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

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Summary

- High concentrations of sulfur dioxide (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 knockout 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 previous 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 the WT, whereas 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 coregulation 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.

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 sulfur dioxide (SO_2) gas as additional sulfur source (De Kok *et al.*, 2007). If, however, atmospheric SO_2 exceeds a critical threshold concentration, 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: (1) the cuticle, which functions as the first barrier for toxic gases, largely restricting the pathway for influx to the stomata (Tamm & Cowling, 1977); (2) active stomatal closure, reducing SO_2 uptake (Rao & Anderson, 1983), and mesophyll resistances to SO_2 flux, mainly determined by metabolism of sulfite, which adjust SO_2 flux into leaves (Pfanz *et al.*, 1987); and (3) 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 nonenzymatic (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 *Arabidopsis thaliana* wildtype (WT) and SO knockout (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 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 nontoxic to WT plants (Randewig *et al.*, 2012). Effects on sulfite detoxification and sulfur assimilation, particularly metabolic coregulation of enzymes

involved in sulfur assimilation, were compared. SO_2 exposure caused a significant increase in sulfate and glutathione (GSH) pool in wildtype Arabidopsis. Conversely, in KO plants the sulfate pool was kept constant, but thiol concentrations were strongly increased (14-fold 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 coregulation 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 vs SO-KO plants before and after SO_2 fumigation would permit (1) a comprehensive analysis of the transcriptional regulation of sulfur metabolism, and (2) 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: (1) a larger and more quantitative dynamic range of the experiment; (2) the ability to estimate absolute transcript numbers; and, therefore, (3) the opportunity to perform more accurate quantification of relative changes in transcript numbers. In the present paper we provide a detailed analysis of consequences of fumigation with *c.* 600 nl l^{-1} SO_2 for 60 h – a nontoxic dosage for *A. thaliana* wildtype plants – and compare the effect on WT and SO-KO plants. Fortunately, we were able to use plant samples that 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 concentrations, a set of enzyme activities, and physiological data.

Materials and Methods

Plant material

For RNAseq experiments *Arabidopsis 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 program (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 86 h. Three hours after the beginning of the dark period during 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, fumigated WT (WT[+]), SO-KO and fumigated SO-KO (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 (an exception to this was that elution was performed using two times 20 µl of RNAse-free H_2O). Total RNA preparations of 10 samples per probe set (WT, WT[+], SO-KO and SO-KO[+]) were pooled. Dynabeads® Plant Oligo$(dT)_{25}$ (Invitrogen, Darmstadt, Germany) were used for final mRNA purification according to the manufacturer's instructions. The 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). 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 associated quality (filename.qual) files and afterwards imported to CLC Genomics Workbench (CLC bio) using the NGS import function. Thus erroneous reads were cropped at the position of the error. Alignment and expression values in reads per kilobase of exon model per million mapped reads (RPKM) were obtained using the 'RNAseq Analysis' feature of CLC Genomics Workbench. RPKM are defined as follows:

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

All four libraries were analyzed separately using standard parameters, that is, 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 20 June 2009. The gene expression values were exported for further analysis.

DEGseq

Identification of differentially expressed genes was done using the R package 'DEGseq' (Wang *et al.*, 2010). 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 nondefault values: method = 'MARS', normal method = none (Supporting Information, Table S5). For each pair of input files, DEGseq provides a list of *P* values to determine significantly differentially expressed genes. Expression was considered significant if the uncorrected *P* value was < 0.001 (corresponding to Benjamini–Hochberg false discovery rate-corrected *P*-values, $P < 0.014$ for SO-KO control vs treated, < 0.017 for WT-treated vs SO-KO-treated, < 0.029 for WT control vs SO-KO 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). There were two data sets used within the studies: (1) absolute expression data and (2) 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 more detailed view of the transcription amounts of different genes. For this purpose, raw data were prepared in GeneSpring GX 11.5, pointing out that they were already log-scaled, which resulted in cutting extremely low data values using the 20th to 100th 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 of absolute transcript data is applicable for each requested gene and its associated splice variants, which is the primary advantage of using RNAseq data compared with microarrays where 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 GeneSpring GX 11.5 generic single-color experiment according to the manual. After the normalization step, experimental data were grouped as genotype × treatment : WT/SO-KO × control/treated with 600 nl l^{-1} SO_2. To identify genes that show differences between treated and control samples or samples with different genotypes, expression ratios (**fold-changes**) were calculated in the following way:

$$\text{Expression ratio} = \text{Fold change} = \frac{\text{Condition 1}}{\textit{Condition2}}.$$

Ratios below or above a determined cutoff show that these genes are *x*-fold up- or down-regulated. Four different pairs of conditions were used within the fold-change analyses: WT vs WT[+]; SO-KO vs SO-KO[+]; WT vs SO-KO; and WT[+] vs SO-KO[+]. Data that were fivefold up- or down-regulated and DEGseq-verified data were used to obtain deeper insights into regulation of other processes beyond sulfur metabolism.

Using the GeneSpring GX 11.5 **cluster analyses** tool, hierarchical clustering was performed for data with a fivefold-change threshold 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 to 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 used for Gene Ontology (GO) analyses.

The GO database (www.geneontology.org) describes connections between gene expression data and defined GO terms. Using the GeneSpring GX 11.5 GO analysis tool, entities of interest obtained from one experiment can be explored, finding matching GO terms. The output of GO analysis is a tree containing GO terms enriched with a *P*-value cutoff of 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 WT plants in this experiment: 23 232 for WT[+], 22 424 for SO-KO, and 22 255 for SO-KO[+]. Quantile-normalized, log_2-scaled and nonbaseline-transformed RPKM of these transcripts were widely spread (Fig. S1). Each analyzed sample consisted of 10 independently prepared RNAs from a total of 20 treated plants. The biggest spread of RPKM, and therefore the greatest change in transcripts, was detected for WT vs SO-KO[+], followed by SO-KO vs SO-KO[+]. A narrower distribution, indicating a weaker response to SO_2 or the genotypic modification, was identified in WT vs WT[+], WT vs SO-KO and SO-KO vs WT[+]. Fig. 1(a,b) present the amounts of differentially expressed genes in relation to the total number of transcripts (different splice variants included) using data within a fivefold-change cutoff, verified with DEGseq (Table S1). With the fivefold-change threshold, between 0.4 and 1.6% of genes were differentially expressed for all condition pairs. Approx. 60% of the **differentially expressed genes** were up-regulated (Fig. 1a), whereas *c.* 40% were down-regulated (Fig. 1b).

Venn diagrams (Fig. 1c,d) were created to investigate several hypotheses concerning the biological evidence of the genotypic variation in SO-KO and the effects caused by SO_2 treatment. The Venn diagrams show the number of fivefold up- (Fig. 1c) or down-regulated (Fig. 1d) transcripts 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 change in transcripts and transcript numbers as does fumigation of the WT. If this were the case there would be 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-regulated

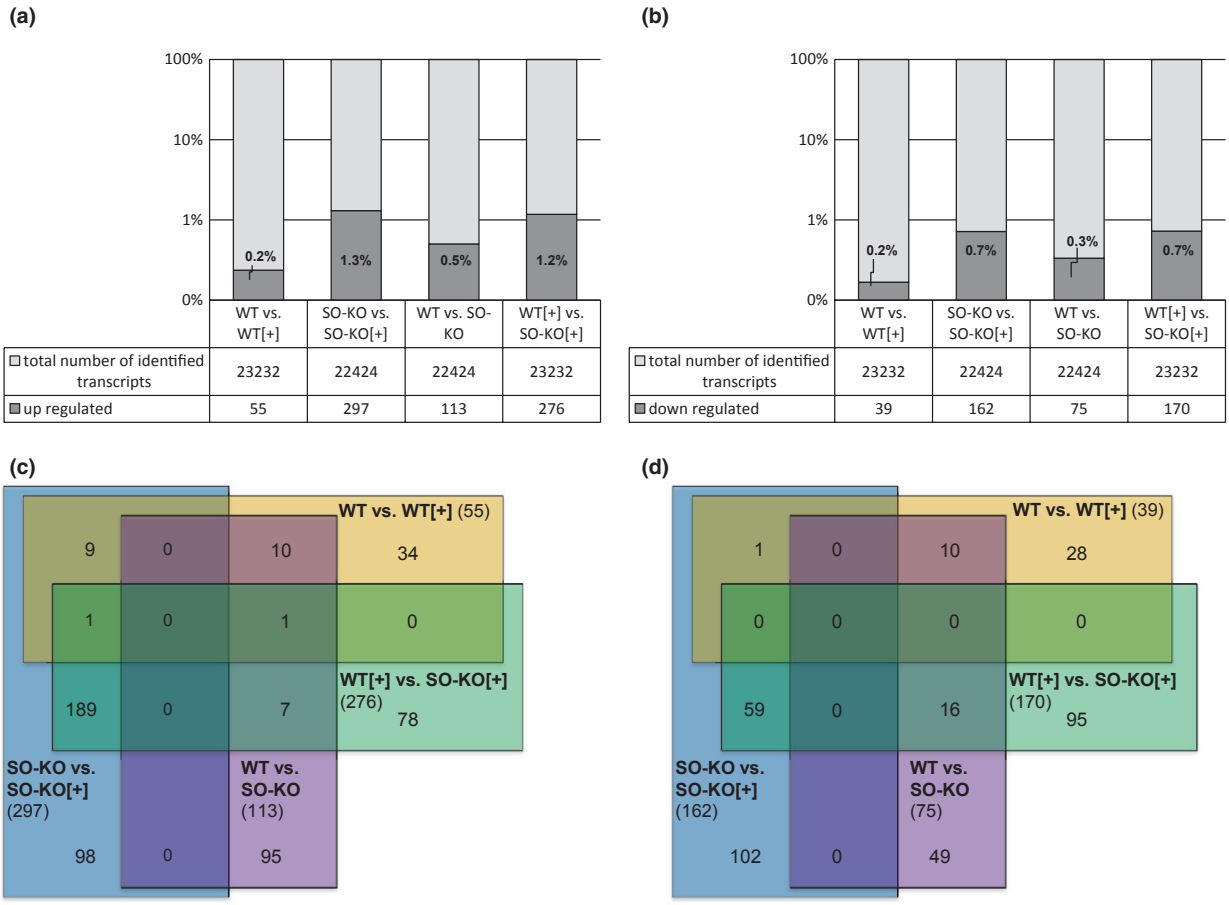

Fig. 1 Differentially expressed transcripts and Venn diagrams presenting intersections after combining the four different *Arabidopsis thaliana* comparison pairs: wildtype (WT) vs SO_2-fumigated WT (WT[+]), sulfite oxidase knockout (SO-KO) vs SO-KO[+], WT vs SO-KO and WT[+] vs SO-KO[+]. The numbers and percentages of differentially expressed transcripts for fivefold up-regulated (a) and down-regulated (b) genes presented the highest abundance 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 fivefold up-regulated (c) and down-regulated (d) 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. [+] indicates SO_2 fumigation.

and 65 transcripts solely down-regulated, and there were 44 up-regulated transcripts and 29 down-regulated transcripts for WT vs WT [+]. These findings ran counter to our hypothesis that the gene expression changes caused by SO knockout were similar to those caused by SO_2 fumigation. This result was, however, in line with the second assumption that we would not find any intersections for WT vs SO-KO and SO-KO vs SO-KO[+], because *SO* gene knockout and fumigation of SO-KO would not have any regulated transcripts in common, and interpretation of our data validated this expectation. Thirdly, SO is predicted to play a key role in SO_2 detoxification, and we therefore hypothesized a higher number of regulated transcripts in SO-KO[+] than in 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[+] confirmed this hypothesis. Only 10 transcripts for up-regulation and one for down-regulation were identified as commonly regulated. Fourthly, we expected that SO-KO plants would have to use different defense mechanisms to detoxify SO_2 than those used by WT plants. If this were the case, we would therefore find only very few transcripts that were commonly up- or down-regulated in WT vs WT[+] and WT[+] vs SO-KO[+]. Transcripts in the overlap represented genes that were already highly regulated in WT[+] and even more highly regulated in SO-KO[+]. This was a small common transcript set resulting from 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 (1.19-fold), whereas the nitrilase was significantly regulated (3.97-fold) 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 would find more genes regulated in SO-KO vs SO-KO[+] than in WT[+] vs SO-KO[+], but overall a high number of commonly regulated transcripts. Counting the transcripts for SO-KO vs SO-KO[+] revealed 297 up-regulated and 162 down-regulated transcripts. For WT[+] vs SO-KO[+] we found 276 up-regulated and 170 down-regulated transcripts: 249 transcripts were commonly regulated in SO-KO vs SO-KO[+] and WT[+] vs

SO-KO[+], and a total of 408 transcripts were solely regulated. 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 [+] than in WT[+] vs SO-KO[+].

To obtain further insights into 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 greater than fivefold transcriptional change 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. In total we were able to identify eight individual gene expression clusters numbered from I to VIII. For further analyses we used the top 20 regulated transcripts and **GO** analyses for each identified cluster.

Application of SO_2 to WT plants should lead to several transcriptional changes, but because of the dosage of 600 nl l^{-1} used and because SO acts as a detoxificating enzyme, we hypothesized a smaller reaction than we would expect for SO-KO treatment. GO analysis of fivefold regulated transcripts in WT vs WT[+] was in line with 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

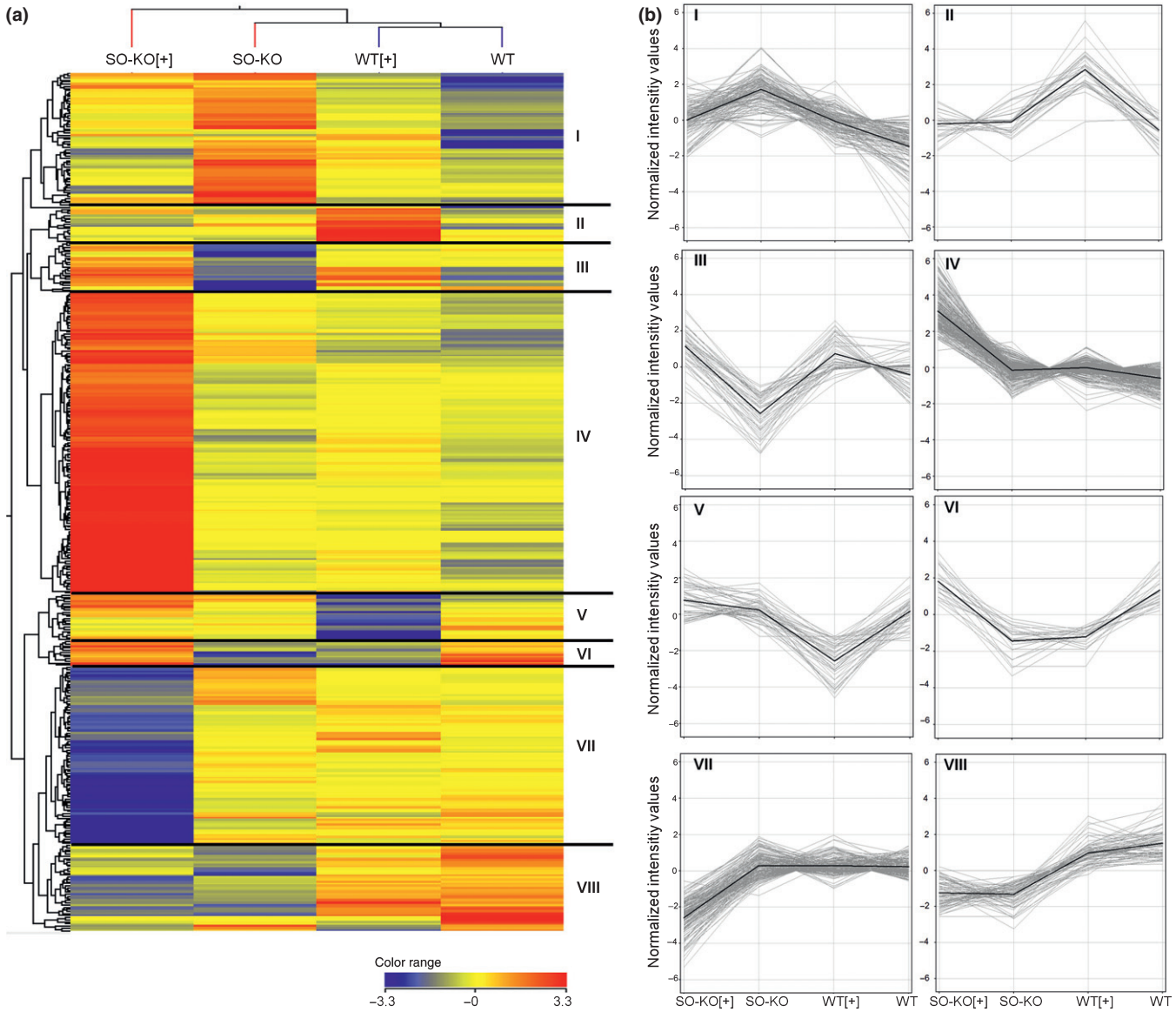

Fig. 2 Hierarchical clustering of fivefold regulated transcripts and profile plots of selected clusters. Colors of the cluster in (a) were assigned based on the normalized, log-scaled RPKM (reads per kilobase of exon model per million mapped reads) values (significance tested using DEGseq). In panel (b), expression profiles of transcripts involved in these eight clusters are depicted. Cluster IV shows transcripts which are solely up-regulated in the SO_2-fumigated *Arabidopsis thaliana* sulfite oxidase knockout (SO-KO[+]); cluster VII includes those which are mutually down-regulated in SO-KO[+]. Both clusters presented the highest number of involved transcripts.

transcripts for WT vs WT[+] in clusters I, II, and III; down-regulation was detected in clusters V, VI, and VIII. The top 20 transcripts in cluster II included four transcripts associated with ribosomal and translation processeses, which indicated an influence of SO_2 fumigation on mRNA synthesis regulation. Cluster II contained transcripts which showed their highest abundances in WT[+] only and which therefore revealed a moderate reaction of WT plants to SO_2 with transcriptional adaptation and thus up-regulation of transcripts belonging to ribosomal processes.

Hypothetically, treatment of SO-KO plants with SO_2 should lead to higher and different transcriptional responses compared with treated WT plants, as this was already verified by Venn diagrams. 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 also clear from the Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses (Fig. S3). GO terms enriched in SO-KO vs SO-KO[+] were principally associated with the terms MOLECULAR FUNCTION and BIOLOGICAL PROCESS. Up-regulation of transcripts was identified in clusters III, IV, V and VI, while down-regulation was identified in clusters 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[+]. The WT[+] top 20 included the greatest number of transcripts associated with transcriptional regulation (cluster I, II), whereas the SO-KO[+] top 20 included transcripts involved in defense processes (cluster IV) and peptidase activity (cluster VI). The top 20 of cluster IV contained nine transcripts associated with 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 resulting from SO_2 and/or genotypic variation of SO knockout

We recently reported enzyme activities and S-metabolites of *A. thaliana* WT 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 combination of transcriptome data with enzyme activities and metabolite concentrations 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, would 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 are summarized in Fig. 3, which presents the regulation of genes for SO-KO vs SO-KO[+]. Raw data and fold changes 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 subfamilies 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 was 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, *SULTR2*;1 transcript abundances were similar in the nonfumigated plant material, but increased in WT[+] by 30% or decreased in SO-KO[+] by 50%. Moreover, the expression of *SULTR2*;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) – *SULTR4*;2 mRNA amounts were two- and 10-fold 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.

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 that was significantly down-regulated (1.3-fold) 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 (3′-phosphoadenosine 5′-phosphosulfate) by one of the four isoforms of **APS kinase (APK)**. Compared with 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 (SOTs)** 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 13-fold 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 as a result of feedback inhibition (Vauclare *et al.*, 2002). In SO-KO control plants, *APR1*, *APR2* and *APR3* transcripts were increased significantly compared with WT control samples (Table S2). Fumigation of these SO-KO plants led to a dramatic decrease in both transcript abundance (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 at the post-translational level. Such a negative feedback inhibition of APR mediated by increasing

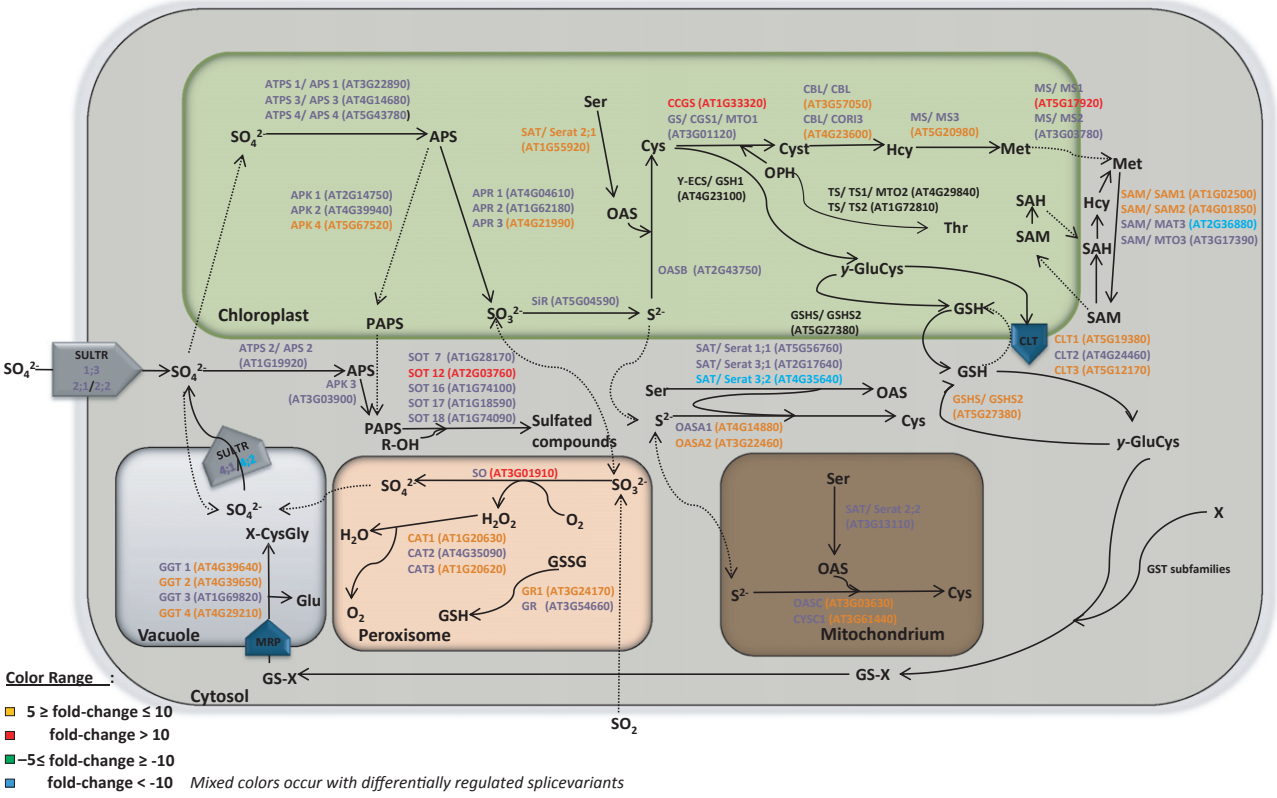

Fig. 3 Alterations in the sulfur metabolism for the *Arabidopsis thaliana* sulfite oxidase knockout (SO-KO) vs SO_2-fumigated SO-KO (SO-KO[+]) comparison. Regulation of sulfur metabolism-associated genes for SO-KO[+] is presented using different colors, as described in the color range. Gene name abbreviations are described in Table S2. To generate this scheme no fold change was applied to the expression values; mostly the glutathione S-transferases (GSTs) and the sulfotransferase (SOT) show a strong reaction in the SO-KO[+], in particular in the up-regulated transcripts.

amounts of thiols (Vauclare *et al.*, 2002) has been discussed previously (for a 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 abundance of definite genes. This has also been proposed by Szalai *et al.* (2009) for abiotic stress conditions either via H_2O_2 or GSH/ oxidized glutathione (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 and 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 was channeled into the direction of cysteine production after SO_2 fumigation, which held true for both fumigated WT and SO-KO plants.

Organic sulfur may flow towards methionine via cystathione or towards GSH. **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 abundances (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-adenosyl-methionine synthetase (SAM-synthetase)** transcripts. MS converts homocysteine into methionine, which could be used by SAM-synthetase to generate S-adenosylmethionine as a methyl group donor in numerous transmethylation reactions (Peleman *et al.*, 1989). Only *MS2* displayed a twofold decrease in SO-KO[+] compared with SO-KO, and a threefold decrease in SO-KO[+] compared with 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 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 abundance of 30 RPKM for WT, WT[+] and SO-KO. Transcript abundances for SO-KO[+] were significantly higher (2.6-fold) compared with other samples, indicating that the higher amounts of produced γ-glutamylcysteine are converted into GSH. **Glutathione reductase (GR)** reduces GSSG to GSH. GR1 had enhanced transcript abundance, especially 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 GSH. 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 affecting 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). However, 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 was 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 10-fold and higher. *GST6* (AT1G02930) was 10.5-fold up-regulated in response to the fumigation stress.

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 for removing 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 the highest transcript abundance 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 immunoblot analysis, because of 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 as a result of the T-DNA insertion and the resulting mRNA is thus noncoding, the transcriptional response apparently attempts to enhance SO production (Figs S4, S5). For *SO*-1 we detected 2.5-fold (and for *SO*-2 *c.* 16-fold) increased transcript abundance after fumigation with 600 nl l^{-1} SO_2 for 60 h. At present, 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).

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 to 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 the senescence processes depending on the dosage, a hypothesis we arrived at because of the initial phenotypic symptoms of injury, with small necrotic spots on the leaf surface

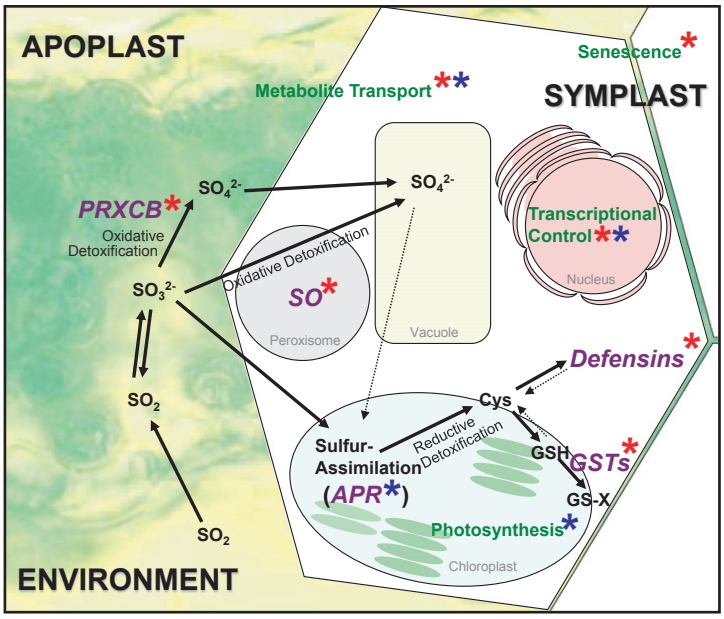

Fig. 4 Current working model derived from RNAseq data interpreting overall plant reaction and detoxification mechanisms in the face of excess SO_2. SO_2 enters the plant cell via the stomata, where it is converted into sulfite: sulfite can be detoxified by an apoplastic peroxidase (PRXCB) and sulfite oxidase (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-regulated (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 a protagonist for plant SO and uncovers plant defensins as a novel mass storage of reduced sulfur. Cys, cysteine; GSH, glutathione; GST, glutathione S-transferase.

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 with senescence. Confirming these expectations, we identified most up-regulated transcripts with the highest fold changes in SO-KO[+]: eight genes with greater than fivefold changes 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-regulated or not regulated in SO-KO vs SO-KO[+]: the *senescence-associated gene 29* (*SAG29*, AT5G13170) was 7.5-fold up-regulated, and the *dark inducible 2* gene (*DIN2*, AT3G60140) was 5.2-fold up-regulated 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 has an effect on senescence processes. Up- or down-regulation of senescence-associated genes was not observed in the WT after fumigation; no transcripts > fivefold regulated were detected.

Transcriptional regulation Cluster analyses revealed different regulation patterns of transcription factors after SO_2 fumigation and in the genotypic comparison. 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 with transcriptional regulation out of 717 fivefold regulated genes. The highest fold-changes were detected for fumigated SO-KO (24 genes more than fivefold regulated), whereas WT vs WT[+] samples revealed three genes, and WT vs SO-KO 12 genes, that were more 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 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 genes regulated over fivefold, 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 amounts of boron (Kasajima & Fujiwara, 2007). We found four MATE efflux transcripts (AT2G04050, AT2G04040, AT3G23550, AT2G04070) of as yet unknown function which showed 30- to 65-fold higher transcript abundance 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 with oxidoreductase activity. We detected 57 genes out of 717 fivefold regulated genes that are involved in oxidoreductase processes. Forty-one genes

were identified as over fivefold regulated for SO-KO vs SO-KO[+], nine for the genotypic comparison and seven for WT vs WT[+]. These data confirmed the hypothesis of a higher regulation of oxidative and reductive processes in fumigated SO-KO plants. Oxidoreductases include the group of peroxidases that were as much as 54.8-fold up-regulated in SO-KO[+], compared with 10-fold 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 reactive oxygen species (ROS) generation under biotic stress reactions (Bindschedler *et al.*, 2006). Moreover, Pfanz and colleagues studied apoplastic 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 have two independent methods of 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 244.5-fold higher transcript abundances were measured. Moreover, for these defensins, the highest RPKM values (Table S3) were measured: 2936.8 RPKM for PDF1.2 in SO-KO [+]. 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 of 45–54 amino acids are cysteine residues.

Conclusion

In the present study, SO_2 at a concentration of 600 nl l^{-1} was applied to Arabidopsis WT and SO-KO for 60 h, which represents neither fully acclimated plants nor immediate stress responses. Before 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 concentrations 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 coregulation between SO and APR plausible, meaning there is a role in keeping the intracellular sulfite pool constant at a low concentration.

To prevent damage from atmospheric SO_2, additional mechanisms play a role *in planta* as well. In this RNAseq experiment, two other factors that are possibly involved in sulfite detoxification, and which therefore assist in coregulation of APR and SO, were identified: PDFs, a group of small cysteine-rich peptides, and a peroxidase which is localized in the apoplastic space (Shah *et al.*, 2004). Up-regulation of PDFs after fumigation seems to be a strikingly new response to excess SO_2 concentrations. Owing to the strong reaction of WT and SO-KO plants to 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). As a result of the apoplastic localization, this enzyme may function as a first line of defense in the detoxification of SO_2. This hypothesis was advanced previously (Pfanz *et al.*, 1990; Pfanz & Oppmann, 1991), but was never tested at the molecular level. The up-regulation of peroxidase CB in SO-KO[+] may indicate it has some role in removing sulfite before it enters the cytoplasm. Both topics will be of great interest in our upcoming investigations.

Our transcript analyses exhibited a set of regulated genes amounting to *c.* 5% (cluster analyses). Giraud *et al.* (2012) reported a strong influence of 1–3 µl l^{-1} SO_2 on grape berries using microarray analyses. Although 600 nl l^{-1} SO_2 is a nontoxic dosage for Arabidopsis (Hänsch & Mendel, 2005), the first responses at the mRNA level were detected in WT[+], though to a lesser extent than detected in SO-KO [+]. WT plants showed a strong and fast reaction to SO_2 (Fig. 1), whereas SO-KO[+] presented the highest RPKM values of several transcripts and a reaction involving a much broader range of transcripts. 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 that categorically tries to produce SO when SO_2 is present.

In conclusion, RNAseq of WT[+] and SO-KO[+] and their controls not only gave quantitative insights into the transcriptional response of Arabidopsis plants to SO_2 fumigation, but also permitted some 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.

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Supporting Information

Additional supporting information may be found in the online version of this article.

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 (PDFs)

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

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

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