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Synthesis of Thiamin-Binding Protein during Seed Development and Its Accumulation in Sesame Seed

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Abstract

The expression of the thiamin-binding protein gene in sesame was found only in seeds during maturation, not in seeds during germination, or in roots, stems and leaves. The amount of mRNA of the thiamin-binding protein increased with the development of sesame seeds. The level of mRNA was maximum in the mature seeds. Also, the thiamin-binding activity from seed proteins increased along with seed development. On the other hand, immunohistochemical analysis using an antibody against the protein and an immunogold-silver enhancement kit detected the thiamin-binding protein only in the albumen cells of seeds. These results suggest that in sesame, the thiamin-binding protein is synthesized in developing seeds and accumulated in the albumen of the seeds.

Key words: seed storage protein, sesame, *Sesamum indicum* L., thiamin, thiamin-binding protein.

Abbreviations

BSA; Bovine albumin, DAF; days after flowering, TBP; thiamin-binding protein.

Introduction

Thiamin-binding proteins (TBPs) found in many kinds of plant seeds are novel storage proteins, because the proteins retain thiamin in dormant seeds and supply nitrogen source and thiamin for germ growth during germination (Shimizu *et al.*, 1996). The engineering of storage proteins in plant seeds, from a nutritional point of view, has recently been given much attention. For example, storage proteins were utilized in the enhancement of the quality of food (Momma *et al.*, 1999). Properties such as molecular mass, subunit structure, amino acid composition, and affinity for thiamin and thiamin-related compounds have been reported for many types of TBPs (Shimizu *et al.*, 1996; Watanabe *et al.*, 1999, 2002). However, there is still no report on the expression of the TBP gene and the accumulation of TBP in plant seeds.

Sesame seeds have TBPs. The TBPs are 2S albumins although the other TBPs of plant seeds are globulins (Watanabe *et al.*, 1999). In addition, the optimal pH of thiamin-binding activity of the TBPs from sesame seeds differs from that of the others, and the molecular masses of the TBPs are low compared with the others (Shimizu *et al.*, 1995). In recent years, the cDNA encoding TBPs from sesame seeds was isolated and characterized (Watanabe *et al.*, 2001). The formation of the final mature proteins by post-translational processing was proposed. Thus, in this study, we investigated the expression of the gene encoding the TBPs using the cDNA and the localization of TBPs in sesame seeds using an antibody against the TBPs, to discuss the synthesis, accumulation and role of the TBPs.

Materials and Methods

Plant materials

Sesame (*Sesamum indicum* L.) was field-grown at the Faculty of Agriculture, Kinki University. The maturation period of sesame seeds was about 45 days in the present experiment. Developing fruits were harvested at the desired times after flowering. Developing seeds were isolated and used immediately or were stored in liquid nitrogen until use.

Extraction of TBPs and assay of thiamin-binding activity

The seeds were ground with a motor and pestle in 0.05 M sodium-phosphate buffer (pH 7.0) con-

taining 1.0% NaCl at 4 ℃. The slurry was centrifuged at 28,000*g* for 15 min at 4 ℃. The protein content of the resulting solution was measured by the Bradford method (Bradford, 1976).

The thiamin-binding activity of the solution was measured by the equilibrium dialysis method as reported previously (Shimizu *et al.*, 1995). The solution was dialyzed against the buffer containing 1 μM thiamin for 24 h at 4 ℃. The thiamin concentrations of the inner and outer solutions were measured by the thiochrome fluorescence method after the equilibration. The thiamin-binding activity was determined by the difference in the thiamin concentration between the two solutions.

RNA extraction and RT-PCR

Total RNA was isolated from seeds, roots, stems, and leaves according to the phenol-SDS method as described previously (Shirzadegan *et al.*, 1991). The amplification of the cDNA encoding TBPs from sesame seeds was carried out on the basis of the method as described previously (Watanabe *et al.*, 2001). The following oligonucleotide primers were designed: 5'-ATATAGATGGCGAGGTTCAG-3' for a forward primer and 5'-ATGATGAGGGTTGAGCCTGC-3' for a reverse primer. The PCR amplification was performed with the following program : an initial denaturation step of 3 min at 94 ℃, followed by 35 cycles of 1 min at 94 ℃, 1 min at 55 ℃ and 1 min at 72 ℃. Amplification products were separated by the electrophoresis (Sambrook *et al.*, 1989). The separated DNA fragments were visualized under UV light after staining with ethidium bromide.

Section preparation for immunohistochemistry

Seed section was prepared according to the method as described previously (Hashimoto *et al.*, 1991). Seeds were fixed in 4% paraformaldehyde and 8% sucrose in 0.1 M potassium-phosphate buffer (pH 7.4), then washed sequentially with the buffer containing 10 to 20% sucrose. Fixed seeds were embedded in paraffin after the dehydration. Sections (10 μm) were cut and mounted on glass-slides that had been coated with egg albumin. The sections were hydrated in an ethanol/water series after the removal of paraffin, and subjected to antibody treatment.

Immunohistochemical analysis

The sections were saturated with 1% BSA and 0.1% Tween 20 in buffer A (10 mM Na_2HPO_4, 3 mM KH_2PO_4, 120 mM NaCl, and 20 mM NaN_3) adjusted to pH 8.2 with 0.1 N NaOH overnight. Then, they were incubated overnight with the primary antibody against sesame thiamin-binding proteins, which was prepared using a Japanese white rabbit as reported previously (Watanabe *et al.*, 1999). For immunogold staining and silver enhancement, reagents from British BioCell Inter. (Ultrasmall Gold Conjugates) and Nanoprobes (HQ Silver ™ Enhancement Kit) were used after washing with buffer A. The secondary antibody and the silver enhancement treatments followed the protocols. After silver enhancement, the sections were observed under a microscope.

Results and Discussion

Change of thiamin-binding activity in sesame seeds during maturation

The amount of protein from sesame seeds increased with the seed development. The thiamin-binding activity of the seeds increased in parallel with the increase in protein level following seed development (**Fig. 1**). A remarkable increase of thiamin-binding activity was observed at 4-6 weeks after flowering. These results suggested that TBP was produced in the seeds during maturation.

Expression of the TBP gene in sesame seeds during maturation

The expression of the TBP gene was analyzed by RT-PCR (**Fig. 2**). The mRNA of the TBP was detected in seeds at 25 DAF. However, the mRNA was not detected in dry seeds imbibed for 1 day, nor was it detected in roots, stems, and leaves of sesame grown in the field. The TBP gene was translated only in developing seeds in sesame. This was the same case for the gene of 2S albumin from *Arabidopsis* seeds (Krebbers *et al.*, 1988), which belongs

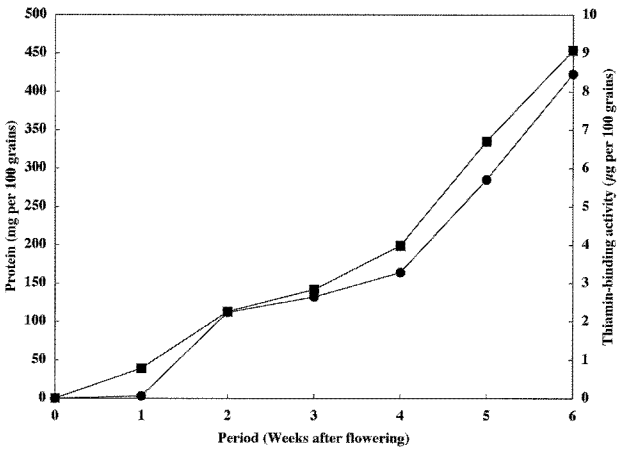


Fig. 1 Changes in total protein content and total thiamin-binding activity of sesame seeds during maturation. The mean value of triplicate experiments is presented. ● , Total protein; ■ , total thiamin-binding activity.

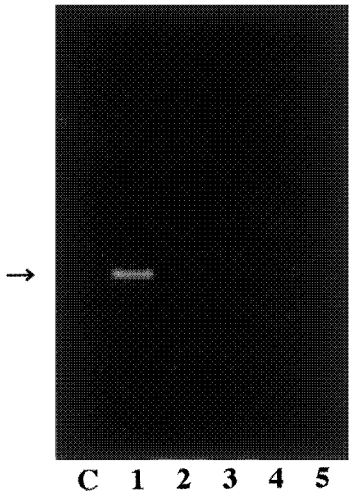


Fig. 2 Expression of mRNA of TBP in sesame. Total RNA (2 μg) was analyzed by RT-PCR as described in Materials and Methods. The arrow shows the mRNA of TBPs from sesame seeds. Lane C; Control (not amplified seeds at 25 DAF), lane 1; seeds at 25 DAF, lane 2; dry mature seeds after imbibition for 1 day, lane 3; roots of sesame grown in field, lane 4; stems, lane 5; leaves.

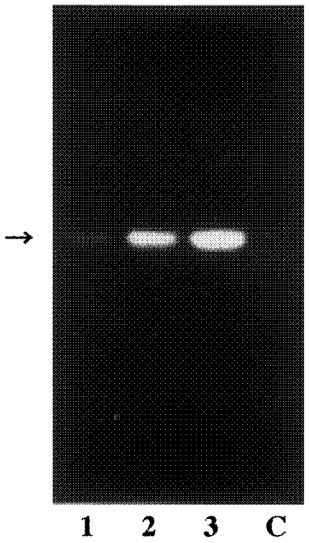
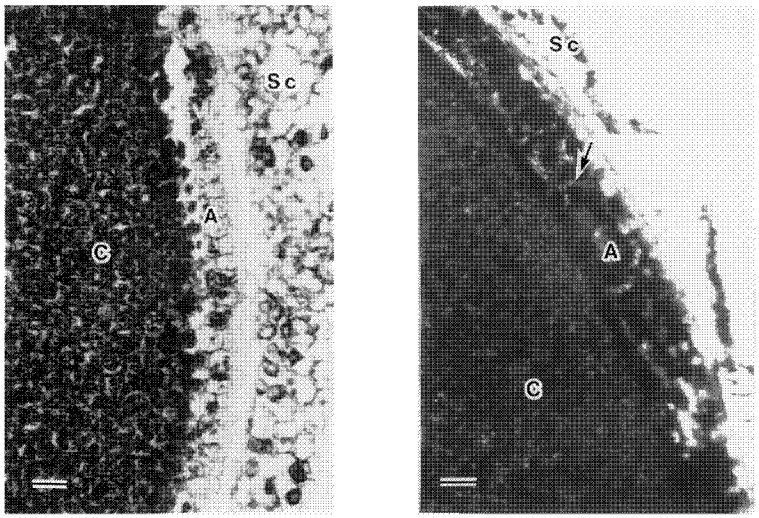


Fig. 3 Level of mRNA of TBP in sesame seeds during maturation. Total RNA (2 μg) was analyzed by RT-PCR as described in Materials and Methods. The arrow shows the mRNA of TBPs from sesame seeds. Lane 1; seeds at 20 DAF, lane 2; seeds at 30 DAF, lane 3; seeds at 40 DAF, lane C; Control (not amplified seeds at 40 DAF).



1 2

Fig. 4 Immunolocalization of TBP in mature sesame seeds. 1; Unstained seed section (control), 2; immunostained seed section. Sc; seed coat, A; albumen, C; cotyledon. Bar = 50 μm. The arrow shows the stained albumen (the localization of the TBPs).

to the same plant protein superfamily as the sesame TBP (Watanabe *et al.*, 1999).

Next, the change in mRNA level during seed development was examined by RT-PCR. The level of mRNA increased along with the seed development. The mRNA was hardly detectable in the seeds at 20 DAF, but it was present at a high level in the seeds at 30 DAF and at 40 DAF (**Fig. 3**). The mRNA level was maximum in the seeds at the later stage of development. Northern hybridization with the non-RI labeled cDNA of the TBPs as a probe gave the same results (data not shown). On the other hand, the thiamin-binding activity from the seeds increased with the seed development (**Fig. 1**). These results showed that the TBP gene was translated only in the seeds during maturation, and that the transcript of the gene was accumulated in the seeds. Consequently, TBPs were accumulated abundantly in mature sesame seeds.

Localization of TBP in sesame seeds

The localization of TBP in seeds was analyzed by immunogold-silver enhancement staining. The immunohistochemical localization of TBP in the sections revealed that TBP was present only in the albumen cells of seeds during maturation (**Fig. 4**). When germinated seeds were analyzed in the same way, they reacted weakly with the antibody against the TBPs (data not shown). No signal was detected in the sections from roots, stems, leaves, and pericarps of sesame grown in the field, nor in any sections when a nonspecific rabbit IgG or an antibody against the TBP from wheat germs was used as the primary antibody (data not shown).

The TBP was accumulated in the albumen cells of mature sesame seeds (**Fig. 4**). Seed storage proteins are accumulated in seeds during maturation, and degraded to serve as nitrogen sources for germ development during seed germination (Tanaka *et al.*, 1980; Mundy *et al.*, 1986). The mRNA of TBP from sesame seeds was detected in the seeds during maturation, but not in the dry mature seeds after imbibition. It was reported that the level of TBP was decreased during seed germination (Mitsunaga *et al.*, 1987). It suggested that TBP was degraded to serve as a nitrogen source and supply thiamin for germ development (Nakayama 1960). These results suggested that TBP was a seed storage protein for germ growth during seed germination. It is the first report on the gene expression and the accumulation of TBP in plant seeds.

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