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Pollen of common ragweed (*Ambrosia artemisiifolia* L.): Illumina-based *de novo* sequencing and differential transcript expression upon elevated NO_2/O_3 ☆

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1. Introduction

Common ragweed (*Ambrosia artemisiifolia*) was described by Carl Linnaeus in the 18th century from North America (Smith et al., 2013; https://de.wikipedia.org/wiki/Traubenkr%C3%A4uter). It is a herbaceous annual plant that belongs to the Asteraceae family and has expanded its distribution out of its native range to Europe, Australia, Asia, South Africa and South America (McFadyen and Weggler-Beaton, 2000; Xu et al., 2006; Cunze et al., 2013; GISD, 2010), and a full-grown plant can produce about 10^9 pollen grains (Fumanal et al., 2007). Moreover, for Europe models indicate a dramatic northward shift of ragweed, accompanied by an increase in pollen production in the future under the expected climate change scenarios (Storkey et al., 2014). The highly allergenic pollen causes hay fever and contributes mainly to allergic rhinitis and asthmatic symptoms in North America (Boulet et al., 1997). Until now, the allergenic proteins, known for *A. artemisiifolia,* can be

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categorized into six biological groups and 34 proteins, including multiple isoforms (Wopfner et al., 2005; Léonard et al., 2010; Allergome, 2014; Gadermaier et al., 2014). Amb a 1, a 38 kDa non-glycosylated protein belonging to the family of pectate lyase proteins, is the major allergen in ragweed pollen (Wopfner et al., 2005; Gadermaier et al., 2014). This highly allergenic protein is recognized by more than 95% of ragweed-sensitized individuals and is regarded as a good detector for specific ragweed sensitization (Gadermaier et al., 2008).

Environmental 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 (Behrendt and Becker, 2001; Traidl-Hoffmann et al., 2003; Rogers et al., 2006; D'Amato et al., 2007; D'Amato and Cecchi, 2008; Ziska et al., 2008, 2011; Ziska and Beggs, 2012; D'Amato et al., 2013). Elevated CO_2 concentrations resulted in an increase of ragweed growth and pollen production (Rogers et al., 2006; Stinson and Bazzaz, 2006; Ziska et al., 2008; El Kelish et al., 2014a). Moreover, it could be shown that the amount of Amb a 1 was increased at the transcriptional, as well as at the protein level under elevated CO_2 and/or drought conditions (Singer et al., 2005; El Kelish et al., 2014b; Frank et al., 2014).

In addition to climate change parameters, automobile exhaust pollution might also influence the allergenicity of common ragweed pollen. In *Cupressus arizonica* pollen air pollution resulted in an increased expression of Cup a 3 allergen (Cortegano et al., 2004; Suárez-Cervera et al., 2008). 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). *Platanus* pollen exposed to gaseous pollutants showed an increased amount of pollen allergen Pla a 1 (Lu et al., 2014). No differences in Bet v 1 content was obvious in birch pollen from urban areas compared to rural areas (Bryce et al., 2010). However, there are also year-to-year and geographical differences in release of Bet v 1 allergen (Buters et al., 2008). Furthermore, depending on the day of pollen release great differences in the amount of Bet v 1 are evident (Buters et al., 2010, 2012). Proteomic profiling of birch pollen extracts from different origins showed significant differences in the content of allergenic, as well as non-allergenic proteins (Erler et al., 2011). Similarly considerable differences in the amount of Bet v 1 and the Bet v 1 isoform compositions exist between different birch species (Schenk et al., 2011), and in different olive cultivars also different amounts of the major allergen Ole e 1 were found (Castro et al., 2003). With respect to air pollution and allergens, the effect of O_3 has been intensively studied. In perennial ryegrass and rye the allergen amount was up-regulated (Masuch et al., 1997; Eckl-Dorna et al., 2010). In contrast, in several cultivars of *Lolium* no differences in the allergen content was obvious upon O_3 fumigation (Galler, 2004). Similarly no altered protein amount of Amb a 1 was found in ragweed upon O_3 fumigation over the whole vegetation period or *in vitro* fumigation of the pollen (Pasqualini et al., 2011; Kanter et al., 2013). However, a positive correlation of the O_3 concentration and the amount of Bet v 1, as well as an enhanced allergenicity was found in birch pollen, sampled from outdoor stands (Beck et al., 2013).

Atmospheric NO_2 has long been known to be either harmful or beneficial to plants depending on the concentration and plant species used (Capron and Mansfield, 1977; Kress and Skelly, 1982; Sandhu and Gupta, 1989; Wellburn, 1990; Honour et al., 2009). Regarding pollen, elevated NO_2 inhibited germination of several plant species, reduced pollen viability in *Pinus nigra* and showed a negative correlation to the pollen amount in *Betula pendula* (Chichiriccò and Picozzi, 2007; Gottardini et al., 2008; Jochner et al., 2013; Cuinica et al., 2014). *In vitro* fumigation of pollen with NO_2 did not induce new allergens in birch or ragweed and had no influence on the allergen release from grass pollen (Behrendt et al., 1997; Aina et al., 2007). Using high concentrations of NO_2 the content of several *Phleum pratense* grass allergens (Phl p) was reduced (Rogerieux et al., 2007). In contrast, polypepetide profiles were not altered in several plant species upon fumigation with moderate NO_2 concentrations, however, immunodetection indicated higher IgE recognition (Sousa et al., 2012; Cuinica et al., 2014). Recently it was shown that an elevated NO_2 level increased the Amb a 1 amount in ragweed pollen (Zhao et al., 2016). These studies suggest that changes of NO_2 concentrations will affect the allergenic potential of pollen and play a role in human health diseases related to allergic rhinitis and asthma. From these perspectives, a comparative transcriptome analysis of allergenic ragweed pollen would not only benefit on the understanding the changes of gene expression in ragweed pollen, but also allow to understand the anticipated changes to pollen allergens in response to elevated NO_2.

In previous studies, we showed that elevated levels of O_3 had no influence on growth and pollen production of ragweed, whereas CO_2 increased and drought reduced the amount of the pollen (El Kelish et al., 2014a). In addition, transcriptional profiling resulted in different transcriptomic patterns, including enhanced levels for allergens and allergen encoding expressed sequence tags (Kanter et al., 2013; El Kelish et al., 2014b). In this study, we altered the gaseous air pollution by linking the transcriptional network changes of ragweed pollen to elevated NO_2, as well as O_3. We emphasize that this environmental change will impact the transcriptome of pollen and will additionally increase the abundance of allergen-related transcripts that are relevant for human health.

2. Material and methods

2.1. Plant growth conditions

Ragweed seeds were collected from a single plant at an outdoor stand (Bad Waldsee, Baden-Württemberg, Germany) to avoid environmental-influenced epigenetic effects (Elwell et al., 2011). Seeds were sown in standard soil (Floradur®, Bayerische Gärtnerei Genossenschaft, München, Germany) in small multiflor palettes (6 × 6 cm) and transferred into four Plexiglass sub-chambers (1,1 m × 0.9 m x 0.8 m) placed within a phytotron walk-in chambers (http://www.helmholtz-muenchen.de/en/eus/environmental-simulation-facilities/phytotron/index.html). Plant growth and NO_2 fumigation were exactly as described by Zhao et al. (2016). Briefly, plants were treated with realistic outdoor concentrations of 40 ppb NO_2 (control) or 80 ppb NO_2 (treatment) for 61 d and pollen was collected during the last 28 d of fumigation (1st generation). The collected pollen samples were stored at −80 °C. In addition seeds from the control were collected and used in a second experiment in the next year (2nd generation), using clean air for technical reasons and 80 ppb NO_2 (Zhao et al., 2016). For the survey of the Illumina data additional fumigation experiments were carried out: 80 ppb O_3 vs. 40 ppb O_3 and 120 ppb O_3 vs. 40 ppb O_3. Growth conditions and O_3 treatment were as described by Kanter et al. (2013), using also the Plexiglas sub-chambers within the phytotrons.

2.2. Quantitative real-time reverse transcription-PCR of major allergen-encoding transcripts (qRT-PCR)

Total RNA was isolated from 50 mg of pollen exactly as described by Kanter et al. (2013). The RNA was quantified using the NanoDrop System (Kisker Biotech, Steinfurt, Germany) at 230, 260 and 280 nm. Only those RNA samples with acceptable ratios of 260/280 (>1.8) and 260/230 (>2.3) were used. An aliquot of 2 μg of total RNA

was used for cDNA synthesis using the SuperScript II Reverse Transcriptase (Life Technologies, Darmstadt, Germany) according to the manufacturer's instructions. Equal amounts of cDNA were diluted 1:20, and PCR was performed in a volume of 20 μl containing 10 μl of the SensiFAST™ SYBR® Lo-ROX Kit (Bioline, Luckenwalde, Germany), 1 μl of each of the forward and reverse primers (10 μM stock), 3 μl of H_2O and 5 μl of diluted cDNA. Amplification was carried out with an ABIPrism 7500 fast real-time PCR system (Applied Biosystems, Darmstadt, Germany). The PCR conditions were as follows: 1 cycle at 95 °C for 2 min and 40 cycles at 95 °C for 15 s and 60 °C for 20 s. As internal standards for normalization, 18S rRNA was used. Three biological replicates (three plants; five inflorescences per plant) were carried out for each group (1st generation control, 1st generation treatment, 2nd generation control and 2nd generation treatment), and each transcript was quantified in triplicate for each sample. The relative expression levels were calculated according to Pfaffl (2001). The gene-specific primers for major and some minor ragweed allergens and the reference genes 18S rRNA are listed in Table S1 and were obtained from Sigma-Aldrich (Steinheim, Germany).

2.3. Illumina sequencing

Pollen of three individual plants (5 inflorescences per plant) from each treatment was randomly sampled, in the case of 80 ppb ozone treated pollen only 2 plants were used. The quality of total RNA was analyzed using the Agilent RNA 6000 Nano Kit and the quality was checked on an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Total RNA had RIN values between 7.6 and 9.2. For each of the libraries three μg of total RNA was applied for processing the Illumina library preparation. Sequencing was carried out with an Illumina HiSeq 2000 sequencing system (Illumina, San Diego, USA).

2.4. Bioinformatic analysis, statistics and data deposition

Bioinformatic analysis was performed using the CLC Genomics Workbench 6.5 (CLC bio a QIAGEN Company, Aarhus, Denmark). To get high quality reads for the *de novo* assembly raw reads were trimmed as follows: short reads <50 bp were removed, trim on quality Q30, on ambiguous bases with max. 2, further one nucleotide on the 3' terminus was removed and adapters were cleaved off. Broken pairs were kept. Before the *de novo* assembly all duplicates were removed from the dataset. The remaining data (Illumina data and 454-data from Kanter et al. (2013)) were then *de novo* assembled to generate a ragweed pollen transcriptome with graph parameters of automatic word size and automatic bubble size, reads were mapped back to the contigs at mismatch cost of 2, insertion cost of 3 and deletion cost of 3, with length fraction of 0.5 and similarity fraction of 0.8. Contigs were updated continuously and scaffolding was performed.

Using BLASTX algorithms the consensus contigs were blasted against a personalized database including NCBI viridiplantae and allergen proteins with an e-value cut-off $\leq 1\ E^{-10}$ and using BLASTN algorithm against known plant allergen sequences with an e-value cut-off $\leq 1\ E^{-20}$ and a minimum length of 210 bp. GO terms were added to the sequences by using Blast2GO plug in from the CLC Genomics Workbench under default settings.

For RNA-Seq analysis trimmed reads were mapped back to the consensus transcriptome (used as reference) and expression values were calculated by using normalized values expressed as RPKM (reads per kilobase of transcript per million mapped reads). Kal-Z test was performed to find significant differentially expressed transcripts with FDR $p \leq 0.05$. Hypergeometric tests, included in the CLC Genomics Workbench were performed to find significantly over-represented GO terms. For this significantly differentially expressed transcripts with a fold-change of at least ±1.5 were compared to the respective abundance in the reference transcriptome. GO terms with a p-value ≤ 0.05 were found to be over-represented.

Raw sequencing data and assembly data were submitted to the European Nucleotide Archive (ENA) with the project accession: PRJEB12820 and PRJEB1470.

3. Results and discussion

3.1. De novo assembly of ragweed pollen transcriptome, annotation and classification

Ragweed is a non-model organism; therefore a *de novo* assembly is the only option for sequence assembly. In *de novo* assemblies, the reads are assembled into contigs without the guidance of a reference sequence (Strickler et al., 2012). In order to cover the ragweed pollen transcripts as complete as possible a *de novo* assembly generated the consensus transcriptome using Illumina sequencing data from ten different conditions together with reads from a 454-sequncing of 80 ppb ozone treated and control-pollen (Kanter et al., 2013). Due to trimming and duplicates removal 78.5% of the 2,390,305,540 reads were ignored and 513,262,497 cleaned reads with an average length of 92.13 bp (Illumina) and 256.75 bp (454), respectively, were used for the *de novo* assembly. 35,136 unique contigs with an average length of 722 bp were generated using the CLC Genomics Workbench (Table 1).

These consensus transcriptome was then blasted against a customized NCBI protein sequence database including sequences "viridiplantae and allergens". BLAST analysis revealed that 60.8% (21,113 contigs) of the reference contigs showed significant BLAST hits by e-value $\leq 1\ E^{-10}$. Annotation of ragweed unique transcripts showed that a total of 18,061 (51.4%) unique transcripts were assigned to at least one GO term, from which 15,647 (44.5%) fitted the annotation criteria and were then used to annotate the reference transcriptome (Fig. 1). Among these 15,647 transcripts, 12,511 were assigned to the biological process category (BP), 11,515 to the molecular function category (MF), and 13,655 to the cellular component category (CC), while most these unique transcripts (9,193) were assigned to GO terms of all three categories (Figs. 1C and 2A). It is worth mentioning that "response to cadmium ion" and "response to salt stress" represented the two most abundant GO terms of the category biological process (Fig. 2B), which is consistent with fact that our transcriptome data were derived from ragweed plants grown under different abiotic stress or better, gives indication that the plants grown under different abiotic conditions

Table 1
De novo assembly statistics.

Parameters	Count
raw reads	2,390,305,540
reads post-trimming	1,937,424,995
readspost-duplicate removal	513,262,497
reads used for assembly	513,262,497
contigs	35,136
average contig length [bp]	722
minimum contig length [bp]	196
maximum contig length [bp]	10,492
N50 [bp]	817
total assembly length [bp]	25,380,299

A pool of RNA samples consisting of differently treated pollen was sequenced on an Illumina HiSeq 2000 platform (2 × 100 bp). Reads were trimmed on quality first, then duplicate sequences were removed, and remaining reads were assembled into contigs using the *de novo* assembly tool in CLC Genomics Workbench.

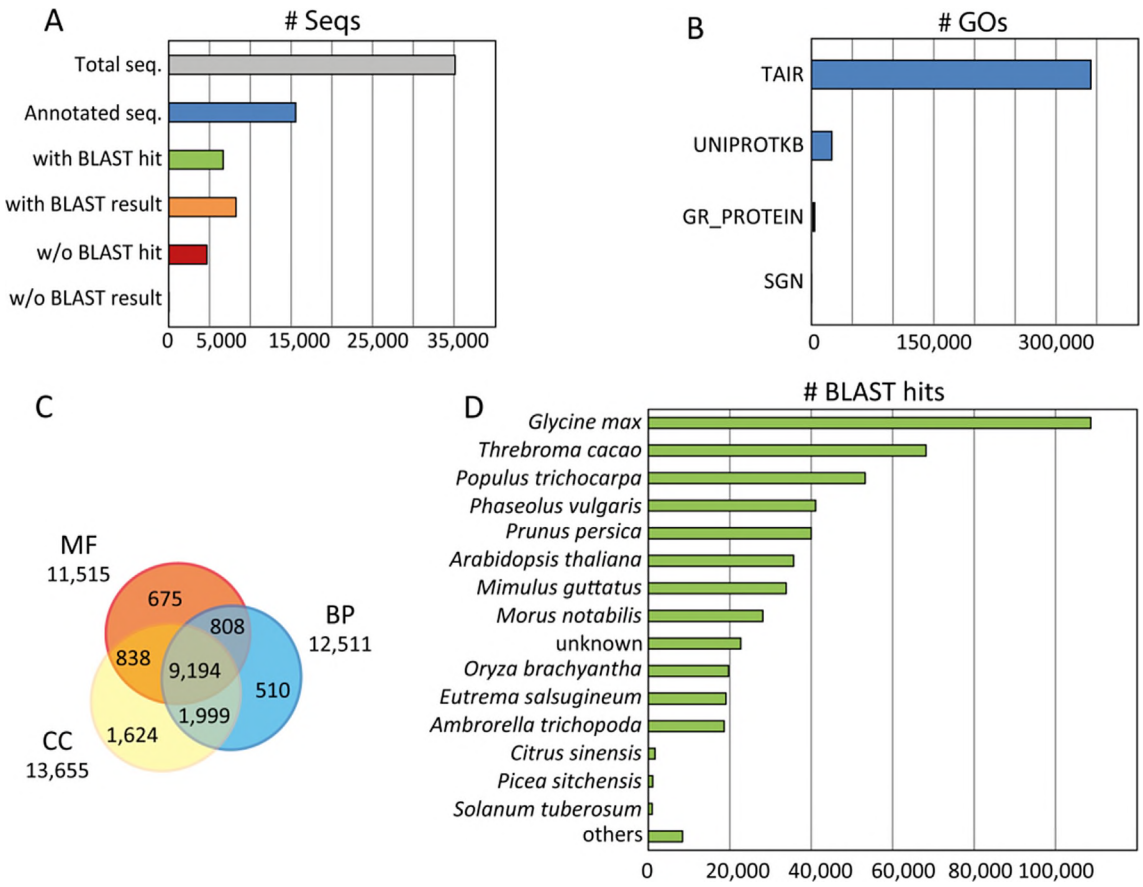

Fig. 1. Annotation statistics. A) gives the annotation statistic for the reference transcriptome. B) shows the mapping data sources distribution and gives the number of GO terms used from the corresponding database. C) VENN diagram of GO terms after GO slim, sorted to molecular function (MF), biological process (BP) and cellular component (CC). D) shows the number of BLAST hits to most common plant species.

have to have a lot of functional proteins to cope with potential stresses. Other interesting highly abundant GO terms of the BP category included “oxidation-reduction process”, “response to cold” and “defense response to bacterium”, which also demonstrated pollen stress in this study.

Ozone is a phytotoxic air pollutant that severely affects plants, resulting in an oxidative stress, formation of reactive oxygen species (ROS) and changes in the redox potential (Sandermann et al., 1998). Antioxidative systems are well known in plants that are important for the detoxification of ROS (Mittler and Zilinskas, 2004). Regarding the *de novo* assembly of the ragweed pollen transcriptome, a part of sequences used, were derived from ragweed pollen treated with O_3, which might stimulate the expression of transcripts involved in “oxidation-reduction process” to reduce the negative effect by O_3. 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 flower 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.

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. 2B). 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 (Uniprot, 2015a); Amb a 3 acts as copper ion binding protein (Wopfner et al., 2005); Amb a 6, a lipid transfer protein, plays a key role in lipid binding (Uniprot, 2015b), 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. E.g. 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 et al. (1994) identified a serine/threonine kinase and suggested that it might play a role in signal transduction events during pollen development and/or pollination. To digest the female integumentary tissue a high level of hydrolases and proteases activity is absolutely necessary for pollen in the fertilization process (Pettitt, 1985; Lazzaro, 1999).

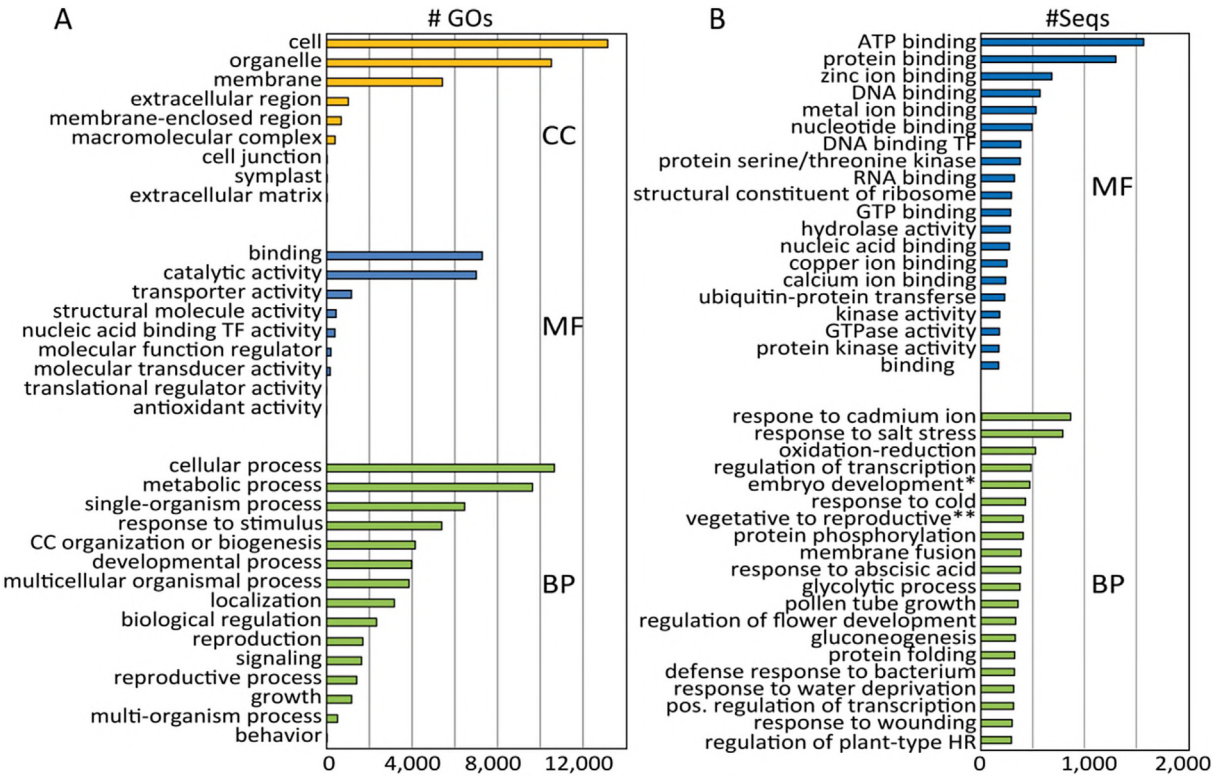

Fig. 2. Annotation distribution of complete ragweed pollen transcriptome. A) Distribution of common GO terms after the usage of GO slim plant included in the Blast2GO tool, sorted to cellular component (CC), molecular function (MF) and biological process (BP), respectively. B) Different GO terms in more detail, according to the direct count of sequences for its corresponding GO term (without GO slim). *ending in seed dormancy; **phase transition of meristem; TF = transcription factor; HR = hypersensitive response.

3.2. *Differentially expressed transcripts upon elevated NO_2 or O_3*

RNA-Seq is also often used in transcript expression analyses (Strickler et al., 2012). RNA-Seq is a sensitive and exact method and has no problems of background signals compared to microarrays ('t Hoen et al., 2008). Typically, in non-model plant transcriptomics 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).

For RNA-Seq analysis trimmed reads from each treatment were mapped back to the reference transcriptome (Table 2) and RPKM values were calculated. Then each treatment was compared to its corresponding control and significantly differentially expressed transcripts were identified by using the Kal-Z test with a FDR p-value of 0.05. Only transcripts which were significantly changed by at least ±1.5-fold were taken into further consideration for hypergeometric tests to calculate whether some GO terms were significantly over-represented or not. In total 796 transcripts (445 up, 351 down) were significantly differently expressed in the first generation of NO_2 treated plants, whereas for the second generation of NO_2 fumigated plant 765 transcripts (507 up, 258 down) were significantly changed. This difference might be caused by using clean air as control in the second generation. Ozone treatment resulted in slightly lower numbers of significantly changed expression 520 (273 up, 247 down) for 80 ppb O_3 fumigation and 634 (335 up, 299 down) for the 120 ppb O_3 fumigation (Table S2).

Table 2
Mapped reads for RNAseq analysis.

Treatment	Mapped reads [%]
40 ppb ozone (1)	77.96
80 ppb ozone (1)	77.75
40 ppb ozone	77.61
120 ppb ozone	76.23
40 ppb NO_2 1st generation	73
80 ppb NO_2 1st generation	73.36
80 ppb NO_2 2nd generation	74.79
air 2nd generation	77.16

3.3. *GO category biological process*

In this study pollen treated with different abiotic stress was analyzed; and there were eight GO terms which were common in all treatments: “response to wounding”, “membrane fusion”, pollen tube growth”, “spermidine biosynthetic process”, “spermine biosynthetic process”, “*S*-adenosylmethioninamine biosynthetic process”, “jasmonic acid biosynthetic process” and “cellular phosphate ion homeostasis” showing that developmental processes as well as the ion homeostasis, the hormone biosynthesis and stress-dependent processes are influenced by elevated NO_2 and O_3, respectively (Fig. 3 A, B). Whereas it seemed that the treatments had mostly a positive effect on the induction of the transcripts for these GO terms, since more upregulated transcripts could be sorted to the corresponding GO terms. E.g. the GO term “pollen tube growth” represented the one of the most abundant up- and down-regulated group of the biological process category. 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 (Sari-Gorla and Frova, 1997). From the number of transcripts corresponding to the GO term “pollen tube growth” it seems that elevated NO_2 had a beneficial effect on pollen tube growth, since around twice the amount of transcripts were found to be up-regulated as compared

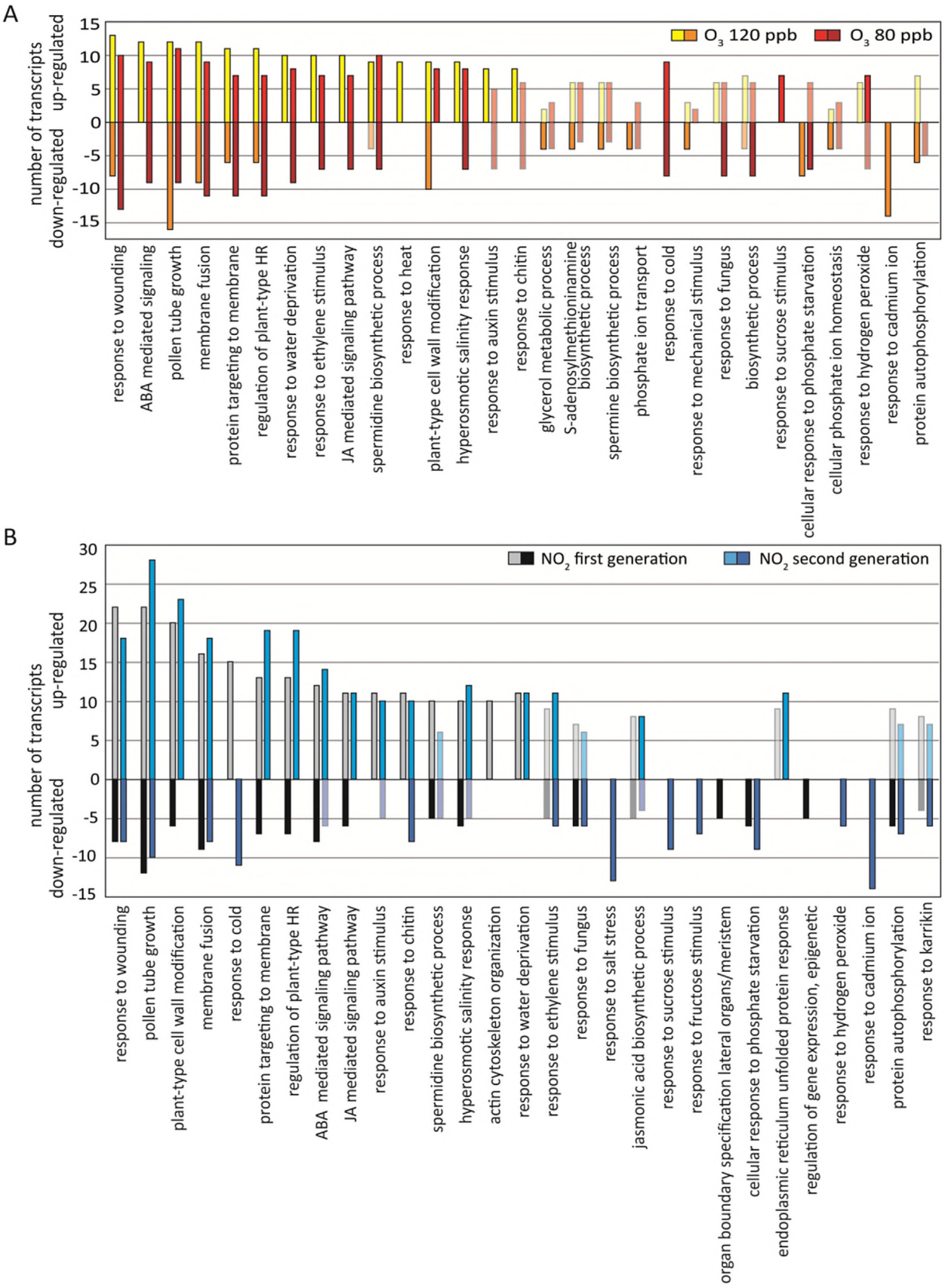

Fig. 3. Over-represented GO terms of the category biological function. A) gives the top 15 overrepresented GO terms for 80 ppb and 120 ppb O_3-fumigated pollen, respectively. B) shows the results for 80 ppb NO_2-fumigated pollen from the 1st and 2nd generation of pollen. Columns which are shown in de-saturated colors are over-represented, but not within the top 15.

to the down-regulated ones (Fig. 3B). A different picture was drawn for ozone; in 80 ppb O_3-treated pollen roughly the same number was up- and down-regulated, with slightly less in the down-regulated ones, whereas fumigation with 120 ppb O_3 resulted in a higher number of down-regulated transcripts as compared to the up-regulated ones (Fig. 3A). This is in accordance to results from tobacco and petunia pollen, where inhibitory effects on pollen tube growth were found after ozone treatment (120 ppb) (Feder and Shrier, 1990). Another interesting group are for instance polyamines, including spermine-, spermidine- and *S*-adenosylmethioninamine biosynthetic processes, which are found to be involved in several developmental processes (Ge et al., 2006). They are able to scavenge radical oxygen species (Das and Misra, 2004) and act stabilizing on macromolecules, proteins and membranes (Verma and Mishra, 2005). As summarized by (Pottosin et al., 2014) polyamines have an impact on the ion transport across the plasma membrane, influence the cell wall loosening and stiffening and help the plant to adapt to different abiotic and biotic stresses. In case of ozone it is known that polyamines such as spermine and spermidine increase in tobacco after ozone-treatment (Langebartels et al., 1991) to protect the plant from reactive oxygen species. Additionally also the "jasmonic acid biosynthesis process" can be connected to the polyamines and wound response, since it was shown, that jasmonic acid promoted a strong increase of e.g. spermidine conjugates in barley leafs, correlated with a reduction of the powdery mildew infection (Walters et al., 2002).

Additionally, 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 fungus" were over-represented in NO_2 and O_3 up- and down-regulated transcripts (Fig. 3). Chitin a major component of fungal cell walls is a general elicitor of plant defense reactions (Boller, 1995). Chitin is degraded by plant's chitinases and the resulting chitooligosaccharides act as elicitors of downstream defense gene responses (Eckardt, 2008). These changes of ragweed pollen in biotic stress response processes indicate the signal crosstalk between response to elevated NO_2/O_3 and to fungal pathogens.

Plant hormones are known to be involved in plant responses to various stresses. In this study, GO terms including "jasmonic acid mediated signaling pathway", "response to ethylene stimulus", "response to auxin stimulus" and "abscisic acid mediated signaling pathway" were highly enriched for all up- and some down-regulated transcripts, corresponding to the respective GO term (Fig. 3). 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 and elevated O_3 stress, respectively. For jasmonic acid, again, it is known that it plays an important role in defense responses to different biotic and abiotic stresses, as well as for development (Creelman and Mullet, 1995).

3.4. GO category molecular function

In principal the results for the GO categories molecular function were not as homogeneous as for the biological process which might be due to the fact that the identified transcripts have different specific molecular functions but interact in same biological processes.

One GO term common within the significant over-represented GOs of up- and down-regulated transcripts under all conditions was "adenosylmethionine decarboxylase activity" (Table S2, Table S3). Adenosylmethionine decarboxylase, an important enzyme in the synthesis of spermine and spermidine and is known to be essential for normal pollen germination in tomato plants (Song et al., 2001). Another GO term "arginine decarboxylase" which is also a key enzyme for the polyamine synthesis was shown to be over-represented in all treatments except for the down-regulated transcripts of the 120 ppb O_3-treatment, where it was also present but not significant (Table S2, Table S3). Additionally the GO term "glycerophosphodiester phosphodiesterase activity" was found to be over-represented in all four treatments. For a *Arabidopsis thaliana* glycerophosphodiester phosphodiesterase (GDPD) it was postulated that they play an important role in maintaining cellular phosphate homeostasis (Cheng et al., 2011).

Regarding the top 15 up- and down-regulated GO terms of molecular function, most of them are involved in binding processes (Table S3), including ATP binding, which was the top altered GO term in the group of repressed transcripts from NO_2-fumigated pollen of the 1st generation. This ATP-binding seems to play also a large role in the other treatments, even if not stated as over-represented, since "ATP-binding" was also very frequently within the other groups containing, dependent on the group, between 14 and 24 transcripts with GO term "ATP-binding". Metal ion binding including "zinc ion binding", "calcium ion binding" and "copper ion binding" was also shown to be over-represented in some treatments. E.g. "Calcium ion binding" was highly enriched within the significantly up-regulated transcripts of elevated NO_2 from the 1st and 2nd generation. Additionally, this GO term was also found in the other treatments, even so not over-represented, but with the tendency of more prominence within the up-regulated transcripts (Table S3). 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 signaling pathways involved in the response to environmental stresses including osmotic, salt, cold, heat and oxidative stress (Bouche et al., 2005).

The GO terms "kinase activity" including e.g. "hydrolase activity", "GTPase activity", "protein serine/threonine kinase" was over-represented in up- or down-regulated transcripts from different treatments in ragweed pollen. For example, the GO term "serine/threonine kinase activity" was shown to be the top over-represented GO term in all ozone-treatments as well as in 2nd generation of NO_2 down-regulated transcripts. Within these, transcripts with homology to integrin-like proteins were found, known to play a role in growth of the pollen tube tip and interaction with the extracellular matrix of style (Sun et al., 2000). Also homologues transcripts to receptor-like cytosolic kinase RBK1-like and RBK2-like proteins were found to be differentially expressed. Both of these kinases interact with Rop GTPases (Molendijk et al., 2008) and for RBK1 it was shown to be pathogen induced in *Arabidopsis* and barley, respectively (Molendijk et al., 2008; Huesmann et al., 2012). 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, and a non-functional RAC/ROP regulation results in disturbed tube polarization (Lazzaro, 1999; Cheung and Wu, 2008; Chen et al., 2013).

Jacobsen et al. found that a *P*-type ATPase MIA is essential for pollen release and subsequent germination in *Arabidopsis* (Jakobsen et al., 2005). Together with the function of serine/threonine kinase and hydrolase discussed before, the NO_2 and O_3 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.

3.5. *Relative expression of different allergen transcripts is changed due to elevated NO_2 and O_3*

The allergenicity of pollen sampled at highway traffic had a higher allergenicity than pollen sampled along roads with low traffic (Chehregani et al., 2004; Cortegano et al., 2004; Suárez-Cervera et al., 2008; Ghiani et al., 2012; Beck et al., 2013). However, different air pollutants, e.g. O_3, NO_2 and CO_2, as well as particulate matter or climatic factors may influence the allergenic potential of pollen diversely. A positive correlation of the amount of the major birch pollen allergen Bet v 1 was found for O_3, whereas temperature showed a negative correlation, and other factors like NO_2 or an urbanization index had no effect. (Beck et al., 2013). As in our chambers all other physical parameters were not changed, we bring forward the argument that the results observed are solely caused by elevated NO_2 or O_3. Different contigs could be detected in our study as follows: Amb a 1 with its corresponding isoforms belongs to the major allergen in *Ambrosia*, a pectate lyase, to which 95% of sensitized patients show IgE reaction. Amb a 3 is thought to be a plastocyanin to which still 51% of the patients react (Adolphson et al., 1978). Further contigs with homology to Amb a 8 (profilin) were detected; Amb a 9, a calcium-binding protein (polcalcin); Amb a 10 another polcalcin-like protein and Amb a 11, a cysteine protease were also identified. As it is seen in Fig. 4 in most cases several contigs could be matched to known allergens, indicating isoform and spliceform expression. From 25 identified contigs with homology to a known *Ambrosia* allergen, 20 showed at least one significant change in expression within the four different treatments and were further analyzed (Fig. 4). In case of elevated ozone (80 ppb), only one transcript, expressed at relatively low level (<RPKM 75) was found to be significantly down-regulated (Amb a 1.1 var. 2; FR669657) whereas a contig with homology to the same gene was significantly elevated, but expressed at a higher level. The highest significant fold-change for 80 ppb O_3-fumigated pollen was found for a transcript with homology to Amb a 10.0101 (2.78-fold). The rest of the detected transcript with homology to ragweed allergens ranged between 1.02-fold and 1.98-fold with highest expression for Amb a 1.0301 (1.07-fold), but only two of them passed the threshold of 1.5-fold (Amb a 8.0101 and Amb a 10.0101. If the threshold was set lower also Amb a 1.0501 (1.37-fold), Amb a 3.0101 (1.44-fold) and Amb a 11.0101 (1.26-fold) showed significant changes. As compared to the 454-sequencing data from Kanter et al. (2013) also in this study a isoform and spliceform specific expression was detected and the expression ranged in comparable scope, and slightly differences in expression might be explained by differences in assembly and contig size. Additionally, also the contigs detected in this study can match to a variety of allergen isoforms, but only the best hit was given for annotation. Interestingly, for the 120 ppb O_3 treated pollen most of the detected ragweed allergen transcripts were repressed due to the treatment, giving indication that 120 ppb O_3-fumigation indeed had an impact on different GO terms, such "pollen tube growth" or "response to wounding", but not on the known allergen induction. This is in contrast to studies on birch, where higher allergen levels were detected due to ozone, whereas in that study only ozone levels up to ca. 45 ppb were detected (Beck et al., 2013), but it is consistent with the fact, that no significant changes were visible in a Amb a 1 specific ELISA (Kanter et al., 2013).

A different picture is drawn for NO_2-fumigated pollen, here we provide strong evidence that elevated NO_2 (80 ppb) results in a clearly changed transcriptional profile of allergen encoding transcripts (Fig. 4). From the 20 analyzed contigs 16 showed a higher expression due to the NO_2-fumigation in both generations. Out of eight contigs corresponding to different isoforms of the major allergen Amb a 1, six were up-regulated due to NO_2-treatment, in both generations, respectively. This is in agreement with increased levels of Amb a 1 proteins upon elevated NO_2 fumigation (Zhao et al., 2016), and indicates an enhanced allergenicity of the pollen upon NO_2 treatment. Concerning the major allergen Amb a 1, the highest significant induction in the first generation was found for Amb a 1.0402 (FR669664; 2.4-fold), followed by Amb a 1.1 (FR669657; 1.95-fold) and Amb a 1.2 precursor (M80559; 1.92-fold), slightly different Amb a 1.1 showed largest changes (1.97-fold), followed by Amb a 1.0402 (1.8-fold) and Amb a 1.2 precursor (1.64-fold). Interestingly both variants Amb a 1.0301 and Amb a 1.0303 were slightly up-regulated in the first generation whereas they were repressed in the second generation, even so not significantly. Highest significant over all induction was found for Amb a 9.0101 (calcium-binding protein; AY894657; 2.5-fold) in the second generation, whereas it was almost not changed in the first

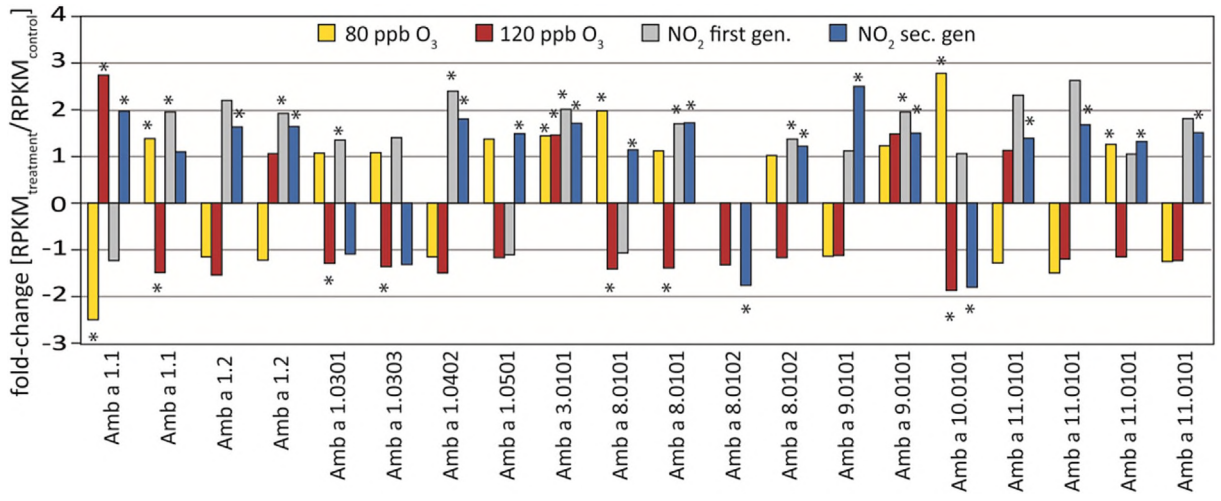

Fig. 4. Fold-change of up- and down regulated allergen transcripts upon elevated O_3/NO_2. * = Kal-Z test; FDR p-value $\leq$ 0.05.

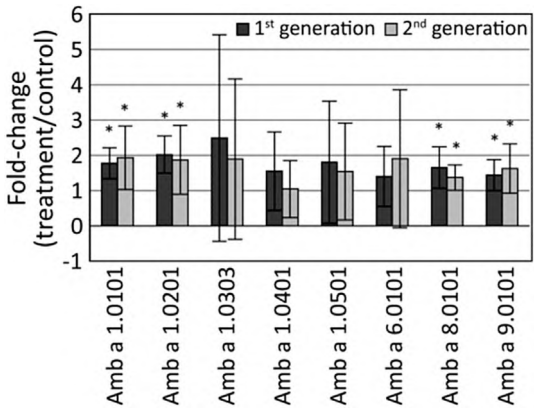

Fig. 5. qRT-PCR analysis of ragweed pollen allergens. 1st generation (control: 40 ppb NO_2, treatment: 80 ppb NO_2) is indicated in dark grey; 2nd generation (control: clean air, treatment: 80 ppb NO_2) is shown in light grey. The transcript levels were normalized relative to the 18S rRNA transcript levels. The mean values were obtained from three independent PCR amplifications (error bars ± SD, T-test * = p-value < 0.05).

generation of NO_2-fumigation. Another contig clearly induced in both generations was a transcript coding for the profilin Amb a 8.0101, as well as several contigs with homology to Amb a 11.0101 (cysteine protease), even if not significant for the first generation. A significant induction of Amb a 3.0101 was found under all four treatments indicating that this plastocyanin might act in stress answers.

To test whether our Illumina RNA-Seq data were reliable, pollen from NO_2-treated plants was chosen to perform quantitative real-time reverse transcription-PCR (qRT-PCR). Therefore qRT-PCR was carried out for selected "Amb a" transcripts to analyze their relative expression and the results were mostly comparable to the Illumina data (Fig. 5).

3.6. Other allergens

Additionally contigs with homology to other known plant allergens have been detected, such as Art v 6.0101 (AY904433) a Amb a 1-like protein from *Artemisia vulgaris* which was found to be significantly increases in NO_2-fumigated pollen (up to 4.5-fold), but was decreased under O_3-treatment. Beside this, similar as recently described (Kanter et al., 2013), homologous to some other known plant allergens have been identified (Table 3, Table S4), from which a few showed significant changes in expression. E.g. transcripts with homology to L-ascorbate oxidase from *Cynodon dactylon* were shown to be increased (up to 3-fold) in NO_2-fumigated pollen whereas they were repressed under 120 ppb ozone (up to −1.4-fold). For these enzymes it is known that they are expressed in developing pollen (Albani et al., 1992) and that they are important to maintain the apoplastic redox state (Pignocchi et al., 2006) and overexpression of a ascorbate oxidase in tobacco resulted in higher sensitivity to ozone (Sanmartin et al., 2003). This could indicate that a reduction of ascorbate oxidase in the 120 ppb fumigated

Table 3
Transcripts with homology to allergens known from other plants.

Protein function	Homology to other plant allergens found in
60s ribosomal protein	*Arabidopsis thaliana*
ABC transporter family	*Arabidopsis thaliana*
abscisic acid receptor pyl4-like	*Arabidopsis lyrata* subsp. *Lyrata*
acid beta-fructofuranosidase-like	*Solanum lycopersicum*
calcium-binding protein	*Medicago truncatula; Olea europaea; Nelumbo nucifera; Solanum lycopersicum; Vitis vinifera; Arabidopsis thaliana*
chlorophyll *a-b* binding protein 2	*A.graveolens*
cyclophilin	*Daucus carota*
endochitinase	*Persea americana*
enolase	*Cynodon dactylon; Hevea brasiliensis*
expansin-like	*Vitis vinifera; Theobroma cacao*
extensin	*Artemisia annua; Populus trichocarpa; Theobroma cacao*
glutathione *S*-transferase	*Betula pendula*
glyoxalase	*Oryza sativa Japonica*
isoflavone reductase-like	*Fraxinus excelsior; Olea europaea*
L-ascorbate oxidase-like	*Cynodon dactylon*
LTP (lipid transfer protein)	*Artemisia vulgaris; Lactuca sativa*
luminal binding protein	*Corylus avellana*
methionine synthase	*Amaranthus retroflexus; Salsola kali*
nucleoredoxin	*Arabidopsis thaliana*
Ole e 10-like	*Nicotiana sylvestris*
PR-1	*Artemisia vulgaris*
PR-10; Bet v 1-like	*Fragaria x ananassa subsp. Ananassa; Lilium regale*
pectate lyase	*Arabidopsis thaliana, Artemisia vulgaris; Ricinus communis*
pectinacetylesterase	*Arabidopsis thaliana*
pectinesterase	*Fraxinus excelsior*
peroxiredoxin	*Arabidopsis thaliana*
polygalacturonase	*Elaeis guineensis*
profilin family protein	*Arabidopsis thaliana*
protein kinase family protein	*Salsola kali*
quinone oxidoreductase-like	*Morus notabilis*
quinone reductase-like protein	*Prunus mume*
regulatory components of aba receptor	*Sorghum bicolor*
s-locus lectin protein kinase family	*Oryza sativa Japonica*
superoxide dismutase	*Hevea brasiliensis; Olea europaea*
thaumatin-like protein	*Actinidia deliciosa*
villin	*Nicotiana tabacum*
xyloglucan endotransglucosylase	*Cryptomeria japonica*

Homologies were detected by BLASTN (green plant + allergen) e-value ≤ E^{-20}.

pollen helps to maintain the redox state and to resist against the ozone. Other contigs, which homology to peroxiredoxin, known to interact with different stresses (Dietz, 2011), were shown to be significantly increased upon 80 ppb O_3 and elevated NO_2 (2nd generation). Also transcripts coding for pectate lyases from other plants e.g. *Artemisia vulgaris* were significantly increased under elevated NO_2 but decreased upon 80 ppb O_3.

4. Conclusions

Our transcriptome study on ragweed plants fumigated with elevated NO_2 and elevated O_3, respectively, over the whole growing season support the idea that NO_2 as well as O_3 induces stress in the plant, as reflected by the stress-related GO terms. NO_2-fumigation leads clearly to enhanced allergen transcript amounts whereas elevated O_3 (80 ppb) did not show this clear effect, and 120 ppb O_3 even resulted in a more pronounced decrease of allergen transcripts than on an increase which is in line with results from grass pollen, showing lower Phl p 5 content after O_3-fumigation (Albertine et al., 2014). The increased allergen transcript amounts due to NO_2-fumigation are in accordance with increased Amb a 1 contents in ragweed and enhanced allergenicity in *Phleum pratense* (Chassard et al., 2015; Zhao et al., 2016), indicating potentially higher allergenicity due to NO_2-influence. This then should be tested in suitable mouse models.

Competing interests

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2017.02.032.

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