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Localization of the main allergy protein in two apple cultivars grown in Sweden

Lokalisering av det främsta allergiframkallande proteinet i två äppelsorter odlade i Sverige

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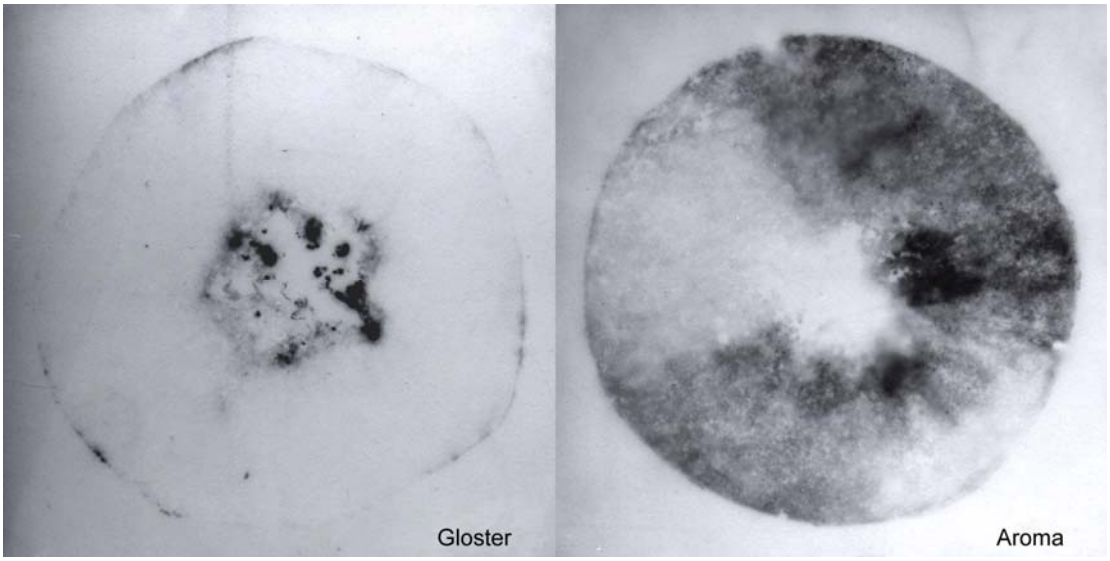

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Abstract

Mal d 1 is an 18 kD protein and identified as a major allergen linked with pollen-associated allergies in apple. Previous studies have indicated that the amount of Mal d 1 is not only strongly dependent on apple cultivar and degree of maturity, but also has a tendency to increase during prolonged storage compared to freshly picked apples. Furthermore, it has been suggested that the most allergenic part of the apple is the peel.

The aim of this study was to evaluate the use of ITP (immunotissue printing) as a method with the purpose of visualizing the localization and distribution of the main apple allergen, Mal d 1, in two apple cultivars grown in Sweden. The method includes the use of antibodies and allows for large quantities to be screened in a relatively short time compared to ILM (immunolight microscopy), which is the commonly practiced method today. In order to make a comparison, both methods were tested and evaluated. In addition, ITP was used to see if and how different storage conditions as well as cultivation methods have an effect on the content of Mal d 1. The chosen material for the study was two apple cultivars grown in Sweden: 'Aroma', known to have a relatively high content of allergen and 'Gloster', known to have a low content of allergen

Comparing immunotissue prints of 'Aroma' and 'Gloster' verified the vast differences in allergen content between the two. 'Aroma' proved to have a relatively high content of allergen distributed both in peel and the pulp. In 'Gloster', the allergen content was low and was mostly concentrated to the peel and core. The highest contents of allergen were found in apples stored in normal atmosphere and the single strongest indication of Mal d 1 was found in the apple from conventional production which was harvested at the later date. Apples from organic production displayed the lowest contents of all.

The experiences made from this study can confirm that ITP is a much more efficient method compared to ILM using immunogold labeling concerning time and labour intensity.

Provided that my results can be confirmed with a larger material, they may help to minimize allergen levels by adjusting storage and cultivation practices. In the future, production of low-allergenic apples can help to support the organic apple farming.

Suggestions for further research in this area could include using whole, transverse slices of apple, combining ITP with a more quantitative method such as ELISA of the same tissue, comparing weather data from different growing seasons, and taking more accurate data for the trees and apples analyzed in terms of e.g. nourishment and exposition to the sun etc.

Sammanfattning

Mal d 1 är det främsta äppleallergenet i Norra Europa. Det tillhör en grupp av allergen som ger upphov till korsallergi mellan pollen och frukt.

Tidigare studier har visat att halten av Mal d 1 skiljer sig beroende på äppelsort och mognadsgrad men också att den är högre i äpplen som lagrats länge jämfört med

nyplockade äpplen. Det har även funnits tendenser till att halten är högre i skalet än i resten av frukten.

Målet med den första delen av studien var att utvärdera ITP (immunotissue printing) som metod för att visualisera var i äppelvävnaden Mal d 1 finns samt hur det är fördelat i två äppelsorter odlade i Sverige.

ITP är en metod som bygger på användandet av antikroppar för att studera specifika ämnen. Den tillåter att väldigt stora material kan studeras på kort tid jämfört med ILM (immunoljusmikroskopi) som är den vanliga metoden för den här typen av studie. Som en del av utvärderingen av ITP testades båda metoderna för att en jämförelse skulle kunna göras.

Målet med den andra delen av studien var att med hjälp av ITP undersöka om och hur olika lagringsmetoder, odlingsmetoder samt skördedatum påverkar innehållet av Mal d 1.

Det valda materialet för studien var sorten ’Gloster’, som är känd för att ha ett lågt allergeninnehåll samt sorten ’Aroma’, som är känd för att ha ett relativt högt allergeninnehåll.

ITP-resultaten kunde visa och därmed verifiera att ’Aroma’ och ’Gloster’ har väldigt olika innehåll av Mal d 1. ’Aroma’ visade sig ha en hög halt av Mal d 1 fördelat både i skal och i fruktkött. ’Gloster’ visade sig ha låga halter av Mal d 1 koncentrerat till kärnhus och skal.

De högsta halterna av Mal d 1 fanns i äpplena som hade lagrats i normal atmosfär. Den absolut högsta halten fanns det äpple som hade odlats konventionellt och skördats vid en sen tidpunkt. Äpplena som hade odlats ekologiskt visade de lägsta halterna. Erfarenheten från den här studien kan även bekräfta att ITP är en mer effektiv metod än ILM vad gäller tid och arbete.

Om dessa resultat framöver kan bekräftas med ett större material skulle det innebära att det faktiskt finns en möjlighet att minska allergeninnehåll genom modifierade lagrings- och odlingsmetoder. I framtiden skulle även produktion av lågallergena äpplen kunna ge ett stöd för ekologisk äppelodling.

Förslag för vidare försök inom detta område skulle kunna vara att använda sig av hela tvärsnitt av äpple, att kombinera ITP med en mer kvantitativ metod såsom ELISA, att använda sig av väderdata från olika odlingssäsonger samt att samla in mer specifik information om träd och frukt såsom position, näringstillgång och solexponering.

Preface

This project was initiated by Dr Salla Marttila and Dr Helena Persson Hovmalm at the department of Crop Science at the Swedish University of Agricultural Sciences, Alnarp, Sweden. The thesis is performed as a Bachelor of Science degree in the frame of the Swedish-Danish Horticulture Programme, of SLU, Alnarp. It serves as a pilot project to give rise to further research in the area. In addition, some of the results have already been presented in a poster (ISHS, Seoul, South Korea, 2006) and in a scientific paper (Nybom *et al*., in press).

Abbreviations
ITP, Immuno tissue printing, ILM, Immuno light microscopy; RAST, radio allergosorbent test; SPT, skin prick test; EAST, enzymes allergosorbent test; CAP, coated allergen particle test; Ig, Immunoglobulin; GRO, Gröna Näringens Riksorganisation; ULO, ultra low oxygen; NA, normal atmosphere; CA, controlled atmosphere; OAS, oral allergy syndrome; BSA, bovine serum albumin; PR-10, pathogenesis-related protein 10 family; ELISA, Enzym-linked Immunosorbent Assay.

Introduction

This study investigates a relatively new method called immunotissue printing (ITP) for screening of apple tissues for allergens. One of the forerunners in this area was Joseph Varner. He worked with the concept of tissue printing which by itself is a method of visualizing cellular material and information through pressing cut surfaces against receptive surfaces. It has allowed biochemical as well as anatomical imaging and except for being economical it also gives a valuable insight of distribution of substances within different tissues (Varner & Ye, 1994).

Immunotissue printing includes the use of antibodies in order to confirm and visualize the occurrence of specific proteins in the chosen tissue. It is a method which allows for large quantities to be screened in a relatively short time compared to immunolight microscopy (ILM). It also gives a detailed overview of the whole tissue and visualizes localization as well as distribution of the chosen substance.

Immunolight microscopy is the common method used in this area today. However, it only gives a picture of a very small part of the tissue as well as being both time consuming and labour-intensive. Other common methods used in this area are immunoblotting and ELISA. They are very valuable for qualitative and quantitative analysis of actual contents but less efficient in producing an instant overview of the tissue.

The chosen material for this study was two apple cultivars grown in Sweden; ‘Aroma’ and ‘Gloster’. ‘Aroma’ is known to have a relatively high content of allergen and ‘Gloster’ is known to have a low content of allergen (Persson Hovmalm *et al*., unpublished observations, Son *et al*., 1999). In some Swedish supermarkets, ‘Gloster’ is even marketed as “the apple for the allergic”.

An ongoing postharvest study of ‘Aroma’ at the SLU research station at Balsgård also gave the opportunity to include some of that apple material. The apples had been grown according to different cultivation standards and stored in different conditions (Tahir, 2004a). Among the cultivars grown commercially in Sweden today, ‘Aroma’ is the second most important. Unfortunately it does not keep well during storage (Tahir, 2004b).

Allergy is an increasing public health problem. To develop analyzing methods like ITP in this area will increase knowledge of the localization and distribution of allergens in different apple cultivars and perhaps also give answers to how, and if, it is possible to influence the occurrence of these substances. For example, by optimizing cultivation methods and post harvest handling of fruits.

Allergy

Allergic diseases are one of the most common public health problems. They can appear in all age groups and in many different organs. The prevalence of these diseases has been increasing since decades ago in Sweden as well as the rest of the Western World (Johansson *et al.,* 2006).

Allergy is a usually lifelong, immunological oversensitivity which causes the body to produce allergen-specific IgE antibodies against allergens in our environment. Antibodies are part of the body's defense system and belong to a group of proteins called immunoglobulins (Ig). The immunoglobulins are named differently based on the structure of the molecule (Johansson *et al.,* 2006).

Today allergy is confirmed by making a skin prick test (SPT) or taking a blood sample and analyzing it for allergen-specific IgE antibodies (RAST). Atopy is a genetical propensity to form IgE antibodies against proteins (allergens), even in small amounts (Formgren, 2006). IgE antibodies can give rise to allergy but the target organ sensitivity, dose of allergen, concurrent infection, and strain are influencing factors. By contrast, IgG antibodies against allergens in our close environment often occur without causing symptoms. If the content of IgG antibodies is not too high it does not necessarily indicate allergy but simply that there has been contact with the allergen (Johansson *et al.,* 2006).

Food allergy is defined as an IgE-mediated reaction (Type 1 allergy) upon repeated exposure to an allergen source. In contrast, food intolerance is defined as a non-immune-system mediated adverse reaction based on an enzymatic defect or pharmacological mechanism (Anonymous, 2006b). The diagnosis of IgE-mediated food allergy is based on combinations of several diagnostic procedures. The identification of allergen-specific IgE present in the serum is performed e.g. with RAST, EAST and CAP. For laboratory use there are further assays like IgE-immunoblots, and RAST-inhibition and histamine release assays (Anonymous, 2006b).

Properly performed epidemiologic studies regarding allergies to plant-derived foods are not available at present. Therefore, the true prevalence of food allergy is still under discussion. European reports indicate that 0.3-0.75% of children and 2% of adults suffer from some type of allergy, while the prevalence among atopic individuals is higher (10%) (Anonymous, 2006b). In Northern Europe, e.g. Sweden, about 70% of atopic people also show reactions towards certain foodstuffs (with apples taking a top position) (Eriksson *et al*., 1982). Due to changes in life style and eating habits, the prevalence of allergic reactions to food seems to have increased over the last ten years (Anonymous, 2006b).

Oral allergy syndrome (OAS) is an allergy towards certain raw fruits, vegetables, seeds, spices and nuts causing allergic reactions in the mouth and throat. These allergic reactions happen mostly in people with hay fever, especially spring hay fever due to birch pollen. This correlation between allergies is called the pollen-fruit syndrome. The pollen-fruit syndrome is the most common form of oral allergy syndrome. A number of pollen allergies may be connected with the condition but the most common is birch pollen (Anonymous, 2006e; Pong, 2000).

Allergen – Mal d 1

An allergen is a special type of antigen which causes an IgE antibody response. It is also usually a protein (Anonymous, 2006c). Antigen is an abbreviation of "antibody generator" and is referred to, within immunology, as a substance foreign to the body. When it enters the body, it causes the immunosystem to react. This reaction leads to the production of antibodies and the recruitment of white blood corpuscles which attack the antigen (Anonymous, 2006d).

Allergens already identified from apple are Mal d 1 (a Bet v 1 homologous allergen), Mal d 2 (apple thaumatin-like protein), Mal d 3 (apple non-specific lipid transfer protein), and Mal d 4 (apple profilin) (Anonymous, 2006a).

The SAFE project, funded by the European commission to stem the rise in food allergies and ensure a healthy diet for all consumers, has made a study where 400 apple allergic patients across Europe were included. They found that Bet v 1 related food allergies which cause oral allergy syndrome with rather mild symptoms, are typical for Northern and Central Europe. Fruit allergies without pollen allergies, often causing more severe symptoms, are typical for Southern Europe. These allergies are most often related to Mal d 4 (Anonymous, 2006a).

Mal d 1 is an 18 kD protein and identified as a major allergen associated with pollen-related allergies in apple. The reason for birch and apple allergy cross reaction is high sequence homology of Mal d 1 to the major birch pollen allergen Bet v 1. As these allergens were cloned and sequenced in previous studies, they were found to display a high degree of sequence identity (Vieths *et al*., 1995, Fritsch *et al.*, 1998). The Mal d 1 sequence has between 62 %, over 25 amino acids and 67,6 %, over 37 amino acids, in common with the sequence of Bet v 1 (Beuning *et al*., 2004, Marzban *et al*., 2005).

The allergic response towards Mal d 1 is usually mild (OAS) and the symptoms are usually restricted to lips, tongue and throat. They only appear after ingestion of fresh fruit since this allergen is rendered harmless by cooking, oxidation and proteolytic digestion (Sancho *et al.*, 2006a).

The most allergenic part of apple may be the peel (Fernandez-Rivas & Cuevas, 1999, Beuning *et al.*, 2004). This means that some people that are allergic to fruits can eat the flesh without reaction if the peel is removed. Some apple cultivars cause more allergic reaction than others. Freshly picked or unripe apples may cause fewer allergic reactions than fruit which is very ripe or which has been stored for several weeks after picking. The allergic reactions are treated by avoidance and sometimes with antihistamines (Pong, 2000). Possibly in line with these observations, Pühringer *et al*. (1999) reported that the amount of Mal d 1 is not only strongly dependent on apple cultivar and degree of maturity, but also has a tendency to increase during prolonged storage compared to freshly picked apples. This has since then been supported by Beuning *et al*. (2004) and Skamstrup Hansen *et al*. (2001).

Mal d 1 also belongs to the pathogenesis-related protein 10 family (PR-10). These proteins are thought to be expressed in response to fungal or bacterial infection and stress and thereby involved in the plant defense. However, the exact biological role of Mal d 1 is still unclear. A recent publication by Sancho *et al.* (2006b), discloses that it may involve binding and transport of plant steroids. It has also been suggested that

Mal d 1 has a role in intracellular signaling since it was found that it can bind the recently detected apple protein, MdAP (Sancho *et al.*, 2006a).

So far, 18 isoforms of Mal d 1 have been detected. They are clustered into four groups, 1.01-1.04, identified to date. In the skin and pulp of the fruit, different isoforms are present at different levels. This can partly explain why there is variability in the allergenic potency of different cultivars. Furthermore, the Mal d 1 isoforms have been found to have different IgE binding capacities (Sancho *et al.*, 2006a).

Antibodies as detection tools

As mentioned, antibodies belong to a group of proteins called immunoglobulins (Ig). Listed in order of decreasing quantity found in plasma or serum, the immunoglobulins comprise five major classes: immunoglobulin G (IgG), IgA, IgM, IgD and IgE. IgG and IgM are the most frequently used antibodies in immunohistochemistry. Each immunoglobulin consists of two identical heavy chains (H) and two identical light chains (L). The H chains differ in antigenic and structural properties and determine the class and subclass of the molecule (Naish *et al.*, 1989).

Antibodies are useful for the detection, measurement, and purification of intracellular and extracellular constituents of animals and plants and of their pathogens. Their high sensitivity coupled with a potential for high specificity, can be used to indicate the presence of a given antigen (allergen) in whole cells, tissues, and in crude and purified extracts of plant material. Antibodies are useful in plant biochemistry, cell and molecular biology to localize key cellular constituents (enzymes, structural proteins, etc.) to quantify developmental or other changes and to identify specific gene products. Antibody-antigen binding also provides a basis for affinity purification of antigens.To produce antibodies, allergen (antigen) is injected into animals such as mice or rabbits. The amounts of antigen required to induce an immune response vary depending on the allergen and the animal (Dewey *et al.*, 1991).

Polyclonal antibodies are produced by different cells and are as a result immunochemically dissimilar. They react with various epitopes on the antigen against which they are raised. Monoclonal antibodies are produced by clones of plasma cells. Therefore they are immunochemically identical and react with a specific epitope on the antigen against which they are raised (Naish *et al*., 1989).

Cultivation and storage of apples in Sweden

Compared to the rest of the world, the apple cultivation in Sweden is very small. The total production area in Sweden today is 1 440 ha and the total harvest is approximately 18000 ton (Anonymous, 2006a).

Approximately 65% of all apples bought in Swedish shops are produced abroad. This is thought to be the result of low prices and an all-year-round supply. However, the season for Swedish apples can, nowadays, be prolonged with the use of ultra low oxygen (ULO) storage (Ekenstierna, 2004).

The use of the ULO storage method for apples is increasing in Sweden. In ULO storage, the composition of the atmosphere is altered to keep the oxygen level reduced and the carbon dioxide level raised. This slows down the respiration rate and the senescence of the fruit (Tahir, 2004b).

Today, there is an ongoing postharvest study of apples at Balsgård research station. The project was initiated by Dr Ibrahim Tahir at The Department of Crop Science, SLU and Eckard Ahner at the fruit section of GRO. The goal of the project is to determine the optimal time for harvest and the optimal storage conditions for cultivars 'Aroma' and 'Amorosa'. Fruit harvested at the optimal date or ten days later has been compared, as also fruit stored in normal atmosphere (NA) or in ULO as well as fruit kept at different gas compositions in ULO. So far, the optimal time for harvest has been determined; harvesting during this period greatly enhances the storage quality and decreases postharvest losses (Tahir, 2004a). The effect of production area on quality and losses was marginal. ULO storage also proved to be superior compared to conventional refrigerator storage in all steps of the experiment concerning total losses, fruit flavor and firmness. Of the apples stored in ULO atmosphere, the best results for both cultivars were found at 3°C and 1% or 3% O_2 and 3% CO_2 . At these conditions the total loss was smaller and the flavor was better. Damage and diseases were much more prevalent in apples stored at 1°C and resembled the result of too low temperature.

Aim

The aim of this study was to evaluate the use of ITP as a method in the purpose of visualizing the localization and distribution of the main apple allergen, Mal d 1, in two apple cultivars grown in Sweden. The cultivars used were 'Aroma' known to contain a relatively high content of allergen and 'Gloster' known to contain a low content of allergen (Persson Hovmalm *et al*., unpublished observations, Son *et al*., 1999). The commonly practiced method today for localization studies is immunolight microscopy. In order to make a comparison, both methods were tested and evaluated. In addition, ITP was used to see if and how different storage conditions as well as cultivation methods have an effect on the localization and quantity of Mal d 1.

It was expected that some of the results would be in line with previous studies which have confirmed that there is a greater expression of Mal d 1 in 'Aroma' compared to 'Gloster' (Persson Hovmalm *et al*., unpublished observations, Son *et al*., 1999). Concerning the apples of the postharvest study at Balsgård, the hypothesis was that storage in normal atmosphere would result in a higher content of allergen than the ULO storage and also that there would be some differences due beween the orchards, where the fruit was harvested, as already reported by Hsieh *et al.* (1995), Skamstrup Hansen *et al.* (2001) and Bolhaar *et al*. (2005).

Materials and methods

Material:
For testing the methods, apples of different cultivars and production areas were used. In the first part of the study, content of Mal d 1 in two different cultivars was compared. The cultivars used were 'Aroma' and 'Gloster' and came from a fruit production farm called Solnäs Gård in Fjelie and a supermarket in Malmö called Hemköp. For comparing the possible influence of storage conditions and cultivation methods on content of Mal d 1, apples of the 'Aroma' cultivar were used. They were obtained from different orchards and had been grown according to different cultivation standards. Apples grown according to IP standards were from Kivik, apples grown according to conventional standards from Lund and organically grown apples from another orchard in Kivik. IP stands for integrated production and is a farming concept with environmental-friendly cultivation methods (IP-Handbok, 2005). The apples were stored in normal atmosphere (NA) at 3°C and in ultra low oxygen atmosphere (ULO) at 3°C and a gas composition of 3% O_2 and 3% CO_2. Furthermore, they have been harvested at optimal and late dates, except for the organic apples, which have only been harvested at an optimal date.

The primary antibody was a gift from Dr. R. Van Ree, The Netherlands. It is a monoclonal antibody against Bet v 1 (clone 5H8).

Immunotissue Printing

Tissue printing:
Before starting the experiment, optimization of the printing technique was necessary. Some information of the procedure was found in the work of Varner & Ye (1994) and Taylor et al. (1993).

Transverse sections of apple of approximately 0.6 cm were made using a kitchen slicer. Tissue prints were made on nitrocellulose membrane using a glass plate and medium pressure for 10 s. Before printing onto membrane, some excess fluid was wiped from the apple slices. The prints were left to dry in room temperature.
To evaluate the amount of pressure and time needed to give an even apple tissue print on the nitrocellulose membrane (Amersham Pharmacia Biotech UK Limited, Buckinghamshire, England), a total protein staining with Coomassie blue was used.

The prints were stained in a solution of 0.1 % Coomassie Blue and 20 % aqueous methanol containing 7% acetic acid for 5 minutes. Then they were destained using distilled water and a solution of 50% methanol, 7% acetic acid for approximately 20 minutes. All steps were performed with gentle agitation (Taylor *et al*., 1993).

After drying in roomtemperature, the prints were evaluated. The best prints were made using a glass plate and both hands with approximately medium pressure for 10 seconds. However, it should be stated that there were still sometimes difficulties getting a completely even print. Attempts to use weights in stead of hand pressure, in order to give continuity and higher reliability, were however even less successful in giving even prints. A prolonged time of pressure (20s, 30s) was also less successful.

Immunostaining of tissue prints:
Transverse sections of apple of approximately 0.6 cm were made using a kitchen slicer. However, due to the limited amount of primary antibody available, only 1 quarter of a slice was used for printing. The process began with washing of the membranes for 2 x 5 min using 1 x TBS (Tris buffered saline, 50mM Tris base; 150 mM NaCl, pH 7.5) on shaker. This was followed by blocking of the membranes for 1 hour on shaker, in a blocking buffer (1% Western blocking reagent, Roche Applied Science, Penzberg, Germany, diluted with 1 x TBS). Thereafter, the membrane was incubated for 1 hour with primary antibody (monoclonal mouse antibody against Bet v 1, 5H8) diluted 1:500 in antibody buffer (1% blocking buffer + 1 volume TBS, 1:1). The membranes were then washed in washing buffer (0.1 % Tween 20 in TBS) 1x 15 minutes plus 2 x 5 minutes on shaker. After this, it was time to incubate the membrane for 1 hour with the secondary antibody (anti-mouse IgG, peroxidase-linked species-specific whole antibody (from sheep) NA 931, Amersham Biosciences UK Limited, Buckinghamshire, England), diluted 1:5000 in antibody buffer. Incubation ended with washing of the membranes with washing buffer (0.1 % Tween 20 in TBS) for 1 x 15 minutes plus 4 x 5 minutes on shaker.

After the final washing of the membranes it was time for detection and developing. The membranes were placed on a plastic plate and detection solution (ECL Plus Western blotting detection reagents, Amersham Biosciences UK Limited, Buckinghamshire, England) was added with an incubation time of 5 minutes. The membranes were then wrapped in SaranWrap and developed on film, in darkroom, using developing substance (G 150, Agfa, Belgium) and fixation substance (G 354, Agfa, Belgium).

Immunolight Microscopy

Paraffin embedding:
Small pieces of pulp were cut out both from the center and the outer layer including the peel of the apple. Five pieces from each area were made. To stop the metabolism, the apple pieces were fixed with 4% paraformaldehyde in PBS (10 mM Na-phosphate, pH 7.2, 150 mM NaCl) for 2 hours at room temperature and then washed with PBS for 6 x 5 minutes. To replace the water with paraffin, dehydration and then infiltration was performed. The dehydration takes place in room temperature, on shaker, and in steps using different concentrations of ethanol. For 30 minutes each, the apple pieces were dehydrated with 30%, 50%, 70%, 90%, and 95% ethanol and last with absolute ethanol for 2 x 30 minutes and over night. The infiltration was made by absolute ethanol and xylene mixed 2:1 on shaker for 1 hour followed by 1 hour with a mixture of 1:2, and then only xylene for 2 x 1 hour. Finally, liquid paraffin was added and the pieces were kept at a temperature of 56-58°C over night and then 2 x 2 hours with new paraffin. Thereafter, casting of the paraffin blocks was performed followed by cutting using a microtome machine (Leitz 1515, Germany). Sections of 10-15 µm were put in a warm water bath to straighten out and then they were collected onto slides (SuperFrost Plus, Menzel Gläzer, Germany). At this stage

it was possible to check and sort out slides under a microscope. The chosen slides were melted on a warm plate and kept in 40°C for 4 hours. The slides were then stored in room temperature.

Immunolocalisation:
Immunogold labeling starts by deparaffinization and hydration of sections and then moves on to the immunolabeling and mounting. The slides were put in a hood and dipped twice in xylene for 10 and 5 minutes, respectively. Thereafter, they were dipped in steps of 5 minutes, starting with xylene and ethanol mixed 1:1, twice in 100% ethanol, followed by 95% ethanol, 90% ethanol, 70% ethanol, 50% ethanol, 30% ethanol and finishing with distilled water (dH_2O).

When performing the immunogold labeling, all dilutions were made in 1% BSA in PBS. Incubations were made in a wet chamber to keep the sections from drying. Washing was made using a slide rack. Blocking was performed by adding 5% normal goat serum to the slides and leaving them for 30 minutes in a wet chamber. The serum was then quickly removed. The primary antibody (monoclonal mouse antibody against Bet v 1, 5H8, 1:100) was added and the slides were left to incubate over night at 4°C. One slide was left for control and was only treated with PBS buffer.

The following day the slides were washed with PBS for 4 x 5 minutes, followed by incubation with secondary antibody (Goat anti-mouse IgG-Gold, 2 nm, British Biocell, International, Cardiff, UK, 1:50) for 60 minutes at 37°C in a wet chamber. Washing was done with distilled water (dH_2O) for 10 x 2 minutes. The slides were then allowed to dry. The silver enhancement was done by mixing two solutions (Silver enhancing kit for Light and Electron Microscopy, BB International, UK, and leaving it for 15-20 minutes. This process was also followed by washing with distilled water (dH_2O) for 6 x 2 minutes. When the slides were dry again they were mounted using a water based mounting media (Mowiol). The slides were photographed with Leica DC300 digital camera on a Leica DMLB light microscope

Results

Coomassie Blue Staining

Staining with Coomassie Blue gives an overview of total protein content and was used in the beginning to evaluate the quality of tissue prints. Figure 1 shows transverse sections of ‘Gloster’ and ‘Aroma’ apples with a typical Coomassie Blue staining. The peel and the core could easily be detected. The pulp was rather evenly stained except for some areas with lighter or no staining. A perfectly even print was almost impossible to achieve.

When comparing the Coomassie Blue stained prints to the immunotissue prints there was no direct correlation between tissue protein distribution and antibody reactivity. In the Coomassie Blue prints, especially of ‘Gloster’, the tissues exhibited an overall high protein content which did not show in the antibody binding of the immunotissue prints. This is due to the fact that Coomassie Blue staining does not detect specific proteins but all proteins of the tissue which in turn can be interpreted

as a verification of antibody specificity. However, it was a useful method when optimizing printing technique.

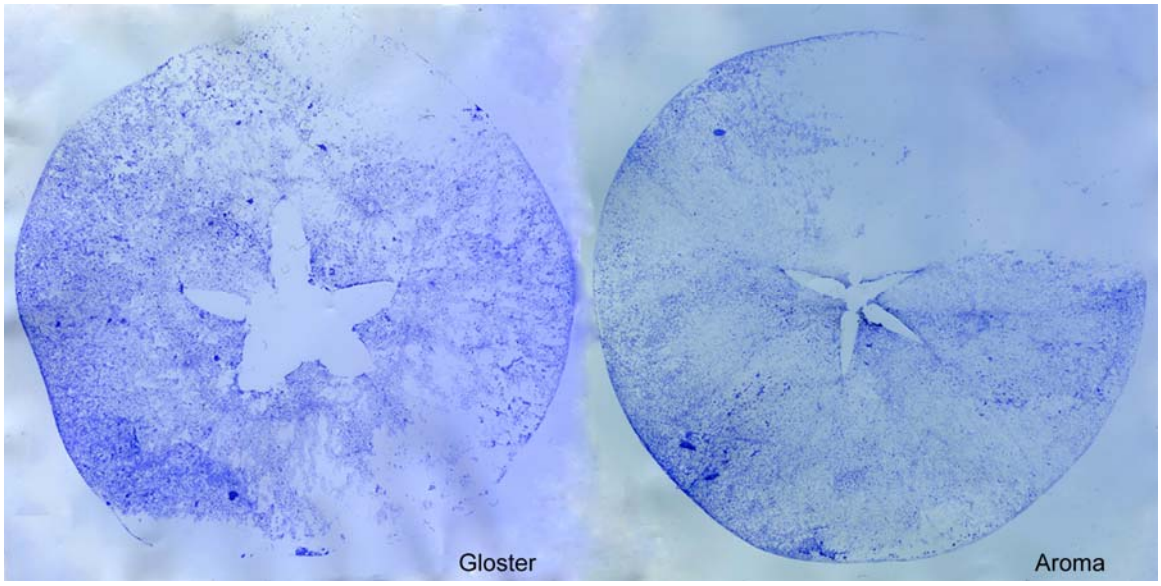

Fig 1. Transverse sections of ‘Gloster’ and ‘Aroma’ apples from Solnäs orchard stained with Coomassie Blue, showing total protein distribution in the tissue prints.

Immunotissue Printing

The first ITPs, comparing ‘Aroma’ and ‘Gloster’, were made with prints of the whole apple slice and the rest where made only with quarters of apple prints due to the limited amount of antibody available.

The immunotissue prints can confirm that the ‘Aroma’ cultivar has a much higher content of allergen than the ‘Gloster’ cultivar. They clearly show a great difference both in allergen content and localization. The immunotissue print of ‘Aroma’ (Fig. 2) showed a somewhat even distribution of allergen in peel compared to pulp. However, this was not the case within the pulp where the distribution of allergen was quite uneven. The print of ‘Gloster’ (Fig. 2) on the other hand showed low levels of Mal d 1 which was mostly found in the peel and core.

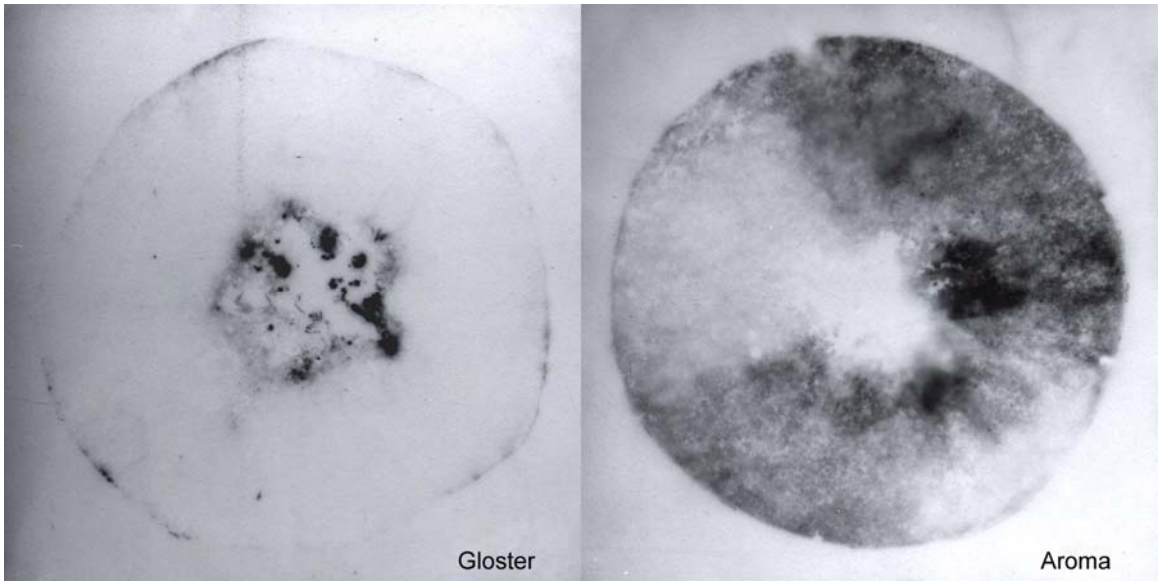

Fig 2. Immunotissue prints of fruits of ‘Gloster’ and ‘Aroma’, bought in a supermarket.

Immunolight Microscopy

The paraffin sections showed that the structure of the parenchyma tissue was somewhat collapsed. No starch was left in the cells and the cell walls were partly broken.

The immunostaining, which is seen as dark silver precipitate, is overall very weak when compared to the ITPs. The most apparent feature is a substantially stronger precipitation in the peel and nearby pulp which indicates a higher amount of Mal d 1 compared to the central pulp (Fig. 3). This is evident in both ‘Aroma’ and ‘Gloster’. The difference between the cultivars is the somewhat thicker area of Mal d 1, from the peel and inwards, in Aroma. This is all in line with the results from the ITPs comparing the two cultivars (Fig. 2). The control sections showed no labeling (not shown).

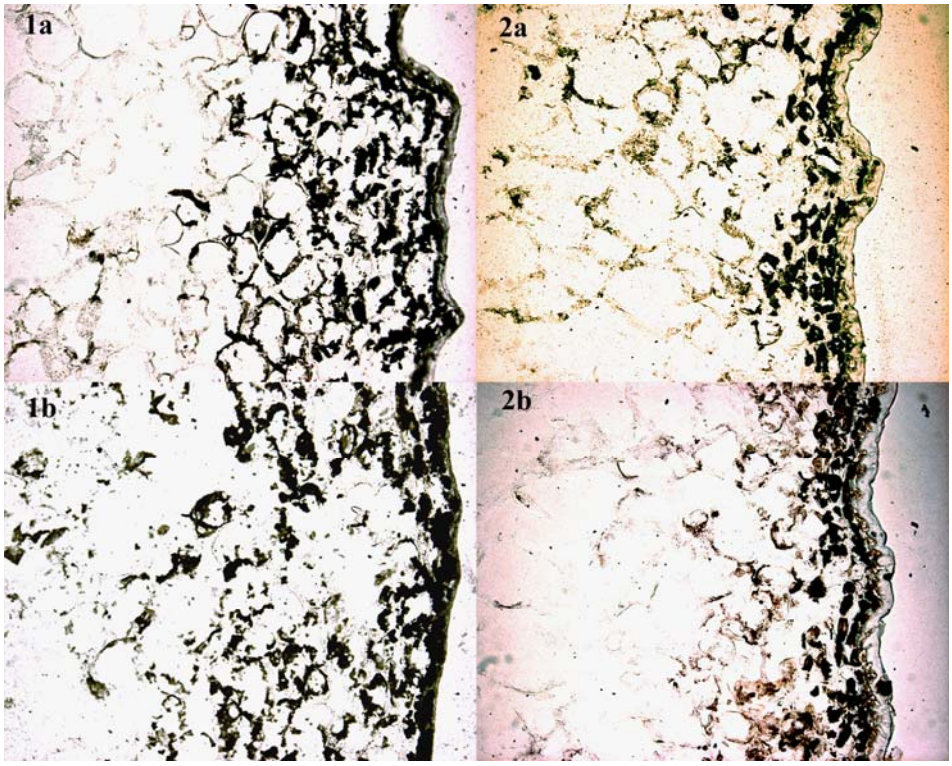

Fig 3. Immunogold labeling of peel from ‘Aroma’ (1a,b) and ‘Gloster’ (2a,b) from Solnäs orchard. The sections were treated with the monoclonal antibody 5H8. Positive labeling is seen as dark precipitate.

ITP – Cultivation and storage conditions

The Aroma apples from the storage experiment at Balsgård, which also included differences in time of harvest showed large differences. Differences were particularly pronounced between apples from a conventional vs. an organic orchard, apples picked at late harvest date vs. optimal harvest date and between apples stored in NA vs. ULO. The differences are highlighted by grouping of the ITPs and can be viewed in Figures 4 and 5.

The results showed that the organic apples had low allergen content regardless of the atmosphere (Fig. 4). Overall, the highest content of allergen was found among apples harvested at the later date (Fig. 5) and the lowest among those harvested at the optimal date (Fig. 4) including the organic apples. The highest content of all was found in apples from a conventional orchard, picked at the optimal harvest date and stored in normal atmosphere. Then came the apples from the IP managed orchard, picked at the later harvest date and stored in normal atmosphere (Fig. 5). For these apples, normal atmosphere storage was the common factor. When evaluating each picture on its own it is evident that the highest content of all is found within the apple from conventional production that was picked at the later date and stored in normal atmosphere. There was also a clear difference between organically and conventionally grown apples. The results are summarized in Table 1.

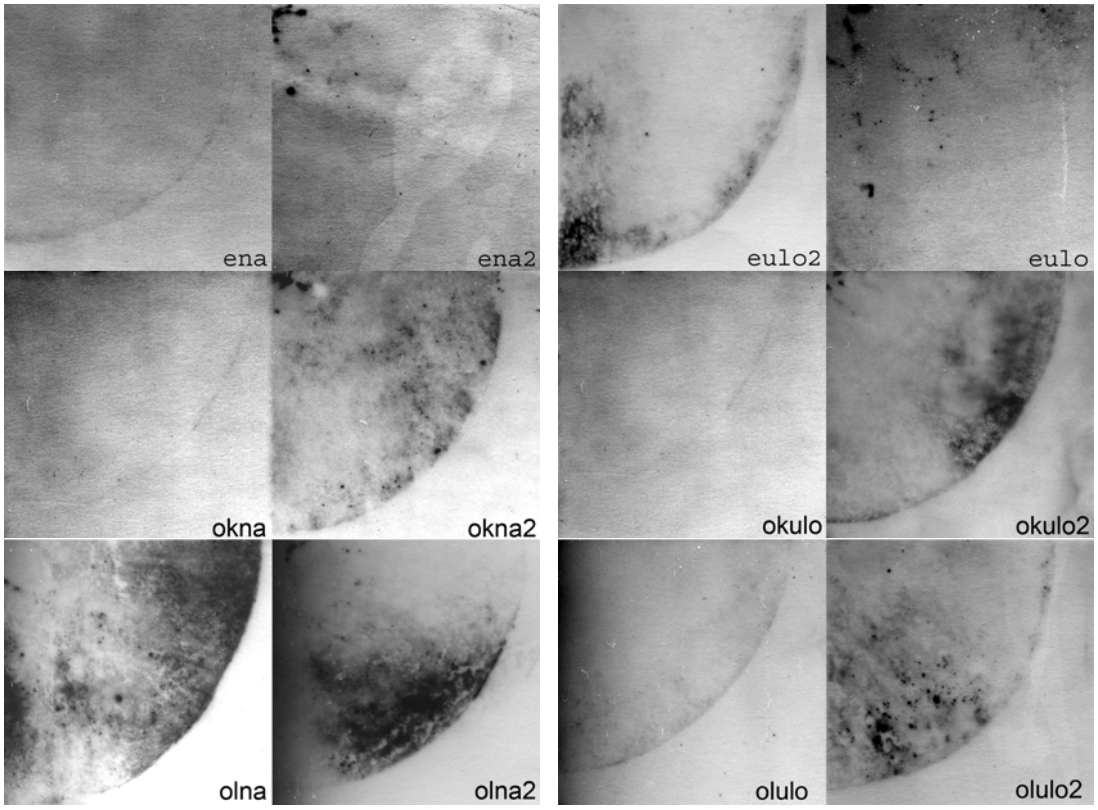

Fig 4. Immunotissue prints of fruits of 'Aroma', picked at optimal harvest date (Sept 16th) and cultivated according to either conventional, IP or organic standards. ENA and ENA2= Prints of 2 different fruits from an organic orchard, picked at the optimum date and stored at 3 °C in NA; EULO and EULO2= Prints of 2 different fruits from an organic orchard, picked at the optimum date and stored at 3 °C in ULO (3% O_2 + 3% CO_2); OKNA and OKNA2= Prints of 2 different fruits from an IP orchard, picked at the optimal date and stored at 3 °C in NA**;** OKULO and OKULO2= Prints of 2 different fruits from an IP orchard, picked at the optimal date and stored at 3 ° in ULO (3% O_2 + 3% CO_2)**;** OLNA and OLNA2= Prints of 2 different fruits from a conventional orchard, picked at the optimal date and stored at 3 °C in NA**;** OLULO and OLULO2= Prints of 2 different fruits from a conventional orchard, picked at the optimal date and stored at 3 °C in ULO (3% O_2 + 3% CO_2). Prints divided into Normal atmosphere (Left) and Ultra low oxygen (Right).

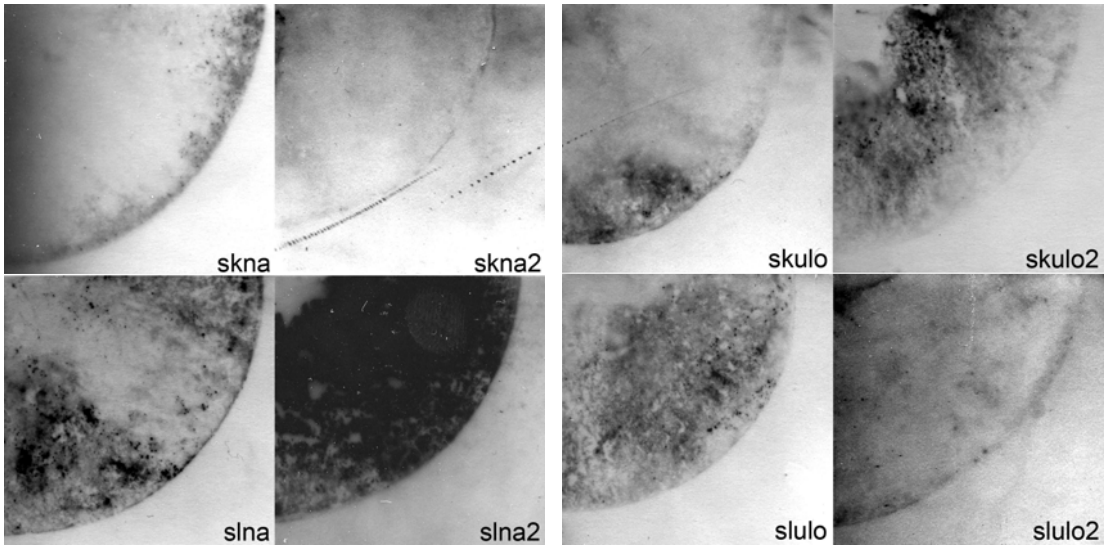

Fig 5. Immunotissue prints of fruits of 'Aroma', picked at a later date (Sept 26th) and cultivated according to either conventional, IP or organic standards.; SKNA and SKNA2= Prints of 2 different fruits from an IP orchard, picked at the later date and stored in 3 °C in NA; SKULO and SKULO2= Prints of 2 different fruits from an IP orchard, picked at the later date and stored at 3 ° in ULO (3% O_2 + 3% CO_2); SLNA and SLNA2= Prints of 2 different fruits from a conventional orchard, picked at the later date and stored at 3 °C in NA; SLULO and SLULO2= Prints of 2 different fruit from a conventional orchard, picked at the later date and stored at 3 °C in ULO (3% O_2 + 3% CO_2). Prints divided into Normal atmosphere (Left) and Ultra low oxygen (Right).

Table 1. Summary of analysis of 'Aroma' immunotissue prints. The symbols +, ++, +++, -, represent little, moderate, intense or no apparent cross reactivity with antibodies and x, no test group available. Optimal (Sept 6th) time of harvest and Late (Sept 26th).

		Conventional	***IP***	***Organic***
Optimal harvest	***ULO***	-/+	-/+	-/+
	NA	++/++	+/-	-/-

		Conventional	***IP***	***Organic***
Late harvest	***ULO***	+/+	+/+	x
	NA	**++/+++**	-/+	x

Discussion/Conclusion

Method

ITP has been used to locate allergens in apple tissue before, for example by Marzban *et al*., in 2005. However, the method has not been used previously on the cultivars ‘Aroma’ and ‘Gloster’, and also not on such well-defined material in terms of storage conditions. The experiences made from this study can confirm that ITP is a much more efficient method compared to ILM using immunogold labeling. ITP is, e.g., less labour-intensive and the results are obtained much faster. However, this method is dependent on the availability of large and expensive antibody quantities, accentuating a possible future need for a commercial production of primary antibodies for detection. Presently, costs for the two methods are probably rather equal in the end. Nevertheless, ITP allows for more accurate screening of a larger material than is possible by using light microscopy.

It should also be stated that quantitative interpretation of antibody reactivity on tissue prints can be difficult without knowing the amount of protein transferred from each tissue (Taylor *et al.*, 1993). ITP is only, at best, a semi-quantitative measure, whereas ELISA provides more accurate information on the actual content. ITP, however, provides an instant overview of the whole tissue including distribution patterns. Possibly, a combination of ITP and ELISA would provide the most extensive and accurate results. The two methods complement each other by providing both detailed data and a valuable overview.

In this study, monoclonal antibodies were used. However, there are both disadvantages and advantages with using monoclonal and polyclonal antibodies, respectively. Monoclonal antibodies are produced in mice as opposed to rabbit in polyclonal antibody production. One advantage of monoclonal antibodies is that they are directed to only one epitope of the antigen. This may also be a disadvantage because changes in the epitope can lead to lack of specificity or to loss of antigen recognition. By contrast, polyclonal antibodies can recognize several of the sites in the antigen. The disadvantage in this case could be the possibility of the polyclonal antibody recognizing the wrong epitope. In addition, production of polyclonal antibodies is more costly (Dewey *et al*., 1991).

Analysis of results

Comparison of immunotissue prints of ‘Aroma’ and ‘Gloster’ verified the large difference in allergen content between the two cultivars. ‘Aroma’ proved to have a relatively high content of allergen distributed both in the peel and the pulp which seems to correspond with previous results by Marzban *et al*. (2005) and unpublished results by Persson Hovmalm. Marzban *et al*. (2005) used ITP to visualize distribution of Mal d 1 in cultivars ‘Golden Delicious’, ‘Idared’, ‘Red Delicious’ and ‘Granny Smith’.

In ‘Gloster’ the allergen was concentrated to the peel and the core. The content of allergen seemed to be very low thereby confirming results by Son et al. (1999) and unpublished results by Persson Hovmalm.

Previous allergy studies have already confirmed that the severity of apple allergy is related to the apple cultivar and the degree of maturity (Hsieh *et al*., 1995). Allergenic potency of different cultivars has also partly been explained by the fact that different isoforms are present at different levels in pulp and skin (Sancho *et al.*, 2006a). When analyzing the rest of the results it appears that differences in content and localization are not only cultivar-dependent, but also a consequence of storage and cultivation methods. This is seen in comparison of ITPs of Aroma/Gloster, ULO/NA, IP/conventional/organic, and late/optimum harvest in the figures above.

There are noticeable variations in allergen content within cultivars as well as within single apples. These differences could be a result of abiotic factors such as stress, position of the tree and of the apple in the tree (sunny or shady). All of which could be referred to Mal d 1 being a PR protein of family 10. This is also in line with the results by Sancho *et al*. (2006b) in a study on the effects of position of the tree, apple maturity and postharvest storage on Mal d 3, which is also a PR protein (PR-14). In that study there was an orchard-dependent increase as apples matured and also a 2-fold increase in apples from the shady site compared to the sunny site (Sancho *et al*., 2006b). These authors also found that time of harvest had an impact and that especially CA storage decreased content of Mal d 3. Possibly similar effects have been acting on Mal d 1 in this study. However, in such a small material as here, the variations could also be a result of uneven transfer of tissue to membrane as well as simply naturally occurring variations in distribution pattern.

The highest contents of allergen were found in apples stored in normal atmosphere. This may be supported by a study made by Bolhaar *et al*. (2005), where apples stored in controlled atmosphere and tested on patients showed lower allergenicity (15 %) compared to apples stored in normal atmosphere. In another study, Sancho *et al*. (2006a) could by using ELISA (enzyme-linked immunosorbent assay), report that different storage and growing conditions had an effect at a translational and transcriptional level. However, these results showed that Mal d 1 gene expression significantly increased during both modified and normal atmosphere storage and that the largest increase of Mal d 1 levels was observed after five months of CA storage. Opposite to our study, different harvesting times did not show any significant differences (Sancho *et al*., 2006a). From results these results it is possible to conclude that assessing allergenicity is complex and that effects are probably highly cultivar-specific.

The fact that the strongest indication of Mal d 1was found in the apple from conventional production which was harvested at the later date and stored in normal atmosphere is interesting because it is the group with the expected highest allergen levels. This is referring to ‘Aroma’, having relatively high allergen content, high level of maturity and high respiration rate. The difference between organically and conventionally cultivated apples is unexpected and interesting. Further investigations are however needed, with fruit harvested from several orchards for each cultivation method in order to rule out effects due to soil, irrigation, fertilization, micro-climate etc. Using prints of the whole apple would also probably have lead to more accurate results.

The tissue printing method on nitrocellulose membrane was optimized through a number of trials but the difficulty to produce and repeat an even pressure remained.

This could partly explain why the ITP prints never were completely even and also why some quarters are very vague while others are much stronger, and the vast variations occurring within the same group of apples. However, the most likely explanation of the variations within the apple tissue of the same apple is differences in allergen content as well as distribution.

Future

Provided that my results can be confirmed with a larger material, they may help to minimize allergen levels by adjusting storage and cultivation practices. In the future, production of low-allergenic apples may also help to support the organic apple farming.

Suggestions for further research in this area could include using whole, transverse slices of apple, combining ITP with ELISA of the same tissue, comparing weather data from different growing seasons, and taking more accurate data for the trees and apples analyzed in terms of e.g. nourishment and exposition to the sun etc.

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