Transgenic Res
DOI 10.1007/s11248-011-9551-5

ORIGINAL PAPER

In planta expression of a mature Der p 1 allergen isolated from an Italian strain of *Dermatophagoides pteronyssinus*

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Received: 7 April 2009 / Accepted: 25 August 2011
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Abstract European (*Dermatophagoides pteronyssinus*) and American (*Dermatophagoides farinae*) house dust mite species are considered the most common causes of asthma and allergic symptoms worldwide. Der p 1 protein, one of the main allergens of *D. pteronyssinus*, is found in high concentration in mites faecal pellets, which can became easily airborne and, when inhaled, can cause perennial rhinitis and bronchial asthma. Here we report the isolation of the Der p 1 gene from an Italian strain of *D. pteronyssinus* and the PVX-mediated expression of its mature form (I-rDer p 1) in *Nicotiana benthamiana* plants. Human sera from characterized allergic patients were used for IgE binding inhibition assays to test the immunological reactivity of I-rDer p 1 produced in *N. benthamiana* plants. The binding properties of *in planta* produced I-rDer p 1 versus the IgE of patients sera were comparable to those obtained on Der p 1 preparation immobilized on a microarray. In this paper we provide a *proof of concept* for the production of an immunologically active form of Der p 1 using a plant viral vector. These results pave the way for the development of diagnostic allergy tests based on *in planta* produced allergens.

Keywords House dust mite allergens · Transient expression · PVX · *Dermatophagoides pteronyssinus*

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Electronic supplementary material The online version of this article (doi:10.1007/s11248-011-9551-5) contains supplementary material, which is available to authorized users.

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Published online: 09 September 2011

Introduction

Forty-five years ago, Voorhorst et al. (1967) remarked the importance of house dust mites (HDM) as the origin of main environmental allergens responsible of several pathologies in sensitized human population (Platts-Mills and de Weck 1989; Steen et al. 1994; Fernandez-Caldas 1997; Robinson et al. 1997; Asturias et al. 1999; Thomas et al. 2002). *Dermatophagoides pteronyssinus* is the source of two classes of major allergens (Der p 1 and Der p 2) (Smith et al. 2001; Thomas et al. 2002; Takai et al. 2005) affecting the allergic people living in tempered climates (Fernandez-Caldas 1997; Platts-Mills et al. 2000). To date, over 30 different IgE-binding proteins have been identified in *D. pteronyssinus* and for 19 of them their gene sequences have been cloned and characterized (Chua et al. 1993, 1996; Lin et al. 1994; Smith et al. 1994; Asturias et al. 1998; Thomas et al. 2002; Lee et al. 2004). Der p 1 protein is found in high concentration in mites faecal pellets, which can became easily airborne and, when inhaled, can cause perennial rhinitis and bronchial asthma (Platts-Mills and Chapman 1988; Jacquet et al. 2000; Platts-Mills and Woodfolk 2011). This allergen (about 25 kDa) is synthesized as a precursor of 320 amino acid residues which includes a 18-amino acid signal peptide and a 80-amino acid N-terminal prosequence (pro-Der p 1) (Chua et al. 1988; Thomas et al. 1988; Jacquet et al. 2000). The protein contains two potential N-glycosylation sites, one located inside the prosequence region and another at position 52–54 (N52) of the mature form (Chua et al. 1988; Jacquet et al. 2000). Der p 1 primary structure displays homologies with the cysteine proteinase archetype papain, including the conservation of the active site residues (Chua et al. 1988; Thomas et al. 1988; Topham et al. 1994; Jacquet et al. 2000). Upon maturation the Der p 1 pro-region is removed by an autocatalytic process which can be mimicked in vitro at 60°C and low pH (Vernet et al. 1991; Mach et al. 1994; Jacquet et al. 2000). The maturation process, but not the glycosylation, seems to be crucial for Der p 1 immunological activity. Greene et al. (1991) have identified at least five distinct regions (residues 1–56, 53–59, 98–140, 166–194 and 188–222) responsible for the reactivity versus IgE and IgG in sera of allergic individuals, demonstrating that they are all located in the mature protein (Greene et al. 1991). In addition, Takai et al. (2005) compared natural Der p 1 against matured Der p 1 and pro-Der p 1 proteins expressed in yeasts. While the mature Der p 1 retained similar molecular weight, secondary structures, and its allergenicity is equivalent to natural Der p 1, the Der p 1 proforms exhibited different secondary structures and a lower allergenicity than the mature form suggesting that the recombinant mature form of Der p 1 could be an useful tool for various in vitro and in vivo analyses (Kraft et al. 1998; Takai et al. 2002; Valenta 2002). Moreover, with the aim of using *Pichia pastoris* for producing recombinant proteins mimicking the natural ones, the same research group (Takai et al. 2002) has generated Der p 1 mutants in which the N-glycosylation motif within the mature sequence was disrupted (Der p 1-N52Q). This study has demonstrated that Der p 1-N52Q protein retains both a molecular weight equivalent to the natural Der p 1 and a full enzymatic and IgE-binding activities. Lienard et al. (2007) produced immunologically active recombinant Der p 1 and Der p 2 proteins in BY-2 tobacco cells suspensions but their attempts for a direct expression of the mature form of Der p 1 without the propeptide domain failed. To date, only Yang et al. (2007) have described the expression of the mature form of Der p 1 in transgenic rice seeds using a GluB promoter and a targeting signal for the endoplasmic reticulum (ER) showing that glycosylated Der p 1 had a significantly reduced IgE binding ability when compared with unglycosylated Der p 1.

Plants are a particularly attractive system for expressing pharmaceutical recombinant proteins (Yusibov et al. 2006; Floss et al. 2007). In fact as economical biomass producer, plants are virtually free of contamination from bacterial toxins or animal pathogens (Cunningham and Porter 1998). Foreign genes can be expressed at high levels in plant cells employing a wide variety of strategies ranging from stable integration in the nucleus or plastid genomes to transient expression mediated by plant viral vectors (Gleba et al. 2005).

Transient expression of foreign genes, mediated by viral vectors such as the tobacco mosaic virus (TMV) and potato virus X (PVX), has become a valuable tool in plant biotechnology. Transient expression systems, based on the PVX, proved very efficient in obtaining high level of expression in less than 6–8 weeks (Bendahmane et al. 1995; Porta and Lomonossoff 1998). Potato virus X, a member of the *Potexviruses*, is a filamentous rod-shaped virus which contains a single plus-sense RNA molecule of about 6400 nucleotides capped at the 5′ end (Sonenberg et al. 1978) and

polyadenylated at the 3′ end (Morozov et al. 2000). Its genome contains five open reading frames (ORFs) encoding for an RdRp (ORF 1) of 166 kDa, a set of three movement proteins (ORF 2–4) of 25, 12 and 8 kDa and the ORF 5 encoding for the 25 kDa virus coat protein (CP). A PVX-based expression vector, named pGR107, was designed to combine the advantages of the *Agrobacterium tumefaciens*-mediated transfection strategy and the infection power of the PVX virus (http://www.tsl.ac.uk/). This engineered plant virus is a binary expression vector derived from pGreen 0000 (Hellens et al. 2000). pGR107 expression vector contains multiple cloning sites where genes of interest (GOIs) can be inserted and which expression is controlled by a duplicated PVX coat protein promoter. The PVX genome, containing the GOI, is surrounded by the left border (LB) and the right border (RB); *A. tumefaciens* transfers the expression construct to plant cells and allows the integration into nuclear genome of plants (Wagner et al. 2004).

Up to now, the functional and molecular characterization of the major *D. pteronyssinus* allergens were obtained using commercial mite strains, although molecular evidences demonstrated the existence of polymorphic variants of Der p 1 and Der p 2 isolated from Australian and Eastern Asia HDM populations respect to those obtained from commercial cultures (Voorhorst et al. 1967; Chua et al. 1993, 1996; HuangFu et al. 2006).

In this paper, the isolation of the I-Der p 1 gene from an Italian strain of *D. pteronyssinus* and the PVX-mediated expression of its mature form (I-rDer p 1) in *N. benthamiana* plants is reported and discussed. The purification of the recombinant protein and the in vitro immunoreactivity of I-rDer p 1 with human allergic sera are also reported. We therefore demonstrate, as *proof of concept,* that by using a plant viral vector it is possible to produce a mature form of I-Der p 1 which could replace complex mixtures of mite extracts to perform specific diagnostic test for allergy.

Materials and methods

RNA extraction cDNA, synthesis and cloning 178 of I-Der p 1 gene

An Italian strain of *D. pteronyssinus* was bred in a pure culture at the CRA-ABP laboratories in Florence, Italy. Thirty specimens were drawn and immediately transferred in liquid nitrogen. Samples were ground in a mortar in presence of liquid nitrogen and total RNA was extracted by adding 200 µl of TRIzol® (Invitrogen, Carlsbad, CA, USA) and following the manufacturer's instruction (Chomczynski and Sacchi 1987). Isolated RNA was resuspended in 30 µl of sterile water treated with diethylpyrocarbonate (DEPC) and conserved at −80°C. RNA purity and concentration were assessed with a GeneQuant apparatus (GE healthcare, Waukesha, WI, USA) and 1% denaturant agarose gel electrophoresis. cDNA was obtained from 1 µg of total RNA using ImPromII Reverse Transcription System (Promega, Madison, WI, USA). Twenty-five ng of the single-stranded cDNA were then amplified using the illustra PuReTaq Ready-To-Go PCR Beads (GE healthcare) and specific primers (forward 5′-ACT AACGCCTGCAGTATCAA-3′; reverse 5′-AGAGA ATGACAACATATGGA-3′), designed on the mature portion of the Australian HDM Der p 1 (accession n. U11695). Amplification protocol was as follow: initial denaturing step at 94°C for 3 min, 30 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, and a final incubation at 72°C for 10 min.

A 670 bp cDNA fragment was visualized on 1.5% agarose gel electrophoresis, purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) and cloned into the pGEM-T Easy vector system I (Promega). Sequencing reactions were performed using the BigDye Terminator Cycle Sequencing kit 1.1 and run on an ABI 377 DNA sequencer (Applied Biosystem, Foster city, CA, USA).

The resulting sequences were used to design specific primers (5′-CCGTGTTGGGAAGCACAA TCGACTA-3′ and 5′-ACGCACAAGGTGTCGAT TATTGG-3′) to be used for 5′and 3′-RACE experiments employing the BD SMART RACE cDNA amplification kit (Clontech, Mountain View, CA). The full-length cDNA clone was reconstructed from 5′ to 3′ RACE fragments using the Vector NTI Suite 8 Contig Express (InforMax). Specific primers and the Advantage 2 PCR mix (Clontech) were used for performing end-to-end amplifications and to obtain the entire transcriptional unit. A fragment of the predicted size (about 1099 bp) corresponding to the I-Der p 1 gene was amplified and directly sequenced. In order to avoid sequence artefacts, cDNA isolation, cloning and sequencing phases were repeated in three independent experiments. Sequence homology was

verified using Mega3 (Kumar et al. 2004) and NCBI-BLAST tools (Altschul et al. 1997).

Construction of pGR107_GAT and pGR107_GAT.Derp1_FLAG_Cterm

The pGR107 vector was modified to make it compatible with the Gateway cloning system. The plasmid was linearized with *Sal*I restriction enzyme (New England Biolabs, Ipswich, MA, USA), purified and treated with Klenow polymerase (New England Biolabs) in order to obtain blunt ends. The resulting plasmid was ligated with reading frame cassette A of the Gateway Vector Conversion System (Invitrogen) and denominated pGR107_GAT destination vector. The mature portion of I-Der p 1 cDNA was amplified using primers DER-5′ATG (5′-ATGACTAACGCC TGCAATATCA-3′) and DER-3′FLAG (5′-TTACT TGTCGTCGTCGTCCTTGTAGTCGAGAATGACAA CATATGG-3′), cloned into PCR8/GW/TOPO TA cloning vector (Invitrogen) and sequenced to confirm the absence of errors. The resulting entry vector was named PCR8/GW.Derp1_FLAG_Cterm.

The FLAG-tag double stranded oligolinker was prepared by mixing 500 pmol of FLAG upper (5′-AA TGGACTACAAGGACGACGACGACAAG-3′) and FLAG lower (5′-ATCACTTGTCGTCGTCGTCCT TGTAGTCCAT-3′) oligos. The oligonucleotide mixtures were heated at 95°C for 10 min and then cooled at room temperature to allow annealing. The oligolinker was cloned into PCR8/GW/TOPO TA cloning vector (Invitrogen) and then the sequence was verified; the resulting entry vector was named PCR8/GW.FLAG.

Gateway LR Clonase II Enzyme Mix (Invitrogen) was used for two indipendent recombination reactions employing pGR107_GAT destination vector and PCR8/GW.Derp1_FLAG_Cterm or PCR8/GW.FLAG entry vectors. The resulting expression vectors were named pGR107_GAT.Derp1_FLAG_Cterm and pGR107_GAT.FLAG (empty vector).

Agroinfiltration

The pGR107_GAT.FLAG and pGR107_GAT.Derp1_FLAG_Cterm vectors were independently introduced into an *A. tumefaciens* strain (GV3101) by electroporation (Nagel et al. 1990), plated on LB agar medium (Tryptone 10 g/l, Yeast extract 5 g/l, Sodium chloride 5 g/l, agar 10 g/l) supplemented with tetracycline (5 μg/ml), gentamicin (25 μg/ml), rifampicin (50 μg/ml) and kanamycin (50 μg/ml), and grown at 28°C for 3 days. A single agrobacterium colony was used to inoculate an overnight 5 ml culture of LB medium with appropriate antibiotics. One ml of agrobacteria culture was then inoculated in 50 ml LB medium containing 10 mM 2-(N-morpholino)-ethanesulfonic acid (MES, pH5.5), 20 μM acetosyringone, tetracycline (5 μg/ml) and kanamycin (50 μg/ml). After overnight culture at 28°C, agrobacterial cells were collected by centrifugation for 15 min at 3,000×*g* and resuspended in 10 mM MES (pH5.5) plus 10 mM $MgCl_2$ and 100 μM acetosyringone. Bacterial suspension was adjusted to a final OD_{600} concentration of 1. *Agrobacterium*-mediated transient transformation was conducted on 30 days old *N. benthamiana* plants grown at 22°C with a light/dark cycle of 16/8 h, respectively. Bacterial suspection was infiltrated into intercellular spaces of intact leaves (2 leaves for each plant) using 1 ml plastic syringe without needle.

cDNA synthesis and RT-PCR analysis

Total RNAs were isolated from 0.1 g of fresh symptomatic leaves 10 days after agroinfection using the GenElute Mammalian Total RNA miniprep kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. Total RNA was purified from residual genomic DNA using the DNA-free kit (Ambion, Norwalk, CT, USA). First strand cDNA was synthesized from 1 μg of total RNA in a volume of 50 μl according to Sambrook and Russell (2001).

For Der p 1 gene detection, specific primers DER_ATG (5′-ATGACTAACGCCTGCAATAT CA-3′) and DER_stop (5′-TTAGAGAATGACAA CATATGGATA-3) were used. *Tubulin* gene was employed as housekeeping and was amplified by using the TUB_for (5′-GTGGAGTGGATCCCCAAC AA-3′) and TUB_rew (5′-AAAGCCTTCCTCCT GAACATGG-3′) specific primers.

RT-PCR reactions were performed in total volume of 25 μl containing 1 μl of the first-strand cDNA, 1 μM of each primer (DER_ATG, DER_stop, TUB_for, TUB_rew), 1× PCR Buffer, 1.5 mM $MgCl_2$, 0.6 μM dNTPs and 2U *Taq* DNA Polymerase (Sigma). Samples were denatured at 94°C for 1 min, and subjected to 25 cycles of denaturation at 94°C

1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min, plus a final step at 72°C for 5 min.

Protein extraction and quantification

Nicotiana benthamiana leaves derived from plant infected either with pGR107_GAT.FLAG or pGR107_GAT.Derp1_FLAG_Cterm and from healthy plants (mock), were ground to a fine powder in liquid nitrogen using mortar and pestel. Resulting powder (0.2 g) was resuspended in 0.5 ml of extraction buffer (EB) (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, pH8) supplemented with complete protease plant inhibitor (Sigma). Homogenates were centrifuged at 15,000×*g* at 4°C for 20 min and the supernatants were quantified. Protein concentrations derived from total soluble protein and immunoprecipitated I-rDer p 1 were determined using the Bradford method according to the Bio-Rad Microassay procedure (Bio-Rad, Berkeley, CA, USA) (Bradford 1976). A standard curve with known concentration of BSA was produced; absorption at OD_{590} nm was used to estimate sample protein concentration while protein quantification was performed using a BECKMAN DU650 spectrophotometer.

Western blot analysis

SDS–PAGE

Equal amounts of total soluble proteins (TPS) were fractioned on 12% SDS-polyacrylamide gels according to Sambrook and Russell (2001). Proteins were transferred to a Hybond C + membrane (GE Healthcare) using a wet-electroblotting apparatus (Bio-Rad) at 20 V for 12 h at 4°C. The resulting blots were blocked with 5% of non-fat dry milk (Bio-Rad), melted in Tween-Tris Buffered Saline (T-TBS), for 1 h at room temperature with gentle agitation. A rabbit polyclonal anti-CP antibody (1:10,000; kindly provided by CNR-IVV) and an anti-rabbit IgG peroxidase conjugated (1:5,000; Sigma) were used as primary and secondary antibodies, respectively in anti-virus coat protein immunoblotting. Indirect and direct antibodies were used for Der p 1 recombinant protein detections. For indirect detection, a mouse monoclonal anti-FLAG antibody (1:5,000; Sigma) and an anti-mouse peroxidase conjugate (1:5,000; Sigma) were used for western blot analysis on TPS samples whereas for immunoprecipitated samples a mouse monoclonal anti-FLAG peroxidase conjugate (1:1,000; Sigma) was used. Direct identification of rDer p 1 was performed using a mouse monoclonal antibody anti-Der p 1 Mite Group 1, clone 4C1 (1:200; Indoor biotechnologies) and an anti-mouse peroxidase conjugate (1:5,000; Sigma) as primary and secondary antibodies, respectively. Hybridized blots were exposed with Kodak AR films (Kodak, Rochester, New York, USA) after the chemiluminescent reaction carried out with the SuperSignal® West Pico HRP Substrate Kit (Thermo scientific, Pierce protein RP, Rockford, IL USA).

For quantification of I-rDer p 1 in agroinfected plants, a N-Terminal Met-FLAG fusion protein of *E. coli* bacterial alkaline phosphatase (BAP) (Sigma) was used as reference standard (25, 50, 75 and 100 ng) in western blot analysis with mouse monoclonal anti-FLAG antibody. Following exposure to film, the blots were analysed using LabImage 1D 2006 software (Kapelan Bio-Imaging GmbH, Leipzig, Germany) by comparison of bands with known quantities of standard (Met-FLAG.BAP; Sigma) and rDer p 1_FLAG_Cterm fusion protein.

Native-PAGE

For western blot analysis total soluble proteins and purified I-rDer p 1 were loaded on 7.5% polyacrylamide gels according to Hames and Rickwood (1990). After a 1 h pre-electrophoresis at 110 V, electrophoresis was carried out at the same voltage for 150 min at 4°C in a Acetic Acid–beta-alanine buffer (pH 4.5). Proteins were transferred using wet-electroblotting apparatus (Bio-Rad) at 400 mA for 1 h at 4°C in a Tris-glycine–methanol buffer (Towbin et al. 1979) to a nitrocellulose membrane (GenScript, Piscataway, NJ, USA). The immunoblot was performed with One-Step complete Western Kit (GenScript) according to manufacturer's instructions using the monoclonal antibody anti-Der p 1 Mite Group 1, clone 4C1 (1:1,000; Indoor biotechnologies). Signals were detected by enhanced chemiluminescence as described above. Purified House dust mite nDer p 1 protein was used as positive control (Stallergenes, SA, Antony, France).

Immunoprecipitation

Leaves derived from plants infected with pGR107_GAT.FLAG, pGR107_GAT.Derp 1_FLAG_Cterm

and healthy plants were used for immunoprecipitation analysis using anti-FLAG M2 affinity Gel (Sigma). Tissues (0.2 g) were homogenized (previously pulverized to a fine powder with a mortar and pestle under liquid nitrogen) using 0.8 ml of EB supplemented with 1× complete protease plant inhibitor (Sigma). The homogenates were centrifuged at 15,000×*g* at 4°C for 30 min and the supernatants were quantified as described above. Anti-FLAG M2 affinity agarose gel was suspended before use to make a uniform suspension of resin in the vials. The ratio of suspension to packed volume should be 2:1. Two hundred microliter of the suspension were transferred into a fresh test tubes and centrifuged at 8,200×*g* for 30 s. Supernatant was removed and the packed gel was washed with 1 ml of TBS solution, centrifuged at 8,200×*g* for 30 s and the supernatants removed again. The resins were washed one more time with 1 ml of TBS before continuing with immunoprecipitation. All samples were added to the washed resins and incubated overnight at 4°C with gentle shaking. After shaking, samples were centrifuged for 30 s at 8,200×*g* and the supernatants removed. Agarose gels were washed 3 times with 1 ml of TBS as described above and final resins were used for the elution step performed at room temperature with 0.1 M glycine HCl (pH 3.5). Samples were gently shook for 5 min and then centrifuged 30 s at 8,200×*g*. Supernatants were transferred to fresh tubes containing 2.5 µl of Tris 0.5 M (pH 11) and used for further analyses.

N-glycosylation analysis

Purified I-rDer p 1 and RNase B (New England Biolabs) were treated to remove carbohydrate residues from protein according to manufacturer's instructions. Briefly, two samples, each composed of 1 µg of I-rDer p 1 and 1 µl of 10× Glycoprotein Denaturing Buffer in 10 µl of reaction mix, were denatured at 100°C for 10 min. After cooling, samples were incubated at 37°C for 12 h in presence or absence of 1,000 U of PNGase F, 1 µl of 10× G7 Reaction Buffer and 2 µl of 10% NP-40 in a final reaction volume of 20 µl. The treated/untreated purified proteins were analyzed by SDS–PAGE Coomassie Brillant Blue Staining and western blotting assay with anti-Der p 1 antibody.

IgE inhibition assay

IgE inhibition experiments were performed as previously reported by Krause et al. (2009). Briefly, 20 µl of pooled patients' sera were incubated overnight with 20 µl of a solution containing the purified I-rDer p 1 allergen preparations at the concentration of 1 mg/ml. After o.n. incubation, the IgE binding inhibition was evaluated by running the ISAC 103 microarray IgE assay (VBC-Genomics, Phadia AB, Uppsala, Sweden), where Der p 1 and Der f 1 allergens are immobilized. Purified proteins derived from plants infected with empty viral vector (pGR107_GAT. FLAG), mock plants were used as control samples. Buffer solution was used as reference value for no inhibition IgE values.

The assay was run as a single point highest inhibition achievable assay (SPHIAa). For control purposes of the specific IgE inhibition obtained on mite molecules, several other allergens that were recognized by the IgE of the same pool were used. A no-inhibition value was required to record the experiment as valuable.

Results

Der p 1 cDNA cloning

A two step strategy was employed to isolate the full-length cDNA of the I-Der p 1 gene (EU881509). A cDNA fragment of 669 bp encoding for the mature Der p 1 enzyme was isolated by RT-PCR with two degenerated primers. Sequence information of this fragment was used to design two specific primers suitable for 5′ and 3′-RACE experiments. A cDNA clone of 1099 bp, compatible with the expected total length of Der p 1 gene (Online resources, Figure 1S) was thus isolated by RACE_PCR. Five prime and 3 prime-end specific primers were designed and used for performing end-to-end amplifications and to obtain the entire transcriptional unit. The obtained fragments were then directly sequenced. Sequence analysis confirmed the isolation of the full-length cDNA isoform which included a 49-base-long 5′UTR, a coding region of 963 bases and a 86 bp 3′UTR. The putative I-Der p 1 is a 320 amino acids polypeptides, composed of a signal peptide of 18 residues, a propeptide of 80 amino acids and a mature portion

of 222 residues with a calculated molecular mass of about 25 kDa (ACG58378).

When compared with the only available Der p 1 full-length cDNA sequence (Online resources, Figure 1S, U11695), a total of eleven polymorphisms were noticed. One synonymous and 4 non synonymous mutations were located in the coding region (Online resources, Figure 2S). In particular, 1 (non silent mutation **Val/Thr-4**), 2 (one silent **Val/Val-54** and one non silent **Phe/Ser-93**) and 1 (non silent mutation **Ser/Asn-103**) mutations were found in signal peptide, propeptide and mature enzyme (Online resources, Figure 2S, AAB60215), respectively. The 5′UTR contained 6 SNPs within the first ten nucleotides whereas no differences were found in the 3′UTR (Online resources, Figure 1S). Moreover, when compared with other available protein sequences derived from Der p 1 and Der f 1 accessions (Online resources, Figure 2S), I-Der p 1 protein showed 3 unique aminoacids: **Thr, Ser** and **Asn** at position +4, +93 and +103, respectively. This confirm that Der p 1 gene isolated from an Italian house dust mite encoding a new specific protein variant.

Construction of PVX expression vectors and agroinfiltration

A plant viral vector, based on the potato virus X, was employed for the expression of the I-Der p 1 allergen in *N. benthamiana* plants. In particular, Gateway cloning sites (Landy 1989) were inserted into the pGR107 vector (www.pbltechnology.com) (Online resources, Figure 3SA) between two copies of the CP sub-genomic promoter; the resulting destination vector was named pGR107_GAT (Online resources, Figure 3SB). Since the mature form of Der p 1 was reported to be immunologically active, it was decided to express only the N-terminal 222 aa which corresponds to the mature form of I-Der p 1 after proteolytic cleavage (Kraft et al. 1998; Takai et al. 2002; Valenta 2002). The corresponding coding sequence was thus amplified with specific primers containing clamp sequences for allowing the insertion of an ATG codon at the 5′ end and a FLAG epitope (DYKDDDDK) at the 3′ end. The amplicon was cloned into PCR8/GW vector (Invitrogen) and sequenced to verify the sequence integrity. The resulting entry vector, named PCR8/GW.Derp1_FLAG_Cterm, was used in a LR recombination with the pGR107_GAT (Online resources, Figure 3SB) destination vector to generate the expression vector pGR107_GAT.Derp1_FLAG_Cterm (Online resources, Figure 3SD). An LR recombination reaction was also performed using PCR8/GW.FLAG entry and the pGR107_GAT vectors to generate the pGR107_GAT.FLAG empty vector (Online resources, Figure 3SC), which was used as a control.

Overnight cultures of *Agrobacterium tumefaciens* cells (strain GV3101) transformed with either pGR107_GAT.Derp1_FLAG_Cterm or pGR107_GAT.FLAG were used to infiltrate 4 weeks old leaves of *N. benthamiana*. Controls were also carried out with mock infections. *N. benthamiana* leaves were collected 10 days after agroinfiltration and stored a −80°C.

I-Der p 1 expression in *Nicotiana benthamiana* plants

The transcription of Der p 1 gene was investigated by RT-PCR with specific primers located at the 5′ and 3′ ends of the I-Der p 1 sequence. An amplification product of the expected size was detected both in cDNA samples derived from plants agroinfiltrated with pGR107_GAT.Derp1_FLAG_Cterm vector and in pGR107_GAT.Derp1_FLAG_Cterm plasmid (positive control) (Online resources, Figure 4SC and D), whereas no amplification products were observed in mock plants or plants infected with the empty vector (Online resources, Figure 4SA and B). No amplification products were observed in samples where RT enzyme was omitted (data not shown). Direct sequencing of amplicons confirmed the integrity of Der p 1 sequence, replicated as additional viral ORF (Online resources, Figure 2S, I-rDer p 1).

In order to verify whether recombinant viral vectors were correctly propagated in plant cells, total soluble protein, extracted from symptomatic leaves collected after agroinfiltration, were analyzed with anti-CP, anti-FLAG (indirect detection) or anti-Der p 1 (direct detection) antibodies through SDS–PAGE and native conditions.

In western blot analysis with polyclonal anti-CP antibody, the coat protein of potato virus X was detectable as a band of about 25 kDa in both pGR107_GAT.FLAG and pGR107_GAT.Derp1_FLAG_Cterm samples (Fig. 1; lanes a2 and a3) but not in mock inoculated plants (Fig. 1; lane a1) suggesting an efficient virus replication.

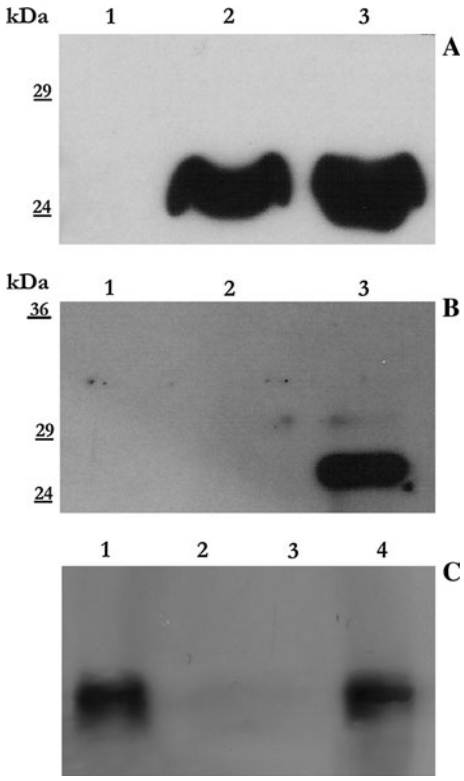


Fig. 1 Der p 1 mature form protein is expressed *in planta*. Western blot analysis were carried out with rabbit polyclonal anti-CP antibody (**a**) and mouse monoclonal anti-FLAG (**b**) under SDS–PAGE conditions and with mouse monoclonal anti-Der p 1 antibody under Native-PAGE conditions (**c**). Total soluble proteins were isolated from mock plants (**a**1, **b**1, **c**2), from plants agroinfiltrated with empty vector (**a**2, **b**2, **c**3) and from plants agroinfiltrated with vector containing I-Der p 1 (**a**3, **b**3, **c**4). House dust mite extracts were used as positive control in Native-PAGE (**c**1). Standard Molecular weights are reported on the left side of figure **a** and **b** (*kDa* kiloDaltons)

Duplicated blots were probed with monoclonal anti-FLAG and anti-Der p 1 antibodies in SDS–PAGE conditions. A single band corresponding to a molecular weight of about 27 kDa was visualized in western blots hybridized with monoclonal anti-FLAG antibody for PVX sample expressing I-Der p 1 (Fig. 1, lane b3). Since the FLAG peptide has a very low molecular weight (about 1 kDa), no signal was detected in samples from plants infected with the empty vector (Fig. 1, lanes b1 and b2). Moreover, western blotting with SDS–PAGE conditions using anti-Der p 1 antibody was able to detect the expected 27 kDa band either in plants or house dust mite extracts (data not shown). Interestingly, under native conditions, anti-Der p 1 antibody detected the expected band in both commercial purified nDer p 1 (Fig. 1; lane c1) and *in planta* protein extracts (Fig. 1; lane c4).

The anti-FLAG antibody also allowed an estimation of the accumulation level of I-rDer p 1 fusion protein (Derp1_FLAG_Cterm) in *N. benthamiana*. Quantifications were performed in western blots, according to Gotoh et al. (2002), by comparing signals in plant samples with known amounts of N-Terminal Met-FLAG bacterial alkaline phosphatase (BAP) fusion protein of *E. coli* (Met-FLAG.BAP, Sigma) using the LabImage 1D 2006 software. The estimated level of accumulation of rDer p 1 in *N. benthamiana* plant cells was about 3% of total soluble proteins.

I-rDer p 1 purification and immunoblotting

The FLAG-tag epitope, fused at the C-terminus region of I-rDer p 1 protein, allowed immunoprecipitations with an anti-FLAG antibody coupled to M2 agarose beads. The efficiency of immunoprecipitation was analyzed by estimating the amount of recombinant protein in the different fractions (Fig. 2a–c) through western blotting with either anti-FLAG antibody and SDS–PAGE conditions (Fig. 2a, b) or anti-Der p 1 antibody and Native-PAGE conditions (Fig. 2c). Purified proteins were also stained with Coomassie Brillant Blue to verify the presence of visible proteins in eluate samples (Fig. 2d).

A single hybridization signal of 27 kDa was visualized in western blotting with anti-FLAG antibody both in eluate (containing the purified protein, Fig. 2, lane a3) and beads fraction after elution (Fig. 2, lane a3) while no signal was detected in supernatant (data not shown). Moreover, a single band was visualized in Coomassie stained gels (Fig. 2, lane d3), and in western blotting with anti-Der p 1 antibody (Fig. 2, lane a4).

As expected, no bands were visualized in immunoprecipitated samples derived either from mock plants (Fig. 2, lanes a1, b1, c2 and d1) or from plants infected with empty viral vector (Fig. 2, lanes a2, b2, c3 and d2). The purification process yielded about 150 µg of I-rDer p 1 per gram of leaves infected with pGR107_GAT.Derp1_FLAG_Cterm vector.

N-glycosylation analysis of I-rDer p 1

To determine whether I-rDer p 1 was glycosylated the purified protein fraction was treated with PNGase F

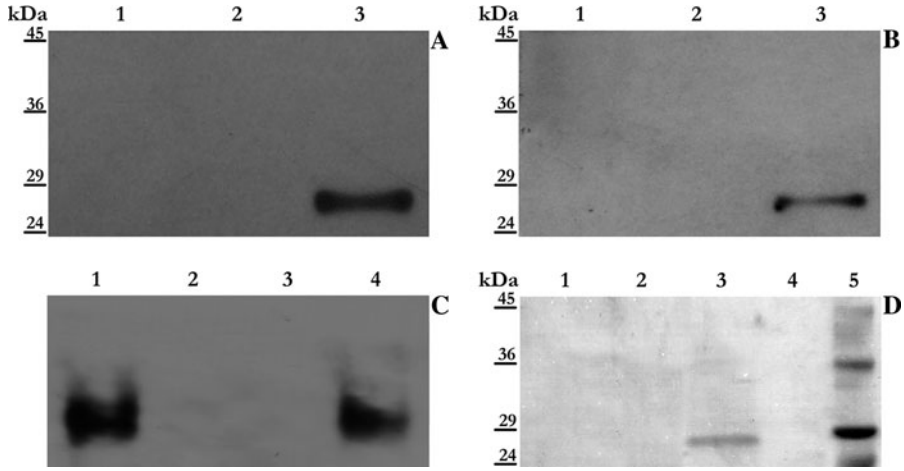


Fig. 2 Der p 1 recombinant protein is immunoprecipitated. After extraction, proteins were subjected to immunoprecipitation with an anti-FLAG antibody coupled to M2 agarose beads and used for western blotting with anti-FLAG monoclonal antibody on both eluate (containing the purified protein, **a**) and beads fraction after elution (**b**). In particular proteins were immunoprecipitated from mock plants (lanes **a**1, **b**1), from plants agroinfiltrated with pGR107_GAT.FLAG (**a**2, **b**2) and with pGR107_GAT.Derp1_FLAG_Cterm (**a**3, **b**3) expression vectors. Western blotting were also carried out under Native-PAGE conditions (**c**) using anti-Der p 1 antibody against positive control (**c**1, commercial purified nDer p 1), immunoprecipitated proteins from mock plants (**c**2), from plants agroinfiltrated with empty vector (**c**3) and pGR107_GAT.Derp1_FLAG_Cterm (**c**4). Staining with Coomassie Brillant Blue (**d**) of immunoprecipitated proteins from mock-inoculated control plants (**d**1), plants infected with empty vector (**d**2) and plants infected with viral vector containing I-Der p 1 (**d**3) was performed. **d**4 is an empty lane while **d**5 is MW marker. Standard Molecular weight (*kDa* kiloDaltons) are reported on the left side of **a**, **b** and **d** figures

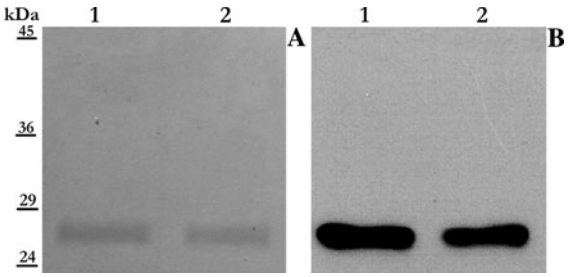


Fig. 3 N-glycosylation analysis of I-rDer p 1. SDS–PAGE coomassie brillant blue staining (**a**) and western blot analysis (**b**) of I-rDer p 1 before (1**a**, 1**b**) and after (2**a**, 2**b**) PNGase F de-glycosylation treatment

enzyme which release all carbohydrate residues from protein. Figure 3a and b shows that the electrophoretic mobility of I-rDer p 1 before (lanes 1a and 1b) and after (lanes 2a and 2b) de-glycosylation does not change. These results suggest that recombinant mature Der p 1 expressed in our study is not glycosylated. RNase B protein was also treated and the results of the SDS–PAGE Coomassie Brilliant Blue before and after de-glycosylation demonstrated the goodness of the experimental procedures (Supplementary material, Figure 5S).

I-rDer p 1 ISAC IgE inhibition assay

IgE inhibition results, obtained by incubating the I-rDer p 1 with pooled sera from mite allergic patients recognizing Der p 1 and Der f 1, are reported in Fig. 4. As clearly shown, an almost total IgE inhibition of both homologous mite molecules immobilized on the ISAC microarray has been achieved, strongly suggesting that the I-rDer p 1 preparation bears all the IgE binding epitopes. As expected no IgE inhibition reaction was observed (data not shown) when purified proteins derived from tissues of plants infected with empty viral vector and mock were used.

Discussion

In developed countries more than 20% of the population is affected by IgE mediated allergic diseases, such as seasonal and perennial rhinitis asthma and atopic dermatitis (Wills-Karp et al. 2001). Diagnosis of IgE mediated allergies is usually conducted by provocation testing with extracts prepared from suspected allergen sources. Alternatively, IgE inhibition

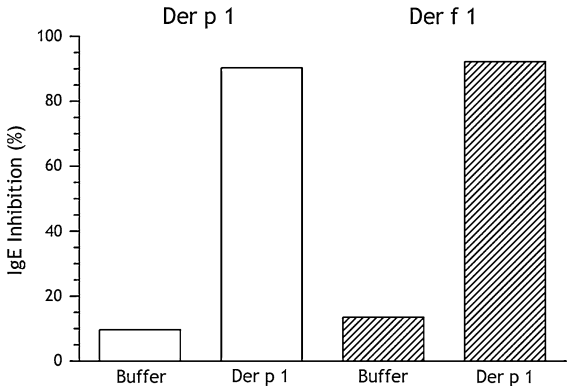


Fig. 4 IgE inhibition analysis. IgE inhibition of Ir-Der p 1 was evaluated by running the ISAC 103 microarray IgE assay where Der p 1 and Der f 1 allergens are immobilized. I-rDer p 1 showed an almost full inhibition of the IgE binding. Buffer solution was used as reference value for no inhibition IgE values

testing can be applied to obtain in vitro measurements of specific serum IgE antibodies. In both cases allergen extracts, consisting of variable mixtures of allergic and non allergic components, are used for in vitro and in vivo diagnosis of allergy. With such diagnostic extracts it is almost impossible to detect whether the patient is sensitized to a variety of unrelated allergens or cross sensitized to few but cross reactive allergens. Moreover, extract-based diagnosis cannot provide information regarding the individual reactivity profiles of allergic patients and thus fail to identify the precise target molecules for specific immunotherapy. Recombinant allergen mimicking the immunological properties of native allergens would be welcome for both diagnostic and therapeutic purposes.

Plants have recently joined the competition for the development of an efficient and cheap system for the production of pharmaceutical proteins (Ma et al. 2005). Compared to other expression systems, such as bacterial or mammalian cell cultures, plants are characterized by a lower risk of getting contaminated by human pathogens (e.g. animal viruses or prions). In the present paper a plant viral vector based on the potato virus X was used to express the mature form of Der p 1, the major allergen of house dust mite. In particular, we have inserted Gateway cloning sites into the pGR107 vector with aiming at agroinfiltrations rather than plasmidic DNA infections. Gateway technology facilitates high throughput cloning of target sequences by making use of the bacteriophage lambda site-specific recombination system (Landy 1989), whereas the agroinfiltration increases the infection efficiency (Gleba et al. 2005).

As far as we know this is the first time that the I-Der p 1 (Der p 1 of an Italian strain of the house dust mite) variant was isolated, sequenced and expressed. The sequence shows several polymorphisms compared to full or partial Der p 1 sequences obtained from house dust mites belonging to other geographical regions (Voorhorst et al. 1967; Chua et al. 1988, 1993; Thomas et al. 1988, 2004; Arlian et al. 2001; Smith et al. 2001; Asokananthan et al. 2002; Takai et al. 2002; De Boer 2003; Wolfowicz et al. 2003; Pichavant et al. 2005). In particular, I-Der p 1 variant shows several mutations at nucleotide level when compared with all the other available sequences. Three amino acid substitutions were observed, one in the signal peptide, one in the propeptide and one in the mature enzyme sequence. These 3 substitutions distinguished the Italian strain from all others. It is worth noting that these substitutions do not pertain neither to the active site (Cys34; His170; Asn190) nor the disulfide bonds (Cys31-Cys71; Cys65-Cys103; Cys4-Cys117) but affect the portions of the molecule located in the signal peptide, in the propeptide and in the NH2-terminal end of the mature enzyme. In particular, amino acidic mutation Phe/Ser-6 is located in the binding region between the propeptide and the mature enzyme.

It is noteworthy that previous studies carried out in *E. coli* and yeasts have failed in expressing the mature form of Der p 1. The same results were reported for tobacco cell suspension transformed with a cDNA coding for the mature form of Der p 1. On the contrary, the pro-Der p1 protein targeted to the endoplasmic reticulum of tobacco BY2 cells was correctly processed to mature form which retained both enzymatic activity and immunogenic properties. Yang et al. (2007) have described the expression of the mature form of Der p 1 in transgenic rice seeds using a construct based on the GluB promoter and a targeting signal for the ER. Possible problems with glycosylation in Golgi were avoided by including a KDEL signal for protein retention in the ER. The authors have also demonstrated that glycosylated Der p 1 had a significantly reduced IgE binding capacity when compared with unglycosylated Der p 1. Based on these results they proposed that the high variability in IgE binding reduction among sera depended on glycosylation levels (Yang et al. 2007). To circumvent this problem, we decided to express a cytoplasmic

version of mature Der p 1 by using an in whole plant transient expression system. Interestingly, we report evidences that anti-Der p 1 antibody (clone 4C1, Indoor biotechnologies) recognized commercial purified n-Der p 1 and our I-rDer p 1 in Native-PAGE suggesting that I-rDer p 1 is correctly folded, at least at the level of epitope recognized by the antibody used.

Comparison between I-rDer p 1 and N-Terminal Met-FLAG fusion protein of *E. coli* Bacterial Alkaline Phosphatase, allowed us to estimate that the recombinant allergen represented about 3% of total soluble proteins in *N. benthamiana* leaves. Our results demonstrate that the execution of immunoprecipitations, using the FLAG-tag for isolating I-rDer p 1 proteins from crude extracts, is very simple and efficient. Nevertheless, FLAG-based immunoprecipitation system for Der p 1 purification need to be optimized since we have demonstrated that a significant aliquot of the bound protein tends to remain attached to the anti-FLAG antibody immobilized to the agarose beads. Our future aims are to test other immunoprecipitation systems employing other tags (e.g. 6HIS or CMyC).

Moreover, when the I-rDer p 1 has been tested for IgE inhibition on a well established immune assay, we obtained an almost full inhibition of the IgE binding. These findings corroborates that the obtained recombinant product mimics the already available ones and thus it could be useful for diagnostic purposes and might be employed for allergen standardization in total mite extracts.

In conclusion, in this paper we have shown that a modified PVX viral vector can be successfully used to express I-Der p 1 protein suitable for diagnostic purposes. A better molecular and immunological characterization is needed to ascertain whether *in planta* produced I-Der p 1 might also be used for therapeutic treatments.

Acknowledgments We owe a big thank to Prof. Luca Santi and Luigi Russi for critical reading of this manuscript and helpful discussions. We are also very grateful to M. Castagnoli of CRA-ABP (Consiglio per la Ricerca e la Sperimentazione in Agricoltura, Centro di ricerca per l'agrobiologia e la pedologia, Florence, Italy) for providing HDM and to Prof. David Baulcombe (The Sainsbury Laboratory, Norwich, UK, www.pbltechnology.com) for kindly providing the pGR107 vector. This work was funded by Progetto PRIN 2005 "Transient expression of human proinsulin and of other therapeutics proteins in *Nicotiana benthamiana*" (EA) and PRIN 2006 "Production, purification and in vitro characterization of some italian-strain recombinant allergens of *Dermatophagoides pteronyssinus*" (FP).

References

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl Acids Res 25:3389–3402

Arlian LG, Neal JS, Morgan MS, Vyszenski-Moher DL, Rapp CM, Alexander AK (2001) Reducing relative humidity is a practical way to control dust mites and their allergens in homes in temperate climates. J Allergy Clin Immunol 107:99–104

Asokananthan N, Graham PT, Stewart DJ, Bakker AJ, Eidne KA, Thompson PJ, Stewart GA (2002) House dust mite allergens induce proinflammatory cytokines from respiratory epithelial cells: the cysteine protease allergen, Der p 1, activates protease-activated receptor (PAR)-2 and inactivates PAR-1. J Immunol 169:4572–4578

Asturias JA, Arilla MC, Gómez-Bayón N, Martínez A, Martínez J, Palacios R (1998) Sequencing and high level expression in *Escherichia coli* of the tropomyosin allergen (Der p 10) from *Dermatophagoides pteronyssinus*. Biochim Biophys Acta Gene Struct Expr 1397:27–30

Asturias J, Gomez-Bayon N, Arilla MC, Martinez A, Palacios R, Sanchez-Gascon F, Martinez J (1999) Molecular characterization of American cockroach tropomyosin (*Periplaneta americana* allergen 7), a cross-reactive allergen. J Immunol 162:4342–4348

Bendahmane A, Köhn BA, Dedi C, Baulcombe DC (1995) The coat protein of potato virus X is a strain-specific elicitor of Rx1-mediated virus resistance in potato. Plant J 8:933–941

Bradford M (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159

Chua KY, Stewart GA, Thomas WR, Simpson RJ, Dilworth RJ, Plozza TM, Turner KJ (1988) Sequence analysis of cDNA coding for a major house dust mite allergen, Der p 1. Homology with cysteine proteases. J Exp Med 167:175–182

Chua KY, Kehal PK, Thomas WR (1993) Sequence polymorphism of the cDNA clones encoding the mite allergen Der p 1. Int Arch Allergy Immunol 101:364–368

Chua KY, Huang CH, Shen HD, Thomas WR (1996) Analysis of sequence polymorphism of a major mite allergen Der p 2. Clin Exp Allergy 26:829–837

Cunningham C, Porter AJR (eds) (1998) Recombinant proteins from plants. Production and isolation of clinically useful compounds. Methods in Biotechnology, vol 3. Humana Press, Totowa, NJ

De Boer R (2003) The effect of sub-floor heating on house-dust-mite populations on floors and in furniture. Exp Appl Acarol 29:315–330

Fernandez-Caldas E (1997) Mite species of allergologic importance in Europe. Allergy 52:383–387

Floss DM, Falkenburg D, Conrad U (2007) Production of vaccines and therapeutic antibodies for veterinary applications in transgenic plants: an overview. Transgenic Res 16:315–332

Gleba Y, Klimyuk V, Marillonnet S (2005) Magnifection: a new platform for expressing recombinant vaccines in plants. Vaccine 23:2042–2048

Gotoh M, Yada T, Sato T, Akashima T, Iwasaki H, Mochizuki H, Inaba N, Togayachi A, Kudo T, Watanabe H, Kimata K, Narimatsu H (2002) Molecular cloning and characterization of a novel chondroitin Sulfate glucuronyltransferase that transfers glucuronic acid to *N*-acetylgalactosamine. J Biol Chem 41:38179–38188

Greene WK, Cyster JG, Chua KY, O'Brien RM, Thomas WR (1991) IgE and IgG binding of peptides expressed from fragments of cDNA encoding the major house dust mite allergen Der p I. J Immunol 147:3768–3773

Hames BD, Rickwood D (1990) Gel electrophoresis of proteins. A practical approach, 2nd edn edn. IRL Press, Oxford, pp 1–270

Hellens RP, Edwards EA, Leyland NR, Bean S, Mullineaux PM (2000) pGreen: a versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation. Plant Mol Biol 42:819–832

HuangFu T, Lim LH, Chua KY (2006) Efficacy evaluation of Der p 1 DNA vaccine for allergic asthma in an experimental mouse model. Vaccine 24:4576–4581

Jacquet A, Haumont M, Massaer M, Daminet V, Garcia L, Mazzu P, Jacobs P, Bollen A (2000) Biochemical and immunological characterization of a recombinant precursor form of the house dust mite allergen Der p 1 produced by *Drosophila* cells. Clin Exp Allergy 30:677–684

Kraft D, Ferreira F, Ebner C, Valenta R, Breiteneder H, Susani M, Breitenbach M, Scheiner O (1998) Recombinant allergens: the future of the diagnosis and treatment of atopic allergy. Allergy 53:62–66

Krause S, Reese G, Randow S, Zennaro D, Quaratino D, Palazzo P, Ciardiello MA, Petersen A, Becker WM, Mari A (2009) Lipid transfer protein (Ara h 9) as a new peanut allergen relevant for a Mediterranean allergic population. J Allergy Clin Immunol 4:771–778

Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 5:150–163

Landy A (1989) Dynamic, structural, and regulatory aspects of lambda site-specific recombination. Annu Rev Biochem 58:913–949

Lee CS, Tsai LC, Chao PL, Lin CY, Hung MW, Chien AI, Chiang YT, Han SH (2004) Protein sequence analysis of a novel 103-kDa *Dermatophagoides pteronyssinus* mite allergen and prevalence of serum immunoglobulin e reactivity to rDer p 11 in allergic adult patients. Clin Exp Allergy 34:354–362

Lienard D, Tran Dinh O, van Oort E, Van Overtvelt L, Bonneau C, Wambre E, Bardor M, Cosette P, Didier-Laurent A, de Borne FD, Delon R, van Ree R, Moingeon P, Faye L, Gomord V (2007) Suspension-cultured BY-2 tobacco cells produce and mature immunologically active house dust mite allergens. Plant Biotechnol J 5:93–108

Lin KL, Hsieh KH, Thomas WR, Chiang BL, Chua KY (1994) Characterization of Der p V allergen, cDNA analysis, and IgE-mediated reactivity to the recombinant protein. J Allergy Clin Immunol 94:989–996

Ma J, Barros E, Bock R, Christou P, Dale PJ, Dix PJ, Fischer R, Irwin J, Mahoney R, Pezzotti M, Schillberg S, Sparrow P, Stoger E, Twyman RM (2005) Molecular farming for new drugs and vaccines. EMBO Rep 6:593–599

Mach L, Mort JS, Glössl J (1994) Maturation of human procathepsin B. Proenzyme activation and proteolytic processing of the precursor to the mature proteinase, in vitro, are primarily unimolecular processes. J Biol Chem 269: 13030–13035

Morozov Sy, Gorbulev Vg, Novlkov Vk, Agranovsky Aa, Kozlov Yv, Atabekov Jg, Aa Baev (2000) The primary structure of the 5′- and 3′-terminal regions of the RNA of potato virus X. Dokl Akad Nauk 259:723–725

Nagel R, Elliot A, Masel A, Birch RG, Manners JM (1990) Electroporation of binary Ti plasmid vector into *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. FEMS Microbiol Lett 67:325–328

Pichavant M, Charbonnier AS, Taront S, Brichet A, Wallaert B, Pestel J, Tonnel AB, Gosset P (2005) Asthmatic bronchial epithelium activated by the proteolytic allergen Der p 1 increases selective dendritic cell recruitment. J Allergy Clin Immunol 115:771–778

Platts-Mills TA, Chapman MD (1988) Dust mites: immunology, allergic disease, and environmental control. J Allergy Clin Immunol 82:841

Platts-Mills TA, de Weck AL (1989) Dust mite allergens and asthma, a worldwide problem. J Allergy Clin Immunol 83:415–427

Platts-Mills TA, Woodfolk JA (2011) Allergens and their role in the allergic immune response. Immunol Rev 242:51–68

Platts-Mills TA, Blumenthal K, Perzanowski M, Woodfolk JA (2000) Determinants of clinical allergic disease. The relevance of indoor allergens to the increase in asthma. Am J Respir Crit Care Med 162:S128–S133

Porta C, Lomonossoff GP (1998) Scope for using plant viruses to present epitopes from animal pathogens. Rev Med Virol 8:25–41

Robinson C, Kalsheker NA, Srinivasan N, King CM, Garrod DR, Thompson PJ, Stewart GA (1997) On the potential significance of the enzymatic activity of mite allergens to immunogenicity. Clues to structure and function revealed by molecular characterization. Clin Exp Allergy 27:10–21

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor, NY., Cold Spring Harbor Laboratory Press

Smith WA, Chua KY, Kuo MC, Rogers BL, Thomas WR (1994) Cloning and sequencing of the *Dermatophagoides pteronyssinus* group III allergen, Der p III. Clin Exp Allergy 24:220–228

Smith WA, Hales BJ, Jarnicki AG, Thomas WR (2001) Allergens of wild house dust mites: environmental Der p 1 and Der p 2 sequence polymorphisms. J Allergy Clin Immunol 107:985–992

Sonenberg N, Shatkin AJ, Ricciardi RP, Rubin M, Goodman RM (1978) Analysis of terminal structures of RNA from potato virus X. Nucleic Acids Res 5:2501–2512

Steen N, Hutchinson A, McColl E, Eccles MP, Hewison J, Meadows KA, Blades SM, Fowler P (1994) Development of a symptom based outcome measure for asthma. Br Med J 309:1065–1068

Takai T, Mineki R, Nakazawa T, Takaoka M, Yasueda H, Murayama K, Okumura K, Ogawa H (2002) Maturation of the activities of recombinant mite allergens Der p 1 and Der

f 1, and its implication in the blockade of proteolytic activity. FEBS Lett 531:265–272

Takai T, Kato T, Yasueda H, Okumura K, Ogawa H (2005) Analysis of the structure and allergenicitiy of recombinant pro- and mature Der p 1 and Der f 1: major conformational IgE-epitopes blocked by prodomains. J Allergy Clin Immunol 115:555–563

Thomas WR, Stewart GA, Simpson RJ, Chua KY, Plozza TM, Dilworth RJ, Nisbet A, Turner KJ (1988) Cloning and expression of DNA coding for the major house dust mite allergen Der p 1 in *Escherichia coli*. Int Arch Allergy Appl Immunol 85:127–129

Thomas WR, Smith WA, Hales BJ, Mills KL, O'Brien RM (2002) Characterization and immunobiology of house dust mite allergens. Int Arch Allergy Immunol 129:1–18

Thomas WR, Smith WA, Hales BJ (2004) The allergenic specificities of the house dust mite. Chang Gung Med J 27:563–569

Topham CM, Srinivasan N, Thorpe CJ, Overington JP, Kalsheker NA (1994) Comparative modelling of major house dust mite allergen Der p I: structure validation using an extended environmental amino acid propensity table. Protein Eng 7:869–894

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354

Valenta R (2002) The future of antigen-specific immunotherapy of allergy. Nat Rev Immunol 2:446–453

Vernet T, Khouri HE, Laflamme P, Tessier DC, Musil R, Gour-Salin BJ, Storer AC, Thomas DY (1991) Processing of the papain precursor. Purification of the zymogen and characterization of its mechanism of processing. J Biol Cem 266:21451–214517

Voorhorst R, Spieksma-Boezeman MIA, Spieksma FTM, Varekamp H, Leupen MJ, Lyklema AW (1967) The house dust mite (*Dermatophagoides pteronyssinus*) and the allergens it produces. Identity with the house dust allergen. J Allergy 39:325–339

Wagner B, Fuchs H, Adhami F, Ma Y, Scheiner O, Breiteneder H (2004) Plant virus expression systems for transient production of recombinant allergens in *Nicotiana benthamiana*. Methods 32:227–234

Wills-Karp M, Santeliz J, Karp CL (2001) The germeless theory of allergic disease: revisiting the hygiene hypothesis. Nat Rev Immunol 1:69–75

Wolfowicz CB, HuangFu T, Chua KY (2003) Expression and immunogenicity of the major house dust mite allergen Der p 1 following DNA immunization. Vaccine 21:1195–1204

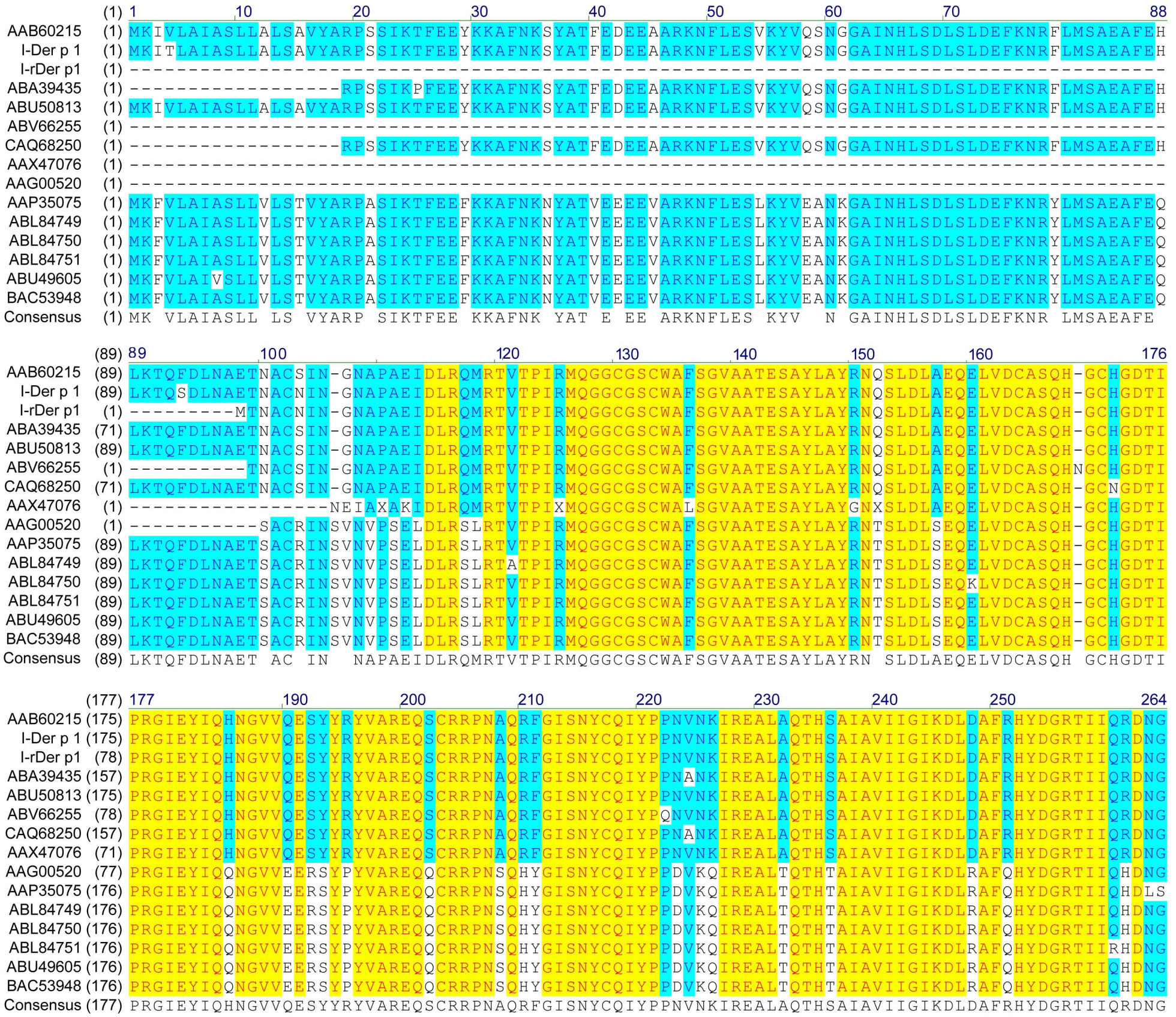
Yang L, Kajiura H, Suzuki K, Hirose S, Fujiyama K (2007) Generation of a transgenic rice seed-based edible vaccine against house dust mite allergy. Biochem Biophys Res Commun 365:334–339

Yusibov V, Rabindran S, Commandeur U, Twyman RM, Fischer R (2006) The potential of plant virus vectors for vaccine production. Drugs R&D 7:203–217

Figure 2S - Amino acid sequence alignment

Alignment of amino acidic sequences of Der p 1 (AAB60215; I-Der p 1, I-rDer p 1_ACG58378; ABA39435; ABU50813; ABV66255; CAQ68250; AAX47076) and Der f 1 (AAG00520; AAP35075; ABL84749; ABL84750; ABL84751; ABU49605; BAC53948) variant proteins.

Black letters on white background indicate non-similar amino acids, blue letters on light blue background indicate conservative amino acids, red letters on yellow background indicate identical amino acids.



Article Title

In planta expression of a mature Der p 1 allergen isolated from an Italian strain of Dermatophagoides pteronyssinus

Transgenic research

Author's names

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Figure 3S - pGR107 derived vectors
Schematic representation of PVX-derived vectors: pGR107 (A), pGR107_GAT (B), pGR107_GAT.FLAG (C) and pGR107_GAT.Derp1_FLAG_Cterm (D).
Primers are indicated in their specific annealing sites. Reading frame cassette A of the Gateway system is represented as Rfa box with Att-R/Att-L recombinantion sites.

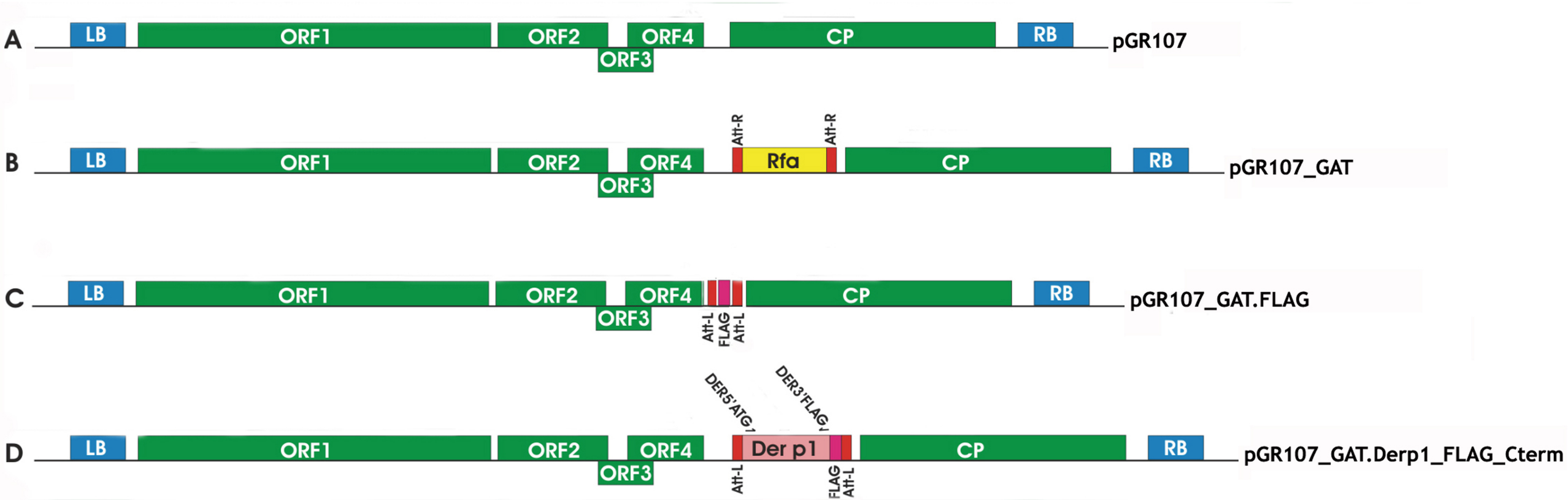

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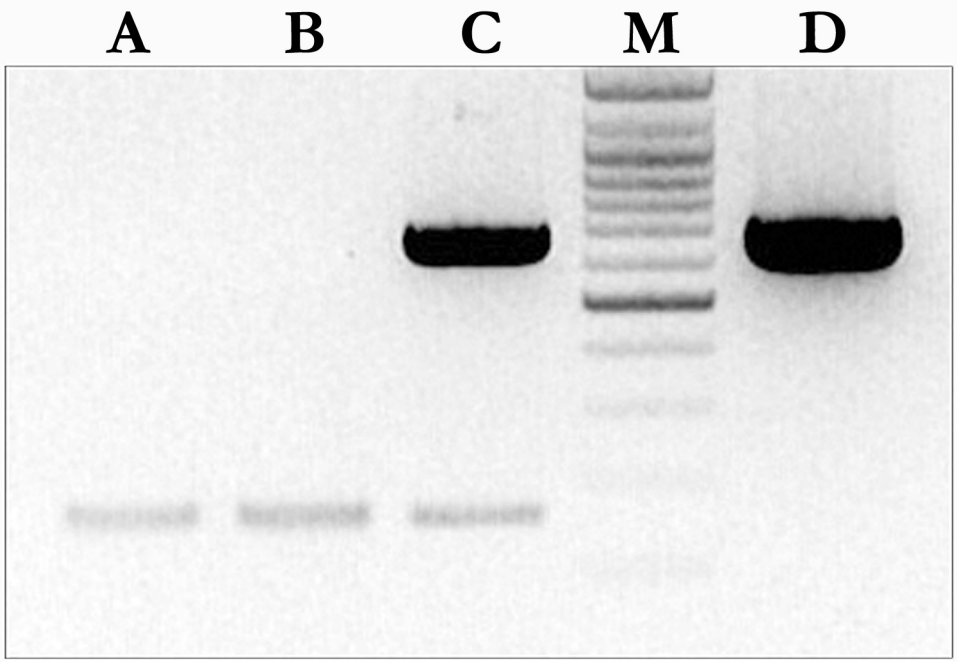
Figure 4S - Expression analysis
RT-PCR carried out with Der p 1-specific primers (amplicon of 669 bp) on samples derived from: mock plants (A), plants agroinfiltrated with pGR107_GAT.FLAG (B) and agroinfiltrated with pGR107_GAT.Derp1_FLAG_Cterm (C).
Amplifications were semi-quantitatively standardized on the basis of a tubulin gene (band of 180 bp) amplified using specific primers. PCR product positive control was obtained using Der p 1 specific primers on pGR107_GAT.Derp1_FLAG_Cterm plasmid (D).



Article Title
In planta expression of a mature Der p 1 allergen isolated from an Italian strain of Dermatophagoides pteronyssinus
Transgenic research

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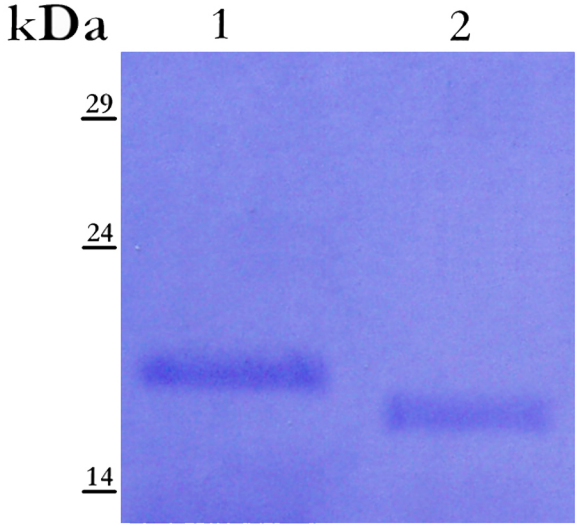
Figure 5S - Control of glycosylation analysis.
SDS-PAGE Coomassie Brillant Blue Staining of RNase B protein (New England biolabs, Ipswich, MA, USA), glycosylated (1) and de-glycosylated (2)
performed to verify the goodness of the experimental procedure.



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