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Production of recombinant allergens in plants

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Abstract

A large percentage of allergenic proteins are of plant origin. Hence, plant-based expression systems are considered ideal for the recombinant production of certain allergens. First attempts to establish production of plant-derived allergens in plants focused on transient expression in *Nicotiana benthamiana* infected with recombinant viral vectors. Accordingly, allergens from birch and mugwort pollen, as well as from apple have been expressed in plants. Production of house dust mite allergens has been achieved by *Agrobacterium*-mediated transformation of tobacco plants. Beside the use of plants as production systems, other approaches have focused on the development of edible vaccines expressing allergens or epitopes thereof, which bypasses the need of allergen purification. The potential of this approach has been convincingly demonstrated for transgenic rice seeds expressing seven dominant human T cell epitopes derived from Japanese

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cedar pollen allergens. Parallel to efforts in developing recombinant-based diagnostic and therapeutic reagents, different gene-silencing approaches have been used to decrease the expression of allergenic proteins in allergen sources. In this way hypoallergenic ryegrass, soybean, rice, apple, and tomato were developed.

Keywords

Allergy; Expression system; Green biotechnology; Molecular farming; Recombinant protein

Introduction

The incidence of atopic diseases such as asthma, allergic rhinitis and atopic eczema has substantially increased over the past few decades, now affecting more than 25% of the European population. Decreasing sources for Th1 immune provocation in the early childhood are assumed to correlate with an increasing Th2 bias of immune responses towards environmental allergens (hygiene hypothesis—Shaheen et al. 1996; Holt et al. 1999). The dysregulation of the Th1 and Th2 lymphocyte subsets leads to the development of distinct T-cell cytokine patterns (interleukins 4, 5 and 13) that favor immunoglobulin (Ig) class switching of specific B cells to IgE and chronic, allergen-driven inflammation (de Vries et al. 1999; Parronchi et al. 1999).

The development of an allergic disease always requires contact with a sensitizing agent, which does not necessarily have to be the elicitor of the allergic symptoms. Allergen cross-reactivity occurs when IgE antibodies originally raised against one allergen binds or recognizes a similar protein from another source (Aalberse et al. 2001). The interaction with such homologous protein can then trigger allergic reactions or can be completely irrelevant for the patient.

The increase in prevalence of allergic diseases has been paralleled by a greater demand on diagnostic and therapeutic products. So far only allergen extracts are routinely applied for diagnostic and therapeutic purposes. These extracts are difficult to standardize regarding their allergen content: several allergens might be under-represented due to degradation, other non-allergenic components are present and there might be even contamination with allergens from other sources. Furthermore, extract-based diagnosis allows the identification of allergenic sources but not of individual allergenic molecules. Consequently, the discrimination between co-sensitization and cross-sensitization in patients showing adverse reactions to more than one allergenic source is also not possible (Pauli 2000; Ferreira et al. 2004a).

The inadequacy of diagnosis with extracts in cases of cross-reactivity is clearly demonstrated by a survey of mite-allergic Orthodox Jews. Due to strict adherence to kosher dietary laws, this population is prohibited the consumption of shellfish. Fernandes and co-workers (Fernandes et al. 2003) showed that IgE antibodies of these mite-allergic individuals cross-reacted with shrimp tropomyosin Pen a 1 despite the fact that direct contact with this aliment did not occur. All these findings point out the necessity of molecule-based diagnosis for allergies.

Allergy diagnosis: from crude extracts to molecule-based approaches

For the establishment of molecule-based allergy diagnosis, well-characterized allergens have to be available in large amounts without batch-to-batch variations. The use of recombinant allergens offers the best prospect for a rational and accurate allergy diagnosis (Valenta et al.

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1999; Chapman et al. 2000; Ferreira et al. 2004b). Over the past few years, allergens from various allergenic sources including pollen, food, house dust mite, cockroach, animal dander, insect venom, latex, moulds and fungi have been cloned, characterized and some of them are now available as recombinant proteins (Arruda et al. 2001; Chapman and Wood 2001; Lorenz et al. 2001; Kurup et al. 2002; Muller 2002; Sussman et al. 2002; Thomas et al. 2002; Andersson and Lidholm 2003; Wopfner et al. 2005). Systems for recombinant production of allergens and genetic engineering techniques offer unique tools for the development of novel molecule-based products to be used in diagnosis and allergen-specific immunotherapy (Ferreira et al. 2004a; Wallner et al. 2004; Valenta and Niederberger 2007; Wallner et al. 2007). Pure and standardized recombinant allergens or cocktails prepared thereof containing most of the IgE-binding epitopes of an allergen source can be formulated to replace natural extracts. The use of recombinant allergens in diagnosis allows the exact identification of the molecules causing the allergic reaction, i.e. a clear association between the disease manifestation and the IgE-mediated immune reactions.

Various heterologous systems for production of recombinant allergens have been used including *E. coli, Pichia pastoris*, insect cells, mammalian cells, and plants (for a review see Singh and Bhalla 2006). Plant systems for the production of recombinant allergens offer a number of advantages such as eukaryotic post-translational modifications, cost-effectiveness and flexibility for rapid scale up, absence of human or animal pathogens. Here we give a short overview on plant expression systems and their use for the production of recombinant allergens.

Genetic engineering of plants

Genetic engineering of plants can be accomplished by a variety of different methods of which biolistic-, *Agrobacterium*- and virus-mediated deliveries are the most commonly used (for detailed protocols and reviews see Newell 2000; Obermeyer et al. 2004; Wagner et al. 2004; Pena 2005; Gleba et al. 2007). In addition, other techniques like electroporation of intact cells or protoplasts (Bates 1995), and the PEG-mediated transformation of plant cell protoplasts are suitable for fast transient expression to test the designed expression plasmids and the cellular localization of the recombinant proteins (Hadlington and Denecke 2001; Baur et al. 2005). *Agrobacteria* may also be used for transient expression (Agro-infiltration) studies to test the constructed gene transfer vectors and to verify that the localization of the recombinant protein is as wanted (Kapila et al. 1997). In any case, the chosen transformation strategy influences the final outcome and should thus be carefully considered. *Agrobacterium*-mediated transformation incorporates the gene of interest and selection marker genes into the nuclear genome allowing the generation of stable transformants (Zupan et al. 2000; Tzfira and Citovsky 2006). However, the integration event into the plant genome cannot be controlled and multiple copies as well as additional integrations of bacterial sequences from the plasmid backbone, e.g. origin of replication, AT-rich sequences may occur. This random integration can promote transgene rearrangements, ‘illegal’ recombinations or methylations, finally leading to gene silencing and loss of the ability to produce the recombinant protein (Matzke et al. 2000; Kohli et al. 2003; Filipecki and Malepszy 2006). Therefore, it is preferable to aim to an insertion of a single copy of the transgene without any additional bacterial sequences. Currently, the only possibility to achieve this is to carry out direct gene transfer (e.g. biolistic delivery) with a linearized and minimized construct and by screening the produced transgenic lines for single copy insertions. Minimized constructs are gene transfer vectors just containing the promoter, the encoding gene, a terminator and optional protective sequences at the 5′- and/or 3′ ends (Fu et al. 2000; Vidal et al. 2006; Yao et al. 2006). Unfortunately, the single copy insertions to nuclear genome are extremely rare and laborious screenings are needed to avoid gene silencing in the progeny. Most importantly, the generated transgenic lines need to be

screened for high expression, since the integration locus in the genome, number of copies, the promoter chosen and the gene itself all affect on the obtained production levels. It is noteworthy that the major drive for high expression is the promoter and that should be very carefully selected (see Choice of expression system: plants and binary vectors).

In contrast to biolistic and *Agrobaterium*-mediated genetic engineering, the use of plant RNA viruses, e.g. tobacco mosaic virus (TMV), allows only transient expression without integration into the genome. Transfection of the entire plant and amplification of the RNA by viral replicases have been shown to yield high levels of the recombinant proteins (Koprowski and Yusibov 2001). First experiments were performed with fully functional viruses that were able to move systemically in the host plant and produced infectious viruses, whereas in the next generation of viral vectors the limitations and undesired functions of the full-virus vectors (e.g. species specificity, the gene size limitation of 1 kb, generation of infectious viral particles) were eliminated leading to 'deconstructed virus' vectors containing only the viral elements required for expression and improved functions provided by non-viral components (Gleba et al. 2004, 2007). A very promising new approach is the 'magnifection', which combines the advantages of two systems: the transfection efficiency of *Agrobacteria* and the high expression yields obtained with viral vectors. In addition, the post-translational capabilities of plants can be fully exploited and the time to achieve gram quantities is reduced to minimum: 4 g GFP per kg leaf fresh weight in *Nicotiana benthamiana* (Marillonnet et al. 2005) or 0.5 g of fully assembled IgG antibody per kg fresh weight in less than 2 weeks (Giritch et al. 2006).

Choice of the expression system: plants and binary vectors

A number of different plant species has been used for the production of recombinant proteins including the moss *Physcomitrella patens*, the algae *Chlamydomonas reinhardtii*, tobacco, potato, lettuce, tomato, carrots, cereals (maize, rice, wheat), soybean and the model plant *Arabidopsis thaliana* (for reviews see Giddings et al. 2000; Daniell et al. 2001; Decker and Reski 2004; Fischer et al. 2004). In addition, production in cell cultures has been evaluated in several plant species e.g. in tobacco, tomato and rice (Doran 2000; Hellwig et al. 2004).

In case of allergens, the production system most probably needs to be contained e.g. to take place in greenhouses or in bioreactors. When the expression host is chosen the biomass production is one important criteria. Therefore, expression in *Arabidopsis* is only appropriate for research interests. For safety and containment aspects, non-food plants might be preferred. On the other hand, food plants do not contain harmful or toxic compounds (e.g. toxic secondary metabolites) and might be preferable in that sense. Plant species with high biomass production like tobacco, maize, and *Brassica* (canola, turnips) might be the plants of choice, also because of the well-established post-harvest procedures.

In most studies, the expression of recombinant proteins has been under the control of a constitutive promoter, e.g. the cauliflower mosaic virus 35S promoter (CaMV 35S) leading to expression in almost all plant tissues. Currently, constitutive promoters of plant origin that enable a high-level expression like the *Chrysanthemum rbcS1* promoter also became available (Outchkourov et al. 2003). Plant tissue-specific promoters enable targeted expression in the preferred tissues, e.g. in edible parts like fruits, seeds or leaves. The use of edible parts as production hosts makes it possible to utilize them as edible vaccines and apply them for allergy therapy (Horvarth et al. 2000; Walmsley and Amtzen 2003; Takagi et al. 2005a; Streatfield 2006). Seeds as production hosts provide a long-time, room temperature storage with less degradation of the recombinant protein. In addition to tissue-specific expression, the final localization of the recombinant allergen is also of importance.

The sub-cellular localization of the recombinant protein determines the set of post-translational modifications it goes through, e.g. a cytosolic protein is less likely to be glycosylated than a membrane, vacuolar or secreted protein. In the case of recombinant allergens, correct glycosylation plays an important role and the sugar chains of the recombinant allergen should mimic or be equal to those of the native allergen, as patient's IgEs might also recognize the glycans of an allergen (Leonard et al. 2005; Altmann 2007). Endogenous trafficking signals, e.g. secretory signal peptides or vacuolar targeting sequences, of the allergen-of-interest may not function in the heterologous expression system and therefore, have to be replaced by the trafficking sequences of the expression system (for a review see Hanton et al. 2006). Nevertheless, the theoretical sub-cellular localization must be checked with a prediction algorithm, e.g. TargetP (Emanuelsson et al. 2007), or tested with fluorescent reporter genes (Hanton and Brandizzi 2006) prior to setting up a production platform.

The plant expression vector should at least have the following features: (1) minimal size, (2) replication in *E. coli* and in case of *Agrobacterium*-mediated deliveries in *Agrobacteria*, (3) modular design allowing a high flexibility to exchange promoters, selection markers, reporter genes and tags or fusion proteins, (4) multiple cloning site also containing rare restriction enzymes, and (5) availability or low license fees for commercial use. Most of the currently used plasmids are derived from the pBIN19 (Bevan 1984) or from the improved pBINPLUS (van Engelen et al. 1995). Note, that the complete sequence of the widely used pBI121 plasmid has been re-investigated (Chen et al. 2003). A general guide for selection criteria of vectors has been published (Hellens et al. 2000). In addition, gateway-compatible vectors have been designed and constructed (Karimi et al. 2002), which allow the expression of tagged recombinant proteins (Earley et al. 2006) and contain inducible promoters (Curtis 2003). Recently, a new set of modular vectors that allow transformation of dicots by *Agrobacterium*-mediated transformation and monocots by particle bombardment (pORE, Coutu et al. 2007) has also been constructed. However, no vector series or system complies with all needs and the chosen vectors have to be optimized for the specific needs.

In general, specific sequences (ω-enhancer of TMV) or features, e.g. codon-usage optimization, can boost the efficiency and yield of the expression system dramatically (Streatfield 2007). Specific tags can also greatly improve the yields and quality especially by making the extraction and purification steps more efficient. An evaluation of different affinity tags for the subsequent purification of the recombinant protein from yeast, *E. coli, Drosophila* and HeLa cells has been published. Particularly, the efficiency–costs relation of different peptide tags (6×His, calmodulin-binding peptide, FLAG, StrepT-agII, heavy chain of protein C) was compared with protein fusion tags (maltose-binding protein, glutathione-S-transferase). The results suggested the StrepTagII was a good compromise for purification with reasonable yields at moderate costs (Lichty et al. 2005). The StrepTagII epitope was also tested for one-step purification of recombinant proteins from plant material (Witte et al. 2004). Fusion of the StrepTagII to the N-terminus of the recombinant protein allowed cleavage by factor X_a protease without any additional amino acids left at the recombinant protein (Skerra and Schmidt 2000).

Plant-based production of recombinant allergens

A large percentage of allergenic proteins are from plant origin, thus plant-based expression systems are regarded ideal for the production of certain recombinant allergens (Breiteneder and Wagner 2002). However, few allergens have been expressed in plants so far, and only two have been purified and further characterized (Krebitz et al. 2003; Lienard et al. 2007). Reasons for that might be that more specialized techniques are required for establishing plant-based expression systems, when compared to systems based on bacteria and yeast.

Beside the use of plants as production hosts for recombinant allergens, the possibility of edible vaccines expressing allergens or epitopes thereof has gained a lot of input, especially because the need for allergen extraction and purification is bypassed. Table 1 summarizes published data on heterologous allergen expression in plants. Additionally, contrasting approaches aiming at the reduction of allergen content in the source plant have been reported.

Expression and purification of recombinant allergens in plants

The first attempts to establish production of plant-derived allergens in plants focused on transient expression in *Nicotiana benthamiana* infected with recombinant viral vectors (Krebitz et al. 2000; Breiteneder et al. 2001; Krebitz et al. 2003; Wagner et al. 2004). A TMV vector was used for the expression of Bet v 1, the major birch pollen allergen, and of Mal d 2, a thaumatin-like allergen of apple. In both cases, fully IgE-reactive proteins were produced. In addition, plant-produced Mal d 2 exhibited antifungal activity, demonstrating the correctness of folding and its function in plant defense against fungal pathogens. It should be mentioned that production of thaumatin or thaumatin-like proteins in bacteria or other non-plant systems has been hampered by several problems such as incorrect processing, incorrect folding, and insolubility (Faus 2000).

The best example in exploring plants as production systems for allergens was carried out with suspension cultures of BY-2 tobacco cells. Lienard and co-workers (Lienard et al. 2007) produced biologically active forms of the two major house mite allergens, Der p 1 and Der p 2. Plant-produced Der p 1 and Der p 2 were physicochemically characterized in regard to (i) identity by mass spectrometry, (ii) folding by circular dichroism, and (iii) homogeneity by size exclusion chromatography. Further, mass spectrometry analysis of oligosaccharides indicated that Der p 1 is N-glycosylated with either high-mannose-type or complex-type N-glycans in both the natural and the plant-produced system. Finally, extensive immunological characterization showed that plant-produced mite allergens displayed all antigenic determinants responsible for IgE reactivity (determined by radio-allergosorbent analysis, ELISA, basophil activation assays and histamine release tests) and T cell recognition (determined by proliferation assays using peripheral blood mononuclear cells from mite allergic patients). This work convincingly demonstrated the usefulness of plants, in particular of suspension cultures, as low-cost and environmentally safe expression system for the production of recombinant allergens suitable for diagnostic and therapeutic applications.

TMV- and *Agrobacterium*-mediated transformation of tobacco plants (Gadermaier et al. 2003) has been reported for Art v 1, the major mugwort pollen allergen. Art v 1 is a basic glycoprotein comprising two domains: an N-terminal cysteine-rich, defensin-like domain and a C-terminal proline/hydroxyproline-rich module. The proline/hydroxyproline-rich domain was recently shown to contain two types of plant-specific glycosylations: (i) a large hydroxyproline-linked arabinogalactan composed of a short β1,6-galactan core substituted by a variable number (5–28) of α-arabinofuranose residues forming branched side chains with 5-, 2,5-, 3,5-, and 2,3,5-substituted arabinoses; and (ii) single and adjacent β-arabinofuranoses linked to hydroxyproline (Himly et al. 2003; Leonard et al. 2005). Therefore, plant expression can be regarded to be the only suitable choice for the production of recombinant Art v 1 for diagnosis purposes. In preliminary experiments, we successfully expressed Art v 1 in entire tobacco plants and the recombinant molecule was recognized by all patient sera investigated so far, including those that failed to recognize the bacteria-produced recombinant Art v 1 (unpublished data).

Edible allergy vaccines

Edible plant or plant products offer a unique opportunity for oral delivery of recombinant proteins (Giddings et al. 2000). This is an attractive therapeutic approach for delivery of recombinant allergens since mucosal immunization has been shown to be safe and effective for treating allergic diseases (Frati et al. 2007).

The model legume *Lotus japonicus* (accession Miyakojima MG-20) was used to produce the major house dust mite allergen Der f 1. Soluble proteins extracted from the leaves of T_1-plants were analyzed by immunoblot using a monoclonal anti Der f 1 antibody. Similarly to natural Der f 1, plant-produced Der f 1 was found to be approximately 25 kDa, suggesting that no post-translational modifications occurred (Kato et al. 2005). No further characterization of this legume-based edible vaccine has been reported.

Encouraging results were reported using the ZYMV vector for expressing large quantities of mite allergen in cucurbit species (Hsu et al. 2004). The recombinant virus carried and expressed Der p 5 for at least 1 year after numerous passages in squash plants. Most importantly, oral treatment of mice with squash extracts containing virus-expressed Der p 5 caused significant inhibition of both allergen-specific IgE synthesis and airway inflammation.

The feasibility of oral immunotherapy using a plant-based edible vaccine has been explored for the treatment of Japanese cedar pollinosis (Okada et al. 2003; Hiroi and Takaiwa 2006; Takagi et al. 2006; Yang et al. 2007). Oral administration of transgenic rice seeds expressing seven dominant human T cell epitopes derived from the Japanese cedar pollen allergens, Cry j 1 and Cry j 2, down-regulated T cell responses and allergen-specific IgE antibodies induced by immunization with whole cedar pollen allergens (Takagi et al. 2005b). This effect was not observed in mice fed with non-transgenic rice seeds. In addition, the development of pollen-induced clinical symptoms was inhibited in an experimental sneezing mouse model (Takagi et al. 2005a). The results of the pre-clinical evaluation of rice-based edible vaccines were very promising and clinical trials should now be performed to evaluate the efficacy and safety profiles in humans.

Designing plants with reduced allergenicity

Parallel to efforts in developing diagnostic and therapeutic reagents, several attempts to reduce the amount of allergen content in the source plant have been reported. Hypoallergenic plants are attractive approaches to reduce allergen exposure, both from inhalant (pollen) and food sources, and thus represent relevant strategies for prevention of allergies. Different gene-silencing approaches have been used to decrease the expression of allergenic proteins in ryegrass pollen (Bhalla et al. 2001), soybean (Herman et al. 2003), rice (Tada et al. 1996), apple (Gilissen et al. 2005), and in tomato (Le et al. 2006a, b).

Grass pollen allergy affects more than 20% of the population in countries with temperate climate conditions. Transgenic ryegrass plants were generated, where the major allergen Lol p 5 expression was down-regulated by antisense technique. The antisense Lol p 5 RNA was controlled by the pollen-specific promoter of *Ori s 1*. Transgenic ryegrass plants showed normal pollen development and generated fertile pollen. Most importantly, the transgenic rye pollen grains showed very low allergenic activity, as determined by IgE immunoblots of pollen extracts (Bhalla et al. 2001; Bhalla and Singh 2004). This study exemplifies the potential of antisense technology in reducing the allergenic features of plants.

Hypoallergenic apple plants were produced by transferring a construct carrying an intron-spliced hairpin RNA containing a Mal d 1-specific inverted repeat sequence separated by a Mal d 1-specific intron sequence into in-vitro grown apple plantlets (Gilissen et al. 2005).

As it takes approximately 5 years to get a fruit-producing apple tree from seed or in vitro culture, evaluation of the transgenic plants was carried out using leaves from young apple shoots grown in-vitro. Skin prick tests of apple-allergic patients confirmed the low allergenicity of the obtained transgenic apple plants in vivo.

Reduction of allergenicity of tomato fruit was achieved by inhibiting expression of Lyc e 1 (profilin) (Le et al. 2006b) and Lyc e 3 (ns-LTP) (Le et al. 2006a) allergens. Transgenic tomato fruit extracts showed strongly reduced skin reactivity in patients allergic to tomatoes. These studies demonstrated the feasibility of creating hypoallergenic tomato fruits by gene silencing. In addition, a *ripening inhibitor (rin)* mutant tomato was shown to carry decreased allergenicity (Kitagawa et al. 2006). Microarray analysis indicated that genes encoding possible allergenic proteins (i.e. ß-fructofuranosidase and polygalacturonase 2A) were expressed at significantly lower levels in the F_1 hybrid of the *rin* mutant tomato. Accordingly, extracts of the *rin* hybrid fruit showed lower IgE-binding activity when tested with serum from a tomato-allergic patient.

Recently, RNA interference (RNAi) was used to reduce the content of the major allergen Ara h 2 in peanuts (Dodo et al. 2007). In comparison with wild type, transgenic peanut seeds showed decreased IgE binding capacity, as determined by ELISA using sera from peanut-allergic patients.

Characterization of recombinant allergens

Before clinical use, purified recombinant allergens should be carefully investigated for their biochemical, biophysical and immunological properties. Ideally, recombinant products should be fully characterized and their properties compared to natural counterparts (Cromwell et al. 2004). A general approach for characterization of recombinant allergens was developed in the frame of CREATE, an EU-funded project aiming at the development of certified reference materials (CRM) based on purified natural or recombinant major allergens (van Ree 2003, 2004). Twenty-nine partners from industry, research labs, regulatory bodies, and clinical centers worked together on 8 relevant allergens.

A critical aspect in the characterization of recombinant allergens concerns physicochemical properties of the molecules, since their production by heterologous systems might result in modifications leading to changes in their immunological properties. Thus, state-of-the-art techniques should be used to address this point (Ferreira et al. 2006). In general, the following physicochemical parameters (Chirino and Mire-Sluis 2004; Ferreira et al. 2006) are considered important for the characterization of protein preparations:

i. *Identity:* The allergen preparations must contain recombinant or natural protein with correct amino acid composition and sequence. Mass spectrometry and amino acid analysis are the methods selected to evaluate this aspect. Mass spectrometry data must show molecular mass according to primary structure. Amino acid analyses must show amino acid content according to primary structure.

ii. *Purity:* The allergen preparations must be pure with respect to the protein content. The method of choice for analysis is SDS-PAGE. More than 95% of the material must be recovered in a single band after SDS-PAGE followed by protein silver-stain. In addition, amino acid analysis can provide information on the purity of a protein sample concerning contamination by other proteins.

iii. *Homogeneity:* The recombinant preparations must be homogenous with respect to the molecular weight and/or comparable to the natural counterparts. One method that is commonly used to evaluate homogeneity is analytical gel filtration. For

monomeric proteins, the sample must contain a single peak of expected molecular weight when analyzed by analytical gel filtration using an appropriate buffer.

iv. *Structure:* The allergen preparations must contain correctly folded protein. Circular dichroism (CD) spectroscopy is commonly used to analyze folding. CD of candidate molecules must show typical spectra of a folded protein with peak amplitudes similar to reference spectra. Small angle X-ray scattering can also provide valuable information as denatured proteins might show altered pair distance distribution function (PDDF).

Below, we give a brief overview on the methods employed in the CREATE project for the physicochemical characterization of the allergen preparations (Ferreira et al. 2006). Recombinant allergens in the CREATE project were produced in *Pichia* or *E. coli* expression systems. However, plant-produced allergens should be subjected to a similar set of analyses.

Amino acid analysis

Although amino acid analysis lacks the speed and sensitivity of mass spectrometry methods, it is still an indispensable tool in the analysis of protein pharmaceuticals. When performed on purified proteins, the technique allows the identification of the protein. It is also an extremely useful method for quantification of proteins, giving more accurate results than colorimetric methods. The accuracy of amino acid analysis depends on the integrity of the sample. Other critical factors affecting accuracy include purity of the reagents used, presence of salts, metals and/or detergents, and sample handling (Tyler 2000).

Peptide mapping, sequencing of peptides by MS/MS

A given protein sequence can be digested with a specific protease, like trypsin, to give rise to a predictable set of peptides. Accurate determination of the masses of a few peptides generated through trypsin digestion (to an accuracy of 1 Dalton) allows the comparison of experimentally determined masses with the masses of peptides obtained through in silico digestion of protein sequences and matching masses can be assigned to predicted peptides. However, the unambiguous assignment of peptide masses is only possible after sequencing. Sequencing of peptides requires tandem mass spectrometry, which is usually denoted MS/MS (Lill 2003; Reinders et al. 2004). In this technique, a given parent (precursor) ion is selected in one mass spectrometer and then fragmented, usually by collisions. The m/z (mass/charge) values for the resulting daughter (product) ions are measured in a second mass spectrometer. Under favorable conditions, this procedure may yield a series of ions containing sufficient information to determine the peptide sequence. Extraction of the peptide sequence from the daughter ion spectra depends on the completeness of the observed series, the accuracy with which the mass differences between the relevant ion peaks are determined and the extent to which the fragmentation spectrum can be correctly interpreted. A peptide resulting from tryptic digestion normally has a basic residue (arginine or lysine) at its C terminus and yields a prominent doubly charged ion peak when ionized by ESI. If this ion is chosen as the parent ion for an MS/MS measurement, the production of a series of y-ion daughters (ions resulting from cleavage at the amide bonds and containing the C terminus) is favored and the resulting spectrum is likely to be easy to interpret. For this reason, ESI has been a popular choice for peptide sequencing.

Analysis of tryptic peptides by LC-MS/MS involved several steps: (a) loading and washing the peptides on a pre-column, (b) gradient elution from the analytical column and acquisition of MS and MS/MS data and finally (c) computational data analysis. Data acquisition can be done in 2 alternating modes of the instrument. In the survey (MS) mode, the quadrupole of the spectrometer was deactivated and ions were analyzed by time-of-flight

spectrometry without fragmentation. The survey mode gives an overview of all ions eluting from the column at a given time. If the software recognized signals of doubly charged ions typical for peptides, the instrument switched from the survey into the sequencing (MS/MS) mode. In the sequencing mode, the quadrupole filters and removes all ions except the peptide ions to be sequenced. The filtered ions are fragmented in the collision cell and the fragment ions are then analyzed by time-of-flight spectrometry. In the final step, the raw data is analyzed and peptide sequences are established from the fragment patterns.

High-performance liquid chromatography–size exclusion chromatography

Separation of molecules using gel filtration is a simple and reliable method to analyze biomolecules on the basis of their relative size. Size exclusion chromatography provides information not only about the molecular weight but also on the aggregation status of the compounds in solution (Goetz et al. 2004). However, detection of aggregates might not be possible depending on the amounts present in the preparation or might go undetectable due to interactions with the column material. On the basis of the elution profile the approximate molecular weight and the homogeneity of the protein preparation can be determined.

Circular dichroism

Circular dichroism (CD) analysis of proteins is based on the differential absorption of left and right circularly polarized light in the far UV region by chiral chromophores. In proteins, mainly peptide bonds serve as chromophores. Absorption characteristics of peptide bonds are measurable depending on the environment and therefore on the secondary structure surrounding the chromophore. Compared to other structural technique such as NMR or X-ray crystallography, CD serves as a low-resolution method for the determination of secondary structure elements of proteins. Its main advantages are the little demands on both time and sample; yet CD spectra give valuable information on the folding of proteins (Verdino and Keller 2004; Kelly et al. 2005).

Small-angle X-ray scattering (SAXS)

The phenomenon of small-angle X-ray scattering (SAXS) resembles the situation when a beam of visible light is scattered by a colloidal suspension (Pilz et al. 1979). Clear solutions of non-aggregated proteins, in contrast, require an electromagnetic beam of much smaller wavelength to interact with. Therefore, monochromatic X-rays are guided through the protein solution and the scattering curve [intensity vs scattering vector] is recorded. By using an indirect Fourier transformation, the pair distance distribution function [distance frequency vs dimension] can be calculated from the scattering curve (Glatter 1977). The pair distance distribution function (PDDF) expresses the frequency of intramolecular distances between electrons. Thus, information on the size, shape, and aggregation behavior of proteins in solution can be obtained (Svergun and Koch 2002). The PDDF of a spherical molecule, for instance, shows a maximum at a distance corresponding to the radius and meets the *x*-axis at the maximal dimension of the molecule thus resembling a normal Gaussian curve. Extended ellipsoids or rod-like aggregates, on the other hand, give asymmetric PDDFs with maxima at rather low dimensions and less steep slopes to larger maximal dimensions. Therefore, information on size and shape of protein molecules in solution can be derived from PDDFs. Assuming a theoretical volume of 20 nm^3 for a spherical 10 kDa protein its aggregation state in solution can be estimated from the maximal dimensions of the PDDF.

Concluding remarks

Expressions systems capable of producing correctly folded allergens are essential for establishing molecule-based diagnosis of allergic diseases. Although the great majority of

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available recombinant allergens can be produced in bacterial or yeast systems, plant-based systems offer a number of unique and attractive features. Particularly, when considering that most allergenic proteins originate from plants and that some of them carry plant-specific post-translational modifications (e.g. glycans), which might be important for IgE recognition. Since IgE-binding is the basis for diagnostic tests in allergy, plant-produced allergens will certainly find their way into routine clinical practices. Finally, the use of edible plants or plant products for oral delivery of recombinant allergens is also being explored for oral immunotherapy of allergic diseases.

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Abbreviations

Art v	Artemisia vulgaris
Bet v	Betula verrucosa
CD	Circular dichroism
Cry j	Cryptomera japonica
Der f	Dermatophagoides farinae
Der p	Dermatophagoides pteronyssinus
ELISA	Enzyme-Linked ImmunoSorbent Assay
GFP	Green fluorescent protein
LC	Liquid chromatography
Lol p	Lolium perenne
Lyc e	Lycopersicon esculentum
Mal d	Malus domestica
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NMR	Nuclear magnetic resonance
ns-LTP	Non-specific Lipid Transfer Protein
PAGE	Polyacrylamide gel electrophoresis
PDDF	Pair distance distribution function
PEG	Polyethylene glycol
Pen a	Penaeus aztecus
SAXS	Small-angle X-ray scattering
SDS	Sodium dodecyl sulfate
SEC	Size exclusion chromatography
T_1	The first progeny generation of transgenic plants (T_0)
Th	T helper lymphocyte

TMV	Tobacco mosaic virus
ZYMV	Zucchini yellow mosaic virus

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Table 1

Examples of recombinant allergens produced in plants

Allergen source	Allergen	Transformation method	Host organism	Purification, tags	IgE reactivity	Comments	Reference
Betula verrucosa (pollen)	Bet v 1	TM virus	*Nicotiana benthamiana*	None	Sandwich ELISA	Immunogenicity tested in mice	Krebitz et al. (2000)
Malus domestica (fruit)	Mal d 2	TM virus	*Nicotiana benthamiana*	His-tag	Immunoblot	Anti-fungal activity	Krebitz et al. (2003)
Dermatophagoides pteronyssisnus (house dust mite)	Der p 1	*Agrobacteria*	Tobacco BY-2 cell culture	His-tag	T-cell proliferation	Glycosylation tested	Lienard et al. (2007)
	Der p 2				Histamine release	Secreted to culture medium	
					IgE reactivity		
Artemisia vulgaris	Art v 1	TM virus and *Agrobacteria*	*Nicotiana tabacum*	None	None	Secreted to the apoplast	Gadermaier et al. (2003)
Dermatophagoides farinae (house dust mite)	Der f 1	*Agrobacteria*	*Lotus japonicus*	None	None	Edible vaccine	Kato et al. (2005)
Dermatophagoides pteronyssisnus (house dust mite)	Der p 5	ZYM virus	*Cucurbita pepo* L. var Zucchini	His-tag	None	Edible vaccine	Hsu et al. (2004)
Cryptomera japonica (pollen)	Cry j 1	*Agrobacteria*	*Oryza sativa*	None	Oral therapy in mice	Expression in seeds	Okada et al. (2003)
	Cry j 2					Edible vaccine	Takagi et al. (2005a)
	7 Cry j 1 epitopes						Takagi et al. (2005b)