Original Paper Plant Biotechnology, **16** (3), 219-224 (1999)

Immunodetection and Characterization of Allergenic Proteins in Common Buckwheat (*Fagopyrum esculentum*)*

Arun NAIR and Taiji ADACHI

Applied Genetics & Biotechnology Division, Faculty of Agriculture, Miyazaki University, Gakuen Kibanadai Nishi 1-1, Miyazaki 889-2192, Japan

Received 18 September 1998; accepted 25 February 1999

Abstract

Allergenic proteins of molecular mass 22 kDa, 36 kDa, 39-40 kDa and 70-72 kDa were identified from water-soluble fractions of common buckwheat cv. "Miyazaki-zairai". The identification was based on the reactivity of the protein with IgE antibodies from patients' allergic to buckwheat. The allergenic proteins fall within the β and γ subunits of the globulin fraction indicating that the allergenic reactions are contributed by the major storage protein in buckwheat. The 22 kDa protein was the most consistent with IgE activity. N-terminal amino acid sequence of 22 kDa protein showed maximum homology with the seed globulins from both dicot and monocot species. Based on the N-terminal amino acid sequence, two degenerate primers were selected to amplify the cDNA encoding the major allergenic protein. A single PCR product obtained was subsequently sequenced. The deduced amino acid sequence was identical in its N-terminal end. Homology search revealed 94 and 93 % homology to two buckwheat legumin like proteins.

1. Introduction

Buckwheat (*Fagopyrum* sp.) is a good source of dietary protein and is rich in essential amino acids such as lysine, threonine and tryptophan. Buckwheat is widely cultivated in Japan. However, Japan imports nearly 80% of her requirement from other countries, viz. China, Canada and the US. Buckwheat is consumed mainly in the form of noodles and dumplings. The seeds contain 15-17% proteins. The high nutritive value of the grains and presence of high content of rutin in foliage makes buckwheat important for human consumption and undoubtedly, is a very important crop in the Japanese diet. Buckwheat related hypersensitivity (allergy), though not very common, is a serious problem among infants as well as adults. It causes serious anaphylatic reactions on ingestion. IgE which is the 200 kDa monomer has been implicated as the antibody involved in mediating the immediate hypersensitivity reactions. IgE-mediated immediate types of allergies are a serious problem for patients with symptoms such as bronchial asthma, perennial rhinitis and dermatitis. It is therefore essential to develop and or select buckwheat lines without allergenic components.

Limited reports are available on the isolation and characterization of allergenic proteins. Buckwheat proteins of molecular mass 100 kDa, 50 kDa and 17 kDa (Yanagihara, 1980), 8-9 kDa (Yano *et al.*, 1989), and 24 kDa (Urisu *et al.*, 1994) have been reported as allergenic proteins with strong IgE binding activity. In this paper, we report the isolation and characterization of the major allergenic protein, the amino acid sequence at the N-terminus and sequencing of an allergen specific cDNA.

2. Materials and Methods

2.1 Electrophoresis

Common buckwheat cv. "Miyazaki-zairai" was field grown at Miyazaki and the seeds were harvested. Bulk seeds (100 seeds/sample) were finely ground using a pestle and mortar. Water soluble proteins were extracted under reducing conditions with 0.125M HCl, 10% SDS (sodium dodecyl sulphate), 1% 2-mercaptoethanol, 30% glycerol and 0.2% bromophenol blue (sample buffer). Ten micro liters of protein extract was used for electrophoresis. SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) was carried out in a discontinuous system as described by Laemlli, (1970). The proteins were separated on 10% acrylamide and 0.1% SDS for about 3h at constant

* Contribution no. 118 from the Laboratory of Plant Breeding, Faculty of Agriculture, Miyazaki University.

current of 25 mA and stained with Coomassie blue.

2.2 Immunoblotting

After SDS-PAGE, the proteins were electroblotted on to polyvinyl difluoride (PVDF) membrane (BIO-RAD) in a transblot system (BIO-RAD) using pre-chilled Towbin buffer (25mM Tris, 192mM glycine, 20% methanol, pH 8.3). Transfer was performed at constant voltage of 60V for 5h at 5 ℃. The electroblotted membrane was blocked in 0.1M tris-buffered saline (TBS) containing 50% glycerol, 1% human serum albumin (HSA, Sigma, USA) and 1% Tween-20 for 1h. The membrane was further incubated with buckwheat allergic patient's serum (kindly provided by Dr. Urisu, Fujita health centre, Japan) diluted 1:1 in TBS + 1% Tween for 16h at room temperature. The blots were washed in TTBS (TBS + 0.5% Tween) and incubated for additional 12h with alkaline phosphatase conjugated monoclonal anti-human IgE (Kir Kegaard & Perry laboratories Inc.) and developed with alkaline phosphatase conjugate substrate kit (BIO-RAD).

2.3 Electroelution, N-terminal amino acid sequence determination and homology search

After identification as the major allergen, the 22 kDa protein band (**Fig. 1-A**) was excised and electrophoretically extracted with Maxyield NP (Atto, Japan) at 7 ℃. The protein was then dialyzed against distilled water for 8h in Desalyser-I (Atto, Japan) followed by precipitation with 4 volumes of

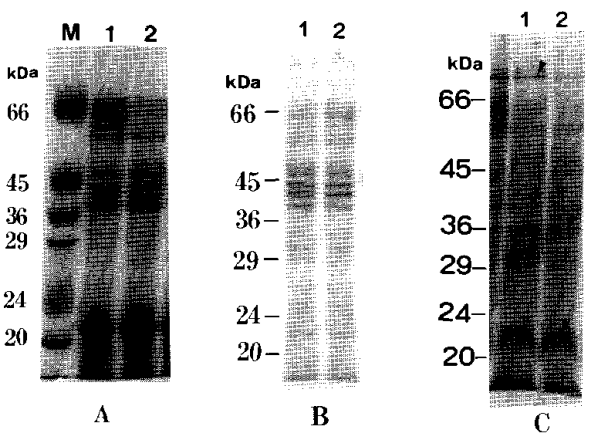

Fig. 1 Immunoblot analysis of buckwheat proteins. (A) SDS-PAGE analysis of water soluble proteins (lanes 1, 2), M- molecular markers. (B) Protein profile after transblotting on to PVDF membrane (lanes 1,2), (C) Immunoblot analysis of buckwheat proteins with IgE binding activity (lanes 1,2). The arrow heads indicate the proteins with maximum IgE binding activity. The standard markers (in kDa) are shown on the left.

cold acetone (−20 ℃) overnight and centrifuged at 10,000 rpm for 30min. The pellet was dissolved in sample buffer. Electrophoresis and blotting was performed as described earlier. After staining the membrane with Coomassie blue, the band was excised and subjected to N-terminal micro-sequencing on an automated Hewlett Packard HP 241 N&C protein sequencer. Homology search was done using the BLASTP, SWISS-PROT system for protein sequence alignment and TBLASTN, GENBANK-NONST for gene sequence alignment.

2.4 Isolation of mRNAs and RT-PCR

Poly $(A)^+$ RNA from 10-15d old maturing buckwheat seeds were isolated with Quick prep micro mRNA purification kit (Pharmacia Biotech). Single stranded cDNAs were synthesized with the First-Strand cDNA synthesis kit (Pharmacia Biotech). Based on the N-terminal amino acid sequence of the 22 kDa buckwheat allergenic protein, two degenerate primers: BWA1 (5′-GGA TTG GAG CAA/G GCT/G TTC/T AGC) and BWA2 (5′-CTG AAA TTC AGA/G CAA/G AAC/T GTT) were synthesized (Life Technologies)

PCR amplification (PCR1) was carried out in a final reaction volume of 25 μl containing 0.5 μl of cDNA solution, 50 pmol of primer BWA1, 10 pmol of primer Not I (5′-TGG AAG AAT TCG CGC CCG CAG) for the reverse direction, 0.2 mM each dNTPs, 1x PCR buffer, 1.5mM $MgCl_2$, and one unit Taq DNA polymerase (Takara). Amplification parameters were 94℃ for 5 min, followed by 35 cycles with 94 ℃ for 45 s, 60 ℃ for 1 min, and 72 ℃ for 1 min 30 s. A 72 ℃ incubation for 10 min as a final extension step was included.

A second PCR (nested) was carried out using 1 μl of the first PCR product as the template. The final reaction volume of 25 μ l consisted of 50 pmol primer BWA2 and 10 pmol of primer Not I. Buffer, dNTPs and Taq DNA polymerase were similar to PCR 1. Amplification parameters were 94 ℃ for 5 min, followed by 10 cycles with 94 ℃ for 30 s, 52 ℃ for 45 s and 72 ℃ for 1 min. This was followed by 25 cycles with 94 ℃ for 30 s, 60 ℃ for 45 s and 72 ℃ for 1 min. A 72 ℃ incubation for 10 min as a final extension step was included. Both PCR reactions were carried out in a Takara PCR thermal cycler MP (TP 3000).

2.5 Cloning and sequencing of PCR products

The PCR product was directly ligated and transformed using Original TA cloning kit (Invitrogen). One microliter of the fresh PCR product was ligated into pCR 2.1 vector and transformed as per manufacturer's instructions. Cells were plated and grown overnight on ampicillin, blue/white selection

dishes at 37 ℃ . White colonies were selected and the plasmid DNA was isolated through alkali SDS-PEG digestion. Positive clones carrying a plasmid with an insert size of approximately 750 base pairs (bp) were selected for sequencing procedures. DNA sequence analysis of the selected plasmids was performed on a Shimadzu DSQ-2000L DNA sequencer using thermosequanase fluorescent labeled primer cycle sequencing kit. Homology search was carried out using EMBL, GenBank database.

3. Results

The protein accumulation in seeds of common buckwheat was found to start from 10 days after pollination (DAP). Up to 15 DAP, the major band was that of 69 kDa protein. The seeds on maturing showed a shift in banding pattern with high intensity bands at 56-57 kDa, 32-34 kDa and 22 kDa (data not shown). Immunoblotting revealed as many as seven proteins with IgE binding activity. The maximum IgE binding activity was detected with protein bands of 22 kDa, 36 kDa, 39-40 kDa and 70-72 kDa (**Fig. 1-C**). As the maximum and most consistent response was from the 22 kDa protein band, this band was electroeluted and subjected to N-terminal microsequencing. The extracted 22 kDa protein on SDS-PAGE gave a single band (**Fig. 2**).

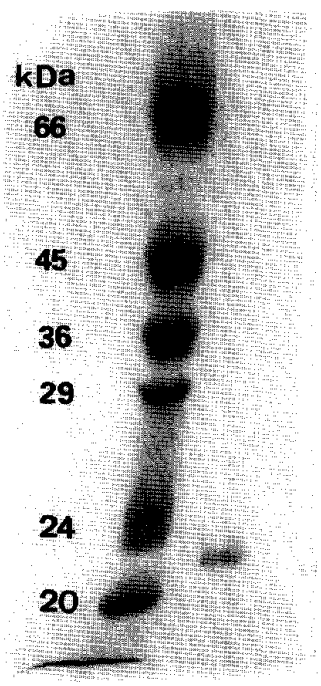

Fig. 2 Isolated 22kDa protein showing a single band after SDS-PAGE.

The N-terminal amino acid sequence of the 22 kDa protein had significant similarity to BW24KD reported by Urisu *et al.*, 1994. Homology search of protein database with BLASTP (Altschul *et al.*, 1990) and SWISS-PROT showed maximum alignment with storage proteins of both dicot and monocot species. Buckwheat allergenic protein showed 60-75% identity in the alignment with 11S globulins from rice, pea, oats, broad bean, sunflower and soybean (**Table 1**). The sequence also showed similarity with proteins from leguminous and non-leguminous plants whose seed meal contains sulphur rich amino acids.

The yield of mRNA from 10-15d old maturing buckwheat seeds varied from 0.3 μg to 0.5 μg per 100 mg of tissue. Single stranded cDNA was synthesized with Not I-$d(T)_{18}$ primer and pd $(N)_6$ (Random hexa deoxy nucleotides) primer. However, for PCR reactions, cDNA synthesized with Not I-$d(T)_{18}$ primer was used. The PCR1 reaction with BWA1 and Not I produced two products approximately 750 bp (**Fig. 3-A**). The second PCR in a nested fashion resulted in one single 750 bp product. (**Fig. 3-B**). Fresh PCR product was directly ligated into pCR 2.1 vector and transformed in *E. coli.* Clones containing an insert of approximately 750 bp were identified and selected for DNA sequencing.

The DNA sequence of the cloned PCR product had the primer sequence (BWA2) and the poly A tail intact. Prediction of amino acid sequence from

Table 1. N-terminal amino acid sequence of the buckwheat allergen (22kDa protein) and its homology.

Crop	Amino acid sequence	Percentage identity
Buckwheat (22kDa)	**GLEQAFSNLKFRQNVNRPSR**	
Cotton (legumin)	**GLEETFCSMRIKENLADPER**	75.0
Rice (glutelin)	**GLDETFCTLRVRQNIDNPNR**	75.0
Sunflower (11S globulin)	**GVEETICSMKFKVNIDNPSQ**	75.0
Garden pea (legumin B)	**GLEETICSAKIRENIARPSR**	70.0
Oat (12S globulin)	**GLEENFCSLEARQNIENPKR**	70.0
Soybean (glycinin)	**GVEENICTLKLHENIARPSR**	65.0
Pumpkin (11S globulin)	**GLEETICTLRLKQNIGRSVR**	60.0

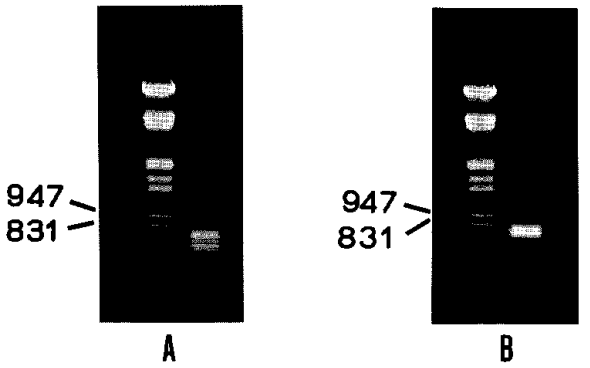

Fig. 3 RT-PCR amplification products of the cDNA of buckwheat. A. PCR products with primers BWA1 and Not I, B. A single PCR product after nested PCR. DNA fragments of molecular marker (lambda DNA/*Eco* R1 + *Hind* III marker) is indicated in base pairs.

```
CTGAAATTCAGGCAAAATGTTAACAGGCCTTCTCGCGCCGACGTCTTCAACCCACGCGCC  60
 L  K  F  R  Q  N  V  N  R  P  S  R  A  D  V  F  N  P  R  A

GGTCGTATCAACACCGTAGACAGCAACAATCTCCCGATCCTCGAATTCATCCAACTTAGC 120
 G  R  I  N  T  V  D  S  N  N  L  P  I  L  E  F  I  Q  L  S

GCCCAGCACGTCGTCCTCTACAAGAATGCGATCCTCGGACCGAGATGGAACTTGAACGCG 180
 A  Q  H  V  V  L  Y  K  N  A  I  L  G  P  R  W  N  L  N  A

CACAGCGCACTGTACGTGACGAGAGGAGAAGGAAGAGTCCAGGTTGTCGGAGATGAAGGA 240
 H  S  A  L  Y  V  T  R  G  E  G  R  V  Q  V  V  G  D  E  G

AGAAGTGTGTTCGACGACAACGTGCAGCGAGGACAGATCCTTGTGGTCCCACAGGGATTC 300
 R  S  V  F  D  D  N  V  Q  R  G  Q  I  L  V  V  P  Q  G  F

GCAGTGGTGTTGAAGGCAGGAAGAGAAGGACTGGAGTGGGTGGAGTTGAAGAACGACGAC 360
 A  V  V  L  K  A  G  R  E  G  L  E  W  V  E  L  K  N  D  D

AACGCCATAACCAGCCCGATTGCCGGGAAGACTTCGGTGTTGAGGGCGATCCCTGTGGAG 420
 N  A  I  T  S  P  I  A  G  K  T  S  V  L  R  A  I  P  V  E

GTTCTTGCCAACTCCTACGATATCTCGACGAAGGAAGCGTTCAGATTGAAGAATGGGAGG 480
 V  L  A  N  S  Y  D  I  S  T  K  E  A  F  R  L  K  N  G  R

CAGGAGGTTGAGGTCTTCCGACCATTCCAGTCCCGAGATGAGAAGGAGAGGGAGCGTTTC 540
 Q  E  V  E  V  F  R  P  F  Q  S  R  D  E  K  E  R  E  R  F

TCCATAGTTTAAGAGAGACAAAGGGTCTATCGTATGCAAAATAAAACAGAAGGAGAAGGA 600
 S  I  V  *

TAAGGGAGTCTTGTCTATCGTTTAGCTAGTCAAGTCCCTCTTCCACTTCTGGGTTATGTT 660

CTATGTTTTTACTTTAGTGTCAATAAAAGAGTGAATTCTCGAAAAAAAAAAAAAAAAAAA 720
```

Fig. 4. Nucleotide and deduced amino acid sequences of the isolated cDNA encoding buckwheat 22 kDa allergenic protein. The sequence identical to the known N-terminal sequence of the allergenic protein is boxed. The AATAAA polyadenylation signals are underlined. The stop codon is indicated with an asterisk.

the DNA sequence data and the protein sequence achieved earlier by N-terminal microsequencing

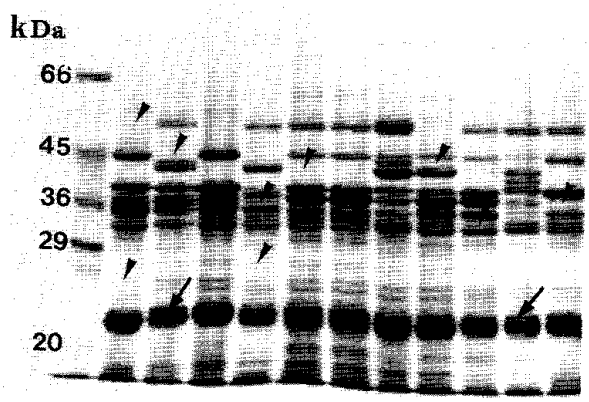

Fig. 5 SDS-PAGE electrophoretogram of seed proteins showing high polymorphism of common buckwheat cv. "Miyazaki-zairai". The arrow heads indicate the absence of respective bands. Consistent 22 kDa protein is visible in all samples (arrows). The standard markers (in kDa) are shown on the left.

showed an overlap of 12 amino acids with all of them being identical (**Fig. 4**). Homology search with BLAST database revealed 94 and 93% homology to two buckwheat mRNAs for legumin like proteins.

Mature single seeds (~600) of common buckwheat were analyzed in order to detect natural mutants for 22 kDa protein. Although the single seed analysis showed very high polymorphism for most of the storage proteins, we were not able to find any natural mutants for allergenic proteins among the seeds tested (**Fig. 5**).

4. Discussion

A high number of protein bands (~11) with IgE activity was reported by Urisu *et al.*, 1994 amongst buckwheat seed proteins. The N-terminal sequence of the 22 kDa protein differed only in three amino acids to that of BW24KD earlier reported by Urisu *et al.*, 1994. This may be due to slightly different isoforms of the allergens from different sources or could also be variety dependent. Partial characterization of 280 kDa globulin, the major storage protein from common buckwheat separated into α, β and γ groups with molecular mass of 55-60 kDa, 32-44 kDa and 16-29 kDa on electrophoresis under reducing conditions (Rout and Chrungoo, 1996). Our candidate proteins, 22 kDa, 36 kDa, 39-40 kDa also fall within the β and γ subunits of the globulin fraction indicating that the allergic reactions are contributed by the major storage protein in buckwheat. Albumins and globulins from rice (Nakamura and Matsuda, 1996), globulins from soybean and other legumes (Lalles and Peltre, 1996) are

known to induce allergy when consumed by humans. The 22 kDa protein of buckwheat also share common characteristics such as the presence of sulphur rich amino acids and being part of the globulin fraction.

The mRNA yield was sufficient enough to proceed with PCR reactions. Nested PCR confirmed that the products are specific to the 22 kDa, the major allergenic protein. The nucleotide sequence covering the open reading frame and the deduced amino acid sequence are shown in **Fig. 4**. The putative nucleotide sequence length from the N-terminus covers 573 nucleotides that encode 191 amino acids. The calculated molecular mass of 21474 correlates well with the apparent molecular mass of allergenic protein (22000) estimated from SDS-PAGE.

A search of the EMBL, GenBank nucleotide and protein sequence database revealed sequence similarities with legumin like protein from buckwheat and other storage proteins from rice, peas and soybeans. Seed storage proteins from these crops are known to cause allergy in human beings (Burks and Brooks, 1988). The results suggest that there must be a precursor protein synthesized at an early stage during seed development in buckwheat as in pumpkin (Inoue *et al.*, 1995). This high molecular precursor must be post-translationally processed to generate the 22 kDa polypeptide.

Our methodology and immunoblotting results are in consensus with previous reports that the most identified food allergens are water soluble glycoproteins that have molecular masses between 18 and 70 kDa (Lemke and Taylor, 1994, Lehrer *et al.*, 1996). The molecular mass of 10 kDa probably represents the lower limit for the immunogenic response. The upper limit is probably the result of restricted mucosal absorption of large molecules (Franck-Oberaspach and Keller, 1997). The PCR strategy coupled with 5′RACE (Rapid Amplification of cDNA Ends) should provide the complete cDNA sequence as in apple (Schoning *et al.*, 1996). We have used the known cDNA sequence of the 22 kDa protein to design primers and are proceeding with RACE reactions. Further, we have developed a self pollinating buckwheat (Woo and Adachi, 1997) which overcomes most of the problems associated with breeding barriers in buckwheat. We hope to ultimately use this material to develop hypoallergenic buckwheat based on an antisense strategy. The data presented in this paper provides the basic information towards this goal.

Acknowledgements

The authors thank Dr. Urisu (Fujita Health University, Aichi, Japan) for kindly providing the buckwheat allergic patients' serum and encouragement. The authors also thank Prof. Franz Hoffmann (University of California, Irvine) for the critical review of the manuscript and valuable suggestions.

References

Altschul, S. F., Gish, W., Miller, W., Myers, W., Lipman, D. J., 1990. Basic local alignment search tool. J. Mol. Biol., **215**: 403-410.

Burks, A.W., Brooks, J.R., 1988. Allergenicity of major component proteins of soybean determined by enzyme-linked immunosorbent assay (ELISA) and immunoblotting in children with atopic dermatitis and positive soy challenge. J. Allergy Clin. Immunol., **81**: 1135-1142.

Franck-Oberaspach, S. L., Keller, B., 1997. Consequences of classical and biotechnological resistance breeding for food toxicology and allergenicity. Plant Breed., **116**: 1-17.

Inoue, K., Motozaki, A., Takeuchi, Y., Nishimura, M., Hara-Nishimura, I., 1995. Molecular characterization of proteins in protein-body membrane that disappear most rapidly during transformation of protein bodies into vacuoles. Plant J., **7**: 235-243.

Laemlli, U. K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature, **227**: 680-685.

Lalles, J. P., Peltre, G., 1996. Biochemical features of grain legume allergens in humans and animals. Nutr. Rev. **54**: 101-107.

Lehrer, B. S., Horner, W. E., Reese, G., 1996. Why are some proteins allergenic? Implications on biotechnology. Crit. Rev. Food Sci. Nutr., **36**: 553-564.

Lemke, P. A., Taylor, S. L., 1994. Allergic reactions and food intolerances. In :Kotsonis, F. N., *et al.* (Eds.) :Nutritional Toxicology, 117-137. Raven Press, New York.

Nakamura, R., Matsuda, T., 1996. Rice allergenic protein and molecular-genetic approach for hypoallergenic rice. Biosci. Biotech. Biochem., **60**: 1215-1221.

Rout, M. K., Chrungoo, N. K., 1996. Partial characterization of the lysine rich 280KD globulin from common buckwheat (*Fagopyrum esculentum* Moench): Its antigenic homology with seed proteins of some other crops. Biochem. Mol. Biol. Int., **40**: 587-595.

Schoning, B., Ziegler, H. G., Veiths, S., Baltes, W., 1996. Apple allergy: The cDNA sequence of the major allergen of apple, determined by performing PCR with a primer based on N-terminal amino acid sequence, is highly homologous to the sequence of the major birch pollen allergen. J. Sci. Food Agric., **71**: 475-482.

Urisu, A., Kondo, Y., Morita, Y., Wada, E., Tsuruta, M., Yasaki, T., Yamada, K., Kuzuya, H., Suzuki, M.,

Titani, K., Kurosawa, K., 1994. Identification of a major allergen of buckwheat seeds by immunoblotting methods. Allerg. Clin. Immunol., **6**: 151-155.

Woo, S. H., Adachi, T., 1997. Production of interspecific hybrids between *Fagopyrum esculentum* and *F. homotropicum* through embryo rescue. SABRAO J., **29**: 89-95.

Yanagihara, Y., 1980. Buckwheat hypersensitivity. Kansensho, **10**: 184-188.(in Japanese).

Yano, M., Nakamura, R., Hayakawa, S., Torii, S., 1989. Purification and properties of allergenic proteins in buckwheat seed. Agric. Biol.Chem., **53**: 2387-2392.