the protein storage vacuoles (Figure 4B-D). No background localization was observed in the control sections incubated with only the secondary antibody.

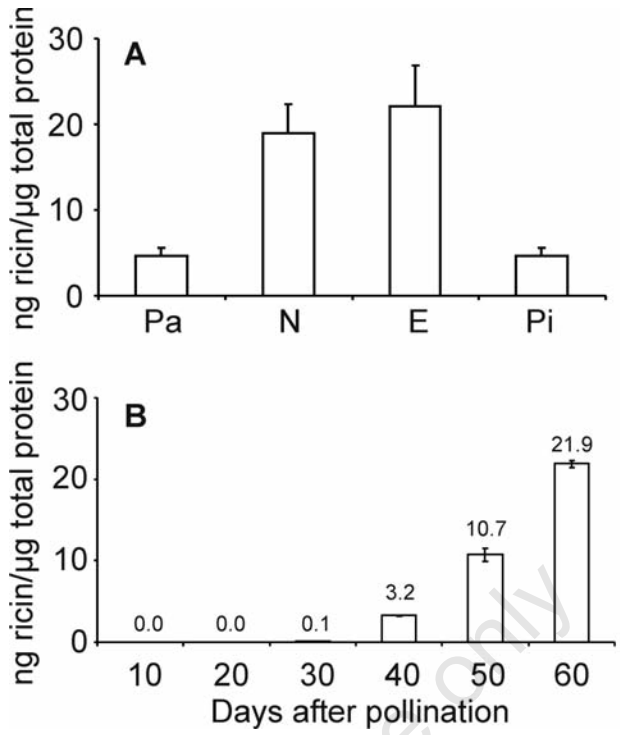


**Figure 2. Ricin quantification in mature seeds of the cvs. Paraguaçu (Pa), Nordestina (N), Energia (E), and Pioneira (Pi) (A), and ricin accumulation in the cv. Energia during seed development (B). The numbers above each bar in (B) represent the mean. Error bars represent the standard deviation from the mean (n=3).**

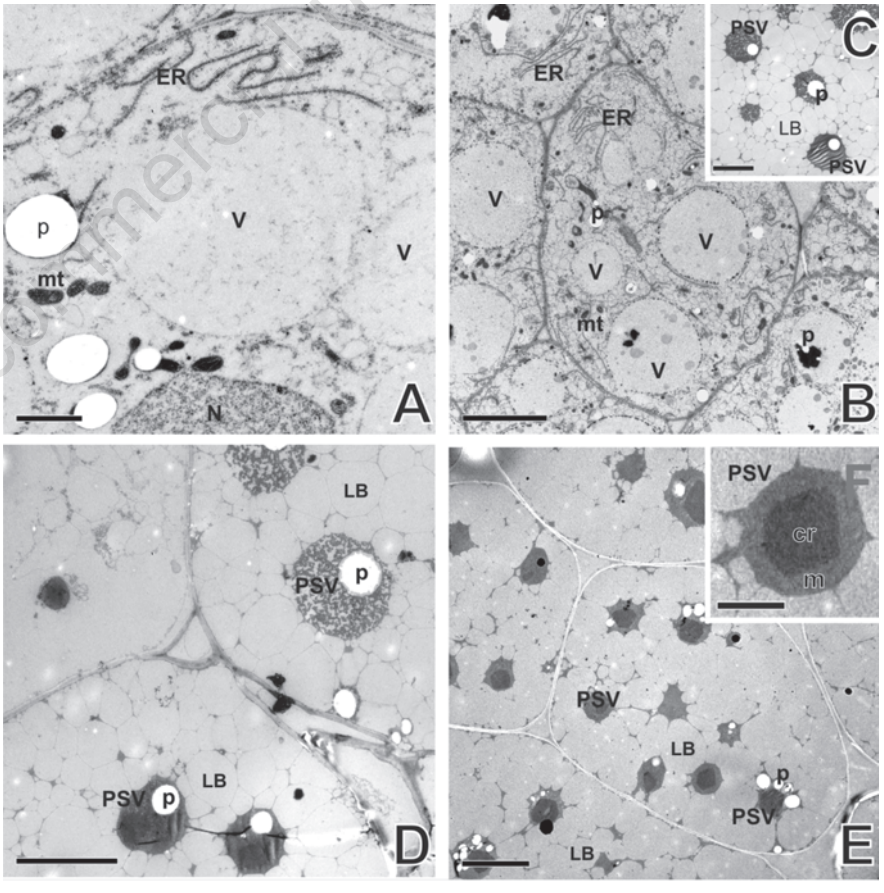


**Figure 3. Endosperm cell development at 30 DAP (A), 40 DAP (B and C), 50 DAP (D), and 60 DAP (D). RE, endoplasmic reticulum; V, vacuoles; mt, mitochondria; N, nucleus; PSV, protein storage vacuoles; LB, lipid bodies; p, phytin globoid cavities; cr, crystalloids; m, matrix. DAP, days after pollination. Bars represent 2 µm (in A and C), 10 µm (in B, D, and E) and 2.5 µm (in the insert F).**

## Discussion

Using ultrastructural immunocytochemistry techniques, the presence and accumulation of ricin in the endospermatic cells of castor bean were characterized during seed development. Castor beans store lipids and proteins in a living endosperm, which is attached laterally to the cotyledons. The endosperm is essentially

free-nuclear in the early stages, at 5 to 10 DAP (stages I and II according to Greenwood and Bewley[17]), and it becomes primarily cellular by stage III (15 DAP). The transition from free-nuclear to cellular endosperm (15 to 20 DAP) proceeds from the micropylar to the chalazal end of the seed.[17] Our analyses confirmed previous observations: in the endosperm cells of mature castor bean most of the cell volume is filled with protein bodies and lipid bodies.[18] In addition, we confirmed that formation of protein storage vacuoles started at about 40 DAP and only a number of cells exhibited that pattern, while others still presented an evident cytosol with ER and mitochondria (Figure 3). As expected, observations of protein and lipid bodies, and the pattern of phytin globoid formation, were consistent with the previous determinations of the amount of proteins, lipids, phytic acid, and phosphorous during the development of castor bean seeds.[19]

Previous analysis of proteins of endosperm sub-organelle fractions demonstrated that there was no overlapping of the protein components between the matrix and the crystalloids, suggesting a clear compartmentalization of the protein components within the protein bodies.[18] In addition, it was established that the crystalloids are composed of storage protein of the globulin type, whereas the matrix contains non-storage albumin proteins, including ricin and phytohemagglutinin.[18,20] In contrast, our results showed that ricin could be found in both matrix and crystalloid after 50 DAP (Figure 4B-D).

Ricin immunolocalization signal and accumulations during endosperm cell development were correlated with the development of endosperm. Our results were consistent with the previous observations on ricin gene transcription. Chen *et al*.[14] used RT-PCR and Northern analysis to examine the transcription of the ricin gene during seed development and stated that no ricin mRNA/cDNA was detected before the endosperm begins to develop (12 and 19 DAP). However, the expression increased significantly in 26-DAP seeds, and the upward trend continued into later stages until 54 DAP. Similar results were obtained by Kermode and Bewley,[13] analyzing a ricin gene expression in developing endosperm at 30 to 60 DAP. In addition, Lu *et al.*[21] have shown that 1.5% of the ESTs derived from a full-length cDNA library of castor developing endosperm corresponded to the ricin gene. Taken together, these results corroborate the observation that ricin gene expression is spatially and temporally regulated.

Owing to its importance for industry and the possibility of using the remaining processed material as fertilizer or animal foodstuffs, there is considerable interest in reducing the ricin content in castor bean seeds using both classical and molecular breeding approaches.

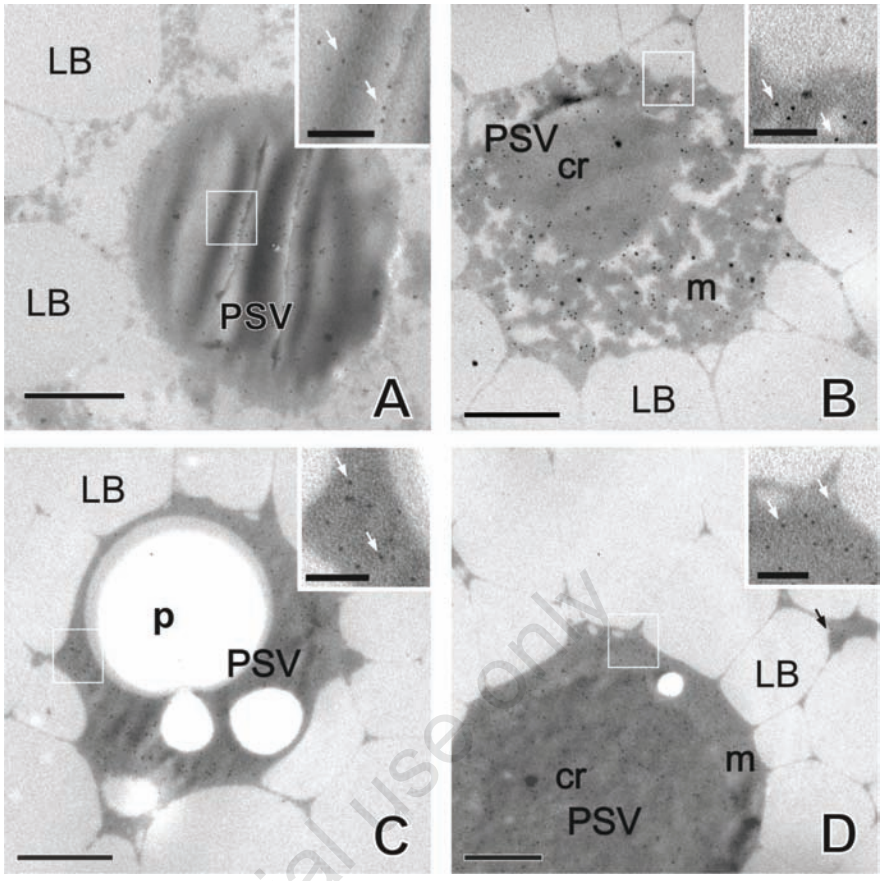


**Figure 4. Immunolocalization of ricin in the endosperm cells during seed development at 40 DAP (A), 50 DAP (B), and 60 DAP (C and D). PSV, protein storage vacuoles; LB, lipid bodies; p, phytin globoid cavities; cr, cristalloids; m, matrix. DAP, days after pollination. Inserts show details of the area marked with the white boxes. Arrows in the inserts indicate the 20 nm gold particles localizing ricin. Bars represent 2.0 µm (in A, B, C, and D) and 0.5 µm (in the inserts).**



Indeed, there are some recent examples in which expressing genes were knocked out.[22-24] However, the understanding of protein expression at cellular level is fundamental for: i) development of constructs with regulatory sequences involving adequate temporal and spatial expression; ii) an effective method of screening individual mutants/transformants to block expression of ricin genes successfully; iii) biosafety analysis of transgenic events.

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