This is a pre- or post-print of an article published in

Hamisch, D., Randewig, D., Schliesky, S., Bräutigam, A., Weber, A.P.M., Geffers, R., Herschbach, C., Rennenberg, H., Mendel, R.R., Hänsch, R.

Impact of SO_2 on Arabidopsis thaliana transcriptome in wildtype and sulfite oxidase knockout plants analyzed by RNA deep sequencing

(2012) New Phytologist, 196 (4), pp. 1074-1085.

Impact of SO_2 on *Arabidopsis thaliana* transcriptome in wildtype and sulfite oxidase knock-out plants analyzed by RNA deep sequencing

Domenica Hamisch[1], Dörte Randewig[2], Simon Schliesky[3], Andrea Bräutigam[3], Andreas P. M. Weber[3], Robert Geffers[4], Cornelia Herschbach[2], Heinz Rennenberg[2,5], Ralf R. Mendel[1], and Robert Hänsch[1]*

[1]Institut für Pflanzenbiologie
Technische Universität Braunschweig
Humboldtstraße 1
D-38106 Braunschweig
Germany

[2]Institut für Forstbotanik und Baumphysiologie
Professur für Baumphysiologie
Albert-Ludwigs-Universität Freiburg
Georges-Köhler Allee 53/54
D-79085 Freiburg
Germany

[3]Institut für Biochemie der Pflanzen
Heinrich-Heine-Universität
Universitätsstraße 1
D-40225 Düsseldorf
Germany

[4]Helmholtz Centre for Infection Research
Genome Analytics
Inhoffenstrasse 7
D-38124 Braunschweig
Germany

[5]King Saud University
P.O.Box 2454
Riyadh 11451
Saudi Arabia

*author for correspondence
Tel. +49 (0) 531 391 5867
Fax. +49-(0) 531 391 8128
e-mail: r.haensch@tu-bs.de

Total word count (6739)
Introduction (680), Materials and Methods (1169), Results plus Discussion (4820), and Acknowledgements (70)

Number of figures: 4 (figures 2 to 4 should be published in color),
Number of tables: 0
Number of supplemental figures: 5
Number of supplemental tables: 5

Summary

- High concentrations of SO_2 as an air pollutant, and its derivative sulfite, cause abiotic stress that can lead to cell death. It is currently unknown to what extent plant fumigation triggers specific transcriptional responses.

- To address this question, and to test the hypothesis that sulfite oxidase (SO) is acting in SO_2 detoxification, we compared Arabidopsis wildtype (WT) and SO knock-out lines (SO-KO) facing the impact of 600 nL L^{-1} SO_2, using RNAseq to quantify absolute transcript abundances. These transcriptome data were correlated to sulfur metabolism-related enzyme activities and metabolites obtained from identical samples in a prior study.

- SO-KO plants exhibited remarkable and broad regulative responses at the mRNA level, especially in transcripts related to sulfur metabolism enzymes, but also in those related to stress response and senescence. Focusing on *SO*-regulation, no alterations were detectable in WT, while in SO-KO plants we found up-regulation of two splice variants of the *SO*-gene, although this gene is not functional in this line.

- Our data provide evidence for the highly specific co-regulation between SO and sulfur-related enzymes like APS reductase, and suggest two novel candidates for involvement in SO_2 detoxification: An apoplastic peroxidase, and defensins as putative cysteine mass storages.

71**Keyword index**

72Sulfite oxidase

73SO_2 fumigation

74RNA-deep-sequencing

75Arabidopsis knock-out mutants

76Sulfate assimilation

77Sulfite detoxification

78Gene ontology

79Cluster Analyses

4

Introduction

Sulfur is an essential nutrient for plant growth. Assimilatory reduction of soil-available sulfate is the main pathway of sulfur acquisition (Rennenberg, 1984), but plants can also use atmospheric SO_2 gas as additional sulfur source (De Kok et al., 2007). If, however, atmospheric SO_2 exceeds a critical threshold level it becomes toxic for the plant and causes irreversible injury. Toxicity of sulfite strongly depends on dosages of SO_2, susceptibility of the plant species, and physiological and environmental factors (Bell, 1980; Ayazloo & Bell, 1981; Rennenberg, 1984; Alscher et al., 1987; De Kok, 1990). Plants as sessile organisms have evolved several protection mechanisms: (i) the cuticle, which functions as first barrier for toxic gases, largely restricting the pathway for influx to the stomata (Tamm & Cowling, 1977); (ii) active stomatal closure, reducing SO_2 uptake (Rao & Anderson, 1983), and mesophyll resistances to, mainly determined by metabolism of sulfite, which adjust SO_2 flux into leaves (Pfanz et al., 1987), and (iii) active detoxification of sulfite or bisulfite. These ions are metabolized within the plant either by feeding into sulfur assimilation, to form cysteine and other sulfur compounds (Filner et al., 1984; Heber & Hüve, 1998), or by oxidation to sulfate by non-enzymatic (Rennenberg, 1984) or enzymatic processes (Pfanz et al., 1990; Eilers et al., 2001). Sulfite conversion to sulfate is catalyzed by the enzyme sulfite oxidase (SO) (Eilers et al., 2001; Hänsch et al., 2006). Loss of SO activity impairs the plant´s ability to survive upon SO_2 exposure; conversely, overexpression of SO helps the plants to withstand even toxic SO_2 concentrations (Brychkova et al., 2007; Lang et al., 2007; Randewig et al., 2012).

Recently, we used *A. thaliana* wildtype (WT) and SO knock-out (SO-KO) plants to decipher in detail responses to SO_2 fumigation in leaf rosettes (Randewig et al., 2012). We identified the significance of SO for the overall shoot response to SO_2 in

5

relation to alterations in plant phenology and physiology (gas exchange, metabolites and enzyme activities involved in assimilatory sulfate reduction). SO-KO and WT plants were exposed to SO_2 dosages that are known to be non-toxic to WT plants (Randewig et al., 2012). Effects on sulfite detoxification and sulfur assimilation, particularly metabolic co-regulation of enzymes involved in sulfur assimilation, were compared. SO_2 exposure caused a significant increase in sulfate and GSH pool in wildtype Arabidopsis. Conversely, in KO plants the sulfate pool was kept constant, but thiol-levels were strongly increased (14fold for cysteine). Moreover, these metabolic changes were connected with a strong regulation of adenosine 5'-phosphosulfate reductase (APR) activity, the key enzyme of sulfate assimilation (Kopriva & Rennenberg, 2004). Based on these results we suggested a tight co-regulation of SO and APR, thus controlling the sulfate assimilation pathway and stabilizing sulfite distribution.

Next, we conducted transcriptome analyses and followed a twofold strategy: The comparison of WT *versus* SO-KO plants before and after SO_2 fumigation would permit (i) a comprehensive analysis of the transcriptional regulation of sulfur metabolism, and (ii) the deciphering of more complex and far reaching reactions of the plant beyond sulfur metabolism. We hypothesized that sulfur metabolism in response to SO_2 is at least partially regulated at the transcriptional level and that an unbiased transcriptome analysis would permit the identification of unknown genes involved in the SO_2 response. For transcriptional profiling, sequencing-based techniques (RNA-deep-sequencing, RNAseq) offer numerous advantages over microarrays, such as (i) a larger and more quantitative dynamic range of the experiment; (ii) the ability to estimate absolute transcript numbers, and therefore (iii) the opportunity to perform more accurate quantification of relative changes in transcript numbers. In the present paper we provide a detailed analysis of

6

consequences of fumigation with approx. 600 nL L^{-1} SO_2 for 60 hours - a non-toxic dosage for *A. thaliana* wildtype plants - and compare the effect on WT and SO-KO plants. Fortunately, we were able to use plant samples which had already been analyzed in a previous study (Randewig et al., 2012) permitting us to compare the transcriptome of WT and SO-KO plants under ambient and elevated SO_2 conditions with sulfur metabolite levels, a set of enzyme activities, and physiological data.

Materials and Methods

Plant material

For RNAseq experiments *A. thaliana* plant samples of two different genotypes were used: *A. thaliana* (L.) *Heynh.* ecotype Columbia (WT plants) and transgenic SO knockout plants (SO-KO, GABI-Kat T-DNA insertion line (850B05) generated within the GABI-Kat programme (Rosso et al. 2003) kindly provided by Bernd Weisshaar (MPI for Breeding Research, Cologne, Germany)). Plantlets were grown in 500 mL plastic boxes at 22/20°C day/night (8 h photoperiod) in controlled environmental chambers (HPS 1500, Voetsch Industrietechnik GmbH, Balingen, Germany). Eight-week-old plants were used for fumigation with 600 ± 15 nL L^{-1} SO_2. Four pots, each with four plants - WT and SO-KO - were placed separately into a single enclosure for 6 hours. Three hours after the beginning of the dark period in the second night, SO_2-exposure was started and finished after 60 h. This treatment was reproduced with a new set of plants at least three times (for more details see Randewig et al., 2012).

Total RNA extraction and mRNA purification

Total RNA for WT, WT[+], SO-KO and SO-KO[+] plants was isolated using the NucleoSpin® RNA Kit (Macherey-Nagel, Düren, Germany). For each genotype/

treatment 10 samples (each consisting of two plants randomly chosen from the three independent fumigation experiments) of 100 mg powdered plant tissue were separately used for total RNA isolation according to the manual (exception: elution was performed by using two times 20 µL of RNAse-free H_2O). Total RNA preparations of ten samples per probe set (WT, WT[+], SO-KO and SO-KO[+]) were pooled. Dynabeads® Plant Oligo$(dT)_{25}$ (Invitrogen Life Technologies GmbH, Darmstadt, Germany) were used for final mRNA purification according to the manual. Quality of total RNA and isolated mRNA was checked using the Agilent 2100 Bioanalyzer RNA chip (Agilent Technologies, Böblingen, Germany). A sequencing library for RNAseq was created from 3 µg of mRNA using the SOLiD Whole Transcriptome Analysis Kit (Applied Biosystems, Carlsbad, California, USA). Thereafter emulsion PCR was performed using SOLiD EZ bead kits. The resulting bead library was divided into three aliquots, loaded in separate flow cells and sequenced for 50 bp on an ABI SOLiD 5500XL system (Applied Biosystems, Carlsbad, California, USA). Using CLC workbench (CLC bio, Mühltal, Germany), transcriptome reads were aligned to whole genome sequences from the TAIR10 *A. thaliana* database (www.arabidopsis.org).

RNAseq Data Analyses

Reads were exported as color-space FASTA (filename.csfasta) and the according quality (filename.qual) files and afterwards imported to CLC Genomics Workbench (CLC bio, Mühltal, Germany) using the NGS-import function. Thus erroneous reads were cropped at the position of the error. Alignment and expression values as RPKM were obtained using the “RNAseq Analysis” feature of CLC Genomics Workbench. RPKM are defined as reads per kilobase of exon model per million mapped reads with the following equation:

8

$$RPKM = \frac{total\ exon\ reads}{mapped\ reads\ (millions) \cdot exonlength\ (kb)}.$$

All four libraries were analyzed separately using standard parameters: i.e. minimum length = 90%, minimum similarity = 80%, maximum number of hits for a read = 10, use color space = yes, type of organism = eukaryote. The reference was set to annotated *A. thaliana* chromosomes from the TAIR10 release Jun/20/2009. The gene expression values were exported for further analysis.

DEGseq (Wang et al., 2010)

Identification of differentially expressed genes was done using the R-Package "DEGseq". This package allowed statistical analysis despite the lack of technical replicates. The underlying algorithm projects a random sampling model to the expression data to estimate the variance and calculates p-values based on this estimation. As input RPKM for each gene were provided. The parameters were set to non-default values: method = "MARS", normal Method = none (Table S5: DEGseq script for R). For each pair of input files, DEGseq provides a list of p-values to determine significantly differentially expressed genes. Significant expression was called if the uncorrected p-value was below alpha = 0.001 (corresponding to Benjamini Hochberg false-discovery rate corrected p-values below 0.014 for SOKO control *vs.* treated, below 0.017 for WT treated *vs.* SOKO treated, below 0.029 for WT control *vs.* SOKO control and below 0.087 for WT control *vs.* WT treated). These genes were marked using the verbalization "TRUE"; those remaining were tagged as "FALSE".

GeneSpring GX

Gene expression values from the RNAseq experiment were used within the GeneSpring GX software version 11.5 (Agilent Technologies, Waldbronn, Germany).

9

There were two data sets used within the studies: (i) absolute expression data and (ii) log scaled data.

Absolute, normalized data, which were not log scaled and not processed using baseline transformation to the median of all sample data, were necessary to get a deeper view into the transcription amounts of different genes. For this purpose raw data were prepared in Gene Spring GX 11.5 pointing out that they were already log scaled which resulted in cutting extremely low data values using the 20^{th} to 100^{th} percentile normalization but no baseline transformation. The resulting data were absolute expression data and used for detailed analysis of transcript abundances involved in the sulfur metabolism. The detailed view into absolute transcript data is applicable for each requested gene and associated splice variants which is the primary advantage of using RNAseq data compared to microarrays were transcript abundances are always given in relation to several control genes and not taken individually.

To prepare **log scaled data** RPKM values were processed using the Gene Spring GX 11.5 generic single color experiment according to the manual. After the normalization step, experiment data were grouped as Genotype - Treatment: WT/SO-KO - control/treated with 600 nL L^{-1} SO_2. To identify genes which show differences between treated and control samples or samples with different genotypes, expression ratios (**fold-changes**) were calculated in the following way:

$$Expression\ ratio = Fold\ change = \frac{Condition\ \mathbf{1}}{Condition\ \mathbf{2}}.$$

Ratios below or above a determined cutoff show that these genes are x-fold up- or down-regulated taking both compared conditions into consideration. Four different pairs of conditions were used within the fold-change analyses: (1) WT *vs*. WT[+], (2) SO-KO *vs*. SO-KO[+], (3) WT *vs*. SO-KO and (4) WT[+] *vs*. SO-KO[+]. Fold-change =

10

five and DEGseq verified data were used to get deeper insights into regulation of other processes beyond sulfur metabolism.

Using the Gene Spring GX 11.5 **Cluster analyses** tool, a hierarchical clustering was performed for data threshold with fivefold-change and verified with DEGseq. Hierarchical clustering was carried out on entities (differentially regulated genes) and conditions (different genotypes and treatments) using combined trees. Merging of entities in different clusters is controlled by applying a certain linkage rule; here we used “complete”. Cluster entities were colored according the numeric values of the normalized, log 2 scaled data. Expression profiles from each of the eight identified clusters were generated; transcripts belonging to the different clusters were exported and further used for GO analyses.

The **Gene ontology** database (GO, www.geneontology.org) describes connections of gene expression data to defined GO terms. Using the Gene Spring GX 11.5 GO analysis tool entities of interest obtained from one experiment can be explored, finding matching GO terms. Output of GO analysis is a tree containing GO terms enriched with a p-value cutoff at 0.1. Transcripts belonging to the different clusters (I to VIII) defined in the cluster analyses were used for GO analysis.

Results and Discussion

General view of the Arabidopsis transcriptome under SO_2 fumigation

A total number of 22,130 genes, including their different splice variants, were identified for wildtype plants (WT) in this experiment, 23,232 for fumigated WT (WT[+]), 22,424 for sulfite oxidase knock-out plants (SO-KO) and 22,255 for fumigated SO-KO (SO-KO[+]). Quantile normalized, log 2 scaled and not–baseline-transformed RPKM of these transcripts were widely spread (Fig. S1). Each analyzed sample consisted of 10 independently prepared RNAs from, in summary, 20 treated

11

plants. The highest spreading of RPKM, and therefore most alterations in transcripts were detected for WT *vs.* SO-KO[+], followed by SO-KO *vs.* SO-KO[+]. A narrower distribution, implicating a less strong response to SO_2 or the genotypic modification, was identified in WT *vs.* WT[+], WT *vs.* SO-KO and SO-KO *vs.* WT[+]. Figs. 1a and 1b present the amounts of **differentially expressed genes** in relation to the total number of transcripts (different splice variants included) using fivefold cut data, verified with DEGseq (Table S1). At the fivefold-threshold, between 0.4% and 1.6% of genes were differentially expressed for all condition pairs. Approximately 60% of the differentially expressed genes were up-regulated (Fig. 1a), whereas approx. 40% were down-regulated (Fig. 1b).

Venn diagrams (Figs. 1c, 1d) were created to answer several hypotheses concerning the biological evidence of the genotypic variation in SO-KO and effects caused by SO_2 treatment. The Venn diagrams show the number of fivefold up- (Fig. 1c) or down-regulated (Fig 1d) transcripts, respectively, which were mutually found in different treatment/genotype combinations and which of those genes were differentially expressed in different condition pairs.

First we asked if knocking out the *SO* leads to the same alteration in transcripts and transcript numbers as the fumigation of WT does. In this case there should exist more transcripts in the overlap of WT *vs*. WT[+] and WT *vs*. SO-KO and fewer in the section of solely regulated transcripts. We identified 11 transcripts for up-regulation and 10 for down-regulation in the overlapping section. For the genotypic comparison we found 102 transcripts up- and 65 transcripts solely down-regulated, as there were 44 upregulated and 29 downregulated transcripts for WT *vs*. WT[+]. These findings negated our hypothesis that the gene expression changes caused by a SO knockout were similar to the gene expression changes caused by SO_2 fumigation. This result implied the second assumption that we should not find any intersections for WT *vs*.

12

SO-KO and SO-KO *vs*. SO-KO[+], because *SO* gene knock-out and fumigation of SO-KO should not have any regulated transcripts in common. Interpretation of our data validated this expectation. Third - SO is predicted to play a key role in SO_2 detoxification, therefore we hypothesized a higher number of regulated transcripts in SO-KO[+] compared to WT[+], since the knockout of *SO* inhibits SO-mediated SO_2 protection. This may further induce other processes. Our findings of 45 up- and 38 down-regulated transcripts in WT *vs*. WT[+] and 287 up- and 161 down- regulated transcripts in SO-KO *vs*. SO-KO[+], respectively, confirmed our hypothesis. Only 10 transcripts for up- and one for down-regulation were identified as commonly regulated. Fourth - we expected that SO-KO plants have to use different defense mechanisms to detoxify SO_2 than WT plants. Therefore we should find only very few transcripts which were commonly up- or down-regulated in WT *vs*. WT[+] and WT[+] *vs*. SO-KO[+]. Transcripts in the overlap represented genes which were already highly regulated in WT[+]and even more highly in SO-KO[+]. This was a small common transcript set due to the different transcript usages of WT[+] and SO-KO[+] during fumigation. For up-regulation we identified two transcripts: AT5G44420, which belongs to the plant defensin family (plant defensin 1.2), and AT3G44310, encoding the nitrilase 1. The genotypic comparison (WT *vs*. SO-KO) marked the defensin as an unregulated transcript (1.19fold); while the nitrilase was significantly regulated (3.97fold) in SO-KO *vs.* SO-KO[+], but this was not visible in the fivefold comparison. For down-regulation there was no transcript detectable in the intersection. Verification of this hypothesis led to the fifth expectation that we should find more genes regulated in SO-KO *vs.* SO-KO[+] compared to WT[+] *vs.* SO-KO[+], but overall a high number of commonly regulated transcripts. Counting of transcripts for SO-KO *vs.* SO-KO[+] revealed 297 up- and 162 down-regulated transcripts. For WT[+] *vs.* SO-KO[+] we found 276 up- and 170 down-regulated transcripts, 249

13

transcripts were commonly regulated in SO-KO *vs*. SO-KO[+] and WT[+] *vs*. SO-KO[+] and 408 transcripts were solely regulated in total. These results confirm a high number of commonly regulated transcripts (50%), but calculations did not verify our hypothesis of a much higher percentage of regulated genes in SO-KO *vs*. SO-KO[+] compared to WT[+] *vs*. SO-KO[+].

To get further insights about the molecular mechanisms affected by knocking out *SO* we identified differentially expressed genes by comparing WT and SO-KO with and without SO_2 fumigation. Significantly regulated genes with a transcriptional change of more than fivefold were selected and significance was determined with the DEGseq tool. We applied **hierarchical clustering** (Fig. 2a) to further delineate associated gene groups with similar expression profiles (Fig. 2b). Most of the transcriptional changes were induced after SO_2 fumigation in the SO-KO mutant plants, represented by the largest clusters IV and VII, respectively. In total we were able to identify eight individual gene expression clusters numbered from I-VIII. For further analyses we used the TOP 20 of regulated transcripts and **Gene ontology** (GO) analyses for each identified cluster.

SO_2 application to WT plants should lead to several transcriptional changes, but due to the used dosage of 600 nL L-1 and SO acting as detoxificating enzyme we hypothesized a lesser reaction than we would expect for SO-KO treatment. GO analysis of fivefold regulated transcripts in WT *vs*. WT[+] fitted this expectation by revealing the lowest number of GO terms. Transcripts could be assigned to the categories BIOLOGICAL PROCESS (16 genes, 36%) and CELLULAR COMPONENT (29 genes, 64%). We identified up-regulated transcripts for WT *vs*. WT[+]in cluster I, II, and III; down-regulation was detected in cluster V, VI and VIII. TOP 20 transcripts in cluster II included four transcripts associated with ribosomal and translation processes, respectively, which indicated an influence of SO_2 fumigation on mRNA

14

synthesis regulation. Cluster II contained transcripts which showed highest transcript abundances only in WT[+] and which therefore described a moderate reaction of WT plants to SO_2 with transcriptional adaptation and thus up-regulation of transcripts belonging to ribosomal processes.

Treatment of SO-KO plants with SO_2 should hypothetically lead to higher and different transcriptional responses compared to treated WT plants. Venn diagrams already verified this. Additionally GO analyses of fivefold regulated transcripts showed the highest numbers of significantly enriched transcripts ($p<0.1$) in SO-KO *vs*. SO-KO[+] (Fig. S2) which was additionally obvious in the KEGG analyses (Fig. S3). GO terms enriched in SO-KO *vs.* SO-KO[+] were principally associated to the terms MOLECULAR FUNCTION and BIOLOGICAL PROCESS. Up-regulation of transcripts was identified in cluster III, IV, V and VI; down-regulation in cluster I and VII. Transcript functions for up-regulation in SO-KO *vs*. SO-KO[+] differed from those identified as up-regulated for WT *vs*. WT[+]. TOP 20 of WT[+] showed most transcripts associated to transcriptional regulation (cluster I, II), whereas SO-KO[+] TOP 20 presented transcripts involved in defense processes (cluster IV) and peptidase activity (cluster VI). TOP 20 of cluster IV contained nine transcripts associated to defense including defensins (AT5G44420, AT5G44430, AT2G26020 and AT2G26010), GSTs (AT1G02930, AT1G02920, AT4G02520) and Peroxidase CB (AT3G49120).

Transcriptional regulation of enzymes related to sulfur metabolism: Effects due to SO_2 and/or genotypic variation of SO knock-out

We recently reported enzyme activities and S-metabolites of *A. thaliana* wild type and transgenic lines subjected to SO_2 fumigation (Randewig et al., 2012). Aliquots of the same plant material were used in this study for the RNAseq experiment. This

15

combination of transcriptome data with enzyme activities and metabolite levels provided new insights into the regulation of sulfate assimilation and related metabolic pathways. We hypothesized that excess SO_2, especially in the absence of SO, will lead to transcriptional down-regulation of the enzymes producing sulfite and transcriptional increases in at least some enzymes mediating reactions downstream of sulfite to sequester the excess organic sulfur produced. The following description and discussion of the results is summarized in Fig. 3, which presents the regulation of genes for SO-KO *vs.* SO-KO[+]. Raw data and fold change levels are presented in Table S2.

Sulfate is taken up by the root and transported *via* the xylem stream into the leaves for further assimilation. Required **sulfate transporters (SULTR)** are divided into sub-families on the basis of their protein sequence similarities ((Hawkesford, 2003), for review see (Davidian & Kopriva, 2010)). With the exception of SULTR3;1, none of the sulfate transporters were significantly regulated in leaves as analyzed by the DEGseq-tool. However, from the group two of the SULTR, responsible for long distance transport and localized in xylem parenchyma cells, *SULTR*2;1 transcript abundances were similar in the non-fumigated plant material but increased in WT[+] by 30% or decreased in SO-KO[+] by 50%. Moreover, the expression of *SULTR*2;2 was down-regulated threefold in SO-KO plants in the fumigation experiment, which possibly reflects a reduction in sulfate uptake and transport. From the SULTR of group four - suggested to function in vacuolar sulfate remobilization to the cytoplasm in roots and leaves (Kataoka et al., 2004) - *SULTR*4;2 mRNA amounts were two- and 10fold decreased during fumigation in WT and SO-KO rosettes, respectively. This led us to the hypothesis that particularly in SO-KO, sulfite cannot be oxidized to sulfate and, hence, there is no requirement for sulfate to be introduced into the assimilatory stream *via* SULTR2 and SULTR4.

16

For assimilation, sulfate has to be activated by the **ATP sulfurylase (APS),** which catalyzes the first step in this pathway. Determination of *APS* transcript abundances revealed that *APS1* was the most abundant (between 244 and 349 quantile normalized RPKM; Table S2) and the only isoform which is significantly down-regulated (1.3fold) in SO-KO[+] and when comparing WT[+] and SO-KO[+]. This supported the hypothesis that sulfate reduction is down-regulated transcriptionally if excess SO_2 is present. The activated sulfate is partly converted into PAPS by one of the four isoforms of **APS kinase (APK)**. Compared to all other samples, a significant decrease (two- to threefold) of *APK1-3* mRNA was detected only in SO-KO[+] plants. Plants possess large numbers of **sulfotransferases (SOT)** that are responsible for sulfonation of small molecules by using PAPS, cysteine, or other reduced S-compounds, as an important component of plant stress responses (Klein & Papenbrock, 2004). SOTs thus can sequester organic sulfur. Fumigation of SO-KO led to an almost 13fold increase of *SOT12* transcript amounts. *SOT12* is known to be stress-inducible and has been described to confer pathogen resistance in *A. thaliana* by sulfonation of salicylic acid (Baek et al., 2010).

The majority of activated sulfate is metabolized further on by **APS reductase (APR).** Three isoforms described in the literature are localized in the chloroplast. APR is known to be the key enzyme of the sulfate assimilation pathway (Kopriva & Rennenberg, 2004) and is regulated transcriptionally and post-translationally, respectively (Kopriva & Koprivova, 2004). Our data confirm these findings: In WT plants, 600 nL L^{-1} SO_2 did not change the transcript abundances of any APR isoform or splice variant. The enzyme activity decreased significantly (Randewig et al. 2012), presumably due to feed-back inhibition (Vauclare et al., 2002). In SO-KO control plants *APR1, APR2 and APR3* transcripts were increased significantly compared to WT control samples (Table S2). Fumigation of these SO-KO plants led to a dramatic

17

decrease both at transcript abundances (Table S2) and enzyme activity levels (Randewig et al., 2012). The strong down-regulation of APR reflects a tight control of sulfite synthesis at the transcriptional level as well as the posttranslational level. Such a negative feedback inhibition of APR mediated by increasing amounts of thiols (Vauclare et al., 2002) has been discussed previously (for review see (Kopriva & Koprivova, 2004; Davidian & Kopriva, 2010)). Moreover GSH itself is involved in cell proliferation of cell cultures and lateral roots of Arabidopsis (Vivancos et al., 2010) as well as in meristem development and embryo maturation of Brassica (Stasolla et al., 2008) by changing the transcript amounts of definite genes. This has also been proposed by (Szalai et al., 2009) for abiotic stress conditions either *via* H_2O_2 or GSH/GSSG in general.

Sulfite reductase (SiR) converts sulfite into sulfide and is a single copy gene. **O-acetylserine(thiol)lyase (OASTL, OASx, CYSC1)** and **serine acetyltransferase (SAT, Seratx;x)** together form the cysteine synthase complex (Wirtz et al., 2010) which produces organic sulfocompounds from sulfide (Fig. 3). For *SIR* transcripts no significant regulation was observed for WT and SO-KO plants after fumigation. OASTL and SAT enzymes occur as different isoforms, which are located in several cellular compartments. OASB and Serat2;1 are localized in chloroplasts, Serat2;2, OASC and CYSC1 were described to act within the mitochondria and OASA1, OASA2, Serat1;1, Serat3;1 as well as Serat3;2 are cytosolic enzymes (Jost et al., 2000; Yamaguchi et al., 2000; Kawashima et al., 2005). We found that after fumigation of SO-KO only transcripts encoding the chloroplastidic *SERAT2;1* and mitochondrial *CYSC1* were significantly increased. This provokes the hypothesis that S-assimilation and therefore cysteine synthesis is possibly induced after fumigation. In summary, *OASTL* and *SAT* transcript abundances showed that additional sulfur

18

was channeled into the direction of cysteine production after SO_2 fumigation, which held true for both fumigated WT and SO-KO plants, respectively.

Organic sulfur may flow towards methionine *via* cystathione or towards gluthathione. **Cysteine gamma-synthase (CGS/MTO1)** is involved in the conversion of cysteine into cystathionine. *CGS* sequencing data did not show any alterations after fumigation regarding WT and SO-KO samples. **Cystathionine beta-lyases (CBL and CORI3)** are involved in the conversion of cystathionine into homocysteine. For SO-KO and SO-KO[+], significantly lower transcript amounts (roughly fivefold) were found for all of the three *CORI3* splice variants. Moreover, after fumigation of WT plants *CORI3* transcripts were up-regulated. *CGS* was expressed at a higher level than *CBL* (Table S2). RNAseq data showed only minor alterations in **methionine synthase (MS)** and **S-adenosylmethionine synthetase (SAM-synthetase)** transcripts. MS converts homocysteine into methionine, which could be used by SAM-synthetase to generate S-adenosylmethionine as methyl group donor in numerous transmethylation reactions (Peleman et al., 1989). Only *MS2* displayed a twofold decrease in SO-KO[+] compared to SO-KO, and a threefold decrease comparing WT *vs.* WT[+]. Based on the transcriptional profile, the excess SO_2 did not flow towards methionine.

Gamma glutamylcysteine synthase (γ-ECS) catalyzes the first step in glutathione (GSH) biosynthesis. We identified a small increase in γ-*ECS* transcript amounts for SO-KO[+]. **Glutathione synthetase (GSHS)** produces GSH from γ-glutamylcysteine and glycine. *GSHS* transcripts showed an average level of 30 RPKM for WT, WT[+] and SO-KO. Transcript abundances for SO-KO[+] were significantly higher (2.6fold) compared to other samples, indicating that the higher amounts of produced γ-glutamylcysteine are converted into GSH. **Glutathione reductase (GR)** converts oxidized GSSG into reduced GSH. GR1 had enhanced transcript amounts, especially

19

in SO-KO[+]. Transcript abundances of SO-KO and SO-KO[+] were higher than those measured for WT and WT[+]. Higher amounts of *GR1* transcripts in SO-KO[+] samples may indicate that higher amounts of GSSG have to be reduced back to GSH during the SO_2 fumigation process. Based on the transcriptional profile, the excess SO_2 flowed towards glutathione. These transcriptional up-regulations of several enzymes in GSH biosynthesis fitted well with the increased amount of γ-glutamylcysteine and GSH measured in these samples previously (Randewig et al., 2012) and confirmed the hypothesis of an enhanced S-flux into the S-assimilation stream. However, accumulation of GSH above a specific threshold could be dangerous to plant cells causing increased oxidative stress in tobacco (Creissen et al., 1999) and affects photosynthesis, growth and sulfur metabolism in poplar (Herschbach et al. 2010). Moreover, GSH is demonstrated to be the sulfur donor in the biosynthesis of glucosinolates in Arabidopsis (Schlaeppi et al. 2008, Geu-Flores et al., 2011). But in our investigation, transcript data of the key enzymes processing GSH conjugates into glucosinolate and camalexin pathways (γ-glutamyl peptidases GGP1 (AT4G30530) and GGP3 (AT4G30550)) were not altered or even slightly decreased (Table S1) suggesting that formation of glucosinolates was only a minor sink for excess sulfur as also shown in previous studies with *Arabidopsis* (Van der Kooij et al. 1997). Therefore a supplemental mass storage for the reduced sulfur should be postulated.

Glutathione S-transferase (GST) proteins are arranged in different subfamilies GSTF, GSTL, GSTT, GSTU and GSTZ (Frova, 2003). In all GST subfamilies isoform transcripts seemed to be regulated after fumigation with SO_2 which was especially obvious for SO-KO[+], with an up-regulation of 10fold and higher. *GST6* (AT1G02930) was 10.5fold up-regulated in response to the fumigation stress.

20

In WT plants, excess sulfite can be detoxified by oxidation to sulfate (Fig. 3). **Sulfite oxidase (SO)** counteracting the APR is supposed to be the most effective tool within the plant cell to remove excess amounts of sulfite (Brychkova et al., 2007; Lang et al., 2007; Randewig et al., 2012). As shown very recently using microarrays, fumigation of grape berries with 1 – 3 µL L^{-1} SO_2 surprisingly resulted in a decrease of *SO* transcripts (Giraud et al., 2012). In our RNAseq-experiment, transcript numbers of all three different SO splice variants were determined. SO splice variant 1 (*SO-1)* showed highest transcript amounts for WT and WT[+] (60 and 57 normalized RPKM) in comparison to the two additional splice variants (0.07 and 0.16 normalized RPKM for *SO-2* and 0.2 and 0.45 normalized RPKM for *SO-3*). SO-KO plants lacked detectable amounts of SO protein, as determined by immuno-blot analysis, due to the T-DNA insertion within this gene (Lang et al., 2007); consequently, no SO activity was detectable (Randewig et al., 2012). Activity measurements applied in Randewig et. al, (2012) showed no alterations in SO activity for WT[+]. RNAseq confirmed this result (Table S2). Surprisingly, RNAseq data showed that in SO-KO the *SO* transcripts were detectable. However, a closer look into the sequencing data (i.e, mapping of sequence reads to Arabidopsis mRNA sequences) showed that SO-KO did not produce a functional transcript. Although the reading frame for the transcript is disrupted due to the T-DNA insertion and the resulting mRNA is thus non-coding, the transcriptional response apparently attempts to enhance SO production (Figs. S4, S5): For *SO*-1 we detected 2.5fold and for *SO*-2 approx. 16fold increased transcript amounts after fumigation with 600 nL L^{-1} SO_2 for 60 hours. At the moment, the physiological relevance of the different splice variants is unclear. The current interpretation of *SO*-1 and *SO*-2 abundances could only be alternative splicing as known from other eukaryotic systems (Graveley, 2005; Smith, 2005).

21

Transcriptional regulation of biological processes beyond sulfur metabolism

We hypothesized that additional lines of defense against SO_2 and additional consequences of SO_2 poisoning could be deduced from the global transcriptome analysis (see also Figs. S3, S4). Therefore RNAseq supported the development of a new regulation model (Fig. 4) based on transcript data, explaining plant reactions facing excess SO_2. Investigations of regulatory mechanisms beyond sulfur metabolism were based on transcripts enriched in specific gene groups or processes for the 717 genes identified as fivefold regulated in at least one of the comparison pairs WT *vs*. WT[+], SO-KO *vs*. SO-KO[+], WT *vs*. SO-KO and WT[+] *vs*. SO-KO[+].

Photosystem components: Several studies describe the down regulation of photosystem components after application of stresses, leading to an inhibition of energy production, increased oxidative stress (Chaves et al., 2009) and the activation of catabolic processes. Comparing the genotypic changes between SO-KO and WT, different components of the photochemical apparatus are down regulated, but here the limit is a twofold down regulation. However, the analysis of SO-KO data after fumigation shows an even stronger down regulation of these transcripts, reaching threefold changes and a higher number of regulated genes. The influence on photosynthesis was also stated in Randewig et al. (2012). The CO_2 assimilation rate was almost halved in SO-KO after SO_2 fumigation, and both the stomatal conductance ($g_{(H2O)}$) and the SO_2 uptake rate were reduced. SO_2 fumigation as well as inhibition of photosynthesis resulted in strong oxidative stress for the plant. As a consequence, genes associated with the oxidative stress response should also be a subject of regulation.

Senescence associated genes: Contact with SO_2 should lead to enhancements of senescence processes depending on the dosage, which we hypothesized because of first phenotypic symptoms of injury with small necrotic spots on the leaf surface

detected in the fumigated plant material (Randewig et al. 2012). RNAseq data confirmed these observations at the transcriptional level: SO-KO plants which are not able to remove SO_2 using SO should present higher numbers of genes associated to senescence. Confirming these expectations, we identified most up-regulated transcripts with the highest fold changes in SO-KO[+]: Eight genes with changes greater than fivefold presenting a maximum at 18.2 normalized RPKM. For the genotypic comparison we found two genes more than fivefold up-regulated, which were further down- or not regulated in SO-KO *vs*. SO-KO[+]: The *senescence-associated gene 29* (*SAG29*, AT5G13170) was 7.5fold up-regulated and for the *dark inducible 2* (*DIN2*, AT3G60140) we detected 5.2 fold up-regulation in SO-KO in the genotypic comparison to WT. Increases in transcript abundances for WT *vs*. SO-KO samples showed that already the genotypic variation lead to senescence processes. Up- or down-regulation of senescence associated genes was not observed in the WT after fumigation; no transcripts more than fivefold regulated were detected.

Transcriptional regulation: Cluster analyses already presented different regulation patterns of transcription factors after SO_2 fumigation and in the genotypic comparison, respectively. The highest enrichment of regulated transcription factors was expected for SO-KO *vs*. SO-KO[+], followed by WT *vs*. WT[+]. Overall we identified 41 genes associated to transcriptional regulation out of 717 fivefold regulated genes. Highest fold changes were detected for fumigated SO-KO (24 over fivefold regulated genes), whereas WT *vs*. WT[+] samples presented three and WT *vs*. SO-KO 12 genes which were more than fivefold up- or down-regulated. Therefore we can assume that transcriptional regulation mainly plays a role in treated SO-KO plants.

Transporters: With respect to the GO analysis we hypothesized an enhanced regulation of transcripts associated with transport after fumigation with SO_2 in all

23

comparisons. An enrichment of genes involved in transport was confirmed by 41 genes out of 717 fivefold regulated genes. For SO-KO *vs*. SO-KO[+] we identified 29 over fivefold regulated genes, six for WT *vs*. SO-KO and WT *vs*. WT[+] samples. A large number of genes belonging to the **multidrug and toxin extrusion (MATE)** family of efflux pumps was identified to be up-regulated in SO-KO[+]. MATE efflux transporters were already identified in *A. thaliana via* microarray and showed an induced transcription in plants treated with high levels of boron (Kasajima & Fujiwara, 2007). We found four MATE efflux transcripts (AT2G04050, AT2G04040, AT3G23550, AT2G04070) of yet unknown function which showed 30- to 65fold higher transcript amounts and were thus remarkably up-regulated in SO-KO[+] plants.

Oxidoreductases and response to oxidative stress: The influence of SO_2 should lead to plant reactions including several oxidative and reductive processes; therefore we expected and confirmed an enrichment of altered transcripts associated to oxidoreductase activity. We detected 57 genes out of 717 fivefold regulated genes which are involved in oxidoreductase processes. 41 genes were identified as over fivefold regulated for SO-KO *vs*. SO-KO[+], nine for the genotypic comparison and seven for WT *vs*. WT[+]. This data confirmed the hypothesis of a higher regulation of oxidative and reductive processes in fumigated SO-KO plants. Oxidoreductases include the group of peroxidases which were as much as 54.8fold up-regulated in SO-KO[+], compared to 10fold up-regulation in WT[+] (Table S4). Peroxidase CB (AT3G49120) which belongs to the class III peroxidases was fivefold up-regulated in SO-KO[+]. This peroxidase is localized in the apoplast (PeroxiBase, http://peroxibase.toulouse.inra.fr, (Shah et al., 2004)) and is involved in cell wall elongation (Irshad et al., 2008) and ROS-generation under biotic stress reactions (Bindschedler et al., 2006). Moreover, Pfanz and colleagues studied apoplastic

24

peroxidases in response to SO_2 fumigation and suggested a role in SO_2 detoxification (Pfanz et al., 1990; Pfanz & Oppmann, 1991). The present interpretation of these findings is that plants use two independent ways for SO_2 detoxification: One in the apoplastic space and the other at the cellular level which will be in the focus of future work.

Defense: Fumigation of *A. thaliana* resulted in higher expression of several defense related genes: We identified 56 out of 717 fivefold regulated genes associated with defense processes. Seven genes in this group are plant defensins (PDFs) or plant defensin-like proteins. Defensins are small (4-6 kDa) peptides, whose three-dimensional structures are stabilized *via* eight disulfide-linked cysteines (Thomma et al., 2002). These peptides represent 0.5% of the whole plant protein content (Stotz et al., 2009) and belong to the family of antimicrobial peptides (Kovaleva et al., 2010). In WT[+] and SO-KO[+] the same PDFs were up-regulated: PDF1.2 (AT5G44420), PDF1.2b (AT2G26020), PDF1.2c (AT5G44430) and PDF1.3 (AT2G26010), a defensin-like protein (AT2G43510), as well as the low-molecular-weight cysteine-rich 67 protein (LCR67, AT1G75830). In general defensin genes were four- to fivefold up-regulated in WT[+], but in SO-KO[+] these transcripts showed the most impressive and highest regulation found in this RNAseq experiment. Here 17.8- to 244.5fold higher transcript amounts were measured. Moreover, for these defensins, the highest RPKM values (Table S3) were measured: SO-KO[+] present 2936.8 RPKM for PDF1.2. Comparing genotypic changes between SO-KO and WT, no defensins were differentially expressed. One possible and logical reason could be the mass storage of reduced sulfur in these cysteine-rich peptides additional to GSH. In defensins, typically four to eight amino acids out 45 to 54 amino acids are cysteine residues.

Conclusion

25

In the present study, SO_2 at a concentration of 600 nL L^{-1} was applied on Arabidopsis WT and SO-KO for 60 hours which represents neither fully acclimated plants nor immediate stress responses. Prior to the current investigations of mRNA alterations, S-metabolism related enzyme activities and S-metabolite concentrations were determined using aliquots of the same plant material (Randewig et al., 2012). Changes in S metabolite levels of WT and SO-KO plants in response to SO_2 were related to enzyme activities and absolute transcript abundances of mRNA. Removal of excess sulfate by conversion into sulfur containing compounds *via* the sulfur assimilatory stream in response to SO_2 exposure - as concluded from S-associated enzyme activities (Randewig et al., 2012) - was supported by transcript data of the present investigations. These results make the hypothesis of a tight co-regulation between SO and APR plausible, which finally results in a function of keeping the intracellular sulfite pool constant at a low level.

To prevent damage due to atmospheric SO_2, additional mechanisms play a role *in planta* as well. Within this RNAseq experiment two other factors which are possibly involved in sulfite detoxification and therefore assist in co-regulating of APR and SO were identified: Plant defensins, a group of small cysteine rich peptides, and a peroxidase which is localized in the apoplastic space (Shah et al., 2004). Up-regulation of plant defensins after fumigation seems to be a strikingly new response to excess SO_2 concentrations. Due to the strong reaction of WT and SO-KO plants facing SO_2, defensins may function in both processes: Excess sulfur storage and sulfite detoxification. Another outstanding finding of this RNAseq analysis is the up-regulation of transcripts encoding a peroxidase (Peroxidase CB, AT3G49120). Due to the apoplastic localization this enzyme may function as a first line of defense in the detoxification of SO_2. This hypothesis was previously voiced (Pfanz et al., 1990; Pfanz & Oppmann, 1991), but was never tested at the molecular level. The up-

26

regulation of Peroxidase CB in SO-KO[+] may indicate relevance in removing sulfite before it enters the cytoplasm. Both topics will be of high interest for our upcoming investigations.

Our transcript analyses exhibited a set of regulated genes amounting to approximately 5% (cluster analyses). Giraud et al. (2012) presented the strong influence of 1 to 3 µL L^{-1} SO_2 on grape berries using microarray analyses. Although 600 nL L^{-1} SO_2 is a non-toxic dosage for Arabidopsis (Hänsch & Mendel, 2005), first responses at the mRNA level were detected in WT[+], but in a less drastic dimension as detected for SO-KO[+]. WT plants showed a strong and fast reaction facing SO_2 (Fig. 1), whereas SO-KO[+] presented highest RPKM values of several transcripts and a reaction concerning a much broader range of transcripts involved. In contrast to microarray approaches, RNAseq enabled us to obtain details on splice variant gene expression and therefore even allowed *SO* transcript observations for SO-KO[+]. Although the resulting protein products are not functional, as determined in SO enzyme activities for SO-KO and SO-KO[+] (Randewig et al., 2012), plants seem to have a driving force which categorically tries to produce SO when SO_2 is present.

In conclusion, RNAseq of WT[+] and SO-KO[+] and their controls gave not only quantitative insights into the transcriptional response of Arabidopsis plants facing SO_2 fumigation, but also permitted first insights into novel putative mechanisms for SO_2 detoxification beyond SO activity and transportation of excess sulfite into the S-assimilation stream. Mainly based on SO-KO, we present in Fig. 4 our new working model for plant reactions to excess SO_2 including the hypothesized SO_2 detoxification mechanisms.

Acknowledgements

27

This work was supported by a grant of the Deutsche Forschungsgemeinschaft to RH under contract no. HA3107/4 and HR under contract no. Re515/32. APMW appreciates support from DFG grants IRTG 1525 and SFB 590. We are grateful to Dr. Jürgen Kreuzwieser, Axel Schwietale and Michael Rienks for excellent technical help. We thank Prof. Rudolf Galensa, Prof. Norbert Käufer and Mariko Matsuda Alexander for discussing and critically reading the manuscript.

28

References

Alscher R, Bower JL, Zipfel W. 1987. The basis for different sensitivities of photosynthesis to SO_2 in two cultivars of pea. *J Exp Bot* **38**: 99-108.

Ayazloo M, Bell JNB. 1981. Studies on the tolerance to sulphur dioxide of grass populations in polluted areas. I. Identification of tolerant populations. *The New Phytologist* **88**: 203-222.

Baek D, Pathange P, Chung JS, Jiang J, Gao L, Oikawa A, Hirai MY, Saito K, Pare PW, Shi H. 2010. A stress-inducible sulphotransferase sulphonates salicylic acid and confers pathogen resistance in Arabidopsis. *Plant Cell Environ* **33**: 1383-1392.

Bell JNB. 1980. Response of plants to sulphur dioxide. *Nature* **284**: 399-400.

Bindschedler LV, Dewdney J, Blee KA, Stone JM, Asai T, Plotnikov J, Denoux C, Hayes T, Gerrish C, Davies DR, Ausubel FM, Bolwell GP. 2006. Peroxidase-dependent apoplastic oxidative burst in Arabidopsis required for pathogen resistance. *The Plant Journal* **47**: 851-863.

Brychkova G, Xia Z, Yang G, Yesbergenova Z, Zhang Z, Davydov O, Fluhr R, Sagi M. 2007. Sulfite oxidase protects plants against sulfur dioxide toxicity. *Plant J* **50**: 696-709.

Chaves MM, Flexas J, Pinheiro C. 2009. Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. *Ann Bot* **103**: 551-560.

Creissen G, Firmin J, Fryer M, Kular B, Leyland N, Reynolds H, Pastori G, Wellburn F, Baker N, Wellburn A, Mullineaux P. 1999. Elevated glutathione biosynthetic capacity in the chloroplasts of transgenic tobacco plants paradoxically causes increased oxidative stress. *Plant Cell* **11**: 1277-1292.

Davidian JC, Kopriva S. 2010. Regulation of sulfate uptake and assimilation - the same or not the same? *Mol Plant* **3**: 314-325.

De Kok L. 1990. Sulfur metabolism in plants exposed to atmospheric sulfur. In sulfur nutrition and sulfur assimilation in higher plants *(eds H. Rennenberg, C. Brunold, L.J. De Kok & I. Stulen),* SPB Academic Publishing, The Hague, the Netherlands, 111-130.

De Kok L, Durenkamp M, L. Y, Stulen I. 2007. Atmospheric sulfur. *Sulfur in Plants - An Ecological Perspective, In: Plant Ecophysiology* **6**: 91-106.

Eilers T, Schwarz G, Brinkmann H, Witt C, Richter T, Nieder J, Koch B, Hille R, Hansch R, Mendel RR. 2001. Identification and biochemical characterization of *Arabidopsis thaliana* sulfite oxidase. A new player in plant sulfur metabolism. *J Biol Chem* **276**: 46989-46994.

Filner P, Rennenberg H, Sekiya J, Bressan RA, Wilson LG, LeCureux L, Shimei T. 1984. Biosynthesis and emission of hydrogen sulfide by higher plants. *In Gaseous Air Pollutants and PlantMetabolism (edsM.J. Koziol & F.R.Whatley),* Butterworth, London, UK, 291-312.

Frova C. 2003. The plant glutathione transferase gene family: Genomic structure, functions, expression and evolution. *Physiol Plant* **119**: 469-479.

Geu-Flores F, Moldrup ME, Bottcher C, Olsen CE, Scheel D, Halkier BA. 2011. Cytosolic gamma-glutamyl peptidases process glutathione conjugates in the biosynthesis of glucosinolates and camalexin in arabidopsis. *Plant Cell* **23**: 2456-2469.

Giraud E, Ivanova A, Gordon CS, Whelan J, Considine MJ. 2012. Sulphur dioxide evokes a large scale reprogramming of the grape berry transcriptome associated with oxidative signalling and biotic defence responses. *Plant Cell Environ*. **35**: 405–417.

Graveley BR. 2005. Mutually exclusive splicing of the insect dscam pre-mRNA directed by competing intronic RNA secondary structures. *Cell* **123**: 65-73.

Hänsch R, Lang C, Riebeseel E, Lindigkeit R, Gessler A, Rennenberg H, Mendel RR. 2006. Plant sulfite oxidase as novel producer of H_2O_2: Combination of enzyme catalysis with a subsequent non-enzymatic reaction step. *J Biol Chem* **281**: 6884-6888.

Hänsch R, Mendel RR. 2005. Sulfite oxidation in plant peroxisomes. *Photosynth Res* **86**: 337-343.

Hawkesford MJ. 2003. Transporter gene families in plants: The sulphate transporter gene family-redundancy or specialization? *Physiol Plantarum* **117**: 155-163.

Heber U, Hüve K. 1998. Action of SO_2 on plants and metabolic detoxification of SO_2. *Int. Rev. Cytochem.* **177**: 255-286.

Herschbach C, Rizzini L, Mult S, Hartmann T, Busch F, Peuke AD, Kopriva S, Ensminger I. 2010. Over-expression of bacterial gamma-glutamylcysteine synthetase (gsh1) in plastids affects photosynthesis, growth and sulphur metabolism in poplar (populus tremula x populus alba) dependent on the resulting gamma-glutamylcysteine and glutathione levels. *Plant Cell Environ* **33**: 1138-1151.

Irshad M, Canut H, Borderies G, Pont-Lezica R, Jamet E. 2008. A new picture of cell wall protein dynamics in elongating cells of *Arabidopsis thaliana*: Confirmed actors and newcomers. *BMC Plant Biol* **8**: 94.

Jost R, Berkowitz O, Wirtz M, Hopkins L, Hawkesford MJ, Hell R. 2000. Genomic and functional characterization of the oas gene family encoding o-acetylserine (thiol) lyases, enzymes catalyzing the final step in cysteine biosynthesis in *Arabidopsis thaliana*. *Gene* **253**: 237-247.

Kasajima I, Fujiwara T. 2007. Identification of novel *Arabidopsis thaliana* genes which are induced by high levels of boron. *Plant Biotechnology* **24**: 355-360.

Kataoka T, Watanabe-Takahashi A, Hayashi N, Ohnishi M, Mimura T, Buchner P, Hawkesford MJ, Yamaya T, Takahashi H. 2004. Vacuolar sulfate transporters are essential determinants controlling internal distribution of sulfate in Arabidopsis. *Plant Cell* **16**: 2693-2704.

Kawashima CG, Berkowitz O, Hell R, Noji M, Saito K. 2005. Characterization and expression analysis of a serine acetyltransferase gene family involved in a key step of the sulfur assimilation pathway in Arabidopsis. *Plant Physiol* **137**: 220-230.

Klein M, Papenbrock J. 2004. The multi-protein family of Arabidopsis sulphotransferases and their relatives in other plant species. *J Exp Bot* **55**: 1809-1820.

Kopriva S, Koprivova A. 2004. Plant adenosine 5'-phosphosulphate reductase: The past, the present, and the future. *J Exp Bot* **55**: 1775-1783.

Kopriva S, Rennenberg H. 2004. Control of sulphate assimilation and glutathione synthesis: Interaction with N and C metabolism. *J Exp Bot* **55**: 1831-1842.

Kovaleva V, Krynytskyy H, Gout I, Gout R. 2010. Recombinant expression, affinity purification and functional characterization of scots pine defensin 1. *Appl Microbiol Biotechnol* **89**: 1093-1101.

Lang C, Popko J, Wirtz M, Hell R, Herschbach C, Kreuzwieser J, Rennenberg H, Mendel RR, Hänsch R. 2007. Sulphite oxidase as key enzyme for protecting plants against sulphur dioxide. *Plant Cell Environ* **30**: 447-455.

Peleman J, Boerjan W, Engler G, Seurinck J, Botterman J, Alliotte T, Van Montagu M, Inze D. 1989. Strong cellular preference in the expression of a housekeeping gene of *Arabidopsis thaliana* encoding s-adenosylmethionine synthetase. *Plant Cell* **1**: 81-93.

Pfanz H, Dietz K-J, Weinerth I, Oppmann B. 1990. Detoxification of sulfur dioxide by apoplastic peroxidases. *In: Sulfur nutrition and sulfur assimilation in higher plants. Rennenberg H, Brunold C, De Kok LJ, and Stulen I, eds.,* SPB Academic Publishing: 229-233.

Pfanz H, Martinoia E, Lange OL, Heber U. 1987. Mesophyll resistances to SO_2 fluxes into leaves. *Plant Physiol* **85**: 922-927.

Pfanz H, Oppmann B. 1991. The possible role of apoplastic peroxidases in detoxifying the air pollutant sulfur dioxide. *In: Lobarzewski J, Greppin H, Penel, C and Gaspar T (eds.) Biochemical, molecular and physiological aspects of plant peroxidases.* University of Geneva, Geneva: 401-417.

Randewig D, Hamisch D, Herschbach C, Eiblmeier M, Gehl C, Jurgeleit J, Skerra J, Mendel RR, Rennenberg H, Hänsch R. 2012. Sulfite oxidase controls sulfur metabolism under SO_2 exposure in *Arabidopsis thaliana*. *Plant Cell Environ*.

Rao IM, Anderson LE. 1983. Light and stomatal metabolism: II. Effects of sulfite and arsenite on stomatal opening and light modulation of enzymes in epidermis. *Plant Physiol* **71**: 456-459.

Rennenberg H. 1984. The fate of excess sulfur in higher plants. *Annual Review of Plant Physiol* **35**: 121-153.

Rosso MG, Li Y, Strizhov N, Reiss B, Dekker K,Weisshaar B. 2003. An *Arabidopsis thaliana* T-DNA mutagenized population (GABI-Kat) for flanking sequence tag-based reverse genetics. *Plant Molecular Biology* **53**, 247–259.

Schlaeppi K, Bodenhausen D, Buchala A, Mausch F, Reymond P. 2008. The glutathione-deficient mutant pad2-1 accumulates lower amounts of glucosinolates and is more susceptible to the insect herbivore *Spodoptera littoralis*. *Plant Journal* **55**: 774-786.

Shah K, Penel C, Gagnon J, Dunand C. 2004. Purification and identification of a Ca^{2+}-pectate binding peroxidase from Arabidopsis leaves. *Phytochemistry* **65**: 307-312.

Smith CW. 2005. Alternative splicing - when two's a crowd. *Cell* **123**: 1-3.

Stasolla C, Belmonte MF, Tahir M, Elhiti M, Khamiss K, Joosen R, Maliepaard C, Sharpe A, Gjetvaj B, Boutilier K. 2008. Buthionine sulfoximine (bso)-mediated improvement in cultured embryo quality in vitro entails changes in ascorbate metabolism, meristem development and embryo maturation. *Planta* **228**: 255-272.

Stotz HU, Thomson JG, Wang Y. 2009. Plant defensins: Defense, development and application. *Plant Signal Behav* **4**: 1010-1012.

Szalai G, Kellos T, Galiba G, Kocsy G. 2009. Glutathione as an antioxidant and regulatory molecule in plants under abiotic stress conditions. *J Plant Growth Regul* **28**: 66-80.

Tamm CO, Cowling EB. 1977. Acid precipitation and forest vegetation. *Water, Air, and Soil Pollution* **7**: 503-511.

Thomma BP, Cammue BP, Thevissen K. 2002. Plant defensins. *Planta* **216**: 193-202.

Van der Kooij TAW, De Kok LJ, Haneklaus S, Schnug E. 1997. Uptake and metabolism of sulfur dioxide by *Arabidopsis thaliana*. *New Phytologist*.**135**: 101-107.

Vauclare P, Kopriva S, Fell D, Suter M, Sticher L, von Ballmoos P, Krahenbuhl U, den Camp RO, Brunold C. 2002. Flux control of sulphate assimilation in *Arabidopsis thaliana*: Adenosine 5'-phosphosulphate reductase is more susceptible than ATP sulphurylase to negative control by thiols. *Plant J* **31**: 729-740.

Vivancos PD, Dong Y, Ziegler K, Markovic J, Pallardo FV, Pellny TK, Verrier PJ, Foyer CH. 2010. Recruitment of glutathione into the nucleus during cell proliferation adjusts whole-cell redox homeostasis in *Arabidopsis thaliana* and lowers the oxidative defence shield. *Plant J* **64**: 825-838.

Wang L, Feng Z, Wang X, Wang X, Zhang X. 2010. DEGseq: An R package for identifying differentially expressed genes from RNA-seq data. *Bioinformatics* **26**: 136-138.

Wirtz M, Birke H, Heeg C, Muller C, Hosp F, Throm C, Konig S, Feldman-Salit A, Rippe K, Petersen G, Wade RC, Rybin V, Scheffzek K, Hell R. 2010. Structure and function of the hetero-oligomeric cysteine synthase complex in plants. *J Biol Chem* **285**: 32810-32817.

Yamaguchi Y, Nakamura T, Kusano T, Sano H. 2000. Three Arabidopsis genes encoding proteins with differential activities for cysteine synthase and beta-cyanoalanine synthase. *Plant Cell Physiol* **41**: 465-476.

32

Figure legends:

Fig. 1 Differentially expressed transcripts and Venn diagrams presenting intersections after combining the four different comparison pairs WT *vs*. WT[+], SO-KO *vs*. SO-KO[+], WT *vs*. SO-KO and WT[+] *vs*. SO-KO[+]. The numbers and percentages of differentially expressed transcripts for (a) fivefold up- and (b) down-regulated genes presented the highest amount of up- and down-regulated transcripts in SO-KO *vs*. SO-KO[+]; the lowest number was observed for WT *vs*. WT[+]. Venn diagrams represent numbers of (c) fivefold up- and (d) down-regulated transcripts when different comparisons overlap. The Venn diagram for up-regulation showed two transcripts which are solely unregulated in SO-KO *vs*. SO-KO[+] and WT *vs*. SO-KO, respectively.

Fig. 2 Hierarchical clustering of fivefold regulated transcripts and profile plots of selected clusters. Colors of the cluster in (a) were assigned based on the normalized, log scaled RPKM values (significance tested using DEGseq). In (b) expression profiles of transcripts involved in these eight clusters are depicted. Cluster IV shows transcripts which are solely up-regulated in SO-KO[+], cluster VII includes those which are mutually down-regulated in SO-KO[+]. Both clusters presented the highest number of involved transcripts.

Fig. 3 Alterations in the sulfur metabolism for the SO-KO *vs.* SO-KO[+] comparison. Regulation of sulfur metabolism associated genes for SO-KO[+] is presented using different colors, as described in the color range. Gene name abbreviations are described in Table S2. To generate this scheme no fold change was applied onto the expression values; mostly GSTs and the SOT show strong reaction in the SO-KO[+] focusing the up-regulated transcripts.

Fig. 4 Current working model derived from RNAseq-data interpreting overall plant reaction and detoxification mechanisms facing excess SO_2. SO_2 enters the plant cell

33

via the stomata, where it is converted into sulfite: Sulfite can be detoxified by an apoplastic peroxidase (PRXCB) and SO in an oxidative reaction or fed into the sulfur assimilation stream (reductive detoxification). These and further specific transcripts (violet) and processes (green) were identified as being up- (red star) or down-regulated (blue star); transcript isoforms involved in specific processes in some cases presented different regulations (red and blue star). In general SO_2 fumigation leads to different reactions including transcriptional control by regulation of transcription factors, changes of metabolite transport, induction of senescence and down-regulation of photosynthetic processes. This model hypothesizes the apoplastic peroxidase PRXCB as protagonist for plant SO and uncovers plant defensins as novel mass storage of reduced sulfur.

34

One sentence legends to Supporting Information

Fig. S1 Scattered matrix plot presenting the distribution RPKM values for different comparisons between WT, WT[+], SO-KO and SO-KO[+].

Fig. S2 GO analysis of fivefold regulated transcripts.

Fig. S3 KEGG Metabolic Pathways of the four different comparison pairs using fivefold data.

Fig. S4 *SO* [At3G01910] splice variants and mRNA fragment mapping for WT, WT[+], SO-KO and SO-KO[+].

Fig. S5 *SO* [At3G01910] mRNA mapping for WT, WT[+], SO-KO and SO-KO[+].

Table S1 Transcript raw data.

Table S2 Raw data for sulfur metabolism associated genes.

Table S3 RPKM values and fold change calculations of highly regulated plant defensins (PDF).

Table S4 RPKM values and fold change calculations of highly regulated peroxidase genes.

Table S5 Script for programming in *R* to execute DEGseq tool.

35