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Differential expression and elution behavior of basic 7S globulin among cultivars under hot water treatment of soybean seeds

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Basic 7S globulin (Bg7S), which accumulates in mature soybean (*Glycine max*) seeds, is an extracellular matrix protein. A large amount of Bg7S is synthesized *de novo* and is eluted from soybean seeds when immersed in 50–60°C water (hot water treatment, HWT). However, the Bg7S elution mechanism remains unclear. Under HWT, the seeds probably undergo heat stress and flooding stress. To obtain fundamental knowledge related to how Bg7S is eluted from hot-water-treated seeds, this study compared Bg7S elution among soybean cultivars having different flooding tolerance during pre-germination. The amounts of Bg7S eluted from seeds varied significantly among cultivars. Elution was suppressed by seed coats regarded as preventing the leakage of seed contents by rapid water imbibition. Furthermore, Bg7S expression levels differed among cultivars, although the difference did not result from any variation in Bg7S promoter sequences. However, the expression levels of Bg7S under HWT were not associated with the flooding tolerance level. Immunoelectron microscopy revealed that the Bg7S accumulated in the intercellular space of hot-water-treated seeds. Plasma membrane shrinkage was observed. The main proteins eluted from seeds under HWT were located in the extracellular space. This study clarified the mechanism of Bg7S elution from seeds under HWT.

[**Key words:** Soybean; Seed; Basic 7S globulin; Stress response; Seed coat]

Soybean (*Glycine max*) seeds accumulate basic 7S globulin (Bg7S) in extracellular matrices (1). In the soybean genome, Bg7S1 (GenBank accession BAA03681, Phytozome accession Glyma03g39940) and Bg7S2 (BAB91077, Glyma19g42490) share 93% identity in amino acid sequences. Bg7S forms a tetramer (168 kDa) that comprising two chains: α (27 kDa) and β (16 kDa) (2). Bg7S is synthesized as a single-chain precursor with an N-terminal signal peptide (3,4). Bg7S precursor is processed into α and β chains, which are bound covalently by disulfide bonds after cleavage of the signal peptide. It is then localized into the middle lamella of the cell wall and the plasma membrane of the cotyledon (1). The crystal structure of Bg7S has been elucidated, but, although the gene has been originated from aspartic proteinases, its physiological role remains unknown (5).

It is particularly interesting that when soybean seeds are immersed in 50–60°C hot water (hot water treatment, HWT), a large amount of Bg7S is synthesized *de novo* and is eluted from soybean seeds (6,7). Furthermore, the percentage of eluted Bg7S for the total proteins in the solution for immersed seeds on the HWT is high. This phenomenon might be common among leguminosae plants because adzuki bean, common bean, winged bean, jack bean, mung bean, and lupin also elute Bg7S-like proteins during HWT (7,8). Because of increased consumption of bioactive and therapeutic proteins including antibodies and vaccines, studies examining mass production of these proteins in plants have increased (9). If the mechanism of Bg7S elution from seeds during HWT were elucidated, the stress response of seeds during HWT might be utilized for the production of bioactive and therapeutic proteins.

Under HWT, soybean seeds undergo severe heat stress. Reportedly, the Bg7S mRNA level increases after treatment at 50°C, although those results were not reproducible (4). Heat-inducible transcription is generally regulated by heat shock transcription factors (HSFs) that bind to the heat shock elements (HSEs) on the promoter regions of heat-inducible genes. The consensus sequence of HSEs comprises contiguous inverted 5′-nGAAn-3′. The HSEs are classified into three types: perfect HSE (nTTCnnGAAnnTTCn), gap-type HSE (nTTCnnGAAn(5b)nGAA), and step-type HSE (nTTCn(5b)nTTCn(5b)nTTC) (10). In *Arabidopsis thaliana*, a major HSF AtHSF-1 also binds to another cis-element, stress responsive element (STRE, core consensus: AGGGG or CCCCT) (11). In *Saccharomyces cerevisiae*, STRE is activated by multiple stressors such as nitrogen starvation, osmotic and oxidative stress, low pH, and heat shock (12,13). The promoter region of the Bg7S1 gene has three heat shock element-like sequences (4). Reportedly both Bg7S promoters contain some cis-elements that control the development of seed-specific expression (14).

In addition to heat stress, soybean seeds are exposed to flooding stress during HWT. The soybean germination rate is highly susceptible to pre-germination flooding stress. The pre-germination flooding tolerance varies considerably among soybean cultivars. Results of several studies have shown that cultivars with a black or brown seed coat tend to be more tolerant than those with a yellow seed coat, suggesting that the seed coat color is associated with pre-

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germination flooding tolerance (15). In addition, the pre-germination flooding tolerance of soybean seeds is probably associated with the seed weight (16). The seed coats of large seeds are physically weak, which engenders the leakage of seed contents (17). The soybean cultivar Peking has especially strong pre-germination flooding tolerance. Its seeds are very small, with hard black seed coats (16). A Japanese soybean cultivar, Enrei, with a yellow seed coat has tolerance against pre-germination stress and has larger seeds than Peking. Another Japanese cultivar, Tamahomare, with yellow seed coats has similar size to that of Enrei, but has weak seed-flooding tolerance.

This study compared the Bg7S expression and elution of four soybean cultivars (Peking, Tamahomare, Enrei, and IOM) under HWT to obtain fundamental knowledge related to how Bg7S is eluted from seeds under such conditions.

MATERIALS AND METHODS

Plant materials Dry mature seeds of soybean (*G. max*, L.) cultivars Peking, Tamahomare, Enrei, and non-GM IOM soybeans (hereafter IOM) were used for this study. IOM is produced in the U.S. states of Indiana, Ohio, and Michigan (18), and is used as the source for food processing in Japan. The average weights of the seeds used in this study were Peking 0.12 g/seed, Tamahomare 0.32 g/seed, Enrei 0.30 g/seed, and IOM 0.15 g/seed.

HWT Dry soybean seeds (5 g) were incubated in a temperature-regulated solution (25 mL) with constant gentle shaking for 8–16 h. 50 mM phosphate buffer (pH 8.0) with 0.2 M NaCl was used to treat the seeds, except in the treatment portrayed in Fig. 1. After the solution volume was measured, the solution was collected for additional analyses. The treated seeds were washed twice with distilled water and stored at −20°C.

Preparation of anti-Bg7S antibody Native Bg7S was prepared as described by Shutov et al. (19). Seeds of soybean cultivar IOM (100 g) were treated with 500 mL of 100 mM sodium acetate (pH 5.0) containing 100 mM NaCl for 6 h at 50°C. Then Bg7S was purified using an SP-sepharose column (GE-Healthcare, UK). Fractions containing Bg7S were concentrated (5000 MWCO, Vivaspin 20; Sartorius Stedim Biotech SA). Bg7S was then immunized on rabbits using standard procedures to obtain anti-Bg7S antibody.

Protein measurement Protein concentrations were quantified using a Protein Assay Rapid Kit (Wako Pure Chemical Industries, Japan) or a 2-D Quant Kit (GE-Healthcare, UK) with bovine serum albumin as the standard. Densitometry of bands was conducted using Image Quant TL (GE-Healthcare, UK). Amounts of Bg7S in solution after the treatment were quantified using SDS-PAGE and were calculated densitometrically. After HWT, the solution was centrifuged at 12,000 ×*g* for 20 min at 4°C. Proteins in the supernatant were precipitated with 25% (w/v) trichloroacetic acid (final concentration), washed with cold acetone, and resuspended in 1× SDS sample buffer [62.5 mM Tris–HCl, pH 6.8, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 0.01% (w/v) BPB] (20). Then, 6 μg of protein was subjected to SDS-PAGE (12% acrylamide gel). Optical density was analyzed after staining with CBB R-250. The amount of Bg7S was calculated based on the total protein amount in the solution. The percentage of Bg7S among total proteins was obtained using densitometric analysis. Bands of Bg7S were confirmed using protein gel immunoblot analysis, as described by Motoyama et al. (21) using rabbit anti-Bg7S antibody and goat anti-rabbit IgG conjugate with alkaline phosphatase.

Amounts of Bg7S in seeds were analyzed using protein gel immunoblot analysis. Seeds were homogenized after treatment using a Multi-Beads Shocker (Yasui Kikai Corp., Japan) according to the manufacturer's instructions. Soybean seed powder was defatted with acetone and hexane. Then protein was extracted from defatted pellets with a 1× SDS sample buffer without bromophenol blue. After centrifugation at 20,000 ×*g* for 15 min, the supernatant was diluted with 1× SDS sample buffer. Then 7.5 μg of protein was subjected to SDS-PAGE (12% acrylamide gel). Immunoblot analysis was performed as described above with quantified Bg7S in solution as the standard. Data are shown for the Bg7S amount in 5 g seeds calculated based on the percentage of Bg7S among 7.5 μg of protein subjected to protein gel immunoblot analysis and the amount of total protein in 5 g seeds. Percentages of proteins contained in seeds of soybean cultivars are Peking 41.8%, Tamahomare 40.3%, Enrei 41.1%, and IOM 40.0% (assumed value) based on data from previous reports (22).

Immunoelectron microscopy Samples for immunoelectron microscopy were prepared as described by Motoyama et al. (21) with some modifications. Both dry (untreated) and treated seeds were cut into 1 mm sections and were fixed using 0.1 M sodium phosphate buffer, pH 7.2 containing 4% (w/v) paraformaldehyde and 0.1% (v/v) glutaraldehyde. Tissue sections were washed with 0.1 M sodium phosphate buffer pH 7.2 and were dehydrated with a series of ethanol [10% (v/v), 30%, 50%, 70% × three times, 90%, 99.9% × three times, 10 min for each wash]. Sections were then placed in LR-white (Electron Microscopy Sciences, USA)/ethanol 1:2 (v/v) for 2–6 h, LR-white/ethanol 2:1 (v/v) for 2–6 h, and finally 100% LR-white for 2 d. After polymerization by UV light at 4°C in beam capsules (Nisshin EM Co. Ltd., Japan) for 2 d, sections were subjected to immunoelectron microscopy. Rabbit-derived anti-Bg7S antibody and goat anti-rabbit IgG conjugated to 15 nm gold particles (BBI International, UK) were used in the immunoassay.

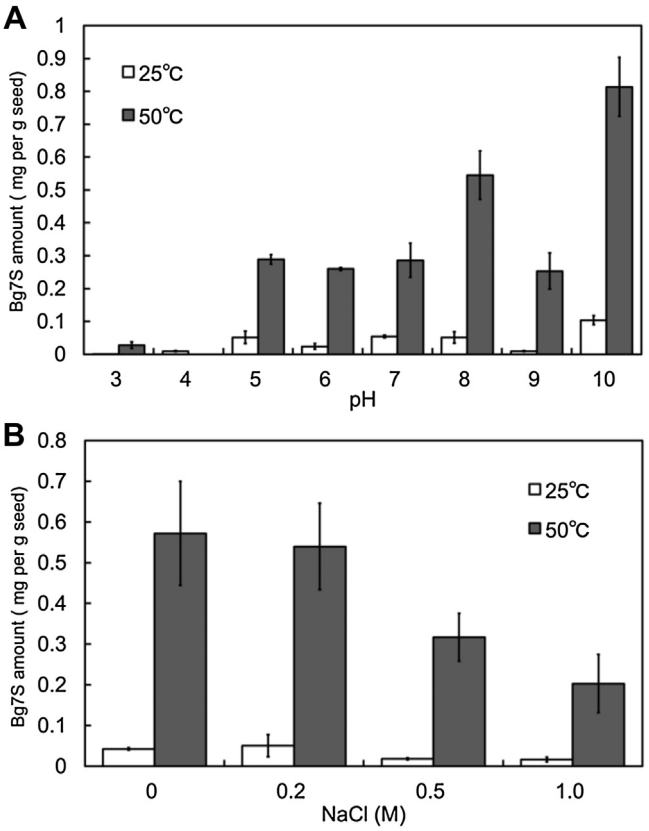

FIG. 1. Amounts of eluted Bg7S in solution under various pH conditions (A) and NaCl concentrations (B). Soybean seeds (5 g) of IOM were treated with various buffer solutions for 8 h at 25°C or 50°C. Amounts of eluted Bg7S per 1 g seed after the treatment were quantified using densitometric analysis. Error bars, SEM ($n = 3$). Used solutions were the following: (A) 0.1 M glycine–HCl buffer (pH 3), 0.1 M sodium acetate buffer (pH 4–5), 0.1 M sodium phosphate buffer (pH 6–8), 0.1 M glycine–NaOH buffer (pH 9–10), (B) 50 mM sodium phosphate buffer (pH 8.0) containing 0, 0.2, 0.5, or 1.0 M NaCl.

Tissue printing Tissue printing was performed as described by Cassab and Varner (23) with some modifications. Nitrocellulose membrane (Whatman, Germany) was treated with 0.2 M $CaCl_2$ for 30 min and dried for 30 min. Dry seeds were cut into halves, washed with distilled water for 2 s, wiped with Kimwipes and blotted on the membrane for 10 s. Seeds stored at −20°C after HWT were also cut, washed with distilled water, frozen with liquid nitrogen, and put onto the membrane for 30 s upon thawing. Immunoassay was performed using anti-Bg7S antibody and anti-Rabbit IgG (Promega Corp., USA) after the membrane was dried.

Cloning and sequencing of Bg7S promoter regions Genomic DNAs of soybean were prepared from young leaves with the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Around 3000 bp of upstream regions of Bg7S1 and Bg7S2 were amplified by PCR. The promoter region of Bg7S1 was amplified with the following primers: forward primer (5′-GGTAAGCTTTTGAAGACTCTTTATCATATATGTTCGG-3′, *Hin*dIII site underlined) and reverse primer (5′-CCTCTAGACATCATTTTGAACGGGTAGAACAA C-3′, *Xba*I site underlined). The promoter region of Bg7S2 was amplified with the following primers: forward primer (5′-TTCTCGAGCAACTTAGCTAGTGTGATTGTGCGG-3′, *Xho*I site underlined) and reverse primer (5′-TTGGATCCTAGTGTGGTGTTGGGGAATGGGGAC-3′, *Bam*HI site underlined). Upstream regions, except for those of Bg7S1 from Enrei and Tamahomare, were cloned into pBluescript SK(−) and sequenced. The Bg7S1 promoter regions of Tamahomare and Enrei were sequenced directly using genomic PCR products as templates.

Purification of Bg7S from dry seeds The purification of Bg7S from dry seeds was performed as described previously with some modifications (19). First, 20 g of powdered seeds were defatted using 200 mL hexane and were washed with distilled water. Then, protein was extracted from defatted soy powder with extraction buffer (0.1 M sodium acetate buffer, pH 5.0, 0.1 M NaCl). After centrifugation, the