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OZONE-INDUCED OXIDATIVE STRESS RESPONSE IN *Arabidopsis*: TRANSCRIPTION PROFILING BY MICROARRAY APPROACH

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Abstract: High ozone concentration generates oxidative stress in plants. To investigate the detailed transcriptional regulation of *Arabidopsis thaliana* genes encoding antioxidant enzymes upon ozone stress, we performed a microarray analysis using *Affymetrix* GeneChip technology. Our transcription profiling revealed a differential expression equal or greater than 2-fold change for 2385 genes (at confidence 99%) in response to 350 ppb ozone dose after 3 and 6 hours of treatment. Among these, we chose 38 genes to be oxidative stress related in ozone treatment: 29 of them were 2 times up-regulated and 9 were shown to be down-regulated in at least one of the time points. Our study revealed a new transcription pattern for catalase genes and showed the first detailed transcriptional analysis of phenylopropanoid-related genes in ozone stress conditions.

Key Words: Oxidative Stress, Microarray, Ozone, Real Time RT-PCR

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Abbreviations used: 3hr & 6hr - 3- & 6-hour time point; 4-CL - 4-coumarate: CoA ligase; APX - L-ascorbate peroxidase; ATP2a - peroxidase; ATR2- NADPH-ferrihemoprotein reductase; C4H - cinnamate-4-hydroxylase; CAD - putative cinnamyl alcohol dehydrogenase; CAT – catalase; CCoAOM - caffeoyl-CoA 3-O-methyltransferase; CCR - cinnamoyl CoA reductase; CHI - chalcone isomerase; CHS - chalcone synthase; GPX - glutathione peroxidase; GR - glutathione reductase; GSH - glutathione synthetase; GST - glutathione transferase; MDHR - monodehydroascorbate reductase; MPK - mitogen-activated protein kinase; PAL- phenylalanine ammonia lyase; PER – peroxidase; ppb – part per billion; PSY - phytoene synthase; Q-PCR - real-time quantitative RT-PCR; RbohD - respiratory burst oxidase protein; SAG - senescence-associated gene.

INTRODUCTION

Ozone, a gaseous pollutant, generates oxidative stress in plants [1-4]. The toxicity of ozone is linked with an increase of reactive oxygen species in the apoplast such as hydrogen peroxide (H_2O_2), hydrogen superoxide and hydroxyl radicals [1,3,4]. O_3 and ozone-derived free radicals oxidize biomolecules initiating a variety of defensive responses. Elevated levels of reactive oxygen species induce the biosynthesis of antioxidant molecules including ascorbate, polyamines, glutathione, phenylpropanoids and carotenoids [1,3,5,6]. Oxidative stress increases the activity of antioxidant enzymes such as superoxide dismutase (SOD) and peroxidase (PRXs), glutathione reductase (GR), catalase (CAT) and glutathione S-transferase (GST) [1,3,5-9]. The antioxidant activity varies depending on plant developmental stages, ozone doses and is found in most cell compartments [10,11]. Previous studies have shed light on the transcription pattern and protein activity of several ROS scavengers upon ozone stress conditions [1, 4-6].

Depending on ozone dose and plant species, O_3 causes two types of responses: acute (>150 ppb) and chronic (<100 ppb) [3]. The acute ozone response seems to be an excellent tool to study ozone tolerance and susceptibility among model plants and their cultivated relatives. To some extent, ozone susceptibility derives from various transcription pattern and protein activity of individual antioxidant biomolecules.

To analyse the detailed transcriptional regulation of *Arabidopsis thaliana* oxidative stress-related genes upon ozone stress conditions, we carried out a microarray analysis using the *Affymetrix* oligonucleotide chip technology, which allows parallel transcriptional study of more than 22,500 specific genes. *Affymetrix* GeneChip uses a set of 11–20 oligonucleotide probes, each 25 bases long, to represent a gene. The perfectmatch probe is designed to hybridise with the target transcript and the mismatch probe is included to measure non-specific and cross-hybridisation.

MATERIAL AND METHODS

Microarray analysis

Arabidopsis thaliana Columbia ecotype was grown on compost, 3-5 plants per pot. Four–week-old plants were exposed to 350 ppb of ozone from 9.00 am to 3.00 pm for one day. Environmental conditions in the ozone chamber included 16 hours photoperiod from 6.00 am to 10.00 pm with 75% humidity and temp 21˚C. O_3 was produced from O_2 with an electric charge. Ozone concentration was determined by an ASIN GDC31 ozone sensor (ASECO). Leaf tissue was harvested directly into liquid nitrogen after 3 or 6 hours of ozone treatment. Total RNA was prepared using the RNeasy Plant Mini kit (Qiagen). The quality of purified total RNA was established by agarose gel electrophoresis using ethydium bromide staining. The concentration of total RNA was determined spectrophotometrically. Then total RNA quality was examined with Lab-on-a-

Chip and analysed with Agilent 2100 Bioanalyzer, version 1.4. Total RNA was extracted in triplicate from 5 non-treated control plants and ozone-treated plants in 3hr and 6hr time points, respectively. Then each corresponding extractions were pooled and fifteen μg of total RNA from pooled samples was used for single microarray hybridisation experiment using ATH1 Genome Array (*Affymetrix*). The microarray experiment was performed twice as technical replicates. cRNA synthesis, labelling, and hybridisation of the chips were carried out according to *Affymetrix* protocols (Microarray Core Facility, University of Manchester). Data obtained from MAS software were normalised with RMA (the robust multi-array analysis; R-package; BioConductor). The RMA normalised data were analysed using GeneSpring 6.1 (Silicon Genetics) software. Genes with signal intensity values below 100 in all conditions were excluded. Genes that were 2-fold over-expressed or 2-fold under-expressed in at least 1 condition were selected. For the evaluation of statistically significant change we used 1-way ANOVA (Welch ANOVA - parametric test, variances not assumed equal) with 1% significance level ($P<0.01$) and Post hoc test. About 1.0% of the identified genes would be expected to pass the restriction by chance.

Real-Time Quantitative RT-PCR using Syber-green

3 μg of total RNA extracted for the GeneChip experiment were treated with RQ1 DNase (Promega) in a final volume 10 μl. First strand cDNA (ABgene kit) was synthesized using half the volume of DNase-treated aliquot with oligo dT primer to a total volume of 33 μl. The cDNA template was then diluted 10 times by adding 297 μl of milli-Q water. A second half of DNase treated RNA sample was subjected to the same dilution conditions (without cDNA synthesis) and treated as the negative control (DNase treatment control). Real time PCR was carried out in an ABI 7700 Detection System (Applied Biosystems). The PCR reaction mix was prepared as follow: 3.4 μl of cDNA template, primer mix solution, containing 1 μM of each primer and 5 μl of 2x QuantiTect SYBR Green PCR Master Mix (Qiagen) to the final volume of 10 μl. Duplicated validation assay was performed in triplicate on each cDNA sample, the negative control and no-template controls for each primer pair. As a reference 18S rRNA and ACT2 were used. Data analyses were performed using the Sequence Detector Software (Applied Biosystems). To determine relative quantification, we performed amplification on standard DNA (a serial dilutions of *A. thaliana* genomic DNA) for both target and reference gene. The normalised value of the target gene was calculated in Microsoft Excel software as follow: the abundance of the target gene transcript was divided by amount of the reference gene transcript. The relative ratio (fold change) for each gene was calculated by the division of the corresponding normalised values of ozone-treated versus non-treated control sample.

RESULTS

We analysed transcriptional changes after 3 and 6 hours of continuous 350 ppb ozone dose treatment. Changes in gene transcription were examined in four-week-old plants of wild type *Arabidopsis thaliana*. The concentration of ozone, time points and *Arabidopsis* ecotype were chosen based on previous studies [3,12]. Ecotype Columbia is regarded as ozone tolerant, this meaning is able to deal with high ozone doses (e.g. 300 ppb) [4,13]. Our earlier experiments indicated that plants grown in conditions as described above (Material and Methods) and fumigated with 350 ppb ozone dose did not exhibit visible necrotic lesions. The choice of 3 and 6 hours ozone treatment was an attempt to capture only longer-term changes in gene expression. The rationale was to try to detect differential gene expression patterns in 3 hours interval for members of specific multigene families. In particular, it was predicted that some known antioxidant-controlling-genes would change their expression upon ozone stress, thus this study was designed to identify gene-specific responses among these multigene families.

To validate the microarray data, we used Real-Time Quantitative RT-PCR. Validation was performed for 15 genes selected with no change or difference in expression level ranging from 1.3- to 18-fold. To investigate the reliability of extremely low (<100) and high intensity signals, the genes for validations were also chosen with various transcript abundance. The same induction or repression trend was observed for 13 genes what is equal to a 86.6% confirmation. Validation experiment confirmed that there was either no change in gene expression or a reliable difference for values equal and above 2-fold. Changes lower than two-fold were not validated and therefore regarded as non-significant. In general, with the exception of genes for which the fold change was entirely consistent between the Affychip and the Q-PCR, the fold changes obtained with the Q-PCR were rather higher than those on the AffyChip. We did not confirm the expression changes for genes whose signal intensity was below 100 in all conditions (non-treated, 3-hour and 6-hour treated samples) (Fig.1).

Using the probe sets representing approximately 23,000 unique *Arabidopsis* genes and based on validation results, our expression profiling revealed differential expression equal or greater than a 2-fold change for 2385 genes (at confidence 99%) in response to ozone (Fig.2). Based on TAIR database annotation and literature [3,4,7,9,12-16], we selected genes that respond to oxidative stress, act as ROS scavengers, takes part in ROS removal or are oxidative stress related in aspect of putative molecular function or biological process they are involved. Among them, for this particular analysis, we selected 38 oxidative stress related genes, 29 of them were revealed to be at least 2 times up-regulated (Tab.1) and 9 were shown to be down-regulated (Tab.2) upon ozone treatment in at least one of the time points. The induction pattern of some genes was consistent with previous reports or with predictions based on homologs in *Arabidopsis* or in other plant species [1,7,8,10,15,17-20]. From the already reported class of genes, we detected a differential expression for *PAL1*

(phenylalanine ammonia lyase) and *GST6* (glutathione S-transferase), that are both gene markers for oxidative stress conditions. Transcript level of the *PAL1* and *GST6* genes reach a peak at 3 hours of ozone treatment and then decrease. For all enzymes of the phenylpropanoid pathway, we detected an increased transcription of *PAL2*, 4-coumarate:CoA ligase 2 and 4-coumarate: CoA ligase 1, cinnamate-4-hydroxylase, NADPH-ferrihemoprotein reductase (*ATR2*) and cinnamoyl CoA reductase - like protein with the highest fold change after 3 hours of ozone treatment.

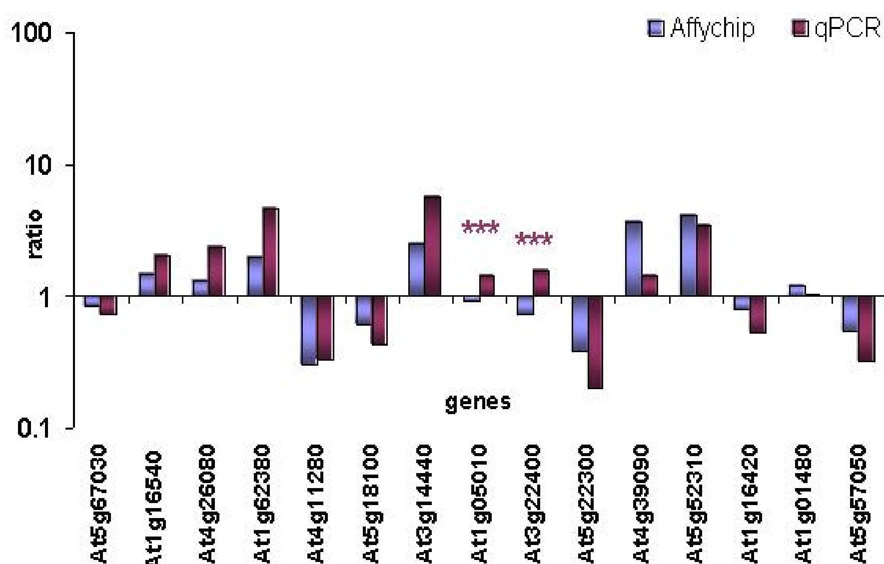

Fig.1. Representative validation data from 6hr time point versus 3hr time point; the Affychip 6hr vs 3hr ratios were plotted with the qPCR relative ratios. Data are shown in logarithmic scale, therefore down-regulated ratios expand to cover the region between 1 to 0.1; the qPCR ratio was determined as described in materials and methods. For validation, we compared the up- or down-regulated trends between the Affychip and the qPCR results: if the trend between the Affychip and the qPCR ratio was consistent, the microarray result was regarded as validated; *** non-validated transcriptions: loci At1g05010 and At3g22400 exhibit very high transcript abundance or low signal intensity values, respectively.

Moreover, 4-coumarate: CoA ligase 2 together with cinnamoyl CoA reductase (*CCR2*) showed up to a 50-fold increase in transcription level compared to non-treated control. Transcript level of two genes coding for cinnamyl alcohol dehydrogenase, one gene for caffeoyl-CoA 3-O-methyltransferase were also increased at both time points, and the highest fold changes were observed after 6-hour of ozone treatment. By contrast, mRNA for chalcone synthase, chalcone isomerase and serine carboxypeptidase I decreased in both time points. Chalcone synthase decreased in abundance 17.5 times and slightly more than 31 times at 3- and 6-hour, respectively.

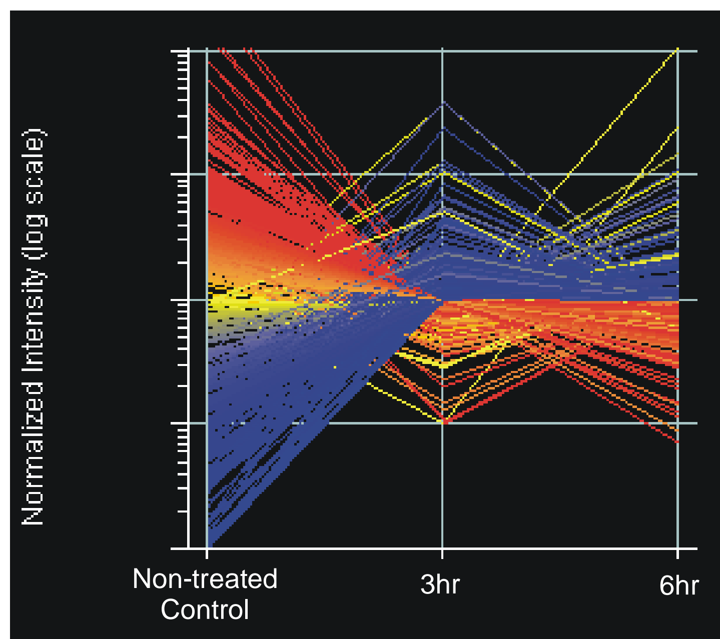

Fig.2. Transcription profiles of 2-fold up- or down-regulating genes, on x axis time points and on y normalised intensity (log base 10) values (GeneSpring output).

Ozone stress affected glutathione–related gene expression. After 3 hours of ozone treatment, compared to non-treated control, we detected a higher transcript level for glutathione synthetase 2 (*GST2*), three genes coding for glutathione transferase and a 2-fold reduction in glutathione reductase (*GR*) abundance. Among these, loci *GST8* and At1g65820 (*GST*) increased their transcription level also after 6 hours of ozone treatment compared to the 3-hour time point. On the contrary, after 6 hours of ozone treatment *GST6* transcript level decreased; however, but still its expression was almost 3 times higher than control level. In addition, exposure to ozone increased the mRNA levels of pyridine nucleotide-disulphide oxidoreductase (12.2 times after 3 hours) and of monodehydroascorbate reductase (more than 8 and 13 times in 3- and 6-hour time points, respectively).

We also observed a constant up-regulation of some genes coding for ascorbate peroxidase isozymes in both time points and two glutathione peroxidases as much as almost 7 times for one glutathione peroxidase (*GPX6*) at 6-hours. Ozone exposure also down-regulates mRNA for chloroplast isoforms of glutathione peroxidase (*GPX1*) and ascorbate peroxidase (*APX4*), slightly more than 2 times after 3 hours and more than 4-fold after 6 hours of treatment.

Tab.1. Genes up-regulated two fold and above in at least one time point.

Genebank	Locus	Description	3hr fold change	6hr fold change
AY088647	At4g11600	glutathione peroxidase, putative *(GPX6)*	4.33	6.98
AY087458	At2g37130	peroxidase, putative *(ATP2a)*	no change	3.26
BZ761857	At2g31570	glutathione peroxidase *(GPX2)*	no change	3.42
	Phenylpropanoid pathway			
BZ384087	At1g09500	putative cinnamyl alcohol dehydrogenase *(CAD)*	2.86	20.18
AV812119	At1g72680	putative cinnamyl-alcohol dehydrogenase *(CAD)*	4.11	8.72
X62747	At2g37040	phenylalanine ammonia lyase *(PAL1)*	8.18	no change
AY088956	At3g53260	phenylalanine ammonia-lyase *(PAL2)*	7.88	no change
BX003958	At3g21230	putative 4-coumarate:CoA ligase 2 *(4-CL2)*	50.49	11.78
AY065145	At2g30490	cinnamate-4-hydroxylase *(C4H)*	4.15	no change
BZ761593	At4g30210	NADPH-ferrihemoprotein reductase *(ATR2)*	2.88	no change
AL764466	At1g67980	caffeoyl-CoA 3-O-methyltransferase, putative *(CCoAOM)*	5.77	9.54
AY133582	At1g51680	4-coumarate:CoA ligase 1 *(4-CL1)*	3.41	no change
BT005826	At1g80820	cinnamoyl CoA reductase, putative *(CCR2)*	55.34	2.81
	Oxidoreductase			
AY120718	At5g16970	quinone oxidoreductase -like protein	8,69	4.22
BZ761914	At5g47910	respiratory burst oxidase protein *(RbohD)*	5.14	2.5
	Catalase			
AY136424	At1g20630	catalase 1 *(CAT1)*	no change	4.2
	Late embryogenesis protein			
AY086267	At4g02380	*SAG21*	6.53	6.44
	Mitogen-activated protein kinase			
BT000007	At3g45640	mitogen-activated protein kinase 3 *(MPK3)*	2.95	no change

Tab.1. continued from previous page.

Genebank	Locus	Description	3hr fold change	6hr fold change
	Glutathione metabolism			
BT000624	At5g27380	glutathione synthetase *(GSH2)*	5.66	3.99
CC054629	At1g78380	glutathione transferase *(GST8)*	2.73	3.67
CB185674	At2g47730	glutathione transferase *(GST6)*	3.35	2.84
AY088618	At1g65820	glutathione transferase *(GST)*	2.64	4.54
AY084651	At3g44190	pyridine nucleotide-disulphide oxidoreductase	12.24	4.63
AY087318	At5g03630	monodehydroascorbate reductase *(MDHR)*	8.65	13.28
	Peroxidase			
BZ352626	At5g05340	peroxidase, putative *(PER52)*	10.85	25.8
AY136364	At4g08770	peroxidase, putative *(PER37)*	23.23	15.03
AY087926	At3g49120	Peroxidase, putative *(PRXCB)*	6.63	10.8
BZ770177	At1g07890	L-ascorbate peroxidase *(APX1)*	3.06	3.02

Tab.2. Genes down-regulated two fold and above in at least one time point.

Genebank	Locus	Description	3hr fold change	6hr fold change
	Catalase			
X64271	At4g35090	catalase 2 *(CAT2)*	2.79	7.57
	Peroxidase			
BZ765698	At2g25080	glutathione peroxidase *(GPX1)*	2.13	4.74
AY089139	At4g09010	ascorbate peroxidase *(APX4)*	2.61	4.41
	Carotenoid biosynthesis			
BT000450	At5g17230	phytoene synthase *(PSY)*	3.62	5.47
	Glutathione metabolism			
D14049	At3g54660	glutathione reductase *(GR)*	2.02	2.98
	Phenylpropanoid metabolism			
AY143880	At2g22990	putative serine carboxypeptidase I	3.63	8.51
AY084729	At1g53520	chalcone isomerase, putative *(CHI)*	3.31	5.33
AY087778	At5g13930	chalcone synthase *(CHS)*	17.56	31.65
	Glycosyl hydrolase			
BZ661669	At3g13750	glycosyl hydrolase family 35	13.91	10.75

Our study differentiates mRNA expression of two catalase genes: *CAT1* transcription increased 4.2-fold after 6 hours of treatment and by contrast *CAT2* mRNA declined approximately 3- and 8-fold after 3 and 6 hours of ozone exposure, respectively.
Transcription of mitogen-activated protein kinase 3 and respiratory burst oxidase protein increased with the highest fold change after 3 hours of ozone treatment. An increase in the transcript level of *SAG21* gene was observed in both time points.

DISCUSSION

Ozone-induced gene expression has been investigated extensively in many plant species [3,21] including *Arabidopsis*. Active oxygen species are also regarded as signalling molecules that initiate transcription of various genes, including those classified as oxidative stress related genes [14,22]. While ozone-modulated oxidative stress responses are widely studied, so far there is only one report of microarray experiment design exclusively to investigate the oxidative stress response upon ozone stress conditions; however, no detailed information has been published yet, thus it is impossible to discuss their data with ours [21]. Ozone stress induced the biosynthesis of plant hormones such as ethylene, salicylic acid and jasmonic acid [1-4]. Macroarray approach has also been used to determine the role of hormonal signalling pathways or cell death regulators in the ozone stress response [12,23] and those aspects of oxidative-stress related transcriptions are not discussed here. Moreover, there is promising report of using cDNA macroarray approach for diagnosis of various environmental stresses in plants, including ozone in field conditions [2].
In the present study we used an *Affymetrix* GeneChip approach to investigate the possible differential transcription of 38 oxidative-stress related genes.
A GeneChip study allows the parallel detection of transcript for approximately 23,000 genes at once with discrimination for members of gene families that are up to 90% identical. Despite the fact that the statistical analyses performed with the Genespring 6.1 software indicated that fold changes as low as 1.5 can be regarded as reliable, our validation results did not confirm absolute change in the 1.3-1.5 range. Therefore, in agreement with previous reports [15,21], we did not regard differences lower than two-fold as significant changes. We considered a two fold induction or repression to be consistent with Q-PCR results and statistically significant at the $P<0.01$ level of significance. Applying those restrictions, we obtained 2385 changes in gene expression in at least one of the time points compared to non-treated control (3hr or 6hr). Analyses, such as Principal Component Analysis (PCA; MaxD software; Manchester Microarray Bioinformatics Group) and outlier detection (dCHIP software; www.dchip.org), confirmed the good quality of the chip experiment and suggested that most changes detected are truly derived from the ozone treatment.
Plant susceptibility to oxidative stress depends on a balance between the production of ROS and of antioxidants. The differential expression of specific

antioxidant enzymes has also been shown to be specific to oxidative stress as opposed to other environmental stresses [6,11,14]. As opposed to previous studies, we used 350 ppb ozone dose, which although it did not trigger visible necrotic changes of the treated plants still induced transcription responses of particular antioxidative enzymes.

Peroxidases are ubiquitous enzymes in plants represented by around 100 different genes in the model organism *Arabidopsis thaliana* [9,11]. Glutathione peroxidase (PXGPX) is a key enzyme in the protection of the membranes exposed to oxidative stress and is inducible under various stress conditions [6,8,9,11]. The function of ascorbate peroxidase is targeted to detoxification of hydrogen peroxide. The induction of both was observed upon many environmental stresses such as ozone, UV, high temperature, salt and cold [9,11,15]. Here we have shown an enhanced transcript level of 9 peroxidase genes upon ozone stress conditions (Tab.1). We detected a differential expression of glutathione peroxidases, ascorbate peroxidases and secretory peroxidase *ATP2a* gene family. In particular, the decline in transcription of the chloroplast isoform of ascorbate peroxidase upon oxidative stress is consistent with previous results [15,19,20], as is an increased mRNA abundance of cytoplasmic peroxidase isoforms [5,20], with the exception of glutathione peroxidase (*GPX6*), which an increase had not been reported yet upon ozone stress conditions. However, the transcript abundance of particular isoforms differed between time points, most exhibiting the highest up- or down-regulation after 6 hours of ozone treatment and this as much as a 25.8-fold up-regulation for *PER52* at the 6hr time point.

The detoxification of H_2O_2 is also mediated by catalases. The *Arabidopsis thaliana* genome encodes 3 catalase genes *CAT1-3*. In plant cells, catalases operate both in the chloroplasts and the cytosol [5-7,18,19]. *Arabidopsis* CAT2 and CAT3 are mostly localised to peroxisomes and CAT1 is known as non-peroxisomal [6]. In previous *Arabidopsis* studies, ozone doses in the range of 150-300 ppb did not influence *CAT2* transcription [1,4,6] and *CAT1* and *CAT3* transcript abundance was uncharacterised [1,6]. Catalases possess a very low affinity with H_2O_2 and their activity is extremely low or not detectable in the cytosol [6]. Our study of catalase activity upon 200-300 ppb ozone treatment analysed by isoelectrofocusing assay (IEF) did not reveal any new catalase isoforms or changes in protein activities (unpublished data), so we did not find any evidences of the postulated posttranscriptional regulation of catalase activity [4,6] upon ozone stress condition. In this paper, we show two different transcription patterns for *CAT2* and *CAT1* genes upon 350 ppb ozone dose [6,14,17]. *CAT1* exhibited higher than 4-fold up-regulation after 6 hours of ozone treatment while *CAT2* transcript level declined approximately 3-fold after 3 hours and 7.5-fold after 6 hours of ozone treatment. In *A. thaliana* acute heat stress reduced *CAT2* transcription and during senescence, all catalase isozymes CAT1-3 had a raised mRNA abundance [16]. Although similar expression pattern for *CAT1* (up-regulation) and *CAT2* (down-regulation) genes in our data could suggest that PCD is induced, we did not observe visible symptoms

indicative of plant death. Moreover, there are no transgenic *Arabidopsis* plants' overexpressing catalase genes or catalase knockouts. Therefore, there is no evidence either of a protective or a detrimental role of catalase upon ozone stress conditions. It is not clear whether the detected transcription profiles for catalase isozymes imply an activated PCD or whether it is derived from a cross-talk between hormonal signalling pathways and instead plays a protecting role against oxidative stress. It remains that this difference is a consequence of 350 ppb ozone dose. Taking this into account, the observed transcription pattern for catalase genes under ozone stress is unexpected when compared to previous results with ozone-induced oxidative stress [1,4,6] and therefore requires further investigation.

Superoxide dismutase (*SOD*) is not discussed in this report, although the SOD responded to the ozone treatment did not pass the $P<0.01$ significance level.

Enhanced lipid peroxidation upon oxidative stress affects membrane properties and activates detoxifying enzymes such as glutathione transferases, glutathione peroxidases and antioxidant molecules such as phenylopropanoids, ascorbate, carotenoids and glutathione [1,3,5,8]. We demonstrated that an ozone treatment increased the transcript level of a vast majority of genes involved in detoxification. As predicted, ozone induces *GST6* and *GST8* localised in chloroplast and cytoplasm, respectively. *GST6* and *GST8* are also induced by others stimuli such as salicylic acid, ethylene, hydrogen peroxide and wounding [1,3,5,8,18,19,24]. This result confirms a protective role for glutathione transferases against the detrimental consequences of ozone. We also detected the induction of the microsomal glutathione S-transferase uncharacterised under ozone stress conditions. We observed an induction of a glutathione synthetase gene (*GSH2*), a rate-limiting enzyme in gluthatione biosynthesis [1,5,8]. This gene reached its highest expression level within 3hr of ozone treatment and had declined by 6hr, which suggested a decrease in the *de novo* glutathione biosynthesis during prolonged ozone treatment. In parallel, we detected an increase in the transcript level of two other cytoplasmatic enzymes involved in glutathione turnover: pyridine nucleotide-disulphide oxidoreductase and monodehydroascorbate reductase (MDHR) that acts to regenerate the active form of glutathione [5]. A decrease in abundance of the chloroplast isoform of glutathione reductase has been linked with a decline in photosynthesis [17] and O_3-induced senescence. As reported recently, enhanced glutathione biosynthesis in tobacco plants potentiates oxidative damage, thus the restriction of glutathione reductase abundance saves NADPH utilisation and protects chloroplast against excessive AOS production [7,17]. Our study revealed that a prolonged ozone treatment decreased the transcription of glutathione biosynthetic genes and increased the transcription of genes involved in glutathione regeneration. It remains unknown whether these transcriptional activities play a crucial role against ozone-induced oxidative stress in *Arabidopsis* plants as well.

We examined in detail the transcription pattern of genes for the phenylopropanoid pathways. Our study revealed various transcription patterns for the genes involved in phenylopropanoid metabolism. As predicted, ozone induced *PAL*, 4-coumarate:CoA ligase and cinnamyl-alcohol dehydrogenase expression [1,2]. *PAL1* transcription is induced by all biotic and abiotic stimuli [1,3,4]. *PAL1* expression and protein activity is also correlated with salicylic acid biosynthesis [3]. In addition to *PAL1*, we have shown induction of *PAL2*, cinnamate-4-hydroxylase (At2g30490) NADPH-ferrihemoprotein reductase (*ATR2*) and 4-coumarate:CoA ligase 1. Those genes are known to be part of the phenylopropanoid biosynthesis pathway and their transcriptional activation translate into an increase abundance of phenolic compounds, including salicylic acid [1,3,4,15]. Other phenylopropanoid pathway-genes cinnamyl alcohol dehydrogenases At1g09500 and At1g72680 and caffeoyl-CoA 3-O-methyltransferase (At1g67980) involved in lignin biosynthesis are up-regulated both within 3 and 6hr of ozone treatment. Among genes that are down-regulated, we identified chalcone isomerase and chalcone synthase; both are connected with flavonoids biosynthesis [14,20] and a putative serine carboxypeptidase I. In agreement with previous results, these expression profiles confirm that not only phenylopropanoid biosynthesis is activated, but also lignin biosynthesis. Flavonoids and anthocyanins exhibit scavenger capacity to protect lipid and membrane integrity under stress conditions [7,14,18]. In this study, the transcription of 2 genes involved in flavonoid biosynthesis drastically decreased, however a detailed analysis of flavonoid content should be carried out to confirm a decline or any change in these metabolites concentration. This is the first report showing transcriptional analysis of genes involved in phenylopropanoids turnover in ozone stress conditions.

In conclusion, the microarray approach undertaken has proven to be a powerful tool to study oxidative stress response upon ozone treatment. Although genes chosen for this analysis are itself markers of oxidative stress and does not explain all aspects of ozone stress induced response our transcript profiling revealed new ozone stress-related genes and suggest a differentiated and time-coordinated response between members of the same multigene family. However, many aspects of this work still require further analysis. Nevertheless, this study already brings into consideration the role of particular antioxidants in the ozone-induced oxidative stress response in *Arabidopsis thaliana* plants.

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