Note

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Brazil nut 2S albumin was synthesized in a transgenic French bean seed with a promoter of the gene for canavalin, 7S globulin from *Canavalia gladiata*

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Abstract

A gene for Brazil nut 2S albumin, a methionine-rich protein, was fused to a promoter region of the canavalin gene. This chimeric gene was introduced into shoot apices of embryonic axes of French bean by particle bombardment and seeds were obtained from plants regenerated from the bombarded axes. Protein in the transgenic seeds was analyzed by immunoblotting with an antiserum against Brazil nut 2S albumin. A 12-kDa immunoreactive polypeptide was detected and its amount in the seed was estimated to comprise less than 1% of the soluble protein.

The nucleotide sequence reported in this paper has been submitted to the DDBJ, GenBank, EMBL under accession number AB044391.

Legume seeds are important crops with a seed protein content higher than that of cereals. However, methionine contents are low compared to animal proteins. Brazil nut 2S albumin contains 18% methionine (Ampe *et al.*, 1986). Its cDNA fused to a promoter region of a gene for a seed storage protein from French bean, phaseolin, was introduced to tobacco (Altenbach *et al.*, 1989). The accumulation of the 2S albumin resulted in a 30% increase in the methionine content of transgenic tobacco seed. To improve the nutritional quality of legume seed protein, the gene was also introduced to *Vicia narbonesis* (Saalbach *et al.*, 1995). Broad bean legumin gene promoter drove 3% 2S albumin in total soluble protein from the seed and the methionine level in the seed reached 42%.

We cloned the gene for canavalin, a 7S globulin from *Canavalia gladiata* seed (Takei *et al.*, 1989). The promoter region fused to the coding region of the β-glucuronidase (GUS) gene from *Escherichia coli* showed seed-specific expression of GUS activity in a transgenic tobacco plant (Yamamoto *et al.*, 1995). The positive and negative regulatory elements in the promoter region were identified by a transient assay system (Yamamoto *et al.*, 1995; Yamauchi, 1997).

French bean is cultivated throughout the world and an important source of protein. Genetic transformation of the plant is expected to improve the quality of the seed protein. *Agrobacteriumu*-mediated transformation is generally used to produce transgenic plants. However, it is unsuccessful in bean plants that are difficult to regenerate (Nagl *et al.*, 1997). The first production of transgenic French bean plants was achieved by particle bombardment that was used to introduce a herbicide-resistant gene driven by a cauliflower mosaic virus 35S RNA promoter (Russell *et al.*, 1993). Kim and Minamikawa also succeeded in producing transgenic French bean plants by particle-bombardment (Kim and Minamikawa, 1996). In addition, they introduced the canavalin gene promoter: GUS fusion gene into embryonic axes of French bean by particle bombardment (Kim and Minamikawa, 1997). GUS activity was detected only in seeds in plants regenerated from the axes. Therefore, we predicted that the canavalin promoter was useful to produce a methionine-rich protein, Brazil nut 2S albumin for improving nutritional quality of protein in a French bean seed.

We report here that the gene for Brazil nut 2S albumin fused to the promoter region of the canavalin gene was delivered into embryonic axes of French bean by particle bombardment. Seeds were obtained from plants regenerated from the axes and protein in the seed was analyzed by immunoblotting.

Seeds of French bean (*Phaseolus vulgaris* L. cv.

Goldstar) were purchased from Sakata Seed (Yokohama, Japan). Brazil nut seeds were kindly provided by Toyo Nut Co. (Kobe, Japan). Brazil nut seeds were powdered with acetone in a homogenizer. An albumin fraction was prepared from the powder according to Minamikawa (1983). Purification of 2S albumin from the albumin fraction was carried out by gel filtration chromatography using a Sephacryl S-200 column (1.6 x 90 cm) equilibrated with 0.5 M KCl and 10 mM 2-mercaptoethanol in 50 mM sodium acetate (pH 5.5). An antiserum against Brazil nut 2S albumin was raised by six subcutaneous injections of 200 μg of the protein emulsified with Freund's adjuvant at two-week intervals. The antiserum from the rabbit was precipitated with 40%-saturated ammonium sulfate and dialyzed against 10 mM sodium phosphate (pH7.4) containing 1.5 M NaCl.

A crude nucleic acid fraction was extracted from seeds of Brazil nut by phenol/chloroform extraction as described by Ishibashi and Minamikawa (1989). Two primers for polymerase chain reaction (PCR) were designed from the sequence of a gene for a 2S albumin from Brazil nut named *BE2S1* (Gander *et al.,* 1991). One primer corresponded to the sequence from +48 to +68 and the other (5'-GTGAGAGCTCTATTAACATTTACA-3') was used for creating a *Sac*I site 37 bp downstream from the stop codon. PCR was carried out with the nucleotide fraction from a Brazil nut seed. A 580-bp fragment of the 2S albumin gene was generated and cloned into pCRII (Clontech). The canavalin gene promoter region was also amplified by PCR with a primer corresponding to the sequence of the canavalin gene from -794 to -774 and a primer (5'-GAAAAAGCCATGGTGGTTTACTAA-3') for creating a *Nco*I site at the initiation codon of the canavalin gene. The amplified fragment was also cloned into pCRII. The promoter region and a 569-bp fragment of the 2S albumin gene were placed in pBI221 (Jefferson *et al.*, 1987) with replacement of the cauliflower mosaic virus 35S RNA promoter and GUS coding region. This fusion gene cut with *Hin*dIII and *Eco*RI was put into pBIN19 (Bevan, 1984) and the resulting plasmid was designated pBI2S1.

Preparation of embryonic axes and particle bombardment for transformation of French bean plants were carried out as described by Kim and Minamikawa (1996). The bombarded embryonic axes were incubated on Murashige and Skoog (MS) agar medium (Murashige and Skoog, 1962) with 3% sucrose. After incubation for three weeks, PCR analysis with a small piece of leaf was carried out as described by Kim and Minamikawa (1996). The reaction mixture was loaded onto a 2% agarose gel for electrophoresis. Total DNA was prepared from leaves of a transgenic plant with Puregene DNA Isolation Kit (Gentra Systems). PCR was carried out with two primers (5'-CTCCTTGTCCTCATGGCCCT-3' and 5'-CTCATGGGACTGAGGTTGCA). After electrophoresis on a 2% agarose gel, DNA was transferred onto a nylon membrane. Hybridization was carried out using the ^{32}P-labeled 2S albumin gene. Immature embryos were collected from regenerated plants. After removal of one side of the cotyledon, embryos were incubated on 1% agar containing 50 mg l^{-1} of kanamycin. Plantlets from the embryos were transferred to soil in a pot. Protein was extracted from cotyledons with 50 mM sodium acetate pH5.5 containing 10 mM 2-mercaptoethanol, and loaded onto a 20% SDS-PAGE gel. Protein in the gel was transferred onto a nitrocellulose filter and immunoreactive polypeptides were detected as described previously (Zhong *et al.,* 1997) except that antiserum against Brazil nut 2S albumin was used.

Brazil nut 2S albumins are encoded by a multigene family (Gander *et al.,* 1991). A Brazil nut 2S albumin gene, *BE2S1*, whose product contains 18% methionine, was isolated by PCR and sequenced (**Fig. 1A**). The gene consists of 1 intron and 2 exons. The sequence is the same as that reported by Gander *et al.* (1991) except for 5 nucleotide residues. These nucleotide changes affected three amino acids residues; Met_{92}, Arg_{93} and Glu_{97} were changed to Arg_{92}, Met_{93} and Lys_{97}, respectively. Mutagenesis with PCR created a *Nco*I site at the start codon of the canavalin gene, because the site was ligated to the *Nco*I site at the initiation codon of the 2S albumin gene. The plasmid, pBI2S1, containing the canavalin: Brazil nut 2S albumin gene, was introduced to shoot apices of embryonic axes of French bean by particle bombardment (**Fig. 1B**). Plantlets were regenerated from embryonic axes on an MS agar medium. Three-hundred-and-fifty plantlets were incubated for 3 weeks and small pieces of young leaves from these plantlets were subjected to analysis by PCR. The promoter region of the canavalin gene was amplified from 2 plantlets (data not shown). These plantlets were transferred to pots and produced maturing seeds (R1). One side of cotyledon of the seed was analyzed by immunoblotting with the antiserum against 2S albumin (**Fig. 2A**). A 12-kDa immunoreactive polypeptide was detected in a transgenic seed. The amount estimated from the band by densitometer, was less than 1% of the soluble protein from the seed. The embryonic axis with the other side of cotyledon of the seed was incubated on 1% agar and regenerated. The DNA fraction was isolated from leaves of the R1 plant

(A)

```
                    20                  40                  60                  80                  100
aatcaccATGGCGAAGATTTCAGTTGCGGCAGCAGCACTCCTTGTCCTCATGGCCCTCGGCCACGCCACCGCCTTCCGGGCCACCGTCACCACCACAGTG
      M  A  K  I  S  V  A  A  A  A  L  L  V  L  M  A  L  G  H  A  T  A  F  R  A  T  V  T  T  T  V

                    120                 140                 160                 180                 200
GTGGAGGAGGAGAACCAGGAGGAGTGTCGCGAGCAGATGCAGAGACAGCAGATGCTCAGCCACTGCCGGATGTACATGAGACAGCAGgttagttattata
V  E  E  E  N  Q  E  E  C  R  E  Q  M  Q  R  Q  Q  M  L  S  H  C  R  M  Y  M  R  Q  Q
                ▲

                    220                 240                 260                 280                 300
caaaagaatatggaatctcttttatatttcttaatttaatgccttatatcttgatgcgaaatgaaccctggtgcagATGGAGGAGAGCCCGTACCAGACC
                                                                                M  E  E  S  P  Y  Q  T
                                                                                            Δ

                    320                 340                 360                 380                 400
ATGCCCAGGCGGGGAATGGAGCCGCATATGAGCGAGTGCTGCGAGCAGCTGGAGGGGATGGACGAGAGCTGCAGATGCGAAGGCTTAAGGATGATGATGA
M  P  R  R  G  M  E  P  H  M  S  E  C  C  E  Q  L  E  G  M  D  E  S  C  R  C  E  G  L  R  M  M  M  R
   ▲                                                                                                   •

•  •                 420                 440                 460                 480                 500
GGATGATGCAACAGAAGGAGATGCAACCCCGAGGGGAGCAGATGCGAAGGATGATGAGGCTGGCCGAGAATATCCCTTCCCGCTGCAACCTCAGTCCCAT
  M  M  Q  Q  K  E  M  Q  P  R  G  E  Q  M  R  R  M  M  R  L  A  E  N  I  P  S  R  C  N  L  S  P  M
  •           •

                    520                 540                 560                 580
GAGATGCCCCATGGGTGGCTCCATTGCCGGGTTCtgaatctgccactagccagtgctgtaaatgttaata gagctctcac
 R  C  P  M  G  G  S  I  A  G  F
                   Δ
```

(B)

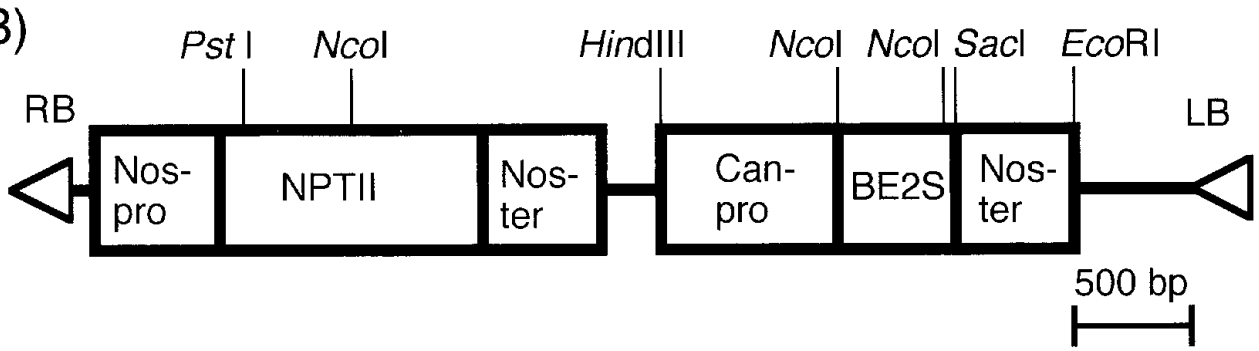

Fig. 1. Nucleotide sequence of the Brazil nut 2S albumin gene and physical map of the T-DNA region of the introduced plasmid designated pBI2S1.
(A) Nucleotide sequence and amino acid sequence of Brazil nut 2S albumin. The nucleotide sequences corresponding to the region of coding and non-coding indicate capital and small letters, respectively. Underlines indicate the sequence of primers for PCR and a created *Sac*I site is shown by italics. The N- and C-terminal amino acids of mature subunits of 2S albumin are shown by closed and open triangles, respectively. The residues different from those reported by Gander *et al.* (1991) are shown by dots. (B) Physical map of the T-DNA region of pBI2S1. RB, right border, LB, left border; Nos-pro, the promoter region of the gene for nopaline synthase, NPTII, kanamycin resistance gene; Nos-ter, the polyadenylation sequence of the gene for nopaline synthase; Can-pro, the promoter region of the gene for canavalin; BE2S, a gene for Brazil nut 2S albumin.

and analyzed by PCR. Brazil nut 2S albumin gene was amplified from the DNA fraction (**Fig. 2B**).

Brazil nut 2S albumin is synthesized as a larger precursor polypeptide of 15 kDa after cotranslational processing (Sun *et al.*, 1987). This precursor was processed to a 3-kDa and 9-kDa polypeptide via a 12-kDa intermediate. Using a broad bean legumin promoter, Brazil nut 2S albumin was synthesized in seeds of transformed *Vicia narbonesis* plants and the 3-kDa and 9-kDa polypeptides were detected in the seeds (Saalbach *et al.*, 1995). Previously, the 2S albumin was synthesized in French bean seeds transiently with particle-bombardment, but the molecular mass was not determined (Aragao *et al.*, 1992). Our results indicate that a transgenic French bean plant accumulated a 12-kDa Brazil nut 2S albumin polypeptide in the seed (**Fig. 2A**). Generally, processing of storage protein precursors is mediated by asparaginyl endopeptidase (Hara-Nishimura *et al.*, 1993). One possibility is that an enzyme in maturing French bean seed may not process the precursor of the 2S albumin effectively. In this study, we used the canavalin gene promoter for synthesis of 2S albumin. However, its amount was estimated to be less than 1% (**Fig. 2A**). The promoter of the legumin gene, *LegB4* from broad

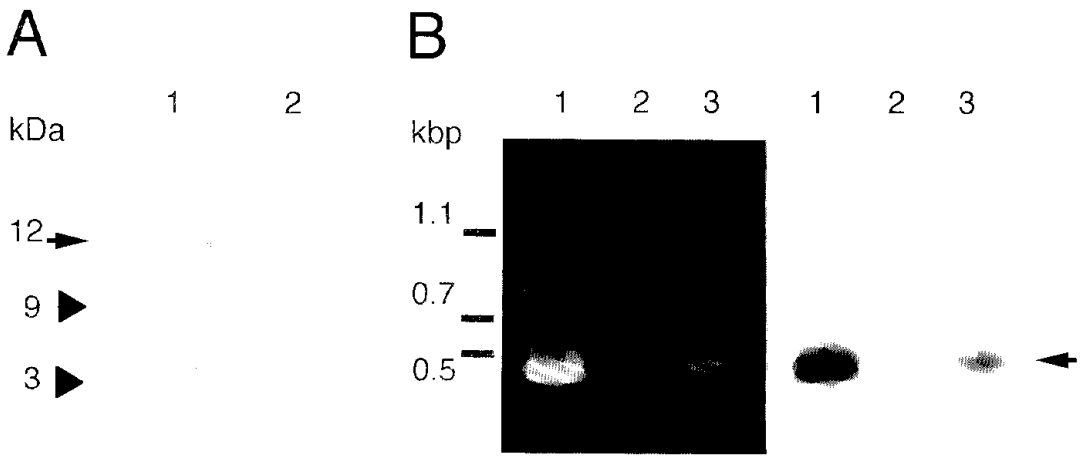

Fig. 2. Characterization of a transgenic plant.
(A) Immunoblot analysis of seed protein. Protein (100 μg) extracted from one side of the cotyledon was loaded on a 20% SDS polylacrylamide gel. Protein was reacted with an antiserum against Brazil nut 2S albumin. Lanes 1 and 2 indicate a transgenic seed (R1) and a non-transgenic seed, respectively. Arrowheads on the left indicate molecular masses of Brazil nut 2S albumin. An arrow denotes a 12-kDa immunoreactive polypeptide. (B) PCR analysis of DNA from leaves of a transgenic plant. Left panel, PCR products separated with a 2% agarose gel for electrophoresis. Right panel, Southern blot analysis of PCR products by hybridization with a ^{32}P-labeled Brazil nut 2S albumin gene. Lanes 1, 2 and 3 indicate a transgenic plant (R1), a non-transgenic plant and pBI2S1, respectively. An arrow indicates the amplified products.

bean drove 3% of the 2S albumin in the soluble protein of a seed of *Vicia narbonesis* (Saalbach *et al.*, 1995). Therefore, we need to improve the canavalin gene promoter for accumulation of methionine-rich protein at high level in French bean seed. The canavalin gene promoter contains negative regulatory elements (Yamamoto *et al.*, 1995; Yama-uchi, 1997). These elements might repress the synthesis of Brazil nut 2S albumin in the French bean seeds.

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