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The expression of a mountain cedar allergen comparing plant-viral apoplastic and yeast expression systems

Marcie H. Moehnke¹, Terumi Midoro-Horiuti^{2,3}, Randall M. Goldblum^{2,3}, and Christopher M. Kearney¹

¹ Institute of Biomedical Studies, Baylor University, One Bear Place #97388, Waco, TX 76798, USA

² Department of Pediatrics, Child Health Research Center, University of Texas Medical Branch, Galveston, TX, USA

³ Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, TX, USA

Abstract

Jun a 3, a major allergenic protein in mountain cedar pollen, causes seasonal allergic rhinitis in hypersensitive individuals. Recombinant Jun a 3 was expressed in *Nicotiana benthamiana* interstitial fluid (300 µg/g leaf material) and *Pichia pastoris* (100 µg/ml media). Polyclonal anti-Jun a 3 and IgE antibodies from the sera of allergic patients both reacted with the recombinant protein. Of the two systems, recombinant protein from the plant apoplast contained fewer contaminating proteins. This method allows for a more convenient and inexpensive expression of the recombinant allergen, which will allow for further structural studies and may prove useful in diagnostic and/or immunotherapeutic strategies for cedar allergy.

Keywords

Jun a 3; *Juniperus ashei*; *Pichia pastoris*; Recombinant allergen; Tobacco mosaic virus

Introduction

Type I hypersensitivity diseases afflict up to a quarter of the world's population. Inadequacies in current therapies are driving efforts to identify alternative sources for allergen vaccines, which might increase the effectiveness and reduce the adverse effects of this therapy (Breiteneder et al. 2001). The use of recombinant allergens could allow modification of the expressed proteins to reduce reactogenicity, and thereby reduce the risk associated with injection of crude allergen extracts (Akdis and Blaser 2000; Valenta et al. 2002; Singh and Bhalla 2006). Finally, recombinant allergens are valuable tools for structural analysis, including that of critical epitopes, and could provide a better understanding of the allergic immune response.

Protein expression systems for producing such allergens must consistently produce adequate amounts of recombinant allergens that retain the immunogenic nature of the native allergen. The yeast *P. pastoris* has been used to express many allergens (Barral et al. 2004; Saarelainen et al. 2004; Barderas et al. 2005). Because *P. pastoris* secretes few of its own proteins, recombinant proteins extracted from the medium are less contaminated with endogenous

proteins than those expressed intracellularly. Several recombinant allergens have also been produced in ground leaves of *N. benthamiana* plants via a tobacco mosaic virus (TMV) vector (Krebitz et al. 2000, 2003). However, extraction from ground leaves can make purification difficult. This can be overcome by expressing the protein in the plant apoplast through the use of a signal peptide. The protein can be extracted from the apoplast by vacuum infiltration of the leaf. Upon centrifugation, the buffer will spin out of the leaf bringing the foreign protein along with it. This approach provides an initial purification step by eliminating most plant cellular proteins (McCormick et al. 1999).

Previous attempts to express Jun a 3 allergen, a 30 kDa protein of *Juniperus ashei* trees, in bacteria were unsuccessful (Goetz et al. 1995; Midoro-Horiuti et al. 2000; Soman et al. 2000). Here we describe the expression of recombinant Jun a 3 (rJun a 3) in *Nicotiana benthamiana* and in *Pichia pastoris*. The recombinant allergen bound polyclonal IgG antibodies raised against native Jun a 3, as well as IgE antibodies in the sera of subjects allergic to mountain cedar pollen. Furthermore, we have demonstrated for the first time that extraction of a recombinant allergen from the plant apoplast via vacuum infiltration is advantageous as an initial purification step, compared to secretion in *P. pastoris*.

Materials and methods

Isolation of *Juniperus ashei* RNA and cDNA synthesis

Juniperus ashei pollen was purchased from Hollister-Stier (Spokane, WA). Total RNA was isolated from 50 mg pollen using Promega's RNagents Total RNA Isolation System. Reverse transcription of 2 µg mRNA was achieved using an oligo-dT primer and an M-MLV reverse transcriptase. Second-strand synthesis was accomplished via PCR using primers specific for the Jun a 3 sequence. The following primers were designed for TMV-plasmid cloning: 5'-GCGGTTAATTAAATGGCCCGAGTATCAGAGCTTGCG-3' (sense, *PacI* restriction site in bold followed by Jun a 3 native signal peptide sequence) and 5'-GGCGCTCGAGTTAGGGACAGAATACTACT-3' (antisense, *XhoI* restriction site bold, stop codon underlined). Primers designed for yeast-plasmid cloning are as follows: 5'-GCGGCTCGAGAAAAGAGAGGCTGCCCGAGTATCAGAGCTTGC-3' (sense, *XhoI* restriction site bold, Kex2/Ste13 cleavage sites underlined followed by Jun a 3 coding sequence) and 5'-GCGGGCGGCCGCTCAAGGGCAGAATACTACTGT-3' (antisense, *NotI* restriction site bold, stop codon underlined).

Production of recombinant Jun a 3 in *N. benthamiana*

Construction of the pBSG1057-Jun a 3 expression vector and inoculation of *N. benthamiana* plants with recombinant infectious RNA—A 30B-based tobacco mosaic virus vector, pBSG1057, (Large Scale Biology Corporation, Vacaville, CA) and the Jun a 3 construct (containing its native signal peptide sequence) were digested with *PacI* and *XhoI*. Following ligation, the pBSG1057-Jun a 3 products were used to transform competent DH5α *E. coli* cells via electroporation. Positive clones were sequenced using a CEQ capillary sequencer (Beckman Coulter).

The pBSG1057-Jun a 3 vector was in vitro transcribed to generate capped infectious RNA using T7 RNA polymerase from the mMessage mMachine kit (Ambion). An equal volume of FES buffer (0.1 M glycine pH 8.9, 0.06 M K₂HPO₄, 1% sodium pyrophosphate, 1% Celite, 1% bentonite) was added to each reaction, and two *N. benthamiana* leaves were mechanically inoculated per plant (10 µl per leaf). Ten days post-inoculation, the virus was passaged to larger plants by grinding 1 g infected leaf material with 1 ml GP-Celite buffer (50 mM glycine pH 8.9, 30 mM K₂HPO₄, 1% w/v celite) and rubbing the inoculum onto two leaves per plant.

Detection of viral RNA and extraction of Jun a 3 protein—Fourteen days post-inoculation, total RNA was extracted from 100 mg systemically infected leaves using Qiagen's RNeasy Plant Mini Kit. The RNA was reverse transcribed using an oligo-dT primer. This served as a template for PCR, in which pBSG1057-specific primers that flanked the inserted gene were used. Twenty-one days post-inoculation, secreted proteins were extracted using a vacuum infiltration method (McCormick et al. 1999). Specifically, 2 g leaf material was submerged in 50 ml infiltration buffer (100 mM Tris/HCl (pH 7.5), 10 mM MgCl₂, 2 mM EDTA). This was placed into a vacuum chamber for 2 min. The vacuum was removed by allowing air to flow into the chamber as a sudden single burst, causing the leaves to turn darker in color and sink. The leaves were removed, dried with paper towels, and then placed into a strainer cup positioned into a 250 ml centrifuge tube. After centrifugation at 2,000g for 10 min (4°C), secreted proteins were collected from the bottom of the centrifuge tube.

Production of rJun a 3 in *P. pastoris*

Construction of the pPICZα-Jun a 3 expression plasmid—Restriction digests were performed on the pPICZα vector (Invitrogen) and the Jun a 3 PCR product using *Xho*I and *Not*I, followed by ligation. The pPICZα-Jun a 3 (pPICZα-J3) ligation products were transformed into competent DH5α *E. coli* cells according to company protocols (Invitrogen, Carlsbad, CA). Transformants were plated on low salt LB plates supplemented with Zeocin (25 µg/ml). PCR was used to select for clones containing the Jun a 3 insert. Positive clones were sequenced using a CEQ capillary sequencer (Beckman Coulter). Ten micrograms of pPICZα-J3 were completely linearized with *Sac*I, and the purified fragments were used to electroporate *P. pastoris* cells, strain GS115, to allow a single crossover recombination event at the AOX1 locus. Transformants were plated on yeast extract peptone dextrose sorbitol medium/Zeocin (100 µg/ml) and incubated at 30°C for 3 days. Colonies were then plated onto minimal methanol histidine (MMH) plates and incubated for 2 days at 30°C to select for the Mut⁺ phenotype.

Expression of rJun a 3—Clones with the Mut⁺ phenotype were inoculated into 50 ml of buffered glycerol complex medium in a 500 ml baffled flask and allowed to grow at 30°C with shaking at 250 rpm for approx 30 h, or until the OD₆₀₀ reached 2–6. The culture was then centrifuged, and the remaining cell pellet was resuspended in buffered methanol complex media to an OD₆₀₀ of 1 to induce expression. Cultures were shaken in 500 ml baffled flasks at 30°C for 96 h. Methanol was added daily to give 0.5%, and samples were taken daily to evaluate expression levels. Cells were cleared from the culture samples by centrifugation.

Immunoblotting experiments

Proteins were analyzed on 15% (v/v) polyacrylamide gels. Protein concentrations were determined by the Bradford method. N-Terminal sequencing was performed on the rJun a 3 protein using Applied Biosystem's Procise sequencing system (Biomedical Resources Laboratory at University of Texas Medical Branch at Galveston).

Proteins separated via SDS-PAGE were transferred to a PVDF membrane. The membranes were blocked and then incubated with rabbit polyclonal anti-Jun a 3 IgG antibody at 1 µg/ml. Bound IgG was detected with anti-rabbit IgG-HRP conjugate (1:1,000, Sigma). Visualization was performed using ECLTM detection reagents (Amersham) followed by immunofluorescence detection.

IgE ELISA

Serum samples were obtained from five *J. ashei* allergic patients in Austin, Texas, USA. Subjects were selected on the basis of clinical history, positive scratch test to *J. ashei* and positive ImmunoCAP to native Jun a 3 (Phadia, Uppsala, Sweden). Normal control serum

samples were obtained from subjects without clinical allergy history and negative scratch test to *J. ashei* and dust mite.

Microtiter plates were coated with 100 µl extract that contained 1, 5, or 10 µg/ml of rJun a 3. After blocking with 5% (v/v) non-fat dry milk, 100 µl pooled sera from patients allergic to *J. ashei* (1:10) or non-allergic individuals was added to each well and incubated at room temperature for 2 h. Bound IgE was detected by adding 100 µl anti-human IgE-HRP conjugate (1:5,000) (Sigma) and incubating at room temperature for 1 h. Colorimetric substrate, TMB, was added and incubated for 1 h at room temperature. The reaction was stopped by adding 100 µl 2 M HCl and the OD_{450 nm} was measured.

Results

Expression and identification rJun a 3 in *N. benthamiana* plants

The Jun a 3 coding sequence, including its signal peptide, was successfully inserted into the pBSG1057 vector under the control of the TMV-U1 coat protein subgenomic promoter. Insertion of the gene, as well as correct orientation were verified with PCR and DNA sequencing. This construct was used to generate infectious viral RNA, which was subsequently used to inoculate *N. benthamiana* plants. Ten days post-inoculation, signs of infection, such as mottled and deformed leaves, were apparent not only in locally inoculated leaves but in systemic leaves as well. RT-PCR products confirmed that the Jun a 3 sequence was stably maintained in the TMV vector. SDS-PAGE revealed a band migrating at 30 kDa from the leaves of plants inoculated with vector carrying Jun a 3, which was not present in the control plants (Fig. 1b). The recombinant proteins were expressed at approx 300 µg from the vacuum infiltrate of 1 g of intact leaves. Western blotting confirmed the identity of the 30 kDa band by its reactivity with an anti-Jun a 3 antiserum (Fig. 2). N-Terminal sequencing of this band revealed that the signal sequence of the rJun a 3 was correctly processed by the plant. Passage of the virus from an infected leaf to larger, uninoculated plants (second passage) resulted in infection of the larger plants. RT-PCR revealed that stable Jun a 3 RNA transcripts were generated, and SDS-PAGE showed rJun a 3 was expressed in these second passage plants. ELISA experiments demonstrated that IgE antibodies from sera of subjects with *J. ashei* pollinosis bound to the rJun a 3 from the proteins extracted by vacuum infiltration. The amount of IgE that bound to wells was proportional to the concentration of the rJun a 3 in the coating solution. IgE in the sera from non-allergic individuals did not bind to rJun a 3 (Fig. 3).

Expression of rJun a 3 in *P. pastoris*

SDS-PAGE analysis of supernatants collected 24 h after induction with methanol revealed a band that approximated the migration of native Jun a 3, which was not seen in the vector control (Fig. 1c). The highest expression levels were seen at 96 h, when rJun a 3 was expressed at approx 100 µg/ml. Western blot analysis revealed the recombinant protein bound polyclonal antibodies to Jun a 3. ELISA experiments showed that the rJun a 3 displayed was also recognized by the IgE in the patient sera, as indicated by a significant increase in IgE binding relative to wells containing sera from non-allergic individuals. Supernatants from yeast transformed with the same vector lacking the Jun a 3 insert bound less of the IgE from patient sera. In addition, IgE in the sera from non-allergic individuals did not significantly bind rJun a 3 (data not shown).

Discussion

This study describes the successful expression of a major *J. ashei* allergen, Jun a 3, in both a virus-mediated plant expression system and a yeast expression system. Both systems are, essentially, secretion expression systems, with the expressed protein being secreted either into

the growth medium with yeast or into the intercellular spaces of the apoplast with the plant system. However, the media from the *Pichia* system contained a larger amount of contaminating proteins. In contrast, the fluid from the vacuum infiltration of the plant expression system yielded a protein product that was approximately 80% pure, which would expedite final purification. This is the first report of apo-plastic extraction of a recombinant allergen. Previously expressed recombinant allergens (Krebitz et al. 2000, 2003) have been extracted by grinding leaves in an extraction buffer. Upon using this extraction method for rJun a 3, several contaminating proteins were extracted as well, making vacuum infiltration a much more valuable tool for extraction from plants (Fig. 1a, b).

The molecular farming strategy is easy to use, relatively inexpensive, and capable of large-scale production. Additionally, because many allergens are of plant origin, plant-based systems provide a means of homologous expression, particularly because of the plant's ability to process the allergen such that it is very similar to the native allergen. Transient expression vectors can also express larger proteins, with the maximum size of an insert at 1.5 kb for a systemically moving TMV vector (G. Pogue, pers. commun.).

There is a great need for recombinant allergens that mimic the native allergens, not only for structural and mutational analysis, but also to develop vaccines for allergy therapy. Recombinant allergens would allow immunotherapy to be performed with pure allergens as opposed to the crude extracts of plant materials (usually pollens) currently in use. This would allow the injections to be standardized such that the exact amount of allergen would be present from batch to batch. Plant and yeast-based expression systems can provide high quantities of soluble, immunogenic recombinant proteins, which can be hard to obtain with other expression systems such as *E. coli*. These systems also provide a means of expressing biologically active allergens at low costs.

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Fig. 1. Coomassie-stained SDS-PAGE of rJun a 3 expression. **(a)** Extraction from two different *N. benthamiana* plants via grinding of leaf material. **(b)** Extraction from three different *N. benthamiana* plants via interstitial fluid collection. **(c)** Expression in *P. pastoris*. Lane 6, culture supernatant from vector control; Lane 7, rJun a 3 culture supernatant

Fig. 2. Western blot using anti-Jun a 3 antibody identifying rJun a 3. Top: *N. benthamiana* extractions. *Lane 1*, vector-inoculated plant; *Lane 2*, uninoculated plant; *Lane 3*, rJun a 3 1st passage plant; *Lane 4*, rJun a 3 2nd passage plant. Bottom: *P. pastoris* extractions. *Lane 5*, supernatant from vector control; *Lane 6*, rJun a 3 from supernatant

Fig. 3. ELISA showing reactivity of IgE from allergic patient sera with rJun a 3 expressed in *N. benthamiana*. ELISA plates were coated with vacuum extracts containing varying amount of rJun a 3 and developed with sera from either allergic or non-allergic subjects and anti-human IgE antibody