

Increased allergen production in turnip (*Brassica rapa*) by treatments activating defense mechanisms

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Background: Practical applications to enhance the productivity of agriculture by using plants with improved resistance to pathogens are expected to increase in the near future. Defense proteins play an important role in pathogen resistance, and some defense-related proteins are significant cross-reacting allergens. For example, cross-allergies are common among patients allergic to natural rubber latex (NRL), which contains many defense-related proteins.

Objective: Using a model plant (ie, turnip), we studied whether allergen contents increase after treatments activating defense mechanisms of the plants.

Methods: Whole or wounded turnips treated with salicylic acid, ethephon, or water were incubated for 2, 4, or 8 days. Allergen content was investigated by IgE immunoblotting with sera from patients allergic to NRL. An induced protein that bound IgE most intensively was purified and further characterized by mass analysis, amino acid sequencing, IgE-ELISA, and skin prick tests.

Results: In immunoblotting, clear IgE-binding bands were discernible only in samples from chemically treated plants. IgE was bound most intensively to a protein with an apparent molecular weight of 25 kd in SDS-PAGE and with a determined molecular weight of 18.7 kd. Sequenced peptides of the 18.7-kd protein showed over 70% homology to prohevein, a major allergen of NRL, and to many other prohevein-like defense proteins. In ELISA, sera from 30 of 34 (88%) adults and 21 of 26 (81%) children previously shown to have IgE against prohevein bound to the purified protein. In skin prick testing with the protein, 4 of 6 patients allergic to NRL had positive reactions.

Conclusion: These results demonstrate that activating defense mechanisms of plants may considerably increase their allergen content. (J Allergy Clin Immunol 1999;104:194-201.)

Key words: Allergy, IgE, cross-reaction, disease resistance, pathogenesis-related protein, prohevein, ethylene, salicylic acid, natural rubber latex, Brassicaceae

Abbreviations used

ACN: Acetonitrile
NRL: Natural rubber latex
SA: Salicylic acid
SAR: Systemic acquired resistance

Different biotic and abiotic stress factors can cause major changes in the protein synthesis of plants.^{1,2} Proteins expressed especially in stress situations often have a defensive function. Such proteins are planned to be used in plant protection through the development of pathogen-resistant transgenic plants and through the use of chemicals that activate defense mechanisms of plants.³ Practical applications to enhance productivity of agriculture with plants highly resistant to various pathogens are expected to increase in the near future.^{4,5}

Many defense-related proteins have been shown to be significant cross-reacting allergens. For example, upon wounding, the rubber tree (*Hevea brasiliensis*) produces liquid latex, which contains allergens very similar to defense-related proteins found in other plants, as exemplified by prohevein (Hev b 6.01),⁶⁻¹⁰ 1,3- β -glucanase (Hev b 2),^{6,11} and patatin-like protein (Hev b 7).¹² Patients allergic to natural rubber latex (NRL) often have allergic symptoms induced by different phylogenetically distant plants, such as avocado, banana, peanuts, melon, tomato, kiwifruit, potato, and papaya.¹³⁻¹⁶ Important allergens in cereal grains, namely α -amylase inhibitors, are defense-related proteins,^{17,18} and the major allergen of birch pollen (Bet v 1) probably also has a defensive function¹⁹ and cross-reacts with similar proteins of many other plants.²⁰

Allergen cross-reactivity is likely to be caused by IgE antibodies that recognize similar epitopes on different proteins. Profilins, the best characterized cross-reacting allergens, display epitope structures that have been conserved during evolution, and profilins of various species may cause cross-reactions to patients allergic to birch pollen.²¹ Defense-related proteins of plants often have evolutionarily conserved structures and are furthermore typically resistant toward proteolytic conditions,²² like many well known food allergens.²³ Because of these properties, defense-related proteins are likely candidates to cause cross-allergies, as discussed recently with regard to NRL.²⁴

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Ethylene and salicylic acid (SA) are widely studied signal molecules known to induce production of defense-related proteins.²⁵ Commercial products for plant protection are being developed from molecules that mimic the action of SA.²⁶ The aim of this study was to investigate experimentally the NRL cross-reactive allergen content of plants treated with SA or ethephon (an ethylene-releasing agent) compared with that of nontreated plants. Here we show, by using vegetable turnip (*Brassica rapa*) as a model plant, that treatments activating defense mechanisms can increase allergen production.

METHODS

Plant material and treatments

Fresh vegetable turnips, purchased as whole, undamaged plants from a local farmer, were dipped in solutions of SA (10 mmol/L 2-hydroxybenzoic acid; Sigma, St Louis, Mo)²⁷ or ethephon (1 mmol/L 2-chloroethyl-phosphonic acid, Sigma) or in water. Ethephon was used as an ethylene-releasing agent. The plants selected to wounding treatment were halved vertically after dipping. Whole or halved plants were wrapped in moistened tissue and incubated at room temperature for 2, 4, and 8 days in tightly sealed plastic bags to prevent evaporation and local increases in concentrations of chemicals.²⁸ Leaves were removed after incubation, and roots were sliced and frozen immediately with dry ice and stored at -20°C .

Patient and control sera

Serum specimens for immunoblotting and ELISA-inhibition experiments were obtained from 12 patients allergic to NRL (11 women and 1 man; mean age, 41 years; range, 22 to 64 years), selected as described previously,²⁹ who had IgE against different NRL allergens. All of these 12 patients had positive skin prick test responses to a commercial latex extract (Stallergènes, Antony, France) and had positive latex RAST results (CAP-RAST, Pharmacia, Sweden). All 12 patients had dermal symptoms of latex allergy, and 2 of them had systemic reactions. For IgE-ELISA, sera from 34 adults (29 women and 5 men; mean age, 37 years; range, 18 to 64 years) and 26 children (11 girls and 15 boys; mean age, 7 years; range, 0 to 16 years) allergic to NRL and previously shown to have IgE antibodies against prohevein were used as patient material. Six of the children and 1 of the adults had histories of multiple (mean, 9; range, 5 to 13) surgical operations at an early age. Sera from 62 individuals (40 females and 22 males; mean age, 31 years; range, 2 to 64 years; 25 atopic subjects) without NRL allergy served as controls.

Extraction, purification, and amino acid sequencing of proteins

To extract proteins for immunoblotting, a method based on organic solvent extraction and reversed-phase chromatography, described previously in detail,³⁰ was used in a slightly modified manner. Slices of frozen turnip root were homogenized in an equal amount of ice-cold water and mixed 1:2 (wt/vol) with 20% acetonitrile (ACN) containing 0.1% trifluoroacetic acid. After stirring and filtering through a 0.45- μm membrane (Millex HV; Millipore, Molsheim, France), the extract was chromatographed (without concentration) on a linear ACN gradient from 15% to 60% in 20 minutes by HPLC.

Affinity binding to and elution from chitin were used as a first step to purify proteins for amino acid sequencing, IgE-ELISA, and skin prick testing. Equal amounts of acetic acid buffer (50 mmol/L acetic acid, 0.2 mol/L NaCl, and 10 mmol/L thiourea; pH 4.0) and pooled turnip homogenate were mixed and stirred overnight at 4°C .

The mixture was centrifuged at 800g for 10 minutes, and the supernatant was filtered through a 0.45- μm membrane (Millex). Then 200 mg of chitin (Sigma), first washed with 1 mol/L acetic acid, was added to 40 mL of turnip extract and incubated in a horizontal shaker for 10 minutes at 4°C . The mixture was centrifuged at 800g for 5 minutes, and the supernatant was discarded. The pellet was washed twice with 30 mL of acetic acid buffer, mixed, and centrifuged at 800g for 5 minutes. One molar acetic acid (10 mL) was added onto the washed pellet and centrifuged at 800g for 5 minutes. Proteins detached from chitin with 1 mol/L acetic acid were purified from the supernatant by reversed-phase chromatography on a ReSource RPC 3-mL column by using a linear gradient of ACN (0% to 60% in 40 minutes) in 0.1% trifluoroacetic acid at a flow rate of 1 mL/min. Elution was monitored at 280 nm, and the collected fractions were analyzed by 13% SDS-PAGE and immunoblotting by using serum from a hevein-immunized rabbit.³⁰ Fractions containing a protein with an apparent molecular weight of 25 kd were pooled for further characterization.

Tryptic cleavage and peptide separation, amino acid sequencing, and sequence comparison were done as described previously in detail.³⁰ Hevein and prohevein were purified from NRL extract as described above for the turnip protein. NRL extract was prepared from fresh NRL of rubber trees as described earlier.⁷ The molecular weight of the purified protein was determined by electrospray mass spectrometry by using a Q-TOF Instrument (Micromass, Manchester, UK).

Protein assays

Total protein concentration of turnip extracts was determined by using the Lowry method. Staining of the proteins transferred to membrane was performed with a commercial kit (Amersham, UK), according to the manufacturer's instructions.

Immunoblotting and immunoblot inhibition

A method described previously was used,³⁰ except that before electrophoresis, antigen samples, corresponding to constant amounts of plant tissue (20 mg/ μL wet weight), were diluted 1:2 in reducing buffer and kept in boiling water for 3 minutes. After electrophoresis, the proteins were transferred from the gels to 0.45- μm pore size polyvinylidene difluoride membranes (Millipore, Immobilon-P). Whole membranes instead of cut strips were used in further steps of immunoblotting.

For immunoblot-inhibition experiments, the antigen preparation was made from pooled turnip homogenates. The antigen extract was chromatographed, and the separated protein peaks were pooled and concentrated under vacuum to correspond to 60 mg/ μL plant tissue. After electrophoresis, the proteins were transferred to a nitrocellulose membrane and subjected to immunodetection as previously described.³⁰ Different concentrations of hevein (0.001, 0.01, 0.1, 1, and 10 $\mu\text{g/mL}$), prohevein (0.01, 0.1, 1, and 10 $\mu\text{g/mL}$) and NRL extract (total protein 0.001, 0.01, 0.1, and 1 $\mu\text{g/mL}$) were used as inhibitors. In control experiments an equal volume of Tris-buffered saline was used instead of inhibitor solution.

IgE-ELISA and ELISA inhibition

Methods described previously were used.³⁰ For ELISA, 96-well polystyrene microtiter plates (Nunc, Roskilde, Denmark) were coated with the purified 18.7-kd turnip protein at a concentration of 4 $\mu\text{g/mL}$. The binding of IgE in sera diluted 1:10 was recorded, and the mean OD value plus 3 SDs of the control sera was regarded as the threshold value for positivity.

ELISA-inhibition experiments were done by using a serum pool (diluted 1:5 in PBS containing 0.02% Tween 20 and 0.2% human serum albumin) from 12 patients allergic to NRL. Sera were diluted before experiments. Microtiter plates were coated with the puri-

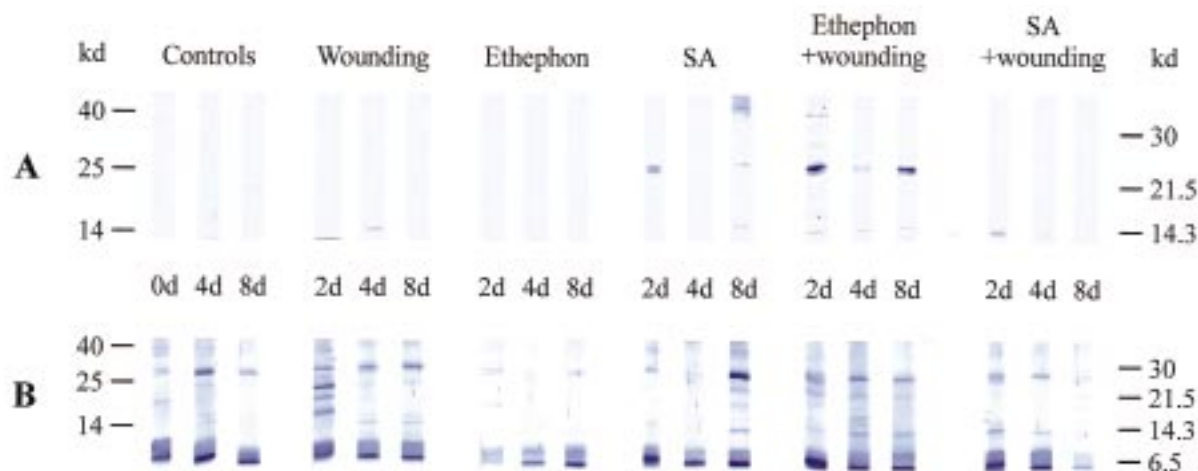


FIG 1. Binding of IgE to proteins of turnip extracts in immunoblotting and total protein staining of corresponding samples. Molecular weights were determined by using standards of Amersham. The electrophoretic mobilities of the molecular weight markers are marked on the right side, and the calculated molecular weights of the IgE-binding proteins are marked on the left side of the illustration. **A**, Immunoblotting shows IgE binding to the 14- and 25-kd turnip protein bands from pooled sera of selected patients allergic to NRL. **B**, Total protein staining shows remarkable variability between the number and intensities of protein bands in samples that were treated differently.

fied turnip protein or prohevein at a concentration of 4 $\mu\text{g/mL}$. Hevein, prohevein, and the turnip protein were used as inhibitors in various concentrations, ranging from 4 pmol/L to 20 $\mu\text{mol/L}$.

Skin prick testing with the purified turnip protein

Skin prick testing with the turnip protein (concentrations of 10 and 100 $\mu\text{g/mL}$ in PBS, pH 7.2) was performed on 6 patients allergic to NRL with IgE antibodies to the protein in ELISA. A wheal larger than half of the reaction caused by histamine dihydrochloride (10 mg/mL; ALK, Copenhagen, Denmark) was regarded as a positive reaction. The study was approved by the Ethical Committee of Tampere University Hospital.

RESULTS

Demonstration and identification of inducible allergens in turnip

Chemical treatments increased the number and intensity of bands visible in IgE immunoblotting recognized by the pooled sera of patients allergic to NRL (Fig 1, A). The most prominent IgE-binding protein induced was a 25-kd band that was found in plants treated with ethephon and wounded immediately after the treatment, as well as in plants that were treated with SA. Additional IgE-binding bands were seen at 14 and 40 kd. No clearly visible IgE-binding proteins were perceptible in the nontreated control turnips. Total protein staining revealed a large number of proteins, but no systematic changes between individual proteins could be observed (Fig 1, B).

In immunoblot inhibition (Fig 2) both the NRL extract and prohevein at a protein concentration of 1 $\mu\text{g/mL}$ totally inhibited IgE binding from pooled sera from patients allergic to NRL to the 25- and 14-kd protein bands. The 25-kd band also bound IgG from the hevein-immunized rabbit serum (Fig 2, C) and IgE from a refer-

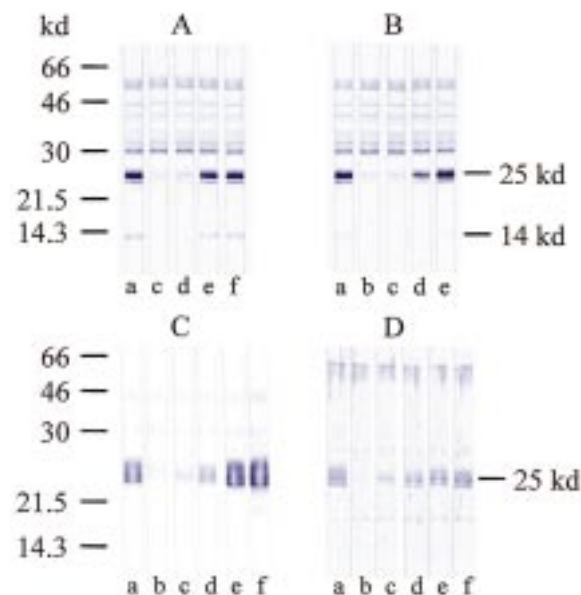


FIG 2. Inhibition of antibody binding to proteins from pooled turnip extracts by different concentrations of NRL, prohevein, and hevein. Concentrations of inhibitor used are, in lanes a to f, 0, 10, 1, 0.1, 0.01, and 0.001 $\mu\text{g/mL}$, respectively. IgE binding from pooled sera of selected patients allergic to NRL to the 14- and 25-kd turnip protein bands is inhibited by NRL (**A**) and purified prohevein (**B**). Purified hevein inhibits the binding of IgE from a patient allergic to hevein to the 25-kd turnip protein band (**C**) and binding of IgG from a hevein-immunized rabbit to the same protein band (**D**).

ence patient with high levels of IgE to hevein (Fig 2, D).⁷ Immunoglobulin binding to the 25-kd protein from both sera was totally inhibited by hevein (10 $\mu\text{g/mL}$).

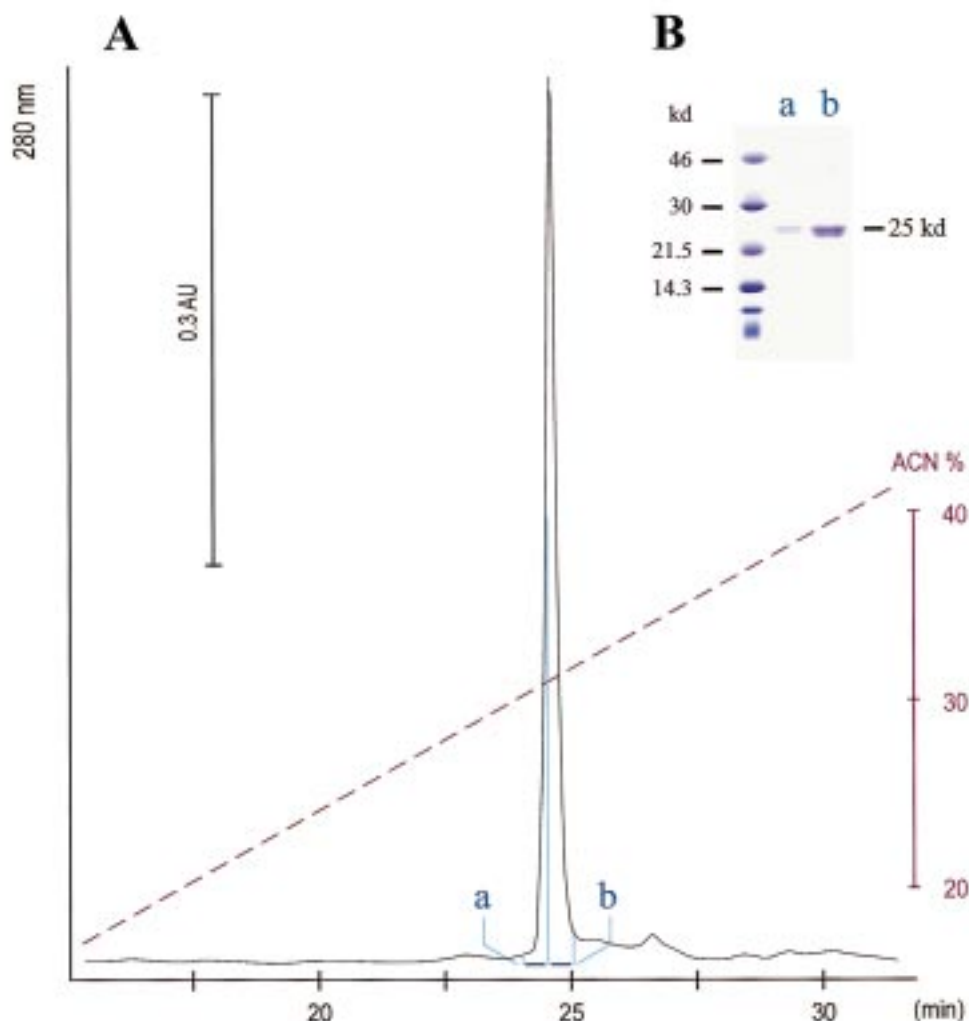


FIG 3. Purification of the most prominently IgE-binding turnip protein. **A**, Reversed-phase chromatography of a chitin affinity-purified turnip extract reveals 1 protein peak. **B**, SDS-PAGE shows that fractions *a* and *b* collected from reversed-phase chromatography contained a protein band of 25 kd (determined mass, 18.7 kd).

Purification and molecular characterization of the most prominent IgE-binding turnip protein

According to the immunoblot-inhibition results, it is likely that the 25-kd turnip protein band contains hevein-like chitin-binding domains. On the basis of this observation, binding to chitin was used as a first step in the purification of the protein.

Reversed-phase chromatography of the turnip proteins that bound to and detached from chitin revealed 1 peak that was collected and analyzed by SDS-PAGE and immunoblotting with rabbit antihevein serum. Two fractions containing the 25-kd protein band (Fig 3), recognized also by the hevein-immunized rabbit serum (data not shown), were electrophoresed and blotted to polyvinylidene difluoride membrane, and the 25-kd band was subjected to tryptic digestion. Internal peptides obtained from the digested protein gave 93% identity to hevein-

like precursor from *Arabidopsis thaliana* and 70% identity to prohevein from rubber tree (Table I) and also showed high homologies to wound-induced proteins of tomato (74%) and potato (71%). Mass spectrometric analysis revealed that the mass of the purified turnip protein was 18,673 d. Because of this fact, the protein corresponding to the 25-kd protein band in SDS-PAGE is heretofore called the 18.7-kd turnip protein.

Binding of IgE from sera of patients allergic to NRL to the purified 18.7-kd turnip protein in ELISA

In ELISA, IgE from 30 of 34 (88%) adults and 21 of 26 (81%) children allergic to NRL with IgE against prohevein bound to the 18.7-kd protein, whereas all 62 control sera were negative (Fig 4). Four of the 7 individuals with histories of multiple operations had ELISA results above the threshold value for positivity.

	23																							
		55																						
Prohevein	Q Y	A C	G S	G P	K D	L H	C N	P C	N Q	N	L	C	C	S	Q	W	G	W	C	G	S	T	D	E
Turnip 18.7 kd	Q Y	A C	G S	G P	Q D	T N	C N	A C	G Q	N		C	C	S	Q	Y	G	Y	C	G	T	T	A	D
<i>Arabidopsis</i>	Q Y	G C	G S	G P	R T	T N	C N	P C	G Q	N	I	C	C	S	Q	Y	G	Y	C	G	T	T	A	D
HEL	27	59																						
	75	108																						
Prohevein	A W	T D	Y A	H N	L K	Y P	N Y	S S	Q W	D R	H	G	W	D	L	N	A	A	S	A	Y	C	S	T
Turnip 18.7 kd	A W	T D	Y A	H D	Y K	Y P	N Y	P S	A W	Q R	N	N	W	D	L	R	A	V	S	A	Y	C	S	T
<i>Arabidopsis</i>	A	T	Y	H	F	Y	N	P	A	Q	N	N	W	D	L	R	A	V	S	A	Y	C	S	T

Hevein, prohevein, and the 18.7-kd turnip protein all inhibited IgE binding of pooled sera from 12 patients allergic to NRL to the purified 18.7-kd protein (Fig 5, *A*) and also to prohevein (Fig 5, *B*). Prohevein inhibited the binding of IgE from patients allergic to NRL to the 18.7-kd turnip protein in a concentration that was about one hundredth part of that of the 18.7-kd protein itself. Hevein inhibited about 80% of the IgE binding to the 18.7-kd protein, whereas the inhibitory capacity of prohevein was near 100%. On the other hand, hevein inhibited only about 25% of IgE binding to prohevein with the same pooled sera.

Four of the 6 patients allergic to NRL tested showed positive reactions to the 18.7-kd turnip protein (100 µg/mL). Diameters of the wheals (mm) caused by the protein/histamine were 8/7, 6/5, 3/5, and 3/5 for the positive reactions and 2/5 and 2/5 for the negative reactions. All of the tested patients had IgE against prohevein and hevein in ELISA with one exception: the patient with a test result of 6/5 had IgE against prohevein only. There were no large local, systemic, or delayed adverse reactions associated with the turnip protein skin prick tests. None of the 5 control subjects not allergic to NRL showed any reactions.

In the present experiments, treatments designed to activate defense mechanisms of plants markedly in-

IgE binding to the 18.7-kd turnip protein was inhibited by hevein and prohevein, suggesting that these proteins have cross-reacting epitopes. Earlier studies have shown that over half of the patients allergic to NRL have IgE against hevein,⁷⁻¹⁰ the chitin-binding N-terminal domain of prohevein. Immunologic and primary structure data show that the 18.7-kd turnip protein contains a hevein-like domain. Hevein-like, chitin-binding domains (about 43 amino acids) exist in many defense proteins of plants,³¹ and it has been reported that NRL,⁷⁻¹⁰ wheat,^{7,32} banana,³⁰ avocado, and chestnut³³ contain proteins with hevein-like domains and IgE-binding properties. Our preliminary observations suggest that a protein closely

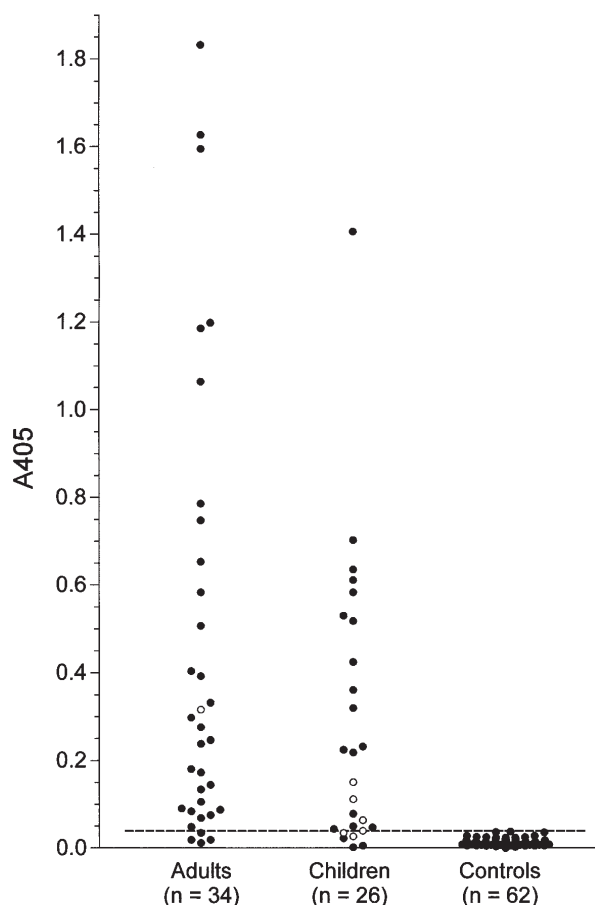


FIG 4. IgE binding to the purified 18.7-kd turnip protein in ELISA. Sera from 30 of 34 (88%) adults and 21 of 26 (81%) children allergic to NRL and previously shown to have IgE against prohevein showed IgE binding, whereas all control sera ($n = 62$) remained negative. Threshold value for positivity (mean OD value + 3 SD of the control sera) is indicated by the dotted line. Patients with histories of multiple surgical operations are marked with open circles.

resembling the 18.7-kd turnip protein may be common to the species of the Brassicaceae family. Several important cultivated plants belong to this family (eg, mustard, cabbage, broccoli, and cauliflower), as well as *Arabidopsis thaliana*, a widely used model plant in studies of plant-pathogen interactions. The immunologic properties and electrophoretic mobility of the proteins found from leaves of Chinese cabbage (*Brassica rapa var pekinensis*) and swede (*Brassica napus*) root are very similar to the 18.7-kd turnip protein (Hänninen et al, unpublished observation).

In ELISA-inhibition studies the IgE antibodies of the pooled sera of 12 patients allergic to NRL had greater affinity to the epitopes of prohevein and hevein than to similar structures of the 18.7-kd turnip protein. It thus seems likely that prohevein and hevein have been the primary sensitizers of the studied patients, and the IgE binding to the 18.7-kd turnip protein is a result of allergen cross-reactivity. Against this background it is of interest

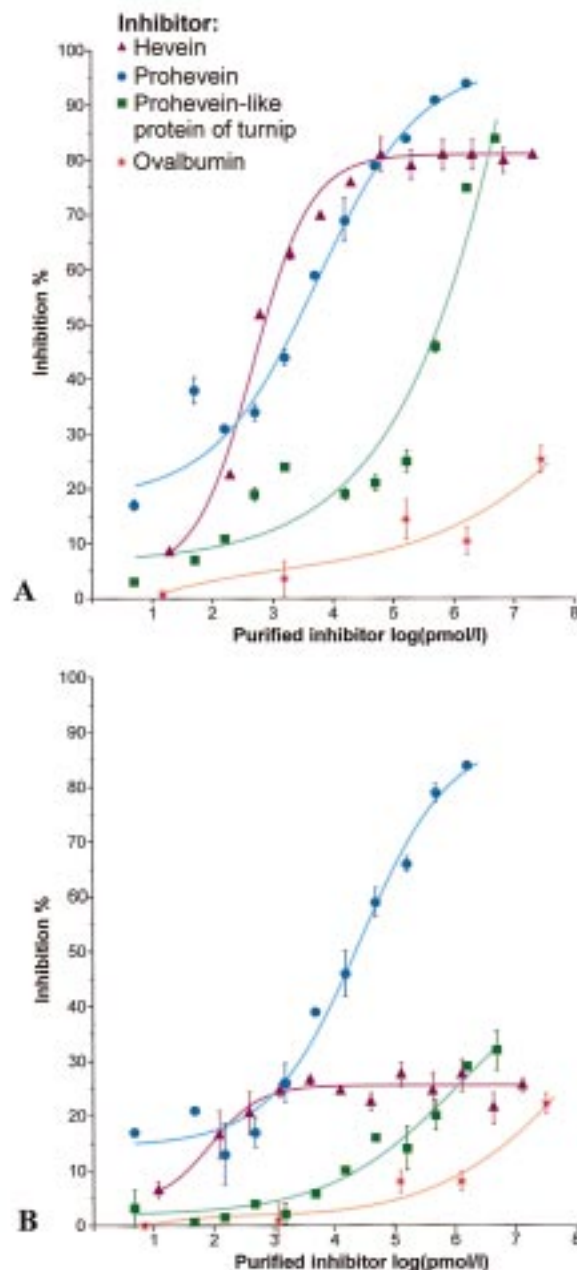


FIG 5. ELISA-inhibition experiments with hevein, prohevein, and the turnip 18.7-kd protein. Inhibition of IgE binding from pooled sera of 12 patients allergic to NRL to the turnip 18.7-kd protein (**A**) and prohevein (**B**) is shown. An unrelated allergen, ovalbumin, was used as a control. Range between duplicate measurements is indicated by vertical bars. Note that hevein, prohevein, and the 18.7-kd turnip protein all inhibited IgE binding to the solid-phase antigens used, but the shapes of the inhibition curves suggest that most of the cross-reacting IgE molecules have stronger affinity to hevein and prohevein than to the 18.7-kd turnip protein. The curves showing the inhibition caused by hevein end in distinct plateau phases at the points of about 80% (**A**) and 25% (**B**) inhibition, indicating that the cross-reactivity between hevein and other antigens is only partial.

that a great majority of patients allergic to prohevein recognized the 18.7-kd turnip protein (Fig 4), suggesting a close structural relationship between these 2 allergens.

Patients allergic to NRL are well suited for examination of the allergenicity of the defense-related proteins because many of the proteins in NRL have a defensive function. The novel 18.7-kd turnip protein may have broader significance apart from latex allergy because we have found patients allergic to different plants but not to NRL with strong IgE reactivity against the 18.7-kd turnip protein (Hänninen et al, unpublished observation).

Induction experiments resulted in marked differences in the appearance of IgE-binding proteins between individual turnips. Occurrence of the IgE-binding pathogenesis-related protein group 4-related 18.7-kd protein is consistent with earlier observations of the expression of this kind of defense protein.^{34,35} On the basis of the immunoblot inhibition experiments, the inducible and IgE-binding 14-kd protein contains antigenic structures similar to prohevein and is thus likely a defense protein, although it does not contain a hevein domain. To assess the role of the 18.7-kd protein in allergies to members of the Brassicaceae family, there must be certainty that skin prick test reagents contain the proper allergen. This emphasizes the importance of taking into account the physiologic state of a plant when preparations are made or plants are chosen for skin prick testing.

Various stressful conditions can significantly increase the production of defense-related proteins in plants.^{1,36} Environmental factors associated with the induction of defense-related protein synthesis include microbial pathogens, many chemical substances, mechanical damage, ultraviolet light, and ozone.³⁷ Ethylene and wounding have a parallel effect on the protein synthesis of plants: both induce production of many pathogenesis-related proteins.² Exogenous SA is able to induce a state called systemic acquired resistance (SAR) and expression of SAR genes to plants.³⁸ During the development of SAR, SA accumulates in plant tissues and systemic changes occur in the physiology of the plant, leading to a long-lasting resistance against different pathogens. Many proteins expressed during SAR are pathogenesis-related proteins.^{36,39}

Recently, there have been studies on possible ways to improve resistance of cultivated plants by increasing their defense protein contents. Genes coding defense proteins have been transferred from one plant to another,⁴⁰ and synthesis of defense proteins has been induced with chemical substances,^{5,26} typically provided by spraying of the developing plants. A synthetic chemical, benzothiadiazole, has been demonstrated to activate SAR and to give protection against a broad spectrum of diseases in crop plants.³⁷ Studies in progress are aimed at cloning genes regulating defense mechanisms of plants, and by means of these clones, genetically modified plants are going to be developed with SAR or other defense responses that are expressed optimally for agricultural production.²⁶

This study shows that activating defense mechanisms of plants may considerably increase their allergen content, demonstrated here through cross-reactive properties of the allergens. To prevent and control possible allergy-related health problems, the allergenic properties of

many inducible defense-related proteins should be taken into account when procedures are planned that might increase the amounts of these proteins in food or environment.

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