

ORIGINAL ARTICLE

Production of native and modified recombinant Der p 1 molecules in tobacco plants

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Summary

Background As a complex molecule requiring post-translational processing, it has been difficult to produce the Der p 1 major allergen from the *Dermatophagoides pteronyssinus* house dust mite in a recombinant form.

Objective Here, we tested whether transgenic tobacco plants are suitable to express Der p 1, either as a wild-type molecule or as variants lacking *N*-glycosylation sites (Gly[−]) and/or cysteine protease activity (Enz[−]).

Methods Using *Agrobacterium tumefaciens*-based transformation, pro Der p 1 molecules bearing mutations within either the *N*-glycosylation sites (N34Q, N150Q) and/or the cysteine protease-active site (C132V) were expressed in tobacco plants. After purification by ion exchange chromatography, allergens were characterized using immunoblotting, circular dichroism (CD), as well as basophil and T lymphocyte stimulation assays.

Results Four forms of recombinant Der p 1 (i.e. wild-type Gly⁺/Enz⁺, as well as Gly[−]/Enz⁺, Gly⁺/Enz[−] or Gly[−]/Enz[−] variants) were successfully expressed in tobacco leaves as pro Der p 1 molecules. Spontaneous cleavage of the pro-peptide was observed in tobacco leaf extracts for all forms of recombinant Der p 1 (r Der p 1). CD confirmed that all r Der p 1 molecules, with the exception of the Gly[−]/Enz[−] variant, exhibited secondary structures comparable to the natural protein. A cysteine protease activity was associated only with the Gly⁺/Enz⁺ form. All these molecules exhibit a profile similar to natural Der p 1 with respect to IgE immunoreactivity, basophil activation and T cell recognition.

Conclusion A tobacco plant expression system allows the production of various forms of mature Der p 1, which could be used for diagnostic or immunotherapeutic purposes.

Keywords allergy vaccine, Der p 1, house dust mite, plant expression, recombinant allergen
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Introduction

Therapeutic vaccines against house dust mite (HDM) allergy are currently based on natural biological extracts [1, 2]. Cloning and successful expression of recombinant allergens allows replacing such allergenic extracts by selected highly purified allergens [3–5]. When compared with natural allergens, recombinant proteins offer several advantages: (i) they can be produced consistently in large quantities, (ii) they are better defined and easier to standardize and (iii) they can be expressed either in a

native conformation to mimic natural allergens, or as modified variants (e.g. hypoallergenic forms with reduced IgE reactivity) [3–9].

A valid recombinant HDM vaccine is likely to include, at a minimum, the two major allergens Der p 1 and Der p 2, both from the *Dermatophagoides pteronyssinus* mite species. Der p 1 is a clinically relevant allergen, because 40–100% of patients allergic to *D. pteronyssinus* are sensitized to it [1]. Also, the molecule is highly homologous to Der f 1, and thus, a vaccine based on recombinant Der p 1 will likely cross-protect against the common

D. farinae mite species. Der p 1 is naturally expressed as a precursor molecule (pre pro Der p 1), yielding the mature form only after cleavage of an 18-amino-acid signal peptide and an 80-amino-acid pro-peptide [10]. Whereas, Der p 2 is easily produced using conventional expression systems (e.g. *Escherichia coli* and *Saccharomyces cerevisiae*), Der p 1 has proven difficult to express in a well-folded conformation, unless in the pro Der p 1 form [11–17]. The pro-peptide appears to allow correct folding of Der p 1, while inhibiting its papain-like cysteine protease activity [13, 17, 18]. The cysteine residue in position 132 within Der p 1 is essential for such enzymatic activity, which likely contributes to the allergenicity of the molecule [19–21]. Two *N*-glycosylation sites, involving the N34 and N150 residues located within the pro-peptide and the mature protein, respectively, may also regulate allergen maturation [10, 15, 22]. Collectively, those biological properties can lead to a considerable level of molecular heterogeneity, making it difficult to produce pharmaceutical-grade Der p 1 for diagnostic and therapeutic purposes.

While candidate recombinant allergy vaccines are often based on hypoallergenic versions of allergens with altered three-dimensional structures [3, 5], our own strategy is rather to use allergens mimicking the conformation of natural allergens, which we believe is better suited for both *in vitro* diagnostic or skin prick tests, as well as sublingual immunotherapy regimens [23]. For this, an ideal expression system should allow appropriate post-translational modifications, ensuring proper folding and stability of the Der p 1 molecule. Given the previously reported successes in producing heterologous proteins in a native conformation using transgenic plants [7, 24, 25] or cultured plant cells [26], we undertook the present study in order to express in a tobacco plant system various forms of Der p 1 with/without pro-peptide, *N*-glycosylation and enzymatic activity, respectively.

Material and methods

Plasmids and constructs for tobacco plant expression

The full-length pro Der p 1 coding sequence (GenBank access number EU092644, with an additional V204A variation) was amplified by PCR/splicing overlap extension (PCR/SOE) to substitute the endogenous signal peptide by the tobacco pathogenesis-related protein signal peptide (PRS), and to introduce *HpaI*, *XhoI* and *XbaI*, *SacI* restriction sites at the 5' and 3' ends, respectively. Plasmids PRS α 1 and pPICZ α A-Der p 1 [15] were used as templates to amplify the PRS and Der p 1 sequences, respectively, using appropriate primers [01271–01273], [01272–01274] and [01271–01274] (Table 1). The *XhoI*- and *SacI*- digested PRS-pro Der p 1 sequence was cloned into *XhoI*-*SacI* sites of pBSKII (Stratagene, La Jolla, CA, USA), leading to plasmid pMRT1831. The pro Der p 1 wild-type (pDWT) construct was created by inserting the *HpaI*-*SacI* coding sequence of pMRT1831 downstream of the constitutive Mpr1163 promoter [27].

Site-directed removal of *N*-glycosylation and/or active sites was performed as follows: the asparagine residues of *N*-glycosylation consensus sequences within the pro-peptide (N34) and mature protein (N150) were replaced by glutamine by PCR/SOE-based site-directed mutagenesis. To remove the *N*-glycosylation site within the Der p 1 pro-peptide, a product amplified by three PCR steps, using [01271–01278], [01277–01279] and [01271–01279] primers (Table 1), was digested by *XhoI*-*PstI* and inserted into the corresponding sites of pMRT1831 to create pMRT1845. The pMRT1819 plasmid, containing an N150Q mutant form, was created by inserting a *PstI*-*SacI*-digested PCR fragment amplified, using [01263–01294], [01293–01264] and [01263–01264] primers (Table 1) and plasmid pMRT1831 as a template, into *PstI*-*SacI*-digested pBSKII. The N150Q fragment from pMRT1819 was subsequently inserted into pMRT1845 to

Table 1. Primers used to amplify coding sequences and introduce mutations

Primer	Sequence	Mutation
01263	TGAATGCTGAACTAACGCC	
01264	GAGAGAGCTCCGATAGTAGCTTCTTGACG	
01271	ATCCGCTCGAGGTTAACAACATGAATTCCTCAAAAGTTTCC	
01272	GTAGCTGTTACTCATGCTCGTCCATCATCGATCAAAAC	
01273	GTTTGTATCGATGATGGACGAGCATGAGTAACAGCTAC	
01274	ATACGAGCTCTCTAGATTAGAGAATGACAACATATGG	
01277	GAATACAAAAAGGCCTTCCAAAAAGTTATGC	N34Q
01278	TAGCATAACTTTTTGGAAGGCCTTTTGTATTCTTC	N34Q
01279	CAAATCGATTTCAGCTGGAGC	
01291	CAAGGAGGCTGTGGGTCCGTTTGGGCTTCTCTGGTG	C132V
01292	CACCAGAGAAAGCCCAACGGACCCACAGCCTCCTTG	C132V
01293	CTTATTTGGCTTACCGTCAACAATCATTAGATCTTGCTGAAC	N150Q
01294	GTTCAGCAAGATCTAATGATTGTTACGGTAAGCCAAATAAG	N150Q

create pMRT1856 containing both mutated glycosylation sites. A PCR-amplified Der p 1 fragment containing the C132V mutation at the enzymatic active site was generated in three steps using [01263–01292], [01264–01291] and [01263–01264] primers (Table 1). The resulting product was digested with *PstI*–*SacI* and cloned into the *PstI*–*SacI*-digested pBSKII to create pMRT1818. The C132V mutation abrogating the enzymatic activity was then introduced into the full-length Der p 1 coding sequence (pMRT1891) by cloning a pMRT1818 *PstI*–*BstXI* fragment into pMRT1831 digested with *PstI*–*BstXI*. A second partial Der p 1 coding sequence containing both C132V and N150Q mutations was amplified by PCR in four steps using combinations of [01263–01292], [01291–01294], [01293–01264] and [01263–01264] primers (Table 1). The amplified product was then digested with *PstI* and *SacI* and cloned into pBSKII digested with the same enzymes to create pMRT1820. The two mutations were then included in the Der p 1 coding sequence (pMRT1857) by ligating a *PstI*–*BstXI* product derived from pMRT1820 into *PstI*–*BstXI*-digested pMRT 1845.

Full-length coding sequences of pMRT1856, pMRT1857 and pMRT1891 were excised with *HpaI* and *XbaI* and inserted downstream of the constitutive Mpr1163 promoter as described above, to create the pDMG (encoding Gly[−]/Enz⁺ Der p 1, with N34Q and N150Q mutations in N-glycosylation sites), pDMAS (encoding Gly⁺/Enz[−] Der p 1 with the C132V mutation in the enzymatic active site) and pDMGAS (encoding Gly[−]/Enz[−] Der p 1 with both N34Q, N150Q and C132V mutations) vectors, respectively. Nucleotide sequences were confirmed for all constructs by DNA sequencing.

Tobacco plant transformation

All pDWT, pDMG, pDMAS and pDMGAS vectors were transferred into *Agrobacterium tumefaciens* (strain LBA4404) by the freeze–thaw method [28]. For transient expression, tobacco plants (*Nicotiana tabacum*) were agroinfiltrated according to Kapila et al. [29]. After a 4-day incubation, plants were harvested, weighed and recombinant proteins were extracted as described below. For stable expression, transgenic plants were generated followed by co-cultivation of tobacco leaf discs with *A. tumefaciens*, harbouring the expression vector according to standard transformation methods [30]. *In vitro* rooted plantlets were transferred into a greenhouse and grown until seed setting. Control untransformed plants were obtained by incubating wild-type leaf explants with water, followed by similar *in vitro* regeneration and greenhouse growth.

Detection of recombinant allergens in transgenic tobacco plants

Six weeks after transferring plants to the greenhouse, leaves from both transgenic and untransformed tobacco

plants were collected, ground in liquid nitrogen and stored at −70 °C until use. Total soluble proteins were solubilized from the frozen powder with 4 volumes (w/v) of extraction buffer (50 mM Tris, pH 8, 150 mM NaCl, 1 mM EDTA and 5 mM L-cysteine). Insoluble material was eliminated by filtration and centrifugation. The clarified crude extract was collected and expression of recombinant Der p 1 was determined with an ELISA assay (Indoor Biotechnologies, Charlottesville, VA, USA) using 4C1 and 5H8 monoclonal antibodies and a naturally purified Der p 1 standard, as per the manufacturer's instructions.

For Western blot analysis, 5 µg soluble proteins obtained from plant extracts were separated by 12% SDS-PAGE and transferred to nitrocellulose. Proteins were detected by Coomassie staining or after incubating membranes with a Der p 1-specific rabbit polyclonal antiserum (PB1) obtained after immunization with the purified native Der p 1 protein. An alkaline phosphatase-conjugated goat anti-rabbit antibody was used as a secondary antibody. Alternatively, membranes were incubated with a Der p 1-specific murine monoclonal antibody (Kori 75) derived against the natural protein.

Recombinant protein purification

Plant material was ground in liquid nitrogen and total proteins were solubilized using the above-described extraction buffer. Insoluble polyvinylpyrrolidone was added to the extraction buffer to prevent oxidation. After filtration on Miracloth, residual insoluble materials were removed by centrifugation. Extracts were further clarified by anion exchange chromatography. The unbound fraction containing recombinant Der p 1 was then concentrated by ammonium sulphate precipitation. Following a centrifugation step, pelleted Der p 1 was resuspended into 1/10 of the initial volume of 50 mM Tris, pH 7.5, 150 mM NaCl buffer and assessed by 12% SDS-PAGE. For radioallergosorbent tests, proteins were purified by immunoaffinity using Sepharose beads coupled to a monoclonal antibody directed against natural Der p 1. After allowing Der p 1 to bind, the column was washed with PBS (pH 7.4) and subsequently eluted with 50% ethylene glycol and 5 mM lysine pH 11. The purity was assessed by SDS-PAGE/silver staining.

Structural and biochemical analyses

Circular dichroism (CD) spectra were measured in far UV (190–260 nm) using a JASCO spectropolarimeter (Cremella, Italy) (model J-810). Measurements were carried out in 1 mm path length quartz cells at a 20 °C constant temperature set by JASCO Peltier, using a 1.5–4 µM protein concentration (determined by UV absorbance at 280 nm). The four recombinant Der p 1 molecules expressed in plants were dialysed against a 50 mM sodium acetate

buffer, pH 6.0, and a blank run was performed using the same buffer. Scans were recorded using a 1 mm bandwidth and a 1-s integration time, with a scan rate of 50 nm/min. Each spectrum represents the average of 10 scans. A spectra manager software was used to calculate the molecular CD ($\Delta\epsilon$) and the mean residue ellipticity $[\theta] = (100 \times \theta_m) / (C \times l \times N)$, where θ_m is the measured ellipticity in degrees, C is the concentration in mol/litre, l is the path length of the cell in centimetre and N is the number of peptide links. $[\theta]$ is expressed in degree square centimetre per decimole residue (deg cm²/dmol res). Secondary structures were determined using the K2d software as described previously [31].

For protein N-terminal sequence determinations, recombinant Der p 1 proteins were separated by SDS-PAGE, and isolated from the gel. Amino acid sequence determination based on Edman degradation was performed using an Applied Biosystems (Les Ulis, France) gas-phase sequencer model 492 (s/n: 9510287J, USA). Phenylthiohydantoin amino acid derivatives generated at each sequencing cycle were identified and quantitated on-line with an Applied Biosystems Model 140C HPLC system using the data analysis system for protein sequencing from Applied Biosystems Model 610A. Chromatography was used to identify and quantify the derivatized amino acid removed at each sequencing cycle. The retention times and integration values of peaks were compared with the chromatographic profile obtained for a standard mixture of derivatized amino acids.

Disulphide bonds were analyzed by mass spectrometry (Maldi-TOF). Briefly, purified proteins were desalted using a ZIP TIP C4 column (Millipore, Molsheim, France) according to the manufacturer's instructions and deglycosylated using PNGase A. Purified proteins were digested with bovine trypsin without denaturation nor reduction in order to protect disulphide bonds. Digested products were further analyzed on a Voyager DE Pro (Applied Biosystems) mass spectrometer in comparison with reduced digests with alkylation of free cysteines in order to establish disulphide bond maps. Analyses were focused on the 2586 Da peptide (MQGGCGSCWAFSGVAATESALAYR) containing cysteine 132 (underlined) from the enzymatic active site. Following engagement of this cysteine in disulphide bonds, the mass of this peptide is reduced to 2584 Da.

Cysteine protease assay

Recombinant or natural Der p 1 molecules were diluted to 25 µg/mL in 50 mM Na phosphate and 1 mM EDTA, pH 7, with or without 1 mM DTT. All samples were incubated for 10 min at 37 °C under constant shaking using a New Brunswick Innova 4200 incubator (Edison NJ, USA). Samples were then mixed with the AMC substrate and fluorescence reading was recorded after 3 min in a BioRad VersaFluor fluorimeter. Results of cysteine protease activ-

ity are reported as change in Relative Fluorescence Units per milligram of allergen.

Radio-allergosorbent test

RAST was performed as described previously [32]. Briefly, purified Der p 1 molecules were coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden) at 250 µg/100 mg. The Sepharose was resuspended to 2 mg/mL in PBS/0.3% BSA/0.1% Tween-20, of which 250 µL was incubated with 50 µL human sera obtained from HDM-allergic patients. After overnight incubation, unbound material was washed away and 50 µL of ¹²⁵I-labelled sheep anti-human IgE antibodies (Sanquin, Amsterdam, the Netherlands) were added. After overnight incubation and washing, bound radioactivity was measured in a γ-counter. Results were expressed as international units/millilitre (IU/mL), which were calculated from a standard curve obtained from serial dilutions of a human/mouse chimeric IgE antibody directed to Der p 2 and Sepharose-coupled r Der p 2 [33].

Basophil activation assay

Measurement of basophil activation following Der p 1 stimulation was performed using an Allergen kit (Beckman Coulter, Marseille, France) according to the manufacturer's instructions. Briefly, heparinized whole-blood samples from HDM-allergic patients were incubated with allergens (2 µg/mL) at 37 °C for 15 min. Basophils were identified based on CRTH2 surface expression. Basophil activation was assessed following detection of the activation marker CD203c in the presence of Der p 1 variants tested at various concentrations. After red blood cell lysis and fixation, CD203c expression was measured using a specific monoclonal antibody and samples were analyzed on a Beckman Coulter FC500 cytofluorometer. The reactivity of whole blood cells with an anti IgE antibody and buffer without allergen were used as positive and negative controls, respectively.

T lymphocyte proliferation assays

Der p 1-specific polyclonal T cell lines were generated from purified blood mononuclear cells (PBMCs) isolated by gradient centrifugation from heparinized venous blood of two HDM-allergic patients. Cells (5×10^6 cells/mL) were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 2 mM mercaptoethanol, penicillin (100 U/mL), streptomycin (100 µg/mL) and 10% fetal calf serum (FCS), in the presence of naturally purified Der p 1 (10 µg/mL). Cells were fed every 3–4 days with 20 U/mL IL-2. T cells were subsequently restimulated three times (every 2 weeks) with natural Der p 1 (10 µg/mL) in the presence of autologous irradiated mononuclear cells.

Cells were starved without IL-2 for at least 5 days, before being used for proliferation assays.

In such assays, 10^7 Der p 1-specific T cells/mL were resuspended in serum-free PBS and incubated with $0.8 \mu\text{M}$ carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Eugene, OR, USA) for 10 min at 37°C . CFSE labelling was stopped after adding an equal volume of heat-inactivated FCS. After several washes, 0.5×10^6 cells/well were plated in 48-well plates in RPMI medium containing 10% FCS and incubated with $10 \mu\text{g/mL}$ of either plant-expressed Der p 1 forms, naturally purified Der p 1 or recombinant Bet v 1 (major allergen from birch pollen used as a negative control), in the presence of 0.5×10^6 irradiated autologous PBMCs as antigen-presenting cells. After 7 days, cells were harvested, washed and incubated with a PerCP-conjugated anti-CD4 antibody for 15 min at 4°C . Proliferating CD4^+ T cells were quantified by flow cytometry as cells with a decreased CFSE fluorescence intensity.

Results

Engineering of cDNAs encoding native or mutated recombinant Der p 1 forms

Different cDNAs encoding various forms of pro Der p 1 were designed for expression into tobacco plants (Fig. 1a). In all constructs, the natural signal peptide was substituted by the tobacco PRS signal peptide to efficiently direct the recombinant protein to the plant secretory pathway. Asparagine residues in positions 34 and 150 within the pro Der p 1 amino acid sequence belong to Asn-X-Thr/Ser recognition sequences used for *N*-glycan attachment. To reduce the potential molecular heterogeneity linked to *N*-glycosylation and to investigate whether glycosylation contributes to Der p 1 maturation and folding, these two *N*-glycosylation sites were abrogated (Gly^- forms) by replacing each of the asparagine residues with a glutamine (N34Q, N150Q). In addition, in order to assess the influence of the enzymatic activity on pro-peptide cleavage and folding, we designed Der p 1 variants lacking cysteine protease activity (Enz^- forms) by mutating the cysteine in position 132 into a valine (C132V). Altogether, four different constructs were made, encoding either a native Der p 1 molecule with preserved *N*-glycosylation sites and intact enzymatic activity (i.e. $\text{Gly}^+/\text{Enz}^+$), or $\text{Gly}^-/\text{Enz}^+$, $\text{Gly}^+/\text{Enz}^-$ and $\text{Gly}^-/\text{Enz}^-$ variants, respectively (Fig. 1a).

Stable expression of pro Der p 1 proteins in tobacco leaves

In preliminary experiments, all constructs were transiently introduced into tobacco leaves by agroinfiltration. We observed that only molecules expressed with the pro-peptide could be detected in tobacco leaf extracts by a

Western blot using Der p 1-specific polyclonal and monoclonal antibodies (data not shown). Individual mutation of either *N*-glycosylation or active sites did not influence expression levels significantly, because comparable amounts of recombinant Der p 1 were detected by ELISA. Only the double $\text{Gly}^-/\text{Enz}^-$ mutant was obtained in lower quantities from tobacco leaves. Thus, we focused on pro Der p 1 cDNAs to develop stable expression systems, as described in methods. Leaves were sampled from transgenic plants grown in a greenhouse, and proteins were purified and analyzed after Coomassie staining (Fig. 1b) or by a Western blot using a Der p 1-specific rabbit polyclonal antiserum (Fig. 1c) or murine monoclonal antibody (Fig. 1d). The four recombinant forms were expressed and reacted with Der p 1-specific antibodies. We estimate that 1–4 mg Der p 1 is expressed per gram of leaf for the various molecules, and that these proteins are purified with a 20–30% recovery rate. As expected, mutation of the two *N*-glycosylation sites led to proteins with a lower molecular weight when compared with natural Der p 1 (Fig. 1b–d, lanes N) or recombinant Gly^+ forms (Fig. 1b–d, lanes 1 and 3 vs. lanes 2 and 4). These results also suggested that maturation of pro Der p 1 occurs in tobacco cells and that the latter is not affected by individual mutation of either *N*-glycosylation or enzymatic sites. Possibly, a proform is still detected in the $\text{Gly}^-/\text{Enz}^-$ variant, which resolved as a double band (Fig. 1b–d, lanes 4).

Biochemical and structural characterization of plant-expressed recombinant Der p 1 molecules

Purified plant-expressed Der p 1 molecules were subsequently used to perform structural and functional studies. We assessed the glycosylation of purified Der p 1 molecules by Western blot, followed by detection with anti-xylose (Fig. 2a)- or anti-fucose (Fig. 2b)-specific antibodies. We confirmed that recombinant Der p 1 molecules expressed in tobacco plants bear characteristic plant-associated complex *N*-glycans. As expected, N34Q and N150Q point mutations substantially reduced such glycosylation (Fig. 2a and b, lanes 2 and 4) even if some residual glycosylation was detected in the $\text{Gly}^-/\text{Enz}^-$ form. MS analysis suggested that the unexpected glycosylation occurs within the asparagin-rich NH2 terminal part of mature Der p 1 (i.e. peptide TNACSINGNAP). The latter observation raises the possibility that it may contain a third 'cryptic' *N*-glycosylation site, unmutated in those variants, which could be used in plant cells. Analysis of the cysteine protease activity using a synthetic fluorogenic substrate revealed that only the $\text{Gly}^+/\text{Enz}^+$ form displays a readily detectable enzymatic activity (Fig. 2c), whereas surprisingly, no such activity is associated with the $\text{Gly}^-/\text{Enz}^+$ form. MS- analyses of disulphide bonds in the two molecules revealed that the $\text{Gly}^+/\text{Enz}^+$ form, like natural

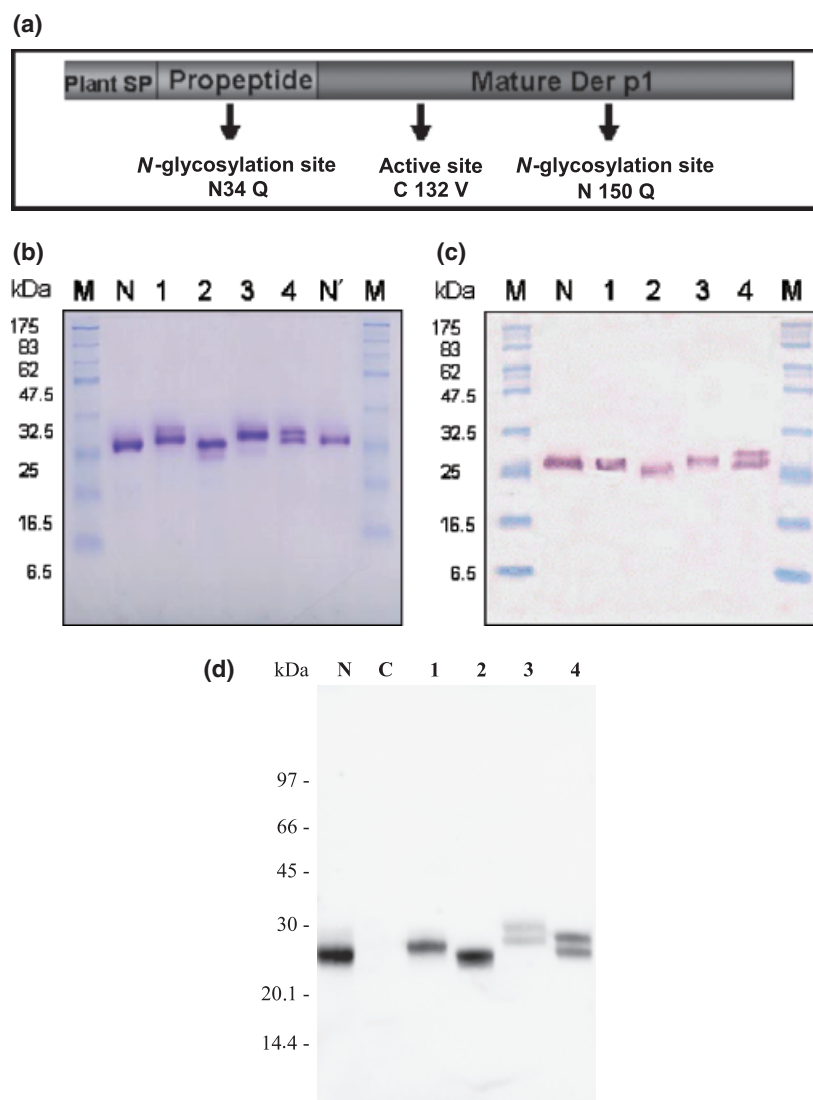


Fig. 1. Expression of recombinant pro Der p 1 forms in tobacco plants. (a) Schematic representation of wild-type and modified Der p 1 molecules. Der p 1 is naturally expressed as an immature protein with a pro-peptide subsequently cleaved off to yield the mature molecule. It contains two potential *N*-glycosylation sites as well as an active enzymatic site associated with papain-like proteolytic activity. The signal peptide from natural Der p 1 was replaced by a plant signal peptide (plant SP). Mutants were made in which either the two *N*-glycosylation sites (Gly⁻, N34Q and N150Q mutations) or the enzymatic activity (Enz⁻, C132V mutation) were abrogated. An additional mutant (Gly⁻/Enz⁻) was designed in which all these modifications were combined. Recombinant proteins purified from tobacco leaves were separated by SDS-PAGE and detected by Coomassie blue staining (b) or analyzed by Western blot and detected using PB1 rabbit polyclonal antibodies (c) or the mouse monoclonal antibody Kori 75 derived against natural Der p 1 (d). MW, molecular weight markers; N, N', natural Der p 1; C, rBet v 1; 1, rDer p 1 Gly⁺/Enz⁺; 2, rDer p 1 Gly⁻/Enz⁺; 3, rDer p 1 Gly⁺/Enz⁻; and 4, rDer p 1 Gly⁻/Enz⁻; 5–10 and 1–3 µg proteins were loaded per lane for experiments in (b) and (c/d), respectively.

Der p 1, has a free cysteine residue within the catalytic site, whereas the latter is engaged in a covalent association with cysteine 129 in the Gly⁻/Enz⁺ form, thus explaining the lack of enzymatic activity in the latter.

Purified recombinant and natural Der p 1 proteins were further analyzed by CD, to compare their secondary structures. As shown in Fig. 3, wild-type Gly⁺/Enz⁺ as well as Gly⁻/Enz⁺ and Gly⁺/Enz⁻ variants contained α -helical and β -sheet folds comparable (although not identical) to natural Der p 1. In contrast, the double variant Gly⁻/Enz⁻ exhibited a very high β -sheet/ α -helix

ratio when compared with other Der p 1 molecules (Table 2). N-terminal amino acid sequencing based on Edman degradation revealed that recombinant native Der p 1 (Gly⁺/Enz⁺) corresponds to two molecules in equal proportions, with either the same N-terminus as natural mature Der p 1 (i.e. TNACSING) or two extra amino acids. Either one or both these sequences were found for the other rDer p 1 molecules (Table 2). Collectively, these data confirmed that pro Der p 1 molecules expressed in plants yield mature proteins with a folding resembling the natural molecule.

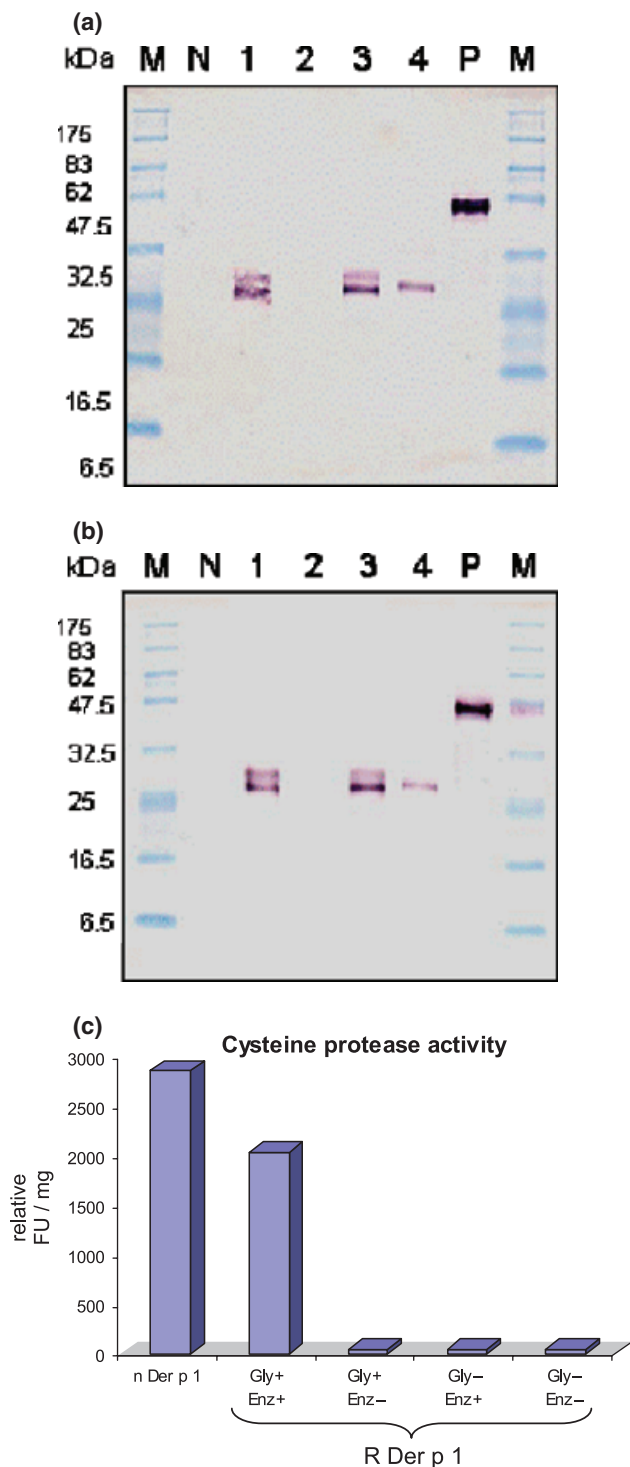


Fig. 2. Analysis of glycosylation patterns and proteolytic activity of plant-expressed recombinant Der p 1 molecules. Recombinant Der p 1 proteins purified from tobacco leaves were separated by SDS-PAGE and analyzed by Western blot using rabbit sera specific for xylose (a) or fucose (b). M, Molecular weight markers; N, native Der p 1; 1, rDer p 1 Gly⁺/Enz⁺; 2, rDer p 1 Gly⁻/Enz⁺; 3, rDer p 1 Gly⁺/Enz⁻; 4, rDer p 1 Gly⁻/Enz⁻; P, horseradish peroxidase; 1 µg proteins were loaded per lane. The cysteine protease activity was determined after 24 h as described in methods (c). Results are expressed as differences in fluorescence units (FU) per mg protein.

Immunoreactivity of plant-expressed recombinant Der p 1 molecules

Purified native and mutated forms of recombinant Der p 1 allergens expressed in tobacco plants were further analyzed for IgE reactivity in comparison with natural Der p 1. IgE binding to the various recombinant allergens was assessed by RAST using sera from 25 patients allergic to HDM. As shown in Fig. 4, naturally purified Der p 1 and plant-expressed recombinant Der p 1 proteins displayed similar IgE-binding capacity. All recombinant Der p 1 molecules were also found to react similarly in quantitative ELISA assays using 4C1 and 5H8 murine monoclonal antibodies directed against natural Der p 1 (data not shown).

The biological activity of recombinant Der p 1 allergens was further assessed using an *in vitro* basophil activation assay. Natural Der p 1 or the various plant-expressed recombinant Der p 1 molecules were incubated with basophils from HDM-allergic patients, and the induction of the CD203c activation marker was evaluated by cytofluorometry. In agreement with the previous IgE-binding results, we did not detect any significant differences between natural Der p 1 or the various plant-expressed recombinant Der p 1 molecules regarding their capacity to activate IgE-sensitized basophils (Fig. 5). This observation was confirmed over the range of allergen concentration tested (i.e. 0.2 ng/mL–2 µg/mL, data not shown).

We subsequently tested recombinant Der p 1 molecules for their capacity to stimulate Der p 1-specific T lymphocytes obtained from HDM-allergic patients. T cells were labelled with the CFSE fluorescent dye and incubated for 2 weeks with 10 µg/mL of each recombinant Der p 1 form. As shown in Fig. 6, recombinant Bet v 1 used as a negative control did not induce any T cell proliferation. In contrast, most plant-expressed Der p 1 recombinant molecules stimulated T lymphocyte proliferation in a manner comparable to naturally purified Der p 1 or recombinant Der p 1 produced in yeast. The significance of the lower stimulation observed in two independent experiments with the double Gly⁻/Enz⁻ mutant is unclear, but could be linked with a lower capture and/or processing of the molecule by antigen-presenting cells, resulting in a decreased presentation of corresponding T cell epitopes.

Discussion

There is a major interest in developing a recombinant vaccine to treat type I respiratory allergies to common *Dermatophagoides* mite species. Such a vaccine will at a minimum comprise a combination of Der p 1 and Der p 2 allergens, which collectively bind 40–100% of IgEs from patients allergic to either *D. pteronyssinus* or *D. farinae* species [1]. Our strategy is to rely on recombinant allergens in the most native configuration in order to perform

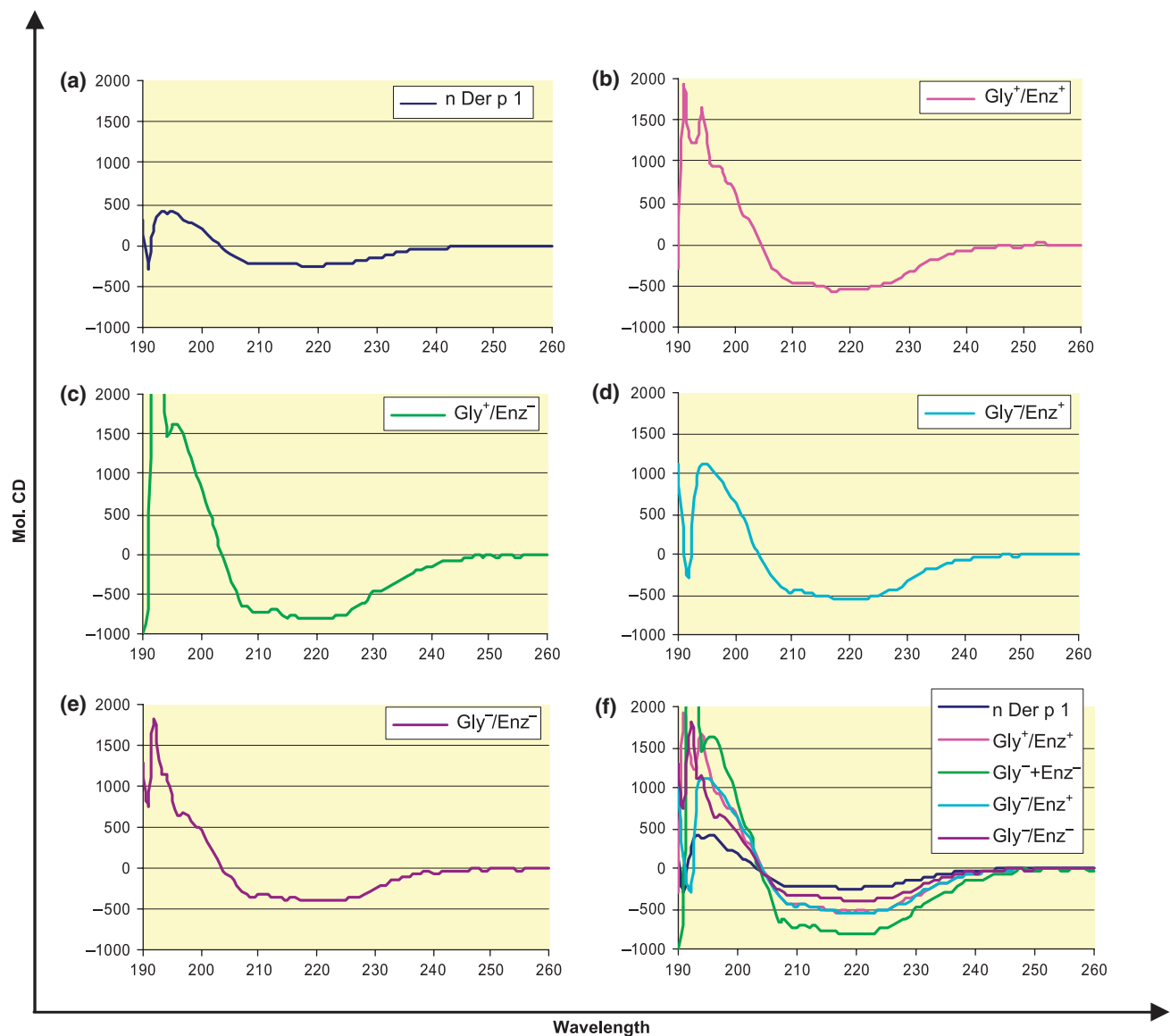


Fig. 3. Circular dichroism analysis of recombinant Der p 1 molecules expressed in tobacco plant. Recombinant Der p 1 purified from tobacco leaves were analyzed individually (a–e) using a Jasco J-810 instrument as described in methods. (f) displays an overlay of spectra obtained with the four plant-expressed Der p 1 molecules and natural Der p 1. Results are expressed in degree square centimetre per decimole residue (ordinate) vs. UV wavelength (nm, abscissa).

Table 2. N-terminal sequence and secondary structure of recombinant Der p 1 molecules expressed in tobacco plants

Molecules	N terminal sequence	% α helix	% β sheet	% random coil
Natural Der p 1	TNACSING	31	19	50
r Der p 1 Gly ⁺ /Enz ⁺	TNACSING (50%) AETNACSING (50%)	20	31	49
r Der p 1 Gly ⁺ /Enz ⁻	AETNACSING	37	13	50
r Der p 1 Gly ⁻ /Enz ⁺	TNACSING (50%) AETNACSING (50%)	21	31	48
r Der p 1 Gly ⁻ /Enz ⁻	TNACSING (50%) AETNACSING (50%)	9	42	49

specific immunotherapy through the sublingual route [23]. In this approach, a preserved IgE binding is expected to facilitate allergen capture by Fc ϵ RI⁺ and CD23⁺ oral Langerhans cells [34]. Also, engagement of Fc receptors for IgEs on oral dendritic cells should induce IL-10 and TGF- β production by those cells, thereby driving allergen-specific T cell responses towards regulatory T cell induction [23, 34].

Whereas Der p 2 has been successfully produced in *E. coli* and yeast, expression of recombinant Der p 1 for diagnostic or therapeutic purposes has been more challenging, due to the complex biology of this molecule [1, 10–16]. Der p 1 is a 27 kDa glycoprotein expressed as a proform, encompassing a pro-peptide subsequently

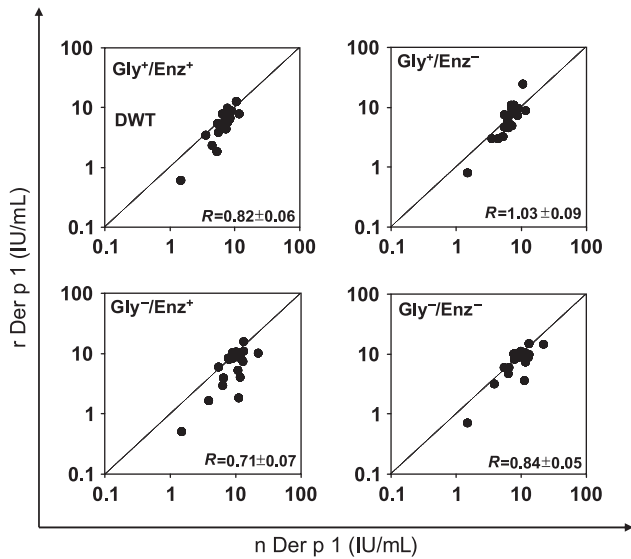


Fig. 4. IgE reactivity (RAST) of recombinant Der p 1 molecules expressed in tobacco plants. IgE reactivity was assessed as described in methods using sera from 25 house dust mite (HDM)-sensitized patients. Data obtained with single recombinant Der p 1 molecules (ordinate) were plotted against the ones obtained with naturally purified Der p 1 (abscissa). Correlation factors (R) are shown in each quadrant.

cleaved off to yield the mature protein [1, 10]. This pro-peptide is thought to facilitate folding of the molecule but also to inhibit its papain-like proteolytic activity by interacting with the substrate-binding cleft [13, 17]. It may also reduce the allergenicity of the molecule by precluding access to conformational or even linear IgE epitopes [15, 18]. Der p 1 also has two potential *N*-glycosylation sites, located within the pro-peptide and the mature protein, respectively, which may regulate the speed of maturation [15, 21]. These features may give rise to substantial molecular heterogeneity, as well as instability, making the Der p 1 mite allergen difficult to produce as a pharmaceutical-grade protein using conventional expression systems. Besides unmodified Der p 1, enzymatically inactive forms should also be considered for vaccination purposes, most particularly because the cysteine protease activity likely contributes to the allergenicity of the molecule. The latter may involve non-mutually exclusive mechanisms, such as the down-regulation of both the alpha chain of the IL-2 receptor and the CD23 low-affinity receptor for IgE, the degradation of gap junctions between epithelial cells, as well as the selective recruitment of dendritic cell [19–21].

In order to produce recombinant Der p 1 in the most native conformation at an industrial scale, we explored

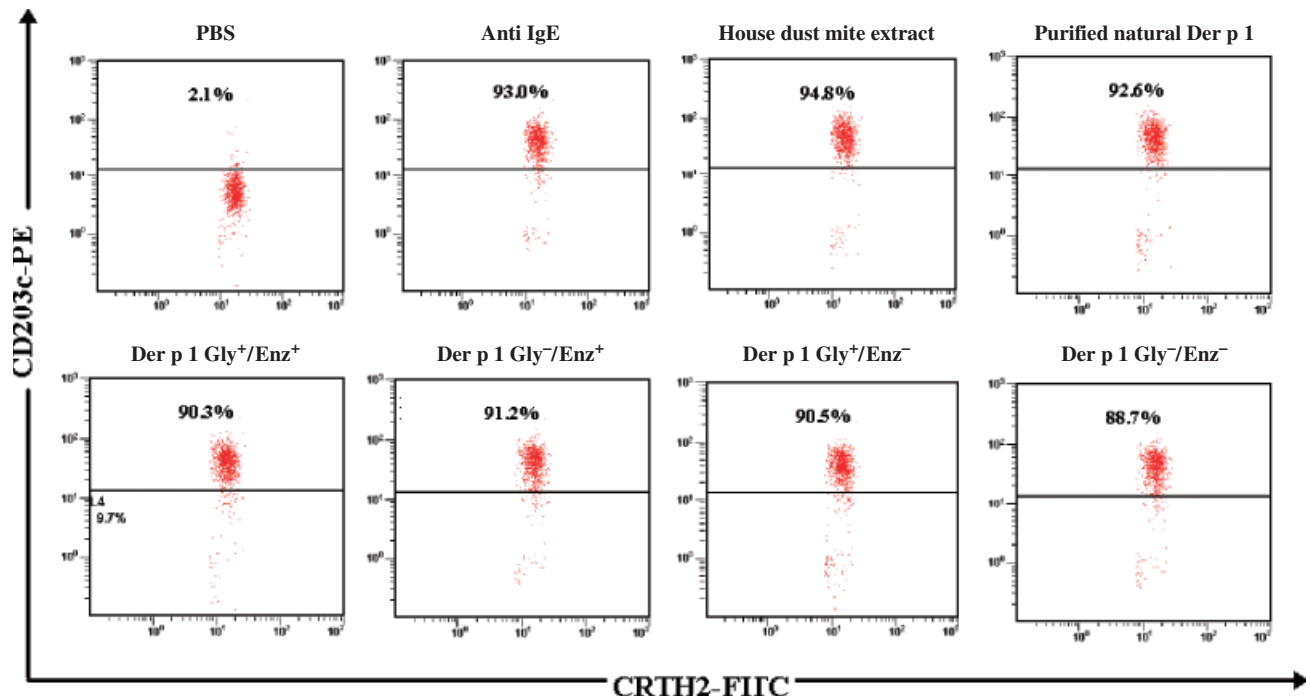


Fig. 5. Basophil activation with recombinant Der p 1 molecules expressed in tobacco plants. Whole blood was obtained from a patient allergic to *Dermatophagoides pteronyssinus*. After excluding $CD3^+$ T lymphocytes, basophils were analysed by cytofluorometry for both CD203c and CRTH2 expression under the following conditions: without stimulation (i.e. PBS-negative control), after anti-IgE cross linking (positive control), in the presence of either a *D. pteronyssinus* extract (2 μ g/mL Der p 1) or 2 μ g/mL purified natural Der p 1 or plant-expressed recombinant Der p 1 molecules. Percentages of activated basophils, defined as CRTH2+ cells expressing CD203c, were detected by flow cytofluorometry and are displayed for each allergen. Results shown are representative of three independent experiments conducted on blood samples from three different donors.

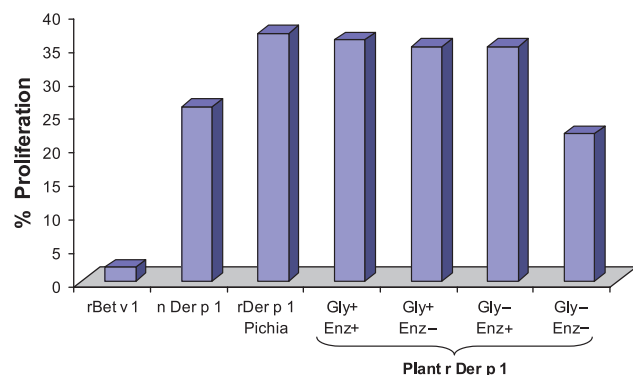


Fig. 6. T lymphocyte stimulation with recombinant Der p 1 molecules expressed in tobacco plants. A Der p 1-specific T cell line was derived from the blood of a *Dermatophagoides pteronyssinus*-allergic patient as described in methods. Cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) and stimulated for 5 days in the presence of irradiated autologous PBMCs with 10 µg/mL of either recombinant Bet v 1 produced in *Escherichia coli* (negative control), naturally purified Der p 1, recombinant Der p 1 produced in *Pichia pastoris* (positive control) or plant-expressed recombinant Der p 1 molecules. Results are representative of two independent experiments conducted with T cell lines generated from two different HDM-allergic patients.

the suitability of transgenic tobacco plants. In the present study, we succeeded in producing various forms of recombinant pro Der p 1 in a stable tobacco plant expression system. This included a non-modified form of the molecule as well as forms in which either *N*-glycosylation, cysteine protease activity or both have been abrogated by point mutations. In contrast to other expression systems, including yeast, *E. coli*, CHO or insect cells [11–18], we observed a spontaneous processing of pro Der p 1 forms in tobacco plants, yielding mature Der p 1 molecules recognized by specific polyclonal and monoclonal antibodies. Recombinant Der p 1 molecules were detected in all leaves, thus demonstrating a good stability of the various recombinant proteins during plant development. We did not detect any significant quantitative differences in terms of expression between the various Der p 1 forms, with the exception of the Gly⁻Enz⁻ double mutant, suggesting that individual modifications introduced in the molecule had no significant impact on expression and/or stability. Pro-peptide cleavage was most probably performed by a tobacco plant protease, because mutation of the enzymatic active site within Der p 1 itself did not impair maturation of the recombinant molecule. Amino-terminal sequencing of Der p 1 processed by the plant cell proteolytic machinery revealed the presence of properly mature Der p 1 molecules, but also additional forms with two residual amino acids from the pro-peptide, in agreement with a previous observation made in cultured plant cells [26].

As expected, glycosylated forms of Der p 1 expressed in tobacco cells bear complex xylose/fucose-rich *N*-glycans

characteristic of plants. Deglycosylation experiments suggest that a third *N*-glycosylation site, unmutated in those variants, possibly located in the NH₂ terminal part of mature Der p 1, can be used in plant cells. The impact of such *N*-glycosylation on Der p 1 immunogenicity in humans remains to be established, but it may facilitate allergen targeting to dendritic cells via specific mannose/fucose receptors [35]. Surprisingly, only the Gly⁺/Enz⁺ form exhibited a readily detectable cysteine protease activity, whereas the Gly⁻Enz⁺ variant showed no such activity, likely because cysteine 132 is engaged in intramolecular bonds in the latter. We subsequently confirmed by CD analysis that the non-modified Der p 1 molecule as well as the Gly⁺Enz⁻ and Gly⁻Enz⁺ mutants exhibited α helical and β sheet secondary structures comparable to natural Der p 1. In agreement with this observation, all plant-expressed Der p 1 molecules bound patients' IgEs as well as polyclonal or monoclonal antibodies similar to naturally purified Der p 1, further suggesting an overall comparable folding for all these molecules. Likewise, all recombinant Der p 1 molecules induced basophil activation and (with the exception of the Gly⁻Enz⁻ variant) stimulated the proliferation of Der p 1-specific T lymphocytes as efficiently as naturally purified Der p 1. It is worth noting that our results are in agreement with another study in which Der p 1 produced in cultured BY-2 tobacco cells was also well recognized by antibodies and immune cells [26]. In contrast, recombinant Der p 1 expressed in transgenic rice seed endosperm was poorly reactive with IgEs, suggesting that it might not be properly folded [36].

Collectively, various forms of properly folded and immunoreactive Der p 1 molecules were produced in tobacco plants. The final choice between the four candidates for inclusion in an HDM recombinant vaccine will be made on the basis of production and purification yields at an industrial scale.

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