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

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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 is 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 2D-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.

Keywords: *Ambrosia artemisiifolia*, allergen, allergenicity, immune serum, immunoblot, NO_2, pollen, proteome, S-nitrosylation, ragweed

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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). Due to 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 characterised, 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 (Gadermaier *et al.* 2014, Wopfner *et al.* 2005).

Air pollution, e.g., NOx 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 to 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 (Gilles *et al.* 2012, Traidl-Hoffmann *et al.* 2003), 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 multi-layered outer wall of sporopollenin (exine) and the pollen coat. The coat is a complex mixture of pigments, lipids, waxes, aromatic

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compounds and proteins that fills 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 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 IgE-binding capacity (Bashir *et al.* 2013b, Vigh-Conrad *et al.* 2010). In addition to proteins, lipids can also interact with the immune system and modify the antigenic properties of allergens (Bashir *et al.* 2013a, Traidl-Hoffmann *et al.* 2003). 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 (Honour *et al.* 2009, Kress & Skelly 1982). 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

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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 (Cuinica *et al.* 2014, Sousa *et al.* 2012).

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, Reinmuth-Selzle *et al.* 2014, Untersmayr *et al.* 2010). 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, Franze *et al.* 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 a lot 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-fumigationon on the pollen allergenicity of ragweed. For that a large scale 2D-difference gel electrophoresis (2D-DIGE) analysis was performed and interesting spots were analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Also immunoblotting with

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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) (1st generation). The seeds were sown in standard soil and germination took place in a 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 Munich region from the 1st of 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 two weeks, and NO_2-treatment began on the 29th of 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 per day. 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-

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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 the 3rd of July until the 29th of July as described by (Kanter *et al.* 2013) and stored on at -80 °C until use. The plants were cultivated further until the 21st of 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 (2nd 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.

SEM

SEM was exactly as described by Kanter et al. 2013

ATR-FTIR

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. 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

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variable when the model classifies the samples into one or two different classes to maximise 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 2D-DIGE

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) were resuspended in acetone containing 10% TCA, 1% DTT and 1% protease inhibitor (Sigma-Aldrich, Taufkirchen, Germany) and placed on a shaker (1400 rpm) 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 precooled 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

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the Bio-Rad Protein Assay with 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.

2D-DIGE

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

Preparative gel electrophoresis, in-gel digestion and LC-MS/MS analysis

Preparative 2-DE 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 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, 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., Oregon, USA) was used to validate the MS/MS-based peptide identifications and

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spectra with the following parameters: one missed cleavage allowed, a parent ion tolerance of 10 ppm, a fragment ion tolerance of 0.6 Da, as well as 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 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) 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 ≥ 7 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, 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.

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Protein S-nitrosylation detection

Protein extraction

From the 1st 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 HEPES-NaOH (pH 7.7), 1 mM 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 homogenised ten 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 rpm) and then centrifuged at 25,000 g for 20 min at 4 °C. The samples were adjusted to 0.8 µg/µl 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

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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 were resolved on 12% SDS-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 (TBS: 10 mM Tris-HCl (pH 7.5), 0.9% NaCl, 1 mM $MgCl_2.6H_20$; 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 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 $MgCl_2.6H_20$), 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).

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Purification and LC-MS/MS analysis of S-nitrosylated proteins

The purification of S-nitrosylated proteins using NeutrAvidin-agarose (Thermo Scientific) was carried out according to (Lindermayr *et al.* 2005). Purified protein samples were resolved on a 12% SDS-PAGE as described above. The gels were cut into 4 equal slices, and in-gel digestion and LC-MS/MS were performed as described above.

Pollen allergen immunoreactivity analysis

Preparation of the sera mixture

The sera of 10 patients suffering from ragweed pollen with CAP classes >3 (Supporting information 1) were pooled to carry out all of the immunochemical analyses. The pooled sera from 9 healthy volunteers provided the control. The serum pool was aliquoted and stored at -20 °C until use.

One- and two-dimensional immunoblot analysis

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

Pollen of three plants from each group (1st generation treatment, 1st generation control, 2nd generation treatment, and 2nd generation control) was randomly sampled, and three independent experiments were performed. For protein extraction pollen samples (125 mg/ml in ddH_2O + protease inhibitor) were mixed, and incubated at RT for 1 h with agitation (1400 rpm). 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 (Bio-Rad) with BSA as a standard.

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

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 1-D 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 3 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 3 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 above.

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Results

ATR-FTIR 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.

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 *vs.* 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 Figure 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 RMSE of 0.2 for calibration and 0.27 for validation and a R^2 of 0.82 and 0.7, respectively).

The predominant spectral differences between the two pollen groups are the result of variations of bands associated with lipids, sporopollenin, protein and carbohydrates (Figure 1B, C). The first PC plot (as a function of wavenumber) which explains differences of 16% of the data from elevated NO_2-treatetd pollen has high positive factor loadings associated predominantly with lipid, sporopollenin and

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protein bands (in PCs 1, Figure 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, as well as proteins are positively correlated to elevated NO_2, however, 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; Figure 1C) and sporopollenin (or more general aromatic groups) vibrational frequencies (band 3, 4, 9; Figure 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 i) 1671 cm^{-1} band (band 1) ii) 1324 cm^{-1} (band 5) and iii) the vibrational absorption range 1125-1105 cm^{-1} (band 6; Figure 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 (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 Chenopodiacea (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

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treatment corroborate 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 scanning electron microscopic analysis (Supporting information 4)

Comparative proteome analysis

2D-DIGE identified increased allergen amounts

2D-DIGE 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 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 (Figure 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 categorised into 14 functional groups according to the

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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 & 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 most affected groups (Supporting information 6 & 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 homologue A and V-type proton ATPase catalytic subunit A.

NO_2-fumigation results in enhanced S-nitrosylation

S-nitrosylation of proteins can have an influence on their structure and function. Therefor 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. 1-D Western blot analysis showed different S-nitrosylation pattern in 80 ppb NO_2-treated samples as compared to the control, indicating that an increase of S-nitrosylation has taken place due to the elevated NO_2 (Supporting information 8), and the relative mean grey value of all band intensities was significantly higher under elevated NO_2 (Figure 3). S-nitrosylated proteins were purified by SDS-PAGE, identified by LC-MS/MS and then further quantitatively analysed. 63 of 73 identified proteins were more abundant in pollen under elevated NO_2, whereas 9 were more pronounced in the control pollen (Table 3, Supporting information 8 & 9). The potentially S-

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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 to 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 significant different abundance, as demonstrated by the 2D-DIGE results, from which 8 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, 3). Additionally, potentially S-nitrosylated proteins detected by biotin-switch were also analysed by the SNO-prediction software GPS-SNO 1.0 (Kovacs & Lindermayr 2013, Xue *et al.* 2010). 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 done (Fares *et al.* 2011).

NO_2-treated pollen show higher IgE recognition in immunoblots

Immuno-dotblot pre-tests of pollen extracts, using a sera mixture from ragweed-sensitised patients, showed significantly higher IgE-binding signals due to elevated NO_2 in pollen of both generations (data not shown). Therefor these different allergenic signals were investigated in more detail by 1-D and 2-D immunoblotting, using the same sera mixture. Figure 4 shows a representative SDS-PAGE and the 1-

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D 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 homologue 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 characterise the allergenic proteins and their isoforms, 2-D Western immunoblots were performed under blotting conditions above. 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-treatement (Figure 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 (Figure 5). Further identification by LC-MS/MS confirmed the 1-D immunoblotting. All five of the Amb a 1 isoforms were found (Table 5). Spot number 5, showing the highest allergenicity, corresponded to a Amb a 1 with the Amb a 1.1 isoform 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 homologue to Hev b 9, reacting also with the sera mixture, was identified

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(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

SEM and ATR-FTIR

SEM pictures clearly indicate no influence of elevated NO_2 on the size or surface of ragweed pollen (Supporting file 3). This result has also been reported for pollen of O_3-treated and of elevated CO_2, and/or drought stress treated plants (El Kelish *et al.* 2014, Kanter *et al.* 2013), clearly indicating that air pollution or climate change scenarios will not alter the pollen size or shape.

ATR-FTIR 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, e.g., where pectin was reduced (Figure 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 (Bashir *et al.* 2013a, Traidl-Hoffmann *et al.* 2003). Moreover, the increase in coat proteins in ragweed pollen (Figure 1) may also contribute to a changed allergenicity, as pollen coat proteins possess IgE-binding capacities (Bashir *et al.* 2013b, Vigh-Conrad *et al.* 2010)

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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 (Beck *et al.* 2013, Chehregani *et al.* 2004, Cortegano *et al.* 2004, Ghiani *et al.* 2012, Suárez-Cervera *et al.* 2008). However, it is not easy to determine the contribution of different air pollutants, e.g., 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 urbanisation 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 (Cuinica *et al.* 2014, Sousa *et al.* 2012), 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 1-D gels, in pollen that was

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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 1-D gels, we also saw no difference in the polypeptide pattern after Coomassie Brilliant Blue staining (Figure 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 homologue to an enolase, Hev b 9, an allergen from rubber tree (Supporting information 5). 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 (Lai *et al.* 2002, Simon-Nobbe *et al.* 2000, Wagner *et al.* 2000). Hev b 9 is found in the milky sap that is produced by the rubber tree. Sensitisation occurs via skin contact; however, sensitisation may also occur via the inhalation of airborne allergens that are released from powdered latex gloves (Wagner *et al.* 2000).

Cytoskeleton dynamics

For pollen germination cytoplasmic and cytoskeletal reorganisation 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 reorganisation and dynamics of these are thought to be controlled by a complex interacting network

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(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 homologues to photosynthesis-related proteins were down-regulated and a homologue to an oxygen-evolving enhancer protein was up-regulator under elevated NO_2 (Table 2). These proteins are located in plastids, and in the majority of angiosperms, these organelles are maternally inherited (Birky 1995, Corriveau & Coleman 1988), 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 (Thyssen *et al.* 2012, Zhang *et al.* 2003), supporting the idea of the presence of plastids in ragweed pollen. This result is further supported by the presence of the homologues 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.

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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 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, homologues to several differentially abundant proteins were identified that belong to metabolism and energy generation. 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

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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 homologue 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 ROS production (Elmayan & Simon-Plas 2007). As the NADPH oxidase/ROS system influences the allergenicity of pollen (Pasqualini *et al.* 2011, Pazmandi *et al.* 2012, Wang *et al.* 2009), the increased amount of a 14-3-3 protein 9 homologue in ragweed pollen upon NO_2-treatment may also contribute to an increased 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 were sampled from urban sites (Bryce *et al.* 2010).

The proteins that are 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 homologue suggest that NO_2-damaged proteins can be effectively degraded.

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Among transporters, a plastid ATP synthase homologue was up-regulated, and an importin homologue was down-regulated. A mitochondrial ATP synthase is important for pollen formation and an *Importin β1* for pollen tube elongation (Han *et al.* 2011, Li *et al.* 2010). These results again suggest the changed interaction of the transport and signalling pattern under elevated NO_2-concentrations.

Stress response

Finally, ten homologues to stress-related proteins were 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 as 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). Reactive oxygen species (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 7). However, the overall nitrosylation was clearly enhanced under 80 ppb NO_2 (Figure 3). This result indicates the S-nitrosylation of new proteins or a different degree of protein S-nitrosylation.

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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 GPS-SNO 1.0 software, indicating the proteins as good candidates (Supporting information 8). As Amb a 3 S-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 (Ackaert *et al.* 2014, Gruijthuijsen *et al.* 2006, Untersmayr *et al.* 2010). 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 homologue 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. Also the possible S-nitrosylation of actin might influence the pollen development, which was enhanced due to elevated NO_2-fumigation. It is known that S-nitrosylation interferes with actin polymerization and changes the actin cytoskeleton structure (Rodriguez-Serrano *et al.* 2014, Yemets *et al.* 2011). 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 (Bachi *et al.* 2013, Hess *et al.* 2005).

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Allergenicity of ragweed pollen is enhanced due to elevated NO_2

The allergenicity of ragweed pollen was clearly enhanced upon elevated NO_2-fumigation (Figure 4 & 5). The allergenicity from the pollen of diverse plant species, including ragweed, also increased in pollen that was collected near high-traffic roads compared to that of more vegetated areas (Chehregani *et al.* 2004, Cortegano *et al.* 2004, Ghiani *et al.* 2012, Suárez-Cervera *et al.* 2008). 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, Cuinica *et al.* 2014, Rogerieux *et al.* 2007, Sousa *et al.* 2012). These experiments have been carried out for from a few hours up to two days, and artificially high NO_2-concentrations (ppm range) were sometimes applied (Rogerieux *et al.* 2007). 1-D Western immunoblot and further 2-D Western immunoblot analysis clearly showed that Amb a 1 isoforms mainly contributed to the increased allergenicity of ragweed pollen upon enhanced NO_2-concentrations (Tables 4 & 5; Figure 5). This result is consistent with the literature, describing Amb a 1 as the major allergen of ragweed (Gadermaier *et al.* 2014, Wopfner *et al.* 2005). Similarly, increased IgE reactivity to not-further-characterised allergens was found in the *in vitro* NO_2-treated pollen of *Acer negundo*, *Betula pendula*, *Ostrya carpinifolia* and *Carpinus betulus* (Cuinica *et al.* 2014, Sousa *et al.* 2012). 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

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collected along high-traffic roads showed a higher allergenicity compared to 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 (Cieslik 2009, Eller & Sparks 2006). 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.* 2011, Schenk *et al.* 2009), or in *Phleum pratense* pollen that were purchased from different companies (Schmidt *et al.* 2010). Interestingly a homologue to Hev b 9 also showed an increased IgE reactivity (Table 5). As the amount of Hev b 9 also

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increased at the protein level (Table 2), these findings support the idea of a new allergen in ragweed, as mentioned above.

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 transcriptional and 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 (Ackaert *et al.* 2014, Reinmuth-Selzle *et al.* 2014, Untersmayr *et al.* 2010). 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. Additionally to the known ragweed allergens, the detected homologue 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.

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Authors' contributions

JD, HB, CT-H, UF and DE conceived and designed the experiments. FZ, AE, PB, CvT, and SMH performed experiments. FZ, CvT, SMH and UF analysed the data. AH and WK were responsible for the SEM analysis. PB was responsible for the ATR-FTIR experiments and the spectra interpretation. CL supervised the S-nitrosylation data. FR supervised the immunological data. JBW was responsible for the Phytotrons. UF and DE wrote the manuscript. All the authors read and approved the final manuscript.

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References

Abat J.K. & Deswal R. (2009) Differential modulation of S-nitrosoproteome of *Brassica juncea* by low temperature: Change in S-nitrosylation of Rubisco is responsible for the inactivation of its carboxylase activity. *PROTEOMICS*, **9**, 4368-4380.

Ackaert C., Kofler S., Horejs-Hoeck J., Zulehner N., Asam C., von Grafenstein S., . . . Duschl A. (2014) The impact of nitration on the structure and immunogenicity of the major birch pollen allergen Bet v 1.0101. *Plos One*, **9**, e104520.

Aina R., Berra E., Marino G., Pastorello E.A. & Citterio S. (2007) Proteomic analysis of birch and ragweed pollen artificially exposed to NO_2. *World Allergy Organization Journal*, S198-S199

Astier J., Rasul S., Koen E., Manzoor H., Besson-Bard A., Lamotte O., . . . Wendehenne D. (2011) S-nitrosylation: An emerging post-translational protein modification in plants. *Plant Science*, **181**, 527-533.

Bachi A., Dalle-Donne I. & Scaloni A. (2013) Redox proteomics: chemical principles, methodological approaches and biological/biomedical promises. *Chemical Reviews*, **113**, 596-698.

Baker H.G. & Baker I. (1979) Starch angiosperm pollen grains and its evolutionary siginificance. *American Journal of Botany*, **66**, 591-600.

Bashir M.E.H., Lui J.H., Palnivelu R., Naclerio R.M. & Preuss D. (2013a) Pollen lipidomics: Lipid profiling exposes a notable diversity in 22 allergenic pollen and potential biomarkers of the allergic immune response. *PLoS ONE*, **8**, e57566.

Bashir M.E.H., Ward J.M., Cummings M., Karrar E.E., Root M., Mohamed A.A., . . . Preuss D. (2013b) Dual function of novel pollen coat (surface) proteins: IgE-binding capacity and proteolytic activity disrupting the airway epithelial barrier. *PLoS ONE*, **8**, e53337.

Beck I., Jochner S., Gilles S., McIntyre M., Buters J.T.M., Schmidt-Weber C., . . . Traidl-Hoffmann C. (2013) High environmental ozone levels lead to enhanced allergenicity of birch pollen. *Plos One*, **8**, e80147.

Behrendt H., Becker W.M., Fritzsche C., Sliwa-Tomczok W., Tomczok J., Friedrichs K.H. & Ring J. (1997) Air pollution and allergy: Experimental studies on modulation of allergen release from pollen by air pollutants. *International Archives of Allergy and Immunology*, **113**, 69-74.

Birky C.W. (1995) Uniparental inheritance of mitochondrial and chloroplast genes: mechanisms and evolution. *Proceedings of the National Academy of Sciences*, **92**, 11331-11338.

Bryce M., Drews O., Schenk M.F., Menzel A., Estrella N., Weichenmeier I., . . . Traidl-Hoffmann C. (2010) Impact of urbanization on the proteome of birch pollen and its chemotactic activity on human granulocytes. *International Archives of Allergy and Immunology*, **151**, 46-55.

Buters J.T.M., Kasche A., Weichenmeier I., Schober W., Klaus S., Traidl-Hoffmann C., . . . Behrendt H. (2008) Year-to-year variation in release of Bet v 1 allergen from birch pollen: Evidence for geographical differences between West and South Germany. *International Archives of Allergy and Immunology*, **145**, 122-130.

Castro A.J., Alché J.D., Cuevas J., Romero P.J., Alché V. & Rodríguez-García M.I. (2003) Pollen from different olive tree cultivars contains varying amounts of the major allergen Ole e 1. *International Archives of Allergy and Immunology*, **131**, 164-173.

Chehregani A., Majde A., Moin M., Gholami M., Shariatzadeh M.A. & Nassiri H. (2004) Increasing allergy potency of *Zinnia* pollen grains in polluted areas. *Ecotoxicology and Environmental Safety*, **58**, 267-272.

Chen W.J., Lo W.J., Cheng B.M. & Lee Y.P. (1992) Photolysis of nitric acid in solid nitrogen. *The Journal of Chemical Physics*, **97**, 7167-7173.

Chichiriccò G. & Picozzi P. (2007) Reversible inhibition of the pollen germination and the stigma penetration in *Crocus vernus* ssp *vernus* (Iridaceae) following fumigations with NO_2, CO, and O_3 gases. *Plant Biology*, **9**, 730-735.

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Cieslik S. (2009) Ozone fluxes over various plant ecosystems in Italy: A review. *Environmental Pollution*, **157**, 1487-1496.

Corriveau J.L. & Coleman A.W. (1988) Rapid screening method to detexct potential biparental inheritence of plastid DNA and resluts for over 200 angiosperm species. *American Journal of Botany*, **75**, 14443-11458.

Cortegano I., Civantos E., Aceituno E., del Moral A., López E., Lombardero M., . . . Lahoz C. (2004) Cloning and expression of a major allergen from *Cupressus arizonica* pollen, Cup a 3, a PR-5 protein expressed under polluted environment. *Allergy*, **59**, 485-490.

Cuinica L.G., Abreu I. & da Silva J.E. (2014) Effect of air pollutant NO_2 on *Betula pendula*, *Ostrya carpinifolia* and *Carpinus betulus* pollen fertility and human allergenicity. *Environmental Pollution*, **186**, 50-55.

D'Amato G., Cecchi L., Bonini S., Nunes C., Annesi-Maesano I., Behrendt H., . . . van Cauwenberge P. (2007) Allergenic pollen and pollen allergy in Europe. *Allergy*, **62**, 976-990.

Dai S., Li L., Chen T., Chong K., Xue Y. & Wang T. (2006) Proteomic analyses of *Oryza sativa* mature pollen reveal novel proteins associated with pollen germination and tube growth. *Proteomics*, **6**, 2504-2529.

Dai S.J., Wang T., Yan X.F. & Chen S.X. (2007) Proteomics of pollen development and germination. *Journal of Proteome Research*, **6**, 4556-4563.

Diethart B., Sam S. & Weber M. (2007) Walls of allergenic pollen: Special reference to the endexine. *Grana*, **46**, 164-175.

Edlund A.F., Swanson R. & Preuss D. (2004) Pollen and stigma structure and function: The role of diversity in pollination. *Plant Cell*, **16**, S84-S97.

Egli B., Kölling K., Köhler C., Zeeman S.C. & Streb S. (2010) Loss of cytosolic phosphoglucomutase compromises gametophyte development in Arabidopsis. *Plant Physiology*, **154**, 1659-1671.

El Kelish A., Winkler J.B., Lang H., Holzinger A., Behrendt H., Durner J., . . . Ernst D. (2014) Effects of ozone, CO_2 and drought stress on the growth and pollen production of common ragweed (*Ambrosia artemisiifolia*) *Julius-Kühn-Archiv*, 139-147.

Eller A.S.D. & Sparks J.P. (2006) Predicting leaf-level fluxes of O_3 and NO_2: the relative roles of diffusion and biochemical processes. *Plant Cell and Environment*, **29**, 1742-1750.

Elmayan T. & Simon-Plas F. (2007) Regulation of plant NADPH oxidase. *Plant Signaling & Behavior*, **2**, 505-507.

Fares A., Rossignol M. & Peltier J.B. (2011) Proteomics investigation of endogenous S-nitrosylation in Arabidopsis. *Biochemical and Biophysical Research Communications*, **416**, 331-336.

Frank I.E. & Friedman J.H. (1993) A statistical view of some chemometrics regression tools. *Technometrics*, **35**, 109-135.

Frank U., El Kelish A., Zhao F., Durner J., Winkler J.B., Behrendt H., . . . Ernst D. (2014) Ragweed (*Ambrosia artemisiifolia*) pollen allergenicity: proteome analysis upon elevated CO_2 and drought stress *Ambroisie*, **29**, 32-43.

Franze T., Weller M.G., Niessner R. & Pöschl U. (2003) Enzyme immunoassays for the investigation of protein nitration by air pollutants. *Analyst*, **128**, 824-831.

Franze T., Weller M.G., Niessner R. & Pöschl U. (2005) Protein nitration by polluted air. *Environmental Science & Technology*, **39**, 1673-1678.

Fratelli M., Demol H., Puype M., Casagrande S., Eberini I., Salmona M., . . . Ghezzi P. (2002) Identification by redox proteomics of glutathionylated proteins in oxidatively stressed human T lymphocytes. *Proceedings of the National Academy of Sciences*, **99**, 3505-3510.

Gadermaier G., Hauser M. & Ferreira F. (2014) Allergens of weed pollen: An overview on recombinant and natural molecules. *Methods*, **66**, 55-66.

Gallois J.-L., Guyon-Debast A., Lécureuil A., Vezon D., Carpentier V., Bonhomme S. & Guerche P. (2009) The *Arabidopsis* proteasome RPT5 subunits are essential for gametophyte development and show accession-dependent redundancy. *Plant Cell*, **21**, 442-459.

This article is protected by copyright. All rights reserved.

Gerber E., Schaffner U., Gassmann A., Hinz H.L., Seier M. & Müller-Schärer H. (2011) Prospects for biological control of *Ambrosia artemisiifolia* in Europe: learning from the past. *Weed Research*, **51**, 559-573.

Ghiani A., Aina R., Asero R., Bellotto E. & Citterio S. (2012) Ragweed pollen collected along high-traffic roads shows a higher allergenicity than pollen sampled in vegetated areas. *Allergy*, **67**, 887-894.

Gilles S., Behrendt H., Ring J. & Traidl-Hoffmann C. (2012) The pollen enigma: Modulation of the allergic immune response by non-allergenic, pollen-derived compounds. *Current Pharmaceutical Design*, **18**, 2314-2319.

Gottardini E., Cristofori A., Cristofolini F., Maccherini S. & Ferretti M. (2008) Ambient levels of nitrogen dioxide (NO_2) may reduce pollen viability in Austrian pine (*Pinus nigra* Arnold) trees - Correlative evidence from a field study. *Science of the Total Environment*, **402**, 299-305.

Grobei M.A., Qeli E., Brunner E., Rehrauer H., Zhang R.X., Roschitzki B., . . . Grossniklaus U. (2009) Deterministic protein inference for shotgun proteomics data provides new insights into *Arabidopsis* pollen development and function. *Genome Research*, **19**, 1786-1800.

Gruijthuijsen Y.K., Grieshuber I., Stöcklinger A., Tischler U., Fehrenbach T., Weller M.G., . . . Duschl A. (2006) Nitration enhances the allergenic potential of proteins. *International Archives of Allergy and Immunology*, **141**, 265-275.

Han M.-J., Jung K.-H., Yi G. & An G. (2011) Rice *Importin β1* gene affects pollen tube elongation. *Molecules and Cells*, **31**, 523-530.

Hastie T., Tibshirani R. & Friiedmann J.H. (2001) *The Elements of Statistical Learning: Data Mining, Inference, and Prediction*. Springer, New York.

Hess D.T., Matsumoto A., Kim S.O., Marshall H.E. & Stamler J.S. (2005) Protein S-nitrosylation: Purview and parameters. *Nature Reviews Molecular Cell Biology*, **6**, 150-166.

Hodgins K.A., Lai Z., Nurkowski K., Huang J. & Rieseberg L.H. (2013) The molecular basis of invasiveness: differences in gene expression of native and introduced common ragweed (*Ambrosia artemisiifolia*) in stressful and benign environments. *Molecular Ecology*, **22**, 2496-2510.

Hodgins K.A. & Rieseberg L. (2011) Genetic differentiation in life-history traits of introduced and native common ragweed (*Ambrosia artemisiifolia*) populations. *Journal of Evolutionary Biology*, **24**, 2731-2749.

Holzmeister C., Fröhlich A., Sarioglu H., Bauer N., Durner J. & Lindermayr C. (2011) Proteomic analysis of defense response of wildtype Arabidopsis thaliana and plants with impaired NO-homeostasis. *Proteomics*, **11**, 1664-1683.

Honour S.L., Bell J.N.B., Ashenden T.W., Cape J.N. & Power S.A. (2009) Responses of herbaceous plants to urban air pollution: Effects on growth, phenology and leaf surface characteristics. *Environmental Pollution*, **157**, 1279-1286.

Huang Z.Y., Gan Z.S., He Y.S., Li Y.H., Liu X.D. & Mu H. (2011) Functional analysis of a rice late pollen-abundant UDP-glucose pyrophosphorylase (OsUgp2) promoter. *Molecular Biology Reports*, **38**, 4291-4302.

Jacquemin B., Sunyer J., Forsberg B., Aguilera I., Briggs D., Garcia-Esteban R., . . . Kunzli N. (2009) Home outdoor NO_2 and new onset of self-reported asthma in adults. *Epidemiology*, **20**, 119-126.

Jaffrey S.R. & Snyder S.H. (2001) The biotin switch method for the detection of S-nitrosylated proteins. *Science Signaling* **2001**, pl1.

Kanter U., Heller W., Durner J., Winkler J.B., Engel M., Behrendt H., . . . Ernst D. (2013) Molecular and immunological characterization of ragweed (*Ambrosia artemisiifolia* L.) pollen after exposure of the plants to elevated ozone over a whole growing season. *PLoS ONE*, **8**, e61518.

Karle A.C., Oostingh G.J., Mutschlechner S., Ferreira F., Lackner P., Bohle B., . . . Duschl A. (2012) Nitration of the pollen allergen Bet v 1.0101 enhances the presentation of Bet v 1-derived peptides by HLA-DR on human dendritic cells. *Plos One*, **7**, e31483.

This article is protected by copyright. All rights reserved.

Kato H., Takemoto D. & Kawakita K. (2013) Proteomic analysis of S-nitrosylated proteins in potato plant. *Physiologia Plantarum*, **148**, 371-386.

Kovacs I. & Lindermayr C. (2013) Nitric oxide-based protein modification: formation and site-specificity of protein S-nitrosylation. *Frontiers in Plant Science*, **4**, 137.

Kress L.W. & Skelly J.M. (1982) Response of several eastern forest tree species to chronic doses of ozone and nitrogen dioxide. *Plant Disease*, **66**, 1149-1152.

Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680-685.

Lai H.-Y., Tam M.F., Tang R.-B., Chou H., Chang C.-Y., Tsai J.-J. & Shen H.-D. (2002) cDNA cloning and immunological characterization of a newly identified enolase allergen from *Penicillium citrinum* and *Aspergillus fumigatus*. *International Archives of Allergy and Immunology*, **127**, 181-190.

Leiblein-Wild M.C., Kaviani R. & Tackenberg O. (2014) Germination and seedling frost tolerance differ between the native and invasive range in common ragweed. *Oecologia*, **174**, 739-750.

Leiblein-Wild M.C. & Tackenberg O. (2014) Phenotypic variation of 38 European *Ambrosia artemisiifolia* populations measured in a common garden experiment. *Biological Invasions*, **16**, 2003-2015.

Li W.-Q., Zhang X.-Q., Xia C., Deng Y. & Ye D. (2010) *MALE GAMETOPHYTE DEFECTIVE 1*, encoding the F_Ad subunit of mitochondrial F_1F_0-ATP synthase, is essential for pollen formation in *Arabidopsis thaliana*. *Plant and Cell Physiology*, **51**, 923-935.

Lin A., Wang Y., Tang J., Xue P., Li C., Liu L., . . . Chu C. (2012) Nitric oxide and protein S-nitrosylation are integral to hydrogen peroxide-induced leaf cell death in rice. *Plant Physiology*, **158**, 451-464.

Lindermayr C., Saalbach G. & Durner J. (2005) Proteomic identification of S-nitrosylated proteins in *Arabidopsis*. *Plant Physiology*, **137**, 921-930.

Lu S., Ren J., Hao X., Liu D., Zhang R., Wu M., . . . Wang Q. (2014) Characterization of protein expression of *Platanus* pollen following exposure to gaseous pollutants and vehicle exhaust particles. *Aerobiologia*, **30**, 281-291.

Maldonado-Alconada A., Echevarría-Zomeño S., Lindermayr C., Redondo-López I., Durner J. & Jorrín-Novo J. (2011) Proteomic analysis of Arabidopsis protein S-nitrosylation in response to inoculation with Pseudomonas syringae. *Acta Physiologiae Plantarum*, **33**, 1493-1514.

Mamun A.N.K. (2007) Reversible male sterility in transgenic tobacco carrying a dominant-negative mutated glutamine synthetase gene under the control of microspore-specific promoter. *Indian Journal of Experimental Biology*, **45**, 1022-1030.

Mascarenhas J.P. (1993) Molecular mechanisms of pollen tube growth and differentiation. *The Plant Cell*, **5**, 1303-1314.

Merl J., Ueffing M., Hauck S.M. & von Toerne C. (2012) Direct comparison of MS-based label-free and SILAC quantitative proteome profiling strategies in primary retinal Müller cells. *Proteomics*, **12**, 1902-1911.

Muñoz-Bertomeu J., Cascales-Miñana B., Irles-Segura A., Mateu I., Nunes-Nesi A., Fernie A.R., . . . Ros R. (2010) The plastidial glyceraldehyde-3-phosphate dehydrogenase is critical for viable pollen development in *Arabidopsis*. *Plant Physiology*, **152**, 1830-1841.

Palevitz B.A., Liu B. & Joshi H.C. (1994) γ-Tubulin in tobacco pollen tubes: association with generative cell and vegetative microtubules. *Sexual Plant Reproduction*, **7**, 209-214.

Pasqualini S., Tedeschini E., Frenguelli G., Wopfner N., Ferreira F., D'Amato G. & Ederli L. (2011) Ozone affects pollen viability and NAD(P)H oxidase release from *Ambrosia artemisiifolia* pollen. *Environmental Pollution*, **159**, 2823-2830.

Pazmandi K., Kumar B.V., Szabo K., Boldogh I., Szoor A., Vereb G., . . . Bacsi A. (2012) Ragweed subpollen particles of respirable size activate human dendritic cells. *PLoS ONE*, **7**, e52085.

Accepted Article

This article is protected by copyright. All rights reserved.

Pertl H., Rittmann S., Schulze W.X. & Obermeyer G. (2011) Identification of lily pollen 14-3-3 isoforms and their subcellular and time-dependent expression profile. *Biological Chemistry*, **392**, 249-262.

Pertl H., Schulze W.X. & Obermeyer G. (2009) The pollen organelle membrane proteome reveals highly spatial-temporal dynamics during germination and tube growth of Lily pollen. *Journal of Proteome Research*, **8**, 5142-5152.

Petersen, Schramm, Schlaak & Becker (1998) Post-translational modifications influence IgE reactivity to the major allergen Phl p 1 of timothy grass pollen. *Clinical & Experimental Allergy*, **28**, 315-321.

Reinmuth-Selzle K., Ackaert C., Kampf C.J., Samonig M., Shiraiwa M., Kofler S., . . . Poschl U. (2014) Nitration of the birch pollen allergen Bet v 1.0101: Efficiency and site-selectivity of liquid and gaseous nitrating agents. *Journal of Proteome Research*, **13**, 1570-1577.

Ribarits A., Mamun A.N.K., Li S., Resch T., Fiers M., Heberle-Bors E., . . . Touraev A. (2007) Combination of reversible male sterility and doubled haploid production by targeted inactivation of cytoplasmic glutamine synthetase in developing anthers and pollen. *Plant Biotechnology Journal*, **5**, 483-494.

Ridley B.L., O'Neill M.A. & Mohnen D. (2001) Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry*, **57**, 929-967.

Ring J., Krämer U., Schäfer T. & Behrendt H. (2001) Why are allergies increasing? *Current Opinion in Immunology*, **13**, 701-708.

Roberts M.R., Salinas J. & Collinge D.B. (2002) 14-3-3 proteins and the response to abiotic and biotic stress. *Plant Molecular Biology*, **50**, 1031-1039.

Rodriguez-Serrano M., Pazmino D.M., Sparkes I., Rochetti A., Hawes C., Romero-Puertas M.C. & Sandalio L.M. (2014) 2,4-Dichlorophenoxyacetic acid promotes S-nitrosylation and oxidation of actin affecting cytoskeleton and peroxisomal dynamics. *Journal of Experimental Botany*, **65**, 4783-4793.

Rogerieux F., Godfrin D., Sénéchal H., Motta A.C., Marlière M., Peltre G. & Lacroix G. (2007) Modifications of *Phleum pratense* grass pollen allergens following artificial exposure to gaseous air pollutants (O_3, NO_2, SO_2). *International Archives of Allergy and Immunology*, **143**, 127-134.

Romero-Puertas M.C., Campostrini N., Mattè A., Righetti P.G., Perazzolli M., Zolla L., . . . Delledonne M. (2008) Proteomic analysis of S-nitrosylated proteins in *Arabidopsis thaliana* undergoing hypersensitive response. *PROTEOMICS*, **8**, 1459-1469.

Sangwan R.S. & Sangwan-Norrell B.S. (1987) Ultrastructural cytology of plastids in pollen of certain androgenic and nonandrogenic plants. *Protoplasma*, **138**, 11-22.

Schenk M.F., Cordewener J.H.G., America A.H.P., Peters J., Smulders M.J.M. & Gilissen L. (2011) Proteomic analysis of the major birch allergen Bet v 1 predicts allergenicity for 15 birch species. *Journal of Proteomics*, **74**, 1290-1300.

Schenk M.F., Cordewener J.H.G., America A.H.P., van't Westende W.P.C., Smulders M.J.M. & Gilissen L. (2009) Characterization of PR-10 genes from eight *Betula species* and detection of Bet v 1 isoforms in birch pollen. *Bmc Plant Biology*, **9**, 24.

Schmidt H., Gelhaus C., Nebendahl M., Janssen O. & Petersen A. (2010) Characterization of *Phleum pratense* pollen extracts by 2-D DIGE and allergen immunoreactivity. *Proteomics*, **10**, 4352-4362.

Simon-Nobbe B., Probst G., Kajava A.V., Oberkofler H., Susani M., Crameri R., . . . Breitenbach M. (2000) IgE-binding epitopes of enolases, a class of highly conserved fungal allergens. *Journal of Allergy and Clinical Immunology*, **106**, 887-895.

Sousa R., Duque L., Duarte A.J., Gomes C.R., Ribeiro H., Cruz A., . . . Abreu I. (2012) In vitro exposure of *Acer negundo* pollen to atmospheric levels of SO_2 and NO_2: Effects on allergenicity and germination. *Environmental Science & Technology*, **46**, 2406-2412.

Accepted Article

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Storkey J., Stratonovitch P., Chapman D.S., Vidotto F. & Semenov M.A. (2014) A Process-Based Approach to Predicting the Effect of Climate Change on the Distribution of an Invasive Allergenic Plant in Europe. *PLoS ONE*, **9**, e88156.

Suárez-Cervera M., Castells T., Vega-Maray A., Civantos E., del Pozo V., Fernández-González D., . . . Seoane-Camba J.A. (2008) Effects of air pollution on Cup a 3 allergen in *Cupressus arizonica* pollen grains. *Annals of Allergy, Asthma & Immunology* **101**, 57-66.

Takahashi M., Furuhashi T., Ishikawa N., Horiguchi G., Sakamoto A., Tsukaya H. & Morikawa H. (2014) Nitrogen dioxide regulates organ growth by controlling cell proliferation and enlargement in Arabidopsis. *New Phytologist*, **201**, 1304-1315.

Takenoue Y., Kaneko T., Miyamae T., Mori M. & Yokota S. (2012) Influence of outdoor NO_2 exposure on asthma in childhood: Meta-analysis. *Pediatrics International*, **54**, 762-769.

Tang L.Y., Matsushima R. & W. S. (2012) Mutations defective in ribonucleotide reductase activity interferw with pollen DNa degradation mediated by DPD1 exonucleause. *The Plant Journal*, **70**, 637-649.

Temple S.J., Vance C.P. & Gantt J.S. (1998) Glutamate synthase and nitrogen assimilation. *Trends in Plant Science*, **3**, 51-56.

Thyssen G., Svab Z. & Maliga P. (2012) Exceptional inheritance of plastids via pollen in *Nicotiana sylvestris* with no detectable paternal mitochondrial DNA in the progeny. *Plant Journal*, **72**, 84-88.

Traidl-Hoffmann C., Kasche A., Menzel A., Jakob T., Thiel M., Ring J. & Behrendt H. (2003) Impact of pollen on human health: More than allergen carriers? *International Archives of Allergy and Immunology*, **131**, 1-13.

Untersmayr E., Diesner S.C., Oostingh G.J., Selzle K., Pfaller T., Schultz C., . . . Duschl A. (2010) Nitration of the egg-allergen ovalbumin enhances protein allergenicity but reduces the risk for oral sensitization in a murine model of food allergy. *Plos One*, **5**, e14210.

Vanzo E., Ghirardo A., Merl-Pham J., Lindermayr C., Heller W., Hauck S.M., . . . Schnitzler J.-P. (2014) S-Nitroso-Proteome in Poplar Leaves in Response to Acute Ozone Stress. *PLoS ONE*, **9**, e106886.

Vidali L. & Hepler P.K. (2001) Actin and pollen tube growth. *Protoplasma*, **215**, 64-76.

Vigh-Conrad K.A., Conrad D.F. & Preuss D. (2010) A protein allergen microarray detects specific IgE to pollen surface, cytoplasmic, and commercial allergen extracts. *PLoS ONE*, **5**, e10174.

Wagner S., Breiteneder H., Simon-Nobbe B., Susani M., Krebitz M., Niggemann B., . . . Hoffmann-Sommergruber K. (2000) Hev b 9, an enolase and a new cross-reactive allergen from *Hevea* latex and molds. *European Journal of Biochemistry*, **267**, 7006-7014.

Wang X.-L., Takai T., Kamijo S., Gunawan H., Ogawa H. & Okumura K. (2009) NADPH oxidase activity in allergenic pollen grains of different plant species. *Biochemical and Biophysical Research Communications*, **387**, 430-434.

Wopfner N., Gadermaier G., Egger M., Asero R., Ebner C., Jahn-Schmid B. & Ferreira F. (2005) The spectrum of allergens in ragweed and mugwort pollen. *International Archives of Allergy and Immunology*, **138**, 337-346.

Xue Y., Liu Z., Gao X., Jin C., Wen L., Yao X. & Ren J. (2010) GPS-SNO: Computational Prediction of Protein *S*-Nitrosylation Sites with a Modified GPS Algorithm. *PLoS ONE*, **5**, e11290.

Yemets A.I., Krasylenko Y.A., Lytvyn D.I., Sheremet Y.A. & Blume Y.B. (2011) Nitric oxide signalling via cytoskeleton in plants. *Plant Science*, **181**, 545-554.

Yu G.-H., Zou J., Feng J., Peng X.-B., Wu J.-Y., Wu Y.-L., . . . Sun M.-X. (2014) Exogenous γ-aminobutyric acid (GABA) affects pollen tube growth via modulating putative Ca^{2+}-permeable membrane channels and is coupled to negative regulation on glutamate decarboxylase. *Journal of Experimental Botany*, **65**, 3235-3248.

Zhang Q., Liu Y. & Sodmergen (2003) Examination of the cytoplasmic DNA in male reproductive cells to determine the potential for cytoplasmic inheritance in 295 angiosperm species. *Plant and Cell Physiology*, **44**, 941-951.

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Zhao Z. & Assmann S.M. (2011) The glycolytic enzyme, phosphoglycerate mutase, has critical roles in stomatal movement, vegetative growth, and pollen production in *Arabidopsis thaliana*. *Journal of Experimental Botany*, **62**, 5179-5189.

Zimmermann B. & Kohler A. (2014) Infrared spectroscopy of pollen identifies plant species and genus as well as environmental conditions. *PLoS ONE*, **9**, e95417.

Ziska L., Knowlton K., Rogers C., Dalan D., Tierney N., Elder M.A., . . . Frenz D. (2011) Recent warming by latitude associated with increased length of ragweed pollen season in central North America. *Proceedings of the National Academy of Sciences of the United States of America*, **108**, 4248-4251.

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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 strech	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

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Table 2. List of LC-MS/MS identified proteins that were differentially abundant by at least ±1.5-fold upon elevated NO_2. The ratio of average spot volume (treatment vs. 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 best protein score, calculated as the sum of the peptide ion scores for each protein, are given.

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 subsp. 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

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	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 subsp. 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 subsp. 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
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 subsp. 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

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.

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

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* subsp. japonica)	Q02897	7	4	267	2.32	
Glutelin type-A 1 (*Oryza sativa* subsp. 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	

[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)

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

			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

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

			Mascot			
Spot #	**Protein name**	**Acc. No.**	**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**

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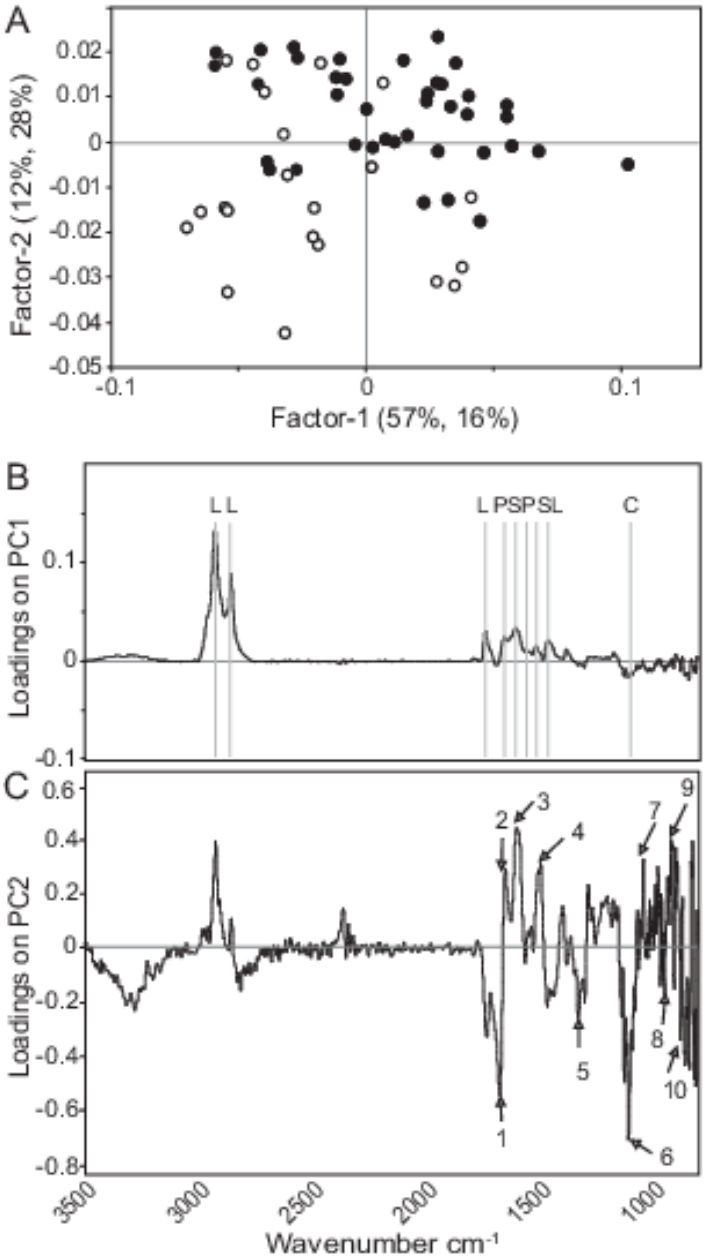

Figure 1 Correlation between spectroscopic data and pollen treatment. **(A)** PLS-DA plot of the ATR-IR spectral data set (60 samples, two spectra per sample; SNV-corrected spectra) with the depiction of the NO_2-fumigated (39 samples, black circles) and control (21 samples, open circles) samples. The 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.

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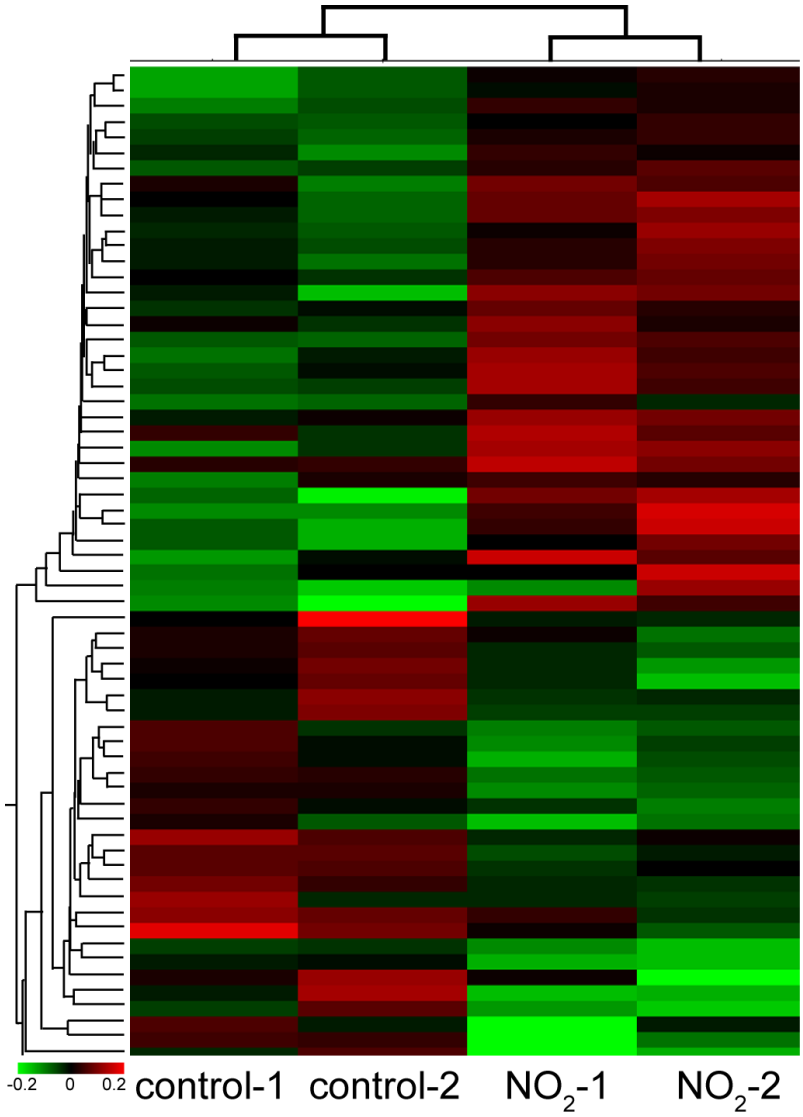

Figure 2 Heat map representation of protein abundances and hierarchical clustering of the abundance profiles. The data are from 2 sub-chambers for 40 ppb NO_2 (control) and 2 sub-chambers for 80 ppb NO_2 (treatment). Red indicates up-regulation, and green indicates down-regulation.

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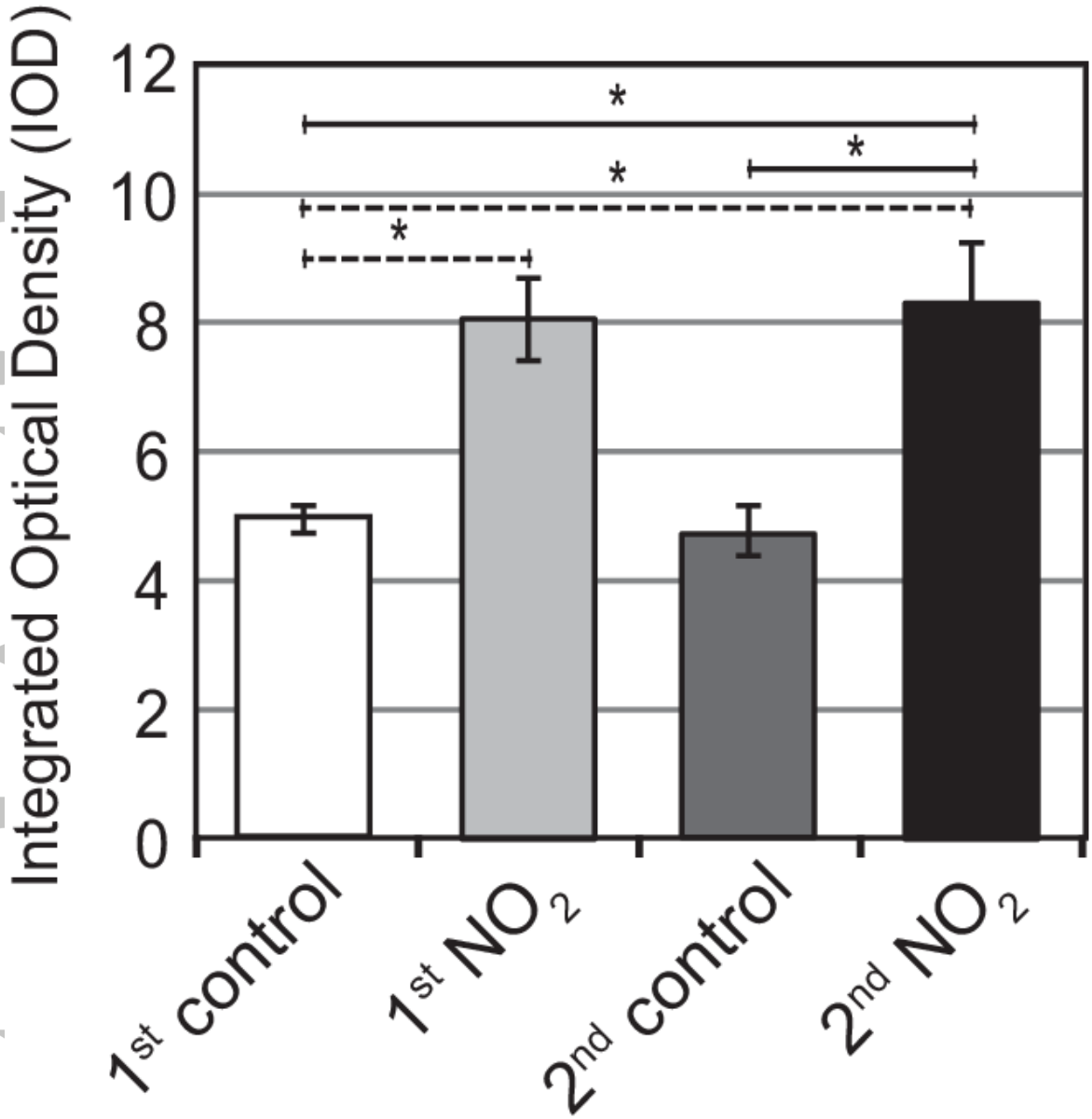

Figure 3 S-nitrosylation of proteins. Relative mean grey value intensities of all of the Western blot bands as calculated by Image J (error bars ±SD, * = p-value < 0.05; One way-ANOVA). 1st control = 40 ppb NO_2; 2nd control = clean air; 1st and 2nd NO_2 = 80 ppb NO_2

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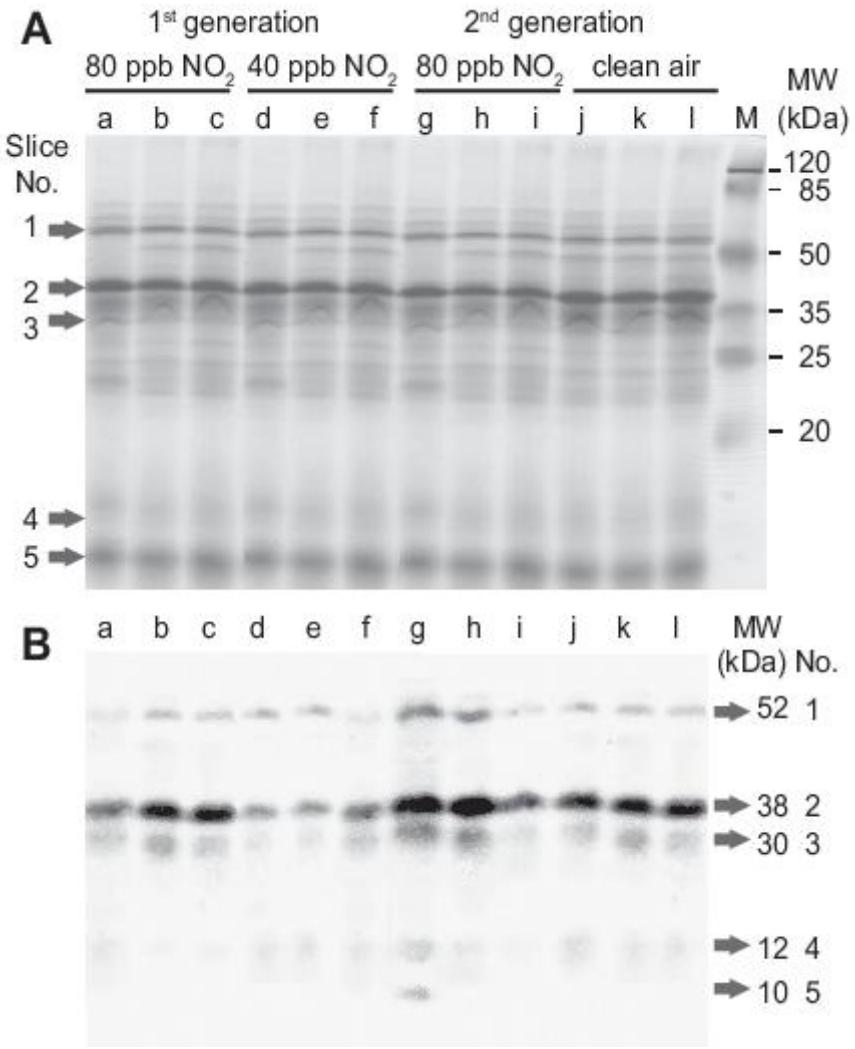

Figure 4 Allergen detection in ragweed pollen. (A) Coomassie brilliant blue-stained SDS-PAGE. **(B)** 1-D Western immunoblot that was probed with sera from ragweed allergic patients. Slice numbers and MW [kDa] are indicated.

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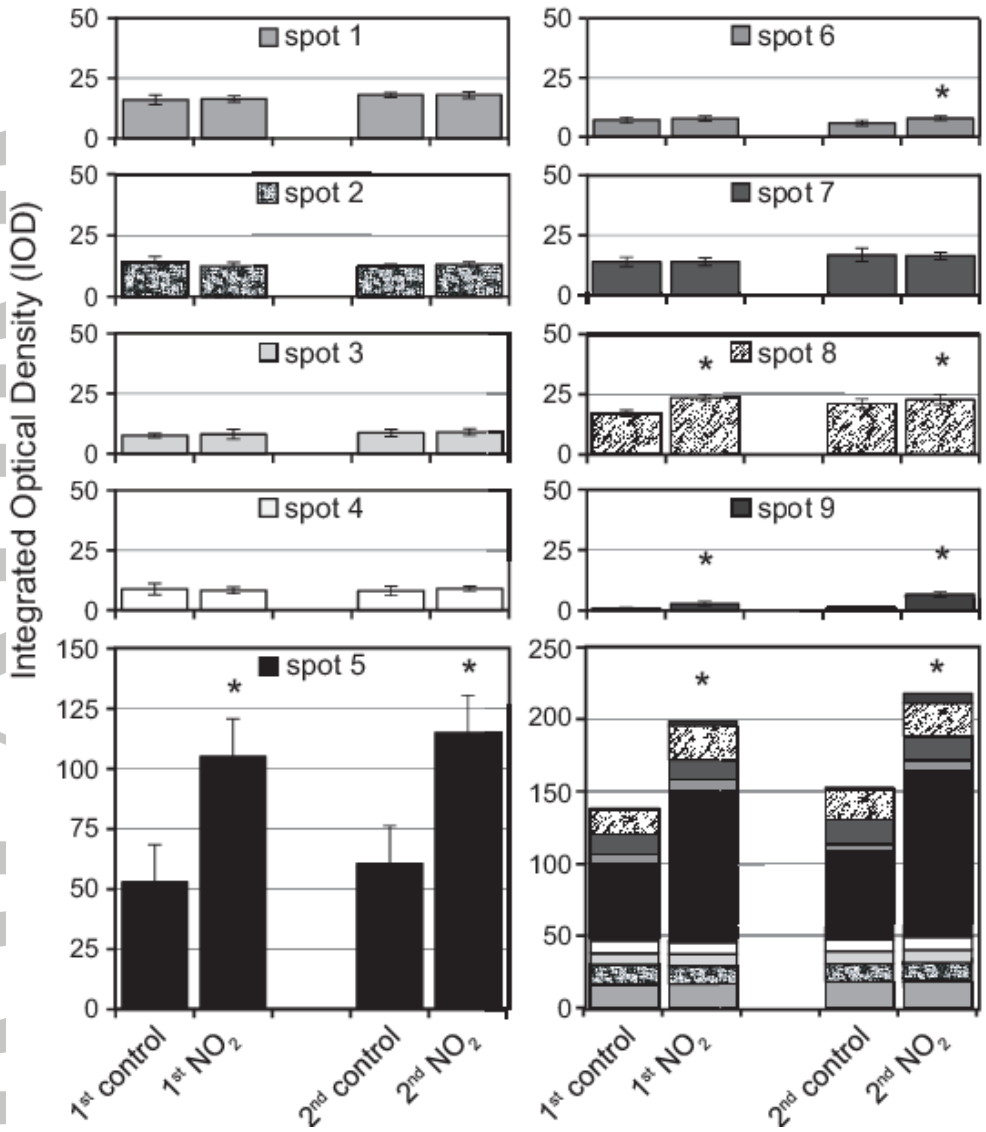

Figure 5 2-D Western immunoblot. Statistical analysis of the major allergens in different pollen extracts and the contribution of individual single spots to the total allergenicity (* = p-value < 0.05; One way-ANOVA). Proteins were identified by LC-MS/MS.

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SUMMARY

Our study shows the influence of abiotic factors on the highly allergenic pollen from ragweed.

Climate change and global warming as well as other environmental conditions have an influence on the plant and therefore might have also an influence on the allergenic potential of their pollen.

We did proteomic studies and infrared spectroscopic-analyses of pollen from the non-model plant Ambrosia artemisiifolia to give more insight to how the pollen reacts to elevated NO2. Allergenic proteins as well as allergen transcripts were increased under elevated NO2 and differences in cell wall components have been detected. Additionally S-nitrosylation of pollen proteins shown and higher allergen recognition by immuno-blot has been detected.

Allergies are increasing and the allergenic potential of the pollen is influenced by environmental factors. We used highly sophisticated methods, such as 2D-DIGE analyses and were able to detect, beside the well-known Ambrosia allergens, a homologue to another plant allergen which reacted also with the sera of ragweed allergenic patients and therefore might modulate the Ambrosia allergenic response.

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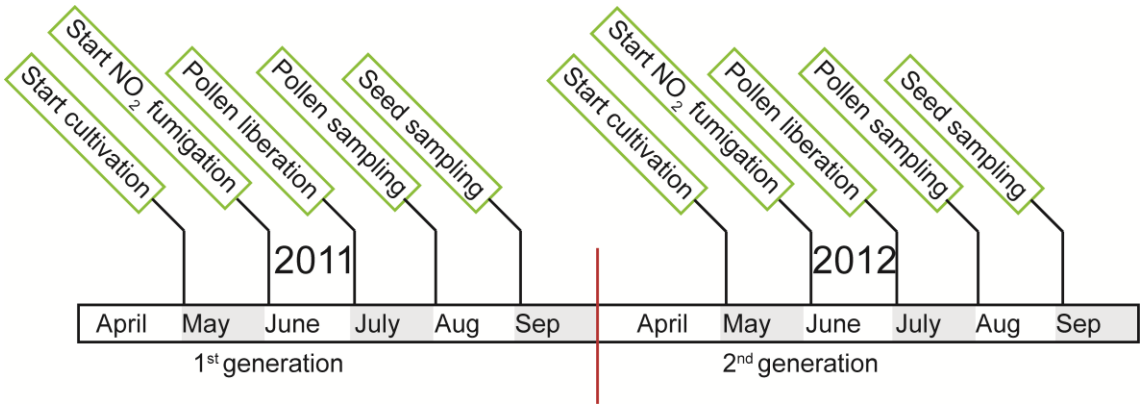
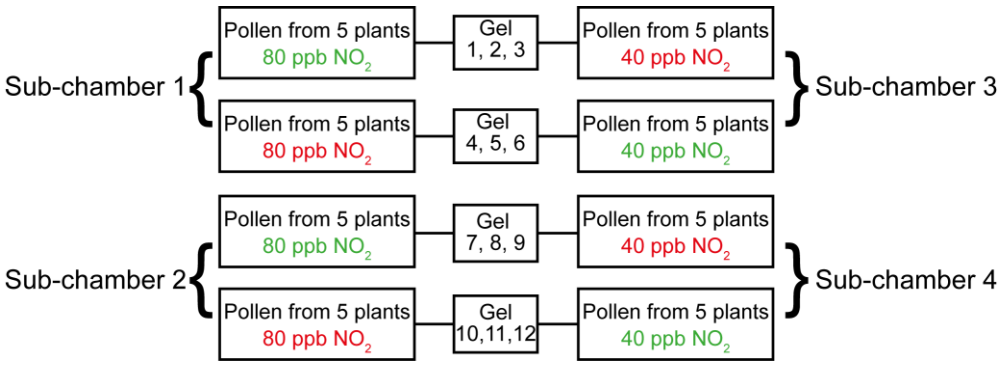
Supporting information 1. ImmunoCAP results for the sera from ragweed sensitized patients, used in this study.

Patient ID	ImmunoCAP specific IgE w1 [kUA/l] (Phadia)
1	6.45
2	13.5
3	12.8
4	11.1
5	20.3
6	6.01
7	4.86
8	4.47
9	6.12
10	10.6

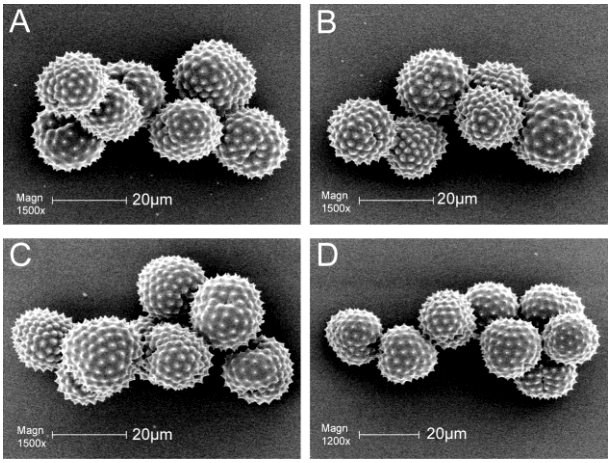
CAP-class 0: <0.35 kUA/l
CAP-class 1: 0.35 - <0.7 kUA/l
CAP-class 2: 0.7 - <3.5 kUA/l
CAP-class 3: 3.5 - < 17.5 kUA/l
CAP- class 4: 17.5 - <50.0 kUA/l
CAP-class 5: 50.0 - <100.0 kUA/l
CAP-class 6: >100 kUA/l



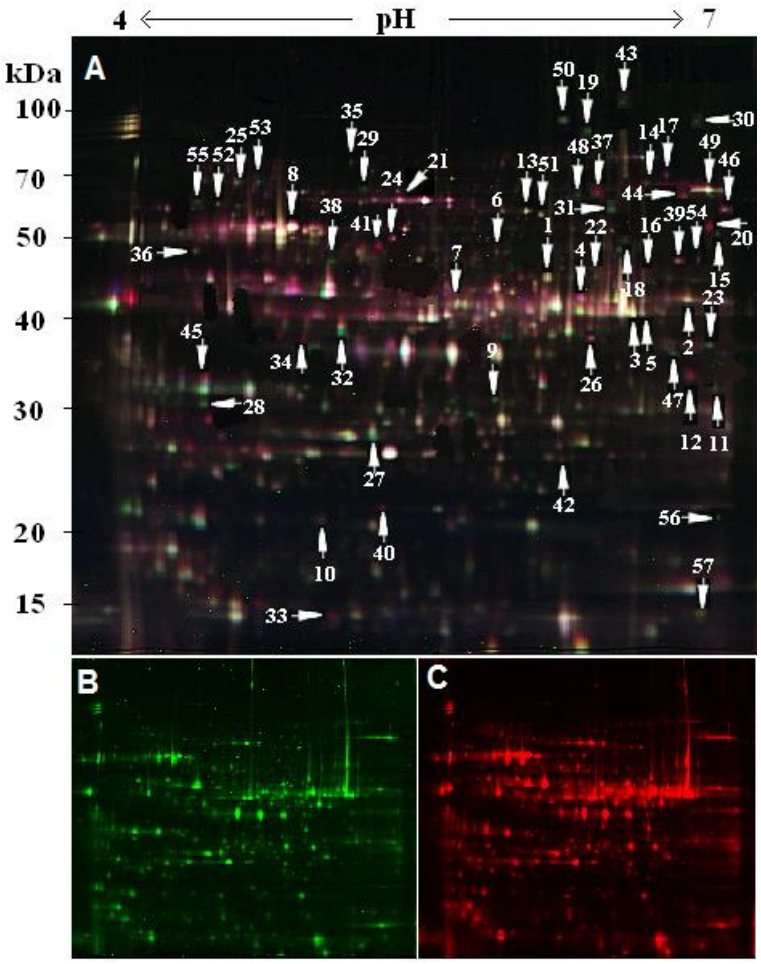
Supporting information 2: Workflow for plant growth, pollen sampling and seed collection. Plants were fumigated with 40 ppb NO_2 (control; 1st generation) and 80 ppb NO_2 (treatment; both generations) in the first year. Due to the fact that not enough NO_2 cartridges were available in the time of experiment, clean air served as control in the second year



Supporting information 3: Sampling workflow and fluorescence labelling of the proteome analysis. Samples labeled with Cy3 are indicated in green, samples labeled with Cy5 are shown in red.



Supporting information 4: SEM of ragweed pollen that was exposed to different NO_2-concentrations. **(A)** 40 ppb NO_2, 1st generation; **(B**) 80 ppb NO_2, 1st generation; **(C)** clean air, 2nd generation; **(D)** 80 ppb NO_2, 2nd generation. Bars indicate 20 µm. SEM was performed to detect possible changes of the pollen shape and/or size. This analysis showed the typical shape of ragweed pollen: coated with small cone-shaped needles. The main size of the pollen ranged from 20-25 µm, and no significant differences between control and treated plants were obvious.



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). Spot numbers 1-57 indicate the identified proteins that were significantly differentially expressed.

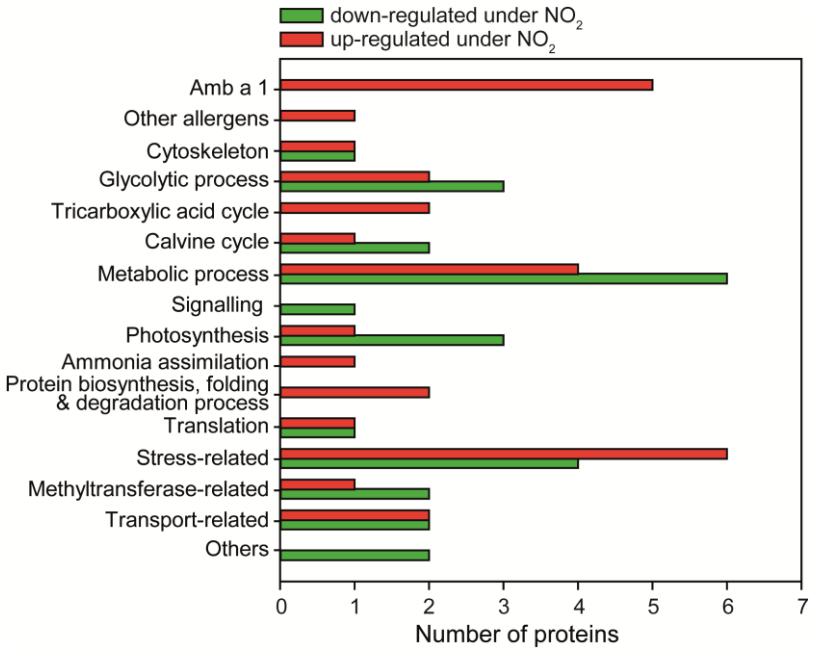
Supporting information 6. List of proteins extracted from 2D-DIGE and identified by LC-MS/MS. Ratio of average spot volume (80 ppb NO_2 vs. 40 ppb NO_2) after application of the processing modules (different in-gel analysis and biological variance analysis) is given. One-way ANOVA was carried out to calculate significance. Protein names and accession numbers are given, as well as the numbers of unique peptides, spectra and the protein scores are given. Only proteins with a protein score difference of less than 100 are shown. Best match per spot is indicated in bold. + = only detected in treated pollen; - = only detected in control pollen.

Spot	Best protein match	Accession	MW [kDa]	Mascot			Spot ratio	p-value
				# unique peptides	# unique spectra	Protein score		
	I: Allergen							
1	**Pollen allergen Amb a 1.1** (*Ambrosia artemisiifolia*)	P27759	43	13	21	1708.0	1.89	1.23E-09
2	**Pollen allergen Amb a 1.2** (*Ambrosia artemisiifolia*)	P27760	44	11	18	1292.5	1.71	5.88E-10
	Pollen allergen Amb a 1.4 (*Ambrosia artemisiifolia*)	P28744	43	5	8	1239.5	1.71	5.88E-10
3	**Pollen allergen Amb a 1.3** (*Ambrosia artemisiifolia*)	P27761	43	10	11	712.1	1.58	2.05E-06
	Pollen allergen Amb a 1.4 (*Ambrosia artemisiifolia*)	P28744	43	8	9	623.2	1.58	2.05E-06
4	**Pollen allergen Amb a 1.4** (*Ambrosia artemisiifolia*)	P28744	43	6	8	823.2	1.56	9.79E-08
5	**Pollen allergen Amb a 1.5** (*Ambrosia artemisiifolia*)	P27762	44	15	19	1162	1.56	2.23E-03
	II: Other allergens							
6	**Enolase 1 (Hev b 9)** (*Hevea brasiliensis*)	Q9LEJ0	48	3	3	203.7	+	5.96E-03
	Glutamine synthetase cytosolic isozyme 1-3 (*Arabidopsis thaliana*)	Q9LVI8	39	3	3	159.4	+	5.96E-03
	III: Cytoskeleton Proteins							
7	**Actin-97** (*Solanum tuberosum*)	P30171	42	8	8	516.4	-2.09	1.13E-02
8	**Tubulin-alpha-1 chain** (*Pisum sativum*)	P46259	50	18	26	2220.4	1.54	3.00E-06
	Tubulin beta-9 (*Arabidopsis thaliana*)	P29517	50	15	17	2134.2	1.54	3.00E-06
	IV: Glycolytic process							
10	**Triosephosphate isomerase** (*Lactuca sativa*)	P48493	21	5	7	324.5	2.05	4.54E-06
	Probable lactoylglutathione lyase (*Arabidopsis thaliana*)	Q8W593	39	2	3	258.8	2.05	4.54E-06
11	**Glyceraldehyde-3-phosphate dehydrogenase** (*Petroselinum crispum*)	P26519	36	3	3	150.2	-1.87	2.59E-04
	V-type proton ATPase catalytic subunit A (*Hordeum vulgare*)	Q40002	64	3	3	97.6	-1.87	2.59E-04
12	**Fructose-bisphosphate aldolase** (*Pisum sativum*)	P46257	38	2	2	140.0	1.56	8.46E-04
13	**2,3-bisphosphoglycerate** (*Arabidopsis thaliana*)	Q9M9K1	61	5	5	287.3	-3.08	6.93E-09
	Phosphoglycerate kinase (*Nicotiana tabacum*)	Q42961	50	3	4	205.6	-3.08	6.93E-09
14	**Glucose-6-phosphate isomerase** (*Arabidopsis thaliana*)	P34795	62	7	9	454	-2.58	2.32E-03

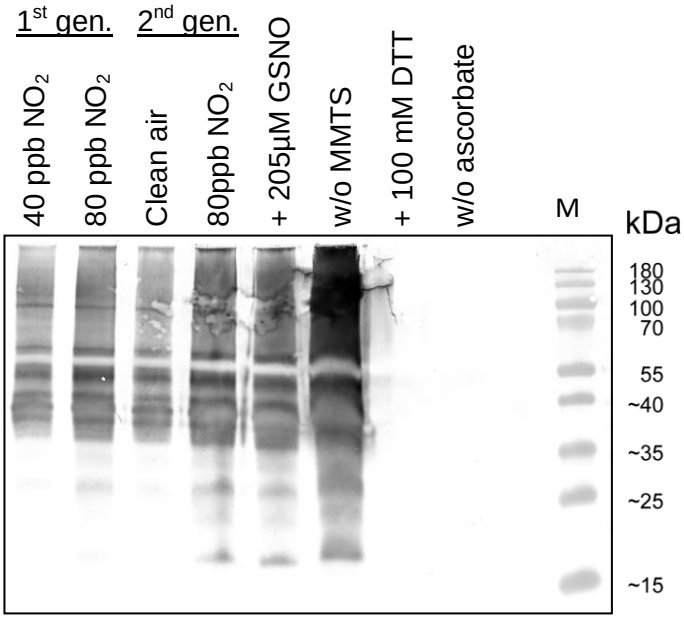
	V Tricarboxylic acid cycle							
16	**Isocitrate dehydrogenase** (*Glycine max*)	Q06197	46	2	3	201	3.02	5.18E-03
	Phosphoglycerate kinase (*Nicotiana tabacum*)	Q42961	50	2	2	117	3.02	5.18E-03
	Heat shock 70 kDa protein 10 (*Arabidopsis thaliana*)	Q9LDZ0	73	2	2	98.7	3.02	5.18E-03
17	**Succinate dehydrogenase flavoprotein subunit 1** (*Arabidopsis thaliana*)	O82663	70	5	5	299.3	1.96	1.89E-06
	VI Calvin cycle							
18	**Ribulose bisphosphate carboxylase large subunit** (*Populus alba*)	Q14FE9	53	2	2	82.6	-2.36	6.30E-03
	GDP-mannose 3,5-epimerase (*Arabidopsis thaliana*)	Q93VR3	43	2	2	78.6	-2.36	6.30E-03
19	**Transketolase** (*Solanum tuberosum*)	Q43848	80	3	4	205.9	-2.55	1.48E-02
20	**Phosphoglycerate kinase** (*Nicotiana tabacum*)	Q42961	50	3	3	251.3	1.88	1.39E-03
	VII Metabolic process							
9	**Glucose and ribitol dehydrogenase homolog** (*Oryza sativa subsp. japonica*)	Q75KH3	32	3	3	165.3	-1.89	5.02E-10
15	**Dihydrolipoyllysine-residue succinyltransferase** (*Arabidopsis thaliana*)	Q8H107	50	3	4	305	-2.69	2.22E-03
	Tubulin beta-5 (*Oryza sativa subsp. japonica*)	P46265	50	5	5	280	-2.69	2.22E-03
	NADP-dependent malic enzyme (*Mesembryanthemum crystallinum*)	P37223	64	3	4	233	-2.69	2.22E-03
21	**Glutamate decarboxylase** (*Petunia hybrida*)	Q07346	57	18	26	2817.7	2.03	2.37E-04
22	**GDP-mannose 3,5-epimerase** (*Arabidopsis thaliana*)	Q93VR3	43	2	2	109.5	-2.33	4.96E-04
23	**Cytochrome c1-1** (*Solanum tuberosum*)	P25076	35	4	7	373.9	-1.63	1.04E-06
24	**Protein disulfide isomerase-like 2-3** (*Oryza sativa ssp. japonica*)	Q67UF5	47	4	4	165.7	2.36	2.77E-04
	Pollen allergen Amb a 1.1 (*Ambrosia artemisiifolia*)	P27759	43	3	3	112.8	2.36	2.77E-04
	Pollen allergen Amb a 1.2 (*Ambrosia artemisiifolia*)	P27760	44	2	3	98.8	2.36	2.77E-04
25	**Beta-fructofuranosidase** (*Arabidopsis thaliana*)	Q8W4S6	62	6	8	435.1	1.99	1.88E-06
	Soluble inorganic pyrophosphatase (*Solanum tuberosum*)	Q43187	24	5	5	373.6	1.99	1.88E-06
	Glutamine synthetase cytosolic isozyme 1-2 (*Arabidopsis thaliana*)	Q8LCE1	39	4	5	353.5	1.99	1.88E-06
	Glutamine synthetase cytosolic isozyme 1-3 (*Arabidopsis thaliana*)	Q9LVI8	39	4	4	340.4	1.99	1.88E-06
26	**Alpha-1,4-glucan-protein synthase** (*Zea mays*)	P80607	41	2	2	85.2	2.21	1.63E-03
27	**NADH dehydrogenase iron-sulfur protein 8** (*Solanum tuberosum*)	P80269	26	4	5	376.7	-1.79	1.79E-03
29	**Phosphoglucomutase** (*Zea mays*)	P93805	63	7	8	661.5	-	1.48E-05
	VIII: Signalling proteins							
28	**14-3-3 protein 9** (*Solanum lycopersicum*)	P93214	29	9	11	1320	-1.63	2.05E-03
	IX: Photosynthesis							
30	**Photosystem I P700 chlorophyll a apoprotein A2** (*Nymphaea alba*)	Q6EW49	82	2	3	123	-2.63	2.33E-03

	Tubulin beta-9 (*Arabidopsis thaliana*)	P29517	50	2	2	110.5	-2.63	2.33E-03
31	**Photosystem II CP47 chlorophyll apoprotein** (*Atropa belladonna*)	Q7FNS4	56	2	2	87.7	-1.95	4.32E-06
	Glucose and ribitol dehydrogenase (*Oryza sativa subsp. japonica*)	Q75KH3	32	2	2	78.5	-1.95	4.32E-06
32	**Photosystem II D2 protein** (*Acorus americanus*)	Q4FFP4	40	2	2	111	-2.44	8.43E-04
	14-3-3-like protein A (*Solanum lycopersicum*)	P93214	29	2	2	109.9	-2.44	8.43E-04
	Phosphoglycerate kinase (*Nicotiana tabacum*)	Q42961	50	2	2	98.7	-2.44	8.43E-04
33	**Oxygen-evolving enhancer protein 1** (*Populus euphratica*)	P84989	11	3	4	201.5	2.77	3.83E-06
	X: Ammonia assimilation							
34	**Glutamine synthetase cytosolic isozyme 1-1** (*Arabidopsis thaliana*)	Q56WN1	39	3	4	243.7	+	1.89E-06
	Glutamine synthetase cytosolic isozyme 1-2 (*Arabidopsis thaliana*)	Q8LCE1	39	3	3	236.8	+	1.89E-06
	Glutamine synthetase cytosolic isozyme 1-3 (*Arabidopsis thaliana*)	Q9LVI8	39	2	3	159.8	+	1.89E-06
	XI: Protein biosynthesis, folding and degradation							
36	**26S protease regulatory subunit 6A homolog A** (*Arabidopsis thaliana*)	Q9SEI2	47	6	6	305.9	2.99	7.23E-06
37	**Chaperonin CPN60-2** (*Cucurbita maxima*)	Q05046	61	10	11	626.1	1.89	1.07E-04
	IX: Translation							
38	**Eukaryotic initiation factor 4A-10** (*Nicotiana tabacum*)	P41382	47	4	4	306.6	-2.04	5.96E-03
	Monodehydroascorbate reductase (*Cucumis sativus*)	Q42711	47	3	3	258.6	-2.04	5.96E-03
39	**Elongation factor 1-gamma 2** (*Oryza sativa subsp. japonica*)	Q6YW46	47	5	5	287.8	2.66	8.88E-05
	Actin-97 (*Solanum tuberosum*)	P30171	42	3	4	225.3	2.66	8.88E-05
	XII: Stress and defence related							
35	**Heat shock 70 kDa protein 9** (*Arabidopsis thaliana*)	Q8GUM2	73	8	11	704.2	-2.36	9.78E-04
	Heat shock 70 kDa protein 10 (*Arabidopsis thaliana*)	Q9LDZ0	73	6	7	683	-2.36	9.78E-04
40	**Soluble inorganic pyrophosphatase** (*Solanum tuberosum*)	Q43187	24	3	3	276.5	2.36	1.77E-02
	Enolase 1 (Hev b 9) (*Hevea brasiliensis*)	Q9LEJ0	48	2	3	225.6	2.36	1.77E-02
41	**Monodehydroascorbate reductase** (*Cucumis sativus*)	Q42711	47	3	4	297.6	2.63	1.57E-11
	Phosphoglycerate kinase (*Nicotiana tabacum*)	Q42961	50	3	3	225.7	2.63	1.57E-11
	Chaperonin CPN60 (*Cucurbita maxima*)	Q05046	61	2	3	198.8	2.63	1.57E-11
42	**Proteasome subunit alpha type-3** (*Oryza sativa subsp. japonica*)	Q9LSU0	27	10	12	1759.4	2.58	1.11E-05
43	**Aconitate hydratase 1** (*Arabidopsis thaliana*)	Q42560	98	14	18	1318	-1.69	1.26E-03
44	**Catalase isozyme 1** (*Cucurbita pepo*)	P48350	57	5	8	623.5	3.03	2.32E-06
	Glutamine synthetase cytosolic isozyme 1-2 (*Arabidopsis thaliana*)	Q8LCE1	39	5	6	557.8	3.03	2.32E-06
45	**Probable protein phosphatase 2C 59** (*Arabidopsis thaliana*)	Q8RXV3	33	2	2	102	1.99	1.48E-05

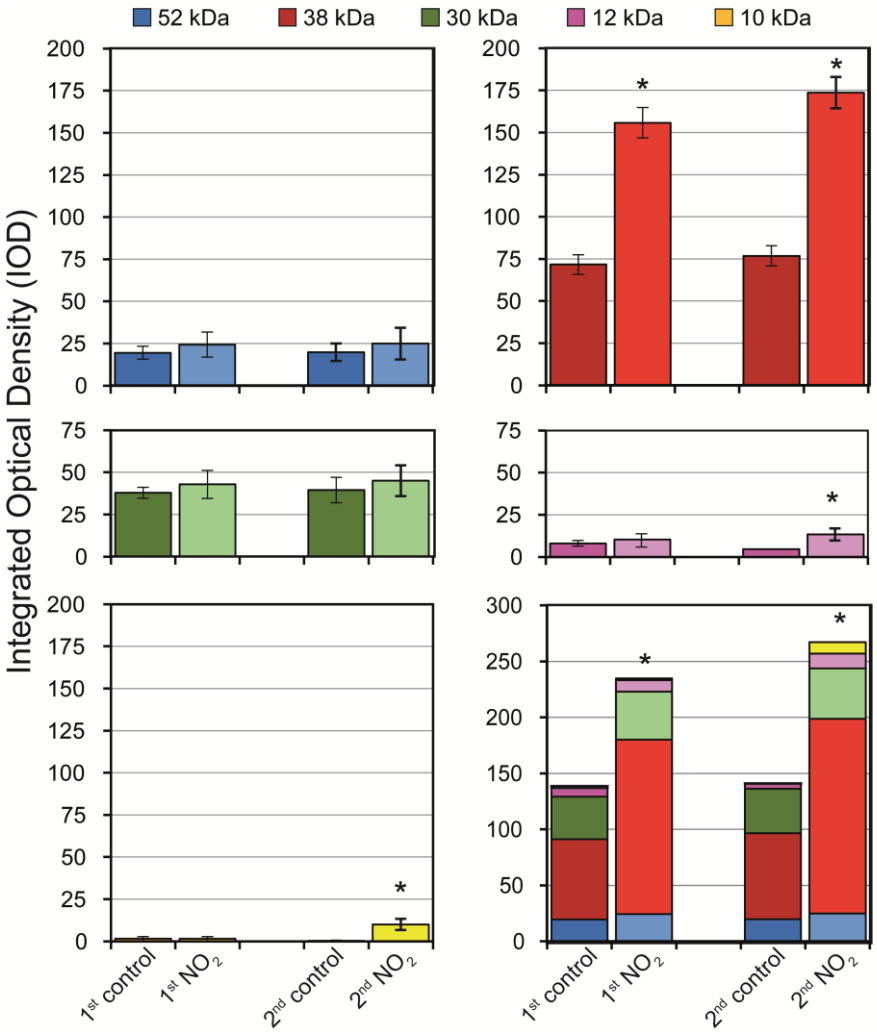
	Phosphoglycerate kinase (*Nicotiana tabacum*)	Q42961	50	2	2	96.3	1.99	1.48E-05
46	**Mitogen-activated protein kinase 9** (*Arabidopsis thaliana*)	Q9LV37	58	4	4	193.9	2.45	3.29E-03
47	**Probable lactoylglutathione lyase** (*Arabidopsis thaliana*)	Q8W593	39	3	3	129.6	-2	5.02E-06
	V-type proton ATPase catalytic subunit A (*Hordeum vulgare*)	Q40002	64	2	3	112.2	-2	5.02E-06
	Actin-97 (*Solanum tuberosum*)	P30171	42	2	2	110	-2	5.02E-06
48	**NADP-dependent malic enzyme** (*Mesembryanthemum crystallinum*)	P37223	64	3	5	269.7	-1.77	2.59E-04
	XIII: Methyltransferase related							
49	**Serine hydroxymethyltransferase 1** (*Flaveria pringlei*)	P49357	57	2	2	107.3	-2.37	2.25E-03
	Tubulin beta-9 (*Arabidopsis thaliana*)	P29517	39	2	2	99.8	-2.37	2.25E-03
	Monodehydroascorbate reductase (*Cucumis sativus*)	Q42711	47	2	2	89.6	-2.37	2.25E-03
50	**5-methyltetrahydropteroyltriglutamat--homocystein methyltransferase** (*Mesembryanthemum crystallinum*)	P93263	85	10	16	1721.1	-1.63	1.39E-03
51	**Adenosylhomocysteinase 1** (*Arabidopsis thaliana*)	O23255	53	5	6	457.8	2.11	5.48E-03
	Isocitrate dehydrogenase (*Glycine max*)	Q06197	46	5	5	396.8	2.11	5.48E-03
	XIV: Transport related							
52	**ATP synthase subunit alpha** (*Eucalyptus globulus*)	Q49L13	56	8	8	497.8	2.09	6.50E-03
53	**V-type proton ATPase catalytic subunit A** (*Hordeum vulgare*)	Q40002	64	11	12	741.3	-2.22	1.46E-05
54	**Sorting nexin 1** (*Arabidopsis thaliana*)	Q9FG38	47	2	2	112.3	2.56	1.76E-07
	Protein disulfide isomerase (*Oryza sativa ssp. japonica*)	Q67UF5	47	2	2	99.6	2.56	1.76E-07
55	**Importin subunit alpha-1A** (*Oryza sativa subsp. japonica*)	Q71VM4	58	2	2	79.1	-1.93	2.36E-10
	GDP-mannose 3,5-epimerase (*Arabidopsis thaliana*)	Q93VR3	43	2	2	68.9	-1.93	2.36E-10
	XV: Others							
56	**Cysteine-rich repeat secretory protein 38** (*Arabidopsis thaliana*)	Q9LRJ9	28	4	4	290.9	-1.88	4.77E-05
	Glucose and ribitol dehydrogenase (*Oryza sativa subsp. japonica*)	Q75KH3	32	3	3	220.6	-1.88	4.77E-05
57	**Histone H4 variant TH011** (*Triticum aestivum*)	P62785	11	2	2	68.8	-1.76	4.09E-04



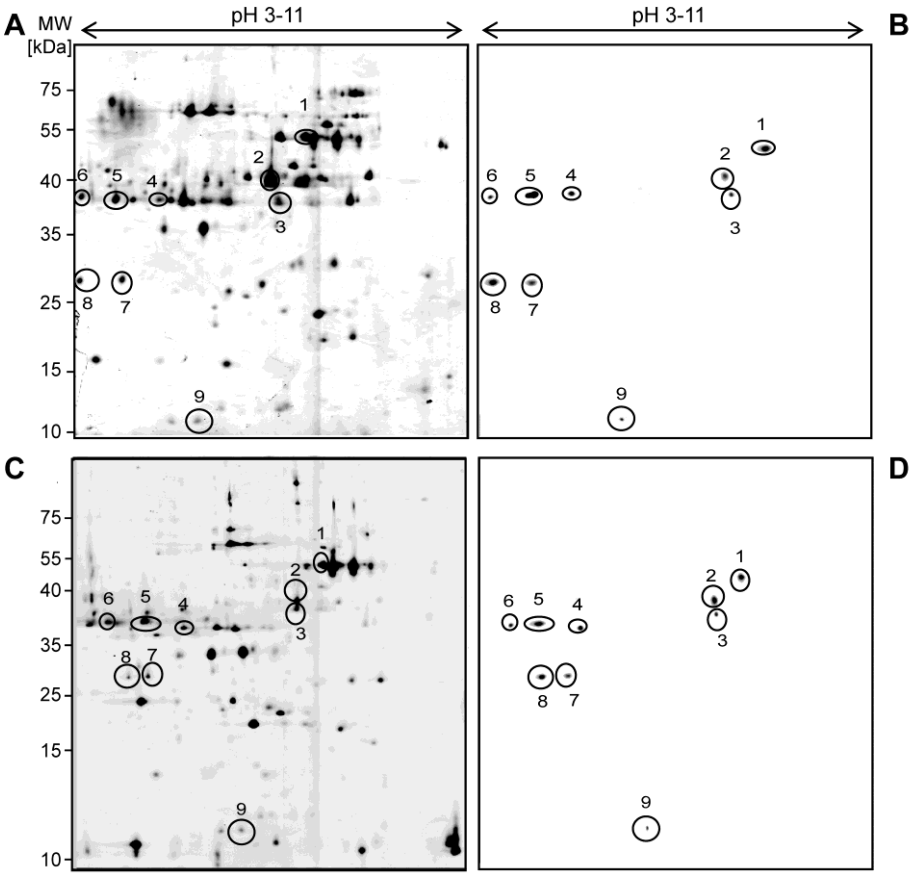
Supporting information 7: Functional classification of LC-MS/MS identified proteins under 40 ppb NO_2 and 80 ppb NO_2. Red indicates up-regulation of protein expression under 80 ppb NO_2. Green gives the number of down-regulated proteins under 80 ppb NO_2 conditions.



Supporting information 8: Western blot detection of S-nitrosylated proteins. *In vivo* S-nitrosylated proteins can be detected in the 1st and 2nd generation of pollen. Examples are given for the 1st control = 40 ppb; the 2nd control = clean air and 80 ppb for the 1st and 2nd generation. The positive control for the biotin switch assay is indicated by 250µM GSNO (GSNO treated pollen proteins). Biotin switch assay without MMTS, without ascorbate or plus 100 mM DTT demonstrate the specificity of the assay. Relative masses of protein standards are shown on the right side.



Supporting information 10: Statistical analysis of 1-D Western immunoblot for ragweed allergens. Statistical analysis of the major allergens in different pollen extracts and the contribution of single bands to the total allergenicity (* = p-value < 0.05; One way-ANOVA).



Supporting information 11: Allergen detection in ragweed pollen by 2-D Western immunoblot analysis. Exemplary two 2-D gels with corresponding Western blots are shown. Equal amounts of protein (300 µg) have been separated on a 2-D gel. Gels were then silver stained. **(A)** 2-D PAGE of 1st generation ragweed pollen extract from plants that were grown under 80 ppb NO_2. **(B)** Corresponding Western immunoblot to A. **(C)** 2-D PAGE of pollen extracts from control plants (40 ppb NO_2, 1st generation). **(D)** Corresponding 2-D Western immunoblot to C.