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Original Article

Common ragweed (*Ambrosia artemisiifolia* L.): allergenicity and molecular characterization of pollen after plant exposure to elevated NO_2

Feng Zhao[1], Amr Elkelish[1,2], Jörg Durner[1,3], Christian Lindermayr[1], J. Barbro Winkler[4], Franziska Ruëff[5], Heidrun Behrendt[6,7], Claudia Traidl-Hoffmann[7,8], Andreas Holzinger[9], Werner Kofler[9], Paula Braun[10], Christine von Toerne[11], Stefanie M. Hauck[11], Dieter Ernst[1,7] & Ulrike Frank[1,7]

[1]Institute of Biochemical Plant Pathology, Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg 85764, Germany, [2]Botany Department, Faculty of Science, Suez Canal University, Ismailia 41522, Egypt, [3]Biochemical Plant Pathology, Technische Universität München, Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt, Freising 85350, Germany, [4]Research Unit Environmental Simulation, Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg 85764, Germany, [5]Clinic and Polyclinic for Dermatology and Allergology, Faculty of Medicine, LMU München, Munich 80337, Germany, [6]Center of Allergy & Environment München (ZAUM), Technische Universität and Helmholtz Zentrum München, Munich 80802, Germany, [7]CK-CARE, Christine Kühne – Center for Allergy Research and Education, Davos 7265, Switzerland, [8]Institute of Environmental Medicine, UNIKA-T, Technische Universität München, Augsburg 86156, Germany, [9]Institute for Botany, Leopold-Franzens Universität Innsbruck, Innsbruck 6020, Austria, [10]Department of Applied Sciences and Mechanotronics, University of Applied Science Munich, Munich 80335, Germany and [11]Research Unit Protein Science, Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg 85764, Germany

ABSTRACT

Ragweed pollen is the main cause of allergenic diseases in Northern America, and the weed has become a spreading neophyte in Europe. Climate change and air pollution are speculated to affect the allergenic potential of pollen. The objective of this study was to investigate the effects of NO_2, a major air pollutant, under controlled conditions, on the allergenicity of ragweed pollen.

Ragweed was exposed to different levels of NO_2 throughout the entire growing season, and its pollen further analysed. Spectroscopic analysis showed increased outer cell wall polymers and decreased amounts of pectin. Proteome studies using two-dimensional difference gel electrophoresis and liquid chromatography–tandem mass spectrometry indicated increased amounts of several Amb a 1 isoforms and of another allergen with great homology to enolase Hev b 9 from rubber tree. Analysis of protein S-nitrosylation identified nitrosylated proteins in pollen from both conditions, including Amb a 1 isoforms. However, elevated NO_2 significantly enhanced the overall nitrosylation. Finally, we demonstrated increased overall pollen allergenicity by immunoblotting using ragweed antisera, showing a significantly higher allergenicity for Amb a 1. The data highlight a direct influence of elevated NO_2 on the increased allergenicity of ragweed pollen and a direct correlation with an increased risk for human health.

Key-words: allergen; immune serum; immunoblot; NO_2; pollen; proteome; ragweed; S-nitrosylation.

Correspondence: U. Frank. e-mail: ulrike.frank@helmholtz-muenchen.de

INTRODUCTION

Ragweed (*Ambrosia artemisiifolia*) pollen is the main cause of hay fever and allergic rhinitis in North America (Wopfner *et al.* 2005; Ziska *et al.* 2011). In Europe, ragweed migrates as a neophyte and has become an increasing problem (Gerber *et al.* 2011; Leiblein-Wild *et al.* 2014; Storkey *et al.* 2014). Because of the late flowering period in Europe, ragweed is a primary cause of allergenic reaction from late summer to autumn (D'Amato *et al.* 2007). The spectrum of allergens in ragweed pollen has been characterized, and so far, 11 different allergenic proteins consisting of different isoforms have been identified (Allergome Platform; http://www.allergome.org/). The major allergen of ragweed is Amb a 1, an acidic 38 kDa protein that belongs to the pectate lyase protein family (Wopfner *et al.* 2005; Gadermaier *et al.* 2014).

Air pollution, for example, NO_x or particulate matter, can influence the morphology of pollen and make pollen allergens more aggressive (Ring *et al.* 2001). *Cupressus arizonica* pollen of air-polluted regions had a higher concentration of allergens and a higher allergenicity compared with that of unpolluted regions (Suárez-Cervera *et al.* 2008). Similarly, ragweed pollen that was collected along traffic roads showed higher allergenicity than pollen from vegetated areas (Ghiani *et al.* 2012). However, year-to-year variations and regional site variations also influence the allergen content, as demonstrated for Bet v 1 from birch pollen (Buters *et al.* 2008).

In addition to intracellular allergenic proteins, non-allergenic pollen-derived compounds can modulate the allergic immune response (Traidl-Hoffmann *et al.* 2003; Gilles *et al.* 2012), and the pollen coat representing the extracellular matrix may also be involved in this inflammatory process. The pollen

surface consists of an internal cellulose layer (intine), a multilayered outer wall of sporopollenin (exine) and the pollen coat. The coat is a complex mixture of pigments, lipids, waxes, aromatic compounds and proteins that fill the cavities of the exine (Edlund *et al.* 2004). In the highly allergenic ragweed pollen, scanning electron microscopy (SEM) indicated that there is no pollen coating and a lack of homogenous, electron-dense pollenkitt (Diethart *et al.* 2007). However, attenuated total reflectance–Fourier transform infrared (IR) spectroscopy (ATR-FTIR) showed that ragweed pollen contains lipid and wax components (Kanter *et al.* 2013). Regarding the allergenic potential of the pollen coat, pollen surface proteins have an immunoglobulin E (IgE)-binding capacity (Vigh-Conrad *et al.* 2010; Bashir *et al.* 2013b). In addition to proteins, lipids can also interact with the immune system and modify the antigenic properties of allergens (Traidl-Hoffmann *et al.* 2003; Bashir *et al.* 2013a). Thus, synergistic actions may play a potential role in the allergic response.

NO_x belongs to the classical and important air pollutants. The main sources of NO_2 are combustion processes during energy production, industrial processes and car traffic. NO_2 affects human health by irritating the bronchial tubes and thus influencing the respiratory function (Jacquemin *et al.* 2009; Takenoue *et al.* 2012). Regarding ecosystems, NO_2 concentrations of around 100 ppb damage plants, resulting in leaf necrosis, reduced growth and premature senescence (Kress & Skelly 1982; Honour *et al.* 2009). But also growth-promoting effects were described, however at lower NO_2 concentrations of 50 ppb (Takahashi *et al.* 2014). The annual limit for the human population is approximately 20 ppb, with a maxima of 100 ppb (hour limit), which should not be reached more often than 18 days per year. To protect vegetation, a critical value of approximately 15 ppb NO_2 is used as an annual average (http://www.umweltbundesamt.de/daten/luftbelastung/aktuelle-luftdaten). However, in urban traffic regions, values of up to 90 ppb can be measured, whereas in rural sites, only values up to 20 ppb are found. Ambient levels of NO_2 may reduce the pollen viability of Austrian pine (Gottardini *et al.* 2008), and the germination of *Crocus vernus* is inhibited by NO_2, albeit only at concentrations of ≥200 ppb (Chichiriccò & Picozzi 2007). There are few studies on modifications of pollen allergens by NO_2. However, these *in vitro* studies were carried out with isolated pollen. In grass pollen, the bioavailability of allergens may be modulated by NO_2 (Behrendt *et al.* 1997). In birch and ragweed pollen, no higher allergen abundance was found (Aina *et al.* 2007), whereas in *Phleum pratense*, a decrease in Phl p allergens was evident (Rogerieux *et al.* 2007). No different polypeptide profiles were revealed in *Acer negundo*, *Betula pendula*, *Ostrya carpinifolia* and *Carpinus betulus*. Nevertheless, immunodetection assays indicated higher IgE recognition (Sousa *et al.* 2012; Cuinica *et al.* 2014).

Another interesting aspect is the nitrosylation and nitration of allergens by NO_2, which can enhance the allergenic responses of pollen and food allergens (Gruijthuijsen *et al.* 2006; Untersmayr *et al.* 2010; Reinmuth-Selzle *et al.* 2014). Protein samples of bovine serum albumin and birch pollen extract that were exposed to ambient air in the region of Munich were efficiently nitrated (Franze *et al.* 2003, 2005). The *in vitro* fumigation of Bet v 1 with NO_2 showed a time-dependent nitration degree of the allergen (Reinmuth-Selzle *et al.* 2014). The nitration of Bet v 1 enhances the presentation of Bet v 1-derived peptides, important for the allergenic response (Karle *et al.* 2012).

Not much is known about the influence of elevated NO_2 on allergenic pollen; thus, in this study, we were interested in the effects of *in vivo* NO_2 fumigation on the pollen allergenicity of ragweed. For that, a large-scale two-dimensional (2D) difference gel electrophoresis (DIGE) analysis was performed, and interesting spots were analysed by liquid chromatography (LC)–tandem mass spectrometry (MS/MS). Furthermore, immunoblotting with specific 'Amb a' antisera was performed to detect allergen levels and potentially new allergens. Additionally, S-nitrosylation studies were carried out to investigate the S-nitrosylation status of pollen proteins.

METHODS

Ethics statement

The use of the sera in this study was under the consent of the probands. The plant material that was used was grown in exposure chambers. The initial ragweed seeds were collected from an outdoor stand, for which no specific permissions were required.

Plant growth conditions

Ragweed seeds were collected from a single plant at an outdoor stand (Bad Waldsee, Baden-Württemberg, Germany) (first generation). The seeds were sown in standard soil, and germination took place in phytotron walk-in chambers (http://www.helmholtz-muenchen.de/en/eus/facilities/phytotron/index.html) as described by Kanter *et al.* (2013) starting with an average climate for the Munich region from 1 May. Fifteen days after germination, the seedlings were transferred into single pots (Ø 17 cm) and cultivated further in the Plexiglas sub-chambers. The plants were allowed to acclimate for a further 2 weeks, and NO_2 treatment began on the 29 May. Two Plexiglas sub-chambers were fumigated with 40 ppb NO_2 (control) and the other two with 80 ppb (treatment). NO_2 was generated by mixing NO with O_2 in a column (0.5 L) that was filled with Raschig rings, and fumigation was performed for 10 h d^{-1}. The equilibrium of NO_2 and NO was approximately 85–90% for NO_2. The light period was 14.5 h; the day/night temperatures were 20–30 °C/10–20 °C, and the relative humidity was 30–50%/80–85% (day/night). The plants were watered automatically using a tube system applying 200 mL of water per pot each day. Pollen was continuously collected from 3 July until 29 July as described by Kanter *et al.* (2013) and stored at −80 °C until use. The plants were cultivated further until 21 August for seed collection (Supporting Information 1), and seeds were stored at 4 °C. In following year, the experiment was repeated using seeds from the 40 ppb NO_2-treated plants (second generation). However, for technical reasons, the second generation was treated with clean air (control) and

80 ppb NO_2 (treatment). The experiment began in May, and pollen was collected until August.

Scanning electron microscopy

Scanning electron microscopy was exactly as described by Kanter *et al.* (2013).

Attenuated total reflectance–Fourier transform infrared spectroscopy

The ATR-FTIR spectra of pollen were recorded according to Kanter *et al.* (2013).

Chemometric data analysis of the single-point spectral data was performed using UNSCRAMBLER 10.2.0 software (Camo ASA, Oslo, Norway). The spectra were exported to the UNSCRAMBLE software programme (version 10.2, Camo ASA) to perform the calibration and validation. A partial least square discriminant analysis (PLS-DA) (Frank & Friedman 1993; Hastie *et al.* 2001) was performed on the spectral data with *Y* as categorical variable. This technique finds the components or latent variables that discriminate as much as possible between two different groups of samples from their spectra (*X* matrix) according to their maximum covariance with a target class defined in the *Y* matrix. The matrix of response *Y* can have one *y* variable when the model classifies the samples into one or two different classes to maximize separation between the two sample groups. The pre-treatment of the data consisted of centre scaling and standard normal variate treatment. Prior to the actual data analysis, the sample set was checked for outliers to obtain robust models.

A test set cross-validation was carried out to verify and validate the results. The model was evaluated by critically assessing the statistical indicators that were used to determine the accuracy of the predictive abilities of the calibration models, including the coefficient of determination of model fitting, R^2, and the standard error of calibration (RMSEC).

Protein extraction for two-dimensional difference gel electrophoresis

Ten individual plants (first generation) from each sub-chamber were randomly selected, and the pollen of five plants from each sub-chamber was pooled to provide four biological samples. Each biological sample was analysed in triplicate, as indicated in Supporting Information 2. The pollen pools (125 mg mL^{-1}) were resuspended in acetone containing 10% trichloroacetic acid (TCA), 1% dithiothreitol (DTT) and 1% protease inhibitor (Sigma-Aldrich, Taufkirchen, Germany) and placed on a shaker (1400 r.p.m.) for 1 h at room temperature (RT). Samples were stored at −20 °C overnight and then centrifuged (25 000 *g*; 20 min; 4 °C). The pellets were washed twice with pre-cooled acetone containing 1% DTT and incubated at −20 °C for 1 h. The vacuum-dried pellets were dissolved directly in labelling buffer (7 M urea, 2 M thiourea, 30 mM Tris–HCl (pH 8.5), 4% CHAPS; pH 8.5) at 20 °C for 1 h. This solution was centrifuged at 25 000 *g* for 20 min at RT, and the supernatant was then used for protein estimation and 2D-DIGE. The protein concentrations were measured using the Bio-Rad Protein Assay with bovine serum albumin (BSA) as a standard (Bio-Rad, Munich, Germany). The 2-D Clean-Up Kit (GE Healthcare, Freiburg, Germany) was used for further protein purification. After purification, the proteins were re-suspended in labelling buffer for 1 h.

Two-dimensional difference gel electrophoresis

Protein labelling with CyDye fluorescence (GE Healthcare), including a dye swap (Supporting Information 2) and 2D-DIGE, using immobilized gradient strips (pH 4–7, 24 cm, GE Healthcare), was exactly performed as described by (Holzmeister *et al.* 2011; Frank *et al.* 2014), except that the maximum current setting was set to 75 mA per strip. Image acquisition and visualization of the DIGE gels was as described by (Frank *et al.* 2014).

Preparative gel electrophoresis, in-gel digestion and liquid chromatography–tandem mass spectrometry analyses

Preparative 2D electrophoresis gels were run with 300 μg of unlabelled proteins from control and treated samples and then silver stained. Protein spots were manually excised from the gel, washed, tryptic digested and analysed by LC-MS/MS, using the previously described method (Frank *et al.* 2014).

Data processing for qualitative analysis

Peptide identification was performed by Mascot version 2.3.02 (Matrix Science, Boston, MA, USA). The spectra were compared against the SwissProt (WB confirmation: release 2012_04; 535 698 sequences; 2D gel spots: release 2014_02; 542 503 sequences) database. Scaffold software version 3_00_03 (Proteome Software Inc., Portland, OR, USA) was used to validate the MS/MS-based peptide identifications and spectra with the following parameters: one missed cleavage allowed, a parent ion tolerance of 10 ppm, a fragment ion tolerance of 0.6 Da and a fixed carbamidomethylation modification and a deamination of glutamine or asparagine or methionine oxidation as variable modifications. Peptides were only accepted and counted for protein identification if the Mascot ion score was >30 and if two or more unique peptides were found for the corresponding protein. Proteins that contained similar peptides but could not be differentiated based on the MS analysis alone were grouped to satisfy the principles of parsimony.

Label-free quantitative analysis based on peak intensities

The RAW files (Thermo Xcalibur file format) were analysed using the Progenesis QI LC-MS software (version 2.0, Nonlinear Dynamics, Newcastle upon Tyne, UK) as described previously (Merl *et al.* 2012). Briefly, for retention time

alignment, 5–10 manual landmarks were set, followed by automatic alignment of all extracted ion chromatograms. Features with one charge or seven or more charges were excluded from further analyses. For peptide identifications, all features were exported to Mascot (Matrix Science, version 2.3) and searched with one missed cleavage allowed, a fragment ion mass tolerance of 0.6 Da and a parent ion tolerance of 10 ppm. Carbamidomethylation was set as fixed modification, and methionine oxidation and asparagine or glutamine deamidations were allowed as variable modifications. Spectra were searched against the SwissProt (release 2014_02; 542 503 sequences) and a Mascot-integrated decoy database calculating an average peptide false discovery rate of <2% and using a *P*-value cut-off score of <0.01. Peptide assignments were re-imported into Progenesis QI. Normalized abundances of all unique peptides were summed up and allocated to the respective protein.

Protein S-nitrosylation detection

Protein extraction

From the first-generation plants, the pollen of five individual control and NO_2-treated plants was sampled, resulting in five biological samples, and each biological sample was analysed in triplicate. The pollen (50 mg per plant) was mixed with 150 μL of HEN buffer [25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-NaOH (pH 7.7), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM neocuproine], transferred to a 2 mL tube containing ceramic spheres (ø 1.4 mm), silica spheres (ø 0.1 mm) and a single glass sphere (ø 4 mm) and homogenized 10 times at 6.5 ms^{-1} for 1 min on dry ice using the FastPrep 24 machine (MP Biomedicals, Eschwege, Germany). The samples were incubated in 500 μL HEN buffer at RT for 1 h with agitation (1400 r.p.m.) and then centrifuged at 25 000 *g* for 20 min at 4 °C. The samples were adjusted to 0.8 $\mu g\,\mu L^{-1}$ by adding HEN buffer.

Biotinylation of S-nitrosylated proteins

For the biotinylation of S-nitrosylated proteins, the biotin switch assay was applied (Jaffrey & Snyder 2001). To prepare a positive and negative control for the assay, S-nitrosoglutathione (GSNO) was used as the NO donor. Then, 25 μL of 10 mM GSNO (250 μM) was added to two control pollen samples (800 μg each) and incubated at RT in the darkness for 20 min (positive control). To one of these samples, 10 μL of 1 M DTT (100 mM) was added and incubated at RT in the darkness for 10 min, resulting in the denitrosylation of proteins (negative control).

Biotinylation of all 800 μg protein samples was performed as described by Lindermayr *et al.* (2005). Finally, 5% of each sample was used for Western blotting and 95% for further protein purification and LC-MS/MS analysis. Proteins were precipitated by two volumes of ice-cold acetone, followed by centrifugation.

Detection of S-nitrosylated proteins by Western blotting

Forty micrograms of protein samples was resolved on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) in a Mini-PROTEAN II Electrophoresis System (Bio-Rad) using SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) (Laemmli 1970). For immunoblotting, the separated proteins were transferred to a nitrocellulose membrane (0.2 μm) (Bio-Rad) using the Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad) with transfer buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, 20% methanol). The membrane was overlaid with blocking buffer TBST-BSA [Tris-buffered saline (TBS): 10 mM Tris–HCl (pH 7.5), 0.9% NaCl, 1 mM $MgCl_2{\cdot}6H_20$; T: 0.5% Tween 20 containing 2% BSA) for 1 h at RT and then was transferred to antibody buffer (TBST-BSA + 0.01% anti-biotin mouse monoclonal antibody, Sigma-Aldrich) for 3 h at RT or overnight at 4 °C. The membrane was washed twice with TBST buffer for 10 min at 4 °C and finally with TBS buffer for 10 min at 4 °C. Then, 10 μL of 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (BCIP) solution (50 mg mL^{-1} BCIP in ddH_2O) and 10 μL of nitrotetrazolium blue chloride (NBT) solution (100 mg NBT in 700 μL dimethylformamide and 300 μL ddH_2O) were mixed with 3 mL of AP buffer (100 mM Tris–HCl (pH 9.5), 100 mM NaCl and 5 mM $MgCl_2{\cdot}6H_20$), and the membrane was incubated for 3 min at RT. After washing with sterile water, the membrane was scanned (Epson Perfection 3170 Photo; Epson, Munich, Germany), and the relative mean grey intensity of the protein bands was calculated by Image J 1.47 (open source).

Purification and liquid chromatography–tandem mass spectrometry analysis of S-nitrosylated proteins

The purification of S-nitrosylated proteins using NeutrAvidin agarose (Thermo Scientific, Braunschweig, Germany) was carried out according to Lindermayr *et al.* (2005). Purified protein samples were resolved on a 12% SDS-PAGE as described earlier. The gels were cut into four equal slices, and in-gel digestion and LC-MS/MS were performed as described earlier.

Pollen allergen immunoreactivity analysis

Preparation of the sera mixture

The sera of 10 patients suffering from ragweed pollen with Immuno CAP® classes >3 (Supporting Information 3) were pooled to carry out all of the immunochemical analyses. The pooled sera from nine healthy volunteers provided the control. The serum pool was aliquoted and stored at −20 °C until use.

One-dimensional and two-dimensional immunoblot analyses

The allergenic potential of ragweed pollen was analysed by immunoblotting of one-dimensional (1D) and 2D SDS-PAGE using atopic patients' immune serum.

Pollen of three plants from each group (first-generation treatment, first-generation control, second-generation treatment and second-generation control) was randomly sampled, and three independent experiments were performed. For protein extraction, pollen samples (125 mg mL^{-1} in ddH_2O + protease inhibitor) were mixed and incubated at RT for 1 h with agitation (1400 r.p.m.). The samples were then centrifuged (14 000 *g*; 10 min), and the supernatants were ready to use. The soluble protein concentrations were measured using the Bio-Rad protein assay with BSA as a standard.

For 1D immunoblot, 5 μg of protein samples was resolved by 15% SDS-PAGE using a Mini-PROTEAN II system (Bio-Rad) as described earlier. To further investigate the different allergenicity signals, also 2D gel electrophoresis immunoblotting was carried out. For this, equal amounts of proteins were loaded onto immobilized non-linear pH gradient strips (pH 3–11, 24 cm; GE Healthcare), and 2D-PAGE was performed as described earlier.

Once separation was completed, proteins were transferred to a nitrocellulose membrane using transfer buffer in a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad) in case of 1D or in a MiliBlot-SDE System (Millipore) in case of 2D-PAGE. Then membranes were transferred into blocking buffer TBST-BSA for 1 h at RT. The sera mixture from ragweed allergic patients and healthy probands was diluted 1:100 in TBST buffer, and membranes were incubated in this solution overnight at 4 °C. After washing three times with TBST buffer for 10 min, the membranes were incubated in antibody buffer [TBST-BSA + 0.01% anti-human IgE (ε-chain-specific)-peroxidase antibody produced in goat (Sigma-Aldrich)] for 1 h at RT. The membranes were washed three times in TBST buffer for 10 min and then developed with Western Lightning ECL Plus (PerkinElmer, Rodgau, Germany) for 1 min. Imaging was carried out with a FUSION-FX7 Spectra System (Vilber, Eberhardzell, Germany) by exposing the membranes for 2 min, and quantification was performed using the programme Image J 1.47 (open source). The bands/spots of interest were cut out of the corresponding gel and further analysed by LC-MS/MS as described earlier.

RESULTS

Attenuated total reflectance–Fourier transform infrared spectroscopy can detect differences in pollen cell wall

Roughly, the IR spectrum of pollen can be divided into specific regions containing signatures of lipids, proteins, carbohydrates and grain wall biopolymers called sporopollenins (Kanter *et al.* 2013; Zimmermann & Kohler 2014) (Table 1). In addition, the vibrational spectra of pollen are rich in information on biochemical constituents such as secondary plant metabolites.

The ATR-FTIR spectra of *Ambrosia* pollen from the elevated NO_2 treatment as well as of control pollen have been explored by chemometric data analysis. In order to obtain separation of treated versus untreated samples based on the IR spectra and to understand which spectral features are responsible for separation, PLS-DA was performed. The signals within 3400–800 cm^{-1} were used as *X* variables, while the *Y* variables were associated with the two differently treated pollen classes (one different *y* variable for each pollen treatment class, with 1 if fumigated with 80 ppb NO_2 or 0 if not). The model obtained in this way was able to discriminate between the two pollen classes, as obvious from the PLS-DA score plot in Fig. 1a. A separation into two clusters, 80 ppb NO_2 fumigated and control, is apparent. By the developed PLS-DA model, 75% of the sample data are explained specifically [with a root mean square error (RMSE) of 0.2 for calibration and 0.27 for validation and an R^2 of 0.82 and 0.7, respectively].

Table 1. Vibrational band associated with lipids, proteins, carbohydrates and sporopollenin, detected in ragweed pollen

Assignments	Wavenumber (cm^{-1})
Lipids, triglycerides and phospholipids	
(CH_2 asymmetrical stretch)	2924
CH_2 symmetrical stretch	2851
C=O stretch, ester bond	1740
CH_2 deformation	1465
CH_2 rocking	722
C–O stretch	1200–1100
Phospholipids	
P=O stretch	1200–1100
Proteins	
Amide I: mostly C=O stretch	1650
Amide II: NH deformation, C–N stretch	1550
Carbohydrates	
C–O–C stretch, C–OH stretch, COH deformation	1200-900
Sporopollenin	
Aromatic rings	1605, 1515, 1171, 833

The predominant spectral differences between the two pollen groups are the result of variations of bands associated with lipids, sporopollenin, protein and carbohydrates (Fig. 1b, c). The first principal component (PC) plot (as a function of wavenumber), which explains differences of 16% of the data from elevated NO_2-treated pollen, has high positive factor loadings associated predominantly with lipid, sporopollenin and protein bands (in PCs 1, Fig. 1b) and negative factor loading associated with carbohydrate bands. This indicates that the outer cell wall polymers, the lipids of cell membrane and/or wax components and proteins are positively correlated to elevated NO_2 but negatively correlated with the polysaccharide absorption. The second and third factors, which explain 40% of the data, have major high positive factor loadings predominantly associated with protein (band 2; Fig. 1c), and sporopollenin (or more general aromatic groups) vibrational frequencies (bands 3, 4 and 9; Fig. 1c) indicate the increase of protein and/or aromatic groups at the pollen wall. In addition, among the major contributing vibrational frequencies arising from the negative factor loadings are the (1) 1671 cm^{-1} band (band 1); (2) 1324 cm^{-1} (band 5); and (3) the vibrational absorption range 1125–1105 cm^{-1} (band 6; Fig. 1c). The band at 1671 cm^{-1} coincides with the protein range of the spectrum, and additionally with the aromatic carbons, C=C, or even the nitric oxide, N=O, stretch vibration

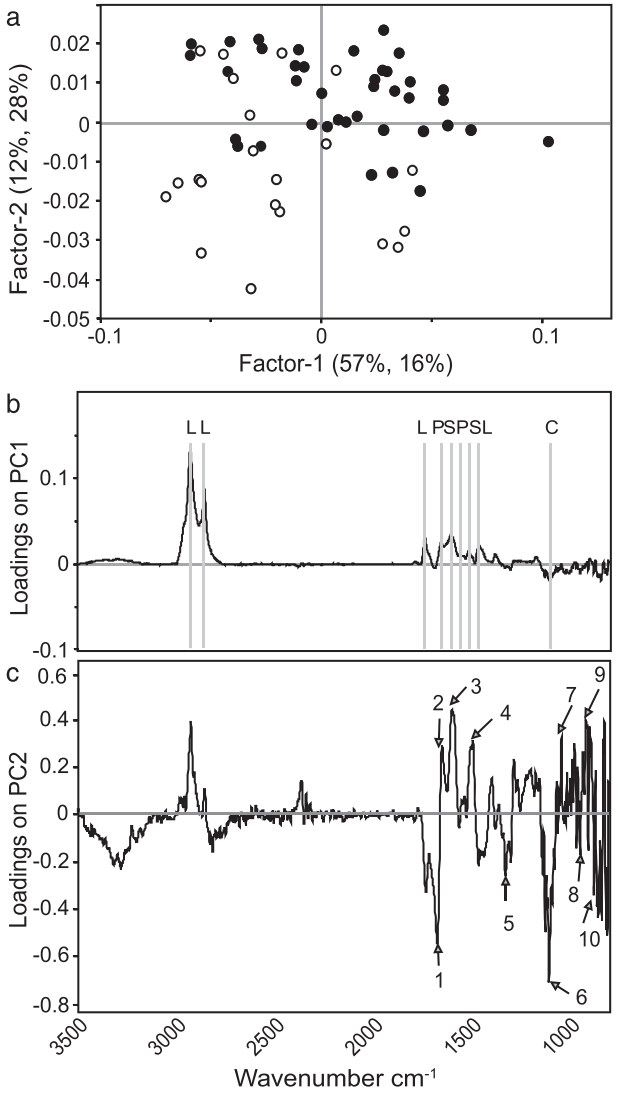

Figure 1. Correlation between spectroscopic data and pollen treatment. (a) PLS-DA plot of the ATR-IR spectral data set (60 samples, two spectra per sample; SNV-corrected spectra) with the depiction of the NO_2-fumigated (39 samples, black circles) and control (21 samples, open circles) samples. The percent *Y* variances for the first five PCs are 16, 28, 12, 8 and 10, and those for *X* variances are 57, 12, 14, 2 and 6; (b) loading plot on the first factor of the PLS-DA (L, lipid; P, protein; S, sporopollenin; C, carbohydrate); (c) loading plot on the second factor of the PLS-DA. The arrows indicate the vibrational frequencies which have major contributions.

(Chen *et al.* 1992). As yet given, that there is a strong positive signal at ~1650 cm^{-1}, and the 1671 cm^{-1} vibration contributes minor to the amid I, is not likely to originate from protein. The vibrational absorptions at 1125 and 1105 cm^{-1} are located in the carbohydrate range, with the prominent 1105 cm^{-1} band corresponding to the backbone vibrations of polygalacturonic acid of the cell wall complex carbohydrate (Kanter *et al.* 2013). Perhaps, the aromatic C=C is contributed by phenylpropanoid compounds such as ferulic or coumaric acid, which have been shown to occur in *Ambrosia* species. These acids have been shown to be esterified to the polysaccharides of the cell wall carbohydrates in case of Chenopodiaceae (Ridley *et al.* 2001). As yet, the exact assignment of these bands, considering the complexity of the pollen spectra, based on single vibrational energies is tentative and has to be treated with care. In any case, the PLS data treatment corroborates the notion that treatment of *Ambrosia* pollen with NO_2 results in alterations of pollen wall constituents, in particular elevation of protein and sporopollenin as opposed to pectin polymers as reflected in the IR spectral variations. In contrast, no visible differences in pollen shape could be detected by SEM analysis (Supporting Information 4).

Comparative proteome analysis

Two-dimensional difference gel electrophoresis identified increased allergen amounts

Two-dimensional difference gel electrophoresis was carried out to record differentially changed soluble proteins from ragweed pollen that were sampled from plants grown under 40 ppb NO_2 (control) or 80 ppb NO_2 (treatment). Approximately 3568 spots were highly resolved over a pH range of 4–7 and a molecular weight (MW) of 10–100 kDa (Supporting Information 5). All of the spots were matched by gel-to-gel comparisons, and the difference of the relative abundance (vol%) of each spot was analysed. Only those spots with a variation of at least ±1.5-fold were considered for further analyses. The relative abundance of 35 spots significantly increased, whereas that of 30 spots significantly decreased under elevated NO_2. The heat map representation of protein abundances and hierarchical clustering that were performed on the abundance profiles indicated common spots that showed statistically significant differences between the two conditions (Fig. 2). Among all of the 65 spots, 30 up-regulated and 27 down-regulated spots could be identified upon LC-MS/MS, followed by a homology-driven identification search (Table 2), and only proteins with the best Mascot protein score were given for each spot. The complete list of identified proteins, including the Mascot protein scores, is given in Supporting Information 6. The identified up-regulated spots were categorized into 14 functional groups according to the predicted protein function, whereat the most affected up-regulated group upon elevated NO_2 was allergenicity and stress/defence related (Table 2, Supporting Information 6 and 7). All of the pollen allergens belong to isoforms of Amb a 1 (pectate lyase family) (Table 2). Interestingly, a homologue to the allergen Hev b 9, an enolase from the rubber tree, was detected only under elevated NO_2 (Table 2). Similarly, the down-regulated spots under elevated NO_2 could be classified into 11 groups, with metabolic process and stress/defence related as the most affected groups (Supporting Information 6 and 7). The gene ontology term 'biological process' showed three proteins that are involved in pollen development: 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (iPGAM), 26S protease regulatory subunit 6A homolog A and V-type proton ATPase catalytic subunit A.

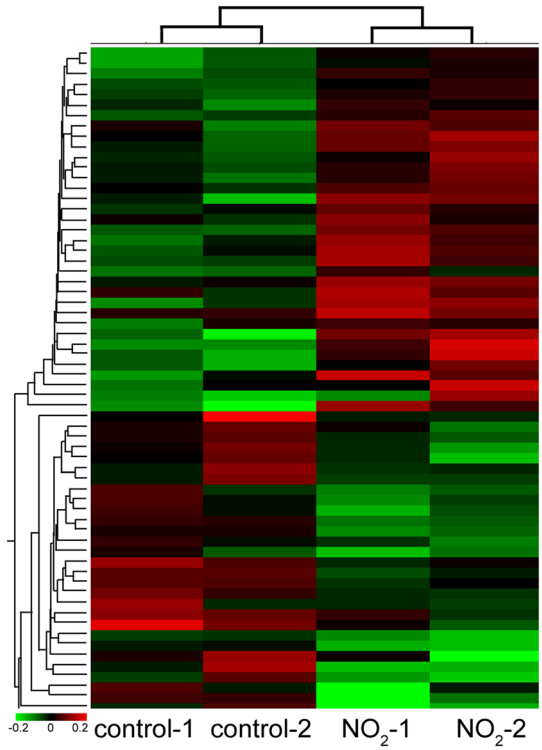

Figure 2. Heat map representation of protein abundances and hierarchical clustering of the abundance profiles. The data are from two sub-chambers for 40 ppb NO_2 (control) and two sub-chambers for 80 ppb NO_2 (treatment). Red indicates up-regulation, and green indicates down-regulation.

NO_2 fumigation results in enhanced S-nitrosylation

S-nitrosylation of proteins can have an influence on their structure and function. Therefore, S-nitrosylation of allergens can have an effect on the allergy response. Protein S-nitrosylation experiments were carried out to examine if ragweed allergens are S-nitrosylated under our experimental conditions. 1D Western blot analysis showed a different S-nitrosylation pattern in 80 ppb NO_2-treated samples as compared with the control, indicating that an increase of S-nitrosylation has taken place as a result of the elevated NO_2 (Supporting Information 8), and the relative mean grey value of all band intensities was significantly higher under elevated NO_2 (Fig. 3). S-nitrosylated proteins were purified by SDS-PAGE, identified by LC-MS/MS and then further quantitatively analysed. Of 73 identified proteins, 63 were more abundant in pollen under elevated NO_2, whereas 9 were more pronounced in the control pollen (Table 3, Supporting Information 8 and 9). The potentially S-nitrosylated proteins could be classified in 13 functional categories from which 8 are shown in Table 3, and *Ambrosia* allergens was the most affected. Hints to known S-nitrosylated proteins are indicated by the corresponding reference. The Amb a 1 isoforms were up to 1.65-fold increased under NO_2 as compared with the control. Amb a 3 increased even stronger and was 5.57-fold more detected in pollen fumigated with elevated NO_2 (Table 3). Interestingly, the six ragweed allergens as well as several other proteins have not been reported as S-nitrosylated. It is noteworthy that nine potentially S-nitrosylated proteins showed a significantly different abundance, as demonstrated by the 2D-DIGE results, from which eight proteins were up-regulated (Amb a 1.1 to Amb a 1.5, triosephosphate isomerase, fructose-bisphosphate aldolase and monodehydroascorbate reductase) and one was down-regulated (glyceraldehyde-3-phosphate dehydrogenase) (Tables 2 and 3). Additionally, potentially S-nitrosylated proteins detected by biotin switch were also analysed by the SNO prediction software GPS-SNO 1.0 (Xue *et al.* 2010, Kovacs & Lindermayr 2013). For most of the detected candidate proteins, also potential S-nitrosylation sites have been predicted by the software, indicating that the detected proteins might be really S-nitrosylated (Supporting Information 9). To identify the correct S-nitrosylation sites, a modified biotin switch assay should be performed (Fares *et al.* 2011).

NO_2-treated pollen shows higher immunoglobulin E recognition in immunoblots

Immuno-dot blot pre-tests of pollen extracts, using a sera mixture from ragweed-sensitized patients, showed significantly higher IgE-binding signals owing to elevated NO_2 in pollen of both generations (data not shown). Therefore, these different allergenic signals were investigated in more detail by 1D and 2D immunoblotting, using the same sera mixture. Figure 4 shows a representative SDS-PAGE and the 1D Western immunoblot. Image analysis again confirmed a statistically higher allergenicity in pollen from the elevated NO_2 fumigation, with highest amounts for the 38-kDa band (Supporting Information 10). LC-MS/MS analysis indicated that Amb a 1 isoforms were present in each band, and the allergenic potential of the 38-kDa band contributed to more than 70% of the total allergenicity of the pollen extract (Table 4, Supporting Information 10). Low amounts of Amb a 3 were also detected in the 12 kDa band. In addition, a homolog to Hev b 9 was also found in the 38 kDa band (Table 4). No protein was found for the 10 kDa band, which passed the filter criteria.

To better characterize the allergenic proteins and their isoforms, 2D Western immunoblots were performed under the aforementioned blotting conditions. Finally, nine spots could be distinguished after immunoreaction (Supporting Information 11). Statistical image analysis revealed a significantly increased allergenicity of pollen from the elevated NO_2 treatment (Fig. 5). Spot number 5 with a MW of 38 kDa contributed almost 50% of the total allergenicity and was strongly increased under elevated NO_2. Further, significantly increased allergenicity in both generations could be observed for spot numbers 8 (30 kDa) and 9 (10 kDa). The allergenic potential of spot number 6 (38 kDa) was only increased in the second generation when exposed to 80 ppb NO_2 (Fig. 5). Further identification by LC-MS/MS confirmed the 1D immunoblotting. All five of the Amb a 1 isoforms were found (Table 5). Spot number 5, showing the highest allergenicity, corresponded to an Amb a 1 with the Amb a 1.1 isoform

Table 2. List of LC-MS/MS identified proteins that were differentially abundant by at least ±1.5-fold upon elevated NO_2

Spot	Identified protein	Acc. no.	MW (kDa)	Spot ratio	*P*-value
I: Pollen allergen					
1	Pollen allergen Amb a 1.1 (*Ambrosia artemisiifolia*)	P27759	43	1.89	1.23E−09
2	Pollen allergen Amb a 1.2 (*Ambrosia artemisiifolia*)	P27760	44	1.71	5.88E−10
3	Pollen allergen Amb a 1.3 (*Ambrosia artemisiifolia*)	P27761	43	1.58	2.05E−06
4	Pollen allergen Amb a 1.4 (*Ambrosia artemisiifolia*)	P28744	43	1.56	9.79E−08
5	Pollen allergen Amb a 1.5 (*Ambrosia artemisiifolia*)	P27762	44	1.56	2.23E−03
II: Other allergen					
6	Enolase 1 (Hev b 9) (*Hevea brasiliensis*)	Q9LEJ0	48	++	5.96E−03
III: Cytoskeleton					
7	Actin-97 (*Solanum tuberosum*)	P30171	42	1.54	1.13E−02
8	Tubulin-alpha-1 chain (*Pisum sativum*)	P46259	50	−2.09	3.00E−06
IV: Glycolytic process					
10	Triosephosphate isomerase (*Lactuca sativa*)	P48493	21	2.05	4.54E−06
11	Glyceraldehyde-3-phosphate dehydrogenase (*Petroselinum crispum*)	P26519	36	−1.87	2.59E−04
12	Fructose-bisphosphate aldolase (*Pisum sativum*)	P46257	38	1.56	8.46E−04
13	2,3-Bisphosphoglycerate-independent phospho-glycerate mutase 2 (iPGAM2)(pollen development) (*Arabidopsis thaliana*)	Q9M9K1	61	−3.08	6.93E−09
14	Glucose-6-phosphate isomerase (*Arabidopsis thaliana*)	P34795	62	−2.58	2.32E−03
V: Tricarboxylic acid cycle					
16	Isocitrate dehydrogenase (*Glycine max*)	Q06197	47	3.02	5.18E−03
17	Succinate dehydrogenase flavoprotein subunit 1 (*Arabidopsis thaliana*)	O82663	70	1.96	1.89E−06
VI: Calvin cycle					
18	Ribulose bisphosphate carboxylase large subunit (*Populus alba*)	Q14FE9	53	−2.36	6.30E−03
19	Transketolase (*Solanum tuberosum*)	Q43848	80	−2.55	1.48E−02
20	Phosphoglycerate kinase (*Nicotiana tabacum*)	Q42961	50	1.88	1.39E−03
VII: Metabolic process					
9	Glucose and ribitol dehydrogenase homolog (*Oryza sativa* ssp. *japonica*)	Q75KH3	32	−1.89	5.02E−10
15	Dihydrolipoyllysine-residue succinyltransferase (*Arabidopsis thaliana*)	Q8H107	50	−2.69	2.22E−03
21	Glutamate decarboxylase (*Petunia hybrida*)	Q07346	57	2.03	2.37E−04
22	GDP-mannose 3,5-epimerase (*Arabidopsis thaliana*)	Q93VR3	43	−2.33	4.96E−04
23	Cytochrome c1-1 (oxidation reduction process) (*Solanum tuberosum*)	P25076	35	−1.63	1.04E−06
24	Protein disulfide isomerase-like 2–3 (protein-folding, oxidation reduction process) (*Oryza sativa* ssp. *japonica*)	Q67UF5	47	2.36	2.77E−04
25	Beta-fructofuranosidase (cell wall) (*Arabidopsis thaliana*)	Q8W4S6	62	1.99	1.88E−06
26	Alpha-1,4-glucan-protein synthase (cell wall) (*Zea mays*)	P80607	41	2.21	1.63E−03
27	NADH dehydrogenase iron-sulfur protein 8 (oxidation reduction process) (*Solanum tuberosum*)	P80269	26	−1.79	1.79E−03
29	Phosphoglucomutase (*Zea mays*)	P93805	63	--	1.48E−05
VIII: Signalling					
28	14-3-3 protein 9 (*Solanum lycopersicum*)	P93214	29	−1.63	2.05E−03
IX: Photosynthesis					
30	Photosystem I P700 chlorophyll a apoprotein A2 (*Nymphaea alba*)	Q6EW49	82	−2.63	2.33E−03
31	Photosystem II CP47 chlorophyll apoprotein (*Atropa belladonna*)	Q7FNS4	56	−1.95	4.32E−06
32	Photosystem II D2 protein (*Acorus americanus*)	Q4FFP4	40	−2.44	8.43E−04
33	Oxygen-evolving enhancer protein 1 (*Populus euphratica*)	P84989	11	2.77	3.83E−06
X: Ammonia assimilation					
34	Glutamine synthase cytosolic isozyme 1-1 (*Arabidopsis thaliana*)	Q56WN1	39	++	1.89E−06
XI: Protein biosynthesis, folding and degradation process					
36	26S protease regulatory subunit 6A homolog A (pollen development) (*Arabidopsis thaliana*)	Q9SEI2	47	2.99	7.23E−06
37	Chaperonin CPN60-2 (*Cucurbita maxima*)	Q05046	61	1.89	1.07E−04
XII: Translation					
38	Eukaryotic initiation factor 4A-10 (*Nicotiana tabacum*)	P41382	47	−2.04	5.96E−03
39	Elongation factor 1-gamma 2 (*Oryza sativa* ssp. *japonica*)	Q6YW46	47	2.66	8.88E−05
XIII: Stress and defence related					
35	Heat shock 70 kDa protein 9 (*Arabidopsis thaliana*)	Q8GUM2	73	−2.36	9.78E−04
40	Soluble inorganic pyrophosphatase (metabolic process) (*Solanum tuberosum*)	Q43187	24	2.36	1.77E−02
41	Monodehydroascorbate reductase (oxidation reduction process) (*Cucumis sativus*)	Q42711	47	2.63	1.57E−11
42	Proteasome subunit alpha type 3 (protein degradation) (*Oryza sativa* ssp. *japonica*)	Q9LSU0	27	2.58	1.11E−05
43	Aconitate hydratase 1 (salt stress, metabolic process) (*Arabidopsis thaliana*)	Q42560	98	−1.69	1.26E−03

(*Continues*)

Table 2. (Continued)

Spot	Identified protein	Acc. no.	MW (kDa)	Spot ratio	*P*-value
44	Catalase isozyme 1 (stress) (*Cucurbita pepo*)	P48350	57	3.03	2.32E−06
45	Probable protein phosphatase 2C 59 (defence) (*Arabidopsis thaliana*)	Q8RXV3	33	1.99	1.48E−05
46	Mitogen-activated protein kinase 9 (stress, defence) (*Arabidopsis thaliana*)	Q9LV37	58	2.45	3.29E−03
47	Probable lactoylglutathione lyase (cold stress, chloroplast) (*Arabidopsis thaliana*)	Q8W593	39	−2.00	5.02E−06
48	NADP-dependent malic enzyme (stress-related) (*Mesembryanthemum crystallinum*)	P37223	64	−1.77	2.59E-04
XIV: Methyltransferase-related					
49	Serine hydroxymethyltransferase 1 (*Flaveria pringlei*)	P49357	57	−2.37	2.25E−03
50	5-Methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (*Mesembryanthemum crystallinum*)	P93263	85	−1.63	1.39E−03
51	Adenosylhomocysteinase 1 (*Arabidopsis thaliana*)	O23255	53	2.11	5.48E−03
XV: Transport related					
52	ATP synthase subunit alpha (*Eucalyptus globulus*)	Q49L13	56	2.09	6.50E−03
53	V-type proton ATPase catalytic subunit A (pollen development) (*Hordeum vulgare*)	Q40002	64	−2.22	1.46E−05
54	Sorting nexin 1 (*Arabidopsis thaliana*)	Q9FG38	47	2.56	1.76E−07
55	Importin subunit alpha-1A (*Oryza sativa* ssp. *japonica*)	Q71VM4	58	−1.93	2.36E−10
XVI: Others					
56	Cysteine-rich repeat secretory protein 38 (*Arabidopsis thaliana*)	Q9LRJ9	28	−1.88	4.77E−05
57	Histone H4 variant TH011 (*Triticum aestivum*)	P62785	11	−1.76	4.09E−04

The ratio of average spot volume (treatment versus control) after application of the processing module (different in-gel analysis and biological variance analysis) is given, together with the accession number (Acc. no; SwissProt), the molecular weight (MW) and the significance level calculated by one-way ANOVA. Only proteins with the best protein score, calculated as the sum of the peptide ion scores for each protein, are given. Spot numbers correspond to Supporting Information 5, and all matched proteins are given in Supporting Information 6. ++, only detected in pollen under elevated NO_2; --, only detected in control pollen.

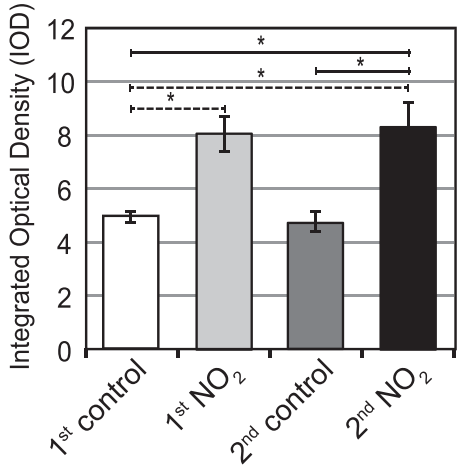

Figure 3. S-nitrosylation of proteins. Relative mean grey value intensities of all of the Western blot bands as calculated by Image J (error bars ± SD, * = *P*-value < 0.05; one-way ANOVA). First control = 40 ppb NO_2; second control = clean air; first and second NO_2 = 80 ppb NO_2.

as a major component (highest protein score), indicating that this 'Amb a' isoform might be the allergen with the highest influence in ragweed pollen. In addition, a homolog to Hev b 9, reacting also with the sera mixture, was identified (Table 5). This result supports the idea of a novel ragweed allergen with homologies to Hev b 9, as also detected by 2D-DIGE analysis.

DISCUSSION

Scanning electron microscopy and attenuated total reflectance–Fourier transform infrared spectroscopy

Scanning electron microscopy pictures clearly indicate no influence of elevated NO_2 on the size or surface of ragweed pollen (Supporting Information 4). This result has also been reported for pollen of O_3-treated and of elevated CO_2 and/or drought stress-treated plants (Kanter *et al.* 2013; El Kelish *et al.* 2014), clearly indicating that air pollution or climate change scenarios will not alter the pollen size or shape.

Attenuated total reflectance–Fourier transform infrared spectroscopy analysis of elevated NO_2-treated and control pollen provided additional insight into the outer cell wall polymers, proteins and carbohydrates. We detected a clear increase in FTIR bands corresponding to lipids and/or wax components, to sporopollenins and to proteins, in contrast to carbohydrate absorption bands, for example, where pectin was reduced (Fig. 1). This result is in contrast to O_3-treated ragweed pollen, which showed increased pectin and decreased lipid wax and sporopollenin (Kanter *et al.* 2013). Although the pollen of ragweed has no pollenkitt, which consists mainly of lipids (Diethart *et al.* 2007), our data clearly confirm previous results (Kanter *et al.* 2013) showing the presence of lipids in the pollen coat of ragweed. This increase in lipids upon elevated NO_2 may modulate the antigenic properties of proteins, thus contributing to allergic responses (Traidl-Hoffmann *et al.* 2003; Bashir *et al.* 2013a). Moreover, the increase in coat proteins in ragweed pollen (Fig. 1) may also contribute to a

Table 3. Identification and quantification of candidates for protein S-nitrosylation from ragweed pollen

Identification		Quantification				
Description	Acc. No	Peptide count	Unique peptides	CS	Fold change	Hints
I: Pollen allergens						
Pollen allergen Amb a 1.1 (*Ambrosia artemisiifolia*)	P27759	14	11	554	1.46	
Pollen allergen Amb a 1.2 (*Ambrosia artemisiifolia*)	P27760	15	11	770	1.24	
Pollen allergen Amb a 1.3 (*Ambrosia artemisiifolia*)	P27761	12	8	681	1.21	
Pollen allergen Amb a 1.4 (*Ambrosia artemisiifolia*)	P28744	16	13	651	1.36	
Pollen allergen Amb a 1.5 (*Ambrosia artemisiifolia*)	P27762	8	8	323	1.65	
Pollen allergen Amb a 3 (*Ambrosia artemisiifolia*)	P00304	3	3	149	5.57	
II: Other allergens						
Profilin (*Helianthus annuus*)	O81982	6	3	283	1.17	1
Profilin-3 (*Ambrosia artemisiifolia*)	Q64LH0	6	3	244	1.20	1
Enolase (*Alnus glutinosa*)	Q43321	6	1	202	1.31	2
Enolase (*Solanum lycopersicum*)	P26300	7	2	196	4.06	2
Enolase 1 (*Zea mays*)	P26301	6	4	171	2.07	2
III: Cytoskeleton						
Actin-1 (*Sorghum bicolor*)	P53504	8	1	391	1.88	2
IV: Glycolytic process						
Triosephosphate isomerase, cytosolic (*Lactuca sativa*)	P48493	4	3	347	1.75	1-6
Glyceraldehyde-3-phosphate dehydro-genase, cytosolic (*Petroselinum crispum*)	P26519	5	1	204	1.50	1, 6-8
VII: Metabolic enzymes						
Glutelin type-B 2 (*Oryza sativa* ssp. japonica)	Q02897	7	4	267	2.32	
Glutelin type-A 1 (*Oryza sativa* ssp. japonica)	P07728	5	2	174	20.40	
VIII: Signalling						
Luminal-binding protein 2 (*Zea mays*)	P24067	9	7	254	2.14	6
14-3-3-like protein A (*Vicia faba*)	P42653	7	3	193	2.11	1
XI: Protein biosynthesis, folding and degradation process						
60S ribosomal protein L12 (*Prunus armeniaca*)	O50003	4	4	131	4.49	
XIII: Stress and defence related						
Heat shock 70 kDa protein 3 (*Arabidopsis thaliana*)	O65719	4	1	158	1.52	
Monodehydroascorbate reductase, seedling isozyme (*Cucumis sativus*)	Q42711	2	1	128	1.16	4, 6, 7
Probable monodehydroascorbate reductase, cytoplasmic isoform 3 (*Arabidopsis thaliana*)	Q9LFA3	2	1	125	1.23	
XV: Transport related						
Peptidyl-prolyl cis-trans isomerase (*Catharanthus roseus*)	Q39613	5	4	246	12.33	1, 6, 8
ATPase 8, plasma membrane-type (*A. thaliana*)	Q9M2A0	6	6	143	3.01	

After the biotin switch biotinylated proteins were affinity purified and analysed by LC-MS/MS. RAW files were analysed using the Progenesis QI LC-MS software (version 2.0, Nonlinear Dynamics) and Mascot search engine (Matrix Science, version 2.3) was used to identify proteins. Protein names and nearest homologue origins, accession number (SwissProt), peptide counts, unique peptides, confidence score (CS) and fold-change of proteins with a CS >125 are given. Redundant proteins were removed as well. Hints for S-nitrosylation to confirm that identified proteins are candidates are indicated in the right column. Categories are according to Table 2.[1](Kato *et al.* 2013); [2](Lindermayr *et al.* 2005); [3](Abat & Deswal 2009); [4](Romero-Puertas *et al.* 2008); [5] (Fratelli *et al.* 2002); [6](Vanzo *et al.* 2014); [7](Lin *et al.* 2012); [8](Maldonado-Alconada *et al.* 2011)

changed allergenicity, as pollen coat proteins possess IgE-binding capacities (Vigh-Conrad *et al.* 2010; Bashir *et al.* 2013b).

Increased protein levels of Amb a 1 isoforms

Pollen that was collected along traffic roads or polluted areas showed higher allergenicity than pollen that was collected from urban regions (Chehregani *et al.* 2004; Cortegano *et al.* 2004; Suárez-Cervera *et al.* 2008; Ghiani *et al.* 2012; Beck *et al.* 2013). However, it is not easy to determine the contribution of different air pollutants, for example, NO_2, O_3 and CO_2, particulate matter or different climatic factors to the changed allergenicity. In birch pollen, O_3 was positively correlated with the Bet v 1 content, whereas the urbanization index or NO_2 concentration showed no significant correlation, and increasing temperature was negatively correlated (Beck *et al.* 2013). In this study, we provide strong evidence that elevated NO_2 (80 ppb) results in a changed proteomic profile, including allergens (Table 2). Because other physical parameters, such as light, temperature, humidity or soil, were identical, we argue that the observed results are specifically caused by NO_2. Proteomic profiling was performed, and an increased protein level was evident for five Amb a 1 isoforms (Table 2). Previous studies on the effect of NO_2 exposure on pollen from different plant species showed no change in the protein profile; however, in these *in vitro* studies, the pollen was fumigated with elevated NO_2 only over a short period of time

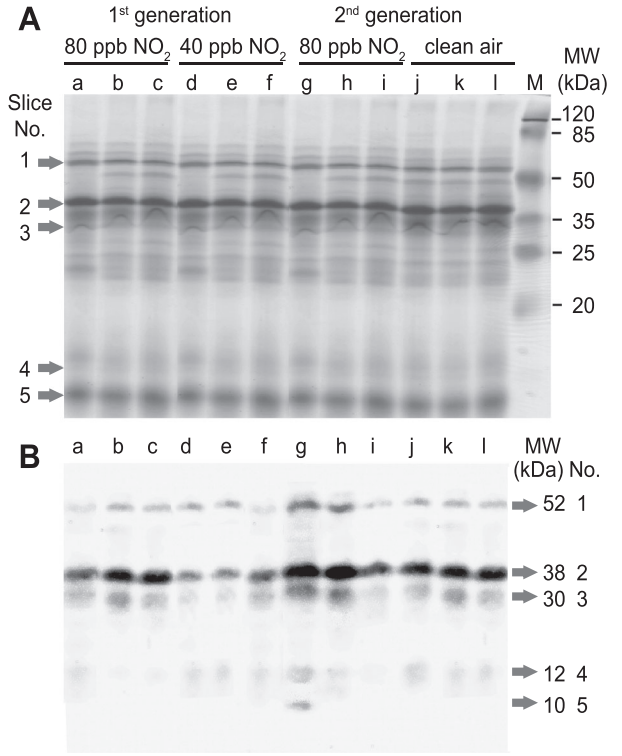

Figure 4. Allergen detection in ragweed pollen. (a) Coomassie brilliant blue-stained SDS-PAGE. (b) 1D Western immunoblot that was probed with sera from ragweed allergic patients. Slice numbers and MW (kDa) are indicated.

(Sousa *et al.* 2012; Cuinica *et al.* 2014), and often, high NO_2 concentrations (ppm) were applied (Rogerieux *et al.* 2007). Similarly, the protein profile of *Zinnia* pollen was not altered when the pollen grains were exposed to polluted air over 20 days (Chehregani *et al.* 2004). In contrast, the *in vitro* exposure of *Platanus* pollen to gaseous pollutants and vehicle exhaust resulted in a higher abundance of the allergen Pla a 1 (Lu *et al.* 2014). Ghiani *et al.* (2012) reported higher protein amounts of Amb a 1 and Amb a 2, as judged from 1D gels, in pollen that was sampled along high-traffic roads. In contrast, Beck *et al.* (2013) found no correlation between the NO_2 concentration and the protein amount of Bet v 1 in the catkins of birch. The increased protein amount of several Amb a 1 isoforms in our study can be attributed to the more sensitive 2D-DIGE analysis that was used. Running only 1D gels, we also saw no difference in the polypeptide pattern after Coomassie Brilliant Blue staining (Fig. 4).

A possible new allergen from ragweed pollen?

The presence of a possible new allergen in ragweed pollen could be assumed from 2D-DIGE analysis and protein identification by LC-MS/MS (Table 2). The best Mascot match for spot 6 resulted in a homolog to an enolase, Hev b 9, an allergen from rubber tree (Supporting Information 6). The MW and isoelectric point (pI) according to the 2D-DIGE are consistent with the MW of 51 kDa and a predicted pI of 5.54. Enolases are important allergens from moulds and some plants and exhibit cross-reactivity to other fungal and plant enolases (Simon-Nobbe *et al.* 2000; Wagner *et al.* 2000; Lai *et al.* 2002). Hev b 9 is found in the milky sap that is produced by the rubber tree. Sensitization occurs via skin contact; however, sensitization may also occur via the inhalation of airborne allergens that are released from powdered latex gloves (Wagner *et al.* 2000).

Table 4. LC-MS/MS identified allergens from a 1D immunoblot

			Mascot			
Slice #	Protein	Accession	MW (kDa)	# unique peptides	# unique spectra	Protein score
L1 52 kDa	**Amb a 1.1 (*Ambrosia artemisiifolia*)**	**P27759**	**43**	**2**	**2**	**92.1**
L2 38 kDa	**Amb a 1.1 (*Ambrosia artemisiifolia*)**	**P27759**	**43**	**20**	**48**	**1044.1**
	Amb a 1.5 / Amb a 2 (*Ambrosia artemisiifolia*)	P27762	44	16	30	709.2
	Amb a 1.3 (*Ambrosia artemisiifolia*)	P27761	43	11	16	537.8
	Amb a 1.2 (*Ambrosia artemisiifolia*)	P27760	44	9	16	439.2
	Amb a 1.4 (*Ambrosia artemisiifolia*)	P28744	43	3	5	115
	Hev b 9 (enolase) (*Hevea brasiliensis*)	Q9LEJ0	48	2	2	71.2
L3 30 kDa	**Amb a 1.3 (*Ambrosia artemisiifolia*)**	**P27761**	**43**	**8**	**15**	**407**
	Amb a 1.2 (*Ambrosia artemisiifolia*)	P27760	44	5	12	304.1
	Amb a 1.1 (*Ambrosia artemisiifolia*)	P27759	43	4	5	170.6
	Amb a 1.4 (*Ambrosia artemisiifolia*)	P28744	43	3	3	141
	Amb a 1.5 (*Ambrosia artemisiifolia*)	P27762	44	2	2	73.7
L4 12 kDa	**Amb a 1.1 (*Ambrosia artemisiifolia*)**	**P27759**	**43**	**8**	**10**	**469.2**
	Amb a 1.2 (*Ambrosia artemisiifolia*)	P27760	44	4	6	186
	Amb a 1.3 (*Ambrosia artemisiifolia*)	P27761	43	2	3	104.6
	Amb a 1.4 (*Ambrosia artemisiifolia*)	P28744	43	2	4	97.8
	Amb a 3 (*Ambrosia artemisiifolia* var. elatior)	P00304	11	2	2	84.8
	Amb a 1.5 (*Ambrosia artemisiifolia*)	P27762	44	2	2	68.3

Only peptides with an ion score from at least 30 were taken into account. Accession number (SwissProt), molecular weight (MW) and numbers for unique peptides and unique spectra, as well as the protein scores, are given. Protein score reflects the sum of best ion scores for each peptide corresponding to the protein. Best matches according to the protein score are given in bold.

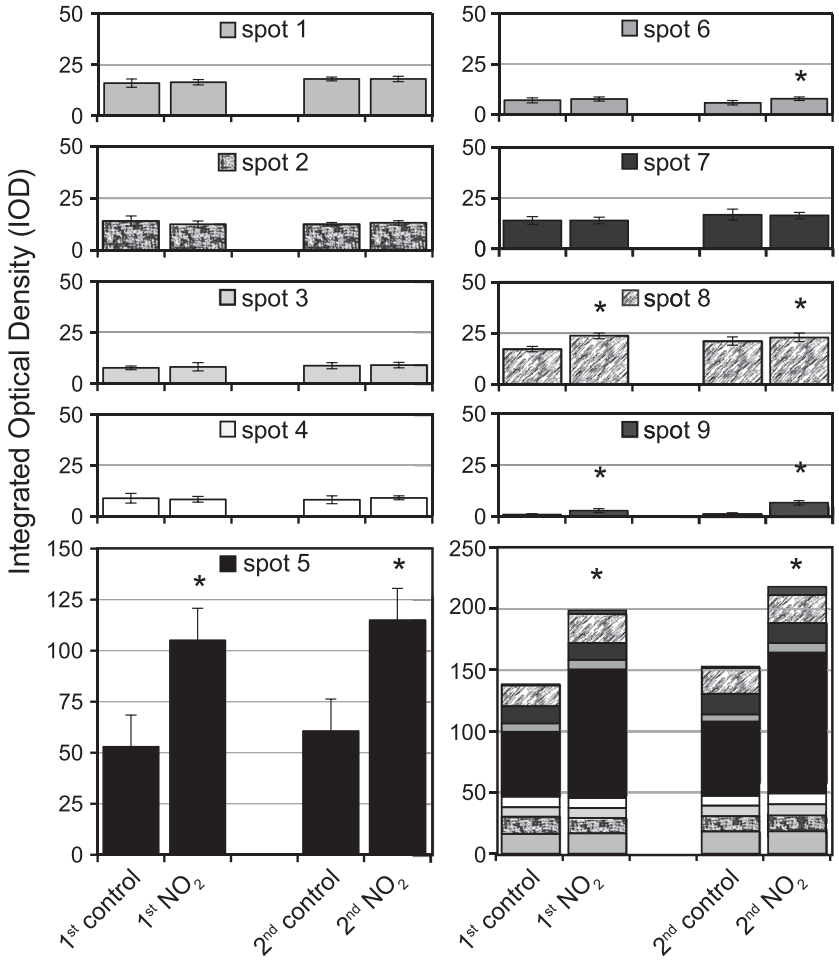

Figure 5. 2D Western immunoblot. Statistical analysis of the major allergens in different pollen extracts and the contribution of individual single spots to the total allergenicity (* = *P*-value < 0.05; one-way ANOVA). Proteins were identified by LC-MS/MS.

Cytoskeleton dynamics

For pollen germination, cytoplasmic and cytoskeletal reorganization is necessary (Mascarenhas 1993). Actin and tubulin are important proteins that are involved in this process (Palevitz *et al.* 1994; Vidali & Hepler 2001), and the reorganization and dynamics of these are thought to be controlled by a complex interacting network (Dai *et al.* 2006). The variation in the amounts of these proteins upon elevated NO_2 (Table 2) indicates that NO_2 affects cytoskeleton dynamics and male gametophyte development, which also agrees with the reduced pollen viability of several plant species upon *in vitro* exposure of the pollen to NO_2 (Cuinica *et al.* 2014).

Photosynthesis-related proteins

Interestingly, three homologs to photosynthesis-related proteins were down-regulated, and a homolog to an oxygen-evolving enhancer protein was up-regulated under elevated NO_2 (Table 2). These proteins are located in plastids, and in the majority of angiosperms, these organelles are maternally inherited (Corriveau & Coleman 1988; Birky 1995), as the organellar DNA is degraded during pollen development (Tang *et al.* 2012). However, there are also reports about the inheritance of plastids via pollen (Zhang *et al.* 2003; Thyssen *et al.* 2012), supporting the idea of the presence of plastids in ragweed pollen. This result is further supported by the presence of the homologs to a chloroplastic lactoylglutathione lyase and an ATP synthase subunit alpha and to three proteins belonging to the Calvin cycle (Table 2), a pathway that is located in plastids. Interestingly, amyloplasts are often present in pollen grains; however, in ragweed, no starch accumulation was found (Baker & Baker 1979; Diethart *et al.* 2007), indicating the absence of amyloplasts. However, as proplastids were found in various plant species (Sangwan & Sangwan-Norrell 1987), they may also be present in ragweed pollen.

Ammonia assimilation

Glutamine synthetase (GS) is a key enzyme for the assimilation of NH_4^+ into amino acids (Temple *et al.* 1998), and elevated NO_2 induced a strong increase of this protein (Table 2). The inactivation of GS resulted in a block of pollen development and male sterility (Ribarits *et al.* 2007). Moreover, pollen viability, as tested by *in vitro* pollen germination, decreased (Mamun 2007). As pollen viability and germination were also reduced

Table 5. LC-MS/MS identified allergens from selected spots from a 2D immunoblot

Spot #	Protein name	Acc. No.	Mascot			
			MW (kDa)	# unique peptides	# unique spectra	protein score
spot 1	**Hev b 9 (enolase) (*Hevea brasiliensis*)**	**Q9LEJ0**	**47.8**	**18**	**42**	**1539.7**
	Amb a 1.1 (*Ambrosia artemisiifolia*)	P27759	42.7	20	34	1381.5
	Amb a 1.4 (*Ambrosia artemisiifolia*)	P28744	42.8	14	19	837.3
	Amb a 1.3 (*Ambrosia artemisiifolia*)	P27761	42.9	12	14	793
spot 2	**Amb a 1.1 (*Ambrosia artemisiifolia*)**	**P27759**	**47.9**	**30**	**73**	**2107.7**
	Amb a 1.3 (*Ambrosia artemisiifolia*)	P27761	48.1	17	35	1033.4
	Amb a 1.4 (*Ambrosia artemisiifolia*)	P28744	47.6	9	14	575.4
	Hev b 9 (enolase) (*Hevea brasiliensis*)	Q9LEJ0	48.0	6	8	399.7
	Amb a 2 (*Ambrosia artemisiifolia*)	P27762	42.7	4	4	208.6
	Amb a 1.2 (*Ambrosia artemisiifolia*)	P27760	42.9	4	5	149.9
spot 3	**Amb a 1.1 (*Ambrosia artemisiifolia*)**	**P27759**	**42.8**	**13**	**15**	**598**
	Amb a 1.4 (*Ambrosia artemisiifolia*)	P28744	47.8	10	18	489.9
	Amb a 1.3 (*Ambrosia artemisiifolia*)	P27761	44.1	8	13	424.7
	Hev b 9 (enolase) (*Hevea brasiliensis*)	Q9LEJ0	43.7	4	5	200.7
spot 4	**Amb a 1.3 (*Ambrosia artemisiifolia*)**	**P27761**	**42.7**	**5**	**7**	**227.3**
	Hev b 9 (enolase) (*Hevea brasiliensis*)	Q9LEJ0	42.8	4	5	195.7
	Amb a 1.2 (*Ambrosia artemisiifolia*)	P27760	42.9	2	2	135.5
	Amb a 1.1 (*Ambrosia artemisiifolia*)	P27759	47.8	3	3	108.9
spot 5	**Amb a 1.1 (*Ambrosia artemisiifolia*)**	**P27759**	**42.9**	**33**	**78**	**1920.6**
	Amb a 1.3 (*Ambrosia artemisiifolia*)	P27761	47.8	18	30	910.5
	Amb a 1.4 (*Ambrosia artemisiifolia*)	P28744	43.7	8	12	436
	Hev b 9 (enolase) (*Hevea brasiliensis*)	Q9LEJ0	42.7	8	13	427.6
	Amb a 2 (*Ambrosia artemisiifolia*)	P27762	42.7	7	9	394.2
	Amb a 1.2 (*Ambrosia artemisiifolia*)	P27760	42.8	2	2	83.8
spot 6	**Amb a 1.2 (*Ambrosia artemisiifolia*)**	**P27760**	**47.8**	**15**	**31**	**841.8**
	Amb a 1.3 (*Ambrosia artemisiifolia*)	P27761	44.1	6	9	404
	Hev b 9 (enolase) (*Hevea brasiliensis*)	Q9LEJ0	47.9	3	5	183.9
	Amb a 1.4 (*Ambrosia artemisiifolia*)	P28744	43.7	2	2	76.6
spot 7	**Amb a 1.2 (*Ambrosia artemisiifolia*)**	**P27760**	**43.7**	**4**	**4**	**204.5**
spot 8	**Hev b 9 (enolase) (*Hevea brasiliensis*)**	**Q9LEJ0**	**42.9**	**3**	**4**	**125.1**
spot 9	**Hev b 9 (enolase) (*Hevea brasiliensis*)**	**Q9LEJ0**	**47.8**	**3**	**4**	**165.4**

Only peptides with a Mascot ion score of ≥ 30 were taken into account. Accession numbers (Acc. No; SwissProt) are indicated. Molecular weight (MW) of the proteins, number of unique peptides and of unique spectra is given. Protein score is the calculated sum of Mascot best ion scores for each peptide corresponding to the protein. Best matches according to the protein scores are indicated in bold.

by elevated NO_2 (Cuinica *et al.* 2014), it might be that the high abundance of GS might partly compensate for these negative effects.

Metabolic processes and energy generation

In plants, pollen germination occurs very rapidly, indicating a very active metabolism and energy production (Mascarenhas 1993). In this study, homologs to several differentially abundant proteins that belong to metabolism and energy generation were identified. Several studies have demonstrated that proteins that are involved in energy and metabolism are highly abundant with up to approximately 40% (Dai *et al.* 2007; Grobei *et al.* 2009; Pertl *et al.* 2009). Similarly, up to 38% of the identified proteins belong to energy and metabolism in this study (Table 2). Of these proteins, approximately 50% were down-regulated under increased NO_2 levels, indicating a negative effect of NO_2 that might not be compensated by the up-regulated ones. Two homologous proteins of the carbohydrate metabolism, iPGAM2 and phosphoglucomutase, were down-regulated. Both of these proteins are important for pollen development (Egli *et al.* 2010; Zhao & Assmann 2011), indicating a negative effect of NO_2 on the development of viable pollen. In addition, the abundance of the homologous protein for a glutamate decarboxylase was increased upon elevated NO_2 (Table 2). This enzyme plays a critical role in the regulation of pollen tube growth and is present in pollen grain extracts (Yu *et al.* 2014), indicating again, similarly to GS, a partial recovery of the damaging effects of NO_2. However, the homolog to the glyceraldehyde-3-phosphate dehydrogenase was down-regulated, and a deficiency of this protein resulted in the male sterility of *Arabidopsis* pollen (Muñoz-Bertomeu *et al.* 2010). Taken together, these results indicate that synergistic/antagonistic partners control the harmful effects of NO_2.

Cellular transport, signalling and protein degradation

14-3-3 proteins are regulators of plant development, including also signal transduction pathways, and are important during pollen grain germination and tube elongation (Pertl *et al.* 2011).

However, these proteins are also involved in stress responses (Roberts *et al.* 2002), and they play a key role in pollen tube elongation. Interestingly, a 14-3-3 protein was shown to interact with NADPH oxidase, thus influencing reactive oxygen species (ROS) production (Elmayan & Simon-Plas 2007). As the NADPH oxidase/ROS system influences the allergenicity of pollen (Wang *et al.* 2009; Pasqualini *et al.* 2011; Pazmandi *et al.* 2012), the changed amount of a 14-3-3 protein 9 homolog in ragweed pollen upon NO_2 treatment may also contribute to an altered allergenicity of the pollen upon NO_2 treatment. This idea is supported by the up-regulation of a 14-3-3 protein in birch pollen that was sampled from urban sites (Bryce *et al.* 2010).

The proteins that involved protein degradation and transport were also influenced by NO_2 (Table 2). In higher plants, a selective protein degradation during gametophyte development is necessary and is carried out by proteasome subunits (Gallois *et al.* 2009). Increased amounts of the 26S protease regulatory subunit homolog suggest that NO_2-damaged proteins can be effectively degraded. Among transporters, a plastid ATP synthase homolog was up-regulated, and an importin homologue was down-regulated. A mitochondrial ATP synthase is important for pollen formation and an *Importin β*1 for pollen tube elongation (Li *et al.* 2010; Han *et al.* 2011). These results again suggest the changed interaction of the transport and signalling pattern under elevated NO_2 concentrations.

Stress response

Finally, 10 homologs to stress-related proteins changed abundance upon the elevated NO_2 treatment (Table 2). Pollen undergoes extracellular stress after release from the anthers and intracellular stress during germination (Dai *et al.* 2006). The changed NO_2-affected stress proteome suggests that mature pollen has the ability to adapt to air pollution, similarly to that found under elevated CO_2 and/or drought (Frank *et al.* 2014).

S-nitrosylation of proteins

In addition, post-translational protein modifications influence IgE reactivity to allergens (Petersen *et al.* 1998). ROS/reactive nitrogen species (RNS) will influence the structure and function of proteins (Astier *et al.* 2011; Bachi *et al.* 2013). In this study, we also analysed the S-nitrosylation of proteins to test the hypothesis as to whether the *in vivo* NO_2 treatment of ragweed plants will result in a changed nitrosylation pattern of the pollen proteome. Treatment with 40 ppb NO_2 and with 80 ppb NO_2 resulted in the S-nitrosylation of many pollen proteins (Supporting Information 8). However, the overall nitrosylation was clearly enhanced under 80 ppb NO_2 (Fig. 3). This result indicates the S-nitrosylation of new proteins or a different degree of protein S-nitrosylation.

Six allergenic proteins were nitrosylated under both NO_2 concentrations; however, we cannot distinguish between possible different degrees of nitrosylation, as the total amount of these allergens also increased (Table 2). Additionally, for the five Amb a 1 allergens, potential S-nitrosylation sites have been predicted by the GPS-SNO 1.0 software, indicating the proteins as good candidates (Supporting Information 9). As Amb a 3S-nitrosylation increased by 5.57-fold under elevated NO_2, this result may indicate increased allergenic potential, similarly as has been reported for the nitration of the egg allergen ovalbumin and Bet v 1 (Gruijthuijsen *et al.* 2006; Untersmayr *et al.* 2010; Ackaert *et al.* 2014). However, as Amb a 3 is a minor allergen of ragweed, this result should not strongly contribute to an enhanced allergenicity of the pollen. A homolog to a potentially S-nitrosylated UTP-glucose-1-phosphate uridyltransferase was strongly increased under elevated NO_2 (17.84-fold). This enzyme plays an important role in late pollen development (Huang *et al.* 2011). This aberrant S-nitrosylation thus might influence pollen germination and may explain in part the reduced pollen viability upon NO_2 fumigation. In addition, the possible S-nitrosylation of actin might influence the pollen development, which was enhanced because of elevated NO_2 fumigation. It is known that S-nitrosylation interferes with actin polymerization and changes the actin cytoskeleton structure (Yemets *et al.* 2011; Rodriguez-Serrano *et al.* 2014). Similarly, the aberrant S-nitrosylation of proteins that are involved in signalling, protein synthesis or folding processes, which increased under elevated NO_2 (Table 3), may have negative effects in redox regulation of cellular processes (Hess *et al.* 2005; Bachi *et al.* 2013).

Allergenicity of ragweed pollen is enhanced because of elevated NO_2

The allergenicity of ragweed pollen was clearly enhanced upon elevated NO_2 fumigation (Figs 4 and 5). The allergenicity from the pollen of diverse plant species, including ragweed, also increased in pollen that was collected near high-traffic roads compared with that of more vegetated areas (Chehregani *et al.* 2004; Cortegano *et al.* 2004; Suárez-Cervera *et al.* 2008; Ghiani *et al.* 2012). However, under outdoor conditions, hundreds of parameters are often changed, and it is difficult to determine the contribution of an individual parameter. To overcome these problems, *in vitro* fumigation with distinct air pollutants has been carried out. Regarding NO_2, there are contradictory reports in the literature with an increase, decrease or no effects on the allergenicity of pollen (Aina *et al.* 2007; Rogerieux *et al.* 2007; Sousa *et al.* 2012; Cuinica *et al.* 2014). These experiments have been carried out for from a few hours up to 2 d, and artificially high NO_2 concentrations (ppm range) were sometimes applied (Rogerieux *et al.* 2007). 1D Western immunoblot and further 2D Western immunoblot analyses clearly showed that Amb a 1 isoforms mainly contributed to the increased allergenicity of ragweed pollen upon enhanced NO_2 concentrations (Tables 4 and 5; Fig. 5). This result is consistent with the literature, describing Amb a 1 as the major allergen of ragweed (Wopfner *et al.* 2005; Gadermaier *et al.* 2014). Similarly, increased IgE reactivity to not-further-characterized allergens was found in the *in vitro* NO_2-treated pollen of *Acer negundo*, *Betula pendula*, *Ostrya carpinifolia* and *Carpinus betulus* (Sousa *et al.* 2012; Cuinica *et al.* 2014). In addition to NO_2, O_3 is a main air pollutant. However, the

O_3 fumigation of ragweed plants did not increase the Amb a 1 content (Kanter *et al.* 2013), and the *in vitro* fumigation of ragweed pollen showed no difference in the amount of Amb a 1 between control and O_3-treated samples (Pasqualini *et al.* 2011). Interestingly, ragweed pollen that was collected along high-traffic roads showed a higher allergenicity compared with that of pollen from vegetated areas, and air pollution measurements showed higher NO_2 concentrations along traffic roads (Ghiani *et al.* 2012). In the same study, no correlation between the allergenicity and O_3 concentration was observed (Ghiani *et al.* 2012). Thus, it can be speculated that ragweed pollen allergenicity is rather increased by NO_2 than by O_3. However, in birch, a correlation of the pollen allergenicity and increasing O_3 concentrations but not NO_2 concentrations was found (Beck *et al.* 2013). This different behaviour between an annual ruderal herbaceous plant and a long-living tree species indicates species-specific effects, including differences in NO_2/O_3 fluxes (Eller & Sparks 2006; Cieslik 2009). Moreover, in addition to the allergen, non-allergenic pollen-derived compounds are important in the modulation of the allergic immune response (Gilles *et al.* 2012). Another important point that has not been addressed yet might be the pollen allergenicity of different ragweed populations. There are great phenotypic variations in European ragweed populations (Leiblein-Wild & Tackenberg 2014) and great genetic differentiation in native and introduced ragweed populations (Hodgins & Rieseberg 2011). Moreover, differences in the gene expression of native and introduced ragweed upon changing environments are known (Hodgins *et al.* 2013). Thus, differences in the amount of 'Amb a' content in different populations has also been considered, similarly as has been reported for differences in the Ole e 1 content in different olive tree cultivars (Castro *et al.* 2003), in different birch species (Schenk *et al.* 2009, 2011) or in *Phleum pratense* pollen that was purchased from different companies (Schmidt *et al.* 2010). Interestingly, a homolog to Hev b 9 also showed an increased IgE reactivity (Table 5). As the amount of Hev b 9 also increased at the protein level (Table 2), these findings support the idea of a new allergen in ragweed, as mentioned before.

CONCLUSIONS

The *in vivo* fumigation of ragweed plants with elevated NO_2 concentrations resulted in a changed proteomic pattern of the pollen. Regarding allergens, an up-regulation of several Amb a 1 isoforms at the protein levels was observed. These allergens were nitrosylated under low and elevated NO_2 conditions, indicating that nitrosylation, similar to nitration, may influence the allergenic potential of the pollen (Untersmayr *et al.* 2010; Ackaert *et al.* 2014; Reinmuth-Selzle *et al.* 2014). Most important for human health, however, is an increased IgE reactivity of the pollen upon elevated NO_2 concentrations, which might also occur under natural outdoor conditions. The increased allergenic potential of ragweed pollen was due to an increased amount of major ragweed Amb a 1 isoforms. In addition to the known ragweed allergens, the detected homolog to Hev b 9, which also reacts with a sera mixture from ragweed allergic patients, indicates the existence of a new allergen in ragweed pollen.

COMPETING INTERESTS

The authors declare no competing interests.

AUTHORS' CONTRIBUTIONS

J.D., H.B., C.T-H., U.F. and D.E. conceived and designed the experiments. F.Z., A.E., P.B., C.v.T. and S.M.H. performed experiments. F.Z., C.v.T., S.M.H. and U.F. analysed the data. A.H. and W.K. were responsible for the SEM analysis. P.B. was responsible for the ATR-FTIR experiments and the spectra interpretation. C.L. supervised the S-nitrosylation data. F.R. supervised the immunological data. J.B.W. was responsible for the phytotrons. U.F. and D.E. wrote the manuscript. All the authors read and approved the final manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Supporting information 1. Workflow for plant growth, pollen sampling and seed collection.
Supporting information 2. Sampling workflow and fluorescence labelling of the proteome analysis.
Supporting information 3. ImmunoCAP results for the sera from ragweed sensitized patients, used in this study.
Supporting information 4. SEM of ragweed pollen that was exposed to different NO_2-concentrations.
Supporting information 5. Representative 2D-DIGE of ragweed pollen proteins. (A) Three overlaid photos; (B) Cy3 image (40 ppb NO_2); (C) Cy5 image (80 ppb NO_2).
Supporting information 6. List of proteins extracted fro,m 2G-DIGE and identified by LC-MS/MS.
Supporting information 7. Functional classification of LC-MS/MS identified proteins under 40 ppb NO_2 and 80 ppb NO_2.
Supporting information 8. Western blot detection of S-nitrosylated proteins. *In vivo* S-nitrosylated proteins can be detected in the 1^{st} and 2^{nd} generation of pollen.
Supporting information 9. Identification and qualification of candidates for proteins S-nitrosylation from *Ambrosia artemisiifolia.*
Supporting information 10. Statistical analysis of 1-D Western immunoblot for ragweed allergens.
Supporting information 11. Allergen detection in ragweed pollen by 2-D Western immunoblot analysis.