



Research paper

High-throughput screening of T7 phage display and protein microarrays as a methodological approach for the identification of IgE-reactive components

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ABSTRACT

Olive pollen and yellow mustard seeds are major allergenic sources with high clinical relevance. To aid with the identification of IgE-reactive components, the development of sensitive methodological approaches is required. Here, we have combined T7 phage display and protein microarrays for the identification of allergenic peptides and mimotopes from olive pollen and mustard seeds. The identification of these allergenic sequences involved the construction and biopanning of T7 phage display libraries of mustard seeds and olive pollen using sera from allergic patients to both biological sources together with the construction of phage microarrays printed with 1536 monoclonal phages from the third/four rounds of biopanning. The screening of the phage microarrays with individual sera from allergic patients enabled the identification of 10 and 9 IgE-reactive unique amino acid sequences from olive pollen and mustard seeds, respectively. Five immunoreactive amino acid sequences displayed on phages were selected for their expression as His6-GST tag fusion proteins and validation. After immunological characterization, we assessed the IgE-reactivity of the constructs. Our results show that protein microarrays printed with T7 phages displaying peptides from allergenic sources might be used to identify allergenic components -peptides, proteins or mimotopes- through their screening with specific IgE antibodies from allergic patients.

1. Introduction

Type I allergy, or immediate hypersensitivity, is a generalized health problem in industrialized countries with an increasing incidence in the last years, which affects about 30% of the population (Asher et al., 2006; Eder et al., 2006; Bousquet et al., 2009). The starting point for triggering the allergic symptoms -rhinitis, conjunctivitis or asthma- consists of IgE overproduction against allergenic substances, innocuous for the rest of the population (Dahl et al., 2004; Stone et al., 2010).

In Mediterranean countries, large areas of California and Australia, olive (*Olea europaea*) pollen is a major allergenic source with a high clinical relevance (Rodríguez et al., 2001; Vereda et al., 2011; Villalba et al., 2014). In Spain, olive pollen is the second cause of pollinosis after grass pollen, and the first one in some localized areas of Extremadura and Andalusia, where olive trees are extensively cultivated. Thirteen

allergens (Ole e 1–13) from olive pollen have been described so far, but its complex allergogram remains to be completed (Rodríguez et al., 2001; Villalba et al., 2014). Indeed, by western blot (WB) analyses there are several IgE-recognized protein bands not yet identified as allergens, and about 20% of patients are negative to the major olive pollen allergens Ole e 1, Ole e 7 and Ole e 9 (Alcántara et al., 2017). On the other hand, yellow mustard (*Sinapis alba*) seeds are responsible for severe allergic symptoms (angioedema and anaphylaxis reactions) in allergic patients in multiple countries (Rance, 2003; Vereda et al., 2011). The use of yellow mustard as a condiment in many foods or a hidden component in many pre-packaged meals produces unexpected reactions and makes mustard an important food allergen in developed countries. Four allergens from mustard seeds have been described (Sin a 1–4), remaining other IgE-reactive components to be identified (Rance, 2003; Sirvent et al., 2009; Vereda et al., 2011).

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Saline protein extracts are commonly used for isolation of allergens and to produce allergenic extracts for skin testing diagnosis and immunotherapy. However, the use of saline protein extracts to identify IgE-reactive components limits this process to those soluble molecules after the extraction procedure. Thus, to identify new IgE-reactive molecules and to contribute to the completion of allergograms of allergenic sources with high clinical relevance, new methodological approaches allowing for the identification of undetectable molecules by conventional methods are needed. The combination of the high-throughput screening of phage display and protein microarrays might offer an interesting approach to identify new allergenic components.

Phage display technology allows for the *E. coli* expression of exogenous peptides on the surface of viral particles using coat proteins as fusion proteins. The construction of phage libraries displaying foreign peptides and their subsequent screening against a desired target are the basis of this method, which has been mainly used for monoclonal antibody production, and epitope mapping, among others (Rhyner et al., 2004; Pande et al., 2010). On the other hand, protein microarrays are a powerful tool for large scale testing of biological samples, which allow for the high-throughput simultaneous screening and rapid analysis of thousands of proteins. These tools permit surveying antigens and antibodies in blood samples or other biological fluids using very low volumes of samples, and have been commonly used for the identification of biomarkers (Kodadek, 2001; Ramachandran et al., 2008). The combination of phage display and protein microarrays increases the throughput of both assays, and has already been used to identify molecules with diagnostic purposes in malignancies different from allergy (Wang et al., 2005; Bradford et al., 2006; Chatterjee et al., 2006a; Freckleton et al., 2009; Babel et al., 2011).

Here, we combined the screening of T7 phage libraries displaying peptides and proteins encoded by cDNA from olive pollen and yellow mustard seeds with protein microarrays as a new methodological approach for the identification of IgE-reactive molecules of these highly relevant clinical allergenic sources. First, two T7 phage libraries displaying amino acid sequences encoded by the cDNA from yellow mustard seeds and olive pollen were constructed. After biopanning of the libraries with sera from allergic patients, 768 monoclonal phages of each library were printed on nitrocellulose microarrays, and probed with sera from both types of allergic patients (Fig. 1). The subsequent screening and bioinformatic analyses of the phage microarrays enabled the identification of allergenic peptides or mimotopes -defined as peptides capable of binding to the paratope of an antibody but unrelated in sequence to the natural protein that the antibody actually recognizes- (Geysen et al., 1986). In total, 10 and 9 unique allergenic peptides or mimotopes from olive pollen and mustard seeds were found, respectively. After expression as His6-GST N-terminal tagged fusion proteins of 5 peptides or mimotopes, we confirmed their allergenicity and observed that the purified allergenic molecules of mustard seeds in combination were able to abrogate about 80% of the specific IgE binding to mustard seeds extract.

2. Materials and methods

2.1. Non-atopic control serum, mustard and olive pollen allergic patients' sera

The Institutional Ethical Review Boards of the Complutense University of Madrid approved this study on the identification of allergenic proteins. Written informed consent was obtained from all patients. Serum samples were obtained from a collection of sera previously characterized from allergic patients to mustard seeds from the Fundación Jiménez Díaz Hospital or olive pollen from the Complejo Hospitalario de Jaén (Sirvent et al., 2009; Vereda et al., 2011; Villalba et al., 2014). In addition, non-atopic controls were obtained from the Fundación Jiménez Díaz Hospital.

For selection of T7 phage libraries, three non-atopic controls sera for

negative selection and allergic patients' sera with > 500 kU/l of total IgE (four sera from mustard seeds and four from olive pollen) for the positive selection of IgE immunoreactive phages were used. The allergic patient's sera were selected for their high specific IgE levels to multiple allergenic protein bands visualized by WB analysis of olive pollen and mustard seeds extracts. For microarray analysis, serum samples from 5 allergic patients to mustard seeds and 5 olive pollen allergic patients' sera were analysed. For validation, an independent cohort of 32 mustard seed allergic patients (Sirvent et al., 2009; Vereda et al., 2011), 92 serum samples from olive pollen allergic patients (Villalba et al., 2014), and three non-atopic control sera were used. As the concentration of IgE in non-atopic controls is minimal, three patients were considered optimal to be used as negative controls in the assays. All samples were handled anonymously according to the ethical and legal guidelines of the Complutense University of Madrid. Skin prick tests (SPT) were performed according to standard procedures. A wheal diameter < 3 mm was considered negative. All patients included in this study reported a clear immediate IgE-mediated allergic reaction with mustard or olive pollen, and had positive SPT to mustard or olive pollen extracts. Olive pollen allergic patients did not report any episode of food allergy and their SPT were negative for commercial food extracts. On the other hand, mustard allergic patients were selected because of a positive SPT to mustard extract, and no positive SPT to pollen extracts. In addition, mustard seeds allergic patients were also positive by SPT to tree nuts and/or peanut. Quantification of specific IgE levels in serum was performed by ELISA using mustard seeds or olive pollen extracts. Each OD value was the mean of 2 determinations after blank subtraction. OD values < 0.1 were considered negative.

2.2. T7 phage display cDNA library synthesis, biopanning, printing and quality control of phage microarrays

We used 4 µg of mRNA isolated from olive pollen and mustard seeds, respectively, for cDNA synthesis. Then, T7 phage cDNA display libraries were constructed using the OrientExpress cDNA synthesis and cloning systems (Novagen) according to manufacturer's instructions. Both cDNAs were synthesized using oligo(dT) primer by RT-PCR, size separated to get rid of small cDNA fragments, and cloned indistinctly into T7Select 415-1 (able to display 415 copies of peptides about 50 amino acids in length) and T7Select 10-3b vectors (able to display an average of 5–15 copies of peptides and proteins up to 1200 amino acids in length) according to the manufacturer instructions. They were grown on *E. coli* BL21 or BLT 5403 strains, respectively. The four T7 libraries on T7Select 415-1 and T7Select 10-3b vectors were titrated using serial dilutions and plaque assays. Library sizes were higher than 10⁶ pfu/mL.

Biopanning was performed in solution using tosil-activated magnetic beads (Invitrogen) covalently attached with an anti-IgE monoclonal antibody (ALK-Abelló) according to the manufacturer instructions. First, a negative selection was performed. Beads were incubated with 120 µL pooled control sera diluted 1:10 at 4 °C overnight. After washing the beads, phage biopanning was performed separately using the 2 libraries constructed with mustard seeds cDNA and the 2 libraries constructed with olive pollen cDNA. In addition, to avoid for the selection of phages displaying sequences from previously known allergens from both allergic sources, the pool of sera of olive pollen allergic patients was preincubated with 10 µg of Ole e 1, profilin, rOle e 3, Ole e 9, rOle e 10, rOle e 11 and rOle e 12, and the pool of sera of mustard allergic patients with 10 µg of profilin, rSin a 1, rSin a 2 and rSin a 3. The flow-through phages were then separately incubated with tosil-activated anti-IgE mAb magnetic beads coated with IgE antibodies captured from the pools of sera of allergic patients in the same conditions. Two different pools of serum samples from mustard seeds or olive pollen allergic patients were used to enrich for IgE reactive phages using respectively phage display cDNA libraries from mustard seeds cDNA or olive pollen cDNA. Finally, after washing with phosphate-buffered saline (PBS) containing Tween 20 0.1%, bound phages were

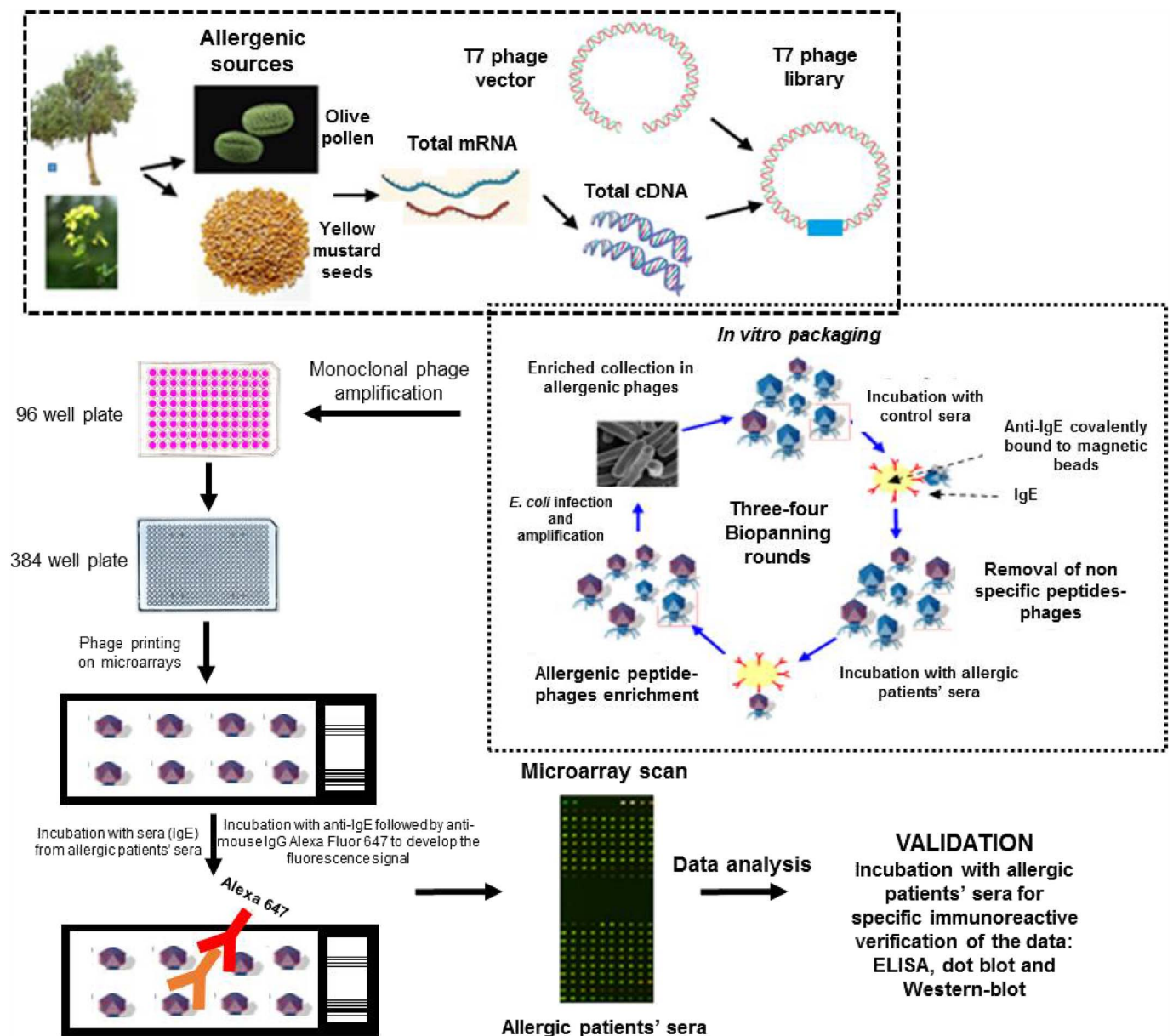


Fig. 1. Schematic representation of the work-flow followed for the identification of allergenic sequences from mustard seeds and olive pollen by phage microarrays. The steps involving the isolation of mRNA and cDNA synthesis for the construction of phage display libraries from mustard seeds and olive pollen are firstly described. Secondly, the biopanning procedure to enrich the libraries using sera from non-atopic controls for a negative selection and sera from allergic patients pre-adsorbed with known allergens from both biological sources to enrich the libraries in non-previously described specific allergenic peptides is summarized. Finally, the schematic of phage microarray construction, screening and data analysis is shown.

eluted with 100 μ L of 1% SDS and amplified in *E. coli* BLT5403 or BL21 strains. The panning was repeated three more times to enrich the four initial libraries in IgE-reactive specific phages.

Following biopanning and amplification of random selected 1536 individual phages from the third and fourth rounds of biopanning of the four selections, monoclonal phage-containing supernatants were clarified by centrifugation. Then, phages were diluted 1:1.5 in PBS containing 0.1% Tween 20 (PBST) and printed in duplicate onto nitrocellulose-coated slides (Whatman/Schleicher and Schuell's) using a Microgrid II Spotter (Babel et al., 2011). Negative controls consisted of BSA (Sigma-Aldrich), PBST or empty spots. Human IgG and mouse IgG (Sigma-Aldrich), and AlexaFluor 555 and AlexaFluor647 (Invitrogen) were also spotted as positive controls to verify the array quality.

Phages printed in nitrocellulose microarrays were detected using an anti-T7 antibody, and the presence/absence of unspecific reactivity analysed using sera from non-atopic controls followed by an anti-human IgE monoclonal antibody and AlexaFluor 647 anti-mouse IgG,

or the secondary antibodies. A homogeneous signal was observed for the anti-T7 antibody, whereas an absence of specific signal was observed for the non-atopic controls or the control of reactivity of the secondary antibodies, indicating the good quality of the microarrays (Supplementary Fig. 1). In addition, to cope with potential concerns related to array reproducibility, we plotted fluorescence intensities of spot duplicates, determining a good correlation among duplicates. Then, we analysed the median of spot duplicates in two different microarrays incubated with anti-T7 antibody followed by AlexaFluor 647 anti-mouse IgG. A good correlation was observed among median fluorescence intensity of duplicates in both arrays (Supplementary Fig. 1).

2.3. Phage microarray screening with sera and scanning

Briefly, slides were equilibrated in PBS at room temperature for 5 min and then blocked with 5% skimmed milk in PBS (MPBS) for 2 h at

room temperature with gentle rocking. Then, 600 μ L of 5% MPBS containing serum of individual mustard seeds allergic patients (1:5 diluted) or olive pollen allergic patients (1:10 diluted), containing 120 μ g of BLT5403 *E. coli* lysate, which also contains the 10B protein of the T7 phage to whom foreign amino acid sequences from mustard seeds or olive pollen are fused on the C-terminal end, to remove unspecific binding to *E. coli* proteins or to the 10B protein were incubated overnight at 4 °C with gentle rocking. The day after, to detect specific human IgE antibodies, slides were subsequently incubated with an anti-human IgE murine monoclonal antibody (ALK Abelló) 1:500 diluted in 5% MPBS for 1 h at room temperature, washed, and then incubated with Alexa Fluor 647-labeled goat anti-mouse IgG (Invitrogen, Carlsbad, CA) diluted 1:2000 in 5% MPBS during 1 h at room temperature. Arrays were then washed five times with PBST, once with PBS, once with MilliQ water and dried by centrifugation at 140g for 10 min. Finally, slides were read on a GenePix 4000B Microarray Scanner (Molecular Devices).

2.4. Bioinformatics and statistical analysis

Analysis, normalization, and quantification of all microarray images were performed using GenepixPro 7.1 software (Axon Laboratories, Boston, MA). The median values of the spots and background were determined, and interarray median normalization was performed (Madoz-Gurpide et al., 2007; Babel et al., 2009; Anderson et al., 2011; Miersch et al., 2013; Yu et al., 2013). To identify immunoreactive phages, a *t*-test using pomelo II (<http://pomelo2.bioinfo.cnio.es/>) was performed, where *p* values were obtained by permutation testing using 200,000 permutations. Pomelo II generated three heatmaps from the statistical analyses using three different algorithms: *limmate*, *paired t-test*, and *regression test*, showing the phages with a false discovery rate (FDR) value below 0.15 and an unadjusted *p* value below 0.05. In addition, we used MultiExperiment Viewer Analysis (MeV) for visualization. Mean and standard error of the mean (SEM) calculations were performed with Microsoft Excel 2010.

2.5. Individual phage analysis by dot-blot

Phages from 5 mL bacterial lysed cultures were centrifuged at 1600g at 4 °C for 30 min, and supernatant containing phages precipitated with 20% polyethylene glycol 8000, 2.5 M NaCl in ddH₂O for their purification (Barderas et al., 2006; Barderas et al., 2008b). Phages were resuspended in 250 μ L of sterile PBS. Dot-blot was performed by spotting in duplicate on nitrocellulose membranes using a 96-well Bio-dot (Bio-Rad) 100 μ L of the amplified phages diluted 1:10 in PBS-Tween 0.1%. Membranes containing blotted phages were incubated with monoclonal anti-T7 antibody (Sigma, diluted 1:2000), directed against the T7 tag located at the N-terminal end of the 10B protein and therefore should bind T7 phage particles. Bound antibodies were detected by horseradish peroxidase (HRP)-labeled rabbit anti-mouse IgG antibody (diluted 1:2500). Alternatively, membranes were incubated with a pool of allergic sera to mustard or olive pollen (diluted 1:5, *n* = 5), or non-atopic control sera (diluted 1:5), preincubated with BLT5403 *E. coli* cell lysate to remove from non-specific binding to *E. coli* proteins and 10B protein of the phage capsid. Binding of specific human IgE was detected by using mouse monoclonal anti-human IgE antibody (diluted 1:5000), followed by HRP-labeled rabbit anti-mouse-IgG antibody (Dako, diluted 1:3000). HRP reaction was detected using the enhanced chemiluminescent substrate (ECL) Pierce ECL Western Blotting Substrate (ThermoFisher Scientific).

2.6. Sequencing of the inserted cDNA in phages

The cDNA sequence inserted in each individual phage was amplified by PCR with TaKaRa DNA Polymerase (TaKaRa Bio Inc.) using 5 μ L of each phage as template and two non-degenerate oligonucleotides, a

sense 5'TGCTAAGGACAACGTTATCGG3' (T7UP New) and an antisense 5'TTGATACCGGACGTTTCAC3' (T7Down New) (Babel et al., 2011). Then, the amplified DNA was purified by precipitation and directly sequenced. Sequence identity and homology searches were performed with Blastp using the amino acid sequences displayed in frame in the T7 phage (Boratyn et al., 2013).

2.7. Cloning and expression of the cDNAs-encoding allergenic peptides as His6-GST fusion proteins

cDNA-encoding the selected sequences displayed on phages (MUST1, MUST3, MUST7, MUST20 and OLI4) were amplified by PCR using the T7UP New and the T7Down New oligonucleotides, digested with *Nde*I and *Hind*III restriction sites, and after purification directly used for subcloning into the pET28aGSTf vector. pET28aGSTf plasmid was developed in this study to express the allergenic peptides as fusion proteins to a His6-GST tag in the N-terminal end of the foreign peptides from mustard seeds or olive pollen. Cultures containing the recombinant constructs were grown overnight and the day after diluted 10-fold in 250 mL LB medium with 30 μ g/mL of Kanamycin. Induction was performed at an optical density (O.D.) of 0.6 at 600 nm by the addition of 0.4 mM of Isopropyl Thio- β -D-Galactoside (IPTG). We analysed protein expression after IPTG induction at 37 °C for 4 h, 30 °C for 16 h and 16 °C for 48 h. We observed the best conditions for expression of MUST1, MUST3, MUST7 and OLI4 at 37 °C for 4 h and MUST20 at 16 °C for 48 h to avoid its extensive degradation observed when expressed at 30 °C and 37 °C. Then, cell cultures were centrifuged at 1600g for 10 min at 4 °C. The pellet was reconstituted in 20 mM Tris-HCl, 0.5 M NaCl, 5 mM Imidazol, 1 mM PMSF, pH 8. After bacterial lysis by five cycles of freezing and thawing at 42 °C, the material was centrifuged at 16,000g for 15 min at 4 °C. Then, both soluble and insoluble fractions of *E. coli* BL21 (DE3) cultures were analysed by Coomassie Blue and immunostaining with an anti-His6 epitope antibody (Pierce, Thermo-Scientific) after SDS-PAGE in 15% polyacrylamide gels under non-reducing conditions as previously performed (Barderas et al., 2008b; Barderas et al., 2012; Mas et al., 2015). Soluble fractions were used as the starting material for the purification of the recombinant proteins, except for OLI4 and MUST20 constructs that were purified after the solubilization of the inclusion bodies (Mas et al., 2017). Recombinant fusion proteins were purified using the QIAexpress Ni-NTA (Nickel-Nitriloacetic Acid) Protein Purification System (Qiagen) following the manufacturer instructions.

2.8. Immunological characterization of the allergenic constructs

Indirect ELISA was performed in 96-well plates coated with 500 ng of the recombinant allergenic constructs, as optimized previously (data not shown). Patients sera were preadsorbed with His6-GST construct to remove non-specific binding to the protein tags and sera from allergic patients to mustard seeds tested at 1:5 dilution and sera from olive pollen allergic patients at 1:10 dilution, as previously optimized (Monsalve et al., 2001; Sirvent et al., 2009; Vereda et al., 2011; Castro et al., 2014; Villalba et al., 2014). Binding of specific human IgE was detected by anti-human IgE mAb (diluted 1:5000) followed by HRP-labeled rabbit anti-mouse IgG antibody (diluted 1:2000) (Mas et al., 2015; Mas et al., 2017). Signal was developed as previously described (Mas et al., 2015; Mas et al., 2017). OD values > 0.1 arbitrary units were considered positive.

ELISA Inhibition assays were performed in 96-well plates coated with 20 μ g of the mustard seeds extract using an equivolumetric pool of human mustard allergic patients' sera, previously adsorbed to 1 μ g or 5 μ g in 50 μ L for the allergenic fusion constructs. Binding of specific human IgE was detected by anti-human IgE mAb (diluted 1:5000) followed by HRP-labeled rabbit anti-mouse IgG antibody (diluted 1:2000). The inhibition percentage was calculated according to the formula: inhibition (%) = [1 - (Abs 492 nm with inhibitor / Abs 492 nm

without inhibitor]] $\times 100$ (Castro et al., 2014).

3. Results

In the present study, we have performed the profiling of T7 phage display libraries from mustard seeds and olive pollen by combining phage display and protein microarrays with the aim to identify IgE-reactive components of these highly relevant allergenic sources. A schematic representation of the workflow of the study is depicted in Fig. 1.

3.1. Identification of IgE-reactive components by the combination of T7 phage display and protein microarrays

Purified mRNA from mustard seeds and olive pollen were separately used to construct four cDNA libraries in the T7Select 415-1 or T7Select 10-3b vectors. Following packaging of the cDNA in phages, four T7 phage display libraries from mustard seeds and olive pollen were obtained. Subsequently, the libraries were separately enriched in specific phages by biopanning using sera from allergic patients to mustard seeds or olive pollen, respectively (Fig. 1).

First, a negative selection using sera from non-atopic individuals was performed to remove non-specific phages. Then, phage libraries were enriched in allergenic-specific phages by their selection with sera from allergic patients and infection of *E. coli* bacterial cells. To avoid for the selection of phages displaying amino acid sequences from previously known allergens, the pools of sera used in the biopanning were preincubated with recombinant allergens from both allergenic sources prior to incubation with phages. After 4 rounds of selection, a specific enrichment of the phage display libraries in allergenic phages was observed by T7 phage titration and phage ELISA (data not shown). Then, a total of 1536 individual phages from the third and four rounds of selection were picked (768 individual phages from phage selection with mustard seeds or olive pollen allergic patients' sera) and printed in duplicate together with positive and negative controls onto nitrocellulose microarrays.

Next, after incubation of the microarrays with specific antibodies for quality control assessment and reproducibility analyses, we observed a good quality of the microarrays (Supplementary Fig. 1). Then, we proceeded to probe the microarrays with sera from 5 mustard seeds and 5 olive pollen allergic patients to identify IgE immunoreactive-specific phages from both allergenic sources. Following image quantification and normalization, as the non-atopic controls showed no specific reactivity, we compared the differential phage immunoreactivity of mustard seeds or olive pollen allergic patients' sera. After bioinformatics analysis using Pomelo II and MeV for visualization, we identified 39 immunoreactive phages showing a clear discrimination and differential reactivity between allergic patients to mustard seeds and olive pollen (Fig. 2). In total, 22 phages displayed peptides from olive pollen and 17 from yellow mustard seeds.

3.2. Identification and confirmation of the IgE-immunoreactivity of the phages

Out of the 39 IgE immunoreactive phages, we obtained nine unique amino acid sequences from mustard seeds and ten from olive pollen (Table 1 and Supplementary Table 1). The length of the displayed peptides as fusion proteins to the C-terminal end of the 10B protein of the capsid of the T7 phage was highly variable from 5 to 58 amino acids, with peptides displayed from olive pollen cDNA considerably shorter than most peptides from mustard seeds cDNA, possibly due to an excess of cDNA fragmentation during the construction of the olive pollen T7 phage display libraries. As expected due to the preincubation of the sera during the biopanning with previously known allergens from both allergic sources, none of the immunoreactive phages displayed amino acid sequences with identity with previously identified mustard

seeds or olive pollen allergens.

Then, we proceeded to verify the IgE immunoreactivity of the amplified individual unique phages by dot-blot (Fig. 3). Importantly, we observed that all phages displaying unique sequences from olive pollen and mustard seeds showed specific IgE-binding capacity at different extents after incubation with sera from allergic patients in comparison to the non-atopic sera control and the negative control of the assay (no sera). On the other hand, although we observed the presence of T7 phages on all spots with the anti-T7 mAb, some differences in the amount of T7 phages displaying peptides from mustard seeds or olive pollen were observed. These differences of T7 phage were attributed to different phage structural restraints during phage amplification.

Among the 19 unique phages displaying allergenic peptides from both allergenic sources, we decided to select for protein expression and characterization those sequences displayed on phages with the highest identity and a lower expect value than 0.001 with proteins deposited in databases, since the lower expect value, or the closer it is to zero, the more significant the match is. In total, we selected four phages from mustard seeds (MUST1, MUST3, MUST7, and MUST20) and one from olive pollen (OLI4) for expression and further immunological analysis using an independent cohort of 32 mustard seed allergic patients' sera and 92 serum samples from olive pollen allergic patients sera than those used in the microarrays (Table 1).

3.3. Cloning, expression, and purification of allergenic fusion proteins

After cloning, transformation of the constructs on BL21 (DE3) *E. coli* cells and induction with IPTG, cell lysates were evaluated by Coomassie Blue staining and WB after SDS-PAGE to analyse the correct protein expression of MUST1, MUST3, MUST7, MUST20 and OLI4 as fusion proteins to a His6-GST in the N-terminal end (Fig. 4). MUST1, MUST3 and MUST7 were mainly produced as soluble proteins. Then, MUST1, MUST3, and MUST7 fusion proteins were purified by using Ni-NTA agarose beads from the soluble fraction, and MUST20 and OLI4 after refolding on-column, as previously done (Barderas et al., 2008a; Barderas et al., 2008b; Mas et al., 2017). After affinity chromatography, fractions containing the recombinant fusion proteins were separately pooled, dialyzed, and used in subsequent experiments. The recombinant proteins were analysed by Coomassie Blue staining and WB (Fig. 4). We confirmed that the molecular mass of the fusion proteins were coincident with the theoretical molecular mass of the peptides plus the His6-GST tag at the N-terminal end.

3.4. Immunological characterization of the allergenic constructs

We then analysed the IgE-binding ability of the purified fusion proteins (Fig. 5). First, we tested the specific IgE-reactivity to all constructs using a collection of 32 sera from mustard seed allergic patients and 92 individual serum olive pollen allergic patients (Fig. 5A,B).

We found by ELISA that the recognition frequency to the different allergenic constructs was variable, ranging from 31 to 59% for mustard seeds allergenic constructs and 8.7% for the olive pollen construct analysed (Fig. 5A). In addition, we observed that mustard allergic patients showed a differential IgE recognition to the different mustard seeds allergenic constructs (Fig. 5B), and thus, overcoming any potential concern related to unspecific reactivity to the His6-GST fusion partner. The most IgE-reactive peptide constructs were MUST7 and MUST20, regarding the potency of the allergenic construct -O.D. mean value of the IgE positive patients sera-, and the number of mustard seeds allergic sera recognizing the constructs (19 and 17 out of 32 allergic patients sera, respectively). On the other hand, OLI4 showed low reactivity in terms of its allergenic potency and regarding to the number of IgE positive patient's sera. OLI4 was recognized by 8 out of the 92 olive pollen allergic patients' sera, probably as a consequence of displaying the smallest allergenic peptide, which might include only one epitope or part of it.

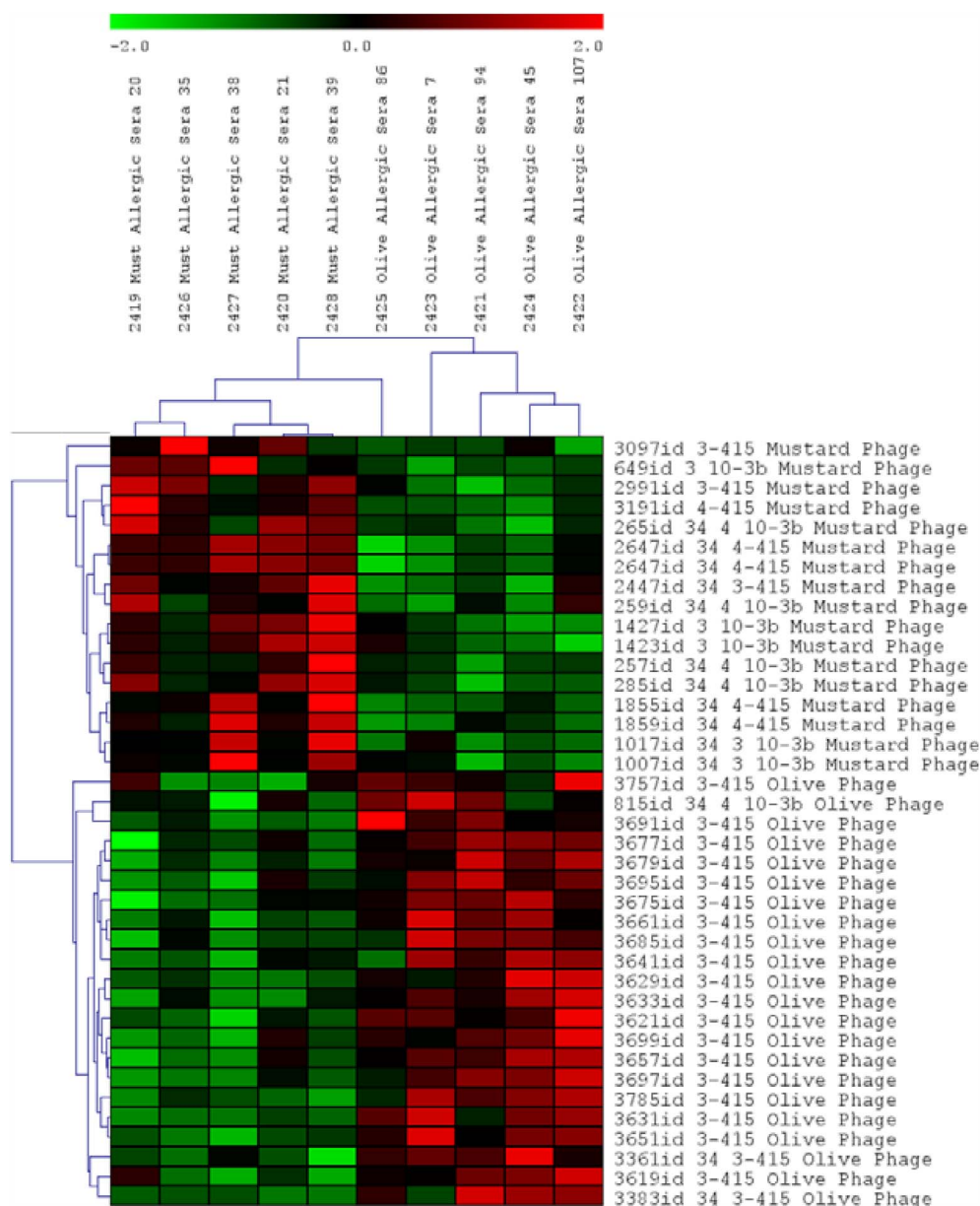


Fig. 2. Bioinformatic analysis. Supervised hierarchical clustering analysis of phages with statistically different reactivity (FDR < 0.15 and unadjusted p value < 0.05) to both types of allergic patients. The color scale is specified in the image. The green and red colors indicate low and high intensity, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1

Unique amino acid sequences displayed on the IgE-immunoreactive phages from *Sinapis alba* and *Olea europaea*.

Phage ^a	Displayed amino acid sequence	Hypothetical protein name	Accession number	Score	Expect	Identity (%)
MUST1	SPDSLEAFSHNPAHGSAFLAFQPSAMTNCANQRFLSY	Putative senescence-associated protein (<i>Trichosantes dioica</i>)	ABN50029.1	128	1E-34	100
MUST3	IKARLRNRSPPRCRKTVA	Uncharacterized protein LOC100846853 (<i>Brachypodium distachyon</i>)	XP_003558790.1	30.3	0.001	89
MUST7	GKETNKDSLNGERTGKSPA	Hypothetical protein (<i>Eutrema salsugineum</i>)	XP_006405913.1	62.6	2E-11	100
MUST20	ITLKVVVFHLRRSSHLFYTSQVISQSRTRVKLNRFVFPFR	Hypothetical protein (<i>Arabidopsis alpina</i>)	BAF01964.1	125	4E-32	97
OLI4	SAKWIRNFGKRIGSE	Hypothetical protein (<i>Pyrus × bretschneideri</i>)	XP_006286106.1	51.5	5E-8	100

^a Phages displaying mustard seeds amino acid sequences are denoted with MUST and phages displaying olive pollen amino acid sequences with OLI.

Since the allergenicity of OLI4 was low, we finally focused on testing the contribution of mustard seeds derived peptide constructs individually or in combination to the allergenicity of mustard seeds pollen protein extract. We analysed their contribution by ELISA inhibition experiments using a random pool of mustard allergic patients'

sera (Fig. 5C). We observed that the individual contribution of mustard seeds allergenic constructs to the whole allergenicity of the extract ranged between 20 and 53%. Importantly, we observed an 80% reduction in the IgE-binding to the whole extract using all the constructs in combination as inhibitors, and thus, demonstrating that the mustard

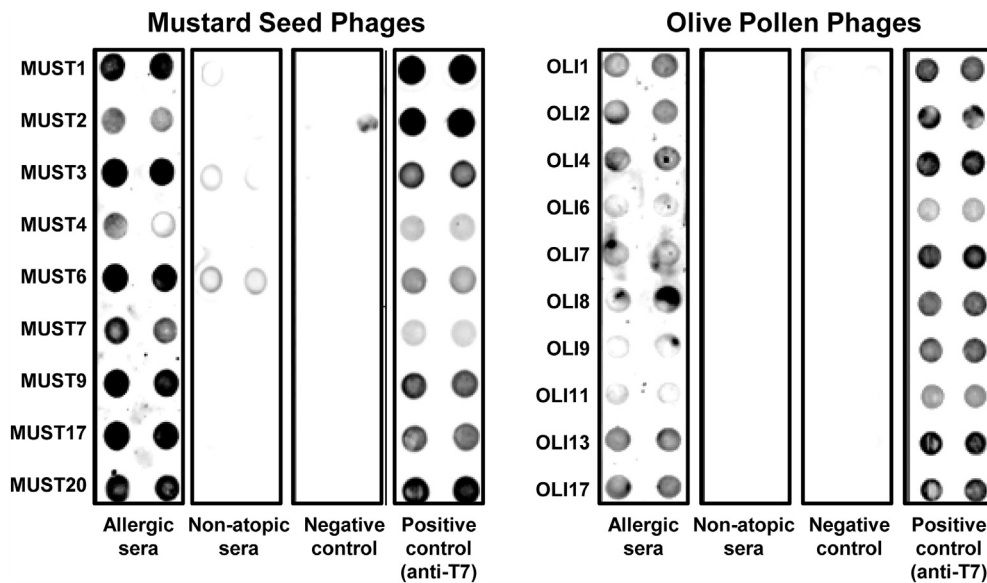


Fig. 3. Dot-blot analysis of the IgE-Immunoreactivity of phages displaying unique amino acid sequences. Phages displaying unique amino acid sequences from mustard seeds (left) or olive pollen (right) were applied on eight equal spots, dividing the nitrocellulose membranes in four equal sections: one for incubation with indicated allergic patient pool of 8 sera (Allergic), one for non-atopic pool of 3 sera (Non-atopic), one for the negative control of the assay: no sera followed by anti-human IgE mAb and HRP-labeled rabbit anti-mouse IgG (Negative control) and one for the monoclonal anti-T7 antibody (Positive control of phage presence).

seeds allergenic constructs were highly IgE-immunoreactive.

Collectively, the immunological characterization data showed that the identified displayed peptides on phages were IgE immunoreactive; and therefore, the combination of T7 phage display and phage microarrays screening has been proved as a new methodological approach for the identification of allergenic peptides or mimotopes.

4. Discussion

In this study, we focused on the combination of phage display and

protein microarrays for the identification of IgE-reactive molecules. We focused on the analysis of olive pollen and mustard seeds as highly relevant clinical allergenic sources for the proof-of-concept of this methodology. We focused on these allergenic sources because of its relevance in Spain. Olive pollen has become the second cause of pollinosis in Spain after grass pollen. On the other hand, the allergy to the condiment yellow mustard is continuously increasing in multiple countries because of its use in multiple processed foods, including Spain, and is responsible for severe allergic symptoms.

Combination of phage display with microarray technologies

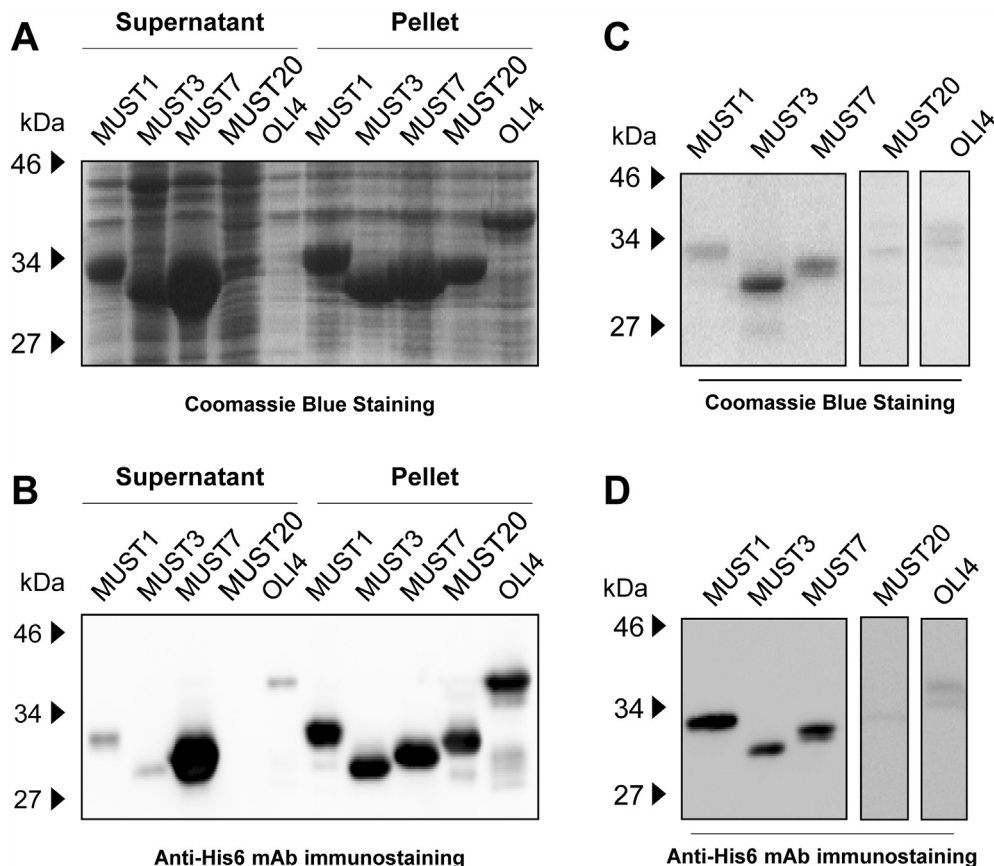


Fig. 4. Analysis of the recombinant *E. coli* expression of His6-GST-tagged allergenic constructs. (A) SDS-PAGE and Coomassie Blue staining of soluble (S) and insoluble (P) fractions of *E. coli* cultures expressing indicated His6-GST-tagged constructs with the allergenic peptide in the C-terminal end of the tag at a 0.4 mM IPTG concentration. (B) WB analysis of the same fractions using an anti-His6 epitope monoclonal antibody. (C) SDS-PAGE and Coomassie Blue staining of purified His6-GST-tagged constructs. (D) WB analysis of the same purified constructs using an anti-His6 epitope monoclonal antibody. (C, D) 1 µg of MUST1, MUST3 and MUST7 and 200 ng of MUST20 and OLI4 were analysed by Coomassie Blue staining and WB on separate experiments due to the lower yield of MUST20 and OLI4 after their purification in comparison to MUST1, MUST3 and MUST7.

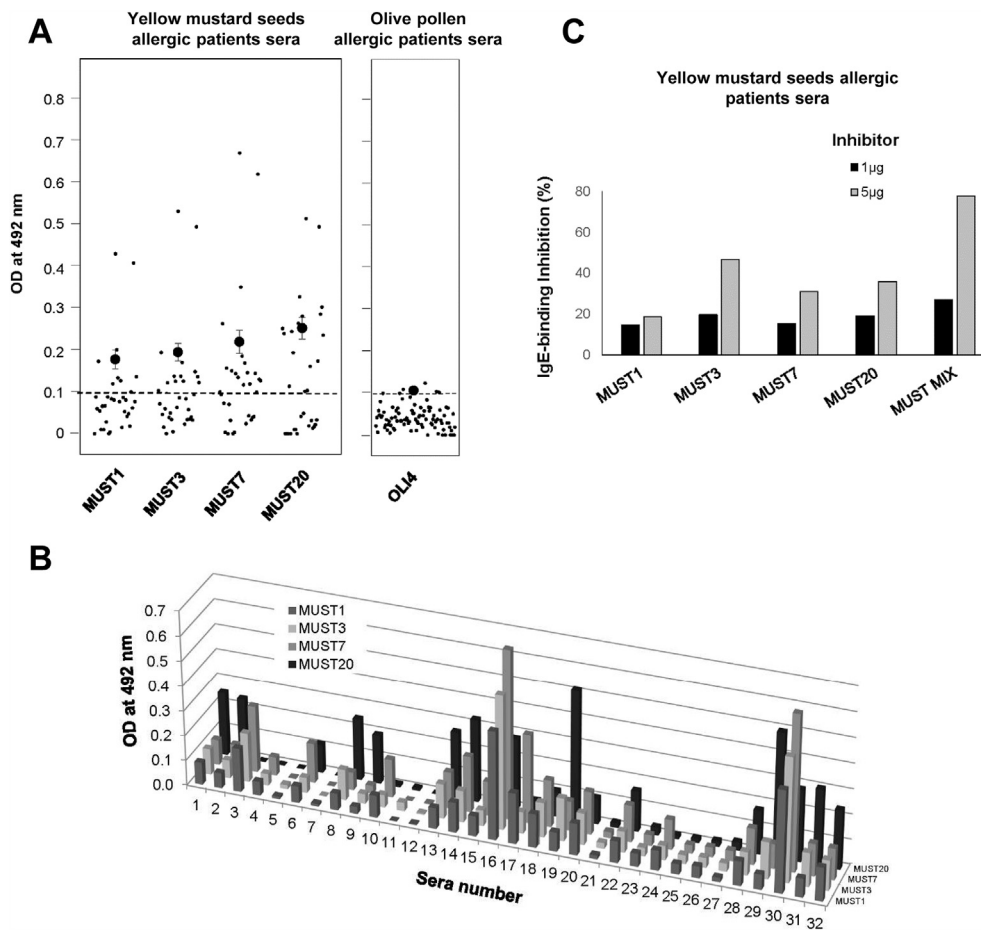


Fig. 5. Immunological characterization of the purified allergenic constructs. (A) Individual ELISA performance of the indicated constructs using 32 sera from allergic patients to mustard seeds or 92 from olive pollen. Mean (circle) and SEM (whiskers) are represented in the figure as a measure of the potency of the allergenic peptides. (B) 3D-bar graph representation showing the individual performance of each patient to the 4 allergenic constructs obtained from mustard seeds phages. (C) An equivolumetric pool of 6 random sera from mustard seeds allergic patients, and the indicated amounts of each protein as inhibitor in 50 µL of PBS containing Tween 20 0.1% and 3% skimmed milk, were tested by ELISA to determine the IgE binding inhibition of each construct individually or in combination to the whole allergenicity obtained to 20 µg of mustard seeds protein extract coated per well.

considerably boosted the throughput of phage display, allowing the testing of thousands of phages with only a few microliters of serum to identify IgE immunoreactive phages. This strategy, however, presents some limitations, such as the presence of mimotopes (Chatterjee et al., 2006b; Kalnina et al., 2008), the potential fragmentation of the cDNA libraries during the construction of the phage display libraries (Babel et al., 2011), or an over-biopanning problem that could permit the selection of phages easily produced in detriment of the selection of phages displaying larger peptides but whose expression could be compromised because of phage structural restraints. In addition, due to the fact that the peptides/proteins are displayed on the C-terminal end *in frame* with the 10B protein of the phage capsid, issues regarding the assignment of peptides/proteins non-corresponding to the inserted cDNA on phages might appear (Chatterjee et al., 2006a; Chatterjee et al., 2006b; Kalnina et al., 2008). In this sense, we here observed the fragmentation of the phage display cDNA library of olive pollen, which has permitted only to identify displayed peptides up to 15 amino acids in length.

Therefore, further refinement of the methodology, especially during the construction of the phage display libraries, or with the inclusion of a second negative selection using allergic patient's sera with high IgE to not cross-reactive sources, will likely improve its utility for clinical and research applications in the allergy field. In addition to its application in the clinic, the phage microarray might also be used as a research tool to quickly collect phenotype data for genetic mapping of susceptibility genes. Genetic studies have implicated several genes in allergy disposition, but most have not been consistently replicated across populations due to the complex nature of the disease (Carlson et al., 2004). Measuring allergen-specific IgE with the phage microarray may facilitate quantitative trait analysis of allergy predisposition in large populations (Lander and Schork, 1994; Lander and Kruglyak, 1995). In this sense, asthma linkage and association studies have benefited from a

similar approach (Cookson and Palmer, 1998).

Previous studies explored the use of phage display for the identification of allergens (for a review see (Appenzeller et al., 2001)). These reports used λ- or M13-phages for the identification of allergens from membrane filters coated with phages or plaque lifts, which required larger volumes of sera than protein microarrays. Here, we have tried to overcome this limitation by combining for the first time the high-throughput capabilities of T7 phage display and protein microarrays aimed at the identification of IgE-reactive molecules. To that end, we constructed two T7 Phage libraries displaying peptides/proteins encoded by the cDNA from olive pollen and yellow mustard seeds. After their enrichment in specific phages to both allergenic sources by biopanning, we picked 1536 unique phages displaying peptides from yellow mustard seeds and olive pollen encoded cDNA for their printing on nitrocellulose microarrays containing a total of 1920 phages and controls printed in duplicate. After bioinformatics analyses and verification of the IgE-immunoreactivity of the unique phages, we found, as the proof-of-concept of the here presented approach, eleven unique allergenic peptides or mimotopes from olive pollen and nine from mustard seeds. Therefore, we have here demonstrated the feasibility of the approach, which has permitted the identification of immunodominant IgE reactive mimotopes using small amounts of sera. Since these mimotopes do imitate conformational IgE epitopes, it would be likely that the identified peptide sequences would actually represent IgE epitopes of known or unknown allergens that should be further addressed by other means.

One of the interesting findings of the work consisted of the identification of a small amino acid sequence also displayed in a larger sequence of another phage -MUST6 displayed peptide (RTGKSPA) and MUST7 displayed peptide (GKETNKDSLNGERTGKSPA)- with both of the peptide sequences showing homology for the same protein

deposited in databases. This finding suggested *a priori* that this sequence might be highly IgE-immunoreactive. Indeed, we further confirmed in this study the high IgE-immunoreactivity by analysing by ELISA the larger sequence as a fusion protein to His6-GST using 32 yellow mustard seeds allergic patients' sera. We found that 19 out of the 32 sera possessed specific IgEs to this allergenic peptide. In addition, we interestingly found that this allergenic construct was able to abrogate the IgE-binding to the whole extract about 38% by ELISA inhibition experiments; and thus confirming the usefulness of this approach to identify highly allergenic peptides or proteins.

On the other hand, we need to remark that in spite of the fact that we found allergenic peptides from olive pollen and mustard seeds, we could only find immunodominant IgE mimotopes from the olive pollen. Regarding yellow mustard seeds, the displayed amino acid sequences *in frame* with the 10B protein of the phage capsid were non concordant with the cDNA inserted on phages, a phenomenon previously described (Chatterjee et al., 2006a; Chatterjee et al., 2006b). In addition, and as a consequence of the fact that the yellow mustard genome is not reported, we could not assign the displayed peptides to any reported protein from this allergenic source. We were then unable to produce the full-length allergens. Accordingly, here for the assessment of the IgE-immunoreactivity of the identified allergenic peptides or mimotopes, those peptides showing the highest sequence identity with proteins from databases were expressed as fusion-proteins to His6-GST (using a new vector here described carrying an N-terminal His6-tag followed by a GST-tag) to decrease solubility issues and obtain enough amounts of recombinant proteins. With this approach, we could verify the IgE immunoreactivity of the constructs by ELISA and ELISA inhibition, and thus, highlighting the important immunoreactivity of the identified peptide sequences from olive pollen and mustard seeds.

5. Concluding remarks

T7 phage display combined with protein microarrays has been proven to be a good technological approach to identify allergenic peptides and/or allergenic mimotopes. The combination of both high-throughput methodologies enabled us the handling of large numbers of individual phages to identify by the screening of protein microarrays immunodominant allergenic peptides from yellow mustard seeds and olive pollen. Thus, the combination of these two powerful technologies is suited to identify immunodominant IgE components.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jim.2018.02.011>.

Conflict of interest

The authors have declared that no competing interests exist.

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References

Alcántara, M., Sáenz de San Pedro, B., Cañada, C., Muñoz, M.A., Jimeno, L., Villalba, M., de la Torre, F., 2017. Steps towards clarifying the clinical relevance of olive minor

- allergens in areas with extremely high levels of olive pollen. *J. Invest. Allergol. Clin. Immunol.* 27.
- Anderson, K.S., Sibani, S., Wallstrom, G., Qiu, J., Mendoza, E.A., Raphael, J., Hainsworth, E., Montor, W.R., Wong, J., Park, J.G., Lokko, N., Logvinenko, T., Ramachandran, N., Godwin, A.K., Marks, J., Engstrom, P., Labaer, J., 2011. Protein microarray signature of autoantibody biomarkers for the early detection of breast cancer. *J. Proteome Res.* 10, 85–96.
- Appenzeller, U., Blaser, K., Cramer, R., 2001. Phage display as a tool for rapid cloning of allergenic proteins. *Arch. Immunol. Ther. Exp.* 49, 19–25.
- Asher, M.I., Montefort, S., Björkstén, B., Lai, C.K., Strachan, D.P., Weiland, S.K., Williams, H., Group, I.P.T.S., 2006. Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. *Lancet* 368, 733–743.
- Babel, I., Barderas, R., Diaz-Urriarte, R., Martinez-Torrecuadrada, J.L., Sanchez-Carbayo, M., Casal, J.I., 2009. Identification of tumor-associated autoantigens for the diagnosis of colorectal cancer in serum using high density protein microarrays. *Mol. Cell. Proteomics* 8, 2382–2395.
- Babel, I., Barderas, R., Diaz-Urriarte, R., Moreno, V., Suarez, A., Fernandez-Acenero, M.J., Salazar, R., Capella, G., Casal, J.I., 2011. Identification of MST1/STK4 and SULF1 proteins as autoantibody targets for the diagnosis of colorectal cancer by using phage microarrays. *Mol. Cell. Proteomics* 10 (M110), 001784.
- Barderas, R., Shochat, S., Martinez-Torrecuadrada, J., Altschuh, D., Meloen, R., Ignacio Casal, J., 2006. A fast mutagenesis procedure to recover soluble and functional scFvs containing amber stop codons from synthetic and semisynthetic antibody libraries. *J. Immunol. Methods* 312, 182–189.
- Barderas, R., Desmet, J., Timmerman, P., Meloen, R., Casal, J.I., 2008a. Affinity maturation of antibodies assisted by *in silico* modeling. *Proc. Natl. Acad. Sci. U. S. A.* 105, 9029–9034.
- Barderas, R., Shochat, S., Timmerman, P., Hollestelle, M.J., Martinez-Torrecuadrada, J.L., Hoppener, J.W., Altschuh, D., Meloen, R., Casal, J.I., 2008b. Designing antibodies for the inhibition of gastrin activity in tumoral cell lines. *Int. J. Cancer* 122, 2351–2359.
- Barderas, R., Babel, I., Diaz-Urriarte, R., Moreno, V., Suarez, A., Bonilla, F., Villar-Vazquez, R., Capella, G., Casal, J.I., 2012. An optimized predictor panel for colorectal cancer diagnosis based on the combination of tumor-associated antigens obtained from protein and phage microarrays. *J. Proteome Res.* 11, 4647–4655.
- Boratyn, G.M., Camacho, C., Cooper, P.S., Coulouris, G., Fong, A., Ma, N., Madden, T.L., Matten, W.T., McGinnis, S.D., Merezuk, Y., Raytselis, Y., Sayers, E.W., Tao, T., Ye, J., Zaretskaya, I., 2013. BLAST: a more efficient report with usability improvements. *Nucleic Acids Res.* 41, W29–33.
- Bousquet, J., Burney, P.G., Zuberbier, T., Cauwenberge, P.V., Akdis, C.A., Bindslev-Jensen, C., Bonini, S., Fokkens, W.J., Kauffmann, F., Kowalski, M.L., Lodrup-Carlson, K., Mullol, J., Nizankowska-Mogilnicka, E., Papadopoulos, N., Toskala, E., Wickman, M., Anto, J., Auvergne, N., Bachert, C., Bousquet, P.J., Brunekreef, B., Canonica, G.W., Carlsen, K.H., Gjomarkaj, M., Haahtela, T., Howarth, P., Lenzen, G., Lotvall, J., Radon, K., Ring, J., Salapatas, M., Schunemann, H.J., Szczeklik, A., Todo-Bom, A., Valovirta, E., von Mutius, E., Zock, J.P., 2009. GA2LEN (Global Allergy and Asthma European Network) addresses the allergy and asthma 'epidemic'. *Allergy* 64, 969–977.
- Bradford, T.J., Wang, X., Chinnaiyan, A.M., 2006. Cancer immunomics: using autoantibody signatures in the early detection of prostate cancer. *Urol. Oncol.* 24, 237–242.
- Carlson, C.S., Eberle, M.A., Kruglyak, L., Nickerson, D.A., 2004. Mapping complex disease loci in whole-genome association studies. *Nature* 429, 446–452.
- Castro, L., Mas, S., Barderas, R., Colas, C., Garcia-Selles, J., Barber, D., Rodriguez, R., Villalba, M., 2014. Sal k 5, a member of the widespread Ole e 1-like protein family, is a new allergen of Russian thistle (*Salsola kali*) pollen. *Int. Arch. Allergy Immunol.* 163, 142–153.
- Chatterjee, M., Ionan, A., Draghici, S., Tainsky, M.A., 2006a. Epitomics: global profiling of immune response to disease using protein microarrays. *OMICS* 10, 499–506.
- Chatterjee, M., Mohapatra, S., Ionan, A., Bawa, G., Ali-Fehmi, R., Wang, X., Nowak, J., Ye, B., Nahhas, F.A., Lu, K., Witkin, S.S., Fishman, D., Munkarah, A., Morris, R., Levin, N.K., Shirley, N.N., Tromp, G., Abrams, J., Draghici, S., Tainsky, M.A., 2006b. Diagnostic markers of ovarian cancer by high-throughput antigen cloning and detection on arrays. *Cancer Res.* 66, 1181–1190.
- Cookson, W.O., Palmer, L.J., 1998. Investigating the asthma phenotype. *Clin. Exp. Allergy* 28 (Suppl. 1), 88–89 (discussion 108–10).
- Dahl, R., Andersen, P.S., Chivato, T., Valovirta, E., de Monchy, J., 2004. National prevalence of respiratory allergic disorders. *Respir. Med.* 98, 398–403.
- Eder, W., Ege, M.J., von Mutius, E., 2006. The asthma epidemic. *N. Engl. J. Med.* 355, 2226–2235.
- Freckleton, G., Lippman, S.I., Broach, J.R., Tavazoie, S., 2009. Microarray profiling of phage-display selections for rapid mapping of transcription factor-DNA interactions. *PLoS Genet.* 5, e1000449.
- Geysen, H.M., Rodda, S.J., Mason, T.J., 1986. A-priori delineation of a peptide which mimics a discontinuous antigenic determinant. *Mol. Immunol.* 23, 709–715.
- Kalina, Z., Silina, K., Meistere, I., Zayakin, P., Rivos, A., Abols, A., Leja, M., Minenkova, O., Schandendorff, D., Line, A., 2008. Evaluation of T7 and lambda phage display systems for survey of autoantibody profiles in cancer patients. *J. Immunol. Methods* 334, 37–50.
- Kodadek, T., 2001. Protein microarrays: prospects and problems. *Chem. Biol.* 8, 105–115.
- Lander, E., Kruglyak, L., 1995. Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. *Nat. Genet.* 11, 241–247.
- Lander, E.S., Schork, N.J., 1994. Genetic dissection of complex traits. *Science* 265, 2037–2048.
- Madoz-Gurpide, J., Canamero, M., Sanchez, L., Solano, J., Alfonso, P., Casal, J.I., 2007. A proteomics analysis of cell signaling alterations in colorectal cancer. *Mol. Cell. Proteomics* 6, 2150–2164.

- Mas, S., Boissy, P., Monsalve, R.I., Cuesta-Herranz, J., Diaz-Perales, A., Fernandez, J., Colas, C., Rodriguez, R., Barderas, R., Villalba, M., 2015. A recombinant Sal k 1 isoform as an alternative to the polymorphic allergen from *Salsola kali* pollen for allergy diagnosis. *Int. Arch. Allergy Immunol.* 167, 83–93.
- Mas, S., Oeo-Santos, C., Cuesta-Herranz, J., Diaz-Perales, A., Colas, C., Fernandez, J., Barber, D., Rodriguez, R., Barderas, R., Villalba, M., 2017. A relevant IgE-reactive 28 kDa protein identified from *Salsola kali* pollen extract by proteomics is a natural degradation product of an integral 47 kDa polygalacturonase. *BBA Proteins Proteomics* 1.
- Miersch, S., Bian, X., Wallstrom, G., Sibani, S., Logvinenko, T., Wasserfall, C.H., Schatz, D., Atkinson, M., Qiu, J., LaBaer, J., 2013. Serological autoantibody profiling of type 1 diabetes by protein arrays. *J. Proteome* 94, 486–496.
- Monsalve, R.I., Villalba, M., Rodriguez, R., 2001. Allergy to mustard seeds: the importance of 2S albumins as food allergens. *Inter. Symp. Food Allergy* 3, 57–69.
- Pande, J., Szewczyk, M.M., Grover, A.K., 2010. Phage display: concept, innovations, applications and future. *Biotechnol. Adv.* 28, 849–858.
- Ramachandran, N., Srivastava, S., Labaer, J., 2008. Applications of protein microarrays for biomarker discovery. *Proteomics Clin. Appl.* 2, 1444–1459.
- Rance, F., 2003. Mustard allergy as a new food allergy. *Allergy* 58, 287–288.
- Rhyner, C., Weichel, M., Fluckiger, S., Hemmann, S., Kleber-Janke, T., Cramer, R., 2004. Cloning allergens via phage display. *Methods* 32, 212–218.
- Rodriguez, R., Villalba, M., Monsalve, R.I., Batanero, E., 2001. The spectrum of olive pollen allergens. *Int. Arch. Allergy Immunol.* 125, 185–195.
- Sirvent, S., Palomares, O., Vereda, A., Villalba, M., Cuesta-Herranz, J., Rodriguez, R., 2009. nsLTP and profilin are allergens in mustard seeds: cloning, sequencing and recombinant production of Sin a 3 and Sin a 4. *Clin. Exp. Allergy* 39, 1929–1936.
- Stone, K.D., Prussin, C., Metcalfe, D.D., 2010. IgE, mast cells, basophils, and eosinophils. *J. Allergy Clin. Immunol.* 125, S73–80.
- Vereda, A., Sirvent, S., Villalba, M., Rodriguez, R., Cuesta-Herranz, J., Palomares, O., 2011. Improvement of mustard (*Sinapis alba*) allergy diagnosis and management by linking clinical features and component-resolved approaches. *J. Allergy Clin. Immunol.* 127, 1304–1307.
- Villalba, M., Rodriguez, R., Batanero, E., 2014. The spectrum of olive pollen allergens. From structures to diagnosis and treatment. *Methods* 66, 44–54.
- Wang, X., Yu, J., Sreekumar, A., Varambally, S., Shen, R., Giacherio, D., Mehra, R., Montie, J.E., Pienta, K.J., Sanda, M.G., Kantoff, P.W., Rubin, M.A., Wei, J.T., Ghosh, D., Chinnaiyan, A.M., 2005. Autoantibody signatures in prostate cancer. *N. Engl. J. Med.* 353, 1224–1235.
- Yu, X.B., Wallstrom, G., Magee, D.M., Qiu, J., Mendoza, D.E.A., Wang, J., Bian, X.F., Graves, M., LaBaer, J., 2013. Quantifying antibody binding on protein microarrays using microarray nonlinear calibration. *BioTechniques* 54 (257–+).

Supplementary Table 1. Unique amino acid sequences displayed on phages from *Sinapis alba* and *Olea europaea* with higher expect value than 0.001 with proteins deposited in databases.

Phage ^a	Displayed Amino Acid Sequence	Hypothetical protein name	NCBI Accession Number ^b	Score ^b	Expect ^b	Identity (%)
MUST2	GRKEVSRRFXRDAETGKPLPRKGP	Putative β -1,3-Galactosyltransferase 19 (<i>Morus notabilis</i>)	EXB29782.1	27.8	0.17	56
MUST4	SSSATYPDLPDNCRHSSAAPSPRGGFLRRVK MRSGQIQTANDCPGRNRPSARCTTDETPS	Predicted protein	CCO20455.1	30.8	2.3	100
MUST5	GGKIRG	Polycomb Group Protein Fertilization-Independent Seed 2 (<i>A. thaliana</i>)	NP_565815.2	24.0	50	100
MUST6	<u>SRTGKSPA*</u>	Hypothetical protein (<i>Phaseolus vulgaris</i>)	XP_007131671.1	25.2	23	100
MUST9	PAGISRELVDKL	Hypothetical protein M569_02388 (<i>Genlisea europea</i>)	EPS72378.1	28.2	3.3	82
MUST17	SRKQA	Hypothetical protein (<i>Selaginella moellendoffii</i>)	XP_002988472.1	24.0	45	100
OLI1	SKKVCA	Hypothetical protein (<i>Erythranthe guttata</i>)	EYU35752.1	21.8	43	100
OLI2	YKKRTS	Hypothetical protein (<i>Erythranthe guttata</i>)	EYU33076	24	89	88
OLI6	AFGGYTS	E3 ubiquitin-protein ligase KEG-like isoform X2 (<i>Jatropha curcas</i>)	XP_012073159.1	27.8	6.1	89
OLI7	FAAGS	BTB/POZ domain-containing protein At5g41330 (<i>Brassica rapa</i>)	XP_009140083.1	23.1	152	100
OLI8	SAKKIK	Predicted Protein (<i>Micromonas pusilla</i>)	XP_003054824.1	24	50	88
OLI9	SMKNAKHL	Hypothetical protein (<i>Cleoma spinosa</i>)	ABD96860.1	26.9	8	53
OLI11	SKKVKG	Predicted protein (<i>Physcomitrella patens</i>)	XP_001755184.1	24	9.6	88
OLI13	SANKNTNKI	Unnamed protein product	CCO14631.1	27.4	5.6	76

		<i>(Bathycoccus prasinus)</i>				
OLI17	SQSLKK	Hypothetical protein <i>(Capsella rubella)</i>	XP_006295180.1	24	47	80

^a, Phages displaying mustard seeds amino acid sequences are denoted with MUST and phages displaying olive pollen amino acid sequences with OLI. ^b, The first hit retrieved from blastp is shown, however as the expect e-value was higher than 0.001, the retrieved sequences from blastp were considered of lower probability sequences than the ones represented in Table 1.* , The underlined amino acid sequence of MUST6 is also observed at the C-terminal end of the displayed amino acid sequence of MUST7 phage.