

BRIEF COMMUNICATION

Transcriptomic/proteomic identification of allergens in the mite *Tyrophagus putrescentiae*

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Abstract

While a number of allergens from house dust mites have been described, much remains to be discovered about allergens from storage mites. Here, next-generation sequencing was combined with MS/MS shotgun proteomics to identify proteins, especially potential allergens from *Tyrophagus putrescentiae*, commonly found in stored food products, especially flour. cDNAs of suspected allergens were cloned and expressed from bacterial cells, and recombinant allergens were tested for binding to IgE in sera from *T. putrescentiae*-sensitive patients. These analyses identified three previously uncharacterized allergens, Tyr p 28, Tyr p 35, and Tyr p 36, which have been officially assigned by the WHO/IUIS Allergen Nomenclature Sub-committee. Recombinant proteins rTyr p 28, rTyr p 35, and rTyr p 36 bound with 47.1%, 82.4%, and 70.6% of *T. putrescentiae*-sensitive sera. We provide here a new method to identify allergens by the combination of transcriptomic and proteomic approaches.

Tyrophagus putrescentiae, a common storage mite, is associated with allergic disorders (1–5) like other domestic mite species. Only four allergens from *T. putrescentiae* have been officially named (1, 3–5). The identification of other allergens in this species is important for clinical diagnosis and therapy. Here, transcriptomic and proteomic approaches were used to investigate *T. putrescentiae* proteins, especially potential allergens, to provide a reference transcriptome and generate the first proteomic data for this mite species. Using a systems biology approach that combined high-throughput next-generation sequencing with MS/MS shotgun proteomics, we identified three previously uncharacterized allergens, named Tyr p 28, Tyr p 35, and Tyr p 36 by the WHO/IUIS Allergen Nomenclature Sub-Committee.

Methods

Mite extracts

Tyrophagus putrescentiae and *Dermatophagoides farinae* extracts were supplied by Greer Laboratories (Lenoir, IL,

USA) as a lyophilized powder from cleaned mites. It was dissolved in phosphate-buffered saline (PBS, pH 7.4), dialyzed against PBS, and diluted to a final concentration of 1 mg/ml.

Human sera

Serum samples were obtained from patients in the allergy clinic, The third People's Hospital of Yancheng. Sera from seventeen *T. putrescentiae*-sensitized patients (allergy diagnosis based on case history and detection of *T. putrescentiae*-specific IgE) and three healthy controls were used to assess the allergenicity of recombinant allergens (Table 1). The study protocol was approved by the Ethics Committee for Clinical Investigation of The Third People's Hospital of Yancheng, Affiliated Yancheng Hospital, School of Medicine, Southeast University (Yancheng, China), and all participants provided written informed consent.

Mite culture

Mites collected from dust samples from the floors of a flour shop were isolated under stereomicroscope. Those identified as

Table 1 Clinical features of the enrolled subjects and their IgE reactivity to three recombinant allergens from *Tyrophagus putrescentiae*

Subjects	Gender	Age (years)	Symptom/ diagnosis	Specific IgE to <i>Dermatophagoides farinae</i> extract by ELISA (OD value, mean $\pm$ SD)	Specific IgE to <i>T. putrescentiae</i> extract by ELISA (OD value, mean $\pm$ SD)	IgE binding by Western blotting using the recombinant protein followed by sera and then anti-IgE as second antibody		
						rTyr p 28	rTyr p 35	rTyr p 36
1	Male	53	Allergic asthma	0.654 $\pm$ 0.013	1.007 $\pm$ 0.002	—	+	—
2	Male	71	Allergic rhinitis	0.956 $\pm$ 0.012	2.104 $\pm$ 0.056	—	—	+
3	Male	68	Allergic asthma/ allergic rhinitis	0.558 $\pm$ 0.009	0.590 $\pm$ 0.016	+	+	+
4	Male	43	Allergic rhinitis	0.457 $\pm$ 0.009	0.924 $\pm$ 0.026	—	+	+
5	Male	61	Allergic asthma/ allergic rhinitis	0.563 $\pm$ 0.012	0.952 $\pm$ 0.044	—	+	+
6	Female	44	Allergic asthma	0.539 $\pm$ 0.044	0.734 $\pm$ 0.032	—	+	+
7	Male	39	Allergic rhinitis	0.862 $\pm$ 0.010	3.175 $\pm$ 0.150	—	—	+
8	Female	20	Allergic rhinitis	0.663 $\pm$ 0.009	1.047 $\pm$ 0.041	—	—	—
9	Female	48	Allergic asthma/ allergic rhinitis	0.675 $\pm$ 0.013	1.485 $\pm$ 0.156	+	+	—
10	Female	21	Allergic rhinitis	0.474 $\pm$ 0.012	0.745 $\pm$ 0.029	+	+	+
11	Female	72	Allergic asthma/ allergic rhinitis	0.284 $\pm$ 0.007	0.500 $\pm$ 0.015	+	+	+
12	Male	59	Allergic asthma/ allergic rhinitis	0.753 $\pm$ 0.007	1.314 $\pm$ 0.087	+	+	+
13	Female	19	Allergic rhinitis	0.469 $\pm$ 0.016	0.729 $\pm$ 0.014	+	+	—
14	Female	20	Allergic rhinitis	0.188 $\pm$ 0.006	0.479 $\pm$ 0.016	—	+	+
15	Female	19	Allergic rhinitis	0.273 $\pm$ 0.013	0.561 $\pm$ 0.148	—	+	—
16	Female	19	Allergic rhinitis	0.256 $\pm$ 0.007	0.508 $\pm$ 0.093	+	+	+
17	Female	19	Allergic rhinitis	0.189 $\pm$ 0.005	0.501 $\pm$ 0.014	+	+	+
18	Female	20	Healthy	0.128 $\pm$ 0.007	0.140 $\pm$ 0.014	—	—	—
19	Female	19	Healthy	0.110 $\pm$ 0.007	0.163 $\pm$ 0.005	—	—	—
20	Female	21	Healthy	0.102 $\pm$ 0.005	0.129 $\pm$ 0.003	—	—	—

'+', positive reaction to the recombinant allergens; '—', negative reaction to the recombinant allergens.

Participants 1–17 with allergic asthma and/or rhinitis are allergic to *T. putrescentiae* extract; Participants 18–20 are mite-insensitive healthy donors included as a negative control.

T. putrescentiae were placed in a large flask (200 ml) of rice bran medium for 9–10 weeks continuous culture in an artificial climate incubator (RXZ-280D; Jiangnan Instrument Factory, Ningbo, China) under $25 \pm 1^\circ\text{C}$ and $85 \pm 5\%$ relative humidity.

Transcriptomics

Total RNA isolated from mites (RNAiso Plus kit 9108Q; TaKaRa, Dalian, China) was used to create a pooled library with the SMART™ cDNA Library Construction Kit (634901; Clontech, Palo Alto, CA, USA). The library was sequenced using Illumina HiSeq™ 2000. Image data were transformed by base calling into sequence data and stored in Fastq format. Raw data were cleaned by removing reads with adaptors, unknown nucleotides $>5\%$, and low-quality reads (base quality ≤ 10) $>20\%$.

Trinity (6) was used to align contigs and generate unigenes. Unigenes were aligned by BLASTX (E -value $\leq 1.0\text{E-}5$) to protein databases in the priority order NR, Swiss-Prot, KEGG, and COG. Highest-ranked proteins were used to determine coding region sequences (CDS), which were translated into amino sequences. Unigenes that could not be aligned to any

database were scanned by ESTScan (7), producing nucleotide sequence (5'–3') direction and amino sequence of the predicted coding region.

Proteomics

Proteins extracted from cultured mites were separated on a 12% polyacrylamide gel by SDS-PAGE followed by in-gel trypsin digestion. Triple TOF 5600 System (AB SCIEX, Concord, ON, Canada) fitted with a Nanospray III source (AB SCIEX) and pulled quartz tip as the emitter (New Objectives, Woburn, MA, USA) were used for proteome identification. Raw data files were converted into MGF files using Proteome Discoverer 1.2 (PD 1.2; Thermo-Fisher Scientific, San Jose, CA, USA) (5600 msconverter). Protein identification was performed with Mascot (version 2.3.02; Matrix Science, London, UK).

Allergen identification

Potential allergens were identified as described in Fig. 1. Briefly, using a protein database constructed from

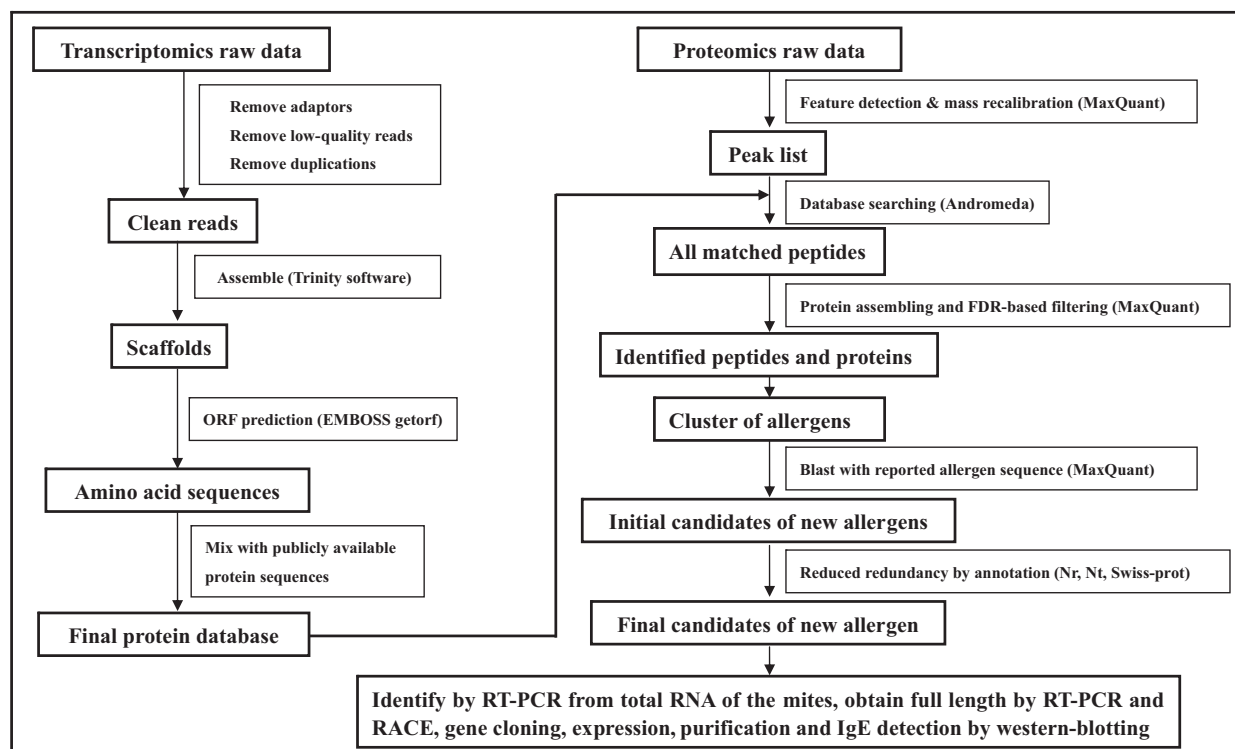


Figure 1 Flow chart outlining approach to identify novel allergens.

Open Reading Frame (ORF) prediction, all matched peptides were obtained using MASCOT software (Matrix Science, Boston, MA, USA). Allergen sequences were selected and clustered to create an Excel table for functional annotation. Allergen sequences deposited in GenBank were removed, leaving initial candidates. Redundant annotations were removed to obtain final candidates. Candidates were confirmed by RT-PCR of total RNA of *T. putrescentiae*.

Recombinant allergens

Full-length allergen sequences were obtained by 5'-rapid amplification of cDNA ends (5'-RACE) and 3' RACE, using SMARTer™ RACE cDNA Amplification Kit (634923 and 634924; Clontech), 3'-Full RACE Core Set with PrimeScript™ RTase (6106; TaKaRa), and PCR. RT-PCR from total RNA was performed with the following primers: Tyr p 28 forward, 5'-AATGGGTCGCGGATCCATGGCCAA CAAGAAGAC GCC-3', Tyr p 28 reverse, 5'-ATCTCAGTGGTGGTGGGGT GGTGCTCGAGTGC GGCCGCTAGTCCACTTCCTCAA TGG; Tyr p 35 forward, 5'-AATGGGTCGCGGATCCATG TCCCAAGTACCGATCAA-3', Tyr p 35 reverse, 5'-ATCTC AGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGT TAC TGCCCGCCAGCAAGCT-3'; and Tyr p 36 forward, 5'-AATGGGTCGCGGA TCCATGTCTTGGCAATCCTACGT-3' and Tyr p 36 reverse, 5'-ATCTCAGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGTGCTCGAGTCAATAGTTGTTGGCTTTCA-3'. PCR products were inserted into expression plasmid pET-28a(+) vector and transformed into *Escherichia coli* BL21 (DE3). Expressed

recombinant proteins were identified with SDS-PAGE (8–16). Recombinant proteins were purified using HiTrap TALON (5 ml TALON Superflow, 28-9537-66; GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) on AKTA Prime Plus (GE) and dialyzed with PBS buffer using Snake-Skin Dialysis Tubing (3500 MWCO, 68035, Lot No. MH 160055). Proteins were identified by SDS-PAGE.

Western blotting

To determine anti-IgE binding of recombinant allergens by Western blotting, recombinant allergens were separated by SDS-PAGE and blotted onto PVDF membranes (IPVH20200; Millipore, Billerica, MA, USA). The membranes were blocked with 1.5% bovine serum albumin and then incubated overnight with *T. putrescentiae*-positive human sera (1 : 10 in blocking buffer) at 4°C. Goat anti-human IgE-HRP (1:2500; Invitrogen, Carlsbad, CA, USA) was used for detection. The membranes were washed and then stained with TrueBlue peroxidase for imaging.

Results

Candidate genes encoding allergens

Using the proteins predicted by the *T. putrescentiae* transcriptome as a database, all matched peptides were obtained from proteomics data, and the peptides and proteins were then identified (Fig. 1). For the annotated allergens, 142

unigenes/contigs (sequence fragments) were clustered to generate composite sequences. Sequences reported in GenBank for Tyr p 1 (GenBank No. DQ983317), Tyr p 2 (EU106626), Tyr p 3 (EU302595), Tyr p 4 (DQ983318), Tyr p 5 (AY800360), Tyr p 7 (DQ983319), Tyr p 8 (JX255733), Tyr p 13 (DQ983316), and Tyr p 34 (FJ534553) were removed; remaining sequences were treated as unnamed allergens. Once redundant candidates were removed, 14 candidates remained. RT-PCR-amplified gene fragments were submitted for 3'RACE and 5'RACE for full-length cDNA encoding previously uncharacterized allergens.

Identification and confirmation of Tyr p allergens

Bioinformatics analyses of the full-length cDNAs encoding potential allergens led to three final candidate sequences, which were then amplified by RT-PCR, yielding products of 1980, 1461, and 396 bp. These sequences were deposited in GenBank as KX060611, KX060612, and KX060615, respectively. Recombinant proteins were generated in bacterial cells for purification and testing against human sera by Western blotting. The recombinant his-fusion proteins encoded by sequence numbers KX060611, KX060612, and KX060615 were recognized by patient IgE at the frequency of 47.1%, 82.4%, and 70.6%, respectively. In contrast, no IgE binding was obtained when recombinant proteins were incubated with control sera from mite-insensitive healthy volunteers (Table 1). These three allergens were submitted to the WHO/IUIS nomenclature subcommittee and officially named as Tyr p 28, Tyr p 35, and Tyr p 36, respectively. CELLO v2.5 (<http://cello.life.nctu.edu.tw/>, Molecular Bioinformatics Center, National Chiao Tung University, HsinChu 30050, Taiwan) prediction indicated these are cytoplasmic proteins.

Discussion

Of 14 groups of allergens identified from *T. putrescentiae* extracts (1), Tyr p 2 reactivity is present in 80% of patients allergic to *T. putrescentiae* extract (3), while Tyr p 3 binds with sera from 30% of mite-allergic individuals (4). Here, we utilized high-throughput transcriptomic and proteomic methods to identify three previously unidentified allergens in this storage mite, all of which showed high reactivity to *T. putrescentiae*-positive sera from allergic individuals.

Tyr p 28 is related to heat-shock protein. The group 28 mite allergen was first identified as a homolog to heat-shock protein in 1994 using *D. farinae* extracts (17). Der f 28 was confirmed by proteomics and two-dimensional immunoblotting of *D. farinae* extracts (18) and later characterized (19). The deduced Tyr p 28 amino acid sequence is 64% similar to Der f 28 (GenBank AGC56218) by VECTOR NIT 11.0 alignment (IBI, New Haven, Conn., USA). Further, high identities were obtained with BLASTp against heat-shock protein of

Nilaparvata lugens (86%, GenBank ADE34170), *Cuculus canorus* (86%, GenBank KFO73662), *Mus musculus* (86%, GenBank BAE30272), *Homo sapiens* (86%, GenBank No. NP_006588), with *Xenopus laevis* (83%, GenBank No. NP_001080068), which suggested they are near in evolutionary level.

Tyr p 35, an aldehyde dehydrogenase (ALDH) homolog, resembles three ALDH allergens deposited in GenBank from *Suidasia medanensis*, *D. farinae*, and *Blomia tropicalis* (ABU97475, AAP35081, and AAQ24547). Among the three recombinant allergens tested in our study, rTyr p 35 had the highest IgE-binding frequency at >80%, implicating it as a potential major allergen. Further, BLAST against the deduced amino acid sequence revealed identities of 68% in *Stegodyphus mimosarum* (GenBank KFM78573), 64% in *Aedes aegypti* (GenBank XP_001648014), 64% in *Homo sapiens* (GenBank NP_003879), and 63% in *Rattus norvegicus* (GenBank NP_446348).

Finally, Tyr p 36 represents the first mite profilin allergen to be officially named, although three other mite profilin sequences exist in GenBank (AAQ24553, AIO08866, AAX34044 from *B. tropicalis*, *D. farinae*, and *S. medanensis*, respectively). Profilin, first recorded as an allergen from birch tree (20), is found in pollen, latex, and plant foods (20). rTyr p 36 reacted with >60% of sera from patients allergic to *T. putrescentiae*, so it warrants further investigation for its role in allergic disease. Similar sequences are found in *S. mimosarum* (76%; GenBank KFM56841), *Choanephora cucurbitarum* (40%; GenBank OBZ91824), *Petroselinum crispum* (40%; GenBank AAW84279), *Zostera marina* (40%; GenBank KMZ59291), and *Pseudodiptomus poplesia* (36%; GenBank ALS04924).

Thus, high-throughput approaches identified three previously unidentified allergens from *T. putrescentiae*, providing a foundation for future investigations of *T. putrescentiae* hypersensitivity.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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