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Stable accumulation of seed storage proteins containing vaccine peptides in transgenic soybean seeds

Nobuyuki Maruyama,[1,*] Keigo Fujiwara,[1] Kazunori Yokoyama,[1] Cerrone Cabanos,[1] Hisakazu Hasegawa,[2] Kyoko Takagi,[3,4,§] Keito Nishizawa,[4,§§] Yuriko Uki,[1] Takeshi Kawarabayashi,[5] Mikio Shouji,[5] Masao Ishimoto,[3,4] and Teruhiko Terakawa[2]

Graduate School of Agriculture, Kyoto University, Gokasho, Uji, Kyoto 611-0011, Japan,[1] Hokko Chemical Industry Co., Ltd., Atsugi, Kanagawa 243-0023, Japan,[2] National Institute of Agrobiological Science, Tsukuba, Ibaraki 305-8602, Japan,[3] National Agricultural Research Center for Hokkaido Region, Sapporo, Hokkaido 062-8555, Japan,[4] and Graduate School of Medicine, Hirosaki University, Hirosaki, Aomori 036-8562, Japan[5]

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There has been a significant increase in the use of transgenic plants for the large-scale production of pharmaceuticals and industrial proteins. Here, we report the stable accumulation of seed storage proteins containing disease vaccine peptides in transgenic soybean seeds. To synthesize vaccine peptides in soybean seeds, we used seed storage proteins as a carrier and a soybean breeding line lacking major seed storage proteins as a host. Vaccine peptides were inserted into the flexible disordered regions in the A1aB1b subunit three-dimensional structure. The A1aB1b subunit containing vaccine peptides in the disordered regions were sorted to the protein storage vacuoles where vaccine peptides are partially cleaved by proteases. In contrast, the endoplasmic reticulum (ER)-retention type of the A1aB1b subunit containing vaccine peptides accumulated in compartments that originated from the ER as an intact pro-form. These results indicate that the ER may be an organelle suitable for the stable accumulation of bioactive peptides using seed storage proteins as carriers.

[**Key words:** Seed storage protein; Three dimensional structure; Endoplasmic reticulum; Vacuole; Vaccine]

In recent years, there has been a significant increase in the use of transgenic plants for the large-scale production of valuable proteins, including antibodies, vaccines, other pharmaceuticals, and industrial proteins (1). The use of plants for this purpose offers many advantages because of their low-price and safety compared to mammalian culture cells. Previous studies have demonstrated the ability of plants to produce complex proteins such as secretory antibodies, which are composed of four different polypeptide chains covalently linked by disulfide bonds (2). Furthermore, engineered proteins containing bioactive compounds may also be produced in crops (3,4). Another advantage of using plant seeds is the possibility of storing pharmaceutical proteins or peptides without requiring a temperature-regulated storage room (5). Legumes, particularly soybeans, represent promising host systems for such molecular farming because they produce more proteins compared to other plants.

Soybeans seeds accumulate around 40% protein by dry weight, which is mostly composed of 7S (β-conglycinin) and 11S globulins (glycinin). Glycinin is composed of 5 subunits. These subunits have been classified into 2 groups according to their amino acid sequences (group I: A1aB1b, A2B1a and A1bB2; group II: A3B4 and A5A4B3). The sequence identity is approximately 80% and 45% within and between groups, respectively. The three-dimensional structures of 11S globulins have been solved (6,7). The structure of the monomer is characterized by a core domain consisting of 2 jelly-roll beta-barrels, and 2 extended helix domains. Highly flexible regions (named disordered regions I through V) are interspersed along the monomer.

In seed cells, constituent subunits are synthesized as a single polypeptide precursor, preproglycinin. The signal polypeptide is removed co-translationally in the endoplasmic reticulum (ER) and the resultant proglycinin assembles into trimers. As proglycinin is sorted into protein storage vacuoles, a specific post-translational cleavage occurs between asparagine and glycine residues, resulting in a mature subunit consisting of acidic (approximately 30–35 kDa) and basic (approximately 20 kDa) chains. These chains are linked by a disulfide bond (8). The cleavage then triggers the hexamer formation of the subunits (9).

The prevalence of Alzheimer's disease, which is the most common form of dementia or loss of cognitive ability, has been steadily increasing, and worsens as it progresses and eventually leads to death. One of the leading theories suggests that the deposition of amyloid-β and subsequent formation of amyloid plaques in neuronal cells trigger this neurodegenerative disease. An epitope vaccine, Phe–Arg–His–Asp–Ser–Gly–Tyr (FRHDSGY), has been

* Corresponding author. Tel.: +81 774 38 3763; fax: +81 884 38 3761.
E-mail address: marunobu@kais.kyoto-u.ac.jp (N. Maruyama).
§ Present address: National Agriculture and Food Research Organization Agricultural Research Center, Tsukuba, Ibaraki 305-8666, Japan.
§§ Present address: NARO Institute of Crop Science, 2-1-18 Kannondai, Tsukuba, Ibaraki 305-8518, Japan.

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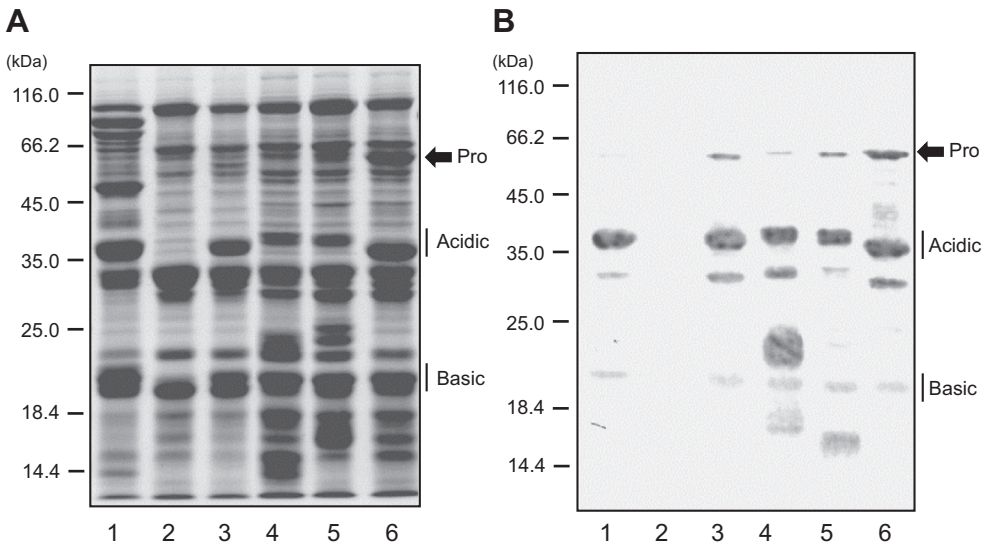

FIG. 1. SDS-PAGE and western blotting of total proteins in the control and transgenic soybean seeds accumulating A1aB1b containing vaccine peptides. (A) SDS-PAGE analysis of total protein from transgenic soybean seeds accumulating the wild type or the mutants. (B) Soybean seed extracts were analyzed by western blotting against the anti-A1aB1b antibody. Lane 1, Jack; 2, JQ; 3, A1aB1bWT; 4, A1aB1bM2; 5, A1aB1bM3; 6, A1aB1bM4. Pro, Acidic, and Basic indicate the pro-forms, acidic chain and basic chain of A1aB1b, respectively.

developed by McLaurin and others (10) against the amyloid protein and has been shown to have therapeutic effects on transgenic mice with Alzheimer's disease. In this study, we aimed to develop a transgenic soybean that accumulates vaccine peptides using Alzheimer's disease vaccine peptides as a model peptide and A1aB1b, the glycinin subunit, as its carrier. Vaccine peptide sequences were inserted into the disordered regions of A1aB1b and their accumulation in transgenic seeds was examined. We also investigated the accumulation of an ER-retention type of A1aB1b containing vaccine peptides.

MATERIALS AND METHODS

Construction and introduction of transgenes into soybean The cDNA for A1aB1b containing vaccine sequences was prepared following the procedure described by Nishizawa et al. (11) and Prak and Utsumi (12). The seed specific glycinin promoter was used for all constructs. Three mutants were structured; specifically, A1aB1bM2, A1aB1bM3, and A1aB1bM4, which contained an FRHDSGY insertion in disordered regions II, III, and IV, respectively. The vaccine peptide was not introduced into region I because of the need to retain the correct cleavage of the A1aB1b signal peptide. Moreover, the wild-type A1aB1b cassette was constructed as the control (A1aB1bWT). The ER retention types of A1aB1bWT and A1aB1bM3 were constructed by adding the ER-retention sequence (His–Asp–Glu–Leu) at the C-terminus (A1aB1bWT-HDEL and A1aB1bM3-HDEL). The resulting plasmids were then transformed into soybean immature embryos using the whisker-supersonic method (13) or the bombardment method (14).

Antibodies Antibodies against A1aB1b, vaccine peptides (antibodies against FRHDSGY peptide), and BiP derived from rabbit were used. Anti-rabbit IgG conjugated with alkaline phosphatase was used as the secondary antibody.

Extraction of total protein Portions of mature dry seeds were scraped with a scalpel and crushed using a Multi-beads Shocker (MB501S, Yasui Kikai, Osaka, Japan). The soybean powder was then defatted in hexane for 10 min and centrifuged at 3000 $\times g$ for 5 min. Defatting was performed 4 times and was followed by air-drying. Twenty microliter of 1× SDS buffer [62.5 mM Tris–HCl, pH 6.8, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2.5% (w/v) SDS] was then added to 1 mg seed powder and vortexed for at least 1 h at room temperature. The mixture was centrifuged at a minimum speed of 12,000 $\times g$ for 15 min at 4°C, and the supernatant was transferred into a new tube.

Extraction of soluble and insoluble fractions Defatted seed powder (100 mg) was added to buffer A [35 mM sodium phosphate, pH 7.4, 0.4 M NaCl, 1 mM EDTA, 0.1 mM (*p*-amidinophenyl)-methanesulfonyl fluoride, 1.2 μM leupeptin, 0.2 μM pepstatin A, 0.02% (w/v) NaN_3]. The mixture was vortexed and mixed with constant stirring at room temperature for 1 h and then centrifuged at a minimum speed of 12,000 $\times g$ for 15 min at 4°C. Then, the supernatant was transferred into a new tube as the soluble fraction. The insoluble fraction was obtained by adding 1× SDS buffer to the remaining pellet, followed by vortexing, mixing and centrifugation.

SDS-PAGE and western blotting SDS-PAGE was performed using 11% polyacrylamide gel. Proteins were stained with Coomassie Brilliant Blue R-250. Western blotting was performed after SDS-PAGE using 11% polyacrylamide gel according to the procedure described by Laemmli (15). The separated proteins on gels were electrophoretically transferred to a nitrocellulose membrane (0.45 μm; Schleicher and Schuell Inc., Dassel, Germany), and recombinant proteins were detected with appropriate rabbit-derived anti-sera followed by goat anti-rabbit IgG–alkaline phosphatase conjugate (Promega, Madison, WI, USA).

Gel-filtration chromatography The soluble protein fraction was applied on to a Hi-Prep 16/60 Sephacryl S-300 HR column (GE Healthcare Life Sciences). The flow rate was set to 0.5 ml/min using buffer A. To check for purity, the fractions were analyzed by SDS-PAGE using 11% polyacrylamide gels. Protein concentration was determined using a Protein Assay Rapid Kit (Wako, Osaka, Japan) with bovine serum albumin as the standard.

Protein analysis Protein fractions containing A1aB1bWT or A1aB1b-vaccine peptides were initially concentrated using a trichloroacetic acid precipitation and quantified using the 2-D Quant kit (GE Healthcare). Proteins were then blotted on a PVDF membrane and subjected to N-terminal amino acid sequencing using a Procise 492 Protein Sequencer (Applied Biosystems).

A1aB1bM3 fragments were analyzed by mass spectrometry. The bands corresponding to A1aB1bM3 acidic chains on SDS-PAGE were digested by trypsin or V8 protease. MALDI TOF MS data was collected by using the AXIMA Performance (Shimadzu Corporation, Kyoto, Japan).

Transmission electron microscopy Dry soybean seeds were cut into 1.0 mm sections and fixed for 2 h in 4% (v/v) formaldehyde, and 0.05% (v/v) glutaraldehyde solution at 4°C. Tissue sections were washed 4 times with buffer (100 mM sodium phosphate, pH 7.2), dehydrated in a graded ethanol series and embedded in LR White resin (London Resin, Basingstoke, UK). Ultrathin sections (70 nm) were cut with a glass knife and placed on formvar/carbon-coated grids. The sections were blocked with 1% (w/v) BSA–PBS and then incubated for 1 h at room temperature with anti-A1aB1b, anti-vaccine peptide, or anti-BiP antibody in 1% (w/v) BSA–PBS. The sections were washed with 1% (w/v) BSA–PBS and then incubated with goat anti-rabbit IgG conjugated to 15 nm gold particles (H + L, Auro Probe EM, Amersham) in 1% (w/v) BSA–PBS at room temperature. After washing with PBS, the sections were washed twice with distilled water, stained for 5 min with 4% (w/v) uranyl acetate, and incubated with 80 mM lead nitrate for 5 min. The grids were examined and photographed using a transmission electron microscope (model H-7100, Hitachi, Tokyo).

RESULTS

Carrier protein accumulation in the soybean line lacking major seed storage proteins In this study, we used a soybean breeding line, JQ, which lacked both glycinin and β-conglycinin, and had high transformation efficiency (16). The JQ line is obtained by crossing the soybean breeding line, QF2, which lacks glycinin and β-conglycinin, with Jack, which is a highly competent variety for