

A molecular-genetic approach for hypoallergenic buckwheat

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Received June 14, 1999; accepted in revised form August 23, 1999

Key words: *Agrobacterium*, allergenic proteins, DNA sequencing, 5' RACE

ABSTRACT

The major allergenic protein with a molecular mass of 22 kDa was identified based on the reactivity with IgE antibodies from patients' allergic to buckwheat. Two degenerate primers were synthesized from the N-terminal amino acid sequence of the 22 kDa protein. The primers were used to amplify the cDNA encoding the protein. The missing 5' end of the cDNA sequence was obtained by a 5' RACE method. The nucleotide sequence of the major allergenic protein had 94 and 93% homology to two buckwheat mRNAs that code for legumin-like proteins. A protein data base search revealed homology to rice, soybean and legume storage proteins that are known to cause allergy in humans. In order to develop self-pollinating type buckwheat, interspecific hybrids between common buckwheat (*Fagopyrum esculentum*) and a wild relative (*Fagopyrum homotropicum*) were produced by cross pollination and embryo rescue which will allow stable transfer of desired genes. To introgress gene(s) from the wild species a back cross program is in progress. The nucleotide sequence information can be effectively used to develop hypoallergenic buckwheat. Towards this, a protocol for regeneration and genetic transformation of buckwheat using *Agrobacterium tumefaciens* was established.

INTRODUCTION

Buckwheat (*Fagopyrum esculentum*) is an important constituent of the Japanese diet. Commonly known as "soba" in Japanese, it is consumed mainly in the form of noodles and dumplings. The seed contains 15–17% protein and is rich in essential amino acids, such as lysine, methionine and tryptophan. Buckwheat is also produced in many other countries where it is exported or consumed locally. Several reports on buckwheat related hypersensitivity (allergy) are available and are well documented in the review by Wieslander (1996).

The allergy on ingestion is caused by the storage proteins in the grains (Yanagihara, 1980; Yano et al., 1989; Urisu et al, 1994; Nair and Adachi, 1999b). The management of food allergy gives only few options and the best choice is to avoid food that causes the allergy. IgE mediated type I allergy, as in buckwheat, requires only a very low amount of the antigen (as low as 1–2 mg) to produce the allergy. Hence, strict avoidance of the offending food may not be practical. The other management alternative is pharmacotherapy. Pharmacotherapy is purely symptomatic and does not offer any means to cure the food allergy. It has no preventive effect and is not specific for food allergies (Nekam, 1998). Extensive information is required on the specific epitopes and cross reactivity of

buckwheat allergens to administer pharmacotherapy or immunotherapy. This is limiting at this stage.

In this report we describe a strategy to develop hypoallergenic buckwheat by combining conventional breeding as well as molecular genetics.

MATERIALS AND METHODS

Electrophoresis, immunoblotting and N-terminal amino acid sequence determination

Common buckwheat cv. "Miyazaki zairai" was field grown at Miyazaki and seeds were harvested. Sampling, Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE), Immunoblotting using the allergic patient's serum and N-terminal microsequencing of the isolated allergenic protein were carried out as described previously (Nair and Adachi, 1999b).

Isolation of mRNAs, RT-PCR and sequencing

Poly (A)⁺RNA from 10–15 days old maturing buckwheat seeds were isolated with Quick prep micro mRNA purification kit (Pharmacia Biotech). Single stranded cDNAs were synthesized using *Not* I dT₍₁₈₎ primer. RT-PCR was carried out in a nested fashion using two degenerate primers and *Not* I primer. The PCR product was directly ligated and transformed (for details, refer Nair and Adachi,

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1999b). The DNA sequence analysis of the selected plasmids was performed on a Shimadzu DSQ-2000L DNA sequencer using thermosequansase fluorescent labeled primer cycle sequencing kit.

5' RACE

Rapid amplification of cDNA ends (RACE) was used for the amplification of the unknown 5' end of the mRNA of the 22 kDa allergenic protein. The amplification was carried out using Takara 5' full RACE core set. The 1st strand cDNA synthesis was carried out using a RT primer phosphorylated at the 5' end (pGCTTCCTCGTCGAGA). The single stranded cDNA was circularized by ligation using T4 RNA ligase. Such circularized cDNAs were subjected to PCR. The technique allows approximately 350 bp extension for each reaction. To get the full length of cDNA, amplification was carried out in steps with different primer sets (primer walking) followed by sequencing.

The sense primers were

BWA6-5'-CAGGTTGTCGGAGATGAAGGA

BWA9-5'-ATTCGCAGTGGTGTGAAGGC

The anti-sense primers were

BWA10-5'-AGGATCGGGAGATTGTTGCTG

BWA11-5'-GCTGTCTACGGTGTGATACG

BWA12-5'-AGCTTGCTGATGGTCTCCTGG

BWA13-5'-ATCTCGTCCTCGAATCCACTC

BWA14-5'-ATTCTTCGCTCTCACTCTGCC

BWA15-5'-AATCTTCTGGTGTGCTGGTCTCC

Production of interspecific hybrids

Self-incompatible short-styled (thrum) plants of common buckwheat cv. "Miyazaki zairai" were crossed with the wild annual species, *F. homotropicum*.

The embryos were rescued at various time intervals after pollination on suitable Murashige and Skoog culture media as described previously (Woo and Adachi, 1997a).

Plant material and culture for transformation

Young seeds of buckwheat, 7–10 days after pollination (DAP) were excised and surface sterilized with ethanol for 1 min. and 2% sodium hypochlorite for 5 min followed by four rinses in sterile distilled water. The testa and endosperm were removed using a binocular microscope and the young embryo was placed on appropriate media. The explants were grown on Murashige and Skoog (MS) basal nutrient medium (Murashige and Skoog, 1962) with 3% sucrose and supplemented with auxin (IAA) and cytokinin (BAP and Kinetin) at different concentrations and combinations (Table 1). The medium was also supplemented with ascorbic acid (100 mg/l) and casein hydrolysate (CH) 1 g/l. The pH was adjusted to 5.8 prior to autoclaving. Gellan gum at 0.2% was used to solidify the medium.

Bacterial strain and vector

The hypervirulent *A. tumefaciens* strain EHA 101 carrying the binary plasmid pBI 101-Hm was used as vector system (Fig. 1). The T-DNA contains both neomycin phosphotransferase II (NPT II) gene for kanamycin resistance and hygromycin phosphotransferase gene (HPT II) for hygromycin B resistance. The NPT II gene is driven by nopaline synthase (Nos) promoter and terminator sequences. The HPT II gene is driven by the cauliflower mosaic virus CaMV 35S promoter and Nos terminator sequences. The *β-galactourousidase* (GUS) gene with an intron, driven

Table 1. Media for culture of buckwheat tissues.

Medium	Composition
MS1	MS basal salts, 2,4-D (1 mg/l), CH (1 g/l), ascorbic acid (100 mg/l), sucrose (3%), gellan gum (0.2%), pH 5.8
MS2	MS basal salts, BAP (2 mg/l), kinetin (0.2 mg/l), CH (1 g/l), ascorbic acid (100 mg/l), glucose (3%), Acetosyringone (39.24 mg/l), pH 5.1
MS3	MS basal salts, BAP (2 mg/l), IAA (0.2 mg/l), CH (1 g/l), ascorbic acid (100 mg/l), glucose (3%), Acetosyringone (39.24 mg/l), pH 5.1
MS4	MS basal salts, 2,4-D (2 mg/l), kinetin (0.2 mg/l), CH (1 g/l), ascorbic acid (100 mg/l), glucose (3%), Acetosyringone (39.24 mg/l), pH 5.1
MS5	MS basal salts, BAP (2 mg/l), kinetin (0.2 mg/l), CH (1 g/l), ascorbic acid (100 mg/l), sucrose (3%), Acetosyringone (39.24 mg/l), cefotaxime (250 mg/l), hygromycin (100 mg/l), gellan gum (0.2%), pH 5.8
MS6	MS basal salts, BAP (2 mg/l), IAA (0.2 mg/l), CH (1 g/l), ascorbic acid (100 mg/l), sucrose (3%), Acetosyringone (39.24 mg/l), cefotaxime (250 mg/l), hygromycin (100 mg/l), gellan gum (0.2%), pH 5.8
MS7	Half-strength MS basal salts, IBA (0.5 mg/l), sucrose (3%), cefotaxime (250 mg/l), hygromycin (100 mg/l), gellan gum (0.2%), pH 5.8

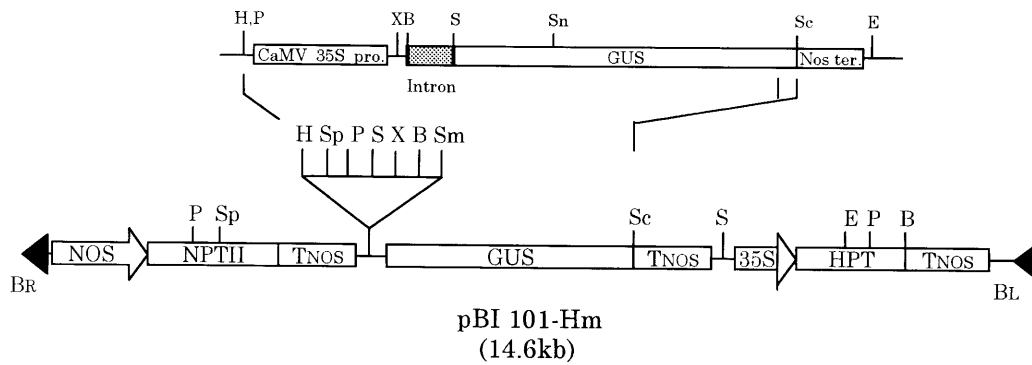


Fig. 1. T-regions of pBI 101-Hm. Abbreviations: BR-right border, BL-left border, NPTII-neomycin phosphotransferase, GUS- β -glucuronidase, HPT-hygromycin phosphotransferase, Nos-Nopaline synthase promoter, 35S-35S promotor, TNos-3' signal of nopaline synthase, B-*Bam*HI, E-*Eco*RI, H-*Hind*III, P-*Pst*I S-*Sal*I, Sc-*Sac*I, Sm-*Sma*I, Sn-*Sna*BI, Sp-*Sph*I, X-*Xba*I

by the CaMV 35S promoter and Nos terminator sequences, was used as the reporter gene. A bacterial strain was grown overnight in a Luria broth (LB) medium with the appropriate antibiotics.

Transformation and regeneration

The pre-cultured young embryos were infected for 30 minutes with the *Agrobacterium* suspension and transferred to MS2, MS3 or MS4 medium and incubated at 25°C in the dark for 3 days. After co-cultivation, the explants were rinsed thoroughly with MS basal medium and placed on MS5 or MS6 supplemented with 250 mg/l cefotaxime and 100 mg/l hygromycin. After first culture, the explants were transferred to fresh MS5 or MS6 medium with cefotaxime and hygromycin for another 15 days. The explants with putative transgenic shoot buds were transferred to MS7 medium supplemented with 250 mg/l cefotaxime and 100 mg/l hygromycin for proper root development.

Histochemical GUS assay

Expression of GUS in leaf segments was assayed essentially as described by Kosugi et al. (1990) with 5-bromo-4-chloro-3-indole glucoronide (X-Gluc) as substrate. The leaf segments were immersed in a 100 mM phosphate buffer that contained 0.3% Triton X-100, 1.9 mM X-Gluc, 0.5 mM Pot. Ferricyanide, 0.5 mM Pot. Ferrocyanide and 20% methanol. The reaction mixture was placed under mild vacuum for 5 minutes and incubated overnight at 37°C. The tissues were examined visually for blue color.

RESULTS AND DISCUSSION

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 (Nair and Adachi, 1999b). As the maximum and most consistent response was from the 22 kDa protein band, this band was electroeluted and subjected to N-terminal microsequencing (data not shown).

Nucleotide sequence of the cDNA encoding the major allergenic protein

RT-PCR combined with 5'-RACE provided the entire sequence of the mRNA coding for the major allergenic protein in buckwheat. A similar strategy to obtain the complete cDNA sequence was reported by Schoning et al. (1996) in apple. As the full length of cDNA was not obtained after one 5'-RACE reaction, the PCR products were cloned and sequenced three times using new primer combinations at the known 5' end. The nucleotide sequence covering the open reading frame and the deduced amino acid sequence is shown in Fig. 2. We have named the nucleotide sequence as FAGAG 1. The complete nucleotide sequence showed an untranslated region of 40 bp followed by an open reading frame of buckwheat protein. The open reading frame consists of 1614 nucleotides with 538 amino acids. The putative nucleotide sequence length of Fag e 1, the allergenic protein, covers 573 nucleotides that encode for 191 amino acids. The calculated molecular mass of 21474 for Fag e 1 correlates well with an apparent molecular mass of 22000 as estimated from the SDS-PAGE (data not shown). The nucleotide sequence of FAGAG 1 is available in the data bank as accession number AF152003.

A protein database search (Altschul et al., 1990), with the predicted amino acid sequence of FAGAG 1, revealed significant homologies with storage proteins of both dicot and monocot species. Buckwheat

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      10      20      30      40      50      60      70      80      90      100      110      120
GATCACCACCTCGACAACACAACCTCAAAACATTCCACCATGTCAACTAAACTCATACTCTCCTTCTCACTGTGCCTTATGGTACTAAGCTGCTCTGCCGAGCTATTGCCATGGCAGAAGG
      M S T K L I L S F S L C L M V L S C S A Q L L P W Q K G

      130      140      150      160      170      180      190      200      210      220      230      240
GACAACGCGCGCGCCCAACCATGGACACCAGCAATTCCAGCATCAGTGTGATATCCAGAGGCTCACCGCCTCTGAGCCCTCTCGTAGAGTCCGGTCCGAGGCGGAGTTACTGAGATTT
      Q R S R P H H G H Q Q F Q H Q C D I Q R L T A S E P S R R V R S E A G V T E I W

      250      260      270      280      290      300      310      320      330      340      350      360
GGGACCATGACACCCCTGAGTTCGATGTGCCGATTGTGCGCGTCCGTGTCGTAATTCAGCCTGGAGGCTCTTGCTTCCTTCTACTCCAACGCGCCCTTACATCACCTTTGTCGAGC
      D H D T P E F R C A G F V A V R V V I Q P G G L L L P S Y S N A P Y I T F V E Q

      370      380      390      400      410      420      430      440      450      460      470      480
AAGGGAGGGGAGTGCAGGAGTGGTCGTGCCAGGATGCCCGGAGACGTTCCAGTCAGGTCGGAATTTGAGTACCTTCGGTCTCAGAGAGACCAACGCTCCAGGCAGAGTGAGAGCGGAG
      G R G V Q G V V V P G C P E T F Q S G S E F E Y P R S Q R D Q R S R Q S E S G E

      490      500      510      520      530      540      550      560      570      580      590      600
AATCCAGCGTGGAGACCAACGCTCCAGGCAGAGTGAGAGCGAAGAAATCCAGCCGTGGAGACCAACGCTCCAGGCAGAGTGAGAGCGAAGAATTCAGCCGTGGAGACCAAGCAGAGA
      S S R G D Q R S R Q S E S E E S S R G D Q R S R Q S E S E E F S R G D Q H Q K I

      610      620      630      640      650      660      670      680      690      700      710      720
TTTTCAGGATCAGAGACGGCGACGTCATCCCATCTCCCGCGGTGTCGTGCAGTGGACCCACAACAACGGTGACAACGATCTCATCAGTATCACTTTTACGATGCCAACAGCTTCCAGA
      F R I R D G D V I P S P A G V V Q W T H N N G D N D L I S I T L Y D A N S F Q N

      730      740      750      760      770      780      790      800      810      820      830      840
ACCAGCTCGATGAGAACGTTAGGAACCTTCTCTAGCTGGTCAGAGCAAGCAGAGCAGGAGGACCGCCGAGCCAGCCAGACTAGGGAGGAAGGCAGTGACCGCCAATCCCGTGAGA
      Q L D E N V R N F F L A G Q S K Q S R E D R R S Q R Q T R E E G S D R Q S R E S

      850      860      870      880      890      900      910      920      930      940      950      960
GCCAAGACGACGAAGCACTTCTCGAAGCAAACTCTGAGTGGATTTCGAGGACGAGATCCTCCAAGAAATCTCCGAAATGTTGACAGGAGACCATCAGCAAGCTCAGAGGTGAGAAGC
      Q D D E A L L E A N I L S G F E D E I L Q E I F R N V D Q E T I S K L R G E N D

      970      980      990      1000      1010      1020      1030      1040      1050      1060      1070      1080
ACCAGAGAGGATTCTCGTCCAGGCTCGGACCTCAAACTCCGGGTCCCAGAGGAGTATGAAGAAGAACTCCAGAGGGAAGAGGTGACAGGAAAAGAGGTGAAGCGGGAGGAGCAATG
      Q R G F I V Q A R D L K L R V P E E Y E E E L Q R E R G D R K R G G S G R S N G

      1090      1100      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
GATTGGAGCAAGCGTTCTGCAACCTGAAATTCAGGCAAAATGTTAACAGGCCTTCTCGCGCCGACGTCTTCAACCCACGCGCCGTCGTATCAACACCGTAGACAGCAACAATCTCCCGA
L E Q A F C N L K F R Q N V N R P S R A D V F N P R A G R I N T V D S N N L P I

      1210      1220      1230      1240      1250      1260      1270      1280      1290      1300      1310      1320
TCCTCGAATTCATCAACTTAGCGCCAGCACGTGCTCCTTACAAGAATGCGATCCTCGGACCGAGATGGAACCTTGAACCGGCACAGCGCACTGTACGTGACGAGAGGAGAAGGAAGAG
      L E F I Q L S A Q H V V L Y K N A I L G P R W N L N A H S A L Y V T R G E G R V

      1330      1340      1350      1360      1370      1380      1390      1400      1410      1420      1430      1440
TCCAGGTTGTCGGAGATGAAGGAAGAAGTGTGTTGACGACACAGTCGACGAGGACAGATCCTTGTGGTCCCACAGGGATTGCAAGTGGTGTGAAGGCAGGAAGAGAAGGACTGGAGT
      Q V V G D E G R S V F D D N V Q R G Q I L V V P Q G F A V V L K A G R E G L E W

      1450      1460      1470      1480      1490      1500      1510      1520      1530      1540      1550      1560
GGGTGGAGTTGAAGAACGACGACACGCCATAACGACCCGATTGCCGGAAGACTTCGGTGTGAGGGCGATCCCTGTGAGGTTCTTGCCAACCTCTACGATATCTCGACGAAGGAAG
      V E L K N D D N A I T S P I A G K T S V L R A I P V E V L A N S Y D I S T K E A

      1570      1580      1590      1600      1610      1620      1630      1640      1650      1660      1670      1680
CGTTCAGATTGAAGAATGGGAGGAGGAGTGTGAGTCTTCGACCAATTCAGTCCCGAGATGAGAAGGAGAGGAGCGTTTCTCCATAGTTTAAGAGAGACAAAGGGTCTATCGTATGC
      F R L K N G R Q E V E V F R P F Q S R D E K E R E R F S I V *

      1690      1700      1710      1720      1730      1740      1750      1760      1770      1780      1790      1800
AAAAATAAAACAGAAGGAGAAGGATAAGGGAGTCTTGTCTATCGTTTAGCTAGTCAAGTCCCTCTCCACTTCTGGGTTATGTTCTATGTTTTACTTTAGTGTCATAAAAGAGTGAATT
      CTGAAAAAAAAAAAAAAAAAAAAA
      1810      1820      1830

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Fig. 2. Nucleotide and deduced amino acid sequences of a cDNA encoding for a buckwheat major allergenic protein (Fag e 1). A start codon ATG is boxed, the poly adenylation signals are double line underlined. The stop codon is indicated by an asterisk. The sequence identical to the known N-terminal sequence of the allergenic protein is underlined.

allergenic protein, Fag e 1, showed 60-75% homology in the alignment with 11S globulins from rice, pea, oats, broad bean, sunflower and soybean. Rice albu-

mins and globulins (Nakamura and Matsuda, 1996), soybean and legume globulins (Lalles and Peltre, 1996) are known to induce allergies when consumed

by humans. FAGAG 1 showed a very high homology to two cDNA clones isolated from a buckwheat cDNA library of developing seeds (Fujino et al., 1997, unpublished; database accession numbers D87980, D87982). Multialignment of FAGAG 1 with the two cDNA clones showed 99% homology, indicating that FAGAG 1 also belongs to the same class of legumin-like proteins. Minor differences in sequence length and deduced amino acid sequences may be due to slightly different isoforms of the protein from different sources or could be variety dependent.

Among the legumin-like storage proteins investigated so far, a high molecular weight precursor was found as the major *in vitro* translation product (Shotwell and Larkins, 1989). However, Maksimovic et al. (1996) in their studies with biosynthesis and *in vitro* translation studies of buckwheat storage proteins, reported that the 13S subunit-polypeptides are not the products of posttranslational processing but are directly encoded by individual mRNAs. Our studies, with the deposition of seed storage protein during developmental stages (Nair and Adachi, 1999a) and the data on nucleotide sequence, suggests the existence of a precursor protein. SDS-PAGE of storage proteins during the developmental stages revealed that a protein at 69 kDa at 10–15 Days after pollination disappeared on maturity. The complete nucleotide sequence encoding the 22 kDa protein extends much beyond the N-terminus, indicating the existence of a precursor protein. The main component of buckwheat seed protein is classified as a 280 kDa globulin. Electrophoresis of this protein under denaturing condition resolved the globulins in α , β and γ subunits with a molecular mass ranges 55–60, 32–44 and 16–29 kDa, respectively (Rout and Chrungoo, 1996). These data support the view that the different subunits are encoded by individual mRNA and that the 22 kDa subunit in buckwheat is also a product of posttranslational processing as in other legumin-like storage proteins.

Development of homostyle buckwheat

Common buckwheat is an outcrossing species with distylic incompatibility. It has two types of flowers : pin (long style) and thrum (short style). Due to allogamy, the individuals in a population are highly heterogeneous in their seed storage proteins. The self-incompatible, distylic system is a limitation for high yield. To overcome this problem, wide hybridization has been attempted in buckwheat by crossing with a homomorphic self-compatible wild species, *F. homotropicum* (Campbell, 1995; Woo and Adachi, 1997a). The hybrids were successfully produced

through embryo rescue and forwarded to successive generations by selfing as well as back crossing. The scheme is shown in Fig. 3.

The F_1 hybrids segregated into a 1 : 1 ratio indicating a single dominant gene for homomorphism. Further analyses revealed that the pin-thrum complex of characters is governed by a single locus S, with three alleles S^h , S and s, which respectively control the *F. homotropicum* homo-type S^hS^h , *F. esculentum* (thrum) Ss and pin ss. In the BC_2F_2 generation, although the expected ratio is 1 : 2 : 1 for S^hS^h (homo) : $S^h s$ (homo) : ss (pin), a few deviations in flower morphology have been observed. Back crossing is being extended for further generations in order to develop a stable population with introgression of the gene(s) from *F. esculentum*.

Agrobacterium-mediated transformation of buckwheat

The complete cDNA sequence now available can be of very high utility in designing experiments to alleviate buckwheat allergy. Antisense RNA with a complementary sequence to mRNA has been used experimentally to inhibit gene expression in both prokaryotes and eukaryotes (Green et al., 1986). Transgenic crop plants, where antisense technology is successfully used to regulate gene expression, are routinely carried out (Sheehy et al., 1988; Smith et al., 1988; Nakamura and Matsuda, 1996). With a proper gene transfer system, it is now possible to repress the allergenic expression in buckwheat.

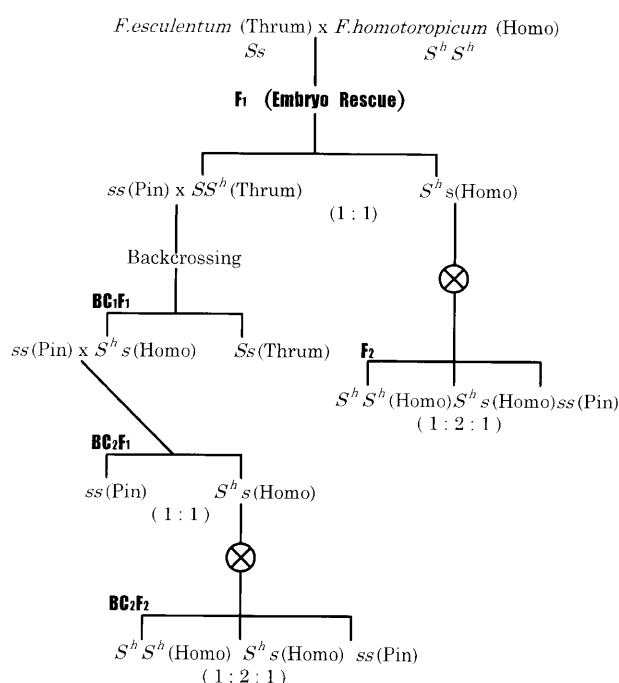


Fig. 3. Scheme of genetic analysis and introgressive hybridization.

Transferring the antisense technology to eliminate the allergenic protein into a self-compatible, high yielding buckwheat population is highly advantageous and stable when compared to self-incompatible, outcrossing buckwheat.

A reliable callus induction and plant regeneration system in *F. homotropicum* have already been reported by Woo et al. (1997b). Standardization of this technique in both common buckwheat and *F. homotropicum* will help us to better understand and apply the gene transfer technology for new self-pollinating buckwheat lines. More than one method is currently available to transfer genes of interest into plants. The *Agrobacterium* mediated gene transfer provides high efficiency and transfer of relatively large segments of DNA. A protocol for regeneration and genetic transformation has been attempted in buckwheat. Two different types of media were selected for regeneration. A medium containing BAP and IAA (MS6) showed better regeneration efficiency than a medium with BAP and kinetin (MS5). A maximum regeneration frequency of 54.16% was obtained with 2 mg/l BAP and 0.2 mg/l IAA (Table 2, Fig. 4a, 4c.). BAP and IAA were successfully used to regenerate shoot buds in *Capsicum* species (Gunay and Rao, 1978; Christopher and Rajam, 1994; Hyde and Phillips, 1996; Manoharan et al., 1998). Efficient rooting was observed when the healthy shoots were transferred to half strength MS medium supplemented with 0.5 mg/l Indole butyric acid (MS7). The transgenic nature of the regenerated plants was confirmed by histochemical staining of the GUS gene (Fig. 4b). Histochemical activity of the leaves for GUS activity revealed the characteristic blue color expression of GUS gene.

Genetic transformation and regeneration of buckwheat using *A. tumefaciens* strain as a vector had earlier been reported by Milju-Djukic et al. (1992). They used cotyledons as explants and the resistance was based on kanamycin. In pBI 101 Hm, both kanamycin and hygromycin resistant genes are included providing a higher selection pressure. Also,

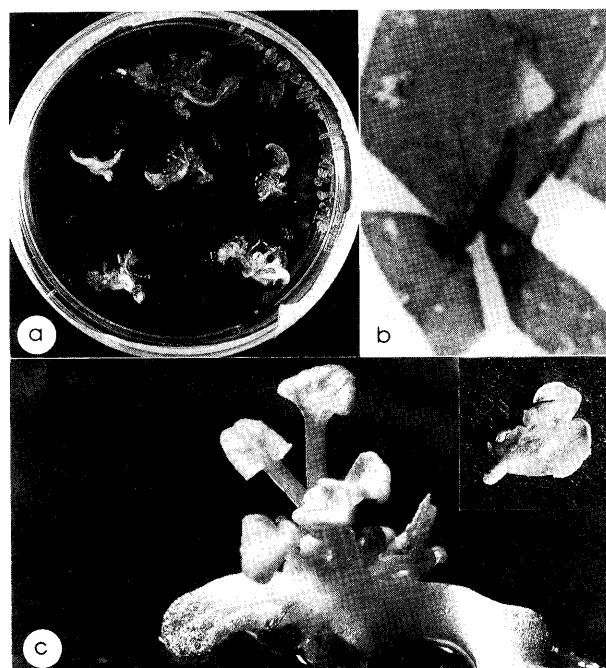


Fig. 4. Regeneration of plants from young embryos (7-10 DAP) of buckwheat after co-cultivation with *Agrobacterium tumefaciens* strain EHA 101 (pBI-101Hm) and GUS expression in the transgenic leaves.

(a) Plant regeneration in media with BAP (2 mg/l) and IAA (0.2 mg/l) and hygromycin (100 mg/l). (b) GUS expression in the leaves of transformants. (c) Hygromycin resistant regenerants from buckwheat tissues. Inset: GUS expression at that stage.

the intron-gus (Ohta et al., 1990) was a convenient marker gene as this gene is strongly expressed in transformed buckwheat cells but not in *A. tumefaciens* cells that attach to the tissues. Further standardization to increase the frequency of transformation and analyses of the transgenic plants are in progress.

Immunotherapy

Another area of application of the known nucleotide sequence encoding the allergenic protein is pharmacotherapy or immunotherapy. By mapping immunodominant IgE epitopes, as in grass pollen

Table 2. Production of hygromycin resistant plants from *A. tumefaciens* inoculated buckwheat young seeds.

SL. No.	Media	No. of young seeds (7-10 DAP) Precultured	No. of explants co-cultured	No. of explants regenerated	Regeneration frequency (%)
1	MS5	25	14	4	28.57
2		25	18	6	33.33
3		30	24	13	54.16
4	MS6	15	7	3	42.85
Total		95	63	26	

allergen Ph1p1 (Ball et al., 1994), specific drugs could be developed by designing non-anaphylactic IgE binding haptens that can locally saturate the effector cells. Recombinant allergens that are extremely useful for antibodies based on *in vitro* or *in vivo* diagnosis require the knowledge of the allergen specific nucleotide sequence. As a result of extensive cross-reactivities among the different allergens, it appears that the number of epitopes needed for diagnosis and specific immunotherapy is less diverse and might soon be covered by recombinant molecules (Valenta et al., 1998).

PCR based screening of buckwheat cultivars

Specific primers could be easily derived from the known sequence. A PCR based method of screening different species or population can be very handy in selecting buckwheat lines that lack the allergenic component. Such allergen less lines could be advanced through conventional breeding via inter specific hybridization or through tissue culture techniques like protoplast fusion and regeneration. We hope that the strategy and the results discussed in this report will aid in the development of hypoallergenic buckwheat.

ACKNOWLEDGEMENTS

The authors thank Dr. Kenzo Nakamura, Nagoya University for the gift of pBI 101-Hm. The technical help, discussion and support from Dr. Yoshihiro Ozeki (Tokyo University of Agriculture and Technology), Dr. Jotaro Aii and Mr. Naonobu Noda (Miyazaki University) are gratefully acknowledged. This work was financially supported by The Iijima memorial foundation for the promotion of food science and technology.

REFERENCES

- Altschul, S. F., W. Gish, W. Miller, W. Myers and D. J. Lipman, 1990. Basic local alignment search tool. *J. Mol. Biol.* 215: 403–410.
- Ball, T., S. Vrtala, W. R. Sperr, P. Valent, M. Susani, K. Dietrich and V. Rudolf, 1994. Isolation of an immunodominant IgE hapten from an epitope expression cDNA library. *J. Biol. Chem.* 269: 28323–28328.
- Campbell, C., 1995. Inter-specific hybridization in the genus *Fagopyrum*. *Current Advances in Buckwheat Research (Proc. 6th Intl. Symp. Buckwheat at Ina)*: 255–263.
- Christopher, T. and M. V. Rajam, 1994. *In vitro* clonal propagation of *Capsicum* sp. *Plant Cell Tissue Organ Cult.* 38: 25–27.
- Green, P. J., O. Pines and M. Inouye, 1986. The role of antisense RNA in gene regulation. *Ann. Rev. Biochem.* 55: 569–597.
- Gunay, A. L. and P. S. Rao, 1978. *In vitro* plant regeneration from hypocotyl and cotyledon explants of red pepper (*Capsicum*). *Plant Sci. Lett.* 11: 365–372.
- Hyde, C. L. and G. C. Phillips, 1996. Silver nitrate promotes shoot development and plant regeneration of chilli pepper (*Capsicum annum* L.). *Plant Sci.* 89: 107–112.
- Kosugi, S., Y. Ohashi and K. Nakajima, 1990. An improved assay for β -glucuronidase in transformed cells: methanol almost completely suppresses a putative endogenous β -glucuronidase activity. *Plant Sci.* 70: 133–140.
- Lalles, J. P. and G. Peltre, 1996. Biochemical features of grain legume allergens in humans and animals. *Nutr. Rev.* 54: 101–107.
- Lehrer, B. S., W. E. Horner and G. Reese, 1996. Why are some proteins allergenic? Implications on biotechnology. *Crit. Rev. Food Sci. Nutr.* 36: 553–564.
- Maksimovic, V. R., E. I. VarkonjiGasic, S. R. Radovic and A. P. Savic, 1996. Biosynthesis of 13S buckwheat seed storage protein. *J. Plant Physiol.* 147: 759–761.
- Manoharan, M., C. S. Sree Vidya and G. Lakshmi Sita, 1998. Agrobacterium-mediated genetic transformation in hot chilli (*Capsicum annum* L. var. *Pusa jwala*). *Plant Sci.* 131: 77–83.
- Miljus-Djukic, M., M. Neskovic, S. Ninkovic and R. Crkvenjakov, 1992. Agrobacterium-mediated transformation and plant regeneration of buckwheat (*Fagopyrum esculentum* Moench.). *Plant Cell Tissue Organ Cult.* 29: 101–108.
- Murashige, T. and F. A. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473–497.
- Nair, A. and T. Adachi, 1999a. Protein at 69 kDa expressed during the initial maturation stage of buckwheat (*Fagopyrum esculentum*) seed development. *Cereal Chem.* 76: 321–322.
- Nair, A. and T. Adachi, 1999b. Immunodetection and characterization of allergenic proteins in common buckwheat (*Fagopyrum esculentum*). *Plant Biotechnology*, In Press.
- Nakamura, R. and T. Matsuda, 1996. Rice allergenic protein and molecular-genetic approach for hypoallergenic rice. *Biosci. Biotech. Biochem.* 60: 1215–1221.
- Nekam, K., 1998. Management of food allergy. *Allergy* 53: 1221–124.
- Rout, M. K. and N. K. Chrungoo, 1996. Partial characterization of the lysine rich 280 KD 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., H. G. Ziegler, S. Veiths, and W. Baltes, 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.
- Sheehy, R. E., M. Kramer, W. R. Hiatt, 1988. Reduction of polygalacturonase activity in tomato fruit by antisense RNA. *Proc. Natl. Acad. Sci. USA* 85: 8805–8809.
- Shotwell, M. A. and B. A. Larkins, 1989. The biochemistry of Plants. In: Marcus, A. (ed.), *Molecular Biology*, pp. 297–345, Academic Press, New York.
- Smith, C. J. S., C. F. Watson, J. Ray, C. R. Bird, P. C. Morris, W. Schuch and D. Grierson, 1988. Antisense RNA inhibition of polygalacturonase gene expression in transgenic tomatoes. *Nature* 334: 724–726.
- Urisu, A., Y. Kondo, Y. Morita, E. Wada, M. Tsuruta, T. Yasaki, K. Yamada, H. Kuzuya, M. Suzuki, K. Titani, and K. Kurosawa, 1994. Identification of a major allergen of buckwheat seeds by immunoblotting methods. *Allerg. Clin. Immunol.* 6: 151–155.

- Valenta R., S. Vrtala, S. Laffer, S. Spitzauer and D. Kraft, 1998. Allergy review series II, Recombinant Allergens. *Allergy* 53: 552-561.
- Wieslander, G., 1996. Review on buckwheat allergy. *Allergy* 51: 661-665.
- Woo, S. H. and T. Adachi, 1997a. Production of interspecific hybrids between *Fagopyrum esculentum* and *F. homotropicum* through embryo rescue. *SABRAO J.* 29: 89-95.
- Woo, S. H., A. Nair, and T. Adachi, 1997b. Somatic embryo-genesis and plant regeneration in tissue cultures of wild buckwheat (*Fagopyrum homotropicum* Ohnishi). *SABRAO J.* 29: 97-102.
- Yanagihara, Y., 1980. Buckwheat hypersensitivity. *Kansensho*, 10: 184-188. (in Japanese).
- Yano, M., R. Nakamura, S. Hayakawa and S. Torii, 1989. Purification and properties of allergenic proteins in buckwheat seed. *Agric. Biol. Chem.* 53: 2387-2392.