Cinnamomin, a type II ribosome-inactivating protein, is a storage protein in the seed of the camphor tree (*Cinnamomum camphora*)

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Cinnamomin is a novel type II ribosome-inactivating protein (RIP) isolated in our laboratory from the seed of the camphor tree (*Cinnamomum camphora*). In this paper the physiological role it plays in the plant cell was studied. Northern and Western blotting revealed that cinnamomin was expressed specifically in cotyledons. It accumulated in large amounts simultaneously with other proteins at the post-stages of seed development. Cinnamomin degraded rapidly during the early stages of seed germination. Endopeptidase was proved to play an important role in the degradation of cinnamomin. Western blotting of total

proteins from the protein body with antibodies against cinnamomin demonstrated that it only existed in this specific cellular organelle as a storage protein. The similar properties of cinnamomin and other seed storage proteins of dicotyledons were compared. We conclude that cinnamomin is a special storage protein in the seed of *C. camphora*.

Key words: cotyledon, endopeptidase, germination, protein body.

INTRODUCTION

Plant ribosome-inactivating proteins (RIPs) are a group of toxic proteins that act on eukaryotic and prokaryotic ribosomes. The molecular mechanism of action of RNA N-glycosidase is to remove a specific adenine from a highly conserved loop ('sarcin/ricin domain') in the largest ribosomal RNA that is responsible for the interaction of both eukaryotic and prokaryotic elongation factors with the ribosome, thereby inhibiting protein synthesis [1,2]. RIPs are classified into three types based on their primary structure. Type I RIP consists of a single, intact polypeptide of about 30 kDa which, in some cases, is processed proteolytically into two shorter polypeptides held together by non-covalent interactions. Type II RIP is composed of an N-terminal domain comparable with type I RIP linked by a disulphide bridge to a C-terminal domain that has carbohydrate-binding activity. Type III RIP consists of a type I RIP-like N-terminal domain and a C-terminal domain of unknown function [3].

Many studies have been performed on the applications of RIPs in drug development and crop-plant biotechnology due to their toxicity to viruses, tumour cells, insects and plant fungal pathogens [4–6]. RIPs have also been used as a powerful probe to study the structure of ribosomes [7]. However, the physiological function that RIPs play in the plant cell is unclear. Previous studies revealed that many RIPs are involved in defence mechanisms in plant cells [4–6] and terminate protein synthesis under appropriate physiological conditions and thus are involved in metabolic regulation [8,9]. In addition, some RIPs accumulate in non-reproductive tissues, such as cotyledon, bark and root. Some researchers have proposed that RIP might play a role as a storage protein in these tissues [4,5,10,11]. However, there has been no conclusive evidence to support the storage role of RIP to date.

Cinnamomin is a novel type II RIP isolated in our laboratory from the seeds of the camphor tree (*Cinnamomum camphora*). Its enzymic mechanism, structure and applications have been investigated intensively [12–14], but the role it plays in the seed cell of

C. camphora is still uncertain. In this article, its expression pattern, metabolic activity and intracellular localization in the plant cell were studied. The results showed that cinnamomin functions as a storage protein *in vivo*.

MATERIALS AND METHODS

Materials

Seeds of *C. camphora* in the different developmental stages were harvested in Shanghai Forest Garden. The collected seeds were used immediately or frozen in liquid nitrogen and stored at -70°C . For the germination experiments, the mature seeds were pretreated with 1 M HCl at room temperature for 1 h to soften the seed coat and washed to neutral pH with distilled water. The *C. camphora* seedlings were grown in a well-controlled growth chamber at 26°C with a light/dark cycle of 16 h light and 8 h dark. Cotyledons were removed from seedlings every 7 days. [α - ^{32}P]dCTP was obtained from Amersham Bioscience. Random-primer labelling kit was purchased from Takara (Osaka, Japan). Alkaline phosphatase-conjugated goat anti-rabbit IgG was the product of Sigma. All other chemicals were of analytical grade.

Extraction of protein

Cotyledons (10 pairs) or material (2 g) from other tissues of *C. camphora* were homogenized in 10 ml of buffer A (0.1 M Tris/HCl, pH 9.1/0.2 M NaCl) in a cold mortar. The homogenates were filtered through four layers of cheesecloth and the filtrate was centrifuged (4°C , 12000 g) for 20 min. For the enzyme assay experiments, the supernatant was dialysed further against buffer B (25 mM citrate/phosphate, pH 5.0).

Northern blotting

Total RNAs were isolated by the method of Krafft et al. [15] and Northern-blot analysis was performed as described by Sambrook et al. [16]. After denaturation with formamide, 5 μg of total RNAs were fractionated on a 1.0% agarose gel, blotted on to a

Abbreviations used: DAF; days after flowering; DAL; days after imbibition; RIP, ribosome-inactivating protein.

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nitrocellulose membrane and then fixed by UV cross-linking. The blotted membrane was treated in blocking solution ($5 \times$ Denhardt's solution/ $5 \times$ SSC/ 0.5% blocking reagent/ 0.5% SDS; where $1 \times$ SSC is 0.15 M NaCl/ 0.015 M sodium citrate) at 65°C for 2 h. Then the RNAs on the membrane were hybridized in the same solution at 65°C overnight with a cDNA probe of cinnamomin A-chain radiolabelled with [α - ^{32}P]dCTP. The membrane was washed twice with $2 \times$ SSC/ 0.1% SDS at 65°C for 1 h, followed by several washes with $0.5 \times$ SSC/ 0.1% SDS under the same conditions. The membrane was exposed to Hyperfilm-MP X-ray films with an intensifying screen at -70°C for 12 h–4 days.

Western-blotting analysis

Antiserum against intact cinnamomin was prepared and polyclonal antibodies were purified by chromatography on a Protein A-Sepharose column as described by Sambrook et al. [16]. After fractionation by SDS/PAGE, proteins were transferred on to a nitrocellulose membrane, which was then incubated with 5% (w/v) non-fat dry milk in TBS (25 mM Tris/HCl, pH 7.4/ 0.8% NaCl/ 0.02% KCl) for 2 h, and then incubated with $10\text{ }\mu\text{g/ml}$ anti-cinnamomin IgG in the same solution for 2 h. The membrane was then washed three times with TBS plus 0.1% Tween 20 (TBST) for 10 min each time, and incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG in TBS containing 5% (w/v) non-fat dry milk for 2 h. The membrane was washed three times with TBST followed by three washes with TBS. Finally, the immunoblotting membrane was visualized by ECL® (Amersham Bioscience).

Measurement of cinnamomin content by ELISA

Anti-cinnamomin IgG ($100\text{ }\mu\text{l}$; $1\text{ }\mu\text{g/well}$) was incubated in a microtitre plate at 4°C overnight and then $200\text{ }\mu\text{l}$ of 5% (w/v) BSA in TBS was added to each well. After incubation for 2 h, the plate was washed three times with $200\text{ }\mu\text{l}$ of TBST. Then $100\text{ }\mu\text{l}$ of solution containing the appropriate amount of cinnamomin was added to each well and the plate was incubated for 2 h. After washing the plate three times with $200\text{ }\mu\text{l}$ of TBST, the horseradish peroxidase-conjugated IgG against cinnamomin was added to each well. After incubation for 1 h, the plate was washed three times with $200\text{ }\mu\text{l}$ of TBST followed by three washings with $200\text{ }\mu\text{l}$ of TBS. The plate was then incubated with $100\text{ }\mu\text{l}$ of substrate solution (3 mM tetramethyl benzidine/ 0.004% H_2O_2 / 0.1 M sodium citrate buffer, pH 5.0) for 15 min. The reaction was terminated by adding $100\text{ }\mu\text{l}$ of 1 M H_2SO_4 and the absorbance was recorded at 450 nm . The amount of cinnamomin was calculated according to the standard curve.

Assay for endopeptidase activity and its effect on the degradation of cinnamomin

Endopeptidase activity was measured using azocoll as the substrate [17]. Extracts (1 ml) from *C. camphora* seeds at different stages of germination were incubated with 2 ml of azocoll solution (5 mg/ml) at 37°C for 3 h and then excess substrate was removed by centrifugation. The absorbance was measured at 520 nm and 1 unit of activity was defined as that necessary to cause an increase of 1 A_{520} unit/h. To observe the effect of endopeptidase activity on the degradation of cinnamomin, the extracts were treated in the presence or absence of 1 mM PMSF at 0°C for 12 h and then incubated at 37°C for 12 h. The content of cinnamomin in the extracts was measured by ELISA.

Preparation of the protein body

The protein body was prepared according to the method of Chrispeels et al. [18]. Ten pairs of mature cotyledons [90 days after flowering (DAF)] were homogenized with 10 ml of buffer C [25 mM citrate/phosphate, pH 4.5/ 40% (w/v) sucrose/ 10 mM 2-mercaptoethanol]. The homogenate was filtered through four layers of cheesecloth, and the filtrate was centrifuged (4°C , 500 g) for 5 min. The supernatant was layered on a step gradient consisting of 20 ml of buffer C and 10 ml of buffer D [25 mM citrate/phosphate, pH 4.5/ 80% (w/v) sucrose/ 10 mM 2-mercaptoethanol]. The gradients were centrifuged (25000 rev./min , 4°C) for 60 min in a SW27 Spinco rotor. The protein-body band above buffer D and the supernatant, containing cytoplasmic organelles, were collected.

RESULTS

Tissue-specific expression of cinnamomin in cotyledons

Total RNA and protein were isolated from various tissues including roots, stems, leaves of seedlings, leaves of adult, fruits and cotyledons of *C. camphora* seeds and the expression of cinnamomin in these tissues was examined. Northern blots

Figure 1 Tissue-specific expression of cinnamomin in cotyledons of *C. camphora*

(A) Total RNA ($10\text{ }\mu\text{g/well}$) from different tissues were extracted and analysed by Northern blotting. (B) Crude proteins ($20\text{ }\mu\text{g}$) of different tissues were separated by SDS/PAGE (12% gel) under non-reducing conditions and then analysed by Western blotting. Lanes 1, fruits; lanes 2, roots of seedlings; lanes 3, stems of seedlings; lanes 4, leaves of seedlings; lanes 5, adult leaves; lanes 6, cotyledons; lane S, $4\text{ }\mu\text{g}$ of cinnamomin; C, cinnamomin; U, unknown protein that cross-reacted with anti-cinnamomin antibody.

Figure 2 Accumulation of cinnamomin in cotyledons of *C. camphora* at different stages of development

Cotyledons were harvested every 15 days over a 120 day period after flowering (from August 15 to December 15). Total proteins were extracted and equal volumes (10 μ l) were separated by SDS/PAGE (12% gel) under non-reducing conditions (A) and analysed by Western blotting (B). For abbreviations see Figure 1.

showed that the mRNA of cinnamomin was expressed exclusively in the cotyledons, a storage organ of the plant (Figure 1A). Western blotting of the total proteins from these tissues using antibodies against cinnamomin showed that it could only be detected in the cotyledons (Figure 1B). These results demonstrated that cinnamomin was expressed specifically in the cotyledons. In addition, an unknown protein that cross-reacted with the antibody against cinnamomin was found in *C. camphora* seeds. Studies of the relationship between cinnamomin and this unknown protein are now in progress.

Synthesis of cinnamomin during seed development

To study the course of the accumulation of cinnamomin during the development of *C. camphora* seeds, proteins extracted from cotyledons at different developmental stages were analysed by SDS/PAGE and Western blotting. As indicated in Figure 2, little protein was synthesized in the early developmental stages (0–60 DAF), and no cinnamomin was detected at these stages. Cinnamomin first appeared at 60 DAF in the cotyledon and subsequently accumulated, together with other proteins, over the following 30 days. Such a pattern of synthesis and accumulation

Figure 3 Degradation of cinnamomin in cotyledons of *C. camphora* at different stages of germination

Cotyledons were harvested every 7 days over a 42 day period after imbibition. Total proteins were extracted and equal volumes (10 μ l) were separated by SDS/PAGE (12% gel) under non-reducing conditions (A) and analysed by Western blotting (B). For abbreviations see Figure 1.

of cinnamomin in the later stages of seed development is very similar to that of other seed storage proteins [17].

Degradation of cinnamomin during seed germination

The change in cinnamomin content during germination of *C. camphora* seeds was monitored for a period of 42 days after imbibition (DAI). By 42 DAI, the plantlets developed three to four leaves and their total dry weight was equal to or larger than that of the initial seeds. Total proteins were extracted from *C. camphora* seeds at different stages of germination and analysed by both SDS/PAGE and Western blotting. The results revealed that the amounts of cinnamomin and other proteins did not change during the first stage (7 DAI), but degraded rapidly during the subsequent stages (7–21 DAI), becoming undetectable at 21 DAI (Figure 3). The rapid degradation of cinnamomin in the early stages of seeds germination was similar to that of seed storage proteins of other species but distinct from two other seed RIPs (grain RIP and ricin), which are degraded slowly during seed germination [19,20].

Effect of endopeptidase activity on cinnamomin degradation

Endopeptidase has been shown to play an important role in the degradation of storage proteins in dicotyledonous seeds [17].

Figure 4 Endopeptidase activity in germinating cotyledons

Proteins were extracted from cotyledons at different germination stages. The content of cinnamomin and activity of endopeptidase were measured as described in the Materials and methods section.

Figure 5 Effects of inhibitors on endopeptidase activity and cinnamomin degradation

Enzyme solutions extracted from cotyledons at different stages of germination were treated with PMSF and then incubated at 37 °C for 12 h. The content of cinnamomin was measured by ELISA.

Changes of the activities of endopeptidase and two exopeptidases, carboxypeptidase and aminopeptidase, during the germination of *C. camphora* seeds were measured. The activities of exopeptidases were very low throughout the germination period (results not shown). However, endopeptidase activity increased dramatically on 7–14 DAI, and was maintained at a high level thereafter (Figure 4). This increment of endopeptidase activity coincided with the rapid breakdown of cinnamomin (7–28 DAI).

The endopeptidase of *C. camphora* seeds was found to be a serine protease, since it was sensitive to PMSF but not to *N*-ethylmaleimide (results not shown). The effect of endopeptidase on the degradation of cinnamomin was studied further by using the protease inhibitor PMSF. Extracts from *C. camphora* seeds at different stages of germination were treated with PMSF and then autolyzed. As shown in Figure 5, the cinnamomin content in extracts treated with PMSF was much higher than

Figure 6 Localization of cinnamomin in the protein body of cotyledons

Protein bodies were prepared by sucrose-density-gradient centrifugation. Proteins were separated by SDS/PAGE (12% gel) under reducing conditions (A) and analysed by Western blotting (B). Lanes 1, protein markers; lanes 2, proteins in the supernatant (40 µl); lanes 3, proteins in protein bodies (10 µl); lanes 4, reduced cinnamomin.

those not treated with PMSF, demonstrating that the inhibition of endopeptidase by PMSF could prevent the degradation of cinnamomin. All of these results suggest that the endopeptidase plays an important role in the degradation of cinnamomin during *C. camphora* seed germination. In this respect, cinnamomin is very similar to other seed storage proteins of dicotyledonous plants [17].

Localization of cinnamomin in the protein body

Most plant storage proteins are located in the protein body of the cell. This highly specialized cellular organelle can be purified by sucrose-density-gradient centrifugation, appearing as a sharp band at the interface of 40% and 80% sucrose. More than 80% of total cotyledon proteins were found in the protein-body fractions. Western blotting showed that cinnamomin also existed in these fractions (Figure 6), whereas no cinnamomin was detected in the component above 40% sucrose, where other cytoplasmic organelles were concentrated. This shows that cinnamomin is localized specifically in protein bodies, consistent with their proposed role as a storage protein.

DISCUSSION

The results presented here indicate that expression of cinnamomin was limited in the cotyledon of *C. camphora* seeds. Its synthesis in large amounts during the latter stages of seed development, as well as its rapid degradation in the early and intermediate stages of seed germination, suggest that cinnamomin may function as a seed storage protein. Cinnamomin is post-translationally processed like other seed storage proteins. These processes include proteolytical cleavage, and the formation of intra- and inter-chain disulphide bonds and glycosyl groups with mannose N-linked to asparagine residues [13,21]. After processing, the mature cinnamomin is transported to the protein body, the specific cellular organelle for storage proteins.

Cinnamomin and other type II RIPs (members of the ricin superfamily) are structurally similar to the leguminous seed storage protein lectin, since they are all composed of a lectin chain linked to another chain via a disulphide bond. However, arguments about the role of the ricin superfamily as seed storage proteins are unresolved [4,5,20,22]. Here we provide evidence that cinnamomin is a storage protein in *C. camphora* seeds. Cinnamomin is one of the most prevalent proteins in *C. camphora* seeds, accounting for about 11 % of total proteins. This content is higher than many other storage proteins, such as soya bean 7 S basic storage proteins (5–10 %) and soya bean lipoxygenases (1–2 %) [22]. Most importantly, the amino acid composition of cinnamomin is very close to that of other storage proteins. It is rich in amide-containing amino acids (Asn, 12.6 %; Gln, 9.4 %). Cinnamomin also has relatively a higher content of sulphur-containing amino acids (Cys, 1.9 %; Met, 2.6 %) than vicilin and legumin [22]. All of these properties suggest cinnamomin functions as a storage protein by which sources of carbon, nitrogen and sulphur are provided during seedling growth and development.

Many RIPs have been reported to play a defence role against plant pathogens such as viruses, fungi and insects [3–6]. Previous studies also showed that cinnamomin is toxic to larvae of mosquito (*Culex pipiens pallens*) and cotton bollworm (*Helioverpa armigra*) [14]. Developing seeds accumulate large quantities of cotyledon-specific cinnamomin and other storage proteins for use as nutrient sources during germination. Thus the abundance of cinnamomin could provide seeds with both nutritional benefits and protection against pathogen invasion.

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