

Lipid Transfer Protein 1 Is a Possible Allergen in *Arabidopsis thaliana*

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Key Words

Allergens · Lipid transfer proteins · *Arabidopsis thaliana*

Abstract

Background: The *Arabidopsis thaliana* genome was recently fully sequenced, and this plant is now considered as the most useful model to study the effects of genetic engineering. The aim of the present study was to identify *A. thaliana* IgE-binding molecules and to localize their genes in order to evaluate the potential effect of gene insertion on the expression of IgE-binding molecules.

Methods: *A. thaliana* flower proteins were separated by two-dimensional gel electrophoresis and transferred onto a nitrocellulose sheet. The nitrocellulose sheet was successively incubated with human sera known to contain IgE that binds to rapeseed proteins, alkaline phosphatase-conjugated goat anti-human IgE and 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. One allergen was further identified by N-terminal amino acid microsequencing. **Results:** The results showed that some individuals possessed IgE that recognized numerous proteins with high molecular masses and various isoelectric points. This binding pattern strongly suggests that the epitopes recognized by these IgE could be, at least partly, sugar residues. Otherwise, out of the 10 sera that possessed IgE to *Arabidopsis* flower proteins, one

serum strongly recognized a unique basic protein with an apparent molecular mass of around 14 kD. This protein was identified by amino acid microsequencing as the lipid transfer protein 1 (LTP1). **Conclusion:** We have demonstrated that *A. thaliana* LTP1 is IgE reactive. The gene encoding this protein is located on chromosome 2, but it has been described that family 1 of *A. thaliana* LTPs constitutes a multigenic family with genes located on various chromosomes.

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Introduction

Since the genome of *Arabidopsis thaliana* has been fully sequenced, it should be possible to localize specific genes and follow the effects of genetic alterations, deletions or insertions on the expression of selected genes. In the field of allergy, *A. thaliana* is not yet known as an allergenic plant, and probably very few people are sensitized to it, mostly those who are professionally exposed. However, the identification of allergens in *Arabidopsis* – whether they are able to induce allergy or only to bind specific IgEs – may serve in establishing a model to study the effects of transgenesis on allergen expression.

It has been previously suggested that the allergenicity of a plant could vary according to the environmental con-

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ditions (e.g. pathogen attacks, physical or chemical stresses) because numerous pathogenesis-related proteins, including lipid transfer proteins (LTPs), are allergens [1, 2]. Thus, the genetic selection of the most resistant plants could result in an increase in plant allergenicity, as the pathogenesis-related proteins will be overexpressed in these plants. Hodgson [3] raised the question of whether gene products that present a threat to human health will occur more often in transgenic plants than in plants produced by traditional genetic crossing. After the identification of a brazil nut allergen in transgenic soybean [4], the possible expression of new allergens in genetically modified organisms has been a permanent matter of debate, and allergenicity has been considered a specific issue in the safety assessment of genetically modified crops. The first classical issue refers to the intrinsic allergenicity of the newly expressed 'foreign' protein(s) encoded by the inserted transgene(s) and possibly marker genes, which are expressed in edible parts of the plants. A decision tree for the assessment of the allergenic potential of novel plant proteins was proposed by FAO-WHO in 2001 [5]. However, this approach only focuses on the product of the transgene. The second issue refers to the host and to indirect, unintended effects of genetic modification on the allergenicity of the whole plant and derived products as a consequence of the random insertion of the transgene into the plant genome. This relates to a possible pleiotropic effect of the transgene and refers to the proteome of the whole plant. The question is whether genetically modified plants can have a different qualitative or quantitative composition of the endogenous allergens naturally present in the conventional plant. There is a need to identify those allergens and then to detect any modified (or increased) levels in the genetically modified plant due to overexpression as an indirect effect of the insertion of a transgene. Such determination of the allergen repertoire of genetically modified versus conventional plants using proteomics requires IgE from the sera of allergic individuals, and very few studies have been performed as yet. In the case of the glyphosate-tolerant genetically modified soybean, Western blotting studies showed that introduction of the transgene did not provoke a visible qualitative or quantitative change in the 'natural' endogenous allergen composition of transgenic soybean, compared with the various classical counterparts on the market [6].

As *A. thaliana* seems to be an appropriate plant material to study the effects of genetic engineering, the aim of the present work was to identify *A. thaliana* proteins that are recognized by human IgE in order to evaluate the possibility of developing a model to study the expression of aller-

gens in genetically modified organisms. Our hypothesis is that, if allergens can be identified, their gene will be localized and it will become possible to modify the genetic environment of the genes (e.g. by transgene insertion or deletion) and to evaluate the impact of such modifications on allergen expression.

Materials and Methods

Human Sera

The human sera were collected from biological analysis laboratories and represented residues of IgE titer evaluations. These sera were selected according to their sensitivity to birch and grass and then tested to detect the presence of IgE to oilseed rape pollen and *Arabidopsis* flower proteins. The first criterion of selection was chosen because the pollinic season of birch and grass overlaps that of oilseed rape, and oilseed rape was chosen because it is a widely cultivated crop related to the *Brassicaceae* family, as is *Arabidopsis*. Out of the 145 sera tested, 45 had IgE to oilseed rape pollen proteins and 10 of these 45 also had IgE to *Arabidopsis* flower proteins.

Extraction of Arabidopsis Flower Proteins

Mature *A. thaliana* flowers (wassilewskija) were provided by the groups of Dr. Pedersen (Danish Veterinary and Food Administration, Denmark) and Dr. Leguay (CEA Cadarache, France).

One hundred and seventy-five milligrams of *Arabidopsis* flowers were incubated with 860 µl of 2% CHAPS in distilled water (1 h, 22°C), and the extract was recovered by centrifugation (16,000 g) followed by filtration (glass wool).

Identification of Arabidopsis Allergens in Two-Dimensional Immunoblots

Arabidopsis flower proteins were separated by an isoelectric-focusing (IEF) pH 2–11 followed by an SDS-PAGE 8–18% gradient gel (ExcelGel, Amersham Biosciences, Uppsala, Sweden). One two-dimensional (2-D) gel was stained with silver nitrate according to the method of Blum et al. [7], and the others were submitted to a Western blot. The proteins separated by 2-D electrophoresis were electroblotted onto a nitrocellulose sheet with a semidry Novablot apparatus (Amersham Biosciences) following the manufacturer's instructions (1 h, 1 mA/cm²). Just after blotting, each nitrocellulose sheet was dried and blocked with PBS containing 1% bovine serum albumin and 0.1% Tween 20 (PBS-BSA-Tw) for 1 h at 22°C. Then, each nitrocellulose sheet was incubated successively with a selected human serum (1:10 in PBS-BSA-Tw, overnight, 22°C) and alkaline phosphatase-conjugated goat anti-human IgE (1:700 in PBS-BSA-Tw, 2 h, 22°C; Sigma Biochemicals, USA). The alkaline phosphatase activity was detected using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Sigma Biochemicals) in 0.1 M Tris buffer, pH 9.5.

After detection on 2-D gels, one allergen was isolated by double one-D [8], and its N-terminal amino acid sequence was determined. Microsequencing was performed by Edman degradation with an Applied Biosystems sequencer (model 473A). Amino acid sequence similarity with already known proteins was sought by using the Basic Local Alignment Search Tool algorithm (PIR, Swiss-Prot and Genpept databases) [9].

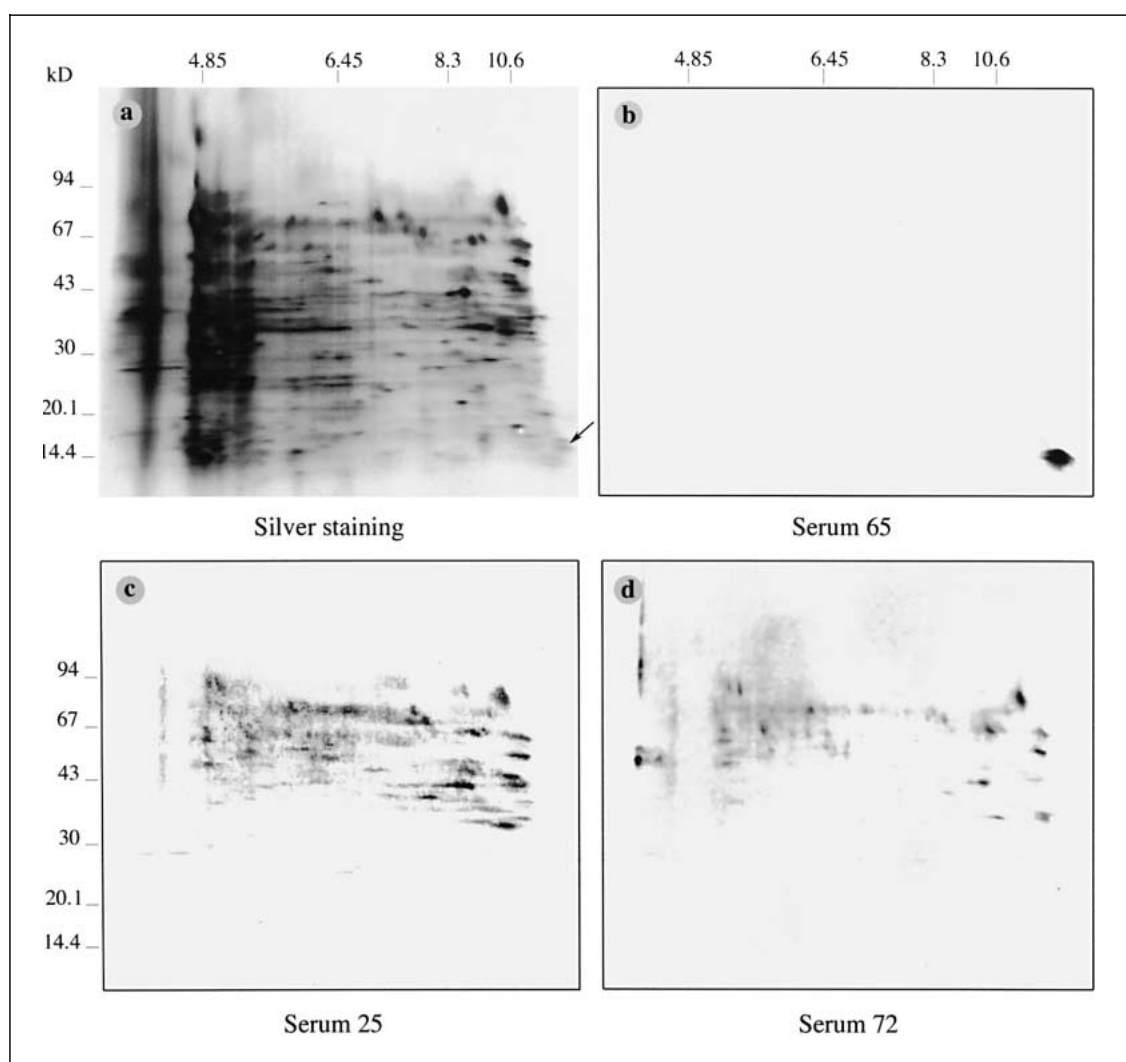


Fig. 1. 2-D analysis of *A. thaliana* flower allergens. Flower proteins were separated by an IEF (pH 2–11), followed by SDS-PAGE (8–18% gradient gel). The 2-D gels were silver stained (**a**) or submitted to a Western blot and incubated with human sera (**b–d**) to detect IgE-binding molecules. Note that sera 25 and 72 displayed similar patterns with numerous spots, while serum 65 was specific to one protein. The protein that bound serum 65 IgEs was located on the 2-D gel (**a**, arrow), sequenced and identified as an LTP1.

Results

The identification of *A. thaliana* allergens was performed using flower extract. Mature flowers were used as a substitute for pollen, which is too difficult to harvest in sufficient amounts to perform the experiments carried out here. In a previous study, we demonstrated that flower extracts may be a good alternative source to study pollen allergens, even though additional allergens could be found in flowers [10]. The 2-D gel analysis of the *Arabidopsis*

flower extract (fig. 1a) shows that 2% CHAPS in water allows good extraction of *Arabidopsis* flower proteins that can be further well separated in 2-D gels.

Out of the 10 human sera positive to *Arabidopsis* flower proteins, four were tested on 2-D blots. One serum was almost negative (weak staining), and the results of the other three 2-D blots are shown in figure 1. Two sera (but also the weak one) recognized numerous proteic spots with molecular masses ranging from 35 to 94 kD and various isoelectric points (fig. 1c, d).

Fig. 2. Sequence alignment of known sequenced LTP allergens. Note that sequence similarities (gray shading) are found particularly around the cysteine residues. *Ara t* = *A. thaliana*; *Pru p* = *Prunus persica* (peach), 55% identity; *Pru av* = *Prunus avium* (sweet cherry), 53% identity; *Pru ar* = *Prunus armeniaca* (apricot), 57% identity; *Pru d* = *Prunus domestica* (garden plum), 54% identity; *Pyr c* = *Pyrus communis* (pear), 52% identity; *Mal d* = *Malus domestica* (apple), 52% identity.

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Ara t  AGVMKLACL--LLACMIVAGPITSNAALSCGVSNSNLAACIGYVLQGGVIPPACCSGVKNLN
Pru p  -----ITCGQVSSALAPCIYVRGGGAVPPACNGIRNVN
Pru av  SAMTKLALV--VALCMVSVPIAQ--ALTCGQVSSNLAPCIAYVRGGGAVPPACNGIRNVN
Pru ar  -----ITCGQVSSSLAPCIYVRGGGAVPPACNGIRNVN
Pru d  --LLKLVLVAVMCCMAVGGP-KAMAAVSCGQVNNLTPCINYYVANGGALNPSCCTGVRSLY
Pyr c  SAVIKLALV--VALCMAV----SVAHAITCSQVSANLAPCINYYVRGGGAVPPACNGIKTIN
Mal d  SAVTKLALV--VALCMAV----SVAHAITCGQVTSSLAPCIYVRSGGAVPPACNGIRTIN

Ara t  SIAKTTPDRQQAACNCIQGAARALG-SGLNAGRAAGIPKACGVNIPYKISTSTNCKTVR
Pru p  NLARTTPDRQAACNCLKQLSASV--PGVNPNNAAALPGKCGVHIPYKISASTNCATVK
Pru av  NLAKTTADRQTACNCLKQLSASV--PGVNANNAALPGKCGVNPYKISPSTNCATVK
Pru ar  NLARTTPDRRTACNCLKQLSGSI--SGVNPNNAAALPGKCGVNPYKISASTNCATVK
Pru d  SLAQTTADRQSICNCLKQAVNGIPYTNANAGLAAGLPGKCGVNPYKISPSTDCSISK
Pyr c  GLAKTTPDRQAACNCLKNLAGSV--SGVNPNGAESLPGKCGVNPYKISTSTNCATVK
Mal d  GLARTTADRQTACNCLKNLAGSI--SGVNPNNAAAGLPGKCGVNPYKISTSTNCATVK

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One serum displayed a more restricted specific pattern and recognized only one spot with a very basic pI (>10) composed of two spots very close together with apparent molecular masses of around 14 kD (fig. 1b). The N-terminal amino acid sequence of this protein was ALSCGVSNSN. This sequence displayed 100% identity with amino acids 26–35 of the LTP1 precursor from *A. thaliana* and corresponds to the N-terminal sequence of the expressed LTP1 obtained after removing the signal peptide. The gene encoding this sequence is located on chromosome 2, position 72cM, bacterial artificial chromosome T6A23 (AC005499, *Arabidopsis* databank, www.arabidopsis.org). The sequence of this *Arabidopsis* LTP was compared to other known allergenic LTPs that displayed more than 50% identity (fig. 2). Similar sequences were mostly found around the cysteine signature.

Discussion

In the present work, we found that some patients allergic to grass and birch pollen possess IgE that recognize *Arabidopsis* flower proteins. Sera from 3 of these patients recognized numerous high-molecular-mass allergens. When tested against rapeseed pollen proteins, these sera displayed a very similar IgE-binding pattern that was abolished when free sugar residues were added to the sera during the incubation. Thus, such a recognition pattern is very likely to involve carbohydrate cross-reacting residues. Otherwise, one patient recognized only one protein identified as an LTP1.

LTPs are small hydrophilic proteins (91–95 amino acids, apparent molecular masses ranging from 7 to 14 kD)

with the characteristic presence of eight cysteines forming four disulfide bridges. Two families of LTPs have been identified: LTP1s have molecular masses of around 9 kD and LTP2s have molecular masses of around 7 kD, and no or low sequence similarities are found between LTP1s and LTP2s, except the cysteine signature [11]. These proteins constitute the family 14 of pathogenesis-related proteins, and play a key role in resistance to biotic and abiotic stresses [2]. Sixteen LTPs have received a nomenclature designation and are included in the allergen bank of the Allergen Nomenclature Sub-Committee of the International Union of Immunological Societies (www.allergen.org). These allergens are mostly implicated in allergy to *Prunoideae* fruits [12–17], but also to maize [18], nuts [19], barley [20] and *Parietaria*, *Artemisia* and *Brassica rapa* pollen [21–23]. All these allergens belong to the LTP1 family, except in turnip (*Brassica rapa*), where an LTP2 was identified as an allergen [23]. Thus, LTPs are considered as potential plant panallergens [24, 25], with more than 90% sequence homology within the *Prunoideae* fruits resulting in frequent cross-sensitizations between plum, apricot and peach [13, 15]. Moreover, Diaz-Perales et al. [21] demonstrated a partial cross-reactivity among LTPs of mugwort pollen, chestnut seeds and apple and peach peel. We also found that serum No. 65 recognized a basic protein with an apparent molecular mass of 14 kD in rapeseed pollen extract (data not shown). This fact can be explained by sequence similarities between this *Arabidopsis* LTP and the three isoforms of *Brassica napus* LTP1 (accession numbers: Q42614, Q42615 and Q42616), which displayed between 68 and 71% identity. The alignment sequence of *Arabidopsis* LTP with other known allergenic LTPs from fruits shows more than 50% identity, especially around the cysteine residues and in

the C-terminal region. Thus, cross-reactions between *Arabidopsis* and previously described allergenic LTPs are likely to occur.

In *A. thaliana*, LTP1s are encoded by a small multigenic family. Fifteen genes have been evidenced in the 9-kD ltp gene family, which could contain a total of 15–20 genes [26]. These genes are located on chromosomes 2, 3, 4 and 5, consisting of small subfamilies of 1–3 genes [26]. The gene coding the LTP identified in this study (ltp1) is located on chromosome 2, position 72cM, bacterial artificial chromosome T6A23 (AC005499), close to the ltp2 gene [26]. When compared to the other amino acid sequences deduced from the ltp genes, the percentage of identical residues between LTP1 and other ltp products varies from 19.4% (ltp15) to 64% (ltp5), and ltp1 and ltp2 exhibit more than 75% identity at the nucleic acid level [26].

The regulation of the expression of the ltp genes displays a temporospatial pattern, and some of them are inducible by biotic or abiotic stresses. In *Arabidopsis*, all these genes are highly expressed in flowers [26], and it has been reported that ltp1 contains expression-controlling motifs that can be induced by various stresses or pathogen attack [27].

In conclusion, this study identifies an LTP1 from *A. thaliana* that is recognized by human IgE. The gene

encoding this allergen is located on chromosome 2. To our knowledge, this is the first time that one locate a gene coding for an allergen in a genome. LTP genes constitute a multigenic family, but only one spot was recognized as an allergen in flowers, suggesting that sometimes a very restricted set of genes may encode for allergens. Taking advantage of the localization of the LTP gene in the fully sequenced genome of *Arabidopsis* together with the probe anti-LTP1 IgE evidenced in this report, LTPs in *Arabidopsis* may be useful to study whether genetic engineering around this specific gene modifies the expression of the allergen. Thus, this model could be valuable to define rules to study the impact of genetic engineering on the allergenicity of transgenic plants.

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