



Expression and epitope analysis of the major allergenic protein Fag e 1 from buckwheat[☆]

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Received 6 December 2003; accepted 29 January 2004

KEYWORDS

Allergy;
Autogamous common
buckwheat;
Critical amino acids;
Epitope;
Seed storage protein

Summary

Seeds of common buckwheat (*Fagopyrum esculentum*) contain valuable nutritive substances but also allergenic proteins that cause hypersensitive reactions. Thus, the development of hypoallergenic buckwheat would make this important pseudo-cereal available to allergic people. A major allergenic protein of buckwheat is Fag e 1. We isolated the respective cDNA, coding for a 22 kDa protein, from a recently developed autogamous strain of common buckwheat and confirmed its immunoglobulin E (IgE)-binding activity using recombinant Fag e 1 and sera of allergic patients. The derived amino acid sequence from Fag e 1 cDNA was used to synthesize an overlapping peptide library on nitrocellulose membranes for the determination of the Fag e 1 epitopes. We identified eight epitopes and the critical amino acids for IgE-binding within the epitopes. This epitope analysis of a major allergenic protein of buckwheat should help therapeutic efforts and aid in the development of hypoallergenic buckwheat.

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Introduction

Buckwheat (*Fagopyrum esculentum*) is a widely consumed pseudo-cereal, especially popular in Asian countries such as Japan, China and Korea

because of its high productivity and outstanding nutritive value. Buckwheat has a short growing period and moderate fertilizing needs. The seeds contain 15–17% protein and are rich in essential amino acids, such as lysine, methionine and

Abbreviations: CBB, Coomassie brilliant blue; Fmoc, *N*-(9-fluorenyl) methoxycarbonyl group; IgE, immunoglobulin E; IPTG, isopropyl- β -thiogalacto-pyranoside; ORF, open reading frame; PVDF, polyvinylidene difluoride; TRX, thioredoxin

[☆]A poster presenting this data won a JPP poster award at the International Colloquium on Plant Biotechnology held in Sakai, Japan, November 2003.

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tryptophan (Eggum, 1980). Furthermore, the seeds hold rutin, a flavonol glycoside, which can function as an antagonist to capillary fragility associated with hemorrhagic diseases or hypertension and also display antioxidant activity (Griffith et al., 1944; Watanabe et al., 1995). On the other hand, buckwheat seeds contain proteins that are known to cause a hypersensitive reaction (allergy), which limits their use as a general food source and additive.

Buckwheat allergy is an immunoglobulin E (IgE)-mediated hypersensitive response capable of causing serious symptoms, including anaphylactic shock. The allergens are seed storage proteins. Electrophoretic immunoblotting confirmed that IgE-antibody binding to the seed storage proteins of buckwheat shows various patterns in different patients (Urisu et al., 1994). In particular, proteins of 17, 50 and 100 kDa (Yanagihara, 1980), 8–9 kDa (Yano et al., 1989), 24 kDa (Urisu et al., 1994), 22, 36, 39–40 and 70–72 kDa (Nair and Adachi, 1999), 14 and 18 kDa (Yoshimasu et al., 2000) and 16 kDa (Tanaka et al., 2002) have been found to bind IgE. The 22 kDa protein, which displayed binding activity with almost all sera from different patients is considered a major allergenic protein (Urisu et al., 1994). This allergen, named Fag e 1, and the respective cDNA were previously isolated (Nair and Adachi, 1999).

Common buckwheat is an allogamous species. This trait constitutes a major part of the breeding barriers in buckwheat, which may have prevented it from becoming a major modern crop. However, the recent discovery of an autogamous wild species, *F. homotropicum* (Ohnishi, 1993), became a starting point in overcoming the self-incompatibility barrier. Interspecific hybridization between common buckwheat and *F. homotropicum*, followed by embryo-rescue culture, resulted in the creation of an autogamous hybrid (Woo and Adachi, 1997). This hybrid was backcrossed to common buckwheat, and autogamous progenies were selected. Continued backcrossing to introgress the self-compatible gene into common buckwheat established an autogamous genotype stably expressing autogamy in the genetic background of common buckwheat.

In the present study, we focused on the molecular characterization of Fag e 1 for the purpose of developing hypoallergenic buckwheat. Fag e 1 cDNA was isolated from autogamous common buckwheat and its antigenicity confirmed by immunoblotting using recombinant protein expressed in *E. coli*. The derived amino acid sequence from Fag e 1 cDNA has been used to construct the synthetic peptides, and we have identified the

major IgE binding epitopes in this allergenic protein. We have also identified the amino acids within each of the IgE binding epitopes that are critical for IgE binding. Our results will be used in future attempts to genetically decrease the allergenic activity of buckwheat storage proteins by modifying its composition or IgE binding sites.

Materials and methods

Plant material

The common buckwheat (*F. esculentum* MOENCH) strain BC₆F₂, established by six backcrosses and one selfing following interspecific hybridization between common buckwheat (cv. Miyazakizairai) and *F. homotropicum* OHNISHI, was used (Woo and Adachi, 1997). The strain has an above 99% putative genetic background of *F. esculentum* and expresses stable self-compatibility.

cDNA isolation

Total RNA from 10 to 15 days old maturing seeds was isolated according to Verwoerd et al. (1989). Two oligonucleotides, FeAg22kDa-F (GAGA-GAGCTCTGGATTGGAGCAAGCGTTCTG) plus Sac I restriction enzyme cassette and FeAg-R (AAGCTA-GACGAGAATTCACCTCTTTTATTGAC) plus Xba I restriction enzyme cassette derived from the 5' and 3' ends of Fag e 1 cDNA, respectively (Nair and Adachi, 1999), served as primers for RT-PCR to amplify the cDNA encoding Fag e 1. RT-PCR was performed by using the Titan™ One Tube RT-PCR System (Roche Molecular Biochemicals). The reverse transcription was performed for 30 min at 50°C, and PCR amplification was followed by denaturation for 2 min at 94°C and 30 cycles of denaturation for 10 s at 94°C, annealing for 30 s at 54°C and elongation for 90 s at 68°C. The amplified product was cloned into Sac I and Xba I multi-cloning sites of the pUC18 cloning vector. The five positive clones were sequenced to confirm their Fag e 1 cDNA nature.

Expression of the fusion protein

The DNA sequence encoding the mature Fag e 1 peptide was PCR amplified from plasmid Fag e 1 (pFag e 1) by using the primer sets Fe22ex-F (5'-AGAGAAGCTTATGGATTGGAGCAAGCGTTC-3') and Fe22ex-R (5'-AGAGCTCGAGCGAGAATTCACCTCTTTATTGAC-3'). The 540-bp PCR product was digested with Hind III and Xho I and ligated between Hind III

and Xho I multi-cloning sites into the pET-32a(+) expression vector (Novagen). This expression vector contains the gene coding for ampicillin resistance and coding sequence for thioredoxin (TRX)-His-Tag produced at the NH₂-terminus of the recombinant protein. The constructed vector (pET-Fag e 1) was transformed into *E. coli* strain BL21 (DE3) pLysS (Novagen) and expression of fusion protein was induced by the addition of isopropyl- β -thiogalacto-pyranoside (IPTG) at a final concentration of 1 mM in LB liquid medium. After 3 h of continuous shaking at 37°C, the fusion protein was purified from bacterial lysates under denaturing conditions using the HisTrap Kit (Amersham Pharmacia Biotech).

Allergen assay of recombinant Fag e 1 by immunoblotting

The purified fusion protein was cleaved between the TRX-His-Tag and Fag e 1 by treatment with enterokinase and electroblotted onto polyvinylidene difluoride (PVDF) membrane after separation on SDS-PAGE. The membrane was incubated with sera from patients hypersensitive to buckwheat for 3 h at room temperature. IgE binding to recombinant Fag e 1 was detected by monoclonal alkaline phosphatase-conjugated anti-human IgE (Sigma, 1:1000 diluted) as secondary antibody. The bound antibodies were detected with the chemiluminescent substrate CDP-Star (Amersham Pharmacia Biotech).

Peptide synthesis and SPOTs assay for the determination of overlapping peptides

Epitopes were identified through analysis of synthetic peptides (SPOTs synthesis, [Frank and Overwin, 1996](#)). The individual peptides were synthesized according to the manufacturer's (Genosys Biotechnologies) instructions ([Fields and Nobel, 1990](#)). Synthesis was monitored with bromophenol blue. In short, the primary sequence was synthesized so that it contained synthetic acids, by coupling *N*-(9-fluorenyl) methoxycarbonyl group (Fmoc) amino acids as per the manufacturer's instruction. The synthesis begins by esterifying a Fmoc amino acid to the cellulose membrane derivatized with a dimer β -alanine-NH₂ groups. Following the coupling of each amino acid, the peptides were rendered unreactive by acetylation with acetic anhydride (4% v/v) in *N,N*-dimethylformamide, following which the Fmoc groups were removed by the addition of piperidine to render the nascent peptides reactive. Once the desired pep-

tides were synthesized, the side chains were deprotected with a solution containing dichloromethane, trifluoroacetic acid, and triisobutylsilane in the ratio 1:1:0.05 (v/v/v). The immobilized synthetic peptides on the membrane were either probed directly or stored at -80°C for later use.

Probing of SPOTs membrane

After washing with methanol, the SPOTs membrane was blocked overnight with blocking buffer (Genosys Biotechnologies) diluted with 9 volumes of TBST (50 mM Tris, pH 8.0, 136 mM NaCl, 2.7 mM KCl and 0.05% Tween 20) with 5% (w/v) sucrose. The membrane was then incubated for 4 h at 25°C with pooled sera obtained from eight patients with buckwheat allergy. The membrane was subsequently washed twice with TBST and incubated with monoclonal alkaline phosphatase-conjugated anti-human IgE (Sigma, 1:1000 diluted) at room temperature for 2 h. After incubation the membrane was washed four times with TBST. The bound antibodies were detected with the chemiluminescent substrate CDP-Star (Boehringer Ingelheim) and enhancer Nitro-Block II (Tropix) both diluted X100 in 0.1 M Tris-HCl, 0.1 M sodium chloride, pH 9.5. The membrane was visualized with a molecular light imager (Berthold Technologies). The image processing and evaluation was performed using WinLight software (Berthold Technologies).

To determine the exact amino acid sequence of the IgE binding regions, detailed epitope mapping was performed by synthesizing overlapping peptides in SPOTs membrane. The overlapping peptides (12 amino acid residues in length), which were stepwise different from the previous peptide by two residues, were prepared and probed with pooled sera from eight allergic patients. The membrane was probed using the technique described above.

Analysis of the amino acids critical for IgE-binding

The critical amino acids of Fag e 1 IgE epitopes were identified by synthesizing multiple peptides with single amino acid changes at each position. Peptides were synthesized on a SPOTs membrane with each amino acid sequentially replaced with either alanine or methionine and probed as described in the previous section.

Results

Fag e 1 cDNA cloning and sequence analysis

Total RNA from immature seeds was used for RT-PCR. The amplified RT-PCR product was inserted into the pUC18 cloning vector and sequenced (Fig. 1). The open reading frame (ORF) is consistent with the 576 bp nucleotides found for Fag e 1 of common buckwheat. However, there are eight nucleotide and two amino acid replacements. Thus, the nucleotide homology is 98.6%.

Expression, antigenicity, and purification of recombinant Fag e 1

The cDNA was subcloned into the pET32a(+) expression vector and used to transform the BL21(DE3)pLysS *E. coli* strain. By the addition of IPTG, expression of the TRX-His-Tag and Fag e 1 fusion protein was induced (Fig. 2). The purified fusion protein was treated with enterokinase, which cleaves fusion protein to TRX-His-Tag and Fag e 1 (Fig. 3a). The IgE-binding activity was observed with recombinant Fag e 1 (Fig. 3b), thus, confirming its allergenicity.

Multiple IgE-binding regions throughout the Fag e 1 protein

Ninety overlapping peptides were synthesized to determine the regions of the Fag e 1 protein that are recognized by serum IgE. Each synthetic peptide was 12 amino acids long and differed from

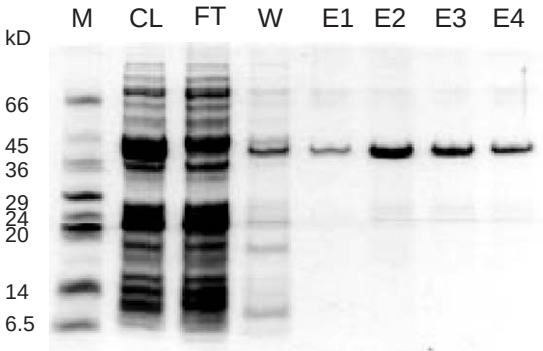


Figure 2. Purification of fusion protein. The fusion protein TRX-His-Tag and Fag e 1 was expressed in *E. coli* and purified by using the His Trap Kit (Amersham Pharmacia Biotech). Proteins were visualized by Coomassie brilliant blue (CBB) staining. CL: cell lysate; FT: flow-through; W: wash; E1: 1st elution; E2: 2nd elution; E3: 3rd elution; 4E: 4th elution.

1	G	L	E	Q	A	F	C	N	L	K	F	K	Q	N	V	N	R	P	S	R	
	GGA	TTG	GAG	CAA	GCG	TTC	TGC	AAC	CTG	AAA	TTC	AAG	CAA	AAT	GTT	AAC	AGG	CCT	TCT	CGC	60
21	A	D	V	F	N	P	R	A	G	R	I	N	T	V	D	S	N	N	L	P	
	GCC	GAC	GTC	TTC	AAC	CCA	CGC	GCT	GGT	CGT	ATC	AAC	ACC	GTT	GAC	AGC	AAC	AAT	CTC	CCA	120
41	I	L	E	F	I	Q	L	S	A	Q	H	V	V	L	Y	K	N	A	I	L	
	ATC	CTC	GAA	TTC	ATC	CAA	CTT	AGC	GCC	CAG	CAC	GTC	GTC	CTC	TAC	AAG	AAT	GCG	ATC	CTC	180
61	G	P	R	W	N	L	N	A	H	S	A	L	Y	V	T	R	G	E	G	R	
	GGA	CCG	AGA	TGG	AAC	TTG	AAC	GCG	CAC	AGC	GCA	CTG	TAC	GTG	ACG	AGA	GGA	GAA	GGA	AGA	240
81	V	Q	V	V	G	D	E	G	R	S	V	F	D	D	N	V	Q	R	G	Q	
	GTC	CAG	GTT	GTC	GGA	GAT	GAA	GGA	AGA	AGT	GTG	TTC	GAC	GAC	AAC	GTG	CAG	AGA	GGA	CAG	300
101	I	L	V	V	P	Q	G	F	A	V	V	L	K	A	G	N	E	G	L	E	
	ATC	CTT	GTG	GTC	CCA	CAG	GGA	TTC	GCA	GTG	GTG	TTG	AAG	GCA	GGA	AAT	GAA	GGA	CTG	GAG	360
121	W	V	E	L	K	N	D	D	N	A	I	T	S	P	I	A	G	K	T	S	
	TGG	GTG	GAG	TTG	AAG	AAC	GAC	GAC	AAC	GCC	ATA	ACC	AGT	CCG	ATT	GCC	GGG	AAG	ACT	TCG	420
141	V	L	R	A	I	P	V	E	V	L	A	N	S	Y	D	I	S	T	K	E	
	GTG	TTG	AGG	GCG	ATC	CCT	GTG	GAG	GTT	CTT	GCC	AAC	TCC	TAC	GAT	ATC	TCG	ACG	AAG	GAA	480
161	A	F	R	L	K	N	G	R	Q	E	V	E	V	F	R	P	F	Q	S	R	
	GCG	TTC	AGA	TTG	AAG	AAT	GGG	AGG	CAG	GAG	GTT	GAG	GTC	TTC	CGA	CCA	TTC	CAG	TCC	CGA	540
181	D	E	K	E	R	E	R	F	S	I	V	*									
	GAT	GAG	AAG	GAG	AGG	GAG	CGT	TTC	TCC	ATA	GTT	TAA									576

Figure 1. Nucleotide sequence and derived amino acids of the Fag e 1 cDNA clone isolated from the BC₆F₂ strain of buckwheat. The numbers on the right indicate the position of the nucleotide sequence relative to the first nucleotide, the numbers on the left the position of the amino acid sequence. The asterisk marks the TAA stop codon.

the previous peptide by two amino acids. These peptides were probed with serum. **Figure 4a** shows the eight IgE-binding regions. **Figure 4b** is an

immunoblot of five synthetic peptides. The amino acid sequence representing this region is shown as well as the amino acid sequences represented by each individual peptide. The boxed area in **Fig. 4b** represents the core epitope. The amino acid sequence of eight IgE-binding epitopes are marked as shaded segments of the total Fag e 1 sequence of 191 amino acids (**Fig. 4c**).

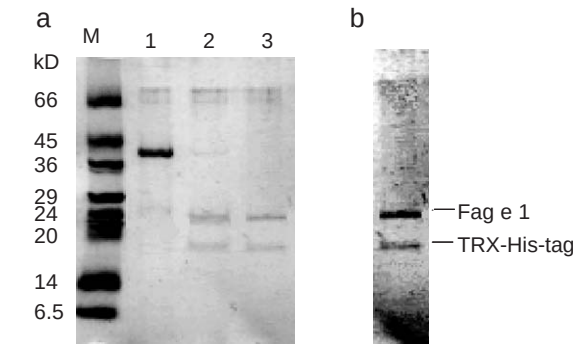


Figure 3. Cleavage of TRX-His-Tag and Fag e 1 and immunoblotting. (a) Purified TRX-His-Tag and Fag e 1 fusion protein treated with enterokinase. (1) Purified TRX-His-Tag and Fag e 1 fusion protein, (2) incubated with enterokinase at room temperature for 8 h, and (3) incubated with enterokinase at room temperature for 16 h. Proteins were visualized by CBB staining. (b) Immunoblotting using patient's sera with cleaved proteins.

Mutations at specific residues eliminate IgE binding

The amino acids essential for IgE binding were determined through the probing of synthetic peptides with single amino acid changes. **Figure 5** shows an immunoblot strip containing the wild type and mutant peptides for epitope 4. The peptide was not recognized or a decrease in binding was observed when alanine was substituted at positions 87, 88, 89, 90, 91 and 92. Interestingly, it appears as if an alanine substitution at positions 87 and 88 can increase IgE binding. The remaining Fag e 1 epitopes were analyzed in the same manner. Some

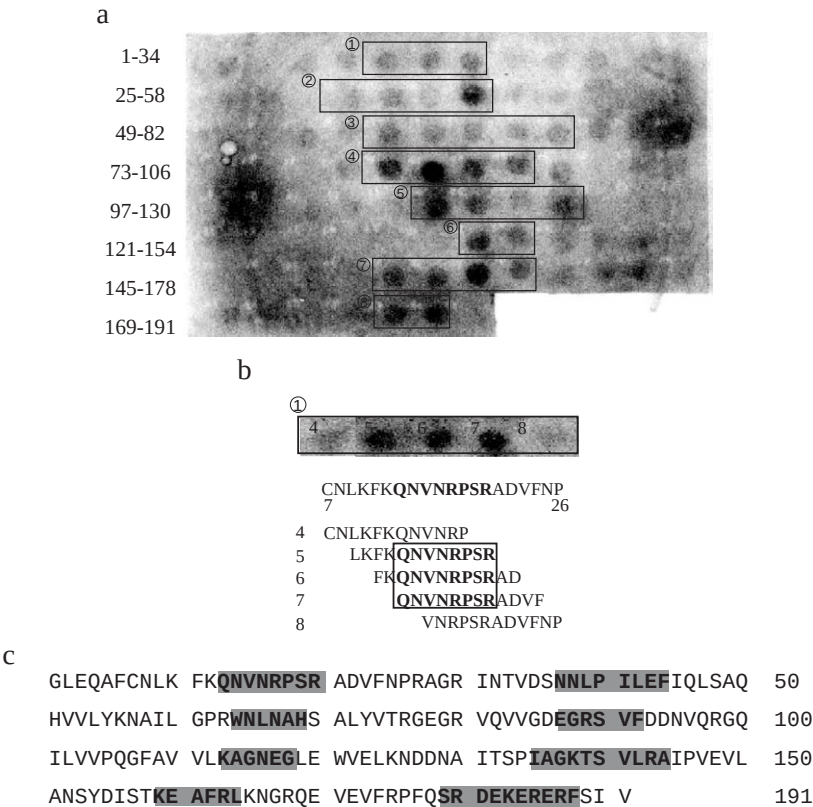


Figure 4. Multiple IgE-binding regions of the Fag e 1 allergen. (a) The Fag e 1 primary sequence was synthesized as 12 amino acid-long peptides, offset from each other by 10 residues. These peptides were probed with a pool of serum IgE from patients. (b) Identification of an IgE-binding epitope. The date shown represent amino acids 7–26. Residues in bold letters corresponding to common IgE-binding amino acids of the spots are shown in the box. (c) The amino acid sequence of the Fag e 1 protein is shown. The shaded areas correspond to the IgE-binding regions.

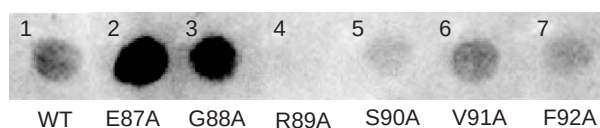


Figure 5. Fag e 1 epitopes can be mutated to non-IgE-binding peptides. Epitope 4 was synthesized with alanine (A) substituted for one of the amino acids in positions 87–92. The synthetic peptides were probed with patient's IgE. The letters on the left of the position number indicate the amino acid normally at that position. WT indicates the wild-type peptide with no amino acid substitution.

Table 1. Fag e 1 IgE-binding epitopes

IgE epitope	Amino acid sequence	Position
1	QNVNRP S R	13–20
2	NNLP I LEF	37–44
3	WNLNA H	64–69
4	EG R SVF	87–92
5	KAGNE G	113–118
6	IAGKTSVL R A	135–144
7	K EAF R L	159–164
8	SRDEKERER F	179–188

The epitopes are shown as a single amino acid code. The position of each peptide with respect to the Fag e 1 primary sequence is indicated in the right column. The amino acids that, when altered, led to a decrease in IgE binding are shown in bold.

epitopes could be altered to a non-IgE-binding peptide by the replacement of the wild-type amino acid residue with alanine or methionine. The critical residues of Fag e 1 epitopes for IgE binding are shown in Table 1. It appears that the central amino acids within each epitope are the most critical ones. All mutations that led to a significant decrease in IgE binding were located at residues found within each core epitope. There was no obvious consensus in the type of amino acid that, when mutated to alanine or methionine, led to a decrease or complete loss in IgE binding.

Discussion

In this paper, we reported cDNA cloning and IgE binding characteristics of a major allergenic protein, Fag e 1, from autogamous common buckwheat. Fag e 1 is the beta subunit of a 13 S globulin found in buckwheat seeds. The globulin has an approximate molecular mass of 62 kDa, and the subunits are chained by disulfide binding and unchained under reducing conditions (Fujino et al., 2001).

The nucleotide sequence homology of Fag e 1 cDNA isolated from the autogamous BC₆F₂ strain of buckwheat and from allogamous common buckwheat (cv. Miyazakizairai) was found to be 98.6%. It is unclear if the observed heterology is a residue of the initial interspecific hybridization or if it must be viewed as an individual genetic difference.

Recombinant Fag e 1, which has modified areas and different dimensions, displayed normal IgE binding activity indicating that the affected areas are not critical for IgE binding. Previously, a 33 kDa rice allergen was shown to display IgE binding activity as recombinant protein and to keep its function as glyoxalase (Usui et al., 2001). For Fag e 1, there is no report of enzymatic activity.

We identified eight epitopes and, in three of the epitopes, we identified a total of six individual amino acids of the buckwheat allergen to be critical for IgE binding. Allergens found in other plant species, for example peanut, soybean or hazelnut, have been shown to share amino acid pattern in critical epitopes (Beardslee et al., 2000; Beyer et al., 2002). However, Fag e 1 critical epitopes have no homology with critical epitopes identified in allergenic proteins from other species suggesting that they may be specific for buckwheat. Three of six critical positions of Fag e 1 are occupied by arginine, which is a positively charged amino acid. This could indicate that the charge is a factor in the binding of IgE to the buckwheat antigen.

Hyposensitization of allergic patients is almost the only therapy available for the cure of allergies. For this treatment, it is preferable to apply disabled allergens with a decreased IgE binding activity. Ikagawa et al. (1996) reported that a single amino acid substitution in an allergenic peptide carrying the T cell epitope might suppress T cell responses and induce IgE in B cells. However, different patients recognize different epitopes, making it necessary that patient-specific modified peptides be used for the hyposensitization treatment. Our epitope analysis of Fag e 1 should make such treatment available for patients with allergy to buckwheat flour. To further this therapeutic approach, we are currently involved with the identification of other allergenic seed proteins in strain BC₆F₂.

Transgenic techniques have been shown to have the potential of modifying, decreasing or even removing allergenic substances in rice and soybean (Tada et al., 1996; Herman et al., 2003). Using similar approaches, we are trying to develop a transgenic hypoallergenic crop from our autogamous strain of common buckwheat.

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