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Proteomic analysis of the major birch allergen Bet v 1 predicts allergenicity for 15 birch species

Martijn F. Schenk[a,*], *Jan H.G. Cordewener*[a], *Antoine H.P. America*[a], *Jeroen Peters*[a], *Marinus J.M. Smulders*[a,b], *Luud J.W.J. Gilissen*[a,b]

[a]*Plant Research International, Wageningen UR, Wageningen, The Netherlands*
[b]*Allergy Consortium Wageningen, Wageningen, The Netherlands*

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ABSTRACT

Pollen of the European and Asian white birch (*Betula pendula* and *B. platyphylla*) causes hay fever in humans. The allergenic potency of other birch species is largely unknown. To identify birch trees with a reduced allergenicity, we assessed the immunochemical characteristics of 15 species and two hybrids, representing four subgenera within the genus *Betula*, while focusing on the major pollen allergen Bet v 1. Antigenic and allergenic profiles of pollen extracts from these species were evaluated by SDS-PAGE and Western blot using pooled sera of birch-allergic individuals. Tryptic digests of the Bet v 1 bands were analyzed by LC-MS^E to determine the abundance of various Bet v 1 isoforms. Bet v 1 was the most abundant pollen protein across all birch species. LC-MS^E confirmed that pollen of all species contained a mixture of multiple Bet v 1 isoforms. Considerable differences in Bet v 1 isoform composition exist between birch species. However, isoforms that are predicted to have a high IgE-reactivity prevailed in pollen of all species. Immunoblotting confirmed that all pollen extracts were similar in immune-reactivity, implying that pollen of all birch species is likely to evoke strong allergic reactions.

1. Introduction

In the boreal and temperate climate zone of the Northern Hemisphere over 30 birch species grow in diverse habitats. Birch trees release large amounts of pollen in spring. This pollen is a major cause of Type I allergies and contains several allergens, including the major allergen Bet v 1. Hay fever caused by birch pollen affects 11–19% of the population in Western European countries (UCB 1997). Bet v 1 belongs to the pathogenesis related-class 10 (PR-10) protein family [1,2]. Pollen from tree species such as alder and hazel also contains PR-10 proteins that share epitopes with Bet v 1, which results in cross-reactivity between these proteins with respect to their antigenic properties [3]. In addition, individuals sensitized to Bet v 1 often become allergic to fruits and vegetables, resulting in the oral allergy syndrome (OAS)[4].

B. pendula occurs in Europe and is currently the only species that has extensively been studied in relation to allergy. Other birch species occur in Europe as well, and sensitization to birch is also reported across Asia and North America, where other *Betula* species predominate [5–8]. Besides, numerous birch species are used as ornamental trees and are planted outside their natural distribution range. These birch species and cultivars might vary in allergenicity. Planting of trees with a reduced allergenicity could potentially reduce the allergenic load. For example, hypoallergenic apple varieties and olive

Abbreviations: LC-MS, liquid chromatography mass spectrometry; LC-MS/MS, liquid chromatography tandem MS; LC-MS^E, alternate scanning LC-MS; MIA, microsphere immuno assay; PR-10, pathogenesis related-class 10; Q-TOF, quadrupole time-of-flight.

* *Corresponding author at:* Institute for Genetics, University of Cologne, Zulpicher Str. 47 a, 50674 Köln, Germany. Tel.: +31 317 483142.
E-mail address: mschenk2@uni-koeln.de (M.F. Schenk).

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trees are known to exist [9–11]. Reduced allergenicity can result from lowered allergen content [9,11,12] or from the absence of specific isoforms with a high allergenicity [13,14].

Multiple PR-10 genes have been identified in *B. pendula*, of which a subset is expressed in pollen and can therefore be considered true Bet v 1 allergens. Similar genes have been identified in other birch species [15], but expression and, moreover, IgE-reactivity of these allergens is unexplored. Bet v 1 is estimated to account for 10% of the total protein content of *B. pendula* pollen [16]. Various Bet v 1 isoforms exist [17,18], including isoforms with low and high IgE-reactivity [13]. A single *B. pendula* tree expresses a mixture of Bet v 1 isoforms with varying IgE-reactivity [15]. The relative abundance of individual isoforms within this mixture influences total allergenicity of the pollen. LC-MS/MS is a widely used technology to identify proteins in an extract by sequencing the peptides obtained after tryptic digestion. Here, a new variant called LC-MS^E was applied which enables both protein identification and quantification in a single run by alternating scans of low and high collision energy [19]. This label-free method was used for a comparative, quantitative analysis of pollen extracts originating from various birch species, thereby mainly focusing on the relative abundance of different Bet v 1 isoforms [15]. Site-directed mutagenesis has shown that several amino acid residues of Bet v 1 are linked to a IgE-reactivity [20,21] and these can be used as markers for high IgE-reactivity. Zaborsky et al. [22] have shown that a single amino acid change has an effect on antigen aggregation and thereby influences the establishment of a protective antibodies [22]. By determining the abundance of these amino acid markers among pollen-expressed Bet v 1, one can predict birch pollen allergenicity of the other birch species.

The allergenicity of 15 birch species and two hybrids was examined to determine whether birch trees with a reduced allergenicity exist in nature. Based on genomic data on Bet v 1 sequences, we applied LC-MS^E to detect and quantify amino acid residues associated with high and low IgE-reactivity among Bet v 1 in the pollen proteome. These data were linked to IgE-reactivity of the pollen by determining the antigenic and allergenic profiles using SDS-PAGE and Western-blotting, and by determining the amount of Bet v 1 protein in a fluorescent array using polyclonal antibodies. The results indicate that all birch species produce pollen that is likely to evoke allergic responses.

2. Material and methods

2.1. Pollen sampling

Pollen was collected from 24 birch trees belonging to 15 species and two hybrids (Table 1). All trees were previously genotyped using AFLP [25] and we constructed a neighbor-joining tree from the subset that was included in this study to visualize phylogenetic relations using NTSYSpc 2.10j (Applied Biostatistics). Two *Alnus* and *Corylus* accessions were included as outgroup in the analysis. Birch species are grouped into five subgenera/four clusters [23–25] that are all represented here by at least one species, except for subgenus *Betulaster* [23], which belongs to cluster IV-C in the AFLP analysis [25]. Sampled trees grew in the Botanical Garden of Wageningen or as ornamental trees along streets. Pollen was collected in the morning and within a 10 km area around Wageningen (the Netherlands) to minimize effects of environmental factors on pollen content. Timing of collection relative to the ripening stage of the pollen influences the Bet v 1 release from the pollen [26] and, therefore, each tree was sampled 2–3 times during its flowering period and these samples were pooled. However, the onset of flowering varied considerably between different species, and hence pollen was collected over a time span of 4–6 weeks (21 April–20 May 2006; 28 March–7 May 2007). During this period all trees were visited every 2–3 days and flowering catkins were shaken inside 50 ml blue caps to collect pollen. As a consequence, pollen was exposed to different weather conditions (humidity, sunlight, and temperature) before sampling. Samples were then sieved through a 90 μm mesh filter to eliminate extraneous material. Light microscopy observations confirmed that other plant parts accounted for <5% of the pollen samples. Samples were freeze dried before storage at 4 °C.

2.2. Flow cytometry and pollen quality

To deduce pollen characteristics and quality, 25 mg of pollen was suspended in 500 μl of 0.1 M PBS (pH 7.4). Samples were subsequently separated on a DakoCytomation CyAn high-performance flow cytometer and analyzed using Summit 4.0 software (DakoCytomation). Scatter characteristics and autofluorescence were recorded.

2.3. Allergen extracts

Extracts were prepared following a previously described protocol [15,27] in which 50 mg pollen was diluted into 1 ml of 0.05 M Tris–HCl buffer (pH 7.5). Total extraction time was 2 h. The supernatant was stored at −20 °C. The total amount of extracted protein was determined using the Bradford assay. The pellet was analyzed by SDS-PAGE to determine the rigorousness of the extraction procedure. We found no 17 kDa (Bet v 1) band in the resuspended pellet, indicating that all Bet v 1 was indeed extracted.

2.4. Patient sera

Sera from ten birch-allergic Dutch individuals (kindly provided by Y. Vissers and M. Bollen, Wageningen University) were stored at −80 °C until use and pooled prior to the Western blotting. These patients all tested positive for birch pollen. Eight patients cross-reacted to other tree pollen allergens. Two patients also displayed a reaction against grass pollen. Sera were characterized for reactivity against Bet v 1, Bet v 2, and Bet v 4 plus Bet v 6 by determining specific IgE levels using the ImmunoCAP system (Phadia, Uppsala, Sweden). Individuals each had total specific IgE levels to birch exceeding 50 kU/l. Reactivity against individual allergens in these sera ranged from 41 to >100 kU/l for Bet v 1, from <0.10 to 2.52 kU/l for Bet v 2, and was <0.10 kU/l for Bet v 4 plus Bet v 6.

2.5. SDS-PAGE and Western blot

Proteins were separated on 15% (w/v) SDS-PAGE and stained with Coomassie BB R-250 (Bio-Rad) or subjected to immunoblot