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"Common ragweed (*Ambrosia artemisiifolia*): Systems biology of the allergenic pollen upon elevated NO_2 concentrations"

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II. ABBREVIATIONS

Acronym	Definition
2D-DIGE	Two-dimensional difference gel electrophoresis
ABA	Abscisic acid
ABC	Ammoniumbicarbonate
ACN	Actetonitrile
AllFam	Database for classifying allergens into protein families
ANOVA	Analysis of variation
BCIP	5-Bromo-4-chloro-3´indolyphosphate-p-toluidine-salt
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
BVA	Biological variance analysis
CaM	Calmodulin
CBPs	Calcium binding proteins
CCD	Charge-coupled device
cDNA	Complementary DNA
ChIP-Seq	Chromatin immunoprecipitation sequencing
CO_2	Carbon dioxide
cpDNA	Chloroplast DNA
CPI	Cystatin Proteinase Inhibitor
Cy2	Cyanine dyes 2
Cy3	Cyanine dyes 3
Cy5	Cyanine dyes 5
Cy-dye	Cyanine dyes
Cys	Cysteinyl residue
ddATP	2',3'-Dideoxyadenosine-5'-triphosphate
ddCTP	2',3'-Dideoxycytidine-5'-triphosphate
ddGTP	2',3'-Dideoxyguanosine-5'-triphosphate
ddTTP	2',3'-Dideoxythymidine-5'-triphosphate
DIA	Differential In gel analysis
DIGE	Difference gel electrophoresis
DPI	Dots per inch
DTT	Dithiothreitol
FAD	Flavin adenine dinucleotide
GA	Genome analyzer
GABA	Fluctuate intracellular γ -Aminobutyric acid
Glu	Glutamate
Gln	Glutamine
GO	Gene Ontology
GS	Glutamine synthetase
GSNO	S-Nitrosoglutathione
H_2O_2	Hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HLA-DR	Human leukocyte antigen DR
HPLC	High-performance liquid chromatography
HNO_3	Nitric acid
IAA	Iodacetamide
IEF	Isoelectric focusing
IgE	Immunoglobulin E
IOD	Integrated optical density
IPG	Immobilized Ph Gradient
LC-MS / MS	Liquid chromatography - mass spectrometry
LEA	Late embryogenesis abundant
MDA	Monodehydroascorbate
MDHAR	Monodehydroascorbate reductase
MMTS	S-Methyl thiomethanesulfonate
MS	Mass electroscopy
MW	Molecular weight
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NBT	Nitrotetrazolium blue chloride
NCBI	National center for biotechnology information
NGS	Next generation sequencing
NH_3	Ammonia
NO	Nitric oxide
NO_2	Nitrogen dioxide
nsLTP	Non specific lipid transferase protein
O_3	ozone
OMI	Ozone monitoring instrument
PALMs	Pollen-associated lipid mediators
PCR	Polymerase chain reaction
pI	Isoelectric point
PRs	Pathogenesis related proteins
PSI	Photosystem I
PSII	Photosystem II
PTM	Post translational modification
qRT-PCR	Real time quantitative PCR
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
RT	Reverse transcriptase
RT-PCR	Reverse transcription-polymerase chain reaction
SBS	Sequencing by synthesis
SDS	Sodium dodecyl sulfate
SE	Standard error
SSH	Suppression subtractive hybridization
TFA	Trifluoroacetic acid

III. SUMMARY

The pollen of ragweed (*Ambrosia artemisiifolia*) is one of the strongest allergy triggers known and can cause allergic reactions such as asthma very quickly. Global warming and changes in the environment (e.g. air pollution, heavy metal ions, etc.) will result in an earlier and longer pollen season, enhanced pollen production and an increase in pollen allergenicity with a negative effect on atopic patients.

In this study, we investigated the impact of elevated NO_2 at the transcriptome and proteome levels of ragweed pollen. In addition the allergenicity of pollen was also analyzed. Plants were exposed to 80 ppb NO_2 over a whole vegetation period and the results from transcriptional analyses of ragweed pollen performed by Illumina sequencing technology, suppression subtractive hybridization and quantitative real-time RT-PCR were described. Two-dimensional difference gel electrophoresis (2D-DIGE) was carried out to analyse the changes and abundances of proteins, and S-nitrosylation assay was performed for the S-nitrosylated protein analysis of ragweed pollen. The variation of pollen allergenicity was revealed by immunoblotting analysis like dot blot, 1D-blotting and 2D-blotting. The influence of elevated NO_2 on morphological changes of ragweed like inflorescence length, 50 seed weight, total seed weight and pollen weight had also been assessed.

The experiment, using realistic outdoor light, temperature and NO_2 fumigation conditions, allowed the investigation of the effects of NO_2 on ragweed over the entire growing season. Elevated NO_2 significantly increased pollen production of the first and second generation plants, while no significant changes could be observed with regard to inflorescence length. Elevated NO_2 decreased the 50 seed weight and total seed weight in both generations.

The qRT-PCR analysis of allergens showed that Amb a 1 is the most abundant of ragweed pollen, and all of the tested allergens were induced by elevated NO_2 except

Amb a CPI as compared to control pollen. Among them, Amb a 1.1, Amb a 1.2, Amb a 8 and Amb a 9 were significantly increased in both generations.

Analysis of the suppression subtractive hybridization showed that most upregulated transcripts were involved in stress resistance whereas most of the downregulated transcripts were involved in reproductive process.

Illumina sequencing of pollen of ragweed generated 587,972,236 and 517,737,064 reads (length 100 bp) in the first and second generation, respectively. *De novo* assembly generated 35,136 unique transcripts with an average length of 722 bp. Further annotation of the ragweed unique transcripts was performed and a total of 16,361 unique transcripts (46.56%) were assigned to at least one GO term. Among them, 6,370 (38.93%) were assigned to the biological process category, 5,832 (35.65%) to the molecular function category, and 6,932 (42.37%) to the cellular component category, while 4,601 (28.12%) unique transcripts were assigned to GO terms of all three categories. A comparative transcriptome analysis by RNA-seq revealed that GO terms of biological process including “response to cadmium”, “response to salt stress” and “pollen tube growth” were the three most enriched groups of both, induced and repressed genes of both generations. In GO term of molecular function, the “ATP binding” was the largest group in both induced and repressed genes of both generations, followed by the “protein binding”, the “zinc ion binding” and the “DNA binding”. The expression patterns of allergens were analyzed by RNA-seq according to the number of reads per contig in the Illumina libraries. Parts of allergens were upregulated and the results showed that although the exact fold changes of the transcripts varied between RNA-seq expression and qRT-PCR analyses, the variation trend indicated good consistency between the two analytical techniques.

Two-dimensional difference gel electrophoresis (2D-DIGE) and proteomic analysis showed that Amb a 1 isoforms as well as another allergen with homology to Hev b 9

from *Hevea brasiliensis* were upregulated in ragweed pollen due to the elevated NO_2. This confirmed the transcriptomic analysis which demonstrated that elevated NO_2 upregulated the expression of genes encoding allergenic proteins in ragweed pollen. A high percentage of the accumulated spots (23%) was related to stress resistance of the NO_2-induced proteins, followed by allergens (17%) and metabolic enzymes (14%). Among the depressed proteins, glycolysis proteins account for the biggest part (15%) followed by photosynthesis and stress proteins both of which account for 11%.

The analysis of protein S-nitrosylation identified 25 and 19 S-nitrosylated proteins in the pollen of ragweed exposed to 80 ppb and 40 ppb NO_2, respectively. Elevated NO_2 significantly increased protein S-nitrosylation of ragweed pollen and this is the first time to show S-nitrosylated allergens in pollen of ragweed.

Moreover, immunoblotting analysis with allergic patients' serum revealed the changes of allergenicity of pollen from ragweed plants exposed to different concentrations of NO_2. The results proved that elevated NO_2 significantly increased the allergenicity of ragweed pollen with Amb a 1.1 contributing more than half of the total allergenicity of ragweed pollen consistent with the results of former researches.

Our study adds a new set of crucial data which will help to further understand the transcriptomic and proteomic changes which occur in ragweed pollen under elevated NO_2 (80 ppb). The whole vegetation period exposure of common ragweed to elevated NO_2 (80 ppb) caused a significant decrease of seed production but at the same time an increase of pollen production and allergenicity.

1. Chapter – INTRODUCTION

1.1 Common ragweed (*Ambrosia artemisiifolia*)

Common ragweed (*Ambrosia artemisiifolia*) was described by Carl Linnaeus in the 18th century from North America (Linnaeus 1753). It is a herbaceous species belonging to the Asteraceae family and has expanded its distribution out of its native range to Europe (Chauvel et al. 2006), Australia (McFadyen 1984), Asia (Xu et al. 2006), Africa and South America (Qbank). In Europe, common ragweed started to attract people's attention in the first decade of the 21st century. It has recently increased in prominence as due to its allergenicity. Its ecological features and anemophilous pollination strategy have also made it an important agricultural weed (Mulligan 1979) as well as an important threat to environmental health (Oswalt and Marshall 2008).

1.1.1 Ecology of common ragweed

Common ragweed is an erect annual broadleaf herb. It has a defined bushy-branched or branchless hairy stem which contains compound pinnately lobed leaves with whitish nerves. It has a superficial, fibrous root system and mature common ragweed can grow up to 250 cm high (Watson and Teshler 2013). Male and female flowers are in separate flower heads on the same monoecious habit plant. The male heads are composed of 10 to 100 arranged flowers at the tops of stems and branches. The female flower heads, however, are green, stemless, and inconspicuous (Simard and Benoit 2011). The main flowering season of common ragweed begins in August and ends in October. Minor amounts of airborne pollen, however, can also be found in June and November (Zink et al. 2012). The seeds of common ragweed are about 3 mm long with several short, thorny juts close to one end. Each plant can easily produce 30,000 to 62,000 seeds which can be easily distributed by rain, birds, burrowing animals, and humans (Smith et al. 2013). The seeds can remain dormant for at least 39 years if conditions are unsuitable for germination (Mulligan 1979).

Common ragweed is widespread in crop fields and it can be abundant in corn, soybean, and some horticultural crops (Simard and Benoit 2012). It can also successionally grow in disturbed areas such as roadsides, waste areas and dwellings under construction (Bazzaz 1974, Lavoie et al. 2007).

Common ragweed was introduced into Europe during the last half of the 19th century. It remained negligible in many of the European countries for more than a century. An accelerated spread of the plant has been observed in the last twenty years and it can now be detected in almost every European country (Dahl et al. 1999, Járai-Komlódi 2000, Rybníček et al. 2000, Taramarcaz et al. 2005, Köhler et al. 2006, Trigo and Garcia-Sanchez 2006, Alberternst and Nawrath 2008). The spreading of common ragweed plants in Germany occurred later, but was extremely fast. In 2007, the infested area in Germany was 10 times larger than that found in 2000 (Alberternst and Nawrath 2008).

1.1.2 Allergenic potential of ragweed pollen

Common ragweed is the reason for the most severe and widespread allergies caused by its pollen (Csepe et al. 2014). The first allergy of common ragweed pollen was characterized as 'autumnal catarrh' by Wyman in the United States during the last half of the 19th century (Wyman 1875). Through the years, ragweed pollen has become the second most important key reason for seasonal asthma and rhinitis in various cities in the United States (White and Bernstein 2003, Salo et al. 2011) and Canada (Chan-Yeung et al. 2010). In Europe, dramatically increased clinical relevance has been observed in the last decades (Burbach et al. 2009). More than 66% of 3,034 patients were allergic to ragweed as reported in a recent European study (Bousquet et al. 2009). A full-grown plant can produce about 10^9 pollen grains during 1 year (Fumanal et al. 2007) and it has been revealed that concentrations lower than 5 – 10 ragweed pollen / m^3 can trigger afflictions for atopic persons (Taramarcaz et al. 2005).

The spatial distribution of airborne ragweed pollen from the most important allergenic taxa in Europe has recently been described in a multi-author publication (Sauliene and Veriankaite 2012, Skjøth et al. 2013) (Fig. 1). As shown, the worst affected areas by common ragweed in Europe is the Pannonian Plain including Ukraine, Hungary and some parts of Serbia, Croatia, Slovenia, Slovakia and Romania, furthermore most parts of France, Italy and the south-east of Germany.

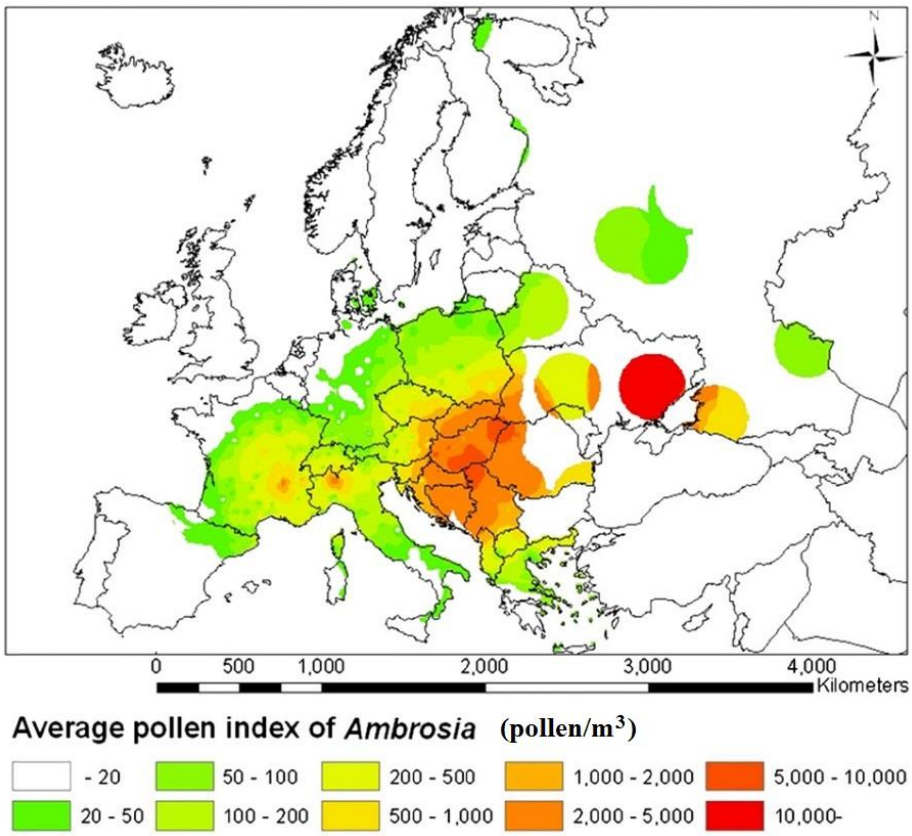


Figure 1. A spatial assessment of the density of naturalised *Ambrosia* populations with flowering potential. The map is based on the mean annual pollen index of *Ambrosia* from 368 stations in Europe (Skjøth et al. 2013).

1.1.2.1 Mechanisms behind pollen allergy

Allergy is also known as hypersensitivity and indicates conditions in which immune responses result in inconvenient consequences for individuals (Traidl-Hoffmann et al. 2009). Pollen has always been considered as allergen carriers. The pollen-induced allergic reaction may arise when pollen reaches the mucosa and proteins of the pollen surface, wall or cytoplasm, among them allergens, dissolve in the mucosal

fluid. There are two steps that can be distinguished in this allergic reaction: the sensibilization phase and the effector phase. 1) **Sensitization phase**: Once exposed to the allergens of pollen, the immune system generates specific immunoglobulin E (IgE) and IgG antibodies, which transmigrate and combine to the surfaces of mast cells and basophils in epithelial tissues. 2) **Effector phase**: On a subsequent exposure, allergens bind and crosslink such specific IgE antibodies existing on the surface of basophils and mast cells, which then release the mediators of the allergic response, such as histamine and cytokines. The stimulation of the nasal nerves with these chemical compounds provokes reflexive sneezing, runny nose, whereas the stimulation of the blood vessels causes swelling of the mucosa, leading to congestion in the nose (the sniffles) (Peter et al. 2011).

Apart from allergens, pollens release bioactive, non-allergenic, pollen-associated lipid mediators (PALMs), which may have immunomodulatory and proinflammatory effects on the cells of the allergic immune response (Behrendt et al. 2001). Furthermore, pollen release non-allergenic reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, which have negative effects on airway epithelial cells by generating reactive oxygen species (ROS) (Boldogh et al. 2005, Dharajiya et al. 2007). Lastly, pollen can also carry biogenic and anthropogenic factors that affect the allergen release, generate novel allergenic epitopes, and regulate the epithelial microenvironment (Traidl-Hoffmann et al. 2009).

1.1.2.2 The physiological roles of allergens in pollen

Structural and architectural features of allergenic proteins appear to play a role in their allergenicity. Researches have shown that pollen allergens from almost all plant species belong to only a few (29 out of 7,868) protein families (Radauer and Breiteneder 2006, Breiteneder 2008).

Interestingly not all members of these families are allergenic. Thus, allergenicity of a given protein is not just dependent on the amino acid sequence or structure of the

protein, but is also associated with the localization and the amount of the protein. Generally, cells keep a tight control over the concentration and localization of proteins. The fact that allergenic proteins are present in high amount indicates that these proteins have physiological roles required by pollen. According to the data from Allfam (http:// www.meduniwien.ac.at/allergens/allfam/), the top 5 families of pollen allergens are profilin, expansins, prolamin, EF-hand domain containing proteins, and Bet v 1-related proteins (Radauer et al. 2008). These seemingly arbitrary families of proteins are indeed playing significant roles in pollen germination and growth (Fig. 2). Profilin binding to actin modulates actin microfilament polymerization (Kandasamy et al. 2007); expansins are involved in cell wall-loosening to allow penetration of the stigma (Cosgrove et al. 1997); calcium signaling pathways required for pollen tube growth are mediated by calcium binding proteins such as EF-hand proteins (Steinhorst and Kudla 2013); the secreted nsLTPs may function in pollen tube guidance down the transmitting tract in an autocrine-like manner (Radauer and Breiteneder 2006); polar growth of pollen tube requires massive cycling of vesicles along actin microfilaments (Songnuan 2013).

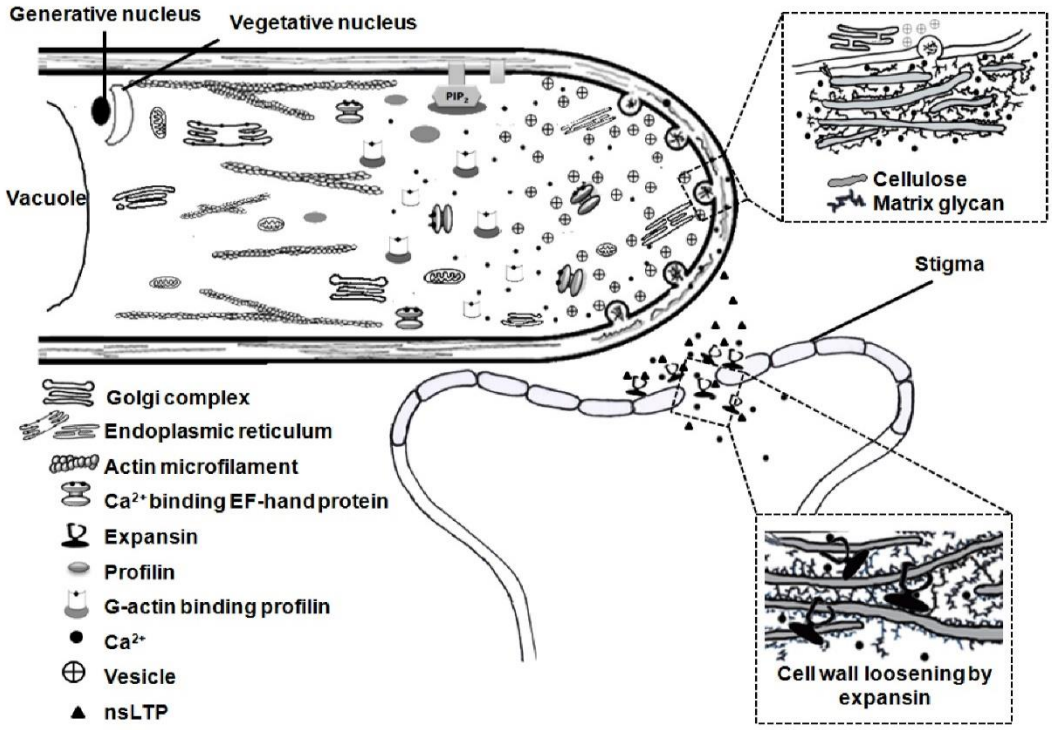


Figure 2. Functions of top allergenic proteins in pollen growth (Songnuan 2013)

1.1.2.3 The major allergens of ragweed pollen

The pollen of common ragweed is recognized as highly allergenic. Amb a 1, a 38-kDa non-glycosylated protein belongs to the family of pectate lyase proteins, is the major allergen in ragweed pollen. It is a highly allergenic molecule that is recognized by 90% of ragweed-sensitized individuals and is regarded as a good detector for specific ragweed sensitization (Gadermaier et al. 2008). At the protein level, Amb a 1 constitutes up to 15% of total protein in ragweed pollen extracts, as identified by quantitative ELISA (Gadermaier et al. 2014). Minor allergens, Amb a 3, Amb a 4, Amb a 5, Amb a 6, Amb a7, Amb a 8, Amb a 9, Amb a 10, Amb a 11 and Amb a CPI have been also identified (Tab. 1). A recent study identified the presence of allergenic terpenoids in common ragweed pollen grains (Taglialatela-Scafati et al. 2012). These secondary metabolites are known to induce potent inflammatory responses and therefore can enhance the sensitizing properties of the allergenic proteins of common ragweed pollen grains.

Allergen	Molecular Weight (kDa)	Allergenicity	Isoforms	Family	Allergenicity reference
			Amb a 1.0101		(Rafnar et al. 1991)
			Amb a 1.0201		(Rafnar et al. 1991)
			Amb a 1.0202		
			Amb a 1.0301		(Rafnar et al. 1991)
			Amb a 1.0302		
Amb a 1	38	Major	Amb a 1.0303	Pectate lyase	(Rafnar et al. 1991)
			Amb a 1.0304		
			Amb a 1.0305		
			Amb a 1.0401		(Rogers et al. 1991)
			Amb a 1.0402		
			Amb a 1.0501		(Rogers et al. 1991)
			Amb a 1.0502		
Amb a 3	11	Minor	Amb a 3.0101	Plastocyanine	(Adolphson et al. 1978)
Amb a 4	30	Minor	Amb a 4.0101	Defensin-like protein	(Leonard et al. 2010)
Amb a 5	5	Minor	Amb a 5.0101	Group 5 ragweed allergen	(Ghosh et al. 1993)
Amb a 6	10	Minor	Amb a 6.0101	Lipid transfer protein (plant)	(Roebber et al. 1983)
Amb a 7	12	Minor	Amb a 7.0101	Plastocyanin	
Amb a 8	14	Minor	Amb a 8.0101	Profilin	(Wopfner et al. 2008)
			Amb a 8.0102		(Wopfner et al. 2008)
Amb a 9	10	Minor	Amb a 9.0101	Polcalcin	(Wopfner et al. 2008)
			Amb a 9.0102		(Wopfner et al. 2008)
Amb a 10	18	Minor	Amb a 10.0101	Polcalcin-like protein (4 EF-hands)	(Wopfner et al. 2008)
Amb a 11	37/52	Minor	Amb a 11.0101	Cysteine protease	
Amb a CPI	11	Minor	—	Cystatin Proteinase Inhibitor	(Rogers et al. 1993)

Table 1. Allergens in common ragweed pollen.

1.1.3 Effect of environmental changes on common ragweed

Climate changes may increase the severity of pollen stimulated atopic disease by influencing the large scale distribution and local incidence of allergenic species, the flowering time, the pollen production and the allergenicity of individual pollen grains (Storkey et al. 2014). An underlying element of climate change is the potential changes in flowering phenology and pollen initiation associated with milder winters and warmer seasonal atmospheric temperatures. The climate change might affect allergic disease by several potential mechanisms. Firstly, extended pollen seasons may perhaps increase the duration of allergy symptoms in individuals with allergic disease. Secondly, the prolongation of human exposure to aeroallergens caused by longer pollen seasons may thus increase allergic sensitization. Finally, increasing atmospherical pollen counts might enhance the severity of allergic symptoms (Ziska et al. 2011, Csepe et al. 2014, Storkey et al. 2014).

Rogers et al. (2006) revealed that ragweed plants derived from seeds breaking dormancy earlier in spring accumulated more resources through the season. The increased biomass and reproductive effort yielded a 54.8% higher pollen production as compared to those released from dormancy 30 days later. However, the ragweed plants released from dormancy later, but grown in elevated levels of CO_2 afterwards, had increased biomass and pollen production, which compensated for any disadvantages associated with a later beginning of the growing season (Rogers et al. 2006, Rueff et al. 2012). Researches have shown that under twice the normal CO_2 concentration, pollen production of individual ragweed plants increases 30% to 90% (Levetin and Van de Water 2001, Rogers et al. 2006).

Ozone can affect plant metabolism. Its toxicity is due to the generation of reactive oxygen species (ROS), such as the superoxide anion radical ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), and singlet oxygen ($^1O_2$) (Mustafa 1990). Pasqualini et al (2011) reported that after fumigation with 100 ppb ozone, pollen viability was decreased. However, a significant enhancement of the ROS-generating

enzyme NAD(P)H oxidase was found, which implied that ozone may increase ragweed pollen allergenicity through stimulation of ROS-generating NAD(P)H oxidase (Pasqualini et al. 2011).

Ragweed in urban locations where air pollutant concentrations and temperatures are higher show earlier flowering and increased pollen production (Ziska et al. 2004). Automobile exhaust pollution might also influence the allergenicity of common ragweed pollen. Pollen from ragweed plants growing in urban parks and along high-traffic roads that are highly exposed to pollution was more allergenic than the pollen of plants from countryside areas (Ghiani et al. 2012).

1.1.4 Control of common ragweed

Common ragweed, which is native to North America, was unintentionally introduced to new regions or brought along with various agricultural produce as an invasive species (Laaidi et al. 2003, Simard and Benoit 2011). It ousts indigenous plant species and its pollen allergens get into respiratory tracts with inhaled air (Taramarcaz et al. 2005, Stach et al. 2007, Tosi et al. 2011). Common ragweed and its pollen cause serious health problems and large sums of losses of the economy and in various fields of daily life. The European and Mediterranean Plant Protection Organization have considered common ragweed to be an invasive alien plant since 2004 (https://www.eppo.int/). The estimated costs of common ragweed in terms of human health and agriculture for 40 European countries had been published in 2010. The human healthcare influences were estimated to affect around 4 million people and the total estimated medical costs was € 2,136 million per year. Moreover, the annual workforce productivity losses due to common ragweed were estimated up to € 529 million (James 2010). Therefore, controlling this weed is not only an agricultural issue, as most weeds are, but also an increasingly important public health concern.

Seeking to ensure environmental quality both in rural and urban territories is a major challenge for ragweed control in Europe (Brunel et al. 2010). Currently, control options are recommended at two levels: (1) The countries still without ragweed populations have to eliminate any vectors that may introduce ragweed *via* contaminated crop seeds; (2) all countries that are already invaded by naturalised ragweed populations should establish strict directives to control these populations and try to avoid further spread at the local or regional scale.

Common ragweed is extremely resistant to atmospheric and soil pollution (Pichtel et al. 2000, Ziska 2002) and it is still able to reproduce after destruction such as mowing and defoliation (Brandes and Nitzsche 2006). Currently, common ragweed is reasonably controlled in almost all major crops by mechanical, chemical or combined measures, but has been revealed to develop resistance to herbicides over time (Louis et al. 2005, Tokarska-Guzik et al. 2011). Another problem is that harvesters and mowers may transport tens of thousands of ragweed seeds from infected areas or roadside verges to places that have not yet been infected (Vitalos and Karrer 2009, Karrer et al. 2012). Complete cleaning of machines after working in infested areas is the most effective way to solve this problem. Additional approaches like crop rotation and flora management can be used for ragweed control on cultivable fields and other heavily infested habitat types like roadsides (Kazinczi et al. 2008).

The long-term control of common ragweed is using biological measures (Gerber et al. 2011, Fukano and Yahara 2012). In Canada, the oomycete *Pustula tragopogonis* has been described from common ragweed (Hartmann and Watson 1980). Attack by *P. tragopogonis* can be very damaging and significantly reduces pollen and seed production if systemic infection is achieved. The beetles *Zygogramma suturalis Fabricius* and *Ophraella communa* LeSage are natural enemies of common ragweed and were studied as inundative biological control agents (Watson and Teshler 2013). Gerber et al (2011) identified 18 insects and 5 fungal pathogens to be promising

candidates for a classical biological control approach of common ragweed in Europe. Virtually, almost all natural enemies that have colonized common ragweed in Europe are polyphagous and cause little damage, making them unsuitable for a system management approach (Smith et al. 2013). A combination of biological agents with other common ragweed control tools will more likely be needed to create acceptable methods for common ragweed management.

1.2 Nitrogen dioxide (NO_2)

Air pollution is a worldwide challenge with increasing importance. Nitrogen dioxide is one of the major pollutants in the atmosphere. It is an important atmospherical trace gas because (1) of its health effects on human beings; (2) it absorbs visible solar radiation and plays a role in reduced atmospheric visibility; and therefore has (3) a potentially direct influence on worldwide climate change as an absorber of visible radiation; (4) it is a main regulator of the oxidizing capacity of the free troposphere by controlling the build-up and fate of radical species, including hydroxyl radicals; and (5) it plays an important role in determining ozone (O_3) concentrations because the photolysis of nitrogen dioxide is the only key initiator of the photochemical formation of ozone (World Health 2000).

1.2.1 General description of nitrogen dioxide

Nitrogen dioxide gas is a strong oxidant of reddish-brown colour, which is soluble in water (Wiberg et al. 2001). Combustion of fossil fuels in stationary sources (power generation, heating) and in motor vehicles (internal combustion engines) is the major source of anthropogenic emissions of nitrogen oxide. Therefore, NO_2 concentrations are increased in industrial, downtown and roadside areas (Koehler et al. 2013). Nitric oxide is another major source of nitrogen dioxide in the atmosphere. In most ambient circumstances, nitric oxide is released and transformed into nitrogen dioxide in the atmosphere. Even at low levels of reactants present in the atmosphere, oxidation of nitric oxide by atmospheric oxidants such as ozone occurs rapidly (World Health 2000). Altshuller (1956) estimated that 50% transformation of

nitric oxide would take less than 1 minute at a nitric oxide concentration of 0.1 ppm in the presence of an ozone concentration of 0.1 ppm.

Nitrogen dioxide, together with nitric oxide, comprise a main types of air pollutants which affect aerosol formation and atmospheric chemistry subsequently influencing the global environment. Atmospheric NO_2 is commonly detrimental to organisms that are exposed to this gaseous free radical because of its reactive nature (Lerdau et al. 2000, Kondo et al. 2008). Additionally, atmospheric NO_2 is a greenhouse gas that affects the radiation budget of the Earth (Solomon et al. 1999) and which is also responsible for agricultural crop reduction and forest ecosystem destruction (Solomon et al. 1999). Finally, other ecological problems are induced when nitrogen dioxide is wahed out of the atmosphere. Most of the nitrogen dioxides are removed from the atmosphere through the formation of nitric acid (HNO_3), which is one of the main components of acid rain. Meanwhile, an increase of nitrogen in surface waters could subsequently bring about eutrophication (Atkins et al. 1992).

To minimize the influences of nitrogen dioxides, worldwide efforts are undertaken to monitor its level and decrease emissions to the environment (Schaap et al. 2013). The mean global tropospheric NO_2 is shown as measured by Ozone Monitoring Instrument (OMI) during the period of September 2013 (Fig. 3). The mean NO_2 concentration in cities from over 141 countries is reported to be 27 ppb (Takahashi and Morikawa 2014). As can be seen in this picture, clearly visible high concentrations are detected in the industrial regions in China, Europe, South-Africa and the USA.

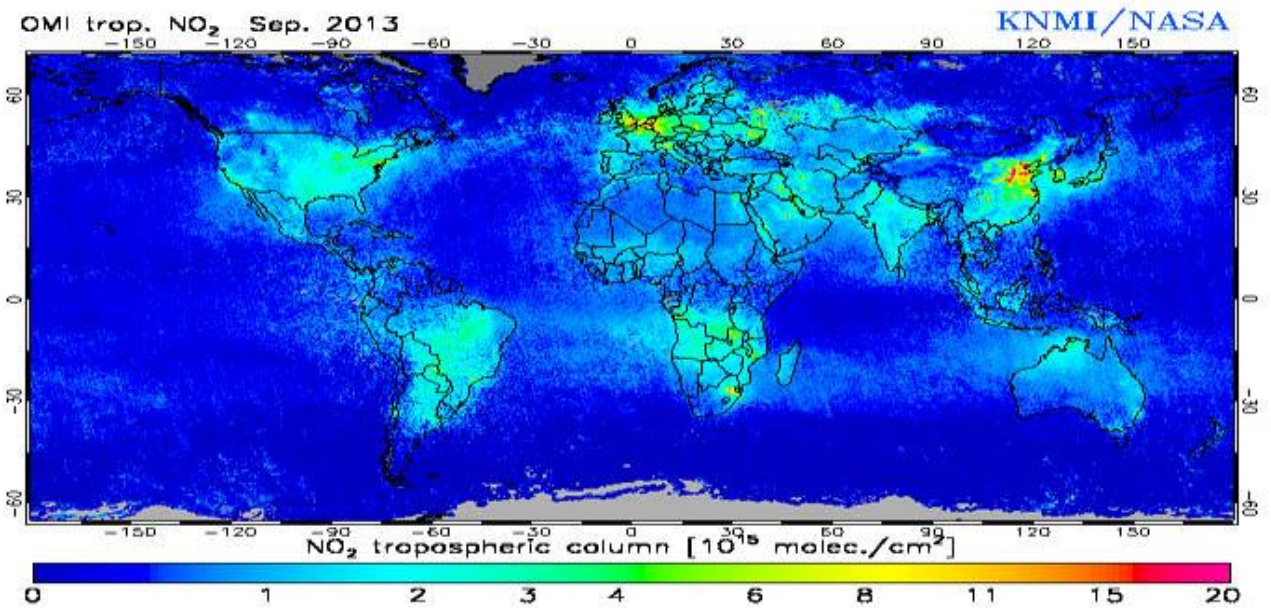


Figure 3. Global Map of Tropospheric Column Density of Nitrogen Dioxide observed by the Ozone Monitoring Instrument (OMI), space-borne sensor of NASA (Unit: 10^{15} molecules/cm^2).

1.2.2 Health effects of nitrogen dioxide

Exposure to NO_2 can lead to health impacts. It can aggravate the lungs and result in lower resistance to respiratory infections like influenza. Frequent exposure to higher concentrations may cause serious respiratory disease. Moreover, NO_2 also leads to aerosol formation, which is also harmful to health (Wiberg et al. 2001). The World Health Organization (WHO) defines the annual limit value of NO_2 as corresponding to 0.02 ppm (World Health 2000). Higher NO_2 levels could be associated with increased asthma symptoms in preschool inner-city children (Peter et al. 2011). A research based on 143 locations with different levels of NO_2 indicated that higher NO_2 correllates to increased respiratory mortality (Behrendt et al. 2001).

The most consistent general impression given by epidemiological studies indicated that nitrogen dioxide may induce increased respiratory disease in youths (5–15 years) and cause new-onset asthma in children (Traidl-Hoffmann et al. 2002). These conclusions are of concern because reiterated respiratory illness in children lead to increased lung damage in later life (Glezen 1989). Therefore, the increase of nitrogen dioxide related illnesses could have later as well as immediate consequences.

On the other hand, NO_2 could lead to genotoxic effects. A study on human nasal epithelium with short exposure durations at a concentration range of 0.01–10 ppm NO_2 showed DNA strand fragmentation. The increase in DNA damage during exposure to NO_2, even at concentrations of 0.01 ppm, was dependent on exposure duration (Koehler et al. 2013). Wiseman and Halliwell (1996) reported that reactive oxygen species (ROS) / reactive nitrogen species (RNS) can lead to ‘‘structural alterations of DNA, e.g. rearrangements, deletions, insertions, base pair mutations and sequence amplification’’. Concerning NO_2, the DNA damage may be explained by the formation of reactive nitrogen species (RNS) that are created by nitrogen oxide which is in equilibrium with NO_2 in the atmosphere.

1.2.3 Effects of nitrogen dioxide on plants

Air pollutants can cause plant deterioration affecting vegetative parts, particularly leaves with typical necrotic or chlorotic areas together with changes of mesophyll, stomata and epidermis (Lorenzini et al. 1999). The air pollutants can also cause morphological and physiological alterations that impact the development of reproductive organs and consequently plant fertility (Rezanejad 2007). Furthermore, pollutants can easily enter the soil and modify its pH, inducing root damage.

Atmospheric NO_2 has long been known to be either harmful or beneficial to plants depending on the concentration and plant species (Capron and Mansfield 1977, Sandhu and Gupta 1989, Wellburn 1990).

There are a variety of reports suggesting that lower concentrations of NO_2 have positive effects on higher plants during long-term exposure studies. Exogenously applied NO_2 has been known to regulate growth and development of the plant (Sandhu and Gupta 1989, Takahashi et al. 2005, Takahashi et al. 2011). Takahashi et al. (2005) reported that prolonged exposure to exogenous NO_2 at ambient concentrations can nearly double the total leaf area, nutrient uptake and shoot

biomass. Similar results have been found in numerous plant species, including *Arabidopsis thaliana* and various horticultural species (Ma et al. 2007, Takahashi et al. 2008, Xu et al. 2010). Additionally, exogenous NO_2 could accelerate flowering time and increases flower number and fruit yield in tomato (Takahashi et al. 2011). At the beginning, these positive impacts of NO_2 to plants were considered to be due to utilization of the nitrogen from the NO_2 as a nutrient (Sandhu and Gupta 1989, Ashenden et al. 1990). Plants were described to assimilate the nitrogen of NO_2 to organic compounds, including amino acids (Wellburn 1990). Conceivably, NO_2 can be an alternative nitrogen source (Morikawa et al. 1998) since some NO_2-philic plants that can even grow with NO_2 as the sole nitrogen source under laboratory conditions (Morikawa et al. 2003).

Nowadays, more evidence showed that exogenous or atmospheric NO_2 is not only a supplemental N source or a pollutant, but instead works as an airborne signalling compound. NO_2-philic plants which grow with exogenously supplied 150 ppb $^{15}NO_2$ as unique nitrogen source, integrated only a minor part of it (< 3%) into their total plant N (Morikawa et al. 2004, Morikawa et al. 2005). This may indicate that the contribution of NO_2-source N to total N was extremely limited (Takahashi et al. 2005). Therefore, it appears probable that the major role of NO_2 is as a multifunctional signal to stimulate plant growth, nutrient uptake and metabolism.

On the other hand, growing evidence shows that high concentration of NO_2 might have a detrimental effect on plants, including reduction and deterioration of crop and vegetable yield and quality (Maggs and Ashmore 1998, Haberer et al. 2006, Han and Naeher 2006). The data from experiments showed that NO_2 at a ppm level may cause visible injury symptoms in some vegetables (Mansfield and Freer-Smith 1981, Nouchi 2002). Ashenden and Mansfield (1978) reported that two grass species showed yield reductions after exposure to a mean concentration of 68 ppb NO_2 for 20 weeks during winter. Similarly, Kress and Skelly found that 62 ppb NO_2 might cause a decrease in the relative growth rate of *Pinus sylvestris* L.(Kress and Skelly

1982), while the same concentration caused an increase in shoot weight in some broad-leaved trees (Freer-Smith 1984).

Gottardini et al. (2008) suggested that NO_2 may perhaps be a factor with a statistically significant influence on the reduction on pollen viability of Austrian pine. Since pollen viability was importantly related to pollen germinability and length of pollen tubes, a potential influence on the reproduction of Austrian pine fumigated with elevated NO_2 may be considered. Honour et al. (2009) have demonstrated a variety of responses of a large number of herbaceous plant species to a NO*x* concentration of 100ppb. The results indicate that plants are likely to show significant changes in delayed flowering time, premature leaf senescence as well as changes in surface waxes. Additionally, young plants are more sensitive to NO_2 than older ones (Plotz et al. 2004).

1.3 Next-Generation Sequencing systems

Nucleic acid sequencing is a technique for identifying the exact order of nucleotides of a particular DNA or RNA molecule. Classical methods were developed in the 1970s, known as Sanger sequencing based on the DNA polymerase-dependent synthesis (Sanger et al. 1977). An intrinsic limitation of Sanger sequencing is its low throughput, because of the requirement of *in vivo* amplification that was usually achieved by cloning into bacterial hosts. Additional limitations of Sanger sequencing are the difficulty of low abundance variant detection due to high background levels and the high cost for each base (Mardis 2011). Nowadays, the Sanger strategy has been partially supplanted by several “next-generation” sequencing (NGS) technologies that offer remarkable improvements in cost-effective sequence throughput, albeit at the expense of read lengths (Liu et al. 2012).

The production of low cost reads by next-generation sequencing technologies renders them valuable in various areas. Significant applications include: (1) *de novo* genome sequencing, whole-genome resequencing or more targeted sequencing for

discovery of mutations or polymorphisms (Bentley 2006, Hodges et al. 2007, Porreca et al. 2007, Velasco et al. 2007, Ossowski et al. 2008, Diguistini et al. 2009, Huang et al. 2009, Xia et al. 2009, Li et al. 2010); (2) transcriptome analysis and cataloguing, in which shotgun libraries derived from small RNAs or mRNA are deeply sequenced (Axtell et al. 2006, Berezikov et al. 2006, Houwing et al. 2007, Sugarbaker et al. 2008, Sultan et al. 2008, Jacquier 2009); (3) large-scale analysis of DNA methylation, by deep sequencing of bisulfite-treated DNA (Cokus et al. 2008, Costello et al. 2009); (4) genome-wide mapping of DNA-protein interactions, by deep sequencing of DNA fragments pulled down by chromatin immunoprecipitation (ChIP-Seq) (Impey et al. 2004); (5) species classification and/or gene discovery by metagenomics and pangenomics (Edwards et al. 2006, Huber et al. 2007, Turnbaugh et al. 2007, Qin et al. 2010).

1.3.1 The Illumina sequencing system

In 2006, Solexa introduced the Genome Analyzer (GA) based on the concept of 'sequencing by synthesis' (SBS) to simultaneously produce 32–40 bp sequence reads from tens of millions of surface-amplified DNA fragments. In 2007 the company was purchased by Illumina (Mardis 2008). The Illumina system utilizes a sequencing-by-synthesis approach (Fig. 4). The library with fixed adaptors is denatured to single strands and grafted to the flowcell. Then, bridge amplification is applied to form clusters which contain clonal DNA fragments. Before sequencing, the library splices into single strands with the help of a linearization enzyme (Mardis 2008) and then four kinds of nucleotides (ddATP, ddGTP, ddCTP, ddTTP) labeled with different cleavable fluorescent dyes complement the template one base at a time, and the signal could be captured by a (charge-coupled device) CCD (Liu et al. 2012).

In early 2010, Illumina launched HiSeq2000, which adopted the same sequencing strategy as GA. The output HiSeq2000 began with 200 G/run and improved to currently 600 G per run which could be finished in 8 days (Rogers et al. 1996, Liu et al. 2012). Even to this day, HiSeq 2000 is the cheapest in sequencing (about 0.02

dollar /million bases) compared to Roche 454 and SOLiD (Liu et al. 2012). With the assistance of Truseq v3 reagents and related softwares, HiSeq 2000 has improved an excellent performance, especially on high GC sequencing (Rogers et al. 1996).

In 2011, a top sequencer bench, MiSeq, was launched which shared most technologies with HiSeq. It is particularly convenient for amplicon and bacterial sample sequencing. It could sequence 150 PE and generate 1.5 G/run in about 10 hours, including sample and library preparation time (Treangen and Salzberg 2012).
Revolutionary changes have happened in the life sciences with technical advances and major breakthroughs in sequencing technologies, hence representing their utility and importance in dealing with complicated biological issues. The spreading and popularization of NGS allowed the investigators to perform projects at large scales which previously had been accessible only in genome centers. It has not only been critical with regard to genome variability clarification, but continues to be crucial for the understanding of living systems and complex phenotypes (Bond et al. 1991).

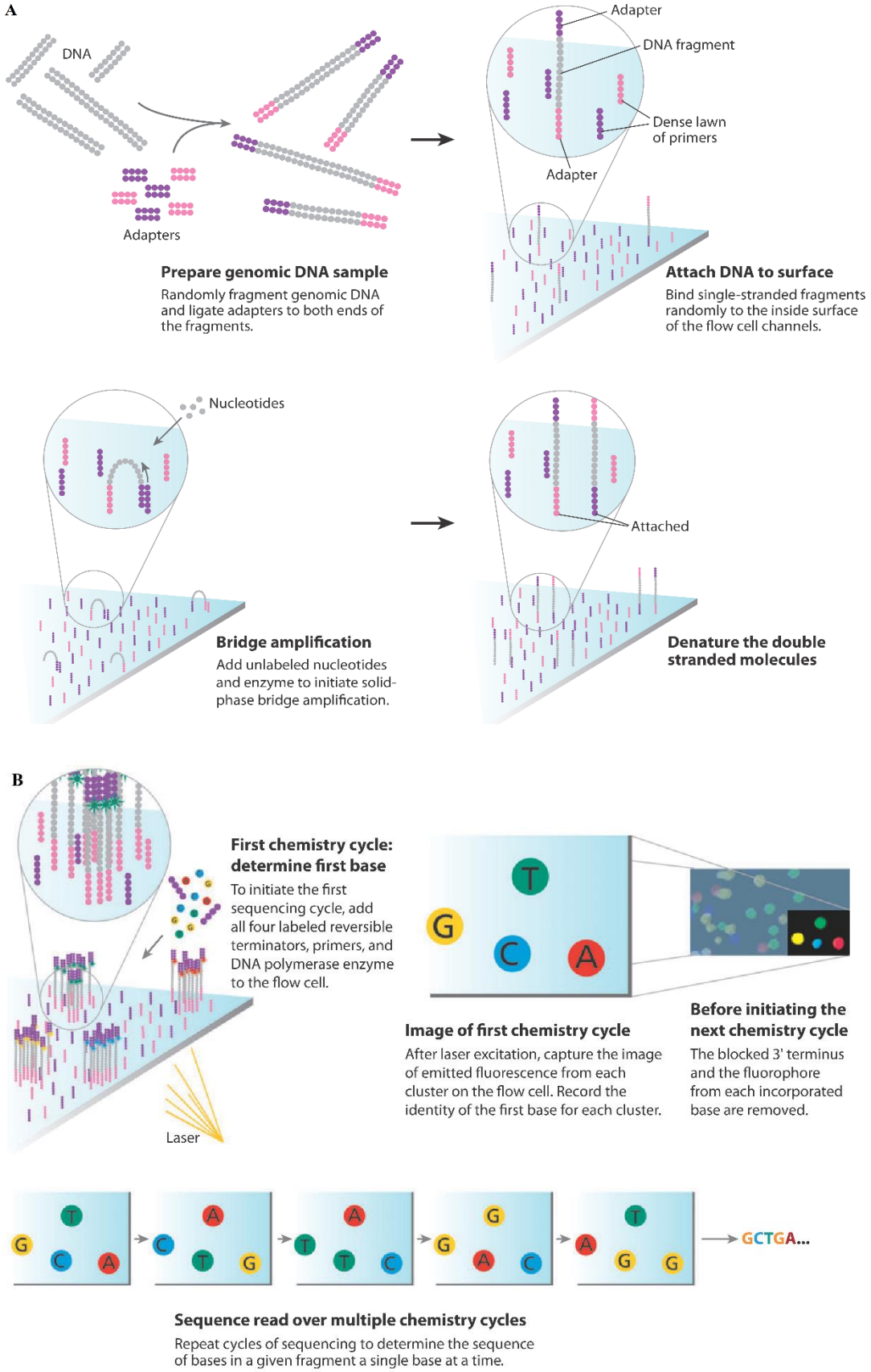


Figure 4. The Illumina sequencing-by-synthesis approach (Mardis 2008).

1.4 Principles and applications of proteomics

In the sequence of events beginning with the expression of genes towards synthesis of the active proteins, proteomics might be regarded as a review of the end product of the genes (Bernard et al. 2004). The goal of proteomics is to discover how particular biological events affect protein expression patterns, structures, post-translational modifications, and subsequently identification of the proteins impacted by the conditions under analysis (Unlu et al. 1997).

1.4.1 Two-dimensional difference gel electrophoresis (2D-DIGE)

Two dimensional difference gel electrophoresis has proven to be a powerful technique for investigating protein expression which allows the simultaneous resolution of thousands of proteins (O'Farrell 1975). The separation of proteins takes place in two steps, in the first dimension separation is dependent on their charge using isoelectric focusing (IEF), and in the second dimension on their molecular weight using SDS-PAGE (Marouga et al. 2005).

The 2D-DIGE technique involves labeling the lysine residues of the proteins in different homogenates using one of three different spectrally resolvable cyanine florescent dyes, called Cy2, Cy3 and Cy5. The use of these dyes allows the multiplex analysis of three differentially labelled protein samples on the same gel. This technique has been employed to identify proteins that are consistently differentially expressed between different treatments (Minden et al. 2009). A pooled mixture that contains an equal amount of each sample being examined is labeled with Cy2 and used as an internal standard to match among multiple DIGE gels. Two different samples individually labelled with Cy3 and Cy5, respectively, are added to the internal standard. Quantitative comparison of expression pattern differences of each sample is available through analysis of the replicate samples relative to the same internal standard (Rozanas and Loyland 2008).

1.4.2 S-nitrosylation

S-nitrosylation has emerged as an important nitric oxide (NO)-dependent post translational modification (PTM) (Hess et al. 2005). S-nitrosylation refers to the reversible incorporation of an NO moiety group to a reactive thiol of Cys residues, forming a nitrosothiol (Hess and Stamler 2012). S-Nitrosylation regulates a wide array of proteins involved in all major cellular activities and understanding of the impact of this PTM provided insights into physiological but also pathophysiological processes (Nakamura and Lipton 2011, Hess and Stamler 2012).

A thousand candidates for S-nitrosylation have been identified, many of them being already characterized as targets for S-nitrosylation in animals (Astier et al. 2011). These proteins are involved in major cellular activities, notably primary and secondary metabolism, photosynthesis, genetic information processing, cellular architecture, and responses to biotic and abiotic stresses (Astier et al. 2012).

Proteomic studies together with transcriptomic analysis could greatly contribute to the building of comparative proteome maps of organisms under normal and altered conditions (Diez 2010). The worthwhile information provided by qualitative and quantitative proteome maps will lead to further identification of mechanisms involved.

1.5 Objectives of the present work

The pollen of ragweed (*Ambrosia artemisiifolia*) is one of the strongest allergy triggers known and can cause allergic reactions such as asthma very quickly (D'Amato et al. 2007). Changes in the environment (global warming, air pollution, heavy metal ions, etc.) will result in an earlier and longer pollen season, enhanced pollen production and an increase in pollen allergenicity with a negative effect on atopic patients (D'Amato and Cecchi 2008). Nitrogen dioxide (NO_2) is regarded as an air pollutant which is reported to originate from the exhaust gas of vehicles. Atmospheric NO_2 has long been known to be either harmful or beneficial to plants depending on the concentration and plant species (Capron and Mansfield 1977, Sandhu and Gupta 1989, Wellburn 1990).

From these perspectives, a comparative transcriptome, proteome and immunohistochemistry analysis of allergenic pollen of ragweed would not only benefit on the understanding the changes of the gene and protein expression in ragweed pollen, but also allow to deeply understand the anticipated changes to pollen allergens in response to elevated NO_2.

The main objective of the present study was to investigate the impact of elevated NO_2 on common ragweed pollen at the following levels:

1. The physiological and morphological levels.

2. The transcriptome level using quantitative qRT-PCR, Suppression Subtractive Hybridization (SSH) libraries and the Illumina sequencing system.

3. The proteome level using 2D-DIGE, S-nitrosylation and LC-MS/MS.

4. In order to investigate the induction of potentially allergic influences on ragweed pollen in response to elevated NO_2, an allergic potential analysis was performed by using dot blotting, 1D Western blotting and 2D Western blotting with atopic serum.

2. Chapter – MATERIAL AND METHODS

2.1 Plant material and NO_2 treatment

Seeds of ragweed (Fig. 5A) were collected from a single plant at an outdoor stand (Waghäusel, Baden Württemberg, Germany) to avoid environmental-dependent epigenetic effects on growth and development (Elwell and Cooper 2011). Seeds were sown in standard soil (Floradur®, Bayerische Gärtnerei Genossenschaft, Munich, Germany) in small multiflor palettes (6 x 6 cm) and transferred into four plexiglass sub-chambers (1,1 m x 0.9 m x 0.8 m) placed within phytotron walk-in chambers (http://www.helmholtz-muenchen.de/en/eus/environmental-simulation-facilities/phytotron/index.htmL) on 12th of April 2011. Fifteen days after germination, the seedlings were transferred into single pots (Ø 17 cm, Fig. 5B) and cultivated further in the plexiglass sub-chambers. Plants were allowed to acclimate for two weeks and NO_2 treatment was started on 10th of May 2011.

Two plexiglass sub-chambers were fumigated with 40 ppb NO_2 as control and the other two with 80 ppb as treatment. The light period was 14.5 h; day/night temperatures were 20-30 °C/10-20 °C and relative humidities were 80-85%/30-50% (day/night). 40 ppb NO_2 (control) and 80 ppb NO_2 (treatment) were applied to the plexiglass sub-chambers, respectively, for 10 h per day. Watering of the plants was carried out automatically by a tube system applying 200 mL water per pot each day. Pollen was sampled from 14th of June until 10th of July 2011 using a modified ARACON system (BETATECH, Ghent, Belgium, Fig. 5D) that covered the inflorescences. Pollen and inflorescences were sampled and stored at -80 °C until analysis. The plants were cultivated further until 2nd August 2011 for seed collection.

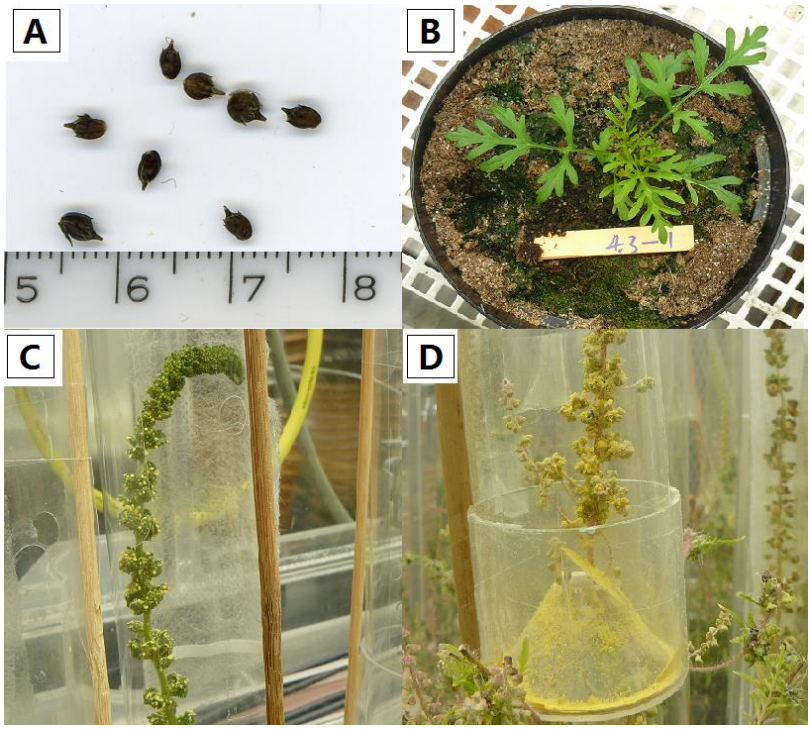


Figure 5. Ragweed was grown in the plexiglass sub-chambers. A: Ragweed seeds before cultivation; B: A ragweed seedling before acclimation in a plexiglass sub-chamber; C: Mature inflorescence and the start of pollen liberation; D: The modified ARACON system used to collect the pollen.

Additionally, seeds that were collected from the plants grown in 40 ppb NO_2 in 2011 were also cultivated and formed the second generation plants (Fig. 6 & 7). However, the second generation was treated with clean air (control) and 80 ppb NO_2 (treatment).

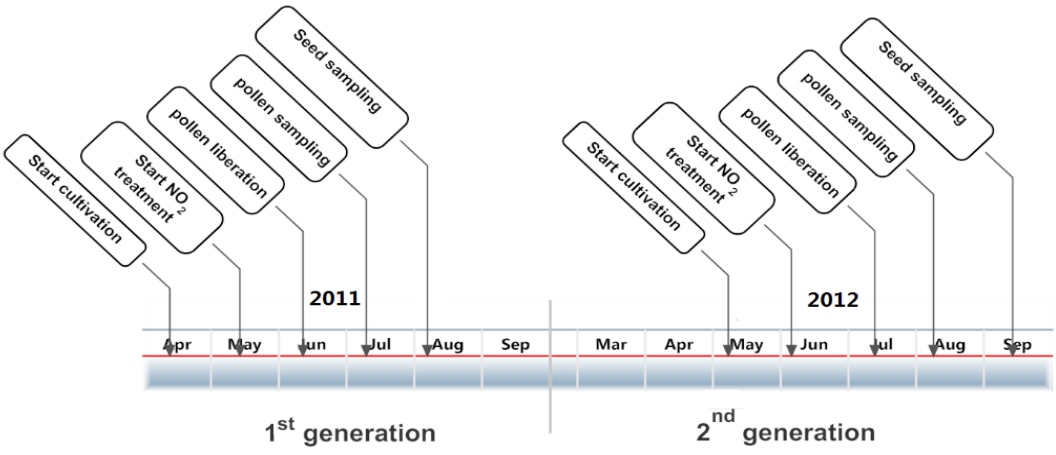


Figure 6. Time line of the plexiglass sub-chambers experiment. Ragweed plants were cultivated for two years; the first generation in 2011, and the second generation in 2012.

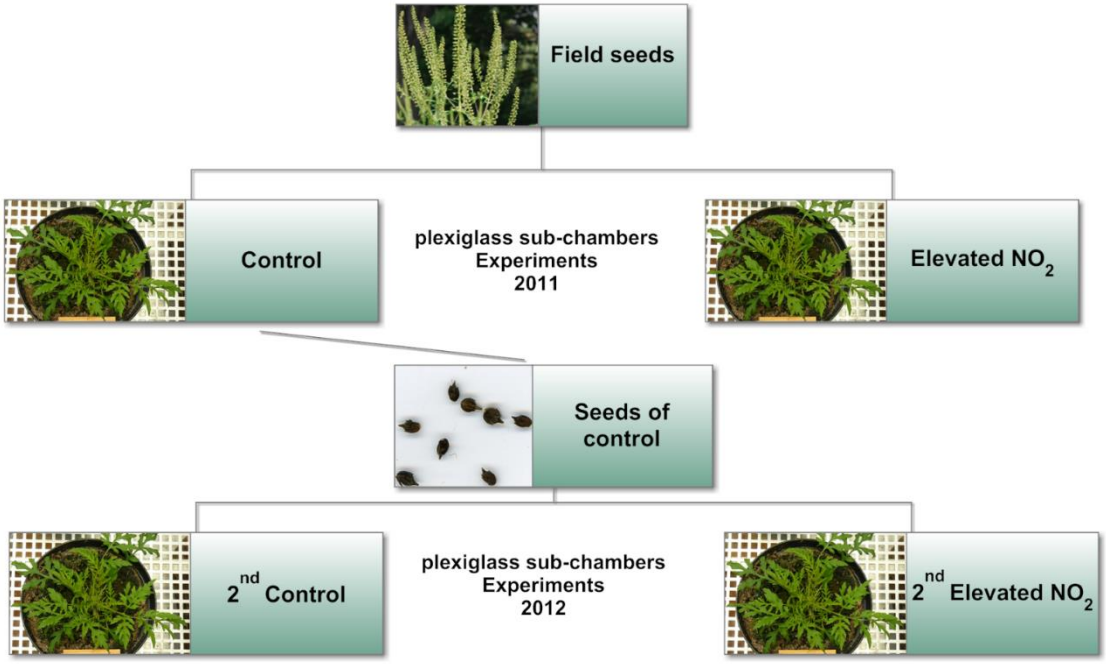


Figure 7. A diagram showing ragweed seeds that were used in 2011 for the first generation and 2012 for the second generation.

2.2 Morphological analysis

2.2.1 Average pollen weight per inflorescence (mg)

Five inflorescences from each plant were randomLy selected and pollen from each inflorescence was weighed. The average weight was calculated.

2.2.2 Total seeds weight and 50 seeds weight (mg)

The total and 50 clean seeds from each plant (broken grains were removed) were randomly sampled, counted and weighed.

2.2.3 Average length of inflorescence (cm)

Five inflorescences from each plant were randomly selected and carefully measured. The average length was calculated.

2.3 Comparative transcriptome analysis

2.3.1 Pollen RNA isolation and cDNA transcription

Pollen samples of 3 plants (5 inflorescences per plant) from each group (1^{st} control, 1^{st} treatment, 2^{nd} control, 2^{nd} treatment) were used for total RNA extraction using a modified Qiagen RNeasy Mini Kit protocol. 150 µL of RLT buffer together with 10 mg pollen was transferred into 2 mL tubes containing ceramic spheres (Ø 1.4 mm), silica spheres (Ø 0.1 mm), and a single glass sphere (Ø 4 mm). The pollen was homogenised ten times at 6.5 ms^{-1} for one minute on dry ice using a FastPrep 24 machine (MP Biomedicals). Chloroform (750 µL) was added and incubated for 10 min on a shaker. Another 600 µL of RTL buffer was added and mixed. After centrifugation, the supernatant was transferred to a new tube and mixed with 0.5 volumes of ethanol by gentle inversion. The solution was transferred to an RNeasy column (RNeasy Mini Kit, Qiagen) and centrifuged at 10,000 g for 15 s. The column was washed with 450 µL of RW1 buffer for 5 min. DNase digestion was carried out using the RNase-Free DNase Set (Qiagen) following the manufacturer's instructions. Then the column was incubated with 500 µL of RPE buffer twice for 2–3 min each and total RNA was eluted according to the user manual of the RNeasy Mini Kit (Qiagen).

Quantification of total RNA was performed using the NanoDrop System at 230, 260 and 280 nm. Only RNA samples with acceptable ratios of 260/280 (> 1.8) and 260/230 (> 2.3) were used and reverse transcribed. Two µg of total RNA was used for cDNA synthesis using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, US) according to the manufacturer’s procedure.

2.3.2 Quantitative real-time RT–PCR of major allergenic genes

The cDNA samples were equally quantified and diluted 1:20. Quantitative real-time RT–PCR (qRT–PCR) was performed in 20 µL reactions, containing 12.5 µL SYBR Green ROX mix (Thermo Scientific QPCR, Surrey, UK), 1.25 µL each of the forward and

reverse primers, 5 µL of diluted cDNA on an ABIPrism 7500 fast real time PCR system (Applied Biosystems, Darmstadt, Germany). The amplification was performed following this program: initial polymerase activation at 50 °C for 2 min and 95 °C for 10 min (1 cycle); followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. α-tubulin and 18S rRNA were used as internal standard genes. Three biological replicates were carried out for each group (1^{st} control, 1^{st} treatment, 2^{nd} control and 2^{nd} treatment) and each transcript was quantified in triplicate for each sample. The relative expression levels were calculated according to Pfaffl et al. (2002), using the REST©software tool (QIAGEN GmbH).

The gene-specific primers for major ragweed allergens and standard genes, α -tubuline, 18S rRNA were synthesised by Sigma-Aldrich (Tab. 2)

Gene	Forward & reverse primers (5’-3’)	Gene Bank Accession No.	Annealing temp. °C	RT-PCR product size/bp
Amb a 1.1	F ggggctggtgacgaaaatattg	M80558	56.3	250
	R caccatgccttcctaggacatt			
Amb a 1.2	F taacatcgttaacgccggtctcac	M62981	59.5	230
	R tgatatcgagcagcccatcggaa			
Amb a 1.3	F ggtcggggaaatcttaccttcagt	M80560	59	188
	R tgaccgtgtagacatcaccccatt			
Amb a 1.4	F tttgacgagcgaggcatgctat	M80562	59	243
	R ctctgacatggcggattcaccata			
Amb a 1.5	F ggagccagaatggatgacttggaa	M80561	59	150
	R tgtggaaccatatctcccggttca			
Amb a 5	F aggatccacagatgaagtcgatga	M84987	58	125
	R aaaccacttgccaaggacagtacc			
Amb a 6	F gtttcatggaggccaacgatgttc	U89793	58.7	167
	R gccacacgatcagctttggttt			
Amba 8	F aacctgaggagatgaaaggca	AY268427	56.5	172
	R gcttggcctgttttcttgatgc			
Amba 9	F aagaatctcggctcggtgtca	AY894657	58.5	141
	R cttgccaacgtccttcattaagcc			
Amba CPI	F gctaaattcgccatcgctgaacac	L16624	59.6	246
	R ccgtccatatggagttaaggtgaggt			
α-Tubulin	F tgcagagggctgtttgcatga	GW917730	58	119
	R acccacgtaccagtgaacaaaag			
18S rRNA	F gaggccttgtcgttgtgtgtctat	EF065545	59	554
	R gcaagacaatgcgtcagggtact			

Table 2. Primer sequences used for quantitative real-time RT–PCR analysis.

2.3.3 Suppression Subtractive Hybridization (SSH) Library (First generation)

Two SSH libraries were constructed for ragweed pollen of the first generation: control (40 ppb NO_2) and treatment (80 ppb NO_2). Pollen of five plants from control and treatment, respectively, were pooled for the SSH analysis.

2.3.3.1 Purification of Poly (A) RNA

Poly (A) RNA was purified from total RNA following the protocol described by the manual of Nucleo Trap® mRNA (MACHEREY-NAGEL). Briefly, the technique involved the following steps:

(1) 100 µg total RNA was dissolved in 100 µL ddH_2O and adjusted to 200 µL with buffer RM0.

(2) 15 µL Oligo(dT) latex beads suspension were added and centrifuged. The supernatant was discarded and the Oligo(dT) latex beads pellet was collected.

(3) Washing steps were performed three times with 600 µL RM2 buffer and the Oligo(dT) latex beads were dried afterwards.

(4) 30 µL pre-warmed (68 °C) RNase-free H_2O was added and Oligo (dT) latex beads were resuspended completely by pipetting up and down.

(5) The eluate was collected for use after 11,000 x g centrifugation for 1 min.

2.3.3.2 Construction of SSH Library

Reverse transcription of poly(A) RNA was performed by SuperScript® II Reverse Transcriptase Kit (Invitrogen). The cDNA pools of control and treatment were quantified in duplicate, one as tester and the other as driver. The procedures were according to the manual of PCR-SelectTM cDNA Subtraction Kit (Clontech) and with the control cDNA (from human skeletal muscle) provided with the kit. The technique involved the following steps:

(1) cDNA synthesis: Tester and driver ds cDNA were prepared from the two mRNA pools under comparison (treatment and control).

(2) Restriction digestion (blunt ends): Tester and driver cDNA were separately digested by Rsa I to obtain shorter, blunt-ended molecules.

(3) Adaptor ligation: Two tester populations were created with different adaptors (Driver cDNA had no adaptors).

(4) First hybridization: Differentially expressed sequences were equalized and enriched.

(5) Second hybridization: Templates for PCR amplification were generated from differentially expressed sequences.

(6) First PCR amplification: Only differentially expressed sequences were exponentially amplified by Suppression PCR.

(7) Second PCR amplification: Background was reduced and differentially expressed sequences were further enriched.

2.3.3.3 Fragments sequencing and data analysis

The second PCR amplification products were ligated to pGEM ®-T vector (Promega) and transformed into *E.coli* for sequencing. An aliquot of competent *E. coli* cells was thawed on ice and mixed with 100-200 ng PCR products in an Eppendorf tube. After incubation on ice for 30 min, the mixture was treated at 42 °C (water bath) for 30 sec, and immediately cooled on ice for 2 min. 500 µL of LB medium was added, and the samples were incubated at 37 °C for 30 min with agitation (1400 rpm). Transformed bacterial suspensions were centrifuged, resuspended with 50 µL LB medium, and plated on selective LB solid medium. Plasmid DNA from *E. coli* was extracted using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After verification of the target fragment being correctly inserted by restriction digests, plasmid DNA was prepared for sequencing according to the manufacturer's instructions (plasmid DNA/water mixture 15 µL and 0.5 µL 10 µM primer) and processed by Eurofins MWG GmbH (Ebersberg, Germany).

Sequencing results were blasted with BLASTX against the NCBI database and also with a local blast (BioEdit) against the *Ambrosia* transcriptome 454-sequencing database (Kanter et al. 2013).

2.3.4 Illumina sequencing

Illumina libraries were constructed by Illumina HiSeq 2000 (illumina®, San Diego, USA) and performed by the Institute of Human Genetics, Helmholtz Zentrum München. Pollen of three individual plants from each group (1st treatment, 1st control, 2nd treatment, and 2nd control) was randomly sampled to produce biological samples for each group.

2.3.4.1 Analysis and quantitation of total RNA

Quantity of total RNA was analysed by Agilent RNA 6000 Nano Kit (Agilent Technologies) on the Agilent 2100 Bioanalyzer (Agilent-Technologies). Finally, 3 µg of high quality total RNA from each sample were prepared for the library construction.

2.3.4.2 Library construction and Illumina sequencing

High quality total RNAs were ready for library construction and the technique involved the following steps:

(1) mRNA purification
(2) cDNA synthesis using a biotinylated oligo-d(T) primer and conversion of single stranded cDNA into double-stranded cDNA.
(3) Adapters were ligated to both ends of randomly fragmented cDNA.
(4) Bind single-stranded fragments randomly to the inside surface of the flow cell channels.
(5) Unlabeled nucleotides and enzyme were added to initiate solid-phase bridge amplification.
(6) The enzyme incorporates nucleotides to build double-stranded bridges on the solid-phase substrate.
(7) Denaturation leaves single-stranded templates anchored to the substrate.

(8) Several million dense clusters of double—stranded DNA are generated in each channel of the flow cell.

(9) The first sequencing cycle begins by adding four labelled reversible terminators, primers, and DNA polymerase.

(10) After laser excitation, the emitted fluorescence from each cluster is captured and the first base is identified.

(11) The next cycle repeats the incorporation of four labeled reversible terminators, primers, and DNA polymerase.

(12) After laser excitation, the image is captured as before, and the identity of the second base is recorded.

(13) The sequencing cycles are repeated to determine the sequence of bases in a fragment, one base at a time.

(14) The data are aligned and compared to a reference and sequencing differences are identified.

2.3.4.3 Bioinformatics analysis

Sequencing data were analysed and visualized by CLC genomics workbench 6.5 (CLC bio®, Aarhus, Denmark).

2.4 Comparative proteome analysis

2.4.1 2D difference gel electrophoresis (2D-DIGE)

2D difference gel electrophoresis (2D-DIGE) was carried out to analyse the differentially expressed proteins in the control (40 ppb NO_2) and treatment (80 ppb NO_2) ragweed pollen. For the first generation plants, 10 individual plants from each sub-chamber were randomly sampled, and five samples from each sub-chamber were pooled to produce four biological samples. Each biological sample was analysed in triplicate, as showed in Fig. 8.

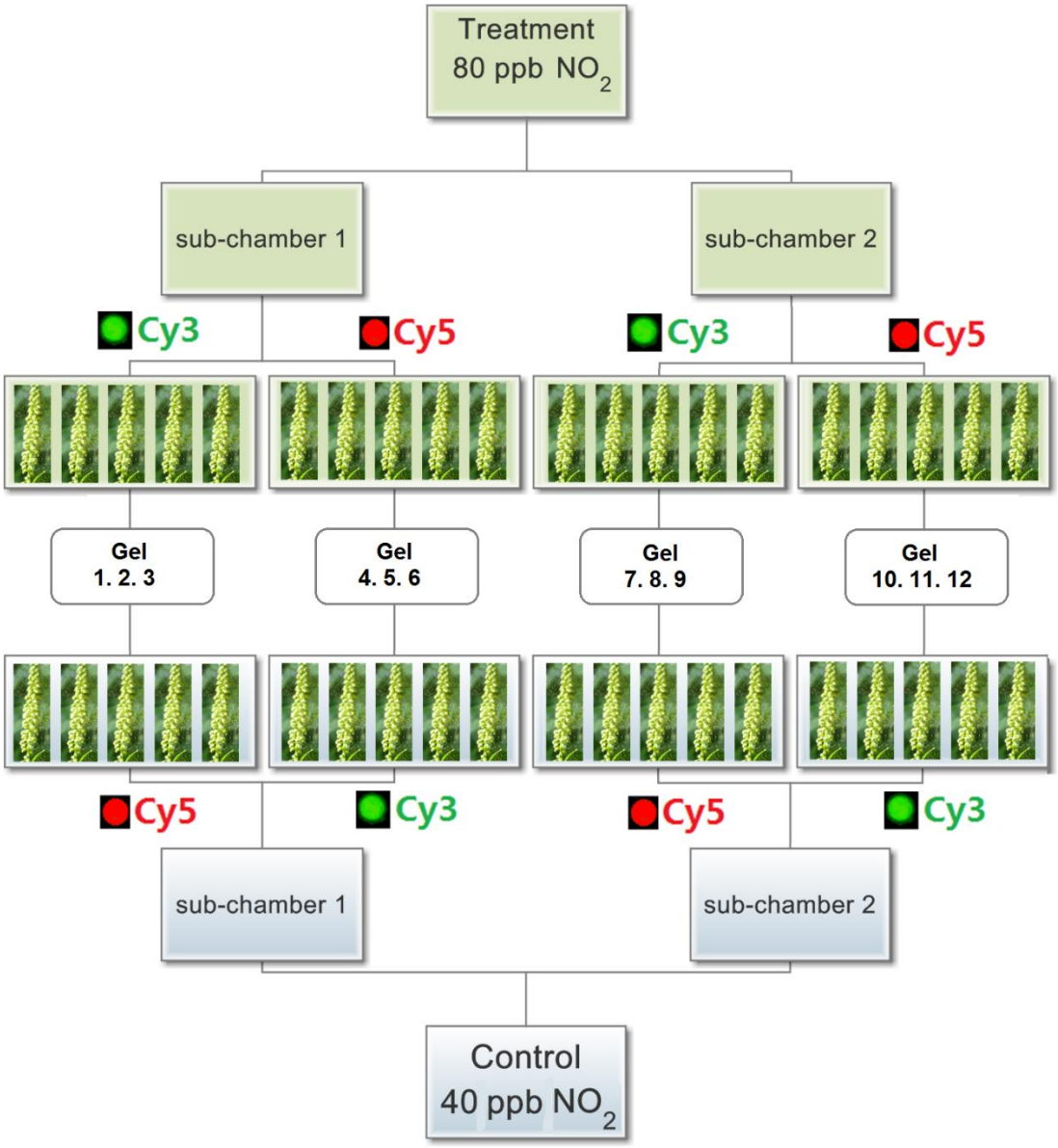


Figure 8. Experimental design of extracted protein samples in 2D-difference-gel electrophoresis (2D-DIGE).

2.4.1.1 Protein extraction

Pollen pools (10 mg) were mixed with 300 µL acetone containing 10% TCA (w/v), 1% DTT (w/v), 1% protease inhibitor (Sigma) and transferred to 2 mL tubes containing ceramic spheres (Ø 1.4 mm), silica spheres (Ø 0.1 mm), and a single glass sphere (Ø 4 mm). The pollen and spheres were homogenised ten times at 6.5 ms^{-1} for one min on dry ice using a FastPrep 24 machine (MP Biomedicals).

The samples were stored at -20 °C overnight and centrifuged at 25,000 g for 20 min at 4 °C. The pellets were washed two times with precooled acetone containing 1% DTT, incubated at -20 °C for 1 hour, and centrifuged. The vacuum dried pellets were dissolved directly in iso-electric focusing (IEF) buffer (8 M urea, 20 mM DTT, 4% CHAPS and 2% ampholyte pH 4–7) at 20 °C for 1 h. This solution was centrifuged at 20 °C for 20 min at 25,000 g and the supernatant was used for protein estimation and electrophoresis analysis. Protein concentrations were measured according to the Bradford assay (BIORAD) using BSA as a standard (Bradford 1976).

A 2D Clean-up Kit (GE Healthcare, Uppsala, Sweden) was used for protein purification. After purification, the proteins were resuspended by incubation for 1 h in labelling buffer (7 M urea, 2 M thiourea, 30 mM Tris-HCl, pH 8.5, 4% [w/v] CHAPS) and adjusted to pH 8.5 using 5 M NaOH.

2.4.1.2 Cyanine dye fluorescent labelling of proteins

Protein labelling was performed using CyDye fluorescence according to the manufacturer's minimal labelling protocol of fluorescence 2-D DIGE technology (GE Healthcare). Cyanine dyes Cy2, Cy3, and Cy5 (1 nmol/µL in dimethylformamide) were diluted to 400 pmol/µL for use.

Fifty µg of protein from each control and treatment sample were labelled with 400 pmol of Cy3 or Cy5 dye. Fifty µg of pooled internal standard which contained equal amounts of each protein sample were labelled with Cy2 dye. Dye swap was used between the different pollen pools to reduce the errors due to the CyDye (Fig. 4). The labelling reaction was first incubated on ice for 30 min in the dark and then terminated by the addition of 1 µL of 10 mM lysine and incubated for 15 min on ice. The differentially labelled samples were immediately mixed in a 1:1:1 ratio (treatment, control and internal standard) and adjusted to a final volume of 150 µL by adding equal amounts of lysis buffer (7 M urea, 2 M thiourea, 2% IPG-buffer, pH 4–7, 2% DTT, 0.04% bromophenol blue, 4% CHAPS).

2.4.1.3 Two-dimensional gel electrophoresis

Immobilized non-linear pH gradient strips (pH 4–7, 24 cm; GE Healthcare) were rehydrated overnight at RT in rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 0.5% IPG buffer, pH 4–7, 0.8% DTT, 0.002% bromophenol blue). The CyDye-labelled samples were applied to the strips by cup-loading and separated at 20 °C. Ettan IPGphor3 Unit (GE Healthcare) was applied with the following settings: 150 V for 3 h, 300 V for 3 h, gradient from 300 to 1000 V for 6 h, gradient from 1000 to 10,000V for 3 h, and 10,000 V for 3 h for a total of 51,750 Vh with a maximum current setting of 75 mA per strip. Afterwards, the stripes were equilibrated in 10 mL equilibration buffer (6 M urea, 2% SDS, 50 mM Tris-HCl, pH 8.8, 30% glycerol, 0.002% bromophenol blue) containing 1% DTT for 15 min and containing 2.5% iodoacetamide for 15 min orderly. After equilibration, the stripes were transferred to 12.5% SDS-PAGE polyacrylamide gels for the second dimensional running at 12 °C at 15 mA per gel for 16–18 h using the Ettan DALT six systems (GE Healthcare).

2.4.1.4 Image acquisition and visualization

The DIGE gels were scanned and visualized after electrophoresis using the Typhoon 9400 variable mode imager (GE Healthcare). Photo-multiplier tubes were set to ensure maximum pixel intensity without spot saturation on each image. All gel images were scanned at 100 nm resolution and analysed using Decyder software (GE Healthcare). Differential in-gel analysis (DIA) module and biological variance analysis (BVA) module were performed for data analysis. DIA was used for spot detection and spot volumes/abundances calculation of the three images (Cy2, 3 and 5) and then normalisation was performed. The spots were manually matched between multiple samples across all the gel images in an experiment *via* BVA. The changes in the abundance of the spots across samples were statistically analysed. The internal standard was employed to facilitate spot matching and allowed a spot-by-spot standardisation for improved reproducibility and accurate quantification of protein abundance changes across the samples. The one-way anova was performed to compare the average standardised abundances of the

replicate samples across the different conditions. Spots with reproducible and significant variations, at least 1.5-fold up- or down-regulated, were considered as differentially expressed spots of interest.

2.4.1.5 Spots picking and in-gel digestion

Preparative 2-DE gels were prepared using 300 µg of the unlabelled sample from the control and treated samples and were then silver stained. The position of interesting protein spots were confirmed according to the 2D-DIGE image. These spots were manually excised, washed with water and further processed by the Research Unit Protein Science (PROT, Helmholtz Zentrum München) for mass electroscopy (MS) analysis. The gel pieces were washed with 200 µL of 60% actetonitrile (ACN) for 10 min followed by a 10 min wash in 200 µL H_2O. Afterwards, the gel pieces were incubated for 10 min with 200 µL of 100% ACN, then 100 µL of 5 mM DTT were added to the gel pieces and incubated for 15 min at 60 °C to reduce the proteins. After an additional dehydration step in 100% ACN, 100 µL of freshly prepared 25 mM iodacetamide (IAA) solution were added and incubated for 15 min at RT in the dark. The gel cubes were washed for 5 min with 100 µL H_2O and dehydrated again by incubation in 100% ACN for 10 min in order to remove the residual DTT and IAA.

After three wash steps in 50 mM ammoniumbicarbonate (ABC) for 10 min, the gel cubes were air-dried for 15 min at 37 °C, then 100 µL of 0.01 µg/µL trypsin (Sigma) in 50 mM ABC were added. After incubation for 10 min, 25 mM ABC was added to cover the gel pieces completely during the overnight digestion at 37 °C. For elution, 100 µL of 60% ACN/0.1% trifluoroacetic acid (TFA) were added to the gel cubes, and incubated for 15 min with shaking. The supernatant was transferred to a new tube, and 100 µL of 99.9% ACN/0.1% TFA were added to the gel pieces, incubated for 30 min. The supernatant was pooled with the first supernatant. The supernatants containing the eluted peptides were dried using a speedvac (UniEquip, San Diego, USA) and stored at -20 °C until analysis.

2.4.1.6 LC-MS/MS analysis

Thawed samples were dissolved in 60 µL of 2% ACN/0.5% trifluoroacetic acid by incubation for 30 min at RT with shaking. The samples were centrifuged for 5 min at 4 °C before loading. LC-MS/MS analysis was performed as described by Hauck et al. (2010). Each sample was automatically injected and loaded onto the trap column at a flow rate of 30 µL/min in 95% buffer A (2% ACN/0.1% formic acid in HPLC-grade water) and 5% buffer B (98% ACN/0.1% formic acid in HPLC-grade water). After 5 min, the peptides were eluted from the trap column and separated on the analytical column using a 120 min gradient which increased from 5% to 31% buffer B at a flow rate of 300 nl/min, followed by a short gradient from 31% to 95% buffer B over 5 min. Between each sample, the column was restored to 5% buffer B and allowed to equilibrate for 20 min.

From the MS pre-scan, the ten most abundant peptide ions were selected for fragmentation in the linear ion trap (if they exceeded an intensity of at least 200 counts and were at least doubly charged). During fragment analysis, a high-resolution (60,000 full-width half maximum) MS spectrum was acquired from the Orbitrap over a mass range from 300 to 1500 Da.

2.4.1.7 Data processing

Peptide identification was performed by Mascot (version 2.3; Matrix Science, Boston, USA). The spectra were compared against the SwissPort database.

Scaffold software (version 3_00_03; Proteome Software Inc., Oregon, USA) was used to validate the MS/MS-based peptide identifications and spectra. Peptide identifications which could be established at greater than 80 % probability were accepted as specified according to the Peptide Prophet algorithm (Keller et al. 2002).

In most cases, the molecular weight (Mw) and theoretical isoelectric point (pI) of the search results correlated well with the 2-DE position of the corresponding spot.

Proteins that contained similar peptides but could not be differentiated based on the MS analysis alone were grouped to satisfy the principles of parsimony.

2.4.2 Protein S-nitrosylation detection

To detect the S-nitrosylated proteins in ragweed pollen, the biotin switch method was chosen, which is based on the labelling of S-nitrosylated proteins with a biotin moiety specifically on S-nitrosylated Cys (Jaffrey et al. 2001). For the first generation plants, five individual plants from the chamber with 40 ppb NO_2 (control) and 80 ppb NO_2 (treatment), respectively, were randomly sampled to produce biological samples and each biological sample was analysed in triplicate.

2.4.2.1 Protein extraction

Fifty mg pollen samples from each plant were used for total protein extraction. The pollen, together with 150 µL of HEN buffer (HEPES 25mM, EDTA 1mM, Neocuprine 0.1m, pH 7.7) was transferred into 2 mL tubes containing ceramic spheres (Ø 1.4 mm), silica spheres (Ø 0.1 mm), and a single glass sphere (Ø 4 mm). The pollen was homogenised ten times at 6.5 ms^{-1} for one min on dry ice using FastPrep 24 machine (MP Biomedicals). Then 500 µL of HEN buffer were added, the samples were incubated at 37 °C for 1h with agitation (1400 rpm) and finally centrifuged at 25,000 g for 20 min at 4 °C. The protein concentration of the supernatant was measured according to the Bradford assay (Bradford 1976) using BSA as a standard. The samples were diluted by HEN buffer to 0.8 µg/µL for use.

2.4.2.2 Biotinylation of S-Nitrosylated proteins

The biotin switch assay was applied for the S-nitrosylation detection and followed the steps below:

(1) 25 µL of S-Nitrosoglutathione (GSNO) (10mM) were added to two standard samples (800 µg) as positive controls. Samples were incubated at room temperature in darkness for 20 min.

(2) 10 µL DTT (1M) were added to one of the positive controls and incubated at room temperature in darkness for 10 min. This sample was the negative control.

(3) 10 µL S-Methyl thiomethanesulfonate (MMTS) (2 mM) and 80 µL Sodium dodecyl sulfate (SDS) (25%, w/v) were added to all the samples (800 µg). The samples were incubated at 50 °C in darkness for 20 min and vortexed every 5 min.

(4) 2 volumes of ice cold acetone were added afterwards and precipitated at -20 °C for at least 20 min and centrifuged at 10000 x g and 4 °C for 20 min.

(5) The pellet was rinsed with ice-cold acetone and resuspend with HENS buffer (HEPES 25 mM, EDTA 1mM, Neocuprine 0.1m, SDS 1%, pH 7.7). 50 µL of HENS buffer were added with 0.8 µL Ascorbate (50mM) and 0.8 µL Biotin HPDP (50 mM).

(6) The samples were incubated at room temperature in darkness for 1 hour and 5% of the sample was used for Western blot, and 95 % for purification.

(7) 2 volumes of ice cold acetone were added to both series of samples and centrifuged at 10000 x g and 4 °C for 20 min. The proteins were then ready for Western blot and purification.

2.4.2.3 Detection of S-nitrosylated proteins by immunoblotting

After the biotin switch assay, all the S-nitrosylated proteins were biotinylated and detected by immunoblotting following the steps below.

(1) Protein samples were combined with nonreducing SDS-PAGE sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% [w/v] SDS, 10% [v/v] glycerol) and resolved by SDS-PAGE using a 4% (w/v) stacking gel and a 12% (w/v) separating gel on a Mini-protean II (Bio-rad, Munich, Germany) vertical electrophoresis system for approximately 70 min at 30 mW in SDS running buffer consisting of 25 mM Tris, 192 mM glycine and 0.05% (w/v) SDS (LaemmLi, 1970).

(2) The electrophoresed samples were transferred to a nitrocellulose membrane (BIORAD, 0.2 µm) for immunoblotting.

(3) The membrane was incubated in blocking buffer (10 mM Tris-HCl pH 7.5, 0.9% [w/v] NaCl, 1mM $MgCl_2.6H_20$, 2% [w/v] BSA, 0.5% [v/v] Tween 20) for 1 hour or overnight in darkness at 4 °C.

(4) Afterwards, the membrane was incubated in antibody buffer (10 mM Tris-HCl pH 7.5, 0.9% [w/v] NaCl, 1mM $MgCl_2.6H_20$, 2% [w/v] BSA, 0.5% [v/v] Tween 20, 0.01% Monoclonal Anti-Biotin antibody produced in mouse [Sigma]) for 3 hours in room temperature or overnight in darkness at 4 °C.

(5) The membrane was washed 2 times with TBST buffer (10 mM Tris-HCl pH 7.5, 0.9% [w/v] NaCl, 1 mM $MgCl_2.6H_20$, 0.5% [v/v] Tween 20) for 10 min and with TBS buffer (10 mM Tris-HCl pH 7.5, 0.9% [w/v] NaCl, 1 mM $MgCl_2.6H_20$) for 10 min in darkness at 4 °C.

(6) 10 µL 5-Bromo-4-chloro-3´indolyphosphate-p-toluidine-salt (BCIP) buffer (50mg BCIP in 1mL sterile ddH_2O) and 10 µL Nitro-blue tetrazolium chloride (NBT) buffer (100 mg NBT in 700 µL DMF and 300 µL ddH_2O) were added in 3 mL AP buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2.6H_20$). The membrane was incubated in it for 3 minutes at room temperature and then washed with water and scanned (Epson Perfection 3170 Photo, Epson).

2.4.2.4 Purification and MS analysis of S-nitrosylated proteins

Purification of S-nitrosylated proteins were carried out according to the protocol of Jaffrey (2001). Protein pellets were resuspended with 100µL of HENS Buffer and 200µL of Neutralization Buffer (20 mM Hepes-NaOH, pH 7.7, 100 mM NaCl, 1 mM EDTA, 0.5% [v/v] Triton X-100). 15 µL of NeutrAvidin Agarose (Thermo) per mg protein were added to purify biotinylated proteins. The biotinylated proteins were incubated with the resin for 1 hour at room temperature. The beads were washed five times with 10 volumes of Neutralization plus NaCl Buffer (20 mM Hepes-NaOH, pH 7.7, 700 mM NaCl, 1 mM EDTA, 0.5% [v/v] Triton X-100). The beads were incubated with Elution Buffer (20 mM Hepes-NaOH, pH 7.7, 100 mM NaCl, 1 mM EDTA, 100 mM β- Mercaptoethanol) to recover the bound proteins. Protein samples were combined with SDS-PAGE Sample Buffer (Nonreducing, 62.5 mM Tris-HCl [pH 6.8], 2% [w/v] SDS, 10% [v/v] glycerol) and resolved by SDS-PAGE using a 4% (w/v) stacking gel and a 12% (w/v) separating gel on a Mini-protean II (BIO-RAD, Munich, Germany) vertical electrophoresis system for approximately 70 min at 30 mW in

running buffer consisting of 25 mM Tris, 192 mM glycine and 0.05% (w/v) SDS (Laemmli, 1970). SDS-PAGE gel was equally divided into four parts and analysed by MS as stated in 2.4.1.5, 2.4.1.6 and 2.4.1.7

2.5 Pollen allergen immunoreactivity analysis

The allergenic potential of ragweed pollen was detected by immunoblotting with atopic patients' immune serum in dotblots, one-dimensional polyacrylamide gel electrophoresis immunoblots and two-dimensional polyacrylamide gel electrophoresis immunoblots.

2.5.1 Preparation of serum mix

Sera were drawn from individuals suffering from ragweed with CAP classes >3 (w1 kUA/L > 3.5) with their informed consent (kindly provided by Prof. Dr.med.Dr.h.c Thomas Ruzicka, Klinikum der Universität München, München, Germany). Ten patients' immune sera were selected for their ability to specifically detect ragweed allergens and were pooled to carry out all immunochemical analyses. Sera from healthy volunteers served as controls. The serum pool was aliquoted and stored at 20 °C until use. Details of atopic patients' immune sera are provided in Tab. 3.

Patients ID	*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**

Table 3. Serum ragweed specific IgE concentration (w1 kUA/L) of patients' immune sera.

2.5.2 Protein extraction

Pollen samples (10 mg) were mixed with 300 µL ddH_2O and incubated at 37 °C for 1h with agitation (1400 rpm). The samples were then centrifuged at 14,000 g for 10 min and the supernatants were ready to use. The soluble protein concentrations of the supernatants were measured using the dye-binding method of Bradford (1976), using bovine serum albumin as a standard.

2.5.3 Dot blot analysis

Dot blot technique was applied to assess the total allergenicity of ragweed pollen. Pollen of six individual plants from each group (1^{st} treatment, 1^{st} control, 2^{nd} treatment, 2^{nd} control) were randomly sampled and three independent experiments were performed.

Equal volumes of protein extracts (5 µg) were bound to nitrocellulose membrane and the evaluation of immunoreactivity was performed using the Bio-Dot Microfiltration Apparatus (BIO-RAD). The procedures for immunoassay followed the protocol described by the manual of Bio-Dot Microfiltration Apparatus with slight changes:

(1) The nitrocellulose membranes were rehydrated by 100 µL TBS (10mM Tris-HCl pH 7.5, 0.9% [w/v] NaCl, 1mM $MgCl_2.6H_20$) per well.

(2) Appropriate wells were filled with 50 µL of protein extracts (5 µg) and the entire sample was allowed to filter through the membrane by gravity flow.

(3) After the antigen samples had completely drained from the apparatus, the membrane was removed from the apparatus and stained with Ponceau S staining solution (0.1% [w/v] Ponceau S in 5% [v/v] acetic acid) to assess the amount of proteins loaded in each well.

(4) The membrane was incubated in blocking buffer (10mM Tris-HCl pH 7.5, 0.9% [w/v] NaCl, 1mM $MgCl_2.6H_20$, 2% [w/v] BSA, 0.5% [v/v] Tween 20) for 1 hour at room temperature.

(5) The serum mix from patients allergic to ragweed was diluted 100 times with TBST buffer (10mM Tris-HCl pH 7.5, 0.9% [w/v] NaCl, 1mM $MgCl_2.6H_20$, 0.5% [v/v] Tween 20) and the membrane was incubated in it overnight in darkness at 4 °C.

(6) The membrane was washed 3 times with TBST buffer for 10 min and incubated in the second antibody buffer (10mM Tris-HCl pH 7.5, 0.9% [w/v] NaCl, 1mM $MgCl_2.6H_20$, 2% [w/v] BSA, 0.5% [v/v] Tween 20, 0.01% Anti-Human IgE (ε-chain specific)−Peroxidase antibody produced in goat [Sigma]) for 1 hour at room temperature.

(7) After 3 washing steps with TBST buffer for 10 min, the membrane was developed with WESTERN LIGHTNING PLUS –ECL (PerkinElmer) for 1min. The signals were caught by FUSION X7 (VILBER) at an exposure time of 2 minutes.

Image analysis was applied to quantify reactivity signals and performed by Image J 1.47.

2.5.4 One-dimensional polyacrylamide gel electrophoresis immunoblot

To investigate the cause of the difference in dot blot allergenicity signals, 1D-immunoblot probed with the same serum mix used for dot blotting was carried out. Pollen of three individual plants from each group (1^{st} treatment, 1^{st} control, 2^{nd} treatment, and 2^{nd} control) were randomly sampled and three independent experiments were performed.

2.5.4.1 One-dimensional polyacrylamide gel electrophoresis

One-dimensional polyacrylamide gel electrophoresis (1-DE) was carried out according to the protocol of Laemmli (1970). Protein samples (50µg) were combined with 25 µL 2x sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% [w/v] SDS, 10% [v/v] glycerol, 5% [v/v] [β-mercaptoethanol], 0.001% [v/v] bromophenol blue) and incubated in a water bath at 100 °C for 3 min prior to loading. The proteins were resolved by SDS-PAGE using a 6% (w/v) stacking gel and a 14% (w/v) separating gel on an Ettan DALTsix Electrophoresis Unit (GE-Healthcare) vertical electrophoresis

system for approximately 18 hours at 30 mW in running buffer consisting of 25 mM Tris, 192 mM glycine and 0.05% (w/v) SDS (pH 8.5). Pierce Prestained Protein Molecular Weight Marker (20 – 120 kDa, Thermo) was utilized to monitor migration and assess molecular masses.

2.5.4.2 1D Western blot

Once separation was completed, Western blot was performed according to the following steps,

(1) The gel, nitrocellulose membrane and 9 pieces of Whatman® filters (Sigma), were Incubated in transfer buffer (25 mM Tris-HCl (pH 7.6), 192 mM glycine, 20% methanol, 0.03% SDS) for 2-5 min.

(2) 6 pieces of filters, membrane, gel and 3 pieces other filters were placed from the bottom up in Trans-Blot® Turbo™ Transfer System (BIO-RAD).

(3) The process of transfer was performed under the electric current of mA equal to the square centimetres of membrane multiplied by the number of membranes multiplied by 2.5 for 1 hour.

(4) The membrane was stained with Ponceau S staining solution (0.1% [w/v] Ponceau S in 5% [v/v] acetic acid) to assess the amount of proteins loaded in each lane.

(5) The membrane was incubated in blocking buffer (10mM Tris-HCl pH 7.5, 0.9% [w/v] NaCl, 1mM $MgCl_2.6H_20$, 2% [w/v] BSA, 0.5% [v/v] Tween 20) for 1 hour at room temperature in the dark.

(6) The serum mix from patients allergic to ragweed was diluted 1:100 with TBST buffer (10mM Tris-HCl pH 7.5, 0.9% [w/v] NaCl, 1mM MgCl2.6H20, 0.5% [v/v] Tween 20) and the membrane was incubated in it overnight in darkness at 4 °C.

(7) The membrane was washed 3 times with TBST buffer for 10 min and incubated in the second antibody buffer (10mM Tris-HCl pH 7.5, 0.9% [w/v] NaCl, 1mM MgCl2.6H20, 2% [w/v] BSA, 0.5% [v/v] Tween 20, 0.01% Anti-Human IgE (ε-chain specific)−Peroxidase antibody produced in goat [Sigma]) for 1 hour at room temperature.

(8) The membrane was washed 3 times with TBST buffer for 10 min and developed with WESTERN LIGHTNING PLUS –ECL (PerkinElmer) for 1min. The signals were caught by FUSION XPRESS (VILBER) at an exposure time of 2 min.

2.5.4.3 Image analysis and MS analysis

Image analysis was applied to quantify reactivity signals and performed by Image J 1.47. Interesting slices were excised and analysed by LC-MS/MS as stated in 2.4.1.5, 2.4.1.6 and 2.4.1.7

2.5.5 Two-dimensional gel electrophoresis immunoblot

To further investigate the different allergenicity signals in dot blots and one-dimensional polyacrylamide gel electrophoresis immunoblots, two-dimensional gel electrophoresis immunoblotting was carried out. Pollen of three individual plants from each group (1^{st} treatment, 1^{st} control, 2^{nd} treatment, and 2^{nd} control) were randomly sampled and three independent experiments were performed.

2.5.5.1 Two-dimensional gel electrophoresis

Equal amounts of protein samples were loaded onto immobilized non-linear pH gradient strips (pH 3 –11, 24 cm; GE Healthcare), which had been rehydrated overnight at RT in rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 0.5% IPG buffer, pH 3 – 11, 0.8% DTT, 0.002% bromophenol blue). The samples were applied to the strips by cup-loading and separated at 20 °C with a maximum current setting of 50 mA per strip using an Ettan IPGphor3 Unit (GE Healthcare) with the following settings: 150 V for 3 h, 300 V for 3 gradients from 300 to 1000 V for 6 h, a gradient from 1000 to 10,000V for 3 h, and 10000 V for 3 h for a total of 51,750 Vh.

Afterwards, the stripes were equilibrated in 10 mL equilibration buffer (6 M urea, 2% SDS, 50 mM Tris-HCl, pH 8.8, 30% glycerol, 0.002% bromophenol blue) containing 1% DTT for 15 min and containing 2.5% iodoacetamide for 15 min, and then transferred to 12.5% SDS-PAGE polyacrylamide gels. Second dimensional SDS-PAGE was

performed in running buffer (250 mM Tris, 1.92 M glycine, 1% SDS) at 12 °C at 15 mA per gel for 16–18 h using the Ettan DALT six systems (GE Healthcare).

2.5.5.2 2D Western blot

The proteins in the gel were transferred to nitrocellulose membranes with equal size following the same procedure as 2.5.4.2.

2.5.5.3 Image analysis and MS analysis

Image analysis was applied to quantify reactivity signals and performed by Image J 1.47. Interesting spots were excised and analysed by LC-MS/MS as stated in 2.4.1.5, 2.4.1.6 and 2.4.1.7.

3. Chapter – RESULTS

3.1 Morphological variations

The influence of variability elevated 80 ppb NO_2 to ragweed was assessed in consecutive years, 2011 and 2012, respectively, through reproductive measures (inflorescence length, pollen production and seed production). The 80 ppb NO_2 was compared with its equivalent control in the same generation to evaluate the influence of elevated NO_2 to ragweed plants.

For both of these two generations (2011, 2012) ragweed plants, elevated NO_2 obviously enhanced the pollen production of the plants. The pollen production per inflorescence was increased by around 67% and 87% for 2011 and 2012 plants, respectively. In contrast, elevated NO_2 reduced seed production not only with regard to plant total seed production but also to single seed weight. Plant total seed production was reduced under 80 ppb NO_2 conditions by 32% and 37 % in 2011 and 2012 plants, respectively, and 50 seed weight was reduced by 14% and 12%. On the other hand, the inflorescence length showed no significant change between the 80 ppb NO_2 and the control (Tab. 4).

In summary, elevated NO_2 dramatically enhanced the pollen production and significantly reduced the total seed production and single seed weight.

NO_2	Inflorescence ± SD	Pollen/ inflorescences ± SD	Seeds/ plant ± SD	Seeds/ 50 grains ± SD
1st 40 ppb (1)	23.73 ± 1.12 cm	79 ± 6 mg	1329 ± 85 mg	229± 18 mg
1st 80 ppb (2)	25.18 ± 1.57 cm	132 ± 11 mg	905 ± 69 mg	199 ± 10 mg
p-value (2 vs 1)	0.327	1.8 x 10E-5*	2.3 x 10E-3*	1.9 x10E-3*
2nd 0 ppb (3)	24.48 ± 1.35 cm	85 ± 8 mg	1637 ± 77 mg	237 ± 16 mg
2nd 80 ppb (4)	24.61 ± 1.77 cm	159 ± 12 mg	1024 ± 53 mg	209 ± 9 mg
p-value (3 vs 4)	0.5682	2.7 x 10E-5*	3.5 x 10E-3*	2.9 x 10E-3*

Table 4. Morphological data of ragweed plants of first and second generation. Mean value of length of the inflorescences, pollen production and seed production (number of plants N = 20; t-test; *= p-value < 0.05).

3.2 Comparative transcriptome analysis

3.2.1 Quantitative real-time RT–PCR of major allergic genes in ragweed pollen

The relative expression variation of 10 major pollen allergens was analyzed by the technique of qRT-PCR using highly-specific primers designed for each pollen allergen. The selection of the optimal reference target was performed by geNorm software. Finally, the housekeeping genes α-tubulin and 18S rRNA were chosen as optimal references.

According to the AllFam database of allergen families, the pollen allergen genes can be grouped into ten classes: pectate lyase(allergen Amb a 1.1, 1.2, 1.3, 1.4 and 1.5); plastocyanine (Amb a 3); defensin-like protein (Amb a 4); group 5 (allergen Amb a 5); non-specific lipid transfers protein (allergen Amb a 6); lastocyanin (Amb a 7); profilin (allergen Amb a 8) ; calcium binding protein (allergen Amb a 9 and Amb a 10); cysteine protease (Amb a 11) and the cystatin family for Amb a CPI (cystatin proteinase inhibitor).

The relative expressions of four different allergens in 80 ppb NO_2 pollen were significantly increased as compared to the control pollen of the first and second generations (Fig. 9 & 10). Amb a 1.1 and Amb a 1.2, which belong to the pectate lyase allergen gene family, showed almost twice the relative expression values as compared to the control, both in the first and the second generation. The pectate lyase allergen gene family contains the most important allergens, which are responsible for skin test reactions of 95% of ragweed-sensitive individuals and which show highest IgE antibody titers (Platts-Mills and Woodfolk 2011). Similarly, the profilin (Amb a 8) and calcium binding protein (Amb a 9) gene families raised nearly 1.5 times, as compared to the control. On the other hand, Amb a CPI slightly decreased, and Amb a 1.3, Amb a 1.4, Amb a 1.5, Amb a 5; Amb a 6 slight increases, however, were not significant (Fig. 9 & 10).

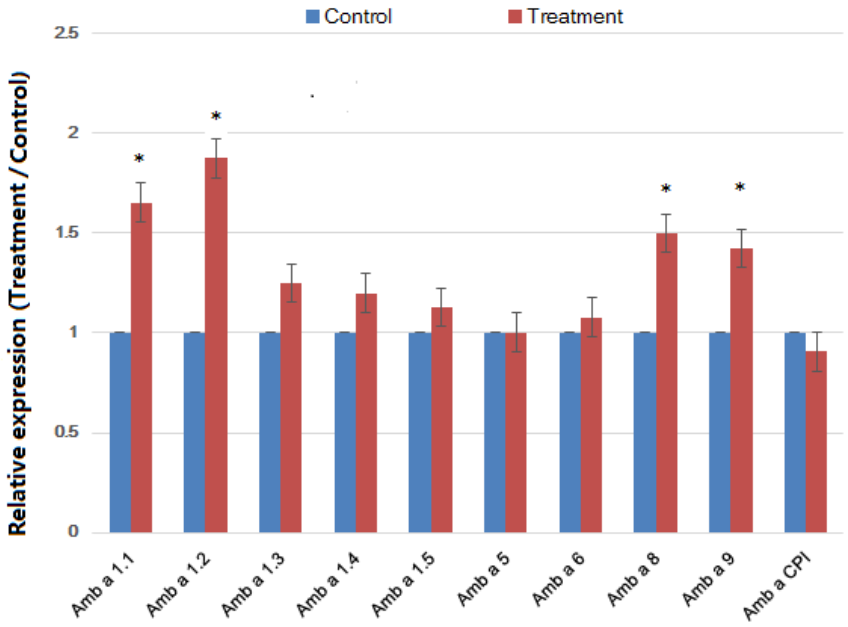


Figure 9. Real-time qRT-PCR analysis of pollen allergen genes of first generation ragweed pollen (2011) of both, treated (80 ppb NO_2) and control (40 ppb NO_2) plants. Transcript levels were normalised with respect to 18S-rRNA and α-tubulin transcript levels. Pair-wise fixed reallocation randomisation test was performed according to Pfaffl et al., (2002). Mean values were obtained from three independent PCR amplifications. * = p-value < 0.05

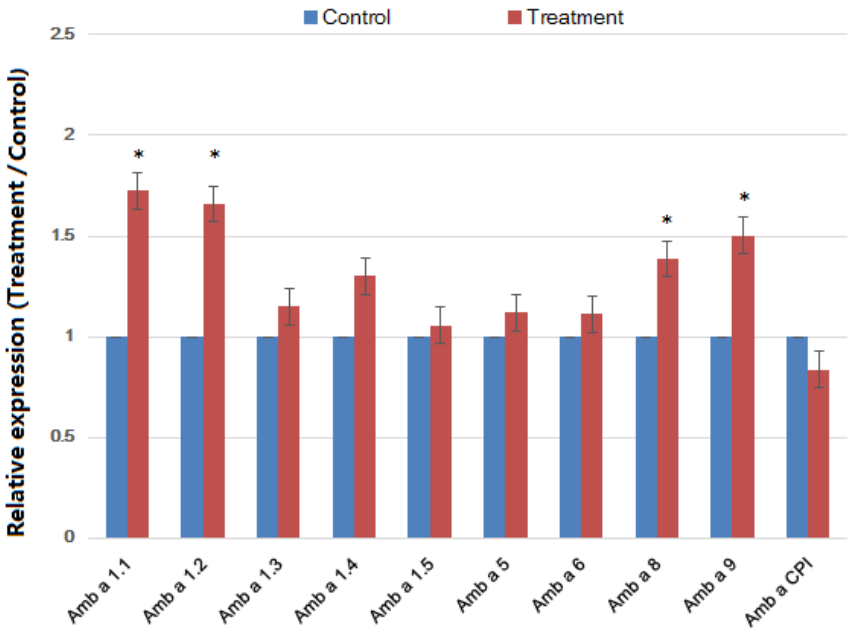


Figure 10. Real-time qRT-PCR analysis for pollen allergen genes of second generation ragweed pollen (2012) of both, treatedt (80 ppb NO_2) and control (clean air) plants. Transcript levels were normalised with respect to 18S-rRNA and α-tubulin transcript levels. Pair-wise fixed reallocation randomisation test was performed according to Pfaffl et al., (2002). Mean values were obtained from three independent PCR amplifications. * = p-value < 0.05

3.2.2 Suppression Subtractive Hybridization (SSH) Library (1st generation)

Suppression subtractive hybridization is a technology that allows a PCR-based amplification of only such cDNA fragments that differ between control and experimental transcriptomes. In the present work, two libraries of control (40 ppb NO_2) and treatment (80 ppb NO_2) were constructed and analysed, respectively. Numerous transcripts present in 80 ppb NO_2 library were up-regulated due to the elevated NO_2.

As a result of two times of hybridization and two times of PCR amplification, only differentially expressed sequences were exponentially amplified and the others were subtracted. The PCR products were obviously different between 80 ppb NO_2 and control SSH libraries and numerous bands disappeared as compared to the non-subtracted PCR products (Fig. 11).

3.2.2.1 Disassociation and identification of differentially expressed sequences

Vector construction was adopted to achieve sequences disassociation with high efficacy. Differentially expressed sequences were ligated to pGEM ®-T vector (Promega) and transformed into *E.coli* for further analysis. One thousand positive single clones from each library were selected and screened by PCR. Large differences in length were shown by PCR (Fig. 12). The clones yielding a single band with lengths of more than 300 bp were sequenced as positive clones. Finally, 190 positive clones from each library were sequenced (Appendix. 1).

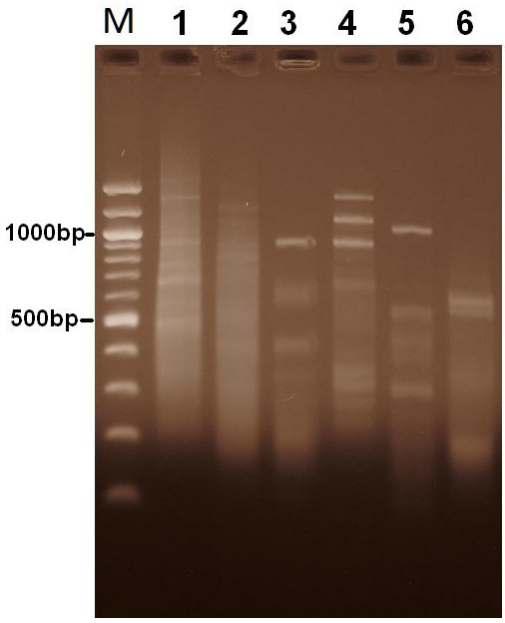


Figure 11. PCR product of control and 80 ppb NO_2 SSH libraries before and after subtraction
M: Marker (100 bp DNA Ladder, Thermo); 1: PCR product of cDNA from human skeletal muscle before subtraction; 2: PCR product of cDNA from control cDNA before subtraction 3: PCR product of cDNA from 80 ppb NO_2 cDNA before subtraction; 4: PCR product of cDNA from human skeletal muscle after subtraction 5: PCR product of cDNA from control cDNA after subtraction; 6: PCR product of cDNA from 80 ppb NO_2 cDNA after subtraction.

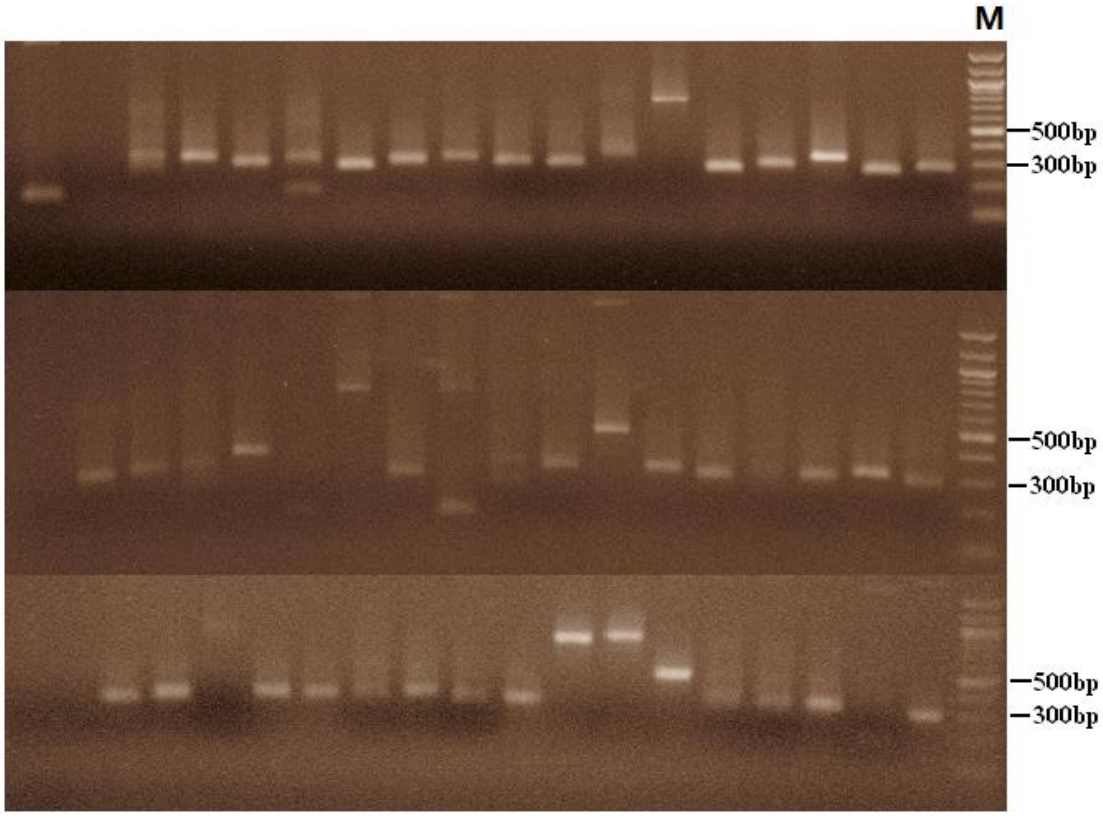


Figure 12. Representative PCR screening result of positive clones. M: Marker (100 bp DNA Ladder, Thermo)

3.2.2.2 Functional annotation of SSH fragments

A significant advantage of SSH is the comparability of differentially expressed sequences between control and experimental groups. On the other hand, investigating the transcriptome of non-model plants reflects a challenge due to the shortage of a complete genome database for accurate annotation, especially with respect to a particular plant part such as pollen. After annotation by *Ambrosia* pollen 454-sequence database (Kanter et al. 2013) and NCBI database, the chart revealed that more than half of the sequences can only match *Ambrosia* pollen 454-sequence database both in 80 ppb NO_2 and control SSH libraries. Moreover, the percentage of non-annotated genes were 6% and 3% in 80 ppb NO_2 and control SSH libraries, respectively (Fig. 13 & 14).

In the elevated NO_2 library, protein transport protein Sec61 beta accounted for the largest share of 17%, whereas in the control library alpha-2 tubulin and SF16 protein accounted for 15% and 12%, respectively (Fig. 13 & 14). The result of annotation indicated that there are no overlapping genes in both libraries. The SSH process thus effectively eliminated the same expressed genes and enriched the differentially expressed genes.

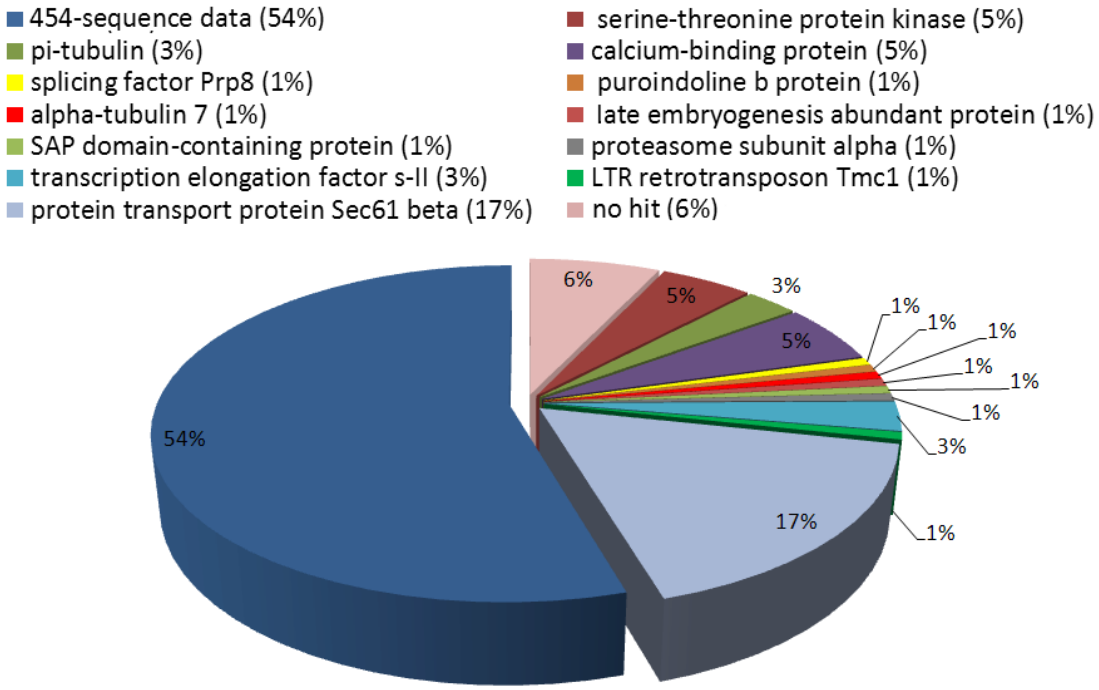


Figure 13. Annotation of the upregulated sequences blasted against ragweed pollen 454-sequence database and NCBI database.

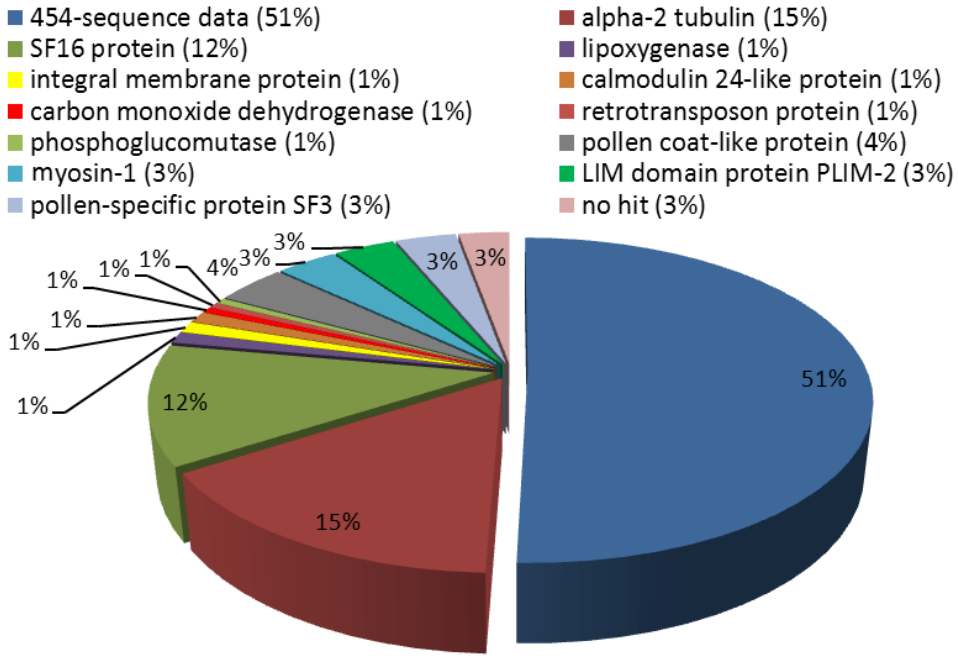


Figure 14. Annotation of the downregulated sequences blasted against the ragweed pollen 454-sequence database and the NCBI database.

3.2.2.2.1 Reproduction related genes

The further description of each gene in these two libraries is provided in Tab.5. Some of the genes in the control library are related to reproductive processes. Knockout mutants of phosphoglucomutase severely impair male and female gametophyte function; SF16 protein acts together with PLIM-1 to regulate the male gamete maturation, pollen tube formation and fertilization; plant mutants without myosins-1 show reduced aerial tissue and number of seeds per silique; pollen coat protein plays an important role in dehydration and rehydration of the pollen grain and pollen-stigma signalling. Since pollen is of crucial importance in plant fertilization and reproduction as male gamete, the suppression of these reproduction related genes by 80 ppb NO_2 at the transcriptome level may subsequently affect the protein expression and even plant propagation.

3.2.2.2.2 Stress related genes

Based on the chemical property of nitrogen dioxide (NO_2), elevated NO_2 is an abiotic stress for plants. However, stress related genes were found in both libraries. Numerous abiotic stress related genes (drought, cold, salt, heat, etc.) were upregulated upon elevated NO_2. Protein transport protein Sec61 beta, which accounts for 17% in the 80 ppb NO_2 library, can increase cold resistance in plant; Serine/threonine protein kinase and Calcium-binding protein which both account for 5% in 80 ppb NO_2 library respond to salt, drought, oxidative stress and hot, anoxia stress, respectively.

3.2.2.2.3 Other genes

Elevated NO_2 has effectively enhanced and restrained the expression of certain genes which are related to reproduction and stress resistance in ragweed pollen. On the other hand, some other noteworthy genes have also been influenced by the environmental change. Transcription elongation factor s-II, the expression of which was upregulated in ragweed pollen, markedly reduced seed dormancy. Similarly, the upregulation of Pi-tubulin may indicate an inhibition of tubulin assembly in pollen.

Some of cytoskeleton and metabolic enzyme genes have been found in the libraries and the changes of these genes may also have effects on plant growth.

Library	Acc.No.	Protein Family	Function	References
Control	CAA52782	SF16 protein	Act together with PLIM-1 to regulate the expression of a number of late pollen-specific genes	Natalia Dudareva, et al. 1994
Control	NP001147673	SF3 Protein (PLIM 1)	Male gamete maturation, pollen tube formation, fertilization	Rachel Balk, et al. 1992
Control	AF116850	LIM domain protein PLIM-2	Male gamete maturation, pollen tube formation, fertilization	Rachel Balk, et al. 1992
Control	ABM66381	Pollen coat protein	Dehydration and rehydration of the pollen grain , pollen-stigma signaling	Mayfield., et al. 2001 Stanchev et al. 1996
Control	AEH27529	Phosphoglucomutase	Knockout mutant of phosphoglucomutase severely impaired male and female gametophyte function	Egli, B., et al. 2010
Control	XP002533960	Myosin-1	Knockout mutant of myosins reduced aerial tissue and number of seeds per silique	Prokhnevsky, A. I., et al. 2008 Sparkes, I. A. 2010
Control	BAD62122	Integral membrane protein	Involved in accumulation and transduction of energy, and proteins responsible for cell adhesion.	Saier, et al. 2009
Control	CAL35827	Alpha-2 tubulin	Cytoskeleton protein	Dutcher. 2001
Control	CAQ58078	Lipoxygenase	Express a variety of cytosolic isozymes as well as what seems to be a chloroplast isozyme in plant	Peng , et al. 1994
Control	ABB47557	Retrotransposon protein	largely responsible for genome expansion	Todorovska. 2007
Control	CCA62925	Carbon monoxide dehydrogenase	Metabolic enzymes	
Control	ADC80732	Calmodulin 24-like protein	Stress related (heat,drought,cold ,salt)	Reddy,et al. 2011
NO_2	ABV55999	Alpha-tubulin 7	Cytoskeleton protein	Dutcher. 2001
NO_2	AAK94059	Pi-tubulin	Inhibited tubulin assembly in pollen.	Evrard, J. L., et al. 2002
NO_2	XP002532142	Transcription elongation factor s-II	Reduced seed dormancy in plant, Increased oxidative stress resistance in yeast	Grasser et al. 2009 Koyama, H., et al. 2003
NO_2	AAZ32862	Splicing factor Prp8	Important regulator of spliceosome activation	Pena, V., et al. 2007
NO_2	ACG31189	Protein transport protein Sec61 beta	Increase cold resistance	Leroch, M., et al. 2008
NO_2	XP002518508	Serine/threonine protein kinase	Increased salt, drought, oxidative stress resistance	Mao, X. G. et al. 2010 Liu, J. P., et al. 2000
NO_2	XP002520716	Late embryogenesis abundant protein	Increased drought, cold and ABA resistance	Ingram, J. and D. Bartels. 1996 Thomashow, M. F. 1999 Hundertmark, M. and D. K. Hincha. 2008
NO_2	CAB63264	Calcium-binding protein	Increased heat ;anoxia, salt stress resistance	Zhu, J. K. 2000 Zielinski, R. E. 1998 Safadi, F., et al. 2000
NO_2	CAQ43070	Puroindoline b protein	Control the endosperm texture (hardness or softness)	Massa, A. N. and C. F. Morris. 2006 Day, L., et al. 2006
NO_2	NP195678	SAP-domain protein		
NO_2	FR692049	LTR retrotransposon Tmc1	Largely responsible for genome expansion	Todorovska. 2007
NO_2	CBJ27423	Proteasome subunit alpha		

Table 5. Functional annotation of sequences from control and treatment SSH libraries

3.2.3 Transcriptome sequencing and RNA-Seq analysis by illumina sequencing

The Illumina system utilizes a sequencing-by-synthesis approach to allow the comparative analysis of whole transcriptomes of two generations of common ragweed pollen from plants exposed to 80 ppb (treatment) and 40 ppb NO_2 (control 2011) / clean air (control 2012), respectively.

3.2.3.1 De novo assembly of common ragweed pollen transcriptome

The transcriptome library of ragweed was prepared from Illumina sequencing data of ragweed pollen. For Illumina sequencing data, three independent biological replicates of each condition were performed and sequenced on the IlluminaHiSeq 2000 system with read lengths of 100 bp.

Items	1st Control	1st Treatment	2nd Control	2nd Treatment	Total
No.of reads	301,139,066	286,833,170	274,805,950	242,931,114	1,105,709,300
No.of assembled transcripts					35,136
Minimum length of transcripts					196
Maximun length of transcripts					10,492
Average length of transcripts					722
Total length of transcripts					25,380,299

Table 6. Summary of common ragweed pollen transcriptome sequencing dataset

For non-model organism studies, a *de novo* assembly is the only option for sequence assembly. In order to cover the ragweed pollen transcripts as completely as possible, *de novo* assembly was performed with the Illumina reads of pollen from ragweed exposure to 40 ppb and 80 ppb NO_2 (2011), clean air and 80 ppb NO_2 (2012), together with Illumina reads of pollen from ragweed exposure to 40 ppb and 80 ppb ozone, 40 ppb and 120 ppb ozone, common air and 100 ppb ozone plus 100 ppb NO_2 and 454-sequencing data of ragweed pollen produced under 40 ppb and 80 ppb ozone (kindly provided by Dr. Ulrike Frank). After removing low quality and adaptor sequences, 35,136 unique transcripts with an average length of 722 bp - the longest transcript measured 10,492 bp - were generated (Tab. 6). This *de novo* assembly result was used as RNA-seq reference in the RNA-seq process.

3.2.3.2 Annotation of ragweed unique transcript sequences

The assembled ragweed unique transcripts were blasted by homologous search against the databases of GenBank *Viridiplantae* database. A total of 86.72% (30,470) unique transcripts had significant hits (E-value $\leqslant$ 1e-6) in the database. Further annotation of the ragweed unique transcripts was performed by assigning them with gene ontology (GO) terms (Blast2Go plugins). A total of 16,361 unique transcripts (46.56%) were assigned with at least one GO term (Fig. 15). Among them, 6,370 (38.93%) were assigned in the biological process category, 5,832 (35.65%) in the molecular function category, and 6,932 (42.37%) in the cellular component category, while 4,601 (28.12%) unique transcripts were assigned GO terms in all three categories. It was also observed that the percentage of genes that could be annotated was positively correlated with the length of genes (Fig. 16).

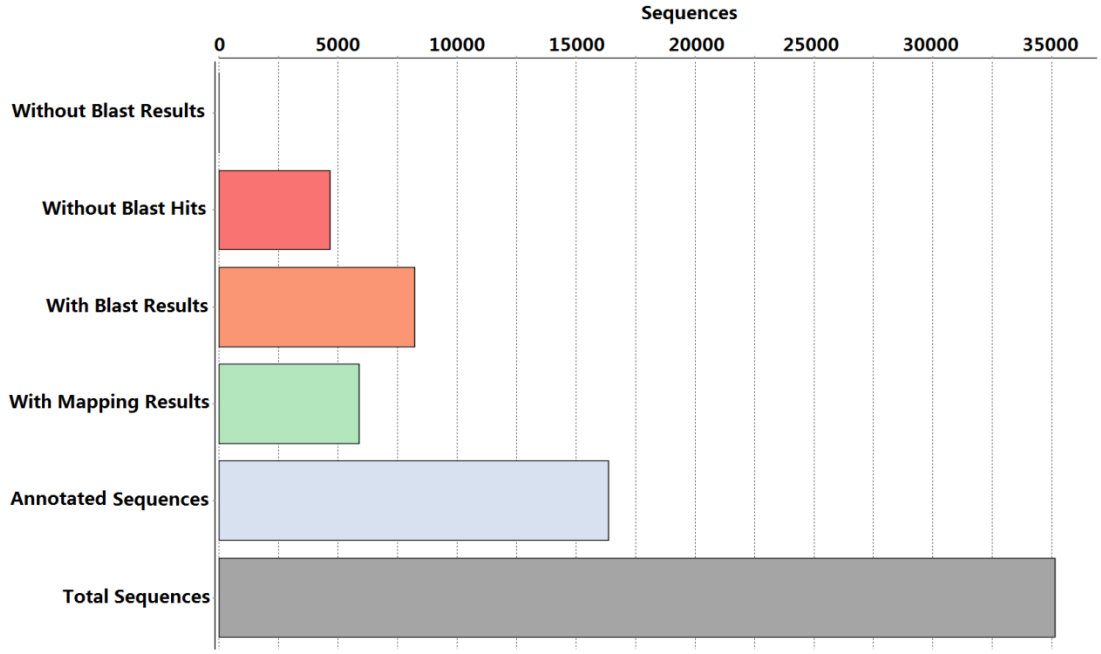


Figure 15. Numbers of annotated, mapped and blasted ragweed unique transcripts.

The ragweed unique transcripts were further classified into different functional categories using a set of plant-specific GO slims (Blast2Go plugins). The top 15 groups of the biological process and molecular function categories are shown in Fig. 17. “Response to cadmium ion” and “response to salt stress” represented the most two abundant groups in the biological process category. Other interesting highly

abundant groups in the biological process category included “response to cold”, “defense response to bacterium”, “pollen tube growth” and “regulation of flower development” (Fig. 17A). In the category of molecular function, the most abundant groups included “ATP binding”, “protein binding” and “zinc ion binding”, and other appealing groups included “DNA binding”, “RNA binding”, “various ions binding”, “kinase activity”, and “hydrolase activity” (Fig. 17B).

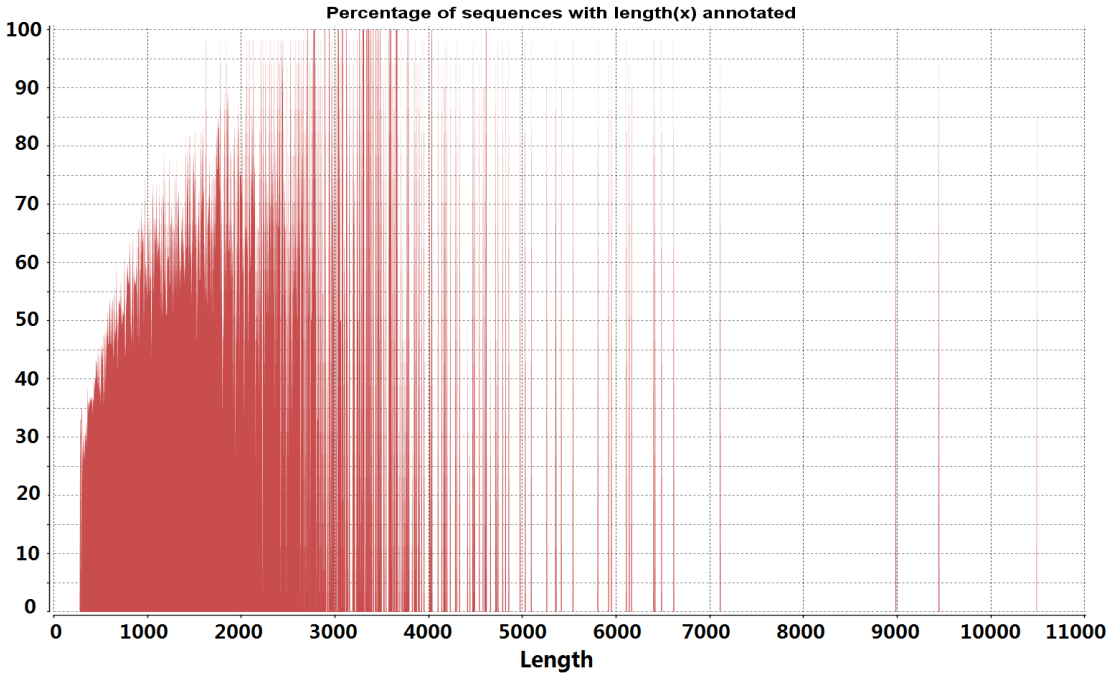


Figure 16. Percentage of annotated sequences with length.

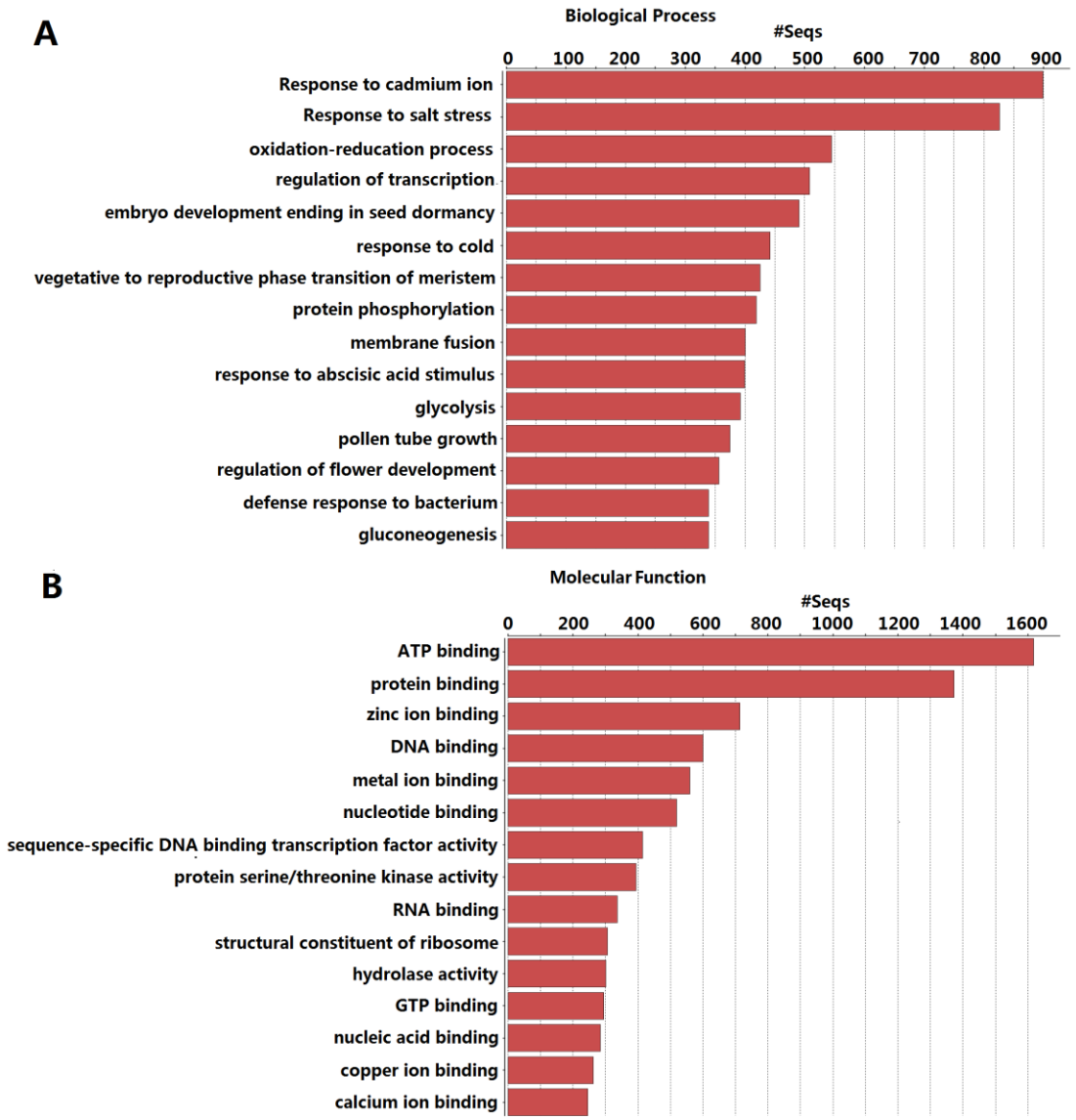


Figure 17. Functional classification of ragweed unique transcripts (A) in the category of biological process and (B) in the category of molecular function.

3.2.3.3 Comparative transcriptome profiles under elevated NO_2 in ragweed pollen

RNA-seq was performed for the comparative analysis of the transcriptomes of ragweed pollen from plants exposed to 80 ppb and 40 ppb NO_2 (control 2011) / clean air (control 2012), respectively. In total, 27,376 and 25,154 differentially expressed unique transcripts were identified in the first and second generation of ragweed pollen, among which 11,866 were induced by 80 ppb NO_2 stress and 15,510 repressed in first generation, while 13,948 were increased and 11,206 decreased in the second generation.

GO terms were identified in the biological process and molecular function categories that were significantly enriched in elevated NO_2-induced and -repressed genes in the first and second generation, respectively. The top 20 groups are shown in Fig. 18 (biological process) and Fig. 19 (molecular function).

In the GO term of biological process, “response to cadmium”, “response to salt stress” and “pollen tube growth” were the most three enriched groups in NO_2 up- and down regulated genes of both generations. Most of the top 20 groups are the same in the first and the second generation with few exceptions like “response to wounding”, “abscisic acid mediated signalling pathway” and “photorespiration”, which are only present in the first generation, whereas “defense response to bacterium”, “gluconeogenesis” and “ubiquitin-dependent protein catabolic process” are only present in the second generation (Fig. 18). The groups of “pollen tube growth”, “membrane fusion”, “response to water deprivation”, “response to chitin” and “plant-type cell wall modification” were highly enriched in NO_2-induced upregulated genes whereas “glycolysis” was highly enriched in NO_2-induced downregulated genes in both generations.

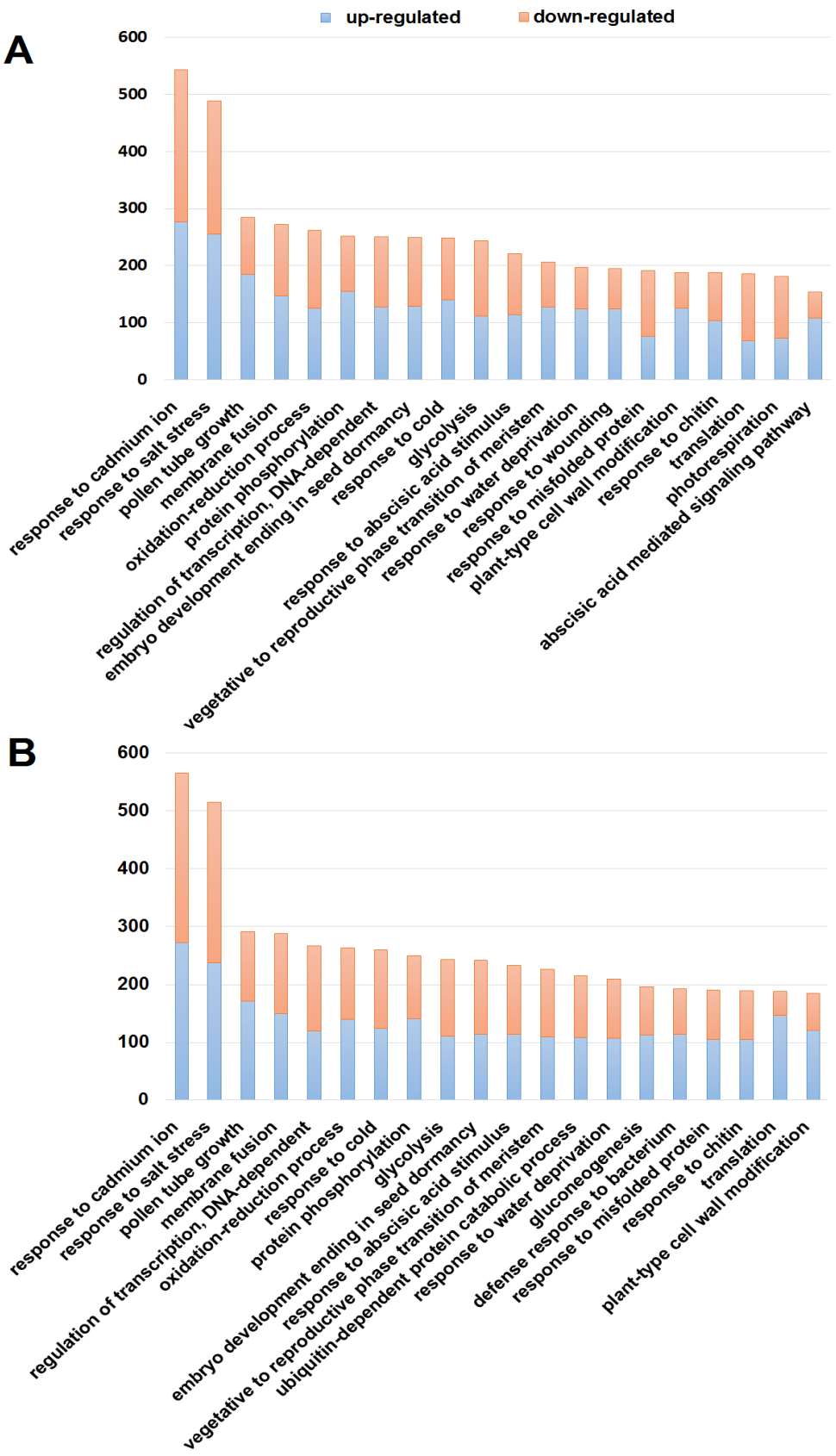


Figure 18. The top 20 up- and downregulated groups of biological process in response to elevated NO_2. A: First generation B: Second generation.

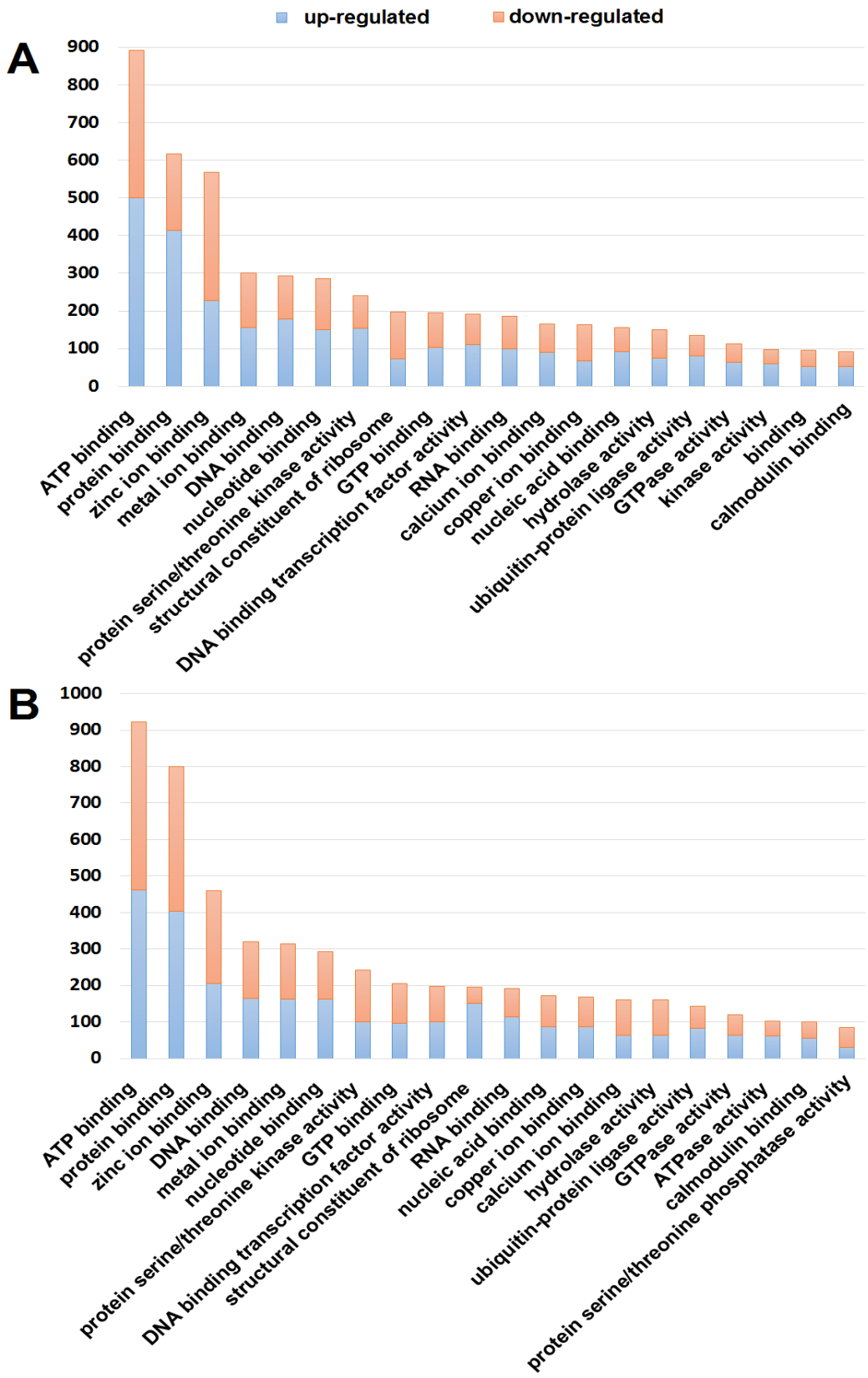


Figure 19. The top 20 up- and downregulated groups of molecular function in response to elevated NO_2. A: First generation B: Second generation.

In the GO term of molecular function, the “ATP binding” was the largest group of both generations, followed by the “protein binding”, the “zinc ion binding” and the “DNA binding”. The “kinase activity” and “binding” were only present in the top 20 groups of the first generation, whereas “protein serine/threonine phosphatase activity” and “ATPase activity” were only in the 20 groups of the second generation. The "protein binding", "DNA binding", "metal ion binding", "nucleotide binding", "DNA binding transcription factor activity", "RNA binding", "ubiquitin-protein ligase activity", "GTPase activity" and "calmodulin binding" were highly enriched in NO_2-induced upregulated genes whereas “zinc ion binding” were highly enriched in NO_2-induced downregulated genes of both generations (Fig. 19)

3.2.3.4 Comparative analysis of allergens

Pollen allergens have always been the most important points of the common ragweed research. In this transcriptome library, 7 ragweed pollen specific allergen isoforms were identified. Three contigs were identified as Amb a 1.1 and all these contigs were upregulated in 80 ppb treated pollen. Contig 525 and 881 were confirmed as Amb a 1.2, all of these two contigs increased in the second generation. However, contig 525 decreased in the first generation. Amb a 1.4 was upregulated in both generations while Amb a 6 was downregulated in the first generation. Three contigs were identified as Amb a 1.3, contig 221 increased in both generations, however, contig 3057 decreased. Amb a 1.5 was composed of 4 contigs, contig 397 was upregulated and contig 5461 was downregulated in both generations. The Amb a 3 and some contigs of other allergens had inconsistent expression patterns between the first and second generation (Tab. 7).

Allergen isoform	contig number	hits	E-value	Transformed change fold (log 10)	
				1st generation	2nd generation
Amb a 1.1	427	20	8,62E-110	1.49	2.45
	428	40	0	1.1	1.3
	1969	20	3,98E-146	1.01	1.69
Amb a 1.2	525	20	7,49E-152	-1.37	1.97
	881	20	2,58E-66	1.29	1.62
Amb a 1.3	221	20	0	1.1	1.01
	3057	20	2,35E-58	-1.11	-1.04
	3634	20	1,11E-39	-1.45	2.1
Amb a 1.4	433	20	0	1.03	1.78
Amb a 1.5	397	20	0	1.04	1.35
	2117	20	2,06E-8	-3.12	6.38
	3264	20	1,05E-29	-1.26	2.05
	5461	20	2,06E-137	-4.23	-7.3
Amb a 3	28	22	9,40E-38	-1.06	1.15
Amb a 6	6178	20	2,12E-79	-0.18	NaN

Table 7. Identification of allergen isoforms in Illumina sequencing library of ragweed. NaN: not present.

3.3 Comparative proteome analysis

3.3.1 2D-Difference-in-gel-electrophoresis (2D-DIGE) (1st generation)

The 2D-DIGE was used to distinguish differentially expressed soluble proteins from ragweed pollen under 80 ppb (treatment) and 40 ppb NO_2 (control). Plants from the same sub-chamber were grouped (control-1, control-2, treatment-1 and treatment-2) for statistical analysis. Around 3568 spots were highly resolved and detected, over a pH range of 4–7 and a molecular weight range of 10–100 kDa. The areas and volumes of spots were quantified by DeCyder 2D software (Fig. 20). These values were expressed as a ratio (fold change) comparing to those corresponding in the internal standard.

All spots were matched by gel-to-gel comparisons and the differences of the relative abundance (vol %) of each spot were analysed. Those spots, the abundance of which varied at least ±1.5-fold between the treated and control pollen, were considered as interesting spots. Finally, 65 interesting spots were selected. The relative abundance of 35 spots significantly increased in the treated ragweed pollen (Fig. 21A), whereas 30 spots decreased (Fig. 21B). Heat map analysis by hierarchical clustering revealing the differential expressions of these 65 spots among the four

groups is given in Fig. 22. It is clear to see the similar expression patterns in the same treated groups and disparate between differently treated groups (Fig. 22).

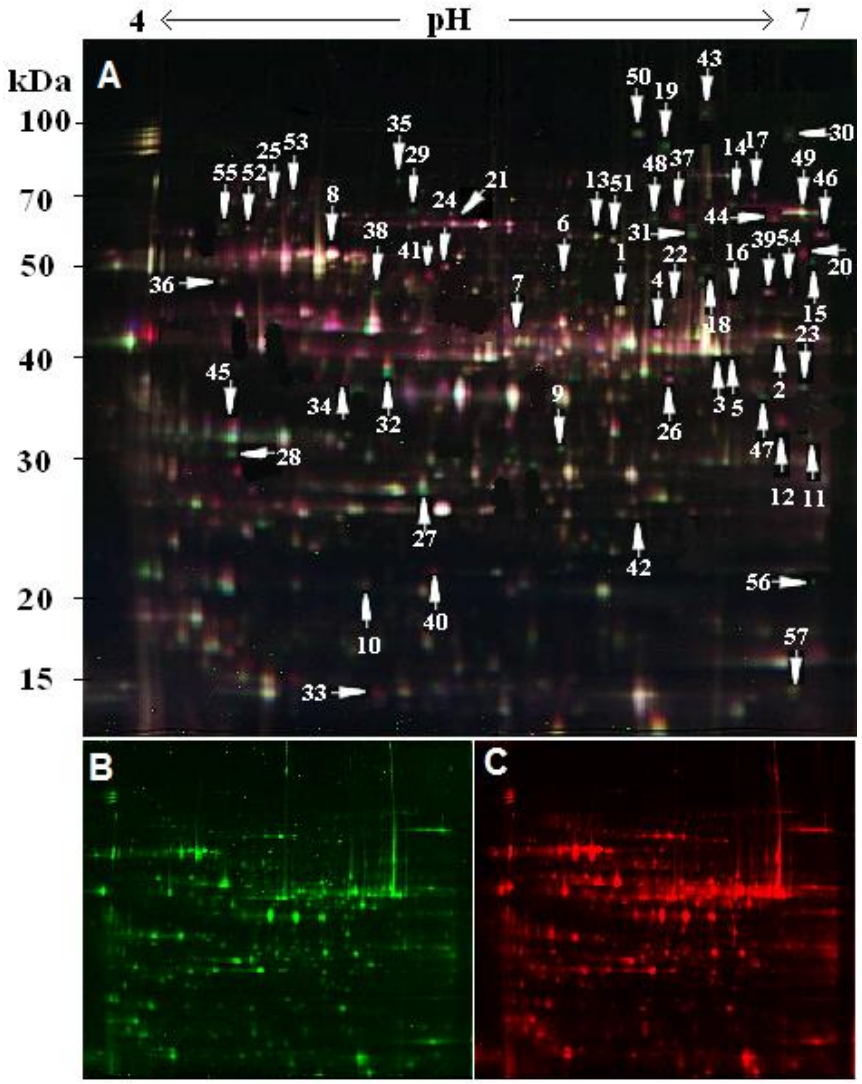


Figure 20. Representative 2D-DIGE images of *Ambrosia* pollen proteins isolated from ragweed pollen labelled with minimal CyDye DIGE fluorescence. A: The three overlaid photos; B: The Cy3 image corresponding to pollen of 40 ppb NO_2; C: The Cy5 image corresponding to pollen of 80 ppb NO_2; Nr. 1-57: Identified spots with significant differential expression.

The spots of interest were digested and analysed by LC-MS/MS, followed by a homology-driven identification search. Thirty upregulated protein spots and 27 downregulated protein spots could be identified. These 57 differentially expressed proteins are marked on the representative 2D-DIGE gel (Fig. 20A). The predicted molecular masses and pIs were generally consistent with the experimental data for

the majority of the identified proteins, as judged from the location of spots on 2-D gels.

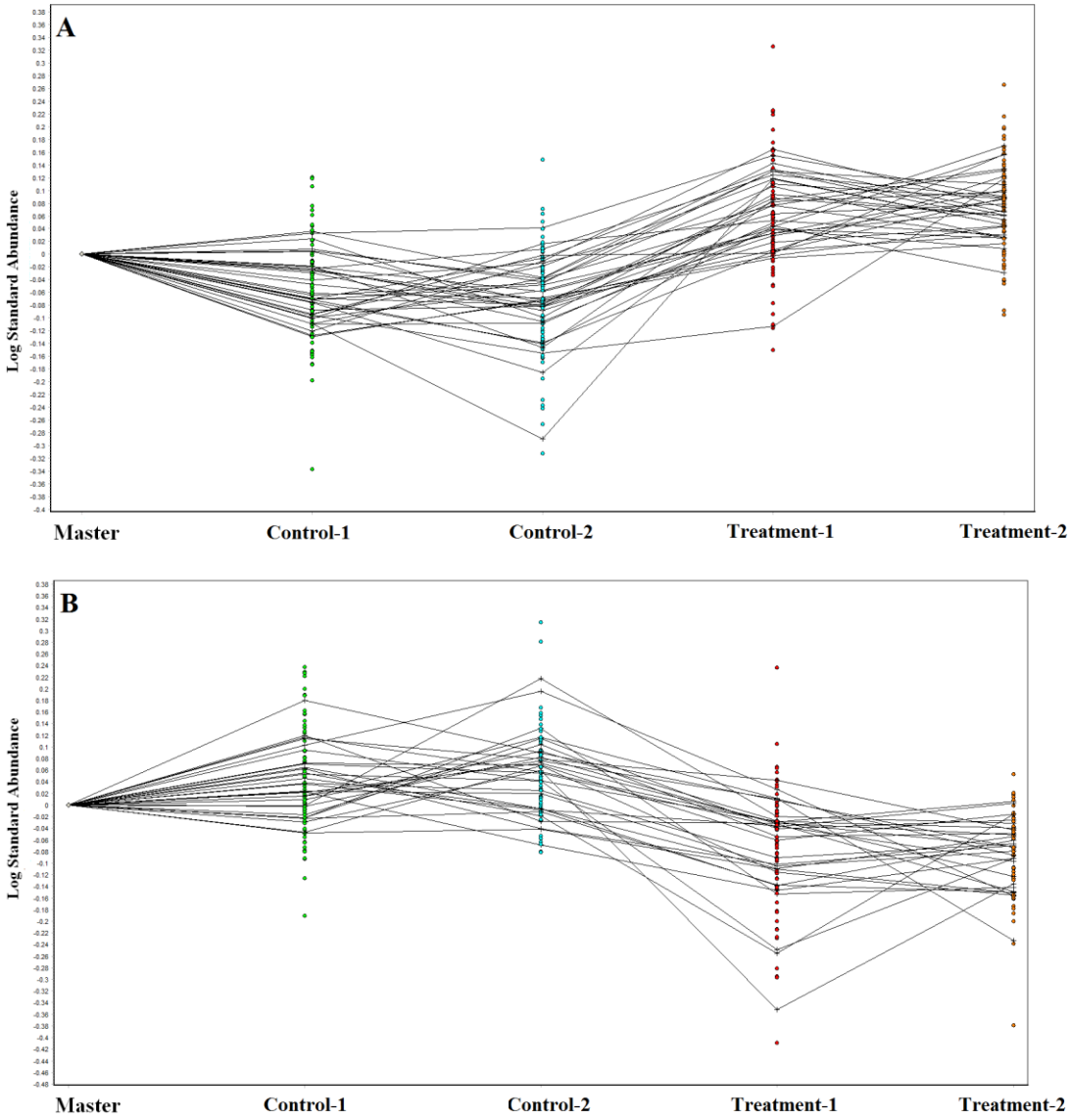


Figure 21: Partitioning clustering analysis of 65 spots with abundance varied at least ±1.5-fold between the treated and control pollen. A: Partitioning clustering of 35 spots with increased abundance upon 80 ppb NO_2; B: Partitioning clustering of 30 spots with decreased abundance upon 80 ppb NO_2.

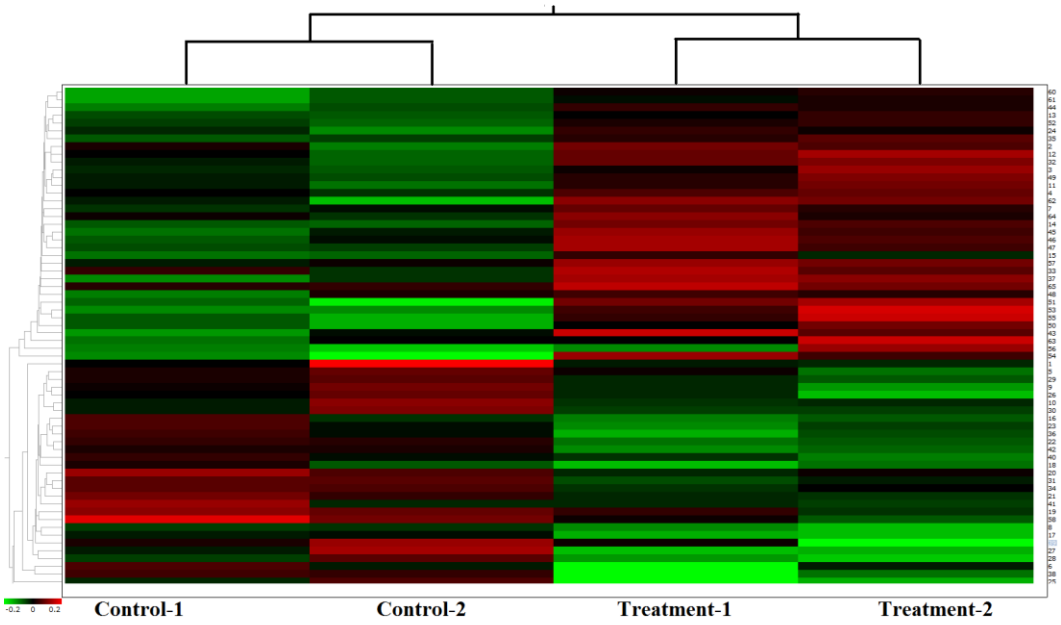


Figure 22: Heat map of hierarchical clustering of 65 spots with abundance varied by group.

The identified proteins were categorised in functional groups according to their predicted protein function (Fig. 23, Tab. 8). The differentially expressed spots, which were upregulated in the first generation of 80 ppb NO_2 treated pollen could be classified into the following functional categories: (1) allergen, (2) cytoskeleton, (3) glycolysis, (4) tricarboxylic acid cycle, (5) calvin cycle, (6) metabolic enzymes, (7) photosynthesis, (8) ammonia assimilation (9) protein folding, (10) stress, (11) methyltransferase and (12) transport (Fig. 23A).

Proteomic analysis of the increased expressed 30 spots revealed that the largest pool comprised 7 spots responsible for stress (23%) and 5 spots (17%) for allergenicity. As shown in Tab. 8, the expression of five allergenic protein spots was significantly upregulated. Interestingly, a homologue to the allergen Hev b 9 from *Hevea* latex was found in the pollen of the treated group but not in the control pollen. Additionally, glutamine synthetase cytosolic isozyme, which is known to be important in the metabolism of nitrogen was also only found in 80 ppb NO_2 treated pollen.

Enlarged and three dimensional images of allergens and other important protein spots are shown in Fig. 24. The spots of allergens are bigger and darker upon 80 ppb NO_2 and their peak values of three dimensional images are significant higher as well. The spots of Hev b 9 and glutamine synthetase cytosolic isozyme disappeared in the figure of control pollen. In contrast, the spot of phosphoglucomutase is absent in the figure of treatment pollen. The spot of 14-3-3 protein A slightly decreased upon 80 ppb NO_2 treatment (Fig. 24).

It is worth noting that the stress-related protein spots, elongation factor and proteasome subunit alpha, the expression ratios of which were 2.66 and 2.58, increased as well at the transcriptional level (Tab. 5).

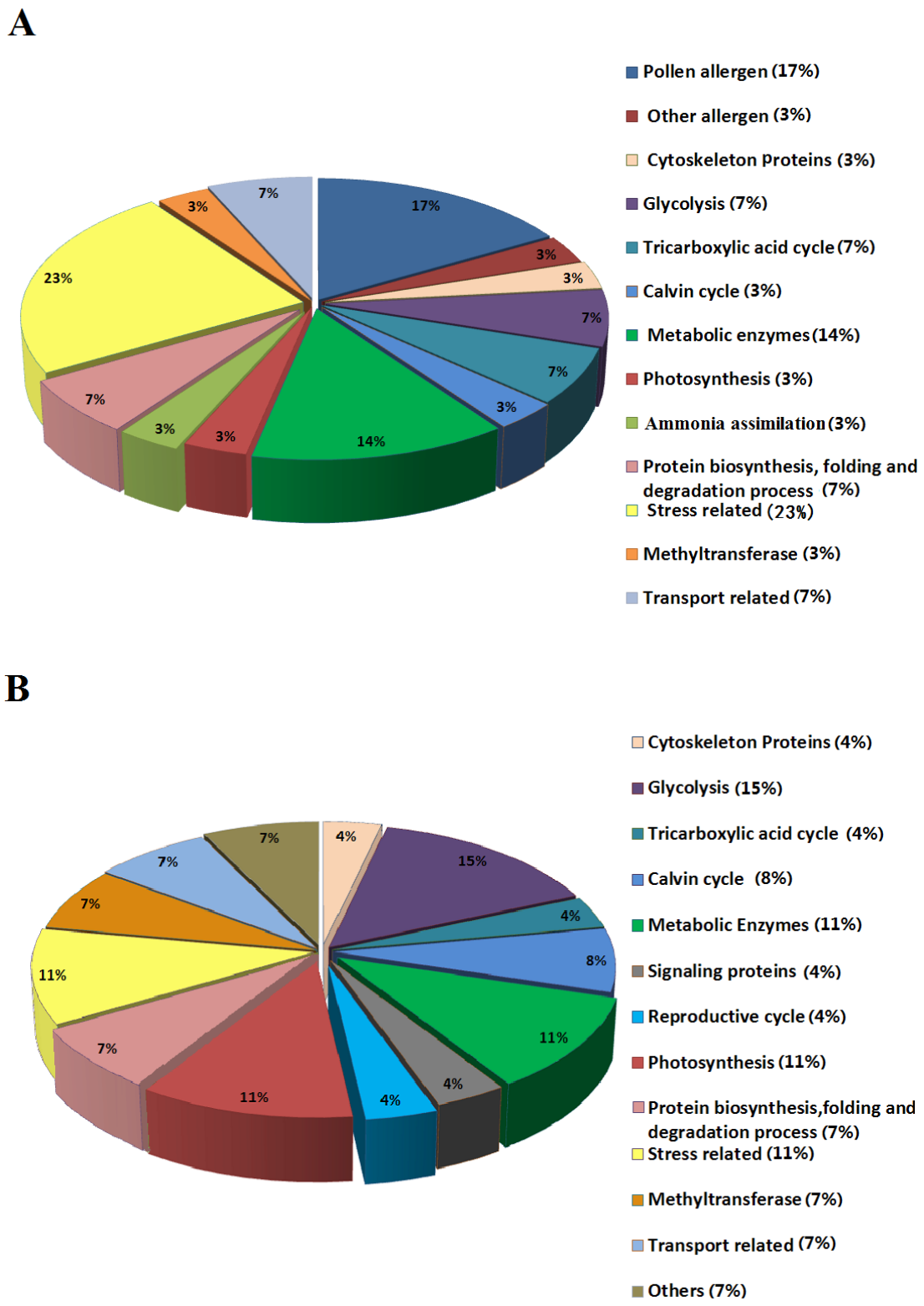


Figure 23. Function classifications of identified proteins of 80 ppb NO_2 treated and control pollen of ragweed. A: Increased expressed protein of 80 ppb NO_2 treated ragweed pollen. B: Decreased expressed protein of 80 ppb NO_2 treated ragweed pollen.

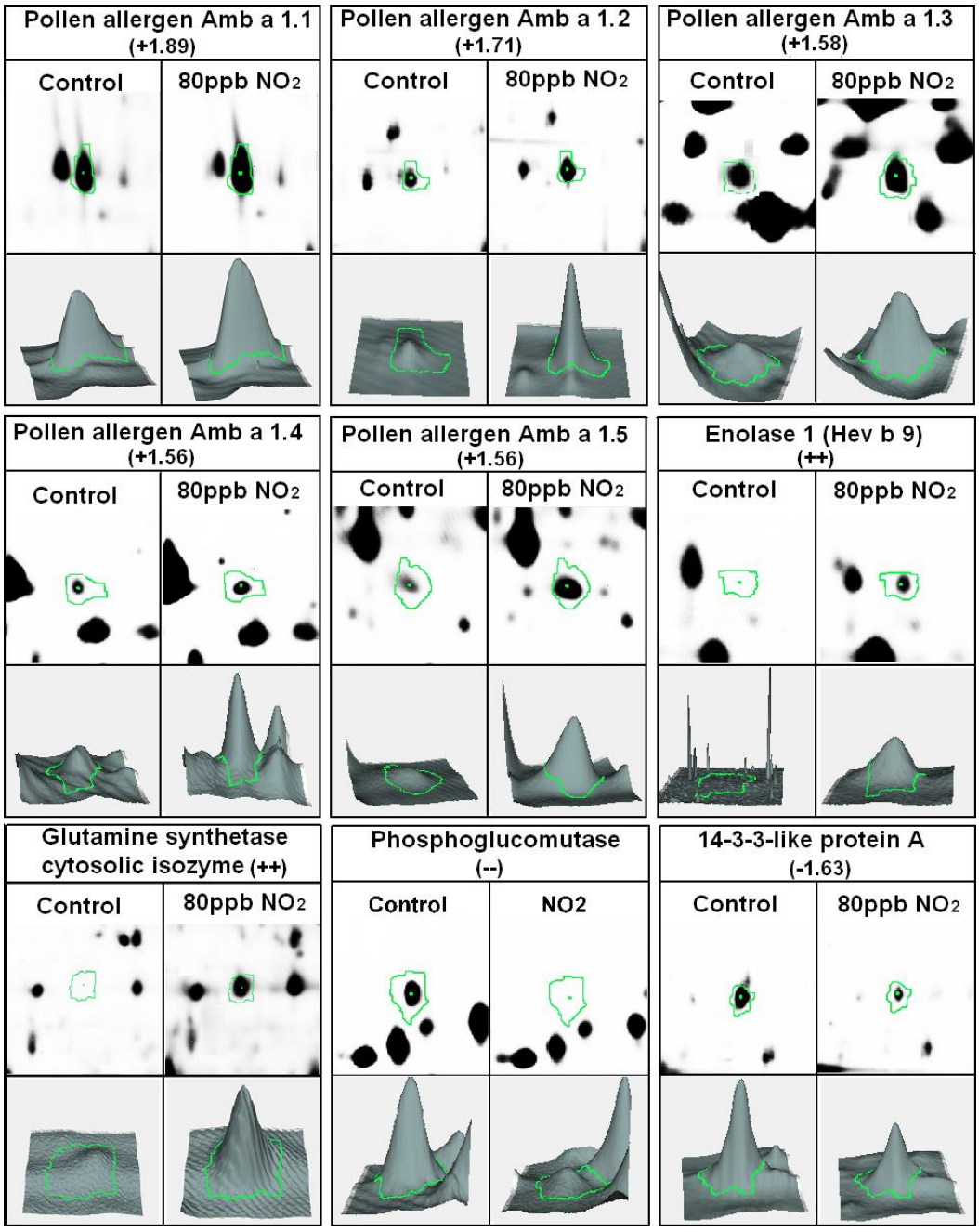


Figure 24. Enlarged and three – dimensional images of selected important spots. The readout of the DeCyder 7 module is shown for pollen allergen Amb a 1.1 (Spot Nr.1), Amb a 1.2 (Spot Nr.2), Amb a 1.3 (Spot Nr.3), Amb a 1.4 (Spot Nr.4), Amb a 1.5 (Spot Nr.5), Enolase 1 (Hev b 9) (Spot Nr.6), Glutamine synthetase cytosolic isozyme (Spot Nr.34), Phosphoglucomutase (Spot Nr.29), and 14-3-3-like protein A (Spot Nr.28).

The downregulated protein spots could be classified in: (1) cytoskeleton, (2) glycolysis, (3) tricarboxylic acid cycle, (4) calvin cycle, (5) metabolic enzymes, (6) photosynthesis, (7) signalling protein, (8) reproduction, (9) protein folding, (10) stress, (11) methyltransferase and (12) transport (Fig. 23B).

Spots	Best matching protein	Acc. No.	Molecular Mass kD	PI	Spot ratio	1-ANOVA
	I: Pollen allergen					
1	Pollen allergen Amb a 1.1	P27759	43	5.68	1.89	1.23E-09
2	Pollen allergen Amb a 1.2	P27760	44	7.09	1.71	5.88E-10
3	Pollen allergen Amb a 1.3	P27761	43	5.97	1.58	2.05E-06
4	Pollen allergen Amb a 1.4	P28744	43	5.83	1.56	9.79E-08
5	Pollen allergen Amb a 1.5	P27762	44	6.44	1.56	2.23E-03
	II: Other allergen					
6	Enolase 1 (Hev b 9)	Q9LEJ0	48	5.54	+	5.96E-03
	III: Cytoskeleton Proteins					
7	Actin-97	P30171	42	5.16	1.54	0.0113
8	Tubulin-alpha-1 chain	P46259	50	4.68	-2.09	3.00E-06
	IV: Glycolysis					
9	Glucose and ribitol dehydrogenase	Q75KH3	32	5.83	-1.89	5.02E-10
10	Triosephosphate isomerase	P48493	21	5.04	2.05	4.54E-06
11	Glyceraldehyde-3-phosphate dehydrogenase	P26519	36	7.61	-1.87	2.59E-04
12	Fructose-bisphosphate aldolase	P46257	38	7.3	1.56	8.46E-04
13	2,3-bisphosphoglycerate	Q9M9K1	61	5.63	-3.08	6.93E-09
14	Glucose-6-phosphate isomerase	P34795	62	6.63	-2.58	2.32E-03
	V: Tricarboxylic acid cycle					
15	Dihydrolipoyllysine-residue succinyltransferase	Q8H107	50	9.63	-2.69	2.22E-03
16	Isocitrate dehydrogenase	Q06197	46	6.14	3.02	5.18E-03
17	Succinate dehydrogenase	O82663	70	6.24	1.96	1.89E-06
	VI: Calvin cycle					
18	Ribulose bisphosphate carboxylase	Q14FE9	53	6.28	-2.36	6.30E-03
19	Transketolase	Q43848	80	6.32	-2.55	1.48E-02
20	Phosphoglycerate kinase	Q42961	50	8.74	1.88	1.39E-03
	VII: Metabolic Enzymes					
21	Glutamate decarboxylase	Q07346	57	5.43	2.03	2.37E-04
22	GDP-mannose 3,5-epimerase	Q93VR3	43	6.15	-2.33	4.96E-04
23	Cytochrome c1-1	P25076	35	7.25	-1.63	1.04E-06
24	Protein disulfide isomerase	Q67UF5	47	5.25	2.36	2.77E-04
25	Beta-fructofuranosidase	Q8W4S6	62	4.79	1.99	1.88E-06
26	Alpha-1,4-glucan-protein synthase	P80607	41	5.98	2.21	1.63E-03
27	NADH dehydrogenase	P80269	26	5.28	-1.79	1.79E-03
	VIII: Signalling proteins					
28	14-3-3-like protein A	P93214	29	4.46	-1.63	2.05E-03
	IX: Reproductive cycle					
29	Phosphoglucomutase	P93805	63	5.4	–	1.48E-05

Table 8. Identified differentially expressed proteins（best matched) of elevated NO_2 treated and control ragweed pollen.
Spot numbers correspond to Fig. 20. + = only detected in treated pollen; - = only detected in control pollen.
All matched proteins are stated in appendix. 2.

Table Cont.

Spots	Best matching protein	Acc. No.	Molecular Mass kD	PI	Spot ratio	1-ANOVA
	X: Photosynthesis					
30	Photosystem I P700 chlorophyll a apoprotein	Q6EW49	82	7.31	-2.63	2.33E-03
31	Photosystem II CP47 chlorophyll apoprotein	Q7FNS4	56	6.76	-1.95	4.32E-06
32	Photosystem II D2 protein	Q4FFP4	40	5.29	-2.44	8.43E-04
33	Oxygen-evolving enhancer protein	P84989	11	5.1	2.77	3.83E-06
	XI: Ammonia assimilation					
34	Glutamine synthetase cytosolic isozyme	Q56WN1	39	5.12	+	1.89E-06
	XII: Protein biosynthesis, folding and degradation process					
35	Heat shock 70 kDa protein	Q9LDZ0	73	5.45	-2.36	9.78E-04
36	26S protease regulatory subunit	Q9SEI2	47	4.65	2.99	7.23E-06
37	Chaperonin CPN60	Q05046	61	6.56	1.89	1.07E-04
38	Eukaryotic initiation factor	P41382	47	5.22	-2.04	5.96E-03
	XIII: Stress related					
39	Elongation factor	Q6YW46	47	6.69	2.66	8.88E-05
40	Inorganic pyrophosphatase	Q43187	24	5.68	2.36	1.77E-02
41	Monodehydroascorbate reductase	Q42711	47	5.06	2.63	1.57E-11
42	Proteasome subunit alpha	Q9LSU0	27	5.97	2.58	1.11E-05
43	Aconitate hydratase	Q42560	98	6.36	-1.69	1.26E-03
44	Catalase isozyme	P48350	57	7.46	3.03	2.32E-06
45	protein phosphatase 2C	Q8RXV3	33	4.43	1.99	1.48E-05
46	Mitogen-activated protein kinase	Q9LV37	58	8.61	2.45	3.29E-03
47	lactoylglutathione lyase	Q8W593	39	7.45	-2	5.02E-06
48	NADP-dependent malic enzyme	P37223	64	6.46	-1.77	2.59E-04
	XIV: Methyltransferase					
49	Serine hydroxymethyltransferase 1	P49357	57	9.05	-2.37	2.25E-03
50	5-methyltetrahydropteroyltriglutamate	P93263	85	6.19	-1.63	1.39E-03
51	Adenosylhomocysteinase	O23255	53	5.83	2.11	5.48E-03
	XV: Transport related					
52	ATP synthase	Q49L13	56	4.86	2.09	6.50E-03
53	V-type proton ATPase catalytic	Q40002	64	5.21	-2.22	1.46E-05
54	Sorting nexin 1	Q9FG38	47	7.28	2.56	1.76E-07
55	Importin subunit alpha	Q71VM4	58	4.85	-1.93	2.36E-10
	XVI: Others					
56	Cysteine-rich repeat secretory protein 38	Q9LRJ9	28	8.9	-1.88	4.77E-05
57	Histone H4	P62785	11	12	-1.76	4.09E-04

Among these proteins, glycolysis proteins account for the biggest part (15%, 4 spots) followed by photosynthesis and stress proteins each accounting for 11% (3 spots). Among the spots related to glycolysis, the expression of four spots were reduced; the most prominent significantly reduced glycolysis protein spot was that of 2,3 bisphosphoglycerate.

All photosynthesis protein spots except oxygen-evolving enhancer protein were reduced. Moreover, expression of three spots related to stress were reduced, whereas 7 spots were significantly accumulated. It deserves to be noted that phosphoglucomutase which showed decreased expression at the transcriptional level (Tab. 5) was only found in the control pollen (Tab. 8).

3.3.2 S-nitrosylation in ragweed pollen (1st generation)

The biotin switch assay was applied for protein S-nitrosylation detection in ragweed pollen under 80 ppb NO_2 and control. As shown in Western blot results of control and treated ragweed pollen, around 20 bands were detected in the pollen exposed to the elevated NO_2 (Fig. 25A) as compared to about 10 bands in the control pollen (Fig.25B). More bands were seen in GSNO-treated pollen (positive control) and the signals were totally subtracted by adding 100mM DTT (negative control) (Fig. 25).

The mean gray value of each lane was measured and calculated by Image J software. As shown in Fig. 26, the band intensities of the pollen, which was exposed to elevated NO_2 were significantly increased, indicating more S-nitrosylated proteins.

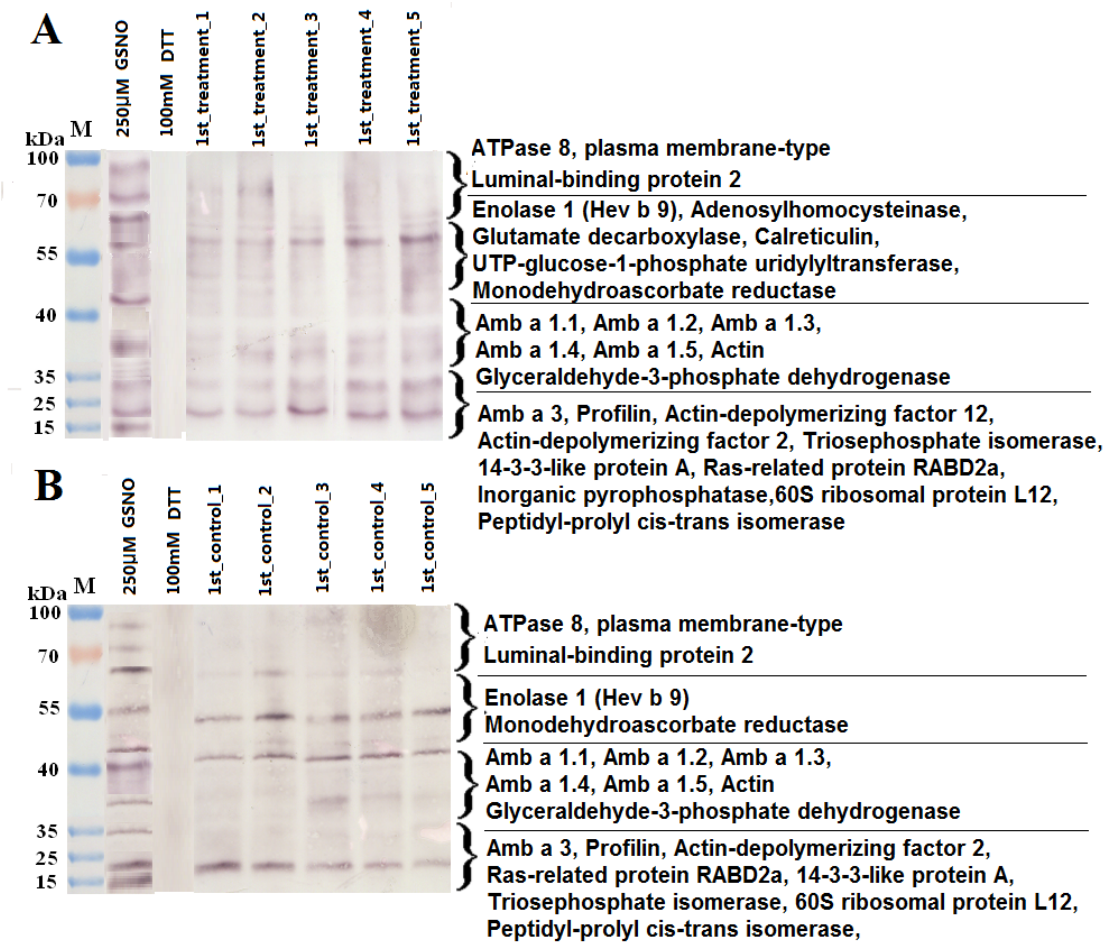


Figure 25. Biotin switch assay western blot result of ragweed pollen. A: Western blot result of ragweed pollen exposed to 80 ppb NO_2 (treatment); B: Western blot result of ragweed pollen exposed to 40 ppb NO_2 (control); 250µl GSNO: 250µl GSNO treated pollen protein as positive control; 100mM DTT: 250µl GSNO + 100mM DTT treated pollen protein as negative control; M: PageRuler Prestained Protein Ladder (10-170 kDa), 1-5: protein of individual plant.

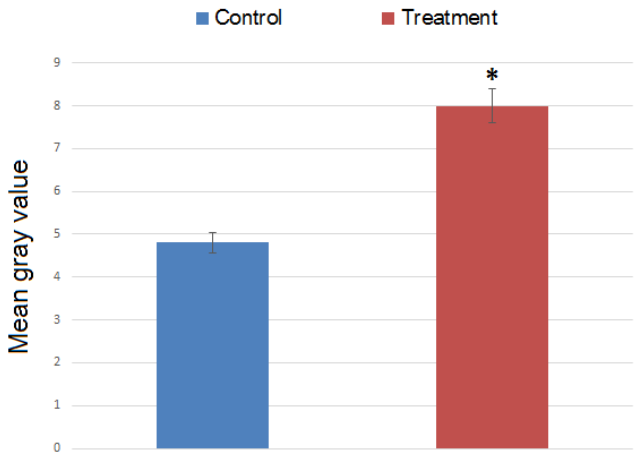


Figure 26. Relative mean gray value of western blot bands intensity calculated by Image J (Error bar=±SE, * = p-value<0.05).

The purified S-nitrosylated proteins from control and treated pollen were separated by SDS-PAGE and the gel was equally divided into four parts and analysed by LC-MS/MS, followed by a homology driven search identification (Fig. 25). Twenty-five proteins were identified in the elevated NO_2 treated ragweed pollen. In contrast, the number of identified proteins was only 19 in control ragweed pollen (Tab. 9).

The S-nitrosylated protein in ragweed pollen could be divided into the following functional categories: (1) allergen, (2) cytoskeleton protein, (3) metabolic enzyme, (4) signalling, (5) stress, (6) reproductive cycle and (7) protein synthesis and folding. Allergens Amb a 1.1, Amb a 1.2, Amb a 1.3, Amb a 1.4, Amb a 1.5, Amb a 3, profilin and Hev b 9 were found in both, 80 ppb NO_2 and control pollen. Some of the detected proteins have been reported in animals and plants. However, six allergens (Amb a 1.1, Amb a 1.2, Amb a 1.3, Amb a 1.4, Amb a 1.5, Amb a 3) and other nine proteins (ATPase 8, glutamate decarboxylase, UTP--glucose-1-phosphate uridylyltransferase, luminal-binding protein 2, Ras-related protein RABD2a, monodehydroascorbate reductase, calreticulin, peptidyl-prolyl cis-trans isomerase and 60S ribosomal protein L12) have never been reported as S-nitrosylated proteins before (Tab. 9). Six proteins (actin-depolymerizing factor 12, adenosylhomocysteinase, glutamate decarboxylase, UTP--glucose-1-phosphate uridylyltransferase, inorganic pyrophosphatase and calreticulin) were only S-nitrosylated in the pollen exposed to elevated NO_2.

It is noteworthy that twelve of these S-nitrosylated proteins displayed significantly different expressions which were detected in 2D-DIGE result. Two proteins (glyceraldehyde-3-phosphate dehydrogenase and 14-3-3-like protein A) were downregulated and 10 proteins (Amb a 1.1, Amb a 1.2, Amb a 1.3, Amb a 1.4, Amb a 1.5, Hev b 9, triosephosphate isomerase, glutamate decarboxylase, inorganic pyrophosphatase, monodehydroascorbate reductase and adenosylhomocysteinase) were upregulated (Tab. 8).

Protein	Acc. No.	Molecular Mass kD	Identified Peptides (Score) Treatment	Identified Peptides (Score) Control	Hints to S-Nitrosylation
I: Pollen allergen					
Pollen allergen Amb a 1.1	P27759	43	WGSYAIGGSASPTILSQGNR (92)	WGSYAIGGSASPTILSQGNR (92)	
Pollen allergen Amb a 1.2	P27760	44	HGDVYTVTSDKDDDVANPK (125)	HGDVYTVTSDKDDDVANPK (125)	
Pollen allergen Amb a 1.3	P27761	43	AILLGADDTHVQDK (94)	AILLGADDTHVQDK (94)	
Pollen allergen Amb a 1.4	P28744	43	ALADcAQGFAK (78)	ALADcAQGFAK (78)	
Pollen allergen Amb a 1.5	P27762	44	QALGNcAQGFGK (84)	QALGNcAQGFGK (84)	
Pollen allergen Amb a 3	P00304	11	GKVYLVGGPELGGWK (58.4)	GKVYLVGGPELGGWK (58.4)	
Profilin	O81982	14	YMVLQGEPGAVIR (70)	YMVLQGEPGAVIR (70)	Fratelli et al. (2002)
II: Other allergy					
Enolase 1 (Hev b 9)	Q9LEJ0	48	IEEELGSEAVYAGANFR (75)	IEEELGSEAVYAGANFR (75)	Fratelli et al. (2002); Lind et al. (2002); Shenton and Grant (2003)
III: Cytoskeleton Proteins					
Actin	P30171	42	SYELPDGQVITIGAER (91)	SYELPDGQVITIGAER (91)	Dalle-Donne et al. (2000,2003); Jaffrey et al. (2001); Fratelli et al. (2002, 2003);Lind et al. (2002)
* Actin-depolymerizing factor 12	Q8LFH6	16	MVYASSKDR (48)		lindermayr et al. (2005)
Actin-depolymerizing factor 2	Q9AY76	17	IFFIAWSPSTSR (58)	IFFIAWSPSTSR (58)	lindermayr et al. (2005)
IV: Metabolic Enzymes					
Triosephosphate isomerase	P48493	21	VASPAQAQEVHAGLRK (108)	VASPAQAQEVHAGLRK (108)	Fratelli et al. (2002) Shenton and Grant (2003)
* Adenosylhomocysteinase	O23255	53	SKFDNLYGcR (44)		lindermayr et al. (2005)
Glyceraldehyde-3-phosphate dehydrogenase	P26519	36	AASFNIIPSSTGAAK (64)	AASFNIIPSSTGAAK (64)	Jaffrey et al. (2001), Teresa Kuncewicz et al. (2003)
ATPase 8, plasma membrane-type	Q9M2A0	104	ADIGIAVADATDAAR (74)	ADIGIAVADATDAAR (74)	
* Glutamate decarboxylase	Q07346	57	LNDLLVEK (34)		
* UTP--glucose-1-phosphate uridylyltransferase	P19595	52	VLQLETAAGAAIK (68)		
V: Signaling proteins					
14-3-3-like protein A	P42653	29	DAAESTLTAYK (61)	DAAESTLTAYK (61)	Lind et al. (2002), Teresa Kuncewicz et al. (2003)
Luminal-binding protein 2	P24067	73	ITPSWVAFTDSER (53)	ITPSWVAFTDSER (53)	
Ras-related protein RABD2a	P28188	23	LQIWDTAGQER (66)	LQIWDTAGQER (66)	
VI: Abiotic stress related					
* Inorganic pyrophosphatase	Q43187	24	TGLIKVDR (45)		Lind et al. (2002)
Monodehydroascorbate reductase	Q42711	47	QGLNPGELAIISK (65)	QGLNPGELAIISK (65)	
VII: reproductive cycle					
* Calreticulin	Q9XF98	48	QTGSLYSDWDLLPAK (27)		
VIII: Protein synthesis and folding process					
Peptidyl-prolyl cis-trans isomerase	Q39613	18	TEWLDGKHVVFGK (49)	TEWLDGKHVVFGK (49)	
60S ribosomal protein L12	O50003	18	VTGGEVGAASSLAPK (56)	VTGGEVGAASSLAPK (56)	

Table 9. Identification and classification of S-nitrosylated proteins in ragweed pollen of the first generation (* = only detected in the pollen of ragweed exposed to 80 ppb NO_2).

3.4 Allergen immunoreactivity analysis

3.4.1 Negative serum screening

Sera from 14 healthy volunteers served as negative controls. A screening of immunoreactivity of these 14 sera to ragweed pollen was performed by dot blot analysis. According to Fig. 27 B, some sera reacted with the pollen (Nr. 3, 5, 7, 13). Therefore, these were not of any use. Finally, a pool comprising the sera of Nr. 1, 2, 4, 6, 8, 9, 10, 11, 12 and 14 was used as negative control in dot blot, 1D immunoblot and 2D immunoblot analyses (Fig. 27).

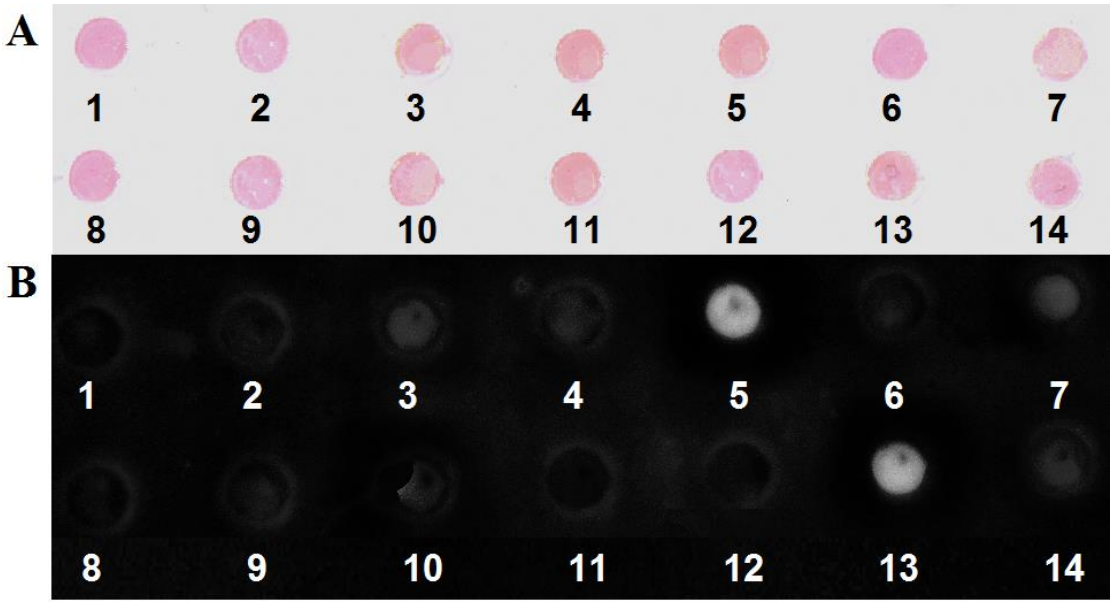


Figure 27. Dot blot of ragweed pollen probed with sera from 14 healthy volunteers. A: stained with Ponceau S dye for protein concentration detection; B: dot blot membrane probed with different sera.

3.4.2 Dot blot analysis

To assess the total allergenicity of pollen samples, protein dot blot analysis was carried out. Identical and comparable volumes of soluble protein extracts were bound on a nitrocellulose membrane and subjected to immunoreaction with a serum mix from 10 ragweed allergic patients.

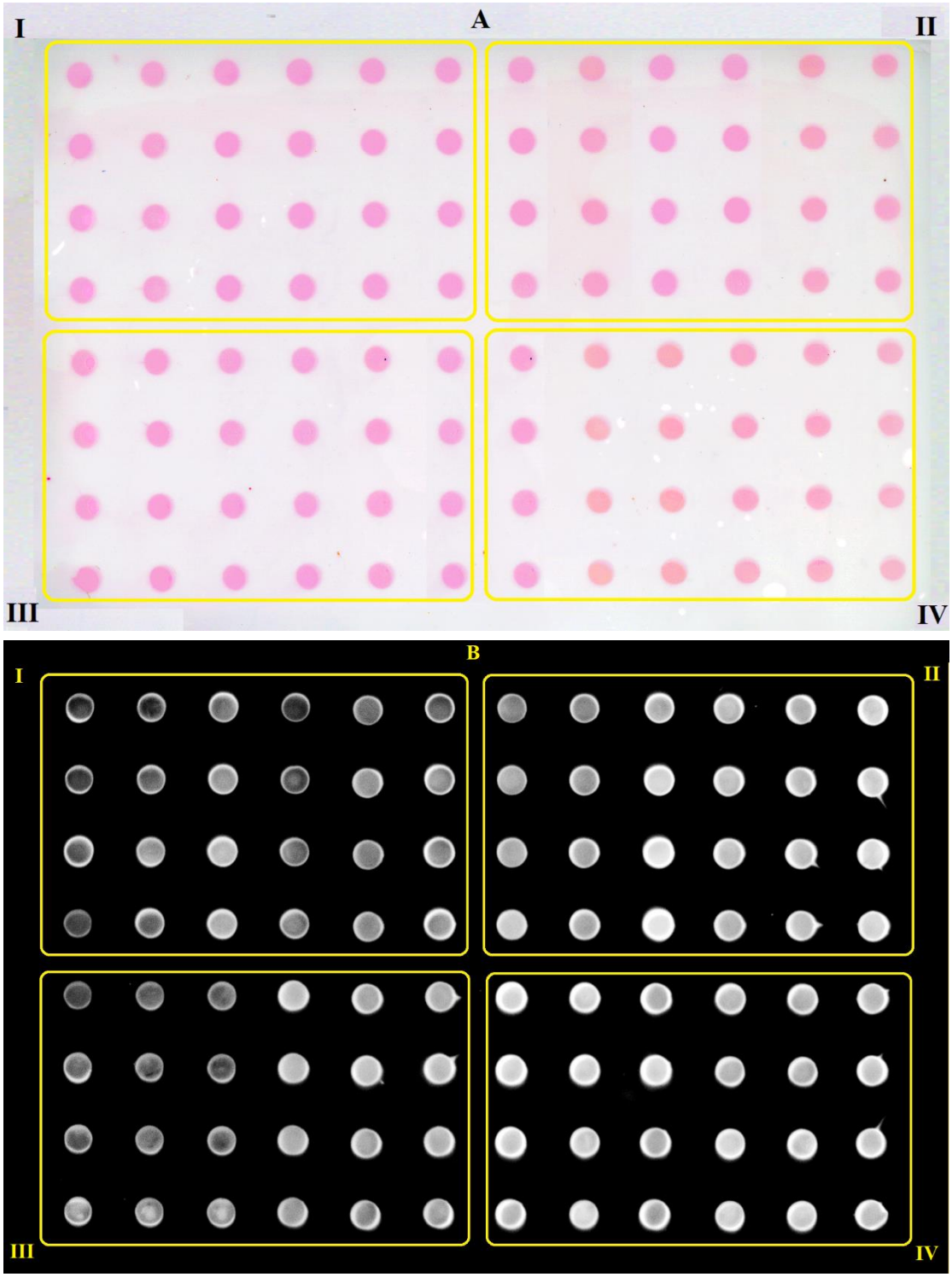


Figure 28. Total allergenicity of pollen samples assessed with dot blotting. A: stained with Ponceau S dye; B: representative dot blot membrane probed with a pool of selected patient sera; I: first generation of ragweed pollen exposed to 40 ppb NO_2; II: first generation of ragweed pollen exposed to 80 ppb NO_2; III: second generation of ragweed pollen exposed to clean air; IV: second generation of ragweed pollen exposed to 80 ppb NO_2.

Fig. 28 shows a representative membrane after immunodetection. Six biological replicates were carried out for each group (horizontal line), and each biological replicate was quantified in quadruplicate (vertical line). The protein amount was shown to be equal in each well by staining with Ponceau S dye (Fig. 28A). Various signal intensities revealed the different total allergenicity of each sample. However, the 80 ppb treated pollen showed a higher allergenic potential (Fig. 28B).

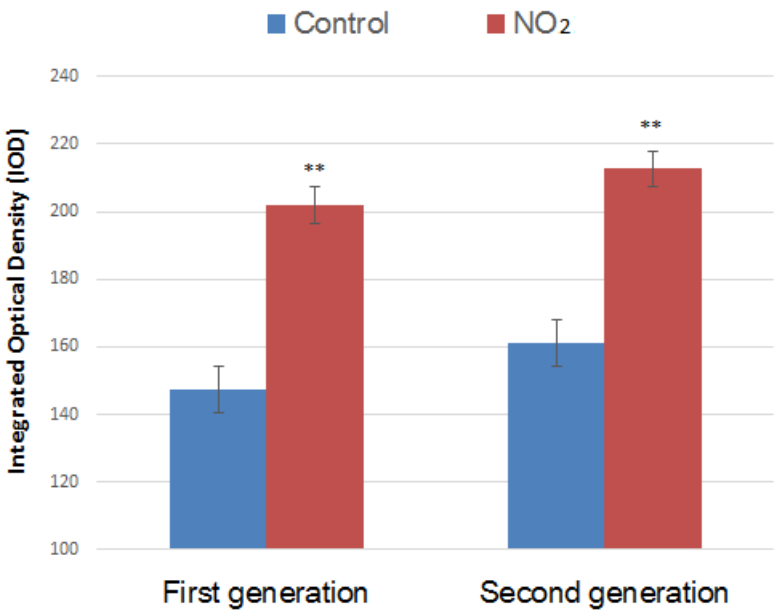


Figure 29. Assessment of total pollen allergenicity through image analysis the integrated optical density (IOD) of immunoreactive spots. ** = p-value < 0.005

Image analysis was applied to quantify immunochemical signals: the integrated optical density (IOD) of immunoreactive spots was measured. The mean results of three independent experiments were calculated and statistically analysed. On average, pollen exposed to elevated NO_2 showed a statistically higher IgE-binding signal both in the first and in the second generation which indicated an increased allergenicity of the 80 ppb NO_2-treated pollen (Fig. 29).

3.4.3 1-D SDS-PAGE and immunoblot analysis

To investigate the differences in dot blot allergenicity signals in more detail, 1-D Western blot, probed with the same sera mix used for dot blotting, was carried out.

Fig. 30 shows a representative 1-D SDS PAGE, and a representative membrane after immunodetection is shown in Fig.31. It can be seen that bands at Mw of 52, 38, 30, 12 and 10 kDa were recognized and the strongest signal was given by the band of 38 kDa.

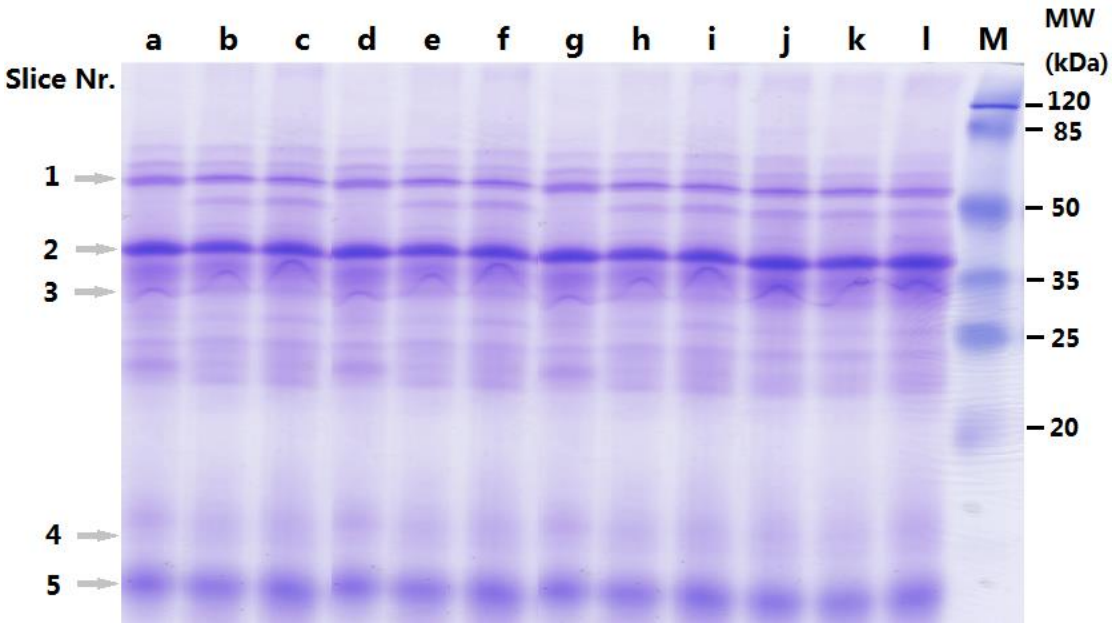


Figure 30. Image of the Coomassie Brilliant Blue stained SDS-PAGE gel. a-c: 1st treatment pollen extract; d-f: 1st control pollen extract; g-i: 2nd treatment pollen extract; j-l: 2nd control pollen extract.

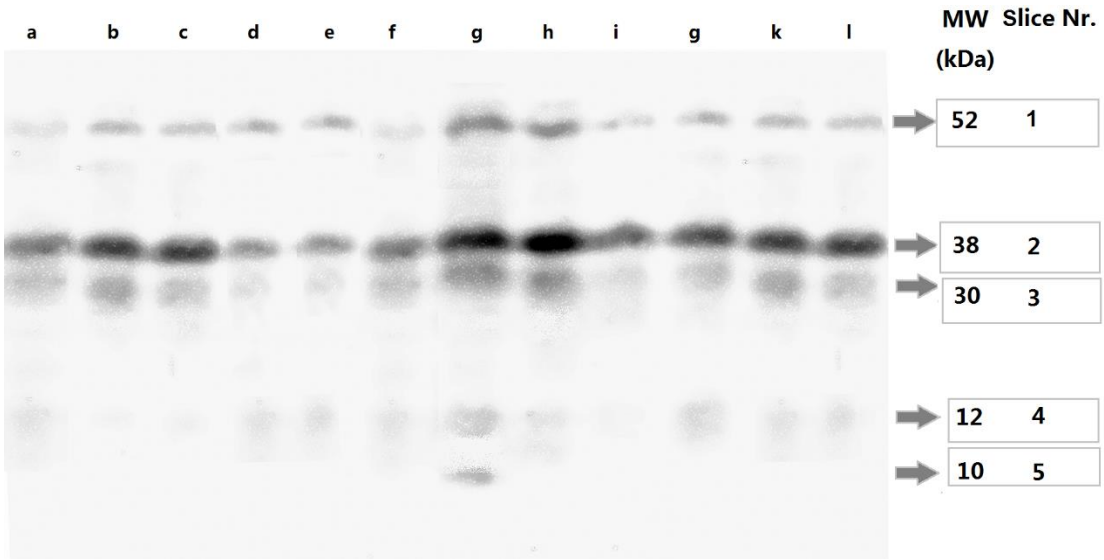


Figure 31. 1-D Western blot analysis of major allergens in different pollen extracts. a-c: 1st treatment pollen extract; d-f: 1st control pollen extract; g-i: 2nd treatment pollen extract; j-l: 2nd control pollen extract.

Image analysis performed on 1-D immunoblots from three independent experiments confirmed the dot blot results indicating a statistically significant higher allergenicity of pollen from the plants exposed to 80 ppb NO_2 in comparison with that of pollen from the plants of control (Fig. 32). The diverse total allergenicity was ascribed to the different allergenic potential of these five allergen bands between treatment and control. As shown by image analysis (Fig. 32A - E), the different allergenic potential of each band was quantified and the contribution of the single allergen bands to the total allergenicity was also analyzed (Fig. 32F).

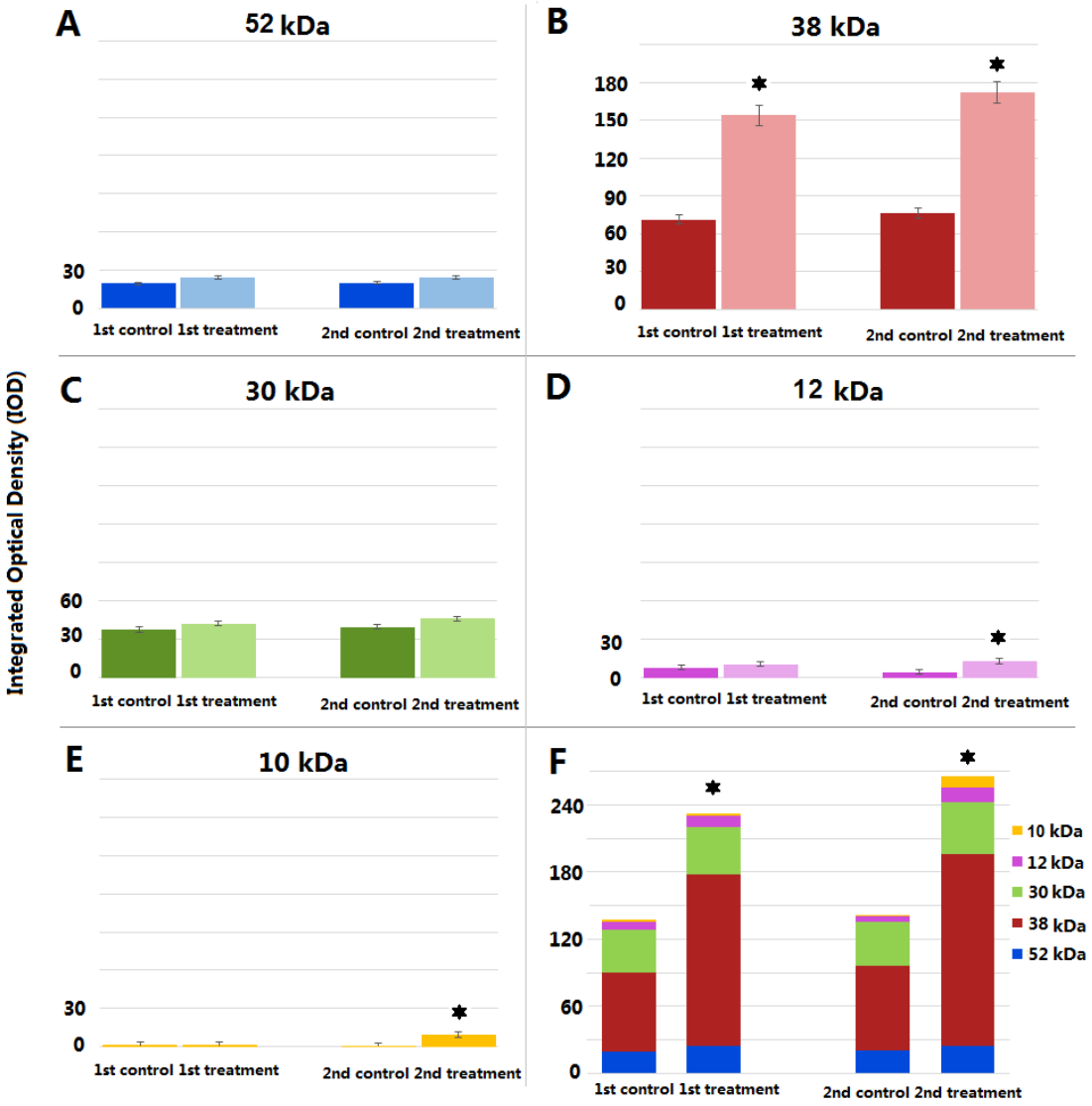


Figure 32. Statistical analysis of major allergens of each individual band in different pollen extracts and contribution of the single allergens to the total allergenicity. * = p-value < 0.05

The allergenic potential of the 38 kDa band was significantly upregulated in both, the first and second generation of pollen exposed to 80 ppb NO_2 and it contributed more than 70 percent of the total allergenicity of pollen extracts. The allergenic potential of 12 and 10 kDa bands significantly increased in 80 ppb NO_2 pollen as compared to the pollen of the control (Fig. 32).

Slice Nr.	Accession Nr. (UniProtKB)	Protein Name	Allergen Name	Percent coverage (%)	Nr. of unique peptide	MW (kDa)
1	P27759	Pectate lyase	Amb a 1.1	3	2	52
	P27760	Pectate lyase	Amb a 1.2	3.5	1	
	Q9LEJ0	Enolase	Hev b 9	3.8	1	
2	P27759	Pectate lyase	Amb a 1.1	57	20	38
	P27760	Pectate lyase	Amb a 1.2	41	11	
	P27761	Pectate lyase	Amb a 1.3	36	9	
	P28744	Pectate lyase	Amb a 1.4	17	3	
	P27762	Pectate lyase	Amb a 1.5	47	16	
3	P27760	Pectate lyase	Amb a 1.2	27	6	30
	P27761	Pectate lyase	Amb a 1.3	24	4	
4	P27759	Pectate lyase	Amb a 1.1	20	8	12
	P27760	Pectate lyase	Amb a 1.2	21	4	
	P00304	Plastocyanine	Amb a 3	23	2	
5	P27762	Pectate lyase	Amb a 1.5	3	1	10

Table 10. Amb a isoforms and Hev b 9 homologue identified by LC–MS/MS in slices excised from 1-D gel of *Ambrosia artemisiifolia* pollen extract.

Further identification of the proteins were carried out by LC–MS/MS of the excised SDS-PAGE (Tab. 10, see also Fig. 30). As shown, Amb a 1 isoforms were present in each band. The specific IgE reactivity seen at 38 kDa in 1-D immunoblots, corresponded to Amb a 1 isoforms: Amb a 1.1, 1.2, 1.3, 1.4 and 1.5. The specific binding appearing at 12 kDa corresponded to Amb a 1.1, 1.2 and Amb a 3. A Hev b 9 homologue was detected in a slice at 52 kDa together with Amb a 1.1 and 1.2.

3.4.4 2-D gel and immunoblot analysis

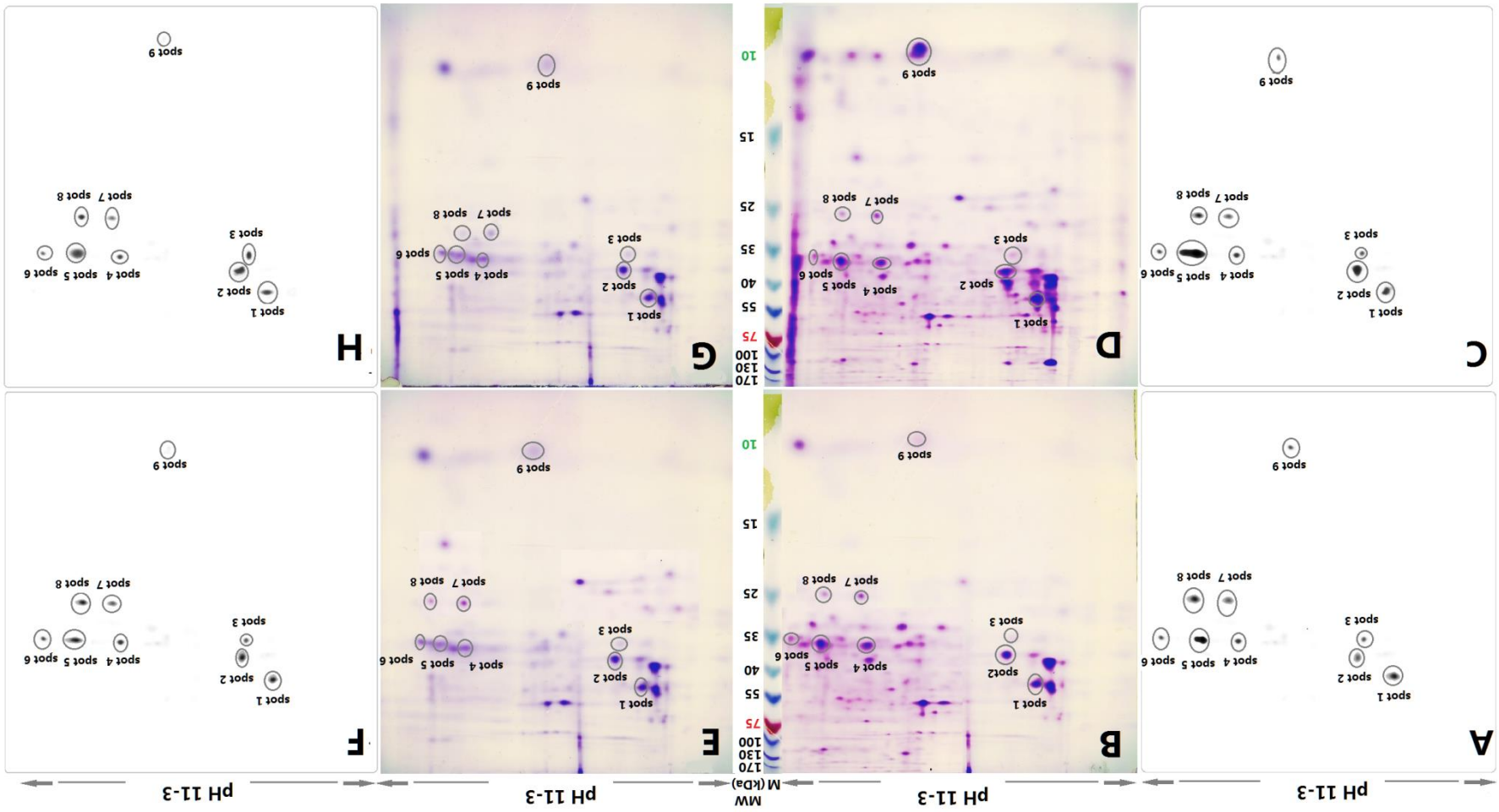
Pollen extracts are complex mixtures of proteins, many of them having similar masses, but different pI values, that cannot be differentiated by 1D SDS-PAGE alone. In order to better resolve and characterize allergen proteins and their isoforms in the ragweed pollen extracts, 2-D gel and western blots were employed with the same serum mix used for dot blotting and 1D Western blotting. The specific IgE reactivity is present in Fig. 33.

Finally, nine spots were distinguished with the Mw of 52 (spot 1), 40 (spot 2), 38 (spot 3, 4, 5 and 6), 30 (spot 7 and 8) and 10 kDa (spot 9). It is known that the major ragweed allergen for humans is Amb a 1, present in five isoallergenforms. All of these isoallergenforms were detected in our 1-D immunoblot at Mw 38 kDa. In Fig 33, 2-D immunoblot shows strong recognition of several spots at Mw 38 kDa and PI thus pointing to Amb a 1 isoforms. The specific bindings detected in 1D-SDS around 38 and 30 kDa were present in 2-D immunoblots as several dots. The detected signal at 12 kDa in 1D-SDS had disappeared in 2-D immunoblots.

The allergenic potential of each spot and the contribution to total allergenicity has also been analyzed and is shown in Fig.34. Spot 5 at Mw 38 kDa contributes almost half of the total allergenicity of pollen extract, and is significantly increased in the first and second generation of pollen exposed to 80 ppb NO_2 (Fig. 34 E). The allergenic potentials of spot 8 at 30 kDa and spot 9 at 10 kDa were significantly upregulated in both first and second generation of treatment pollen (Fig. 34 H & I). The allergenic potential of spot 6 at 38 kDa was significantly upregulated only in the second generation exposed to 80 ppb NO_2 (Fig. 34 F). Taken together, pollen sampled from 80 ppb treated plants showed a stronger allergenic potential than pollen sampled from control plants of both first and second generation (Fig. 34 J). These results confirmed the dot blot and 1-D immunoblotting analyses above.

These 9 IgE binding spots were further identified by LC–MS/MS (Tab. 11). Similar to 1-D immunoblotting results, all five Amb a 1 isoforms were found in 2-D immunoblotting spots (spot 2, 3, 4, 5, 6 and 7). The strongest signal spot 5 could correspond to a single allergen isoform Amb a 1.1 which would indicate that the Amb a 1.1 was the largest contributor to the total allergenicity of ragweed pollen. It's worth noting that spot 1 (52 kDa), spot 8 (30 kDa) and spot 9 (10 kDa) correspond to the same allergen Hev b 9. This indicates new allergens in ragweed pollen, which are homologous to Hev b 9, reacting also against human IgE.

Figure 33. 2-D Western blot analysis of major allergens in different pollen extracts with patients' sera.
M: PageRuler Prestained Protein Ladder (10-170 kDa, Thermo); A: Western blot result of 1st treatment pollen extract; B: Coomassie Brilliant Blue stained 2D-gel result of 1st treatment pollen extract; C: Western blot result of 2nd treatment pollen extract; D: Coomassie Brilliant Blue stained 2D-gel result of 2nd treatment pollen extract; E: Coomassie Brilliant Blue stained 2D-gel result of 1st control pollen extract; F: Western blot result of 1st control pollen extract; G: Coomassie Brilliant Blue stained 2D-gel result of 2nd control pollen extract; H: Western blot result of 2nd control pollen extract.



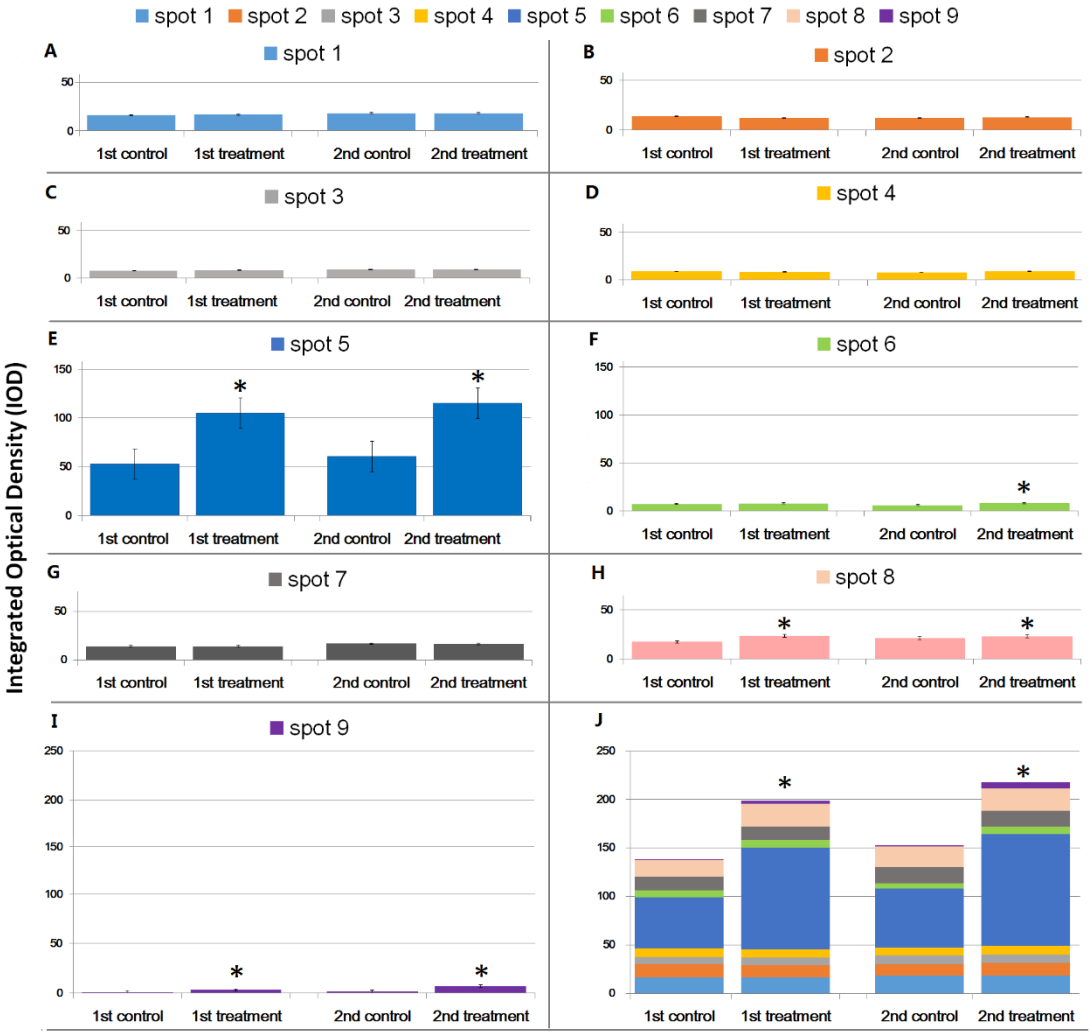


Figure 34. Statistical analysis of major allergens of each individual spot of different pollen extracts and contribution of single allergens to total allergenicity. * = p-value < 0.05

Spot Nr.	Accession Nr. (UniProtKB)	Protein Name	Allergen Name	Percent coverage (%)	Nr. of unique peptide	MW (kDa)
1	Q9LEJ0	Enolase	Hev b 9	44	18	52
2	P27759	Pectate lyase	Amb a 1.1	63	30	40
	P27762	Pectate lyase	Amb a 1.5	14	4	
	P27761	Pectate lyase	Amb a 1.3	49	17	
3	P27759	Pectate lyase	Amb a 1.1	37	13	38
	P28744	Pectate lyase	Amb a 1.4	33	10	
4	P27760	Pectate lyase	Amb a 1.2	13	2	38
	P27761	Pectate lyase	Amb a 1.3	17	5	
5	P27759	Pectate lyase	Amb a 1.1	64	33	38
6	P27760	Pectate lyase	Amb a 1.2	51	15	38
	P27761	Pectate lyase	Amb a 1.3	16	6	
7	P27760	Pectate lyase	Amb a 1.2	12	2	30
	P27761	Pectate lyase	Amb a 1.3	5.3	2	
8	Q9LEJ0	Enolase	Hev b 9	10	2	30
9	Q9LEJ0	Enolase	Hev b 9	15	3	10

Table 11. Amb a isoforms and Hev b 9 homologues identified by LC–MS/MS in spots excised from 2-D gel of *Ambrosia artemisiifolia* pollen extract.

4. Chapter – DISCUSSION

4.1 Morphological analysis of common ragweed upon NO_2 treatment

4.1.1 Effect of NO_2 on the pollen production of common ragweed

Generally, atmospheric NO_2 has long been known to be either harmful or beneficial to plants depending on concentration and plant species (Capron and Mansfield 1977, Sandhu and Gupta 1989, Wellburn 1990). This study demonstrated that the pollen production per inflorescence increased by around 67% and 87% in 2011 and 2012 plants, respectively, upon NO_2 fumigation. There is not direct report of NO_2 influencing pollen yield, whereas other NO_2 induced morphological changes revealed by previous reports might have effects on pollen production. Takahashi et al (2011) showed that prolonged exposure to atmospheric nitrogen dioxide at 50 ppb appeared to stimulate the acceleration of flowering time by 3.2 days in tomato, which subsequently increased the flowering duration. Furthermore, exposure to NO_2 increased flower numbers per plant by a maximum of 60%. These increments of flower number and flowering duration may lead to an increase of pollen grains. Moreover, NO_2 could also enhance the shoot biomass, total leaf area, and contents per shoot of carbon (C), nitrogen (N), sulphur (S), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), free amino acids, and crude proteins in seedlings of *Nicotiana plumbaginifolia* (Takahashi et al. 2005). These positive effects on nutrient uptake of the plant might enhance the plant's physiological status, thereby boosting the pollen yield. The increased pollen yield of common ragweed may result in a higher concentration in the air with a negative effect on atopic patients (D'Amato and Cecchi 2008).

4.1.2 Effect of NO_2 on seeds of common ragweed

High concentrations of NO_2 might have detrimental effects on plants, including reduction and deterioration of crop and vegetable yield and quality (Maggs and Ashmore 1998, Haberer et al. 2006, Han and Naeher 2006). The 50 seeds mass and plant total seed mass of ragweed significantly decreased upon elevated NO_2 in both

generations, and the amount of seeds per plant decreased as well as compared to the control plants. Although many species produce more seeds at low concentrations of NO_2 (Sandhu and Gupta 1989), decreased seed numbers reported under NO_2, on the other hand, often correlated with ozone and other air pollutions. Wahid and colleagues (1995) showed a reduction of 42% and 37% in the grain yield of two cultivars of rice (*Oryza sativa L.*) in polluted air containing 12 ppb NO_2 and 30-55 ppb O_3. Another study described significant yield reduction in two successive seasons which ranged from 33% to 46% in wheat and from 37% to 51% in rice in ambient polluted air with 20-25 ppb NO_2 (Maggs et al. 1995).

Decreased 50 seeds weight indicates lower seed nutrition and seed viability, which may further influence the sexual reproduction of common ragweed. The polluted air, which contained 12 ppb NO_2 proved to significantly reduce the 1000 grain weight of rice (Wahid et al. 1995). The seed weight is determined by the process of seed filling. Several processes are probably involved in this process, including storage, phloem loading, carbohydrate hydrolysis, and phloem unloading into the seed (Prasad et al. 2008). Premature plant senescence is the main cause of losses in grain filling and seed biomass yield due to leaf yellowing and deteriorated photosynthesis (Gepstein and Glick 2013). NO_2 at higher levels may show visible injury symptoms in some vegetables, which may further lead to premature plant senescence (Mansfield and Freer-Smith 1981, Nouchi 2002). Additionally, various genes and transcription factors are involved in seed filling. A study showed that exposure to NO_2, even at concentrations as low as 10 ppb could increase DNA damage (DNA strand fragmentation) (Koehler et al. 2013). This kind of negative effect of NO_2 on DNA structure might impact the expression of genes and further influence the seed filling of ragweed.

Furthermore, recent studies suggest that basal abscisic acid (ABA) levels play an important role during seed set and grain filling events (Govind et al. 2011). Sandhu et al (1990) reported a significant increase of ABA (160%) in plants that were treated

with 400 ppb NO_2 for 5 days as compared to an increase of only 82% on exposure for just 7 h. Although the concentration of NO_2 in this study was significantly less than 400 ppb, the exposure time was much longer (over a whole vegetation period), which might also induce in a disorder of ABA concentrations in the plant that could further affect the grain filling process.

In both generations of ragweed plants, elevated NO_2 led to similar trends in decreasing the seed number. Similarly, small effects on grains per ear/panicle of rice and wheat induced by NO_2 have been reported (Maggs et al. 1995, Wahid et al. 1995). The seeds number of spermatophytes normally is determined by two factors, ovule number or/and development and pollen viability or/and quality. Understanding the factors that control ovule initiation is of great importance as the ovule number and development will determine the number of seeds (Cucinotta et al. 2014). Various genes and transcription factors are important in the regulation of the number and development of ovules in plants (Elliott et al. 1996, Schneitz et al. 1997). Furthermore, hormones play a key role in the ovule primordia formation and number. Auxin is required for ovule primordia formation (Benkova et al. 2009) while cytokinin positively regulates the number of ovules (Hwang et al. 2012)(Hwang et al. 2012)(Hwang et al. 2012). The treatment of plant with elevated NO_2 may regulate the expression of related genes and/or transcription factors as well as stimulate the level of hormones, thus influencing the quantity and/or quality of the ovules in common ragweed.

Pollen viability is generally considered to indicate the ability of the pollen grain to perform its function of delivering the sperm cells to the embryo sac following compatible pollination (Shivanna et al. 1991). The quality and viability of pollen play a pivotal role in determining seed yield and quality. Matthews and Bramlett (1986) showed that the yields of developed and filled seeds, as well as apparent pollination effectiveness were more strongly due to increasing viability than to increasing pollen quantity. Chichiriccó and Picozzi (2007) exposed plants of *Crocus vernus* to 200 –

2000 ppb NO_2 and observed a pronounced inhibition of pollen germination. Similarly, Masaru et al (1976) found a marked inhibition of tube elongation in *Lilium longiflorum* pollen when exposed to high concentration of NO_2. Another study suggested that NO_2 might perhaps be a factor with a statistically significant influence on reduction of pollen viability (Gottardini et al. 2008). All things considered, the reduction of seed weight and seed number in this study may be partly considered as a result of NO_2 induced pollen viability decrease.

4.2 Comparative transcriptomic analysis

4.2.1 Transcriptional analysis of allergen genes of ragweed pollen

Several highly expressed proteins in pollen behave as allergens for humans. The close interaction between allergens and the immune system may explain the allergenicity of an allergen. Profilins, calcium binding proteins (CBPs), β-expansins, lipid transfer proteins (LTPs), pathogenesis related proteins (PRs), and a few enzymatic proteins are the main allergen families of pollen grains, and profilins and CBPs are most common (Radauer and Breiteneder 2006).

Common ragweed is the best known weed responsible for the most severe and widespread allergies caused by its pollen (Csepe et al. 2014). A full-grown plant can produce about 10^9 pollen grains during 1 year (Fumanal et al. 2007) and it has been revealed that concentrations lower than 5 – 10 ragweed pollen / m^3 can trigger afflictions for atopic persons (Taramarcaz et al. 2005).

4.2.1.1 Quantitative real-time RT–PCR of allergen genes of ragweed pollen

In this study, quantitative real-time RT-PCR of ragweed pollen allergens showed that the elevated NO_2 significantly increased the allergen encoding transcripts. Amb a 1.1 and Amb a 1.2, which belong to the pectate lyase allergen gene family, showed almost a 2-fold increased transcript level both in the first and second generation (Fig. 9). The pectate lyase allergen gene family is the most important ragweed allergen, showing highest Ig E antibody titers and positive skin tests in 95% of

ragweed-sensitive patients (Platts-Mills and Woodfolk 2011). Similarly, the profilin (Amb a 8) and calcium binding protein (Amb a 9) gene families were raised about 1.5 times as compared to the control. On the other hand, Amb a CPI slightly decreased, whereas Amb a 1.3, Amb a 1.4, Amb a 1.5, Amb a 5; Amb a 6 slightly increased, however, not significantly (Fig. 9 & 10).

NO_2 is one of the major pollutants in the atmosphere and NO_2 concentrations are increased in industrial, downtown and roadside areas (Koehler et al. 2013). Allergen patterns can change in response to air pollution, which can modify the allergenic potential of pollen (D'Amato et al. 2013). Real-time PCR based quantification of silver birch allergen Bet v1 revealed a higher expression level in samples from central parts of urbanized area when compared to the samples from the countryside (Ziarovska et al. 2013). The same result was found by Cortegano et al. (2004), who reported that the expression of a major allergen Cup a 3 from *Cupressus arizonica* pollen was increased due to the pollution in the area.

4.2.1.2 RNA-seq analysis of allergens in ragweed pollen

The expression patterns of allergens were analyzed by RNA-seq according to the number of reads per contig in the Illumina libraries (Tab. 7).

Four contigs were identified as Amb a 1.5, three contigs for Amb a1.1 and Amb a 1.3, respectively. Amb a 1.2 is composed of two contigs, whereas Amb a 1.4, Amb a 3 and Amb a 6 have only one contig each. All the three contigs of Amb a 1 were upregulated in the first and second generation of ragweed pollen and all of these contigs were upregulated in 80 ppb treated pollen, consistent with the result of qRT-PCR. The qRT-PCR results revealed a significant increase of Amb a 1.2 in both generations, whereas the two contigs of Amb a 1.2 increased in the second generation. Amb a 1.4 increased in qRT-PCR and RNA-seq in both generations, whereas Amb a 8 and Amb a 9 were not found in the RNA-seq results. Amb a 1.3, Amb a 1.5, Amb a 3 and Amb a 6 were slightly upregulated in qRT-PCR results of both

generations, whereas their expression pattern in RNA-seq were more complicated (Tab. 7). The results showed that although the exact fold changes of the transcripts varied between RNA-seq expression and qRT-PCR analyses, the variation trend indicated a good consistency between the two analysis techniques (Fig. 9 & 10, Tab. 7).

The Illumina sequencing technique has like all methods both, benefits and shortcomings. In combination with qRT-PCR, however, it is a powerful tool that can and will advance the procedure of analyzing gene expression variation. The combination of multiple techniques, especially those adding complementary information, has proven to be beneficial in terms of data consistency (Loewe 2013). Next generation sequencing has become an integral part of molecular biology. A technique like RNA-Seq seems to be quite a challenge, but by utilizing qRT-PCR at various steps, the workflow can be significantly improved, either in terms of reproducibility or overall speed. In some cases the use of qRT-PCR and NGS improved and enriched data (Perkins et al. 2009, Meyer et al. 2011).

Overall, together with the qRT-PCR result, it is evident that elevated NO_2 resulted in an increased level of allergen en coding transcripts.

4.2.2 Suppression Subtractive Hybridization (SSH) Library of of ragweed pollen

Suppression subtractive hybridization is a technology that allows a PCR-based amplification of only cDNA fragments that differ between a control and an experimental transcriptome. It is one of the most powerful and popular methods for generating subtracted cDNA or genomic DNA libraries.

In the present work, two pollen libraries of control (40 ppb NO_2) and treatment (80 ppb NO_2) of the first generation of ragweed plants were constructed and analysed and an enrichment of more than 1000-fold for rare sequences was achieved.

4.2.2.1 Upregulated transcripts

Several transcripts were upregulated at the elevated NO_2 concentrations (Tab. 5). These transcripts include tubulin, transcription elongation factor, splicing factor, protein transport protein, serine/threonine protein kinase, late embryogenesis abundant protein, calcium-binding protein, puroindoline b protein, SAP-domain protein, proteasome, and LTR retrotransposon.

4.2.2.1.1 Stress related genes

Interestingly, most genes can be related to abiotic stress resistance, which implies that 80 ppb NO_2 is a stress for ragweed plants. Protein transport protein Sec61 beta, which accounts for the largest part of 17% in the upregulated library, significantly increases the cold resistance of *Arabidopsis* (Leroch et al. 2008). Serine/threonine protein kinase, which accounts for 5% in the upregulated library is a kinase enzyme that phosphorylates the OH group of serine or threonine. Protein phosphorylation/dephosphorylation are major signalling events and are also induced by osmotic stress in higher plants (Mao et al. 2010). Mao and colleagues showed that transgenic *Arabidopsis* overexpressing a serine/threonine protein kinase had enhanced tolerance to drought, salt, and freezing stresses, which were simultaneously supported by physiological results, including decreased rates of water loss, enhanced relative water content, strengthened cell membrane stability, improved photosynthesis potential, and significantly increased osmotic potential.

Late embryogenesis abundant (LEA) protein, which is abundant in seeds and pollen, increased in the pollen of ragweed exposed to 80 ppb NO_2. Several studies demonstrated that overexpression of LEA proteins could dramatically increase the cold and dehydration tolerance (Houde et al. 1992, Ingram and Bartels 1996, Thomashow 1999).

In this study, the expression of a calcium-binding protein was upregulated by 80 ppb NO_2 at the transcriptional level. Calcium is essential for pollen germination and

pollen tube growth. The action of Ca^{2+} is primarily mediated by Ca^{2+}-binding proteins and a large body of information has established a link between elevation of cytosolic Ca^{2+} at the pollen tube tip and its growth (Safadi et al. 2000). On the other hand, signalling pathways involved in the response to environmental stresses form interconnected networks in which Ca^{2+} plays a major role (Bouche et al. 2005). It has been shown that Ca^{2+}-binding proteins play a key role in plant salt tolerance, as well as in heat and anoxia resistance (Zielinski 1998, Zhu 2000).

4.2.2.1.2 Other upregulated genes

Furthermore, various genes that are involved in different biological process in ragweed pollen have been upregulated by the elevated NO_2. Pi-tubulin, the expression of which is restricted to the male gametophyte, has been found in a complex with beta-tubulin in mature sunflower pollen and may inhibit tubulin assembly in pollen (Evrard et al. 2002). Splicing factor Prp8 and LTR retrotransposon Tmc1 have been reported as important regulators of spliceosome activation and genome expansion (Pena et al. 2007, Todorovska 2007). Transcription elongation factor s-II is a transcript elongation factor that facilitates transcription by RNA polymerase II, as it assists the enzyme to bypass blocks to mRNA synthesis (Saunders et al. 2006). It has been proven to be involved in the seed dormancy process. Grasser et al (2009) reported that *Arabidopsis* lines harbouring T-DNA insertions in the coding sequence of transcription elongation factors displayed essentially normal development. However a slightly earlier flowering and a clearly reduced seed dormancy were evident, as compared to control plants. It has been noted that another upregulated protein – puroindoline - which has a role in determining the grain softness (Massa and Morris 2006) has been found to be allergenic in IgE-mediated food allergy test in European patients (Pastorello et al. 2007).

4.2.2.2 Down regulated transcripts

Elevated 80 ppb NO_2 also caused a decrease of several transcripts of ragweed pollen (Tab. 5). These transcripts include SF16 protein, SF3 Protein (PLIM 1), LIM domain

protein PLIM-2, Pollen coat protein, Phosphoglucomutase, Myosin-1, Integral membrane protein, Alpha-2 tubulin, Lipoxygenase, Retrotransposon protein, Carbon monoxide dehydrogenase and Calmodulin 24-like protein.

4.2.2.2.1 Reproduction related genes

Different from the upregulated transcripts, the downregulated transcripts were more related to reproductive processes.

SF16 protein which accounts for the second biggest part of 12% in the downregulated library was reported to be pollen-specific and normally expressed late in pollen development (Dudareva et al. 1994). Pollen-specific protein SF3 and protein PLIM-2 contain 2 LIM zinc-binding domains, and act together with SF16 protein in the regulation of pollen-specific processes such as male gamete maturation, pollen tube formation, or even fertilization (Baltz et al. 1992).

The pollen extracellular matrix contains proteins mediating species specificity and components needed for efficient pollination (Mayfield et al. 2001). In this study, a pollen coat protein was downregulated under elevated NO_2. Mayfield and colleagues reported that elimination of a pollen coat protein GRP17, resulted in delayed pollen hydration that further affected the initiation of pollination in *Arabidopsis* (Mayfield and Preuss 2000). Another pollen coat protein PCP1 has been proven to be involved in pollen-stigma interactions (Stanchev et al. 1996). The decrease of pollen coat protein in pollen of ragweed at the transcriptional level might have a negative effect on pollen hydration and interactions with the stigma, which would further influence the reproductive process of ragweed.

Phosphoglucomutase is a key enzyme of central metabolism which interconverts glucose-6-phosphate and glucose-1-phosphate. In this study, 80 ppb NO_2 significantly decreased phosphoglucomutase transcripts in pollen of ragweed. In *Arabidopsis*, phosphoglucomutase plays a key role in reproduction. Egli et al (2010) have shown

that the double mutant of two cytosolic phosphoglucomutase PGM2 and PGM3 has normal ovules but approximately half remain unfertilized 2 days after pollination. They attribute these phenotypes to an inability to effectively distribute carbohydrate from imported or stored substrates (eg. sucrose) into the major biosynthetic (eg. cell wall biosynthesis) and respiratory pathways (eg. glycolysis and the oxidative pentose phosphate pathway). Disturbing these pathways is expected to have dramatic consequences for germinating pollen grains, which have high metabolic and biosynthetic activities (Egli et al. 2010).

Plant myosins have been implicated in several roles such as organelle movement, endocytosis, plasmodesmata function and cytoplasmic stiffness (Sparkes 2010). The expression of a myosin was suppressed at the transcriptional level in ragweed pollen by the elevated NO_2. Prokhnevsky et al (2008) reported that the knockout mutant of two myosins xik/mya1 resulted in a significantly stunted phenotype with narrower rosette diameter, and reduced aerial tissue and number of seeds per silique. Similarly, an Oryza sativa (rice) T-DNA insertional line of a myosin displayed male sterility under short-day length resulting in defective embryos (Jiang et al. 2007).

As discussed before, the 80 ppb NO_2 significantly suppressed the seed production not only with regard to seed grain weight, but also the seed numbers of common ragweed of both generations. With the down-regulation of reproduction related genes at the transcriptional level, pollen development might be affected, leading to male sterility and seeds reduction.

4.2.2.2.2 Other downregulated genes

Furthermore, various genes which are involved in different biological process in ragweed pollen were downregulated by elevated NO_2. Retrotransposons, the most mobile genomic components, vary greatly in copy number over a relatively short evolutionary timescale. They represent one of the most important factors affecting the structural evolution of plant genomes, especially those of the higher plants

(Todorovska 2007). The integral membrane proteins (IMP) are permanently attached to the biological membranes (Saier et al. 2009). These proteins are involved in the accumulation and transduction of energy and cell adhesion (Saier et al. 2009). A calmodulin 24-like protein was found to be downregulated in pollen of ragweed exposed to 80 ppb NO_2. Calmodulin (CaM) is one of the best characterized Ca^{2+}-binding proteins, a highly conserved and multifunctional regulatory protein in eukaryotes. Its regulatory activities are triggered by its ability to modulate the activity of a certain set of CaM-binding proteins after binding to Ca^{2+}, and thereby generating physiological responses to various stimuli like heat, drought, cold and salt (Poovaiah and Reddy 1993, Reddy et al. 2011).

4.2.3 Illumina sequencing libraries of ragweed pollen grains

4.2.3.1 Whole transcriptome expression profiling of ragweed pollen

The application of next-generation sequencing (NGS) to transcriptomics, commonly called RNA-seq, allows the nearly complete characterization of transcriptomic events occurring in a specific tissue. It has proven particularly useful in non-model species, which often lack the resources available for sequenced organisms (Strickler et al. 2012).

4.2.3.1.1 Sequence preprocessing

In this study, the Illumina sequencing libraries of ragweed pollen were composed of pollen from ragweed plants exposed to 40 ppb (control 2011) /clean air (control 2012) and 80 ppb NO_2, respectively. An assessment of the unprocessed reads is critical to check for sequence biases and contamination. Some other important measures to consider are overall quality of the reads, length, duplication level, and overabundant sequences. Additionally, the raw reads may contain the adaptor and/or linker sequence used in the sequencing reaction, that need to be removed before assembly (Schroder et al. 2010). In this study, after removing low quality, adaptor and barcode sequences, a total of 587,972,236 and 517,737,064 reads of lengths of 100 bp were obtained in the first and second generation, respectively.

4.2.3.1.2 De novo assembly of ragweed pollen transcriptome

For nonmodel organism studies, a *de novo* assembly is the only option for sequence assembly. In *de novo* assembly, the reads are assembled into contigs without the guidance of a reference sequence. In addition, alternative splicing or mis-annotation can be discovered (Strickler et al. 2012).

In order to cover the ragweed pollen transcripts as complete as possible, *de novo* assembly in this study was performed with Illumina reads of pollen from ragweed exposed to 40 ppb and 80 ppb NO_2 (2011), clean air and 80 ppb NO_2 (2012) together with the Illumina reads of pollen from ragweed exposed to 40 ppb and 80 ppb ozone, 40 ppb and 120 ppb ozone, common air and 100 ppb ozone plus 100 ppb NO_2 and 454-sequencing data of ragweed pollen (Kanter et al. 2013). Since the Illumina sequencing platform generates reads shorter than 454-sequencing, a combination of 454- sequencing and Illumina may assist *de novo* assembly, based on NGS data from *Eschscholzia californica*, *Persea americana*, and *Arabidopsis thaliana* (Wall et al. 2009). Finally, *de novo* assembly generated 35,136 unique transcripts with an average length of 722 bp, the longest transcript measuring 10,492 bp.

4.2.3.1.3 Annotation of ragweed pollen unique transcripts

Annotation of ragweed unique transcripts showed that a total of 16,361 unique transcripts (46.56%) were assigned to at least one GO term. Among them, 6,370 (38.93%) were assigned to the biological process category, 5,832 (35.65%) to the molecular function category, and 6,932 (42.37%) to the cellular component category, while 4,601 (28.12%) unique transcripts were assigned GO terms of all three categories. Gene annotation is usually performed by using BLAST to find significant matches to annotated genes.

4.2.3.1.4 Annotated transcripts in biological process

In this study, the whole ragweed pollen transcriptome data were gathered from pollen of ragweed plants exposed to NO_2, O_3 and NO_2 plus O_3. A total of 16,361

unique transcripts (46.56%) were assigned to at least one GO term. Among them, 6,370 (38.93%) were assigned to the biological process category (Fig. 17). It is worth mentioning that “response to cadmium ion” and “response to salt stress” represented the most two abundant groups of the biological process category, consistent with the fact that our transcriptome data were derived from ragweed plants under abiotic stress. Other interesting highly abundant groups of the biological process category included “oxidation-reduction process”, “response to cold” and “defense response to bacterium” which also demonstrated pollen stress in this study.

Ozone is a strong oxidising agent, the phytotoxicity of O_3 results primarily from the oxidative stress imposed by the pollutant on sensitive components of the plasmalemma (Zheng et al. 2000). The apoplastic antioxidants will react with the O_3 within the wall space, presumably protecting the next level of organization (the membrane) from injury (van Hove et al. 2001). Regarding the *de novo* assembly of the transcriptome of ragweed pollen, part of the ragweed pollen were treated with O_3, which might stimulate the expression of transcripts involved in “oxidation-reduction process” to reduce the negative effect by O_3.

Usually one kind of stress is accompanied or followed by another stress. Putative separate abiotic stress signalling pathways are also likely to interact in a similar manner. In addition, several abiotic stress pathways share common elements that are potential 'nodes' for crosstalk (Knight and Knight 2001). For instance, Ca^{2+} signalling has been implicated in plant responses to a number of abiotic stresses including low temperature, osmotic stress, heat, oxidative stress, anoxia, and mechanical perturbation (Knight 2000). In this study, elevated NO_2 and O_3 enhanced the expression of numerous transcripts which play key roles in other abiotic stress as well.

Pollen grains play a vital role in the reproductive process of flowering plants as male gametes. It's no surprise to find transcripts belonging to “pollen tube growth”,

“regulation of flower development”, “embryo development ending in seed dormancy”, and “vegetative to reproductive phase transition of meristem”. The growing pollen tube delivers the sperm cells to the ovule in higher plants and thus is central to the process of fertilization and sexual reproduction (Hepler et al. 2013). The process of “vegetative to reproductive phase transition of meristem “ is involved in transforming a meristem that produces reproductive structures, such as a flowers or an inflorescence (Huijser and Schmid 2011). Therefore the high expression of genes involved in reproductive processes hits well to the function of pollen as male gametes.

4.2.3.1.5 Annotated transcripts in molecular function

In the category of molecular function, the most abundant groups included ATP binding, protein binding, zinc ion binding and other appealing groups including binding and kinase activity like metal ion binding, copper ion binding, calcium ion binding, serine/threonine kinase activity, hydrolase activity and so on (Fig. 17).

It should be noted that allergenic proteins of ragweed pollen have specific molecular functions and most of them are related to binding processes. Amb a 1, the major allergen in ragweed pollen has the molecular function of metal ion binding (Rafnar et al. 1991); Amb a 3 acts as copper ion binding protein (Klapper et al. 1980); Amb a 6, a lipid transfer protein, plays a key role in lipid binding (Hiller et al. 1998), whereas Amb a 9 and Amb a 10 belong to the calcium ion binding group (Wopfner et al. 2008) . The fact that allergenic proteins play a key role in binding processes implies their vital roles in ragweed pollen. Pollen-specific proteins SF3 and PLIM-2, which both contain 2 LIM zinc-binding domains, act together with SF16 protein to regulate pollen-specific processes such as male gamete maturation, pollen tube formation, or even fertilization (Baltz et al. 1992).

Kinase activity is known to be indispensable for pollen viability and quality. Mu and colleagues (1994) identified a serine/threonine kinase and suggested that it might

play a role in signal transduction events during pollen development and/or pollination. A High level of hydrolases activity is absolutely necessary for pollen in the fertilization process. Pollen lands in the pollination droplet and germinates in the micropyle. The pollen tube tip secretes proteases and hydrolases (Pettitt 1985) as it slowly digests through integumentary tissue to reach the egg cell within the female gametophyte (Singh 1978).

4.2.3.2 Comparative transcriptome profiles under elevated NO_2 in ragweed pollen

Expression analysis is another important application of RNA-Seq. By looking at changes in gene expression between tissues, over time, or by treatments, a greater understanding of the genes critical in certain responses may be gained. It has proven to be accurate and sensitive, without the problem of background signals from nonspecific binding found in array-based measures of expression (Hoen et al. 2008). Typically, in non-model plant transcripomics where no reference genome exists, the reads are assembled *de novo*, and the number of reads per contig is used as an indicator of expression (Alagna et al. 2009, Barakat et al. 2009).

In total, 27,376 and 25,154 differentially expressed unique transcripts were identified in the first and second generation of ragweed pollen, among which 13,948 were induced by 80 ppb NO_2 stress and 11,206 repressed in first generation, while 11,866 increased and 15,510 decreased in the second generation. Then, these NO_2-induced and repressed genes were identified in GO terms in the biological process and molecular function categories, respectively (Fig. 18 & 19).

4.2.3.2.1 Differentially expressed genes in biological process

As expected, GO terms including “response to salt stress”, “response to cold”, “response to water deprivation”, “response to wounding”, and “oxidation-reduction process” were highly enriched in both induced and repressed genes in both generations (Fig. 18). This further validated the efficiency of the elevated NO_2 stress treatments and the reliability of gene expression data in this study. In addition, GO

terms related to responses to various other types of abiotic stresses, indicated the crosstalk of different stress responses in ragweed pollen to be the same as those reported in other plant species (Rodriguez et al. 2010).

In addition to the responses to abiotic stresses, biotic stress response processes were also found. GO terms including “response to chitin” and “defense response to bacterium” were highly enriched in NO_2 downregulated genes. Chitin is a polymer of –N-acetyl-D-glucosamine that is a major component of fungal cell walls and has been recognized as a general elicitor of plant defense responses for many years (Boller 1995). Fungal infection induces the expression of chitinases in plant cells, and these chitin-degrading enzymes accumulate at the site of invasion. In addition, the resulting chitin fragments (chitooligosaccharides) also appear to function as elicitors of numerous downstream defense response genes (Eckardt 2008). These changes of ragweed pollen in biotic stress response processes indicate the signal crosstalk between response to elevated NO_2 and to fungal & bacterial pathogens.

Plant hormones are known to be involved in plant responses to various stresses. In this study, GO terms including “response to abscisic acid stimulus” and “abscisic acid mediated signalling pathway” were highly enriched in both up- and downregulated genes. Knowledge of the various stressors capable of altering endogenous abscisic acid (ABA) concentration has also been extended. Thus, water deficit, water logging, osmotica, high and low temperatures, mineral deprivation, wounding, and long photoperiod have all been reported to induce ABA accumulation in affected plants, plant parts and tissues (Cowan et al. 1997). These results imply that ABA was also involved in ragweed pollen responses to the elevated NO_2 stress.

Interestingly, the GO term “pollen tube growth” represented the third most abundant up- and downregulated group of the biological process category. It is worth mentioning that in the SSH libraries, SF 3 protein and PLIM-2 protein, both related to pollen tube formation, were depressed by elevated NO_2 treatment. Sexual

reproduction in plants requires elongation of the pollen tube through the transmitting tissues toward the ovary. Therefore, tube growth rate is an important determinant of pollen competitive ability, which describes the reproductive success of a pollen grain (Gorla and Frova 1997). The expression changes of pollen tube growth related genes at the transcriptional level is consistent with the fact that the seeds of ragweed plants under elevated NO_2 showed reduced seeds weight and seed number in both generations.

4.2.3.2.2 Differential expressed genes in molecular function

Regarding the top 20 up- and downregulated GO terms of molecular function, most of them are involved in binding processes (Fig. 19).

ATP binding is biggest group altered in both generations. The ATP binding process was proved to be of great concern in pollen. Quilichini and colleagues (2010) reported ABCG26, a member of the ATP-binding cassette (ABC) transporter superfamily, is required for pollen exine formation in *Arabidopsis.* Mutants of ABCG26 are severely reduced in fertility, with most siliques failing to produce seeds by self-fertilization and mature anthers failing to release pollen (Quilichini et al. 2010). Another paper revealed that the ATP-binding cassette transporter OsABCG15 is required for anther development and pollen fertility in rice (Niu et al. 2013). In ragweed pollen, this large variation of gene expression in the ATP binding process may lead to negative effects on pollen viability and fertility which further impact the quality and quantity of seeds. ATP-binding process is also involved in abiotic stress resistance. A paper identified a new DEAD-box helicase ATP-binding protein (OsABP) from rice which is upregulated in response to multiple abiotic stress treatments including NaCl, dehydration, ABA, blue and red light (Macovei et al. 2012).

Metal ion binding including “zinc ion binding”, “calcium ion binding”, “copper ion binding” and “calmodulin (CaM) binding” were highly enriched in both induced and repressed genes of both generations. Calcium is essential for pollen germination and

pollen tube growth. Since the action of Ca^{2+} is primarily mediated by Ca^{2+} binding proteins such as calmodulin (CaM), the CaM binding proteins in pollen should also be important in Ca^{2+} regulated pollen germination and tube growth. Exogenous CaM enhances pollen germination and pollen tube growth (Ma et al. 1999), whereas CaM antagonists and anti-CaM serum inhibit pollen germination and tube growth and stop cytoplasmic streaming in a concentration-dependent manner (Obermeyer and Weisenseel 1991). On the other hand, Ca^{2+} plays a major role in signalling pathways involved in the response to environmental stresses including osmotic, salt, cold,heat and oxidative stress (Bouche et al. 2005).

The GO term of “Nucleotide binding” including “DNA binding” and “RNA-binding” were highly and differently enriched in both induced and repressed genes of ragweed pollen exposed to 80 ppb NO_2. DNA and RNA binding proteins in eukaryotes have crucial roles in all aspects of post-transcriptional gene regulation. Lorkovic (2009) provided clear evidence for the involvement of glycine-rich RNA-binding proteins in the plant response to various stress conditions (cold, dehydration, salt and ABA stress). Two DNA binding proteins, DDB1A and DDB2, were proved to contribute to UV tolerance, heat tolerance and floral patterning (Ly et al. 2013).

The kinase activity including hydrolase activity, ATPase activity, GTPase activity, ubiquitin-protein ligase activity, protein serine/threonine kinase and protein serine/threonine kinase phosphatase activity were enriched in 80 ppb treated ragweed pollen. An E3 ubiquitin ligase ARC1 was demonstrated to promote the ubiquitination of proteins during the rejection of self-Incompatible *Brassica* pollen (Stone et al. 2003). ATPase and GTPase are essential for pollen viability. The RAC/ROP GTPases of plants are molecular switches that pivotally control the polarized pollen tube growth process. Perturbing RAC/ROP regulation results in pollen tube depolarization defects ranging from broader and slower-growing to balloon-tipped and growth-arrested pollen tubes (Cheung and Wu 2008). Jakobsen et al. (2005) found that a P-type ATPase MIA is essential for pollen release and subsequent

germination in *Arabidopsis*. Together with the function of serine/threonine kinase and hydrolase discussed before, the NO_2 induced expression variations of kinase at the transcriptional level may have negative effects on pollen tube growth and consequent low level of fertilization of ragweed female gametes.

4.3 Proteome analysis

Although the analysis of gene expression at the transcriptional level is beneficial for identifying candidates for further functional studies, the up- or down-regulation of certain transcript does not necessarily have predictive power for the expression changes of the protein (Noir et al. 2005). For instance, regulation of post-translational and/or post-transcriptional may have effects on the accumulation of proteins encoded by high levels of transcripts, whereas low levels of a transcript may be sufficient for adequate accumulation of the specific protein (Noir et al. 2005). With regard to ragweed pollen, Kanter and colleagues found that the transcription level of the major allergen Amb a 1 was elevated in the pollen from ozone-treated (80 ppb) plants but no significant difference was seen at the protein level, as tested by direct ELISA (Kanter et al. 2013).

Therefore, in addition to transcriptomics, proteomics are also important in a systems biology approach.

4.3.1 Functional categories of mature pollen proteins

Proteome analysis of the differentially expressed spots in ragweed pollen upon NO_2 treatment assigned to the different functional categories.

4.3.1.1 Allergens

Although the concentration of pollen can be used as an indicator of the allergenic potential, the underlying mechanism of allergenicity depends on the specific protein allergens of pollen (Ahlholm et al. 1998, Beggs 2010). Recent studies have examined the effects of environmental variables (such as temperature and precipitation) and

air pollutants on the concentration of various proteins (mainly allergenic proteins) of pollen or their overall allergenicity (Sheffield et al. 2011).

In this study, the 2D-DIGE results showed an increase of Amb a 1.1, Amb a 1.2, Amb a 1.3, Amb a 1.4, Amb a 1.5 as well as of another allergen with homology to Hev b 9 from *Hevea brasiliensis*. Ozone resulted in an allergen increase in *Lolium perenne* and rye (Masuch et al. 1997, Eckl-Dorna et al. 2010), and elevated CO_2 resulted in an increased Amb a 1 level in ragweed pollen (Singer et al. 2005).
The increase of major pollen allergen proteins upon elevated NO_2 is in accordance with the transcriptional data and may further impact the allergenic potential of ragweed pollen.

4.3.1.2 Cytoskeleton dynamics

Polarization by cytoplasmic and cytoskeletal reorganization is crucial for pollen germination. It requires a continuous deposition of new cell wall and plasma membrane after pollen tube growth reached the maximum tip region (Mascarenhas 1993). Considering the ragweed pollen spot annotation in this study, a small proportion of identified proteins were categorized as related to cytoskeleton dynamics (>3%) (Fig. 15). An actin-97 protein was proved to be upregulated upon 80 ppb NO_2, whereas a tubulin-alpha-1 decreased. The main function of these cytoskeletal proteins is their contribution to the deposition of cell-wall components (Cai et al. 2005). These expression changes of cytoskeletal proteins suggest complex machinery available in mature pollen to intercede reorganization leading to pollen germination and rapid tube elongation and orientation (Palevitz et al. 1994, Vidali and Hepler 2001).

4.3.1.3 Photosynthesis related proteins

Photosystems are functional and structural units of protein complexes involved in photosynthesis and are located in the chloroplasts (Pakrasi 1995). Interestingly, in the pollen from plants exposed to 80 ppb NO_2, three photosystem proteins (Photosystem

I P700 chlorophyll a apoprotein, Photosystem II CP47 chlorophyll apoprotein and Photosystem II D2 protein) were downregulated, whereas an oxygen-evolving enhancer protein was upregulated (Tab. 5).

Photosystem I (PSI) is a multisubunit protein complex initiating one of the first steps of solar energy conversion by light-driven electron transport (Bengis and Nelson 1975). In plants, the PSI complex consists of at least 19 protein subunits, approximately 175 chlorophyll molecules, 2 phylloquinones and 3 Fe_4S_4 clusters (Ben-Shem et al. 2003) . Photosystem II (PSII) functions as a light energy-driven water–plastoquinone oxidoreductase (Bricker et al. 2012). In higher plants at least seven major intrinsic proteins appear to be absolutely required for PSII (Burnap and Sherman 1991). These are CP47, CP43, the D1 protein, the D2 protein, α and β subunits of cytochrome b559, and the 4.8 kDa *psbI* gene product. Any unexpected disorder of these components leads to a loss of normal PS II assembly and a loss of the ability to evolve oxygen (Bricker 1992, Bricker et al. 2012).

The majority of flowering plants exhibit maternal inheritance of chloroplast DNA (cpDNA) (Corriveau and Coleman 1988, Birky 1995), although there are numerous exceptions, i.e. paternally or biparental biased inheritance (Shore and Triassi 1998). A landmark study by Corriveau and Coleman (1988) examined the potential for paternal plastid inheritance in 235 species of angiosperms from 80 different families. They detected plastid DNA in either the generative cells or/and sperm cells in pollen from 26 genera, indicating the potential for the paternal chloroplast genome to be transmitted to offspring. Paternal chloroplast inheritance has subsequently been documented in a wide range of angiosperms (Clement and Pacini 2001, Ji et al. 2004). Meanwhile large-scale studies have screened pollen grains for evidence of plastids or plastid DNA in generative or sperm cells (Zhang et al. 2003).

Although the function of theses photosystem proteins has not been studied yet in pollen, these results demonstrate the presence of photosystem proteins in pollen

and suggest that these photosystem proteins may play important roles in NO_2 tolerance of ragweed pollen.

4.3.1.4 NH_4^+ assimilation

Glutamine synthetase (GS) is the key enzyme involved in ammonia (NH_3) assimilation by plants (Temple et al. 1998). GS takes charge of primary NH_4^+ assimilation in plants by catalyzing the ATP-dependent conversion of glutamate (Glu) into glutamine (Gln) scavenging NH_4^+ (Coruzzi 2003). The amino acid glutamine is essential for pollen development (Moreno et al. 1988).

In this study, 80 ppb NO_2 increased the expression of a glutamine synthetase cytosolic isozyme in ragweed pollen (Tab. 5). This protein is absent in control pollen which implies the high concentration of NO_2 acts as a trigger to switch on its expression. Ribarits et al (2007) reported that inactivation of a gene of glutamine synthetase resulted in failure of the first pollen mitosis, resulting in male sterility in tobacco plants. Targeted inactivation of glutamine synthetase has been used as a novel and reversible male sterility system in transgenic tobacco plants (Mamun 2007, Ribarits et al. 2009). As discussed before, elevated NO_2 may produce adverse effects on pollen viability. Since glutamine synthetase plays a key role in pollen development, the extra expression of glutamine synthetase might partly compensate the NO_2-induced pollen viability damage in common ragweed.

4.3.1.5 Metabolism and energy generation

The task of the pollen grain in plant reproduction includes the production of a tube to deliver the sperm cells to the embryo sac. Therefore, it seems normal that the pollen grain saves proteins required for pollen tube germination and early growth, as well as proteins for energy metabolism (Mascarenhas 1993). In this study, several differential expressed proteins were identified to be involved in energy generation (including glycolysis, tricarboxylic acid cycle and calvin cycle) and metabolic processes. Studies suggest that the majority of the *Arabidopsis* and *Oryza sativa* pollen polypeptides are involved in metabolism (at least 42%) and high percentages of

energy generation proteins have been identified (at least 17%) as well (Noir et al. 2005, Dai et al. 2006).

It is worth noting that glutamate decarboxylase, which increased about 2 times in the treated pollen, is involved in feedback controls of Ca^{2+}-permeable channels to fluctuate intracellular γ -aminobutyric acid (GABA) levels and thus modulates pollen tube growth (Yu et al. 2014). Elevated NO_2 decreased the amount of glycolytic glyceraldehyde-3-phosphate dehydrogenase. Interestingly, deficiency of this protein resulted in male sterility of *Arabidopsis* (Munoz-Bertomeu et al. 2010).

4.3.1.6 Cellular transport and signaling

In view of the vast physiological and metabolic events that takes place in the mature pollen grain it is no surprise to detect transport and signalling molecules in ragweed mature pollen proteomes (Tab. 5).

14-3-3 proteins are major regulators in plant development and physiology including primary metabolism and signal transduction pathways, and typically there is a phosphorylation-dependent interaction with the target protein (Pertl et al. 2011). In this study, a 14-3-3 protein decreased 1.6-fold in the ragweed pollen exposed to 80 ppb NO_2. Elmayan and Simon (2007) suggested that 14-3-3 proteins may interact directly with the C-terminal part of NADPH oxidase, resulting in reduced ROS production which may further influence the allergenic potential of ragweed pollen. Another work reported that 14-3-3 proteins also played a key role in the pollen tube elongation of conifers (Bhatt and Lazzaro 2003). 14-3-3 proteins are also involved in responses to biotic and abiotic stresses (Roberts et al. 2002, Chen et al. 2006).

The proteins involved in transport processes in this work were importin, sorting nexin, ATP synthase and V-type ATP synthase catalytic. Importin proteins which decreased 1.93 times mediate the nuclear import and export pathways that regulate diverse cellular functions (Dingwall and Laskey 1991). It has been demonstrated to be

specifically required for pollen tube elongation by *in vitro* assays in mutant rice grains (Han et al. 2011). On the other hand, ATP synthase which was upregulated by 2.09 times had been proved to be highly expressed in pollen grains at the later developmental stage and played an important role in pollen formation in *Arabidopsis* (Li et al. 2010). The expression changes of proteins involved in the transport and signalling process suggest that the ragweed pollen changed the transport and signaling patterns to better adapted to the high concentration of NO_2.

4.3.1.7 Stress response

A clear portion of stress related proteins were identified in the ragweed pollen proteome analyses (Tab. 5). Among them, 7 proteins were upregulated whereas 3 proteins were downregulated. As mentioned by Dai et al. (2006), the pollen, as a highly compact tri-cellular organism, must have gained the ability during evolution to deal with extracellular stresses after release from the anther as well as intracellular stresses resulting from the active metabolism of germinating pollen and its interaction with cells of the stigma and the style.

The expression of an inorganic pyrophosphatase was upregulated due to the elevated NO_2 treatment. Plants with reduced inorganic pyrophosphatase were less able to cope with drought stress due to an inability to synthesize sufficient ABA (George et al. 2010). In another report, two inorganic pyrophosphatases which were expressed in pollen tubes led to an inhibition of growth, when they were phosphorylated in a Ca^{2+} – dependent manner (de Graaf et al. 2006).

Interestingly, an elongation factor and a proteasome subunit alpha which were found to be increased in the 2D-DIGE results were also found to be upregulated in the SSH data.

The differential expression of stress related proteins led to the suggestion that the plants recognize even 80 ppb NO_2 as stress and stimulate related proteins in pollen grains to protect themselves against the stress.

4.3.1.8 Reproduction related protein

In the SSH results, phosphoglucomutase was downregulated in ragweed pollen due to the elevated NO_2. In 2D-DIGE, phosphoglucomutase was only found in the control spots which indicated a significant decrease in the pollen after elevated NO_2 treatment. As discussed before, lack of phosphoglucomutase may have dramatic consequences for germinating pollen grains and reproduction processes (Egli et al. 2010).

4.3.2 Protein S-nitrosylation in ragweed pollen

S-nitrosylation, also known as S-nitrosation, constitutes the most studied and best described NO-dependent posttranscriptional modification in plants. It refers to the reversible covalent binding of a NO moiety to the thiol group of a cysteinyl residue (Cys) of a target protein, leading to the formation of an S-nitrosothiol (Astier et al. 2011). Identification of plant S-nitrosylated proteins has been achieved on a proteome-wide scale from plant tissues, cell suspensions, cellular extracts, or organelles treated with nitrosylating agents, thus leading to elevated levels of S-nitrosylated proteins (Astier et al. 2012). A thousand candidates for S-nitrosylation have been identified, many of them being already characterized as targets for S-nitrosylation in animals. These proteins are involved in major cellular activities, notably primary and secondary metabolism, photosynthesis, genetic information processing, cellular architecture, and response to biotic and abiotic stresses (Astier et al. 2012).

In this work, the proteomic analysis of ragweed pollen by LC-MS/MS resulted in the identification of 25 S-nitrosylated proteins belonging to allergens, cytoskeleton proteins, metabolic proteins, stress-related proteins, signalling proteins and protein

folding proteins (Tab. 9). Elevated NO_2 significantly enhanced the protein S-nitrosylation in ragweed pollen and 6 additional proteins were only S-nitrosylated by elevated NO_2.

4.3.2.1 S-nitrosylation of allergens in ragweed pollen

Since the conversion between NO and NO_2 is rather fast and they always maintain a balance in the atmosphere, elevated NO_2 may also lead to an increase in the concentration of NO (Düring et al. 2011).

In this study, 25 and 19 S-nitrosylated proteins were found in the pollen of ragweed plants exposed to 80 ppb NO_2 and 40 ppb NO_2, respectively. Among them, 7 pollen allergens including Amb a 1.1, Amb a 1.2, Amb a 1.3, Amb a 1.4, Amb a 1.5 Amb a 3, profiling and Hev b 9 were confirmed to be S-nitrosylated which has never been reported before. As can be seen, the mean gray value of Western blot bands intensity was upregulated upon high concentration of NO_2 which implied an increase of S-nitrosylated allergens in ragweed pollen (Fig. 25). As the expression of all these S-nitrosylated allergens increased at the protein level, it is not clear if the upregulation of S-nitrosylation was due to the high expression of protein or due to more S-nitrosylated sites of them. Recently it was shown that "*in vivo*" nitration of allergens enhanced the allergenic potential of proteins by antigen processing (Gruijthuijsen et al. 2006, Karle et al. 2012). Therefore the increase of structural modification of allergen proteins by NO_2 might also have an effect on the allergenicity of ragweed pollen.

4.3.2.2 S-nitrosylation of cytoskeleton proteins

Ragweed pollen contained the cytoskeleton proteins actin and actin-depolymerizing factors, which had already been described to undergo S-nitrosylation in animal and plant systems (Jaffrey et al. 2001). The dynamic nature of the cytoskeleton filaments allows cells a rapid response to intracellular and extracellular signals by changing shape and translocating intracellular organelles or vesicles. Conformational changes

of cytoskeleton components due to S-nitrosylation might be involved in directing vesicles loaded with toxic metabolites to the infection site and deflating the contents into the extracellular space (Collins et al. 2003).

4.3.2.3 S-nitrosylation of metabolic proteins

In this work, 6 metabolic enzymes were identified as potential candidates for S-nitrosylation. Interestingly, an adenosylhomocysteinase, a UTP--glucose-1 -phosphate uridylyltransferase and a glutamate decarboxylase were only S-nitrosylated in the pollen of plants exposed to 80 ppb NO_2. Lindermayr et al (2006) reported that S-nitrosylation of adenosyltransferases in *Arabidopsis* will significantly inhibit the enzyme activity and markedly reduce ethylene emission of *Arabidopsis* cell cultures, which may further regulate plant growth, plant development, and plant defense reactions. UTP--glucose-1-phosphate uridylyltransferase was proved to play an important role during pollen maturation especially with regard to pollen starch accumulation. It belongs to the “late” pollen gene as it preferentially expressed in maturing pollens (Huang et al. 2011). A dsRNA-mediated transcriptional gene silencing of UTP--glucose-1-phosphate uridylyltransferase showed the failure of starch accumulation in pollen which thus led to sterile pollen in rice (Mu et al. 2009). As discussed before, glutamate decarboxylase was proved to play a key role in modulating pollen tube growth by regulating the GABA biosynthesis (Yu et al. 2014). Conformational changes of metabolic enzymes due to S-nitrosylation might lead to changes of enzyme activity and further influence on pollen viability in ragweed pollen.

4.3.2.4 S-nitrosylation of stress-related proteins

Within the stress-related proteins, inorganic pyrophosphatase and monodehydroascorbate reductase (MDHAR) were found to be S-nitrosylated in ragweed pollen. An interesting find is monodehydroascorbate reductase, a flavin adenine dinucleotide (FAD) enzyme that catalyzes the reduction of monodehydroascorbate (MDA) radical to ascorbate using NAD(P)H as an electron

donor. MDHAR is part of the ascorbate-glutathione enzymatic antioxidant cycle and its activity is inhibited by thiol-modifying reagents (Sano and Asada 1994), which suggest that S-nitrosylation could be involved in the regulation of enzymatic activity. The inorganic pyrophosphatase was only S-nitrosylated in the treated pollen and it was related to the synthesis of abscisic acid and pollen tube growth.

4.3.2.5 S-nitrosylation of signalling proteins

Another cluster of S-nitrosylated proteins in ragweed pollen includes several signalling proteins like 14-3-3-like protein, Luminal-binding protein and Ras-related protein RABD2a. Ras is a molecular switch that cycles between an inactive GDP-bound state and an active GTP-bound state, to regulate a number of cellular processes, including cell growth, differentiation and apoptosis (Wittinghofer 1998). Many proteins within the Ras superfamily contain redox-sensitive Cys residues that are susceptible to S–nitrosylation (Raines et al. 2007). In mammals, Ras S-nitrosylation is also implicated in the initiation of tumorigenesis and maintenance of established tumors (Lander et al. 1995). S-nitrosylation of wild-type Ras by endothelial NO synthase has been shown to promote pancreatic tumor growth (Lim et al. 2008). The S-nitrosylation of Ras has not been reported in plants until now. A study reported that lacking Ras-related protein RABD2 resulted in short and bushy seedling with low fertility in *Arabidopsis* (Pinheiro et al. 2009).

S-nitrosylation of plant proteins is now the subject of increasing research effort but there are several challenges for the future. In this work, the elevated concentration of NO_2 significantly enhances protein S-nitrosylation of pollen of common ragweed. Identification of ragweed pollen proteins being potential targets for S-nitrosylation is a promising starting point to get insight in physiological as well as regulatory functions of NO_2 in ragweed. The effect of S-nitrosylation on ragweed pollen proteins, if enzyme activities are inhibited or enhanced due to S-nitrosylation or if a structural alteration followed by change of the protein function is the result of the modification, will have to be analyzed.

4.3.2.6 Influence of post-translational modification on pollen allergenic potential

Post-translational modification (PTM) is the chemical modification of a protein after its translation, and there are several different PTMs, such as phosphorylation, tyrosine nitration, S-nitrosylation, ADP-ribosylation, myristoylation, isoprenylation, glycosylation (Aebersold and Goodlett 2001). PTMs have been shown to affect almost every aspect of protein activity, including function, localization, stability, and dynamic interactions with other molecules (Webster and Thomas 2012).

Elevated NO_2 may lead to protein tyrosine nitration and protein S-nitrosylation (NO_2/NO conversion). Protein tyrosine nitration (PTN) is a post-translational modification occurring under the action of a nitrating agent. Tyrosine is modified in the 3-position of the phenolic ring by the addition of a nitro group (NO_2) (Abello et al. 2009). The heterogeneous reaction of the protein with the gaseous reactants NO_2 was found to form nitrated proteins as well as protein degradation and aggregation products (Shiraiwa et al. 2012). Interestingly, tyrosine residues of pollen allergens are efficiently nitrated by the air pollutants nitrogen dioxide and ozone at levels reached in urban air (Franze et al. 2003). Franze et al (2005) demonstrated that birch pollen proteins including the allergen Bet v 1 could be efficiently nitrated by traffic-related air pollution (high concentrations of nitrogen oxides and ozone).

In this study, elevated NO_2 concentrations significantly enhanced the allergenicity of ragweed pollen, which was demonstrated by dot blot, 1D-immunoblot and 2D-immunoblot. Nitration of tyrosine residues may alter immunogenicity and allergenicity of proteins. A recent experiment found a higher amount of specific IgE against nitrated than against untreated allergen Bet v 1 in sera from birch pollen-allergic patients indicateing that allergen nitration is relevant *in vivo* and can contribute to allergenicity in polluted environments (Gruijthuijsen et al. 2006). The detection of IgE specific for nitrated Bet v 1a, which does not bind unmodified Bet v 1 or nitrated unrelated proteins, implies that nitration generates novel allergenic epitopes (Gruijthuijsen et al. 2006). Another study showed that nitration of Bet v 1

alters antigen processing and presentation *via* Human Leukocyte Antigen DR (HLA-DR), by enhancing both the quality and the quantity of the Bet v 1-specific peptide repertoire (Karle et al. 2012). It is not clear for which purpose this nitration reaction occurs in biological systems. A possible explanation is that NO_2 groups may serve as markers for foreign proteins and guide the immune system (Franze et al. 2005).

Since protein nitration may play a central role in the promotion of allergies, a possible mechanism would be that the elevated concentration of NO_2 leads to further nitration of allergen protein or other proteins that boost the allergenic potential of allergens or even generate new allergens in ragweed pollen.

In this study, 7 pollen allergens were confirmed to be S-nitrosylated in the pollen of ragweed plants exposed to 80 ppb NO_2 and 40 ppb NO_2. As can be seen, the mean gray value of Western blot bands intensity was upregulated at the high concentration of NO_2 which implies an increase of S-nitrosylated allergens in ragweed pollen. Another recent important work allowing a better comprehension of the NO signalling in plants and involving S-nitrosylation concerns the NAD(P)H oxidase and reactive oxygen species (ROS) synthesis (Yun et al. 2011). This observation raises the possibility that modification of this specific Cys residue might regulate the activity of NAD(P)H oxidase. The activity of NAD(P)H oxidase and further ROS synthesis in pollen may affect the allergenic potential which will be discussed below.

As discussed before, due to the balance of NO_2 and NO in the atmosphere, elevated NO_2 may lead to an increase of NO concentration. This exogenous NO may further result in more S-nitrosylation proteins in ragweed pollen and the modification of protein might regulate the NAD(P)H oxidase and reactive oxygen species (ROS) synthesis (Yun et al. 2011).

Furthermore, a recent study showed that a protein of the 14-3-3 family was able to interact directly with the C-terminal part of NADPH oxidase, and that modification of

its expression in tobacco cells led to reduced ROS production (Elmayan and Simon-Plas 2007). In this study, the expression of a 14-3-3 family protein significantly decreased 1.63 fold in 2D-DIGE result. This down-regulation may have an impact on the expression of NADPH oxidase and further increase the ROS production of ragweed pollen.

4.4 Immunoblotting analysis of ragweed pollen

Several highly expressed proteins in pollen behave as allergens for humans. The close interaction between allergens and the immune system may explain the allergenicity of an allergen (Gadermaier et al. 2014).

When pollen grains enter the upper respiratory tract and land on the mucosa, allergens are released upon rehydration. In some cases, primarily in grasses, the allergens are carried by microscopic (5.0 µm) sub-pollen particles, which may be derived from pollen grain bursting in the atmosphere during rainfall. The particles are subsequently able to directly reach the lower respiratory tract without further assistance. Mast cells serve as one of the most important mediators in the pathogenesis of respiratory allergies, as well as in other chronic inflammatory diseases. The commonly accepted view is that allergens trigger immunoglobulin E (IgE) antibody production from B-lymphocytes, and IgE molecules bind them to the corresponding receptors on the surface of mast cells. The specific IgE antibodies are subsequently bound and cross-linked by allergens onto the surface of mast cells; this triggers the exocytotic release of cytoplasmic granules, which contain an array of preformed and newly synthesized mediators involved in the allergic inflammatory response (e.g., histamine, proteases, prostaglandins, leukotriene, and cytokines) specifically from mast cells (Swindle and Metcalfe 2007).

In order to compare the allergenicity of pollen of ragweed plants exposed to different concentrations of NO_2, immunoblotting analysis with allergic patients' sera was applied. The dot blot results showed that 80 ppb NO_2 significantly increased the total

allergenicity of the pollen (Fig. 28 & 29) whereas Western blotting revealed the IgE reactivity changes of specific allergens (Fig. 31-33). As expected, Amba 1.1 contributed more than half of the total allergenicity of ragweed pollen, which is consistent with the literature (Gadermaier et al. 2008, Gadermaier et al. 2014).

Urban air pollutants, like nitrogen dioxide (NO_2), can influence the allergenicity of pollen. Cuinica indicated higher IgE recognition by patient sera sensitized to the pollen extracts from NO_2 exposed samples in comparison to the non-exposed samples (Cuinica et al. 2014). Similar results were presented by Sousa et al (2002), who observed high IgE reactivity to allergens in *Acernegundo* pollen after *in vitro* exposure to SO_2 and NO_2. Other authors showed that SO_2 and NO_2 increased the allergenic potential of pollen (Behrendt et al. 1997). A field experiment demonstrates that ragweed pollen collected along high-traffic roads showed a higher allergenicity than pollen from low-traffic roads and vegetated areas (Ghiani et al. 2012).

4.4.1 Putative mechanisms involved in NO_2 induced allergenic potential increase

Elevated NO_2 clearly increased the allergenicity of ragweed pollen. The reasons might be various aspects like expression pattern, protein post-translational modification, reactive oxygen species (ROS) and other influences on ragweed pollen due to the elevated NO_2.

4.4.1.1 Changes in expression patterns of pollen allergens

Allergen patterns can change in response to air pollution, which can modify the allergenic potential of pollen (D'Amato et al. 2013). In this study, allergens were upregulated both at the transcriptional and protein levels. These enhanced expression patterns will certainly increase the allergenic potential which may have negative effects on atopic patients.

4.4.1.2 A potential new allergen from ragweed pollen with homology to Hev b 9 from *Hevea brasiliensis*

The pollen of common ragweed (*Ambrosia artemisiifolia*) is a major cause of hay fever and associated asthma in North America and Europe. Proteins from natural latex (*Hevea brasiliensis*) have been recognized as a potent source for type I allergic reactions (cutaneous, nasal, bronchial, and systemic reactions) (Axelsson et al. 1987, Spaner et al. 1989). It was further demonstrated that the sera of latex allergic patients show considerable cross-reactivity with ragweed pollen extracts. IgE-binding to latex allergens in immunoblots was inhibited effectively by the ragweed pollen extract, which indicated close homology of the essential allergens in ragweed pollen and latex (Fuchs et al. 1997).

In this work, certain allergens which could bind to ragweed allergic human IgE antibodies were detected by 1D-immunoblots and 2D-immunoblots. Further analysis of LC-MS/MS revealed the presence of the expected allergens of ragweed pollen (e.g. Amb a 1 isoforms and Amb a 3) as well as another allergen with homology to Hev b 9 from *Hevea brasiliensis*. This Hev b 9 homologue was detected in the band of 52 kDa in 1D-immunoblots and in the spots with molecular weight of 52, 15 and 10 kDa in 2D-immunoblots. The molecular weight of Hev b 9 from *Hevea brasiliensis* is 51 kDa which matches the assumed molecular weight of the Hev b 9 homologue in ragweed pollen of around 52 kDa. On this basis, the detected presence of Hev b 9 homologue at a molecular weight of 15 and 10 kDa may either indicate a different fold pattern or fragments which could also reveal an allergenic potential with the allergic human IgE antibodies.

An increasing number of allergens will presumably be found by future studies, requiring a lot more work with regard to allergen identification. However, this study identified a new putative allergen from ragweed pollen.

4.4.1.3 Other influences on ragweed pollen

Besides the issues discussed above, atmospheric pollution could also enhance the pollen allergenic potential through some peculiar ways. By attaching to the surface of pollen grains and of plant-derived paucimicronic particles, pollutants can modify the morphology of these antigen-carrying agents and alter their allergenic potential (D'Amato 2002). A scanning electron microscopy study of pollen grains indicated that in polluted areas, airborne particles accumulated on the surface of pollen grains and changed the shape and tectum of pollen (Chehregani et al. 2004). Pollutants were also demonstrated to interact directly with pollen grains, affecting cell wall structure and leading to increased release of allergens or sub-pollen particles containing allergens. Motta et al. (2006) demonstrated that exposure to a mix of O_3 and NO_2 clearly resulted in damage of the pollen, which induced the release of allergen-containing cytoplasmic granules from grass pollen.

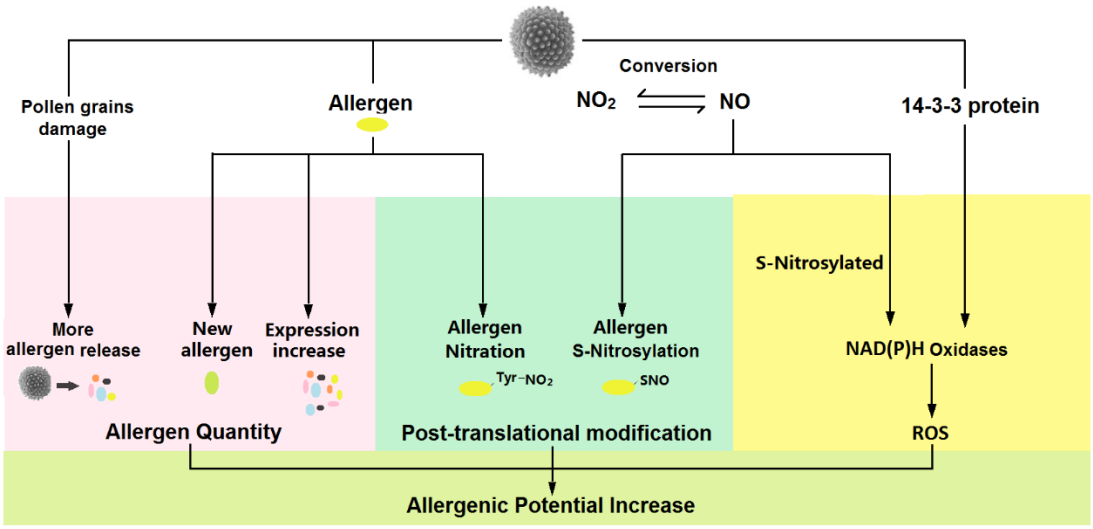


Figure 35. Putative mechanisms involved in NO_2 induced allergenic potential increase of pollen of common ragweed.

In this study, pollen from ragweed plants exposed to 80 ppb NO_2 showed a significant increase of allergenic potential, which was demonstrated by immunoblot assay with ragweed allergic human IgE antibodies. The mechanisms involved in this phenomenon are discussed in different ways (Fig. 35) and might lead to a preliminary understanding of the impact of NO_2 on ragweed pollen allergenicity and even

provide a new link between air pollution and the increased prevalence of respiratory allergic diseases. However, the underlaying mechanisms remain to be elucidated.

4.5 Conclusion

This dissertation has investigated the effects of exposure of common ragweed to elevated NO_2 (80 ppb) over the whole vegetation period. Significant decreases of the seed production were observed, whereas the pollen production was increased. Furthermore, the pollen allerginicity as tested by immunoreaction was also increased. The allergen Amb a 1.1, which corresponds to the 38 kDa protein was proved to be the major allergen (it contributed more than half of IgE binding ability) of pollen of ragweed and it was upregulated at the transcriptional and protein level.

The whole transcriptome and proteome of ragweed pollen was analyzed by Illumina sequencing and 2D-DIGE. The annotation and GO term analysis indicated the major genes involved in different biological processes and molecular functions. The change of gene expression patterns were also studied by RNA-seq. The expression of some allergens were upregulated in the RNA-seq result and further confirmed by the qRT-PCR.

The significant effect of S-nitrosylation observed in this work showed that NO_2 affected the post-translational modification of pollen proteins. Since high concentrations of NO_2 may lead to nitration of proteins and thus further increase the allergenicity, the protein S-nitrosylation of ragweed pollen observed in this work should be further analyzed.

Putative mechanisms involved in NO_2 induced allergenic potential increase of pollen of common ragweed have been discussed, however, the real mechanisms remain to be elucidated. Taking into consideration that the results were obtained at NO_2 concentrations of 80 ppb, which may be present in seriously polluted areas, this NO_2

influence on ragweed pollen may also happen in the natural environment and induce negative effects on atopic patients.

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APPENDIX

Appendix 1. Sequence data of SSH libraries

Library	Acc.No.	Protein Family
Control	**CAA52782**	**SF16 protein**

> control 17

TAGCGTGGTCGCGGCCGAGGTAAGTCAACCACACCAGATCAAACTCGCGTTTCCTAGGTAGAACAAGGGAGGAAGTAGGT
GCCATCAAGATTCAGGCGGCTTATCGTGGATACATGGCAACAAGGGCTTTCCGGTCTTTAAGAGCAATGAGGAGGCTGAAC
CTGTGGCTTCAAGGACAGGCAGTCAAACGTCAAACGACGTTCGCCTTAATGCGCATACAGACAATGGGTCGTGTCCAGTCT
CAGGTTCGGGTCAGGAGGATTCGAATGGCTGAAGTGAATGAGGCTCTCCAACGACAGCTTATCCAAAGGCGACTAAAGAT
TCTTGACAAACAAGCTTTTGATCTAAGTCCCAGATCAAAGGAACAAGTAGAAGCCAGCTTACGAAGCAAAAAGGAGGCTG
CTGAAAGGAGATTAAAGGCATTGGCTTATGCGTACCTGCCCGGGCGGCCGCTCGAA

> control 31

TAGCGTGGTCGCGGCCGAGGTAAGTCAACCACACCAGATCAAACTCGCGTTTCCTAGGTAGAACAAGGGAGGAAGTAGGT
GCCATCAAGATTCAGGCGGCTTATCGTGGATACATGGCAACAAGGGCTTTCCGGTCTTTAAGAGCAATGAGGAGGCTGAAC
CTGTGGCTTCAAGGACAGGCAGTCAAACGTCAAACGACGTTCGCCTTAATGCGCATACAGACAATGGGTCGTGTCCAGTCT
CAGGTTCGGGTCAGGAGGATTCGAATGGCTGAAGTGAATGAGGCTCTCCAACGACAGCTTATCCAAAGGCGGCTAAAGAT
TCTTGACAAACAAGCTTTTGATCTAAGTCCCAGATCAAAGGAACAAGTAGAAGCCAGCTTACGAAGCAAAAAGGAGGCTG
CTGAAAGGAGATTAAAGGCATTGGCTTATGCGTACCTGCCCGGGCGGCCGCTA

> control 105

TAGCGTGGTCGCGGCCGAGGTAAGTCAACCACACCAGATCAAACTCGCGTTTCCTAGGTAGAACAAGGGAGGAAGTAGGT
GCCATCAAGATTCAGGCGGCTTATCGTGGATACATGGCAACAAGGGCTTTCCGGTCTTTAAGAGCAATGAGGAGGCTGAAC
CTGTGGCTTCAAGGACAGGCAGTCAAACGTCAAACGACGTTCGCCTTAATGCGCATACAGACAATGGGTCGTGTCCAGTCT
CAGGTTCGGGTCAGGAGGATTCGAATGGCTGAAGTGAATGAGGCTCTCCAACGACAGCTTATCCAAAGGCGGCTAAAGAT
TCTTGACAAACAAGCTTTTGATCTAAGTCCCAGATCAAAGGAACAAGTAGAAGCCAGCTTACGAAGCAAAAAGGAGGCTG
CTGAAAGGAGATTAAAGGCATTGGCTTATGCGTACCTGCCCGGGCGGCCGCTCGAA

> control 153

TAGCGTGGTCGCGGCCGAGGTAAGTCAACCACACCAGATCAAACTCGCGTTTCCTAGGTAGAACAAGGGAGGAAGTAGGT
GCCATCAAGATTCAGGCGGCTTATCGTGGATACATGGCAACAAGGGCTTTCCGGTCTTTAAGAGCAATGAGGAGGCTGAAC
CTGTGGCTTCAAGGACAGGCAGTCAAACGTCAAACGACGTTCGCCTTAATGCGCATACAGACAATGGGTCGTGTCCAGTCT
CAGGTTCGGGTCAGGAGGATTCGAATGGCTGAAGTGAATGAGGCTCTCCAACGACAGCTTATCCAAAGGCGGCTAAAGAT
TCTTGACAAACAAGCTTTTGATCTAAGTCCCAGATCAAAGGAACAAGTAGAAGCCAGCTTACGAAGCAAAAAGGAGGCTG
CTGAAAGGAGATTAAAGGCATTGGCTTATGCGTACCTGCCCGGGCGGCCGCTA

Control	NP001147673	SF3 Protein (PLIM 1)

> control 18

TTCGAGCGGCCGCCCGGGCAGGTACCACAAACAATGTTTTAGGTGTATTCACGGAGGGTGCCCTTTAACACACTCTTCGTAT
GCCGCCCTCAATGGCGTTCTTTACTGCAGACACCATTTTGCTCAACTCTTCCTGGAAAAAGGCACATTGTCTCATGTTCTCAT
GGCTGCAGACCGCAAGAAAAACACAGTCCCAGTCGATCAAGAAACCCAACCTGCTGATGACAAGCTACTCAAACAGGAGG
AAACTCAAGTTGACAAGCTGGTAACCCAACAGGAAACTGATCAAGTTGACAATGTAATCCAACAGGAAGGTGAAGTTGAA

AGCAAACCCGAGACTACAGAAAAAACGAATGAATTTGAAGAGCAAGAATCCTAGACACGAGACGTTTGTCACAAATCTAG
CTTGTTAATTGGTCGGTGTTACTGAAAAGCCATATACAGTAGTACATCGGCCGCGACCACGCTA

> control 48
TCAATGGCGTTCTTTACTGCAGACACCATTTTGCTCAACTATTCCTGGAAAAAGGCACATTGTCTCATGTTCTCATGGCTGCA
GACCGCAAGAAAAACACAGTCCCAGTCGATCAAGAAACCCAACCTGCTGATGACAAGCTACTCAAACAGGAGGAAACTCA
AGTTGACAAGCTGGTAACCCAACAGGAAACTGATCAAGTTGACAATGTAATCCAACAGGAAGGTGAAGTTGAAAGCAAAC
CCGAGACTACAGAAAAAACGAATGAATTTGAAGAGCAAGAATCCTAGACACGAGACGTTTGTCACAAATCTAGCTTGTTAA
TTGGTCGGTGTTACTGAAAAGCCATATACAGTAGTACCTCGGCCGCGACCACGCTAATCAACTAGTGCGGCCGCCTGCAGGT
CGACCATATGGGAG

> control 130
TAGCGTGGTCGCGGCCGATGTACTACTGTATATGGCTTTTCAGTAACACCGACCAATTAACAAGCTAGATTTGTGACAAACGT
CTCGTGTCTAGGATTCTTGCTCTTCAAATTCATTCGTTTTTTCTGTAGTCTCGGGTTTGCTTTCAACTTCACCTTCCTGTTGGAT
TACATTGTCAACTTGATCAGTTTCCTGTTGGGTTACCAGCTTGTCAACTTGAGTTTCCTCCTGTTTGAGTAGCTTGTCATCAGC
AGGTTGGGTTTCTTGATCGACTGGGACTGTGTTTTTCTTGCGGTCTGCAGCCATGAGAACATGAGACAATGTGCCTTTTTCC
AGGAAGAGTTGAGCAAAATGGTGTCTGCAGTAAAGAACGCCATTGAGGGCGGCATACGAAGAGTGTGTTAAAGGGCACC
CTCCGTGAATACACCTAAAACATTGTTTGTGGTACCTGCCCGGGCGGCCGCTCGA

> control 21
CGAGCGGCCGCCCGGGCAGGTACCACAAACAATGTTTTAGGTGTATTCACGGAGGGTGCCCTTTAACACACTCTTCGTATG
CCGCCCTCAATGGCGTTCTTTACTGCAGACACCATTTTGCTCAACTCTTCCTGGAAAAAGGCACATTGTCTCATGTTCTCATG
GCTGCAGACCGCAAGAAAAACACAGTCCCAGTCGATCAAGAAACCCAACCTGCTGATGACAAGCTACTCAAACAGGAGGA
AACTCAAGTTGACAAGCTGGTAACCCAACAGGAAACTGATCAAGTTGACAATGTAATCCAACAGGAAGGTGAAGTTGAAA
GCAAACCCGAGACTACAGAAAAAACGAATGAATTTGAAGAGCAAGAATCCTAGACACGAGACGTTTGCCACAAATCTAGC
TTGTTAATTGGTCGGTGTTACTGAAAAGCCATATACAGTAGTACATCGGCCGCGACCACGCTA

> control 33
TTCGAGCGGCCGCCCGGGCAGGTACCACAAACAATGTTTTAGGTGTATTCACGGAGGGTGCCCTTTAACACACTCTTCGTAT
GCCGCCCTCAATGGCGTTCTTTACTGCAGACACCATTTTGCTCAACTCTTCCTGGAAAAAGGCACATTGTCTCATGTTCTCAT
GGCTGCAGACCGCAAGAAAAACACAGTCCCAGTCGATCAAGAAACCCAACCTGCTGATGACAAGCTACTCAAACAGGAGG
AAACTCAAGTTGACAAGCTGGTAACCCAACAGGAAACTGATCAAGTTGACAATGTAATCCAACAGGAAGGTGAAGTTGAA
AGCAAACCCGAGACTACAGAAAAAACGAATGAATTTGAAGAGCAAGAATCCTAGACACGAGACGTTTGTCACAAATCTAG
CTTGTTAATTGGTCGGTGTTACTGAAAAGCCATATACAGTAGTACATCGGCCGCGACCACGCTA

> control 71
CGAGCGGCCGCCCGGGCAGGTACCACAAACAATGTTTTAGGTGTATTCACGGAGGGTGCCCTTTAACACACTCTTCGTATG
CCGCCCTCAATGGCGTTCTTTACTGCAGACACCATTTTGCTCAACTCTTCCTGGAAAAAGGCACATTGTCTCATGTTCTCATG
GCTGCAGACCGCAAGAAAAACACAGTCCCAGTCGATCAAGAAACCCAACCTGCTGATGACAAGCTACTCAAACAGGAGGA
AACTCAAGTTGACAAGCTGGTAACCCAACAGGAAACTGATCAAGTTGACAATGTAATCCAACAGGAAGGTGAAGTTGAAA
GCAAACCCGAGACTACAGAAAAAACGAATGAATTTGAAGAGCAAGAATCCTAGACACGAGACGTTTGCCACAAATCTAGC
TTGTTAATTGGTCGGTGTTACTGAAAAGCCATATACAGTAGTACATCGGCCGCGACCACGCTA

Control	AF116850	LIM domain protein PLIM-2

> control 10

AACACCGACCAATTAACAAGCTAGATTTGTGACAAACGTCTCGTGTCTAGGATTCTTGCTCTTCAAATTCATTCGTTTTTTCTG
TAGTCTCGGGTTTGCTTTCAACTTCACCTTCCTGTTGGATTACATTGTCAACTTGATCAGTTTCCTGTTGGGTTACCAGCTTGT
CAACTTGAGTTTCCTCCTGTTTGAGTAGCTTGTCATCATCAGGT

Control	ABM66381	Pollen coat protein

> control 49

TAGCGTGGTCGCGGCCGAGGTACATTATGATTCTCTTTTTTGTTCGAGTGTATATAAAATAAAACAAAACAAAATGAAATAAA
ATACAAAAAAAGAATTGGTTATGTTAATTGAATAGATAGTTATGATGGTTGATGAAATGGTCCCTAGAGGTTCATTTGTTCATTC
CAGTTGCATCTTTGGCTGCATCTACTGCTCCTTGTGCACTTGCCACCTGCCCGGGCGGGCCGCTCGAAATCACATAGTGCGG
CCGCCTGCAAGGTCGAAC

> control 85

TAGCGTGGTCGCGGCCGAGGTACATTATGATTCTCTTTTTTGTTCGAGTGTATATAAAATAAAACAAAACAAAATGAAATAAA
ATACAAAAAAAGAATTGGTTATGTTAATTGAATAGATAGTTATGATGGTTGATGAAATGGTCCCTAGAGGTTCATTTGTTCATTC
CAGTTGCATCTTTGGCTGCATCTACTGCTCCTTGTGCACTTGCCACCTGCCCGGGCGGCCGCTCGAA

> control 163

TAGCGTGGTCGCGGCCGAGGTACATTATGATTCTCTTTTTTGTTCGAGTGTATATAAAATAAAACAAAACAAAATGAAATAAA
ATACAAAAAAAGAATTGGTTATGTTAATTGAATAGATAGTTATGATGGTTGATGAAATGGTCCCTAGAGGTTCATTTGTTCATTC
CAGTTGCATCTTTGGCTGCATCTACTGCTCCTTGTGCACTTGCCACCTGCCCGGGCGGCCGCTCGAA

Control	AEH27529	Phosphoglucomutase

> control 137

AGATGCTCCTGGACTTTTGATGGGGATATGTCCCAGTAAACCCATTGTAAGTTGAAGATATTGTAAGACAAAAATGCACTAAT
ACACCTAACCTCGGCCGCGACCACGCTA

Control	XP002533960	Myosin-1

> control 8

TTCGAGCGGCCGCCCGGGCAGGTACATAAATTCAAGAAACAAGTGAAGGATACGTTGGAAGAGGTTAAACAAGTTGAAGC
CAAGGGACTCACAGATTTACTATCCGAGATCAAGCCGATTTACAAACATCTTCACAAGATAAACACTAGACTTGCATCATGGT
TGCAACAAAGCATATTGCTCAACAATGAACTCGACACAATAAACTCGTATTTGTGCAACATCCAGGAAGAAATAACACAGGA
AGAAATCAAGTTCAGTGCACATCAGGCTGCAAAGTTCAAAGGTGATATATTAAACATGAAACAAGAAAACAACAAAGTTAA
TGAAGAACTTGAAGCAGGTTTAGATCATGCCATTGCCCTAAAACATGAAATAGAGCAAACTCTAGAAAGACTAGAAAGAAA
GTTTGGTCTTTCTGCCAATCAAAATCAGCCACGTAAGAGATGGTCATCAAGGCGGTCACTAAGCTTGAGGTCATTACTTTTT
GGTGGTACCTCGGCCGCGACCACGCTAATCAACTAGTGCGGCCGCCTGCAGGTCGAACCATAT

> control 12

TTCGAGCGGCCGCCCGGGCAGGTACATAAATTCAAGAAACAAGTGAAGGATACGTTGGAAGAGGTTAAACAAGTTGAAGC
CAAGGGACTCACAGATCTACTATCCGAGATCAAGCCGATTTACAAACATCTTCACAAGATAAACACTAGACTTGCATCATGGT
TGCAACAAAGCATATTGCTCAACAATGAACTCGACACAATAAACTCGTATTTGTGCAACATCCAGGAAGAAATAACACAGGA
AGAAATCAAGTTCAGTGCACATCAGGCTGCAAAGTTCAAAGGTGATATATTAAACATGAAACAAGAAAACAACAAAGTTAA

TGAAGAACTTGAAGCAGGTTTAGATCATGCCATTGCCCTAAAACATGAAATAGAGCAAACTCTAGAAAGACTAGAAAGAAA
GTTTGGTCTTTCTGCCAATCAAAATCAGCCACGTAAGAGATGGTCATCAAGGCGGTCACTAAGCTTGAGGTCATTACTTTTT
GGTGGTACCTCGGCCGCGACCACGCTA

> control 25

TTCGAGCGGCCGCCCGGGCAGGTACATAAATTCAAGAAACAAGTGAAGGATACGTTGGAAGAGGTTAAACAAGTTGAAGC
CAAGGGACTCACAGATCTACTATCCGAGATCAAGCCGATTTACAAACATCTTCACAAGATAAACACTAGACTTGCATCATGGT
TGCAACAAAGCATATTGCTCAACAATGAACTCGACACAATAAACTCGTATTTGTGCAACATCCAGGAAGAAATAACACAGGA
AGAAATCAAGTTCAGTGCACATCAGGCTGCAAAGTTCAAAGGTGATATATTAAACATGAAACAAGAAAACAACAAAGTTAA
TGAAGAACTTGAAGCAGGTTTAGATCATGCCATTGCCCTAAAACATGAAATAGAGCAAACTCTAGAAAGACTAGAAAGAAA
GTTTGGTCTTTCTGCCAATCAAAATCAGCCACGTAAGAGATGGTCATCAAGGCGGTCACTAAGCTTGAGGTCATTACTTTTT
GGTGGTACCTCGGCCGCGACCACGCTA

>control 92

TTCGAGCGGCCGTCCGGGCAGGTACATAAATTCAAGAAACAAGTGAAGGATACGTTGGAAGAGGTTAAACAAGTTGAAGC
CAAGGGACTCACAGATCTACTATCCGAGATCAAGCCGATTTACAAACATATTCACAAGATAAACACTAGACTTGCATCATGGT
TGCAACAAAGCATATTGCTCAACAATGAACTCGACACAATAAACTCGTATTTGTGCAACATCCAGGAAGAAATAACACAGGA
AGAAATCAAGTTCAGTGCACATCAGGCTGCAAAGTTCAAAGGTGATATATTAAACATGAAACAAGAAAACAACAAAGTTAA
TGAAGAACTTGAAGCAGGTTTAGATCATGCCATTGCCCTAAAACATGAAATAGAGCAAACTCTAGAAAGACTAGAAAGAAA
GTTTGGTCTTTCTGCCAATCAAAATCAGCCACGTAAGAGATGGTCATCAAGGCGGTCAGTAAGCTTGAGGTCATTACTTTTT
GGTGGTACCTCGGCCGCGACCACGCTA

>control 114

TGAGCGGCCGCCCGGGCAGGTACATAAATTCAAGAAACAAGTGAAGGATACGTTGGAAGAGGTTAAACAAGTTGAAGCC
AAGGGACTCACAGATCTACTATCCGAGATCAAGCCGATTTACAAACATCTTCACAAGATAAACACTAGACTTGCATCATGGTT
GCAACAAAGCATATTGCTCAACAATGAACTCGACACAATAAACTCGTATTTGTGCAACATCCAGGAAGAAATAACGCAGGAA
GAAATCAAGTTCAGTGCACATCAGGCTGCAAAGTTCAAAGGTGATATATTAAACATGAAACAAGAAAACAACAAAGTTAAT
GAAGAACTTGAAGCAGGTTTAGATCATGCCATTGCCCTAAAACATGAAATAGAGCAAACTCTAGAAAGACTAGAAAGAAAG
TTTGGTCTTTCTGCCAATCAAAATCAGCCACGTAAGAGATGGTCATCAAGGCGGTCACTAAGCTTGAGGTCATTACTTTTTG
GTGGTACCTCGGCCGCGACCACGCTA

> control 121

GAAACAAGTGAAGGATACGTTGGAAGAGGTTAAACAAGTTGAAGCCAAGGGACTCACAGATCTACTATCCGAGATCAAGC
CGATTTACAAACATCTTCACAAGATAAACACTAGACTTGCATCATGGTTGCAACAAAGCATATTGCTCAACAATGAACTCGAC
ACAATAAACTCGTATTTGTGCAACATCCAGGAAGAAATAACACAGGAAGAAATCAAGTTCAGTGCACATCAGGCTGCAAAG
TTCAAAGGTGATATATTAAACATGAAACAAGAAAACAACAAAGTTAATGAAGAACTTGAAGCAGGTTTAGATCATGCCATTG
CCCTAAAACATGAAATAGAGCAAACTCTAGAAAGACTAGAAAGAAAGTTTGGTCTTTCTGCCAATCAAAATCAGCCACGTAA
GAGATGGTCATCAAGGCGGTCACTAAGCTTGAGGTCATTACTTTTTGGTGGTACCTCGGCCGCGACCACGCTA

Control	**BAD62122**	**Integral membrane protein**

> control 128

TTCGAGCGGCCGCCCGGGCAGGTACTAGTGCAACTAAAATCAGGGCAACCAAGCTGTCCGCAATTATACATCTATAGCAGTG
CAGATAAAGTCATTCCTGCAGGATCTGTAGAGTCTTTCATAGAGGTGCAGCAGAGAATGGGACGTGTTGTCAGGTCATGCA
ACTTTAAATCAACACCTCATGTAGATCATTTTAGACACGAACCCGAGTTGTACCTCGGCCGCGACCACGCTA

> control 172

TTCGAGCGGCCGCCCGGGCAGGTACTAGTGCAACTAAAATCAGGGCAACCAAGCTGTCCGCAATTATACATCTATAGCAGTG
CAGATAAAGTCATTCCTGCAGGATCTGTAGAGTCTTTCATAGAGGTGCAGCAGAGAATGGGACGTGTTGTCAGGTCATGCA
ACTTTGAATCAACACCTCATGTAGATCATTTTAGACATGAACCCGAGTTGTACCTCGGCCGCGACCACGCTA

Control	**CAL35827**	**Alpha-2 tubulin**

> control 2

TAGCGTGGTCGCGGCCGAGGTACCAGTGCACAAACGCCCTTTTCGCAAACATCATATCGAATTTATGATCAATCCGACTGAA
AACCTCCGAAACTGCAGTGTTGTTGCTGATCATACATACTGCGCGTTTCACCTTCGCCAAATCTCCGCCAGGCACCACCGTAG
GAGCCTGGTAGTTGATGCCGCACTTGAATCCCGTAGGACACCAGTCGACGAATTGAACCGTCTTTTTTGTTTTGATTGATCC
AACGGCATTGTTAACGTCTTTTGGAGCAATGTCTCCACGGTACCTGCCCGGGCGGCCGCTCGAA

> control 34

TCGAGCGGCCGCCCGGGCAGGTACCGTGGAGACATTGCTCCAAAAGACGTTAACAATGCCGTTGGATCAATCAAAACAAA
AAAGACGGTTCAATTCGTCGACTGGTGTCCTACGGGATTCAAGTGCGGCATCAACTACCAGGCTCCTACGGTGGTGCCTGG
CGGAGATTTGGCGAAGGTGAAACGCGCAGTATGTATGATCAGCAACAACACTGCAGTTTCGGAGGTTTTCAGTCGGATTGA
TCATAAATTCGATATGATGTTTGCGAAAAGGGCGTTTGTGCACTGGTACCTCGGCCGCGACCACGCTAATCACCTAGTGCGG
CCGCCTGCAGGTCGACCATATGGGAGAG

> control 38

TAGCGTGGTCGCGGCCGAGGTACCGTGGAGACATTGCTCCAAAAGACGTTAACAATGCCGTTGGATCAATCAAAACAAAAA
AGACGGTTCAATTCGTCGATGGTGTCCTACGGGATTCAAGTGCGGCATCAACTACCAGGCTCCTACGGTGGTGCCTGGCGG
AGATTTGGCGAAGGTGAAACGCGCAGTATGTATGATCAGCAACAACACTGCAGTTTCGGAGGTTTTCAGTCGGATTGATCAT
AAATTCGATATGATGTTTGCGAAAAGGGCGTTTGTGCACTGGTACCTGCCCGGGCGGCCGCTCGA

> control 67

TAGCGTGGTCGCGGCCGAGGTACCAGTGCACAAACGCCCTTTTCACAAACATCATATCGAATTTATGATCAATCCGACTGAA
AACCTCCGAAACTGCAGTGTTGTTGCTGATCATACATACTGCGCGTTTCACCTTCGCCAAATCTCCGCCAGGCACCACCGTAG
GAGCCTGGTAGTTGATGCCGCACTTGAATCCCGTAGGACACCAGTCGACGAATTGAACCGTCTTTTTTGTTTTGATTGATCC
AACGGCATTGTTAACGTCTTTTGGAGCAATGTCTCCACGGTACCTGCCCGGGCGGCCGCTCGAA

> control 69

TCGAGCGGCCGCCCGGGCAGGTACCGTGGAGACATTGCTCCAAAAGACGTTAACAATGCCGTTGGATCAATCAAAACAAA
AAAGACGGTTCAATTCGTCGACTGGTGTCCTACGGGATTCAAGTGCGGCATCAACTACCAGGCTCCTACGGTGGTGCCTGG
CGGAGATTTGGCGAAGGTGAAACGCGCAGTATGTATGATCAGCAACAACACTGCAGTTTCGGAGGTTTTCAGTCGGATTGA
TCATAAATTCGATATGATGTTTGCGAAAAGGGCGTTTGTGCACTGGTACCTCGGCCGCGACCACGCTA

> control 72

TCGAGCGGCCGCCCGGGCAGGTACCGTGGAGACATTGCTCCAAAAGACGTTAACAATGCCGTTGGATCAATCAAAACAAA

AAAGACGGTTCAATTCGTCGACTGGTGTCCTACGGGATTCAAGTGCGGCATCAACTACCAGGCTCCTACGGTGGTGCCTGG
CGGAGATTTGGCGAAGGTGAAACGCGCAGTATGTATGATCAGCAACAACACTGCAGTTTCGGAGGTTTTCAGTCGGATTGA
TCATAAATTCGATATGATGTTTGCGAAAAGGGCGTTTGTGCACTGGTACCTCGGCCGCGACCACGCTA

> control 78
TAGCGTGGTCGCGGCCGAGGTACCAGTGCACAAACGCCCTTTTCGCAAACATCATATCGAATTTATGATCAATCCGACTGAA
AACCTCCGAAACTGCAGTGTTGTTGCTGATCATACATACTGCGCGTTTCACCTTCGCCAAATCTCCGCCAGGCACCACCGTAG
GAGCCTGGTAGTTGATGCCGCACTTGAATCCCGTAGGACACCAGTCGACGAATTGAACCGTCTTTTTTGTTTTGATTGATCC
AACGGCATTGTTAACGTCTTTTGGAGCAATGTCTCCACGGTACCTGCCCGGGCGGCCGCTCTGAA

> control 41
TAGCGTGGTCGCGGCCGAGGTACCGTGGAGACATTGCTCCAAAAGACGTTAACAATGCCGTTGGATCAATCAAAACAAAAA
AGACGGTTCAATTCGTCGACTGGTGTCCTACGGGATTCAAGTGCGGCATCAACTACCAGGCTCCTACGGTGGTGCCTGGCG
GAGATTTGGCGAAGGTGAAACGCGCAGTATGTATGATCAGCAACAACACTGCAGTTTCGGAGGTTTTCAGTCGGATTGATC
ATAAATTCGATATGATGTTTGCGAAAAGGGCGTTTGTGCACTGGTACCTCGGCCGCGACCACGCTA

> control 45
TTCGAGCGGCCGCCCGGGCAGGTACCGTGGAGACATTGCTCCAAAAGACGTTAACAATGCCGTTGGATCGATCAAAACGA
AAAAGACGGTTCAATTCGTCGACTGGTGTCCTACGGGATTCAAGTGCGGGATCAACTACCAGGCTCCTACGGTGGTGCCTG
GCGGAGATCTGGCGAAGGTGAAACGTGCAGTATGTATGATCAGCAACAACACTGCAGTTTCGGAGGTTTTTAGTCGGATTG
ATCATAAATTCGATATGATGTTCGCTAAACGGGCGTTTGTGCACTGGTATTTGGGTGAAGGGATGGAAGAAGGCGAGTTCTC
GGAGGCGCGAGAAGATCTTGCAGCCCTGGAGAAAGATTATGAAGAAGTTGTTCAAGACAGTCAGCAGGAGGAGTACCTC
GGCCGCGACCACGCTA

> control 59
TAGCGTGGTCGCGGCCGAGGTACCAGTGCACAAACGCCCTTTTCGCAAACATCATATCGAATTTATGATCAATCCGACTGAA
AACCTCCGAAACTGCAGTGTTGTTGCTGATCATACATACTGCGCGTTTCACCTTCGCCAAATCTCCGCCAGGCACCACCGTAG
GAGCCTGGTAGTTGATGCCGCACTTGAATCCCGTAGGACACCAGTCGACGAATTGAACCGTCTTTTTTGTTTTGATTGATCC
AACGGCATTGTTAACGTCTTTTGGAGCAATGTCTCCACGGTACCTGCCCGGGCGGCCGCTCGAA

> control 63
TAGCGTGGTCGCGGCCGAGGTACCAGTGCACAAACGCCCTTTTCGCAAACATCATATCGAATTTATGATCAATCCGACTGAA
AACCTCCGAAACTGCAGTGTTGTTGCTGATCATACATACTGCGCGTTTCACCTTCGCCAAATCTCCGCCAGGCACCACCGTAG
GAGCCTGGTAGTTGATGCCGCACTTGAATCCCGTAGGACACCAGTCGACGAATTGAACCGTCTTTTTTGTTTTGATTGATCC
AACGGCATTGTTAACGTCTTTTGGAGCAATGTCTCCACGGTACCTGCCCGGGCGGCCGCTCGAAATCACTAAGTGCGGCCG
CCTGCAGGTCGACCATATGGGAGAG

> control 75
TAGCGTGGTCGCGGCCGAGGTACCAGTGCACAAACGCCCTTTTCGCAAACATCATATCGAATTTATGATCAATCCGACTGAA
AACCTCCGAAACTGCAGTGTTGTTGCTGATCATACATACTGCGCGTTTCACCTTCGCCAAATCTCCGCCAGGCACCACCGTAG
GAGCCTGGTAGTTGATGCCGCACTTGAATCCCGTAGGACACCAGTCGACGAATTGAACCGTCTTTTTTGTTTTGATTGATCC
AACGGCATTGTTAACGTCTTTTGGAGCAATGTCTCCACGGTACCTGCCCGGGCGGCCGCTCA
> control 84
TAGCGTGGTCGCGGCCGAGGCACCAGTGCACAAACGCCCTTTTCGCAAACATCATATCGAATTTATGATCAATCCGACTGAA

AACCTCCGAAACTGCAGTGTTGTTGCTGATCATACATACTGCGCGTTTCACCTTCGCCAAATCTCCGCCAGGCACCACCGTAG
GAGCCTGGTAGTTGATGCCGCACTTGAATCCCGTAGGACACCAGTCGACGAATTGAACCGTCTTTTTTGTTTTGATTGATCC
AACGGCATTGTTAACGTCTTTTGGAGCAATGTCTCCACGGTACCTGGCC

> control 101
TAGCGTGGTCGCGGCCGAGGTACCGTGGAGACATTGCTCCAAAAGACGTTAACAATGCCGTTGGATCAATCAAAACAAAAA
AGACGGTTCAATTCGTCGACTGGTGTCCTACGGGATTCAAGTGCGGCATCAACTACCAGGCTCCTACGGTGGTGCCTGGCG
GAGATTTGGCGAAGGTGAAACGCGCAGTATGTATGATCAGCAACAACACTGCAGTTTCGGAGGTTTTCAGTCGGATTGATC
ATAAATTCGATATGATGTTTGCGAAAAGGGCGTTTGTGCACTGGTACCTGCCCGGGCGGCCGCTCA

> control 112
TCGAGCGGCCGCCCGGGCAGGTACCAGTGCACAAACGCCCTTTTCGCAAACATCATATCGAATTTATGATCAATCCGACTGA
AAACCTCCGAAACTGCAGTGTTGTTGCTGATCATACATACTGCGCGTTTCACCTTCGCCAAATCTCCGCCAGGCACCACCGTA
GGAGCCTGGTAGTTGATGCCGCACTTGAATCCCGTAGGACACCAGTCGACGAATTGAACCGTCTTTTTTGTTTTGATTGATC
CAACGGCATTGTTAACGTCTTTTGGAGCAATGTCTCCACGGTACCTCGGCCGCGACCACGCTA

> control 119
TTGAGCGGCCGCCCGGGCAGGTACCGTGGAGACATTGCTCCAAAAGACGTTAACAATGCCGTTGGATCAATCAAAACAAA
AAAGACGGTTCAATTCGTCGACTGGTGTCCTACGGGATTCAAGTGCGGCATCAACTACCAGGCTCCTACGGTGGTGCCTGG
CGGAGATTTGGCGAAGGTGAAACGCGCAGTATGTATGATCAGCAACAACACTGCAGTTTCGGAGGTTTTCAGTCGGATTGA
TCATAAATTCGATATGATGTTTGCGAAAAGGGCGTTTGTGCACTGGTACCTCGGCCGCGACCACGCTA

> control 120
TCGAGCGGCCGCCCGGGCAGGTACCGTGGAGACATTGCTCCAAAAGACGTTAACAATGCCGTTGGATCAATCAAAACAAA
AAAGACGGTTCAATTCGTCGACTGGTGTCCTACGGGATTCAAGTGCGGCATCAACTACCAGGCTCCTACGGTGATGCCTGG
CGGAGATTTGGCGAAGGTGAAACGCGCAGTATGTATGATCAGCAACAACACTGCAGTTTCGGAGGTTTTCAGTCGGATTGA
TCATAAATTCGATATGATGTTTGCGAAAAGGGCGTTTGTGCACTGGTACCTCGGCCGCGACCACGCTA

> control 150
TCGAGCGGCCGCCCGGGCAGGTACCGTGGAGACATTGCTCCAAAAGACGTTAACAATGCCGTTGGATCAATCAAAACAAA
AAAGACGGTTCAATTCGTCGACTGGTGTCCTACGGGATTCAAGTGCGGCATCAACTACCAGGCTCCTACGGTGGTGCCTGG
CGGAGATTTGGCGAAGGTGAAACGCGCAGTATGTATGATCAGCAACAACACTGCAGTTTCGGAGGTTTTCAGTCGGATTGA
TCATAAATTCGATATGATGTTTGCGAAAAGGGCGTTTGTGCACTGGTACCTCGGCCGCGACCACGCTA

> control 152
TCGAGCGGCCGCCCGGGCAGGTACCGTGGAGACATTGCTCCAAAAGACGTTAACAATGCCGTTGGATCAATCAAAACAAA
AAAGACGGTTCAATTCGTCGACTGGTGTCCTACGGGATTCAAGTGCGGCATCAACTACCAGGCTCCTACGGTGGTGCCTGG
CGGAGATTTGGCGAAGGTGAAACGCGCAGTATGTATGATCAGCAACAACACTGCAGTTTCGGAGGTTTTCAGTCGGATTGA
TCATAAATTCGATATGATGTTTGCGAAAAGGGCGTTTGTGCACTGGTACCTCGGCCGCGACCACGCTA

> control 162
CGAGCGGCCGCCCGGGCAGGTACCGTGGAGACATTGCTCCAAAAGACGTTAACAATGCCGTTGGATCGATCAAAACGAAA

AAGACGGTTCAATTCGTCGACTGGTGTCCTACGGGATTCAAGTGCGGGATCAACTACCAGGCTCCTACGGTGGTGCCTGGC GGAGATCTGGCGAAGGTGAAACGTGCAGTATGTATGATCAGCAACAACACTGCAGTTTCGGAGGTTTTTAGTCGGATTGAT CATAAATTCGATATGATGTTCGCTAAACGGGCGTTTGTGCACTGGTATTTGGGTGAAGGGATGGAAGAAGGCGAGTTCTCG GAGGCGCGAGAAGATCTTGCAGCCCTGGAGAAAGATTATGAAGAAGTTGTTCAAGACAGTCAGCAGGAGGAGTACCTCG GCCGCGACCACGCTA > control 179 TAGCGTGGTCGCGGCCGAGGTACCAGTGCACAAACGCCCTTTTCGCAAACATCATATCGAATTTATGATCAATCCGACTGAA AACCTCCGAAACTGCAGTGTTGTTGCTGATCATACATACTGCGCGTTTCACCTTCGCCAAATCTCCGCCAGGCACCACCGTAG GAGCCTGGTAGTTGATGCCGCACTTGAATCCCGTAGGACACCAGTCGACGAATTGAACCGTCTTTTTTGTTTTGATTGATCC AACGGCATTGTTAACGTCTTTTGGAGCAATGTCTCCACGGTACCTGCCCGGGCGGCCGCTA > control 188 TCAAGTGCGGCATCAACTACCAGGCTCCTACGGTGGTGCCTGGCGGAGATTTGGCGAAGGTGAAACGCGCAGTATGTATGA TCAGCAACAACACTGCAGTTTCGGAGGTTTTCAGTCGGATTGATCATAAATTCGATATGATGTTTGCGAAAAGGGCGTTTGT GCACTGGTACCTCGGCCGCGACCACGCTA > control 189 TAGCGTGGTCGCGGCCGAGGCACCAGTGCACAAACGCCCTTTTCGCAAACATCATATCGAATTTATGATCAATCCGCCTGAA AACCTCCGCAACTGCAGTGTCGTTGCTGATCATACATACTGCGCGTTTCACCTTCGCCAAATCTCCCCCAGGCACCCCCGTAG GAGCCTGGTAGTTGATGCCGCACTTGAATCCCGTAGGACACCAGTCGACGAACTGAACCGTCTTTTTTGTTTTGATTGATCC AACGGCATTGTTAACGTCTTTTGGAGCAATGTCTCCACGGTCCCTGCCCGGGCGCCCGCTCA		
Control	**CAQ58078**	**Lipoxygenase**
> control 16 TTGGGAGCTCTCCCATATGGTCGACCTGCAGGCGGCCG > control 20 ACTAGTGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACG		
Control	**ABB47557**	**Retrotransposon protein**
> control 26 CGGGGGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCAAAGTCAA AACGACATAAACCATTTCTATTAGTATTAGTTATTAATGTTGTATCACTTGAGAAATCAAGTTTAGTAGAAACGACAACTCAAT GCCACAACCACTTTCATCCTTTACTTCACCAACACCCTTTCTCATATTCATTTCACCTGCCCGGGCGGCCGCTCGAA		
Control	**CCA62925**	**Carbon monoxide dehydrogenase**
> control 155 CTGCCCGGGCGGCCGCTAATCACTAGTGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAAACG		
Control	**ADC80732**	**Calmodulin 24-like protein**

> control 39 TCGGTAGGAAAGGCACCACCTTTCATCATCTGCCTAAACTCTTTATAATCGACCATCCCATCTCCATCCGCGTCTACTTTCTTCA CCATGATCCGGCAATCCTCAACGGTTCGGCCTTGTCCCAAGCCAAGAGAAGTCAAGACACCTGCCCGGGCGGCCGCTCGA A > control 136 GGTGGAAAGGCACCACCTTTCATCATCTGCCTAAACTCTTTATAATCGACCATCCCATCTCCATCCGCGTCTACTTTCTTCACCA TGATCCGGCAATCCTCAACGGTTCGGCCTTGTCCCAAGCCAAGAGAAGTCAAGACACCTGCCCGGGCGGCCGCTCGAA		
NO2	**ABV55999**	**Alpha-tubulin 7**
> treatment 30 AAAAAAGACGGTTCAATTCTTCTACTGGTGTCCTACGGTATTCAAGTGCGGCATCAACTACCAGGCTCCTACGGTGGTGCC		
NO2	**AAK94059**	**Pi-tubulin**
> treatment 6 TTCGAGCGGCCGCCCGGGCAGGTACTTGGGTGAAGGGATGGAAGAAGGCGAGTTCTCGGAGGCGCGAGAAGATCTTGC AGCCCTGGAGAAAGATTATGAAGAAGTTGTTCAAGACAGTCAGCAGGAGGAGTACCTCGGCCGCGACCACGCTA > treatment 77 TAGCGGCCGCCCGGGCAGGTACTTGGGTGAAGGGATGGAAGAAGGCGAGTTCTCGGAGGCGCGAGAAGATCTTGCAGC CCTGGAGAAAGATTATGAAGAAGTTGTTCAAGACAGTCAGCAGGAGGAGTACCTCGGCCGCGACCACGCTA > treatment 86 TTCGAGCGGCCGCCCGGGCAGGTACTTGGGTGAAGGGATGGAAGAAGGCGAGTTCTCGGAGGCGCGAGAAGATCTTGC AGCCCTGGAGAAAGATTATGAAGAAGTTGTTCAAGACAGTCAGCAGGAGGAGTACCTCGGCCGCGACCACGCTA		
NO2	**XP002532142**	**Transcription elongation factor s-II**
> treatment 31 CGGCCGCGACCACGCTAATACAATAGTGCGGCCGCCTGCAGGTCGACCATATGGAGAGCTCCCAACGCG		
NO2	**AAZ32862**	**Splicing factor Prp8**
> treatment 37 CCCGGGCGGCCGCTCGAAATCACTAGTGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAAAC		
NO2	**ACG31189**	**Protein transport protein Sec61 beta**
> treatment 4 CCAAGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACCTCGGCCGCGACCACGCT A > treatment 57 TTCGAGCGGCCCCCGGGCAGGTACAAGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT TTTTTTTTTTTTTTTTTACCTCGGCCGCGACCACGCTA		

> treatment 58
TTGAGCGGCCGCCCGGGCAGGTCAAGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACCTCGGCCG
CGACCACGCTA

> treatment 61
ACAAGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACCTG
CCCGGGCGGCCGCTCGA

> treatment 89
TAGCGGCCGCCCGGGCAGGTCCGGGCAGGGGTACAAGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
TTTTTTTTTTTTTTTTTTTTTTTTTTACCTCGGCCGCGACCACGCTA

> treatment 95
TTCGAGCGGCCGCCCGGGCAGGTACAAGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
TTTTTTTTACCTCGGCCGCGACCACGCTA

> treatment 2
TCGAGCGGCCGCCCGGGCAGGTCCGGGCAGGGGTACAAGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
TTTTTTTTTTTTTTTTTTTTTTTTTTACCTCGGCCGCGACCACGCTA

> treatment 37
TTCGAGCGGCCGCCCGGGCAGGGGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
TACCTCGGCCGCGACCACGCTA

> treatment 65
TTCGAGCGGCCGCCCGGGCAGGTCCGGGCAGGGGTCCAAGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACCTCGGCCGCGACCACGCTA

> treatment 66
GACAAGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACCTGCCCGGGC
GGCCGCTCGA

> treatment 69
TCGAGCGGCCGCCCGGGCAGGTCAAGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
TTTTACCTCGGCCGCGACCACGCTA

> treatment 75
ACCAACCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACCTCGGCCGCGACCACGCTA

> treatment 82
TACAAGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACC
TGCCCGGGCGGCCGCTCGA

> treatment 102
TAGGGTGGTCGCGGCCGAGGTACAAGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTAC
CTGCCCGGGCGGCCGCTCGA

> treatment 103
TTGAGCGGCCGCCCGGGCAGGTACAAAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
TTTTACCTCGGCCGCGACCACGCTA

> treatment 106
CAAGGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACCTCGGCCG
CGACCACGCTA

> treatment 110
CAAGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACCTCGGCCGCGACCACGCTA

> treatment 112
CCCCCCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACCT
CGGCCGCGACCACGCTA

> treatment 129
TACAAGCATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACCTCGGCCGCGAC
CACGCTA

> treatment 150
CAGGGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACCTCGGCCGCGAC
CACGCTA

> treatment 154
ACAACTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACCTCGGCCGCGACCA
CGCTA

> treatment 155
CGGCCTTCCGGGCAGGTACAAGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACCTCGG
CCGCGACCACGCTA

> treatment 152
CCCGAAGTCGCATGTTCCCGGCCGCCATGGCCCCGGGATTAGCGGCCGCCCGGGCAGGGACAAGCTTTTTTTTTTTTTTTTTTTT
TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACCTCGGCCGCGACCACGCTA

> treatment 174
AACACCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTGTAATACGACTCACTATAGGGCGAATTGGG
CCCGACGTCGCATGCTCCCGGCCGCCATGGCCGCGGGATTTCGAGCGGCCGCCCGGGCAGGTACAAGCTTTTTTTTTTTTTTTTT

TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACCTCGGCCGCGACCACGCTA

> treatment 17

TAGCGGCCGCCCGGGCAGGTACAAGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
ACCTCGGCCGCGACCACGCTA

> treatment 20

TAGCGTGGTCGCGGCCGAGGTACAAGCCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
TTTTTACCTGCCCGGGCGGCCGCTCGA

> treatment 22

GGGGATTTGCTGCAAGGCGATTAAGTTGGGTAACCCCAGGGTTTTCCCAGTCCCGACGTTGTAAAACGGCGGCCAGTGAA
TTGTAATTCGACTCCCTATAGGGGGAATTGGGCCCGACGTTGCATGTTCCCGGCCCCCATGGCCGCGGGATTTCGAGCGGC
CGCCCGGGCAGGTACAAGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTA
CCTCGGCCGCGACCACGCTA

> treatment 28

ATACAGGGCGCGTCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTTTTCGCTATTACGCC
AGCTGGGGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACCCCAGGGTTTTCCCAGTCACGACGTTGTAAAACG
ACGGCCAGGGAATTGTAATTCGACTCCCTATAGGGCGAATTGGGCCCGACGTCGCATGCTCCCGGCCCCCATGGCCGCGGG
ATTAGCGTGGTCGCGGCCGAGGTACAAACTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACCTGC
CCGGGCGGCCGCTCGA

> treatment 29

TCGAGCGGCCGCCCGGGCAGGTACAAGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
ACCTCGGCCGCGACCACGCTA

> treatment 39

CGAGCGGCCGCCCGGGCAGGTACAAACTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
TTTTTTTTTTACCTCGGCCGCGACCACGCTA

> treatment 40

TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTATTATTATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACGACCTCGGCCGCGACCACGC
TA

> treatment 66

TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACCTGCCCGGGCGGCCGCTCGA

> treatment 74

TTCGAGCGGCCCCCCGGGCAGGTACAAGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACCTCGGCCGCGACCACGCTA

> treatment 87

TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACCTCGGCCGCGACCACGCTA

> treatment 89
TTCGAGCGGCCGCCCGGGCAGGTACAAGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
TTTTTTTTTTTTTTTACCTCGGCCGCGACCACGCTA

> treatment 7
CCAAGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCCAAGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
ACCTCGGCCGCGACCACGCTA

> treatment 36
TAGGGTGGTCGCGGCCGAGGTACAAGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
TTTTTTTACCTGCCCGGGCGGCCGCTCGAA

> treatment 55
TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACCTGCCCGGGCGGCCG
CTCGA

NO2	XP002518508	Serine/threonine protein kinase

> treatment 96
GAGCGGCCGCCCGGGCAGGTACTTTTTTACTTGCTACACAAAACAAATGTTAGCGTGTGGATTTTTTCACTCACTTCGCCTT
TTACTGTGTGTTTGTGTAAGAACATCATACGACCAATTTGTCAACTCCCCATTTTCGACAAGTTGGTTGTGATGACCATGTAAC
CCCTTCTGTTACTTAAATGTCGACAAAGGCAGTGTTACTTTCAAGGGCAGAACAGTCATTTTGATGGTATTGGATCATGCGCA
AGGGACTCGAGGGCTGCAACCACATCACTGATTGATGGGCGGATCGATGGATCCGTATTGAGACACATGGCTGCTATAGCC
AACACTTGGCTCAAACTAGCCTGTGAATAATTTCCATCAAGAAGTGGGTCAACCAGTTCGGAGTATTTCTTTGGGTCCTTGAT
CCTTGTCTCAGCCCAACGAACGAGATGTAGCTCTTCGCTTTCCCTTGTCAAATCAATGGCCCTCCTTCCTGTAATGAGTTCCA
GCAACACAACTCCGTAGCTGTAAATATCTGATTTTACGACCTCGGCCGCGACCACGCTA

> treatment 101
TAGCGTGGTCGCGGCCGAGGTCGTAAAATCAGATATTTACAGCTACGGAGTTGTGTTGCTGGAACTCATTACAGGAAGGAG
GGCCATTGATTTGACAAGGGAAAGCGAAGAGCTACATCTCGTTCGTTGGGCTGAGACAAGGATCAAGGACCCAAAGAAAT
ACTCCGAACTGGTTGACCCACTTCTTGATGGAAATTATCCACAGGCTAGTTTGAGCCAAGTGTTGGCTATAGCAGCCATGTG
TCTCAATACGGATCCATCGATCCGCCCATCAATCAGTGATGTGGTTGCAGCCCTCGAGTCCCTTGCGCATGATCCAATACCATC
AAAATGACTGTTCTGCCCTTGAAAGTAACACTGCCTTTGTCGACATTTAAGTAACAGAAGGGGTTACATGGTCATCACAACC
AACTTGTCGAAAATGGGGAGTTGACAAATTGGTCGTATGATGTTCTTACACAAACACACAGTAAAAGGCGAAGTGAGTGAA
AAAATCCACACGCTAACATTTGTTTTGTGTAGCAAGTAAAAAAGTACCTGCCCGGGCGGCCGCTCGAA

> treatment 105
TAGCGTGGTCGCGGCCGAGGTCGTAAAATCAGATATTTACAGCTACGGAGTTGTGTTGCTGGAACTCATTACAGGAAGGAG
GGCCATTGATTTGACAAGGGAAAGCGAAGAGCTACATCTCGTTCGTTGGGCTGAGACAAGGATCAAGGACCCAAAGAAAT
ACTCCGAACTGGTTGACCCACTTCTTGATGGAAATTATCCACAGGCTAGTTTGAGCCAAGTGTTGGCTATAGCAGCCATGTG
TCTCAATACGGATCCATCGATCCGCCCATCAATCAGTGATGTGGTTGCAGCCCTCGAGTCCCTTGCGCATGATCCAATACCATC
AAAATGACTGTTCTGCCCTTGAAAGTAACACTGCCTTTGTCGACATTTAAGTAACAGAAGGGGTTACATGGTCATCACAACC
AACTTGTCGAAAATGGGGAGTTGACAAATTGGTCGTATGATGTTCTTACACAAACACACAGTAAAAGGCGAAGTGAGTGAA
AAAATCCACACGCTAACATTTGTTTTGTGTAGCAAGTAAAAAAGTACCTGCCCGGGCGGCCGCTCGAA

> treatment 169

TCGAGCGGCCGCCCGGGCAGGTACTTTTTTACTTGCTACACAAAACAAATGTTAGCGTGTGGATTTTTTCACTCACTTCGCC
TTTTACTGTGTGTTTGTGTAAGAACATCATACGACCAATTTGTCAACTCCCCATTTTCGACAAGTTGGTTGTGATGACCATGTA
ACCCCTTCTGTTACTTAAATGTCGACAAAGGCAGTGTTACTTTCAAGGGCAGAACAGTCATTTTGATGGTATTGGATCATGCG
CAAGGGACTCGAGGGCTGCAACCACATCACTGATTGATGGGCGGATCGATGGATCCGTATTGAGACACATGGCTGCTATAG
CCAACACTTGGCTCAAACTAGCCTGTGGATAATTTCCATCAAGAAGTGGGTCAACCAGTTCGGAGTATTTCTTTGGGTCCTT
GATCCTTGTCTCAGCCCAACGAACGAGATGTAGCTCTTCGCTTTCCCTTGTCAAATCAATGGCCCTCCTTCCTGTAATGAGTT
CCAGCAACACAACTCCGTAGCTGTAAATATCTGATTTTACGACCTCGGCCGCGACCACGCTA

> treatment 70

TTCGAGCGGCCGCCCGGGCAGGTACTTTTTTACTTGCTACACAAAACAAATGTTAGCGTGTGGATTTTTTCACTCACTTCGC
CTTTTACTGTGTGTTTGTGTAAGAACATCATACGACCAATTTGTCAACTCCCCATTTTCGACAAGTTGGTTGTGATGACCATGT
AACCCCTTCTGTTACTTAAATGTCGACAAAGGCAGTGTTACTTTCAAGGGCAGAACAGTCATTTTGATGGTATTGGATCATGC
GCAAGGGACTCGAGGGCTGCAACCACATCACTGATTGATGGGCGGATCGATGGATCCGTATTGAGACACATGGCTGCTATA
GCCAACACTTGGCTCAAACTAGCCTGTGGATAATTTCCATCAAGAAGTGGGTCAACCAGTTCGGAGTATTTCTTTGGGTCCT
TGATCCTTGTCTCAGCCCAACGAACGAGATGTAGCTCTTCGCTTTCCCTTGTCAAATCAATGGCCCTCCTTCCTGTAATGA
GTTCCAGCAACACAACTCCGTAGCTGTAAATATCTGATTTTACGACCTCGGCCGCGACCACGCTA

> treatment 134

TAGCGTGGTCGCGGCCGAGGTCGTAAAATCAGATATTTACAGCTACGGAGTTGTGTTGCTGGAACTCATTACAGGAAGGAG
GGCCATTGATTTGACAAGGGAAAGCGAAGAGCTACATATCGTTCGTTGGGCTGAGACAAGGATCAAGGACCCAAAGAAAT
ACTCCGAACTGGTTGACCCACTTCTTGATGGAAATTATCCACAGGCTAGTTTGAGCCAAGTGTTGGCTATAGCAGCCATGTG
TCTCAATACGGATCCATCGATCCGCCCATCAATCAGTGATGTGGTTGCAGCCCTCGAGTCCCTTGCGCATGATCCAATACCATC
AAAATGACTGTTCTGCCCTTGAAAGTAACACTGCCTTTGTCGACATTTAAGTAACAGAAGGGGTTACATGGTCATCACAACC
AACTTGTCGAAAATGGGGAGTTGACAAATTGGTCGTATGATGTTCTTACACAAACACACAGTAAAAGGCGAAGTGAGTGAA
AAAATCCACACGCTAACATTTGTTTTGTGTAGCAAGTAAAAAAGTACCTGCCCGGGCGGCCGCTCA

NO2	XP002520716	Late embryogenesis abundant protein

> treatment 38

TAGCGTGGTCGCGGCCGAGGTACCGTGGAGACATTGCTCCAAAAGACGTTAACAATGCCGTTGGATCAATCAAAACAAAAA
AGACGGTTCAATTCGTCGACTGGTGTCCTACGGGATTCAAGTGCGGCATCAACTACCAGGCTCCTACGGTGGTGCCTGGCG
GAGATTTGGCGAAGGTGAAACGCGCAGTATGTATGATCAGCAACAACACTGCAGTTTCGGAGGTTTTCAGTCGGATTGATC
ATAAATTCGATATGATGTTTGCGAAAAGGGCGTTTGTGCACTGGTACCTGCCCGGGCGGCCGCTCGA

NO2	CAB63264	Calcium-binding protein

> treatment 99

ATTCGAGCGGCCGCCCCGGCAGGTACCAGCCTTTTTTTTTTTTTTTTTTTTTTTTTTTTGGTTTTTTTTTTTTTTTTTTTTTTT
TTTTTTTTTTTTAGAGAACTTATGATTCTTTATTCAATTTGAATTCCCATAAAGATACATGGTGATGAACAAGATGGTATAGAAA
AAAAAAAGTTCCAATAGATGGAAAAATCAAGTGGGATCTTTGGTTGTAAAGGCACCACCTTTCATCATCTGCCTAAACTCTTT
ATAATCGACCATCCCATCTCCATCCGCGTCTACTTTCTTCACCATGATCCGGCAATCCTCAACGGTTCGGCCTTGTCCCAAGCC

AAGAGAAGTCAAGACTGCCCGAAGCTCCTCAACCGTGATGAACCCATCTCCATTTTTGTCGAACACATTAAACGCATCTTTC
ATATCCTTTTCTTCGTCCCTCTCTTCCATTATTATTGCTTCATACAACCTCTCAAATTCCTCCATGTTCACTGACCCATCACCATCC
GTATCAATATGATCAATCATGTGTTCAAGATCCTTCTCGGGTATAACAAACCCAAGACGTTCAAGAGAGTTGGATAGCTCACC
TCGGCCGCGACCACGCT

> treatment 121

ATCGAGCGGCCGCCCCGGCAGGTACAAACCTTTTTTTTTTTTTTTTTTTTTTTTTTTTGGTTTTTTTTTTTTTTTTTTTTTTT
TTTTTTTTTTTTTAGAGAACTTATGATTCTTTATTCAATTTGAATTCCCATAAAGATACATGGTGATGAACAAGATGGTATAGAA
AAAAAAAAGTTCCAATAGATGGAAAAATCAAGTGGGATCTTTGGTTGTAAAGGCACCACCTTTCATCATCTGCCTAAACTCT
TTATAATCGACCATCCCATCTCCATCCGCGTCTACTTTCTTCACCATGATCCGGCAATCCTCAACGGTTCGGCCTTGTCCCAAG
CCAAGAGAAGTCAAGACTGCCCGAAGCTCCTCAACCGTGATGAACCCATCTCCATTTTTGTCGAACACATTAAACGCATCTT
TCATATCCTTTTCTTCGTCCCTCTCTTCCATTATTATTGCTTCATACAACCTCTCAAATTCCTCCATGTTCACTGACCCATCACCAT
CCGTATCAATATGATCAATCATGTGTTCAAGATCCTTCTCGGGTATAACAAACCCAAGACGTTCAAGAGAGTTGGATAGCTCA
CCTCGGCCGCGACCACGCTA

> treatment 123

TTCGAGCGGCCGCCCCGGCAGGTACAAGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTGTTTTTTTTTTTTTTTTTTTTTTTTTTT
TTTTAGAGAACTTATGATTCTTTATTCAATTTGAATTCCCATAAAGATACATGGTGATGAACAAGATGGTATAGAAAAAAAAGT
TCCAATAGATGGAAAAATCAAGTGGGATCTTTGGTTGTAAAGGCACCACCTTTCATCATCTGCCTAAACTCTTTATAATCGACC
ATCCCATCTCCATCCGCGTCTACTTTCTTCACCATGATCCGGCAATCCTCAACGGTTCGGCCTTGTCCCAAGCCAAGAGAAGT
CAAGACTGCCCGAAGCTCCTCAACCGTGATGAACCCATCTCCATTTTTGTCGAACACATTAAACGCATCTTTCATATCCTTTTC
TTCGTCCCTCTCTTCCATTATTATTGCTTCATACAACCTCTCAAATTCCTCCATGTTCACTGACCCATCACCATCCGTATCAATATG
ATCAATCATGTGTTCAAGATCCTTCTCGGGTATAACAAACCCAAGACGTTCAAGAGAGTTGGATAGCTCACCTCGGCCGCGA
CCACGCTA

NO2	**CAQ43070**	**Puroindoline b protein**

> treatment 62

TGCTGCAAGGCGATTAAGATGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTGTAATA
CGACTCCCTATAGGGGGAATTGGGCCCGACGTCGCATGCTCCCGGCCCCCATGGCCCCGGGATTTCGAGCGGCCGCCCGG
GCAGG

NO2	**NP195678**	**SAP-domain protein**

> treatment 83

CGACCGAAGCTCCTCAACCGTGATGAACCCATCTCCACTTTCGTCGAGCACAATAAACGCATCTTTCATATCCTTTTCTTCGTC
CCTCTCTTCCATTATTATTGCTTCATACAACCTCTCAAATTCCTCCATGTTCACTGACCCATCACCATCCGTATCAATATGATCAAT
CATGTGTTCAAGATCCTTCTCGGGTATAACAAACCCAAGACGTTCAAGAGAGTTGGATAGCTCACCTCGGCCGCGACCACG
CTA

NO2	**CBJ27423**	**Proteasome subunit alpha**

> treatment 149

ATCCCGCGGCCATGGCGGCCGGGAGCATGCGACGTCGGGCCCAATTCGCCCTATAGTGAGTCGAATTACAATTCACTGGCC
GTCGTTTTACAACGTCGTGACTGGAAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCT
GGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGACGCGCCCTGTAT
CGGCGC

NO2	**FR692049**	**LTR retrotransposon Tmc1**

> treatment 108

CGGGGGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCAAAGTCAA
AACGACATAAACCATTTCTATTAGTATTAGTTATTAATGTTGTATCACTTGAGAAATCAAGTTTAGTAGAAACGACAACTCAAT
GCCACAACCACTTTCATCCTTTACTTCACCAACACCCTTTCTCATATTCATTTCACCTGCCCGGGCGGCCGCTCGAA

Appendix 2. List of proteins 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. Only proteins with a protein score difference of less than 100 are shown. Best match per spot is indicated in bold. * MW was calculated by ExPaSy; + = only detected in treated pollen; - = only detected in control pollen.

Spot #	Best protein match	Accession	MW	Mascot			Spot ratio	p-value
				# unique peptides	# unique spectra	Protein		
	I: Allergen							
1	**Pollen allergen Amb a 1.1**	P27759	43	13	21	1708	1.89	1.23E-0
2	**Pollen allergen Amb a 1.2**	P27760	44	11	18	1292.5	1.71	5.88E-1
	Pollen allergen Amb a 1.4	P28744	43	5	8	1239.5	1.71	5.88E-1
3	**Pollen allergen Amb a 1.3**	P27761	43	10	11	712.1	1.58	2.05E-0
	Pollen allergen Amb a 1.4	P28744	43	8	9	623.2	1.58	2.05E-0
4	**Pollen allergen Amb a 1.4**	P28744	43	6	8	823.2	1.56	9.79E-0
5	**Pollen allergen Amb a 1.5**	P27762	44	15	19	1162	1.56	2.23E-0
6	**Enolase 1 (Hev b 9)**	Q9LEJ0	48	3	3	203.7	+	5.96E-0
	Glutamine synthetase cytosolic isozyme 1-3	Q9LVI8	39	3	3	159.4	+	5.96E-03
	II: Cytoskeleton Proteins							
7	**Actin-97**	P30171	42	8	8	516.4	-2.09	1.13E-0
	Actin-related protein 3	P61158	47	3	3	428.6	-2.09	1.13E-0
8	**Tubulin-alpha-1 chain**	P46259	50	18	26	2220.4	1.54	3.00E-0
	Tubulin beta-2	P61857	50	16	22	2150.7	1.54	3.00E-0
	Tubulin beta-9	P29517	50	15	17	2134.2	1.54	3.00E-0
	III: Metabolism							
9	**Glucose and ribitol dehydrogenase**	Q75KH3	32	3	3	165.3	-1.89	5.02E-1
10	**Triosephosphate isomerase**	P48493	21	5	7	324.5	2.05	4.54E-06
	Probable lactoylglutathione lyase	Q8W593	39	2	3	258.8	2.05	4.54E-06
11	**Glyceraldehyde-3-phosphate dehydrogenase**	P26519	36	3	3	150.2	-1.87	2.59E-04
	V-type proton ATPase catalytic	Q40002	64	3	3	97.6	-1.87	2.59E-04
12	**Fructose-bisphosphate aldolase**	P46257	38	2	2	140	1.56	8.46E-04
13	**2,3-bisphosphoglycerate**	Q9M9K1	61	5	5	287.3	-3.08	6.93E-0
	Phosphoglycerate kinase	Q42961	50	3	4	205.6	-3.08	6.93E-0
14	**Glucose-6-phosphate isomerase**	P34795	62	7	9	454	-2.58	2.32E-03
15	**Dihydrolipoyllysine-residue succinyltransferase**	Q8H107	50	3	4	305	-2.69	2.22E-03
	Tubulin beta-5	P46265	50	5	5	280	-2.69	2.22E-0
	NADP-dependent malic enzyme	P37223	64	3	4	233	-2.69	2.22E-03

Spot #	Best protein match	Accession	MW	Mascot			Spot ratio	p-value
				# unique peptides	# unique spectra	Protein score		
16	**Isocitrate dehydrogenase**	Q06197	46	2	3	201	3.02	5.18E-03
	Phosphoglycerate kinase	Q42961	50	2	2	117	3.02	5.18E-03
	Heat shock 70 kDa protein 10	Q9LDZ0	73	2	2	98.7	3.02	5.18E-03
17	**Succinate dehydrogenase**	O82663	70	5	5	299.3	1.96	1.89E-06
18	**Ribulose bisphosphate carboxylase**	Q14FE9	53	2	2	82.6	-2.36	6.30E-03
	GDP-mannose 3,5-epimerase	Q93VR3	43	2	2	78.6	-2.36	6.30E-03
19	**Transketolase**	Q43848	80	3	4	205.9	-2.55	1.48E-02
20	**Phosphoglycerate kinase**	Q42961	50	3	3	251.3	1.88	1.39E-03
	50S ribosomal protein L9	P0A7R1	16	2	3	207	1.88	1.39E-03
21	**Glutamate decarboxylase**	Q07346	57	18	26	2817.7	2.03	2.37E-04
22	**GDP-mannose 3,5-epimerase**	Q93VR3	43	2	2	109.5	-2.33	4.96E-04
23	**Cytochrome c1-1**	P25076	35	4	7	373.9	-1.63	1.04E-06
24	**Protein disulfide isomerase**	Q67UF5	47	4	4	165.7	2.36	2.77E-04
	Pollen allergen Amb a 1.1	P27759	43	3	3	112.8	2.36	2.77E-04
	Pollen allergen Amb a 1.2	P27760	44	2	3	98.8	2.36	2.77E-04
25	**Beta-fructofuranosidase**	Q8W4S6	62	6	8	435.1	1.99	1.88E-06
	Soluble inorganic pyrophosphatase	Q43187	24	5	5	373.6	1.99	1.88E-06
	Glutamine synthetase cytosolic isozyme 1-2	Q8LCE1	39	4	5	353.5	1.99	1.88E-06
	Glutamine synthetase cytosolic isozyme 1-3	Q9LVI8	39	4	4	340.4	1.99	1.88E-06
26	**Alpha-1,4-glucan-protein synthase**	P80607	41	2	2	85.2	2.21	1.63E-03
27	**NADH dehydrogenase**	P80269	26	4	5	376.7	-1.79	1.79E-03
	IV: Signalling proteins							
28	**14-3-3-like protein A**	P93214	29	9	11	1320	-1.63	2.05E-03
	V: Reproductive cycle							
29	**Phosphoglucomutase**	P93805	63	7	8	661.5	-	1.48E-05

Spot #	Best protein match	Accession	MW	Mascot # unique peptides	Mascot # unique spectra	Mascot Protein score	Spot ratio	p-value
	VI: Photosynthesis							
30	**Photosystem I P700 chlorophyll a apoprotein**	Q6EW49	82	2	3	123	-2.63	2.33E-03
	Tubulin beta-9	P29517	50	2	2	110.5	-2.63	2.33E-03
31	**Photosystem II CP47 chlorophyll apoprotein**	Q7FNS4	56	2	2	87.7	-1.95	4.32E-06
	Glucose and ribitol dehydrogenase	Q75KH3	32	2	2	78.5	-1.95	4.32E-06
32	**Photosystem II D2 protein**	Q4FFP4	40	2	2	111	-2.44	8.43E-04
	14-3-3-like protein A	P93214	29	2	2	109.9	-2.44	8.43E-04
	Phosphoglycerate kinase	Q42961	50	2	2	98.7	-2.44	8.43E-04
33	**Oxygen-evolving enhancer protein**	P84989	11	3	4	201.5	2.77	3.83E-06
	VII: Ammonia assimilation							
34	**Glutamine synthetase cytosolic isozyme 1-1**	Q56WN1	39	3	4	243.7	+	1.89E-06
	Glutamine synthetase cytosolic isozyme 1-2	Q8LCE1	39	3	3	236.8	+	1.89E-06
	Glutamine synthetase cytosolic isozyme 1-3	Q9LVI8	39	2	3	159.8	+	1.89E-06
	VIII: Protein biosynthesis, folding and degradation							
35	**Heat shock 70 kDa protein 9**	Q8GUM2	73	8	11	704.2	-2.36	9.78E-04
	Heat shock 70 kDa protein 10	Q9LDZ0	73	6	7	683	-2.36	9.78E-04
36	**26S protease regulatory subunit**	Q9SEI2	47	6	6	305.9	2.99	7.23E-06
37	**Chaperonin CPN60**	Q05046	61	10	11	626.1	1.89	1.07E-04
38	**Eukaryotic initiation factor**	P41382	47	4	4	306.6	-2.04	5.96E-03
	Monodehydroascorbate reductase	Q42711	47	3	3	258.6	-2.04	5.96E-03
	IX: Stress related							
39	**Elongation factor**	Q6YW46	47	5	5	287.8	2.66	8.88E-05
	Actin-97	P30171	42	3	4	225.3	2.66	8.88E-05

Spot #	Best protein match	Accession	MW	Mascot			Spot ratio	p-value
				# unique peptides	# unique spectra	Protein score		
40	**Soluble inorganic pyrophosphatase**	Q43187	24	3	3	276.5	2.36	1.77E-02
	Enolase 1 (Hev b 9)	Q9LEJ0	48	2	3	225.6	2.36	1.77E-02
41	**Monodehydroascorbate reductase**	Q42711	47	3	4	297.6	2.63	1.57E-11
	Phosphoglycerate kinase	Q42961	50	3	3	225.7	2.63	1.57E-11
	Chaperonin CPN60	Q05046	61	2	3	198.8	2.63	1.57E-11
42	**Proteasome subunit alpha**	Q9LSU0	27	10	12	1759.4	2.58	1.11E-05
43	**Aconitate hydratase**	Q42560	98	14	18	1318	-1.69	1.26E-03
44	**Catalase isozyme**	P48350	57	5	8	623.5	3.03	2.32E-06
	Glutamine synthetase cytosolic isozyme 1-2	Q8LCE1	39	5	6	557.8	3.03	2.32E-06
45	**Protein phosphatase 2C**	Q8RXV3	33	2	2	102	1.99	1.48E-05
	Phosphoglycerate kinase	Q42961	50	2	2	96.3	1.99	1.48E-05
46	**Mitogen-activated protein kinase**	Q9LV37	58	4	4	193.9	2.45	3.29E-03
47	**Lactoylglutathione lyase**	Q8W593	39	3	3	129.6	-2	5.02E-06
	V-type proton ATPase catalytic	Q40002	64	2	3	112.2	-2	5.02E-06
	Actin-97	P30171	42	2	2	110	-2	5.02E-06
48	**NADP-dependent malic enzyme**	P37223	64	3	5	269.7	-1.77	2.59E-04
	X: Methyltransferase							
49	**Serine hydroxymethyltransferase 1**	P49357	57	2	2	107.3	-2.37	2.25E-03
	Tubulin beta-9	P29517	39	2	2	99.8	-2.37	2.25E-03
	Monodehydroascorbate reductase	Q42711	47	2	2	89.6	-2.37	2.25E-03
50	**5-methyltetrahydropteroyltriglutamate**	P93263	85	10	16	1721.1	-1.63	1.39E-03
51	**Adenosylhomocysteinase**	O23255	53	5	6	457.8	2.11	5.48E-03
	Isocitrate dehydrogenase	Q06197	46	5	5	396.8	2.11	5.48E-03

Spot #	Best protein match	Accession	MW	Mascot			Spot ratio	p-value
				# unique peptides	# unique spectra	Protein score		
	XI: Transport related							
52	**ATP synthase**	Q49L13	56	8	8	497.8	2.09	6.50E-03
53	**V-type proton ATPase catalytic**	Q40002	64	11	12	741.3	-2.22	1.46E-05
54	**Sorting nexin 1**	Q9FG38	47	2	2	112.3	2.56	1.76E-07
	Protein disulfide isomerase	Q67UF5	47	2	2	99.6	2.56	1.76E-07
55	**Importin subunit alpha**	Q71VM4	58	2	2	79.1	-1.93	2.36E-10
	GDP-mannose 3,5-epimerase	Q93VR3	43	2	2	68.9	-1.93	2.36E-10
	XII: Others							
56	**Cysteine-rich repeat secretory protein 38**	Q9LRJ9	28	4	4	290.9	-1.88	4.77E-05
	Glucose and ribitol dehydrogenase	Q75KH3	32	3	3	220.6	-1.88	4.77E-05
57	**Histone H4**	P62785	11	2	2	68.8	-1.76	4.09E-04

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