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ORIGINAL ARTICLE

Differences in detoxification mechanism and gene expression changes of sulfur metabolism in coping with the air pollutant SO_2 between the resistant and ordinary poplar variety

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Abstract

In this study, two poplar varieties with different resistance to sulfur dioxide were selected for a comparative experiment. SO_2 fumigation to the poplars was carried out under controlled conditions to determine the variation in the activity of enzymes, the content of metabolites and the expression of enzymes genes in pathways of sulfur metabolism in plants. The results showed that the activity of enzymes and the content of sulfur metabolites were closely related to the response of the poplars to SO_2 stress. *Populus*×*euramericana* cv. 'Purui' had two ways of detoxification: oxidation detoxification, oxidizing sulfite (SO_3^{2-}) to sulfate SO_4^{2-} by sulfite oxidase; reductive detoxification, SO_3^{2-} being reduced to S^{2-} by sulfite reductase (SiR). Moreover, OASTL and SAT activity, and levels of cysteine (CYS) and glutathione (GSH) also increased in *P.*×*euramericana* cv. 'Purui' in response to SO_2 fumigation, and the gene expression encoding Glutathione S-transferases (GST), and some enzymes in cysteine and methionine metabolism was up-regulated. For *Populus*×*euramericana* cv. '74/76' with weaker resistance to sulfur dioxide, it only detoxified by increasing the activity of SiR, and but down-regulated the expression of gene encoding 3'-phosphoadenosine 5'-phosphosulfate synthase (PAPSS), which could affect the consumption of sulfite in the exposure to SO_2. Thus, the SO_2-resistant difference of the two poplar varieties is mainly attributed to variation in activity of the enzymes and content of their metabolites in pathways of sulfur metabolism, and gene expression of some enzymes in cysteine and methionine metabolism also plays a role in the resistant difference.

Keywords SO_2 resistance · Sulfite oxidase · Sulfite reductase · Sulfur metabolites · Gene transcripts · Poplar

Abbreviations

SO_2	Sulfur dioxide
SO	Sulfite oxidase
SiR	Sulfite reductase
SAT	Serine acetyltransferase
OAS	O-acetylserine
OASTL	O-acetylserine (thiol) lyase
APR	Adenosine 5′-phosphosulfate reductase
APS	Adenosine 5′-phosphosulfate
Cys	Cysteine
γ-EC	γ-Glutamylcysteine
GSH	Glutathione
PAPSS	3'-Phosphoadenosine 5'-phosphosulfate synthase
ATCYSC1	L-3-cyanoalanine synthase
cysK	Cysteine synthase
mmuM	Homocysteine S-methyltransferase
metE	5-Methyltetrahydropteroyltriglutamate-homocysteine methyltransferase
E4.4.1.11	Methionine-gamma-lyase
ACS	1-Aminocyclopropane-1-carboxylate synthase
DNMT1	DNA (cytosine-5)-methltransferase 1
ahcY	Sdenosylhomocysteinase
TAT	Tyrosine aminotransferase
ggt	Gamma-glutamyltranspeptidase (E2.3.2.2) or glutathione hydrolase (E3.4.19.13)
GST	Glutathione S-transferase
Met	L-methionine
SAM	S-adenosyl-L-methionine
ACC	1-Aminocyclopropane-1-carboxylate

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Introduction

Sulfur dioxide (SO_2) is one of the hazardous environmental pollutant gases and mainly emitted from the combustion of fossil fuel and eruption of volcanoes. SO_2 enters plant leaves mainly through stomata. On the cell surface or in the cytoplasm, SO_2 is converted into bisulfite and sulfite ions after encountering water. These ions can lead to the production of free radical ions such as reactive oxygen species (ROS) in plants (Asada and Kiso 1975; Alscher 1984). They initiate a series of reactions in plants, leading to metabolic changes. Some of these changes will cause serious harm to plants. High concentration of SO_2 may even lead to acute leaf necrosis or even plant death. To maintain physiological activity and survival, plants have evolved effective protective mechanisms against sulfur dioxide stress (Kondo 2002). These bisulfite and sulfite ions enter the sulfur assimilation process in plants, during which they are reduced and converted to cysteine and other sulfur compounds (Filner et al. 1984; Heber and Hüve 1998), or oxidized to sulfate (Pfanz et al. 1990; Eilers et al. 2001).

In peroxisome, sulfite oxidase (so) catalyzes the conversion of sulfite (so_3^{2-}) to sulfate (so_4^{2-}) (Rennenberg and Herschbach 2014, refer there). The generated sulfate can be stored in vacuoles or directly entered the assimilation pathway. In chloroplast or cytoplasm, sulfate is activated by ATP sulfonylase (ATPS) to form adenosine 5´-phosphate sulfate (APS). APS can then be further phosphorylated to 3'-adenosine phosphate 5'-phosphate sulfate (PAPS) by APS kinase (APK) or reduced to sulfite (so_3^{2-}) by APS reductase (APR). On the other hand, sulfite can be reduced to sulfide (s^{2-}) by sulfite reductase (SIR). In chloroplasts, mitochondria, and cytoplasm, sulfide combines with o-acetylserine (OAS) to form cysteine (Cys), which is the precursor of all sulfur-containing organic compounds. Serine acetyltransferase (SAT) synthesizes o-acetylserine (OAS), a precursor of Cys, and then o-acetylserine (mercaptan) lyase (oastl) exchanges acetate of OAS for sulfide. Cysteine is mainly used to synthesize proteins, In addition to protein synthesis, glutathione (GSH) is considered to be the main product of cysteine consumption.

If sulfites persist in plant cells for a long time, plants will suffer serious stress and damage. The key to detoxification is to convert bisulfite and sulfite as soon as possible. A previous study has found that sulfite oxidation and sulfite reduction as well as the assimilation all contribute to SO_2 detoxification of Poplar that is exposed to SO_2 (Randewig et al. 2014). Although the detoxification mechanisms of plants to sulfite are clear, whether these attributes are related to the ability of plants to resist sulfur dioxide has not been reported. As we know there are different resistances to SO_2 over various plant species. Is the resistance to sulfur dioxide reflected in these attributes? In addition, SO_2 evokes a comprehensive reprogramming of metabolic pathways, and there are significant changes in the transcript abundance of genes that participate in SO_2 metabolic pathways in Arabidopsis (Zhao and Yi 2014). The transcriptome analysis of poplars under SO_2 fumigation is not yet clarified.

Before 2017, China was the largest emitter of sulfur dioxide in the world due to the rapid development of industrialization (Li 2017). In a previous study, we found that the poplar variety of *Populus*×*euramericana* cv. 'Purui' expressed stronger resistance to SO_2 contamination compared with another similar poplar variety of *Populus*×*euramericana* cv. '74/76' (Xu et al. 2011). *P.*×*euramericana* cv. 'Purui' has some characteristics of coping with SO_2 stress compared with *P.*×*euramericana* cv. '74/76' as follows: 1) maintains relatively higher net assimilation rate after fumigation for 2 h with 9.1 μL L^{-1} SO_2 fumigation; 2) has higher reduced glutathione (GSH) content and superoxide dismutase (SOD) activity; 3) has thicker cuticle, and larger size of stomata with a lower density (Xu et al. 2011). The cuticle can function as the first barrier for toxic gases (Tamm and Cowling 1977), but sulfur dioxide intake into leaves through cuticle cannot be fully neglected, although it is a proceeding at low rates (Lendzian 1984). As mentioned above, SO_2 enters plants mainly through stomata; thus, stomatal density and aperture are important factors that determine SO_2 entering plants. When subjected to SO_2 stress, some plants avoid excessive toxic gas inhalation through sensitive stomatal movement (Robinson et al. 1998; Grulke et al. 2007). However, it is not clear whether the variety brings into play in its resistance to SO_2 pollution by suppressing the entrance of the pollutant or reducing the toxic of SO_2 in leaves. In a preliminary experiment, we have found that there is more sulfate content in *P.*×*euramericana* cv. 'Purui' leaves compared with *P.*×*euramericana* cv. '74/76' in the environment with mild SO_2 pollution, suggesting that *P.*×*euramericana* cv. 'Purui' may not suppress the entrance of SO_2 into the leaves. To further study the resistant mechanism of *P.*×*euramericana* cv. 'Purui' to SO_2 pollution, in this study, we compared the differences in the detoxification mechanism, including effects of SO_2 on leaf enzyme activities of the sulfate assimilation pathway, sulfur metabolites, and transcriptional regulation of enzymes related to sulfur metabolism, between *P.*×*euramericana* cv. 'Purui' and *P.*×*euramericana* cv. '74/76'. Under SO_2 fumigation, the co-regulation of enzymes involved in the sulfur assimilation pathway and the impact on the transcript levels of genes involved in the sulfur metabolite, cysteine and methionine metabolite, and glutathione metabolite were investigated in *P.*×*euramericana* cv. 'Purui' and *P.*×*euramericana* cv. '74/76' varieties. The main aim of the present study is to identify the differences

in detoxification mechanism and gene expression changes of sulfur metabolism between *P.* × *euramericana* cv. 'Purui' and *P.* × *euramericana* cv. '74/76', and whether these features are related to the ability of the poplars to resist sulfur dioxide.

Materials and methods

Plant material

This experiment was performed with 1-year-old cuttings of two poplar varieties, *Populus* × *euramericana* CV. 'Purui' and *Populus* × *euramericana* CV. '74/76'. The cuttings were grown in a greenhouse (26 ± 5 °C) until 6–8 expanded leaves were produced and then were placed into a growth chamber (BIC-400, Boxun, Shanghai, China) for 1 week to acclimate to the conditions of 25/16 °C (day/night), a photoperiod of 16 h light (6 a.m.–10 p.m.) with a photosynthetic photon flux density (PPFD) of 125 ± 5 μmol m^{-2} s^{-1} at plant level, and 60% relative air humidity. To avoid SO_2 absorbance by the soil substrate, pots were wrapped by the air tight plastic before starting the fumigation experiments.

Experimental design

Six poplar plants of each variety were placed in a Plexiglass enclosure (40 × 50 × 90 cm^3) and exposed to SO_2 using an experimental fumigation system referred to the design by Randewig et al. (2012). The enclosure was equipped with a fan (ACF-120, Golden Filed, Beijing, China), and leaf temperature was measured with a thermocouple (copper-constantan thermocouple, Type 'T') positioned on the lower leaf surface. The temperature and relative humidity in the enclosure were monitored with a combined humidity and temperature sensor (HMP155, Vaisala, Finland). Light in the controlled-environment chamber was provided by fluorescent lamps (12 V, 58 W, Philips Master TLD Leuchtstoffampen, Germany). The enclosure was flushed with 10 L min^{-1} of filtered ambient air provided by a gas cylinder. Defined SO_2 concentrations were adjusted by adding a defined amount of concentrated SO_2 (250 μL L^{-1}, Yiyang CO., Beijing, China) to the airstream by means of a mass flow controller (21S-1–55-1–5-KMB05, ALICATA, USA). Mass flow meters (21–1-00–1-20-KMB05, ALICATA, USA) were used to control the flow rates into the enclosure. The airstream was connected to a SO_2 analyzer (AP-G008, Anpaer, Shenzhen, China). Leaf temperature, enclosure temperature, and relative humidity were recorded in a PC-based data acquisition system (CR1000, Campbell Scientific, USA) with intervals of 24 min.

The plants in the Plexiglass enclosure were exposed to 0.7, 1.4, and 2.1 μL L^{-1} SO_2 for 5 h. Under the highest concentration of SO_2 (2.1 μL L^{-1} SO_2), the susceptible variety of *P.* × *euramericana* cv. '74/76' exhibited visible symptoms of injury induced by SO_2 (Fig. 1), while the resistant variety of *P.* × *euramericana* cv. 'Purui' did not have visible symptoms. The moderate concentration of 1.4 μL L^{-1} SO_2 caused phenotypical symptoms of injury with small necrotic spots on the leaves of the susceptible variety. The lower concentration of SO_2 did not induce visible symptoms regardless resistant or susceptible variety. Later on, we chose the moderate concentration of 1.4 μL L^{-1} SO_2 to treat the plants, and compared their responses in the activity of enzymes, the content of metabolites, and the expression of enzymatic genes in pathways of sulfur metabolism. The leaf samples exposed to 1.4 μL L^{-1} SO_2 were harvested and immediately frozen in liquid nitrogen and stored at – 80 °C for RNA extraction and biochemical analysis. The 5th and the 6th expanded leaf, counted from the apex, were pooled for biochemical analysis, and the 4th and the 5th leaf were pooled for RNA extraction. The treatment was repeated three times with a new set of plants. A parallel fumigation experiment with air served as the control.

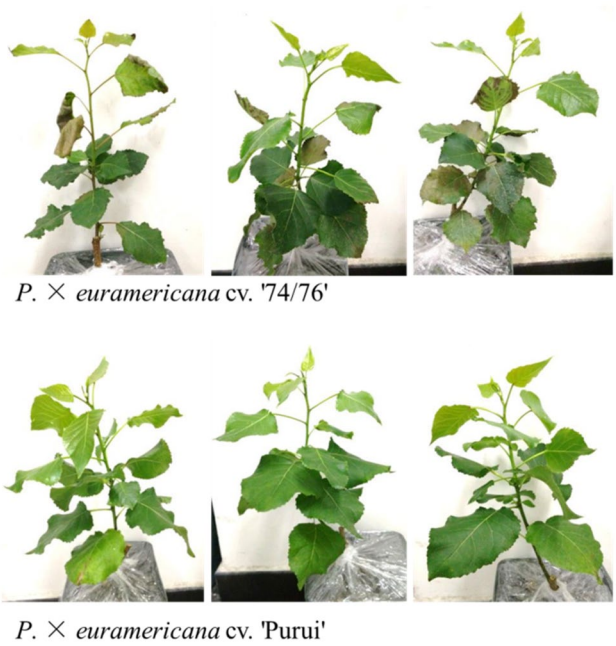


Fig. 1 Visible symptoms of injury induced by SO_2. Upper row is susceptible variety of *P.* × *euramericana* cv. '74/76', and lower row is resistant variety of *P.* × *euramericana* cv. 'Purui'. Both varieties were subjected to 2.1 μL L^{-1} SO_2 for 5 h

RNA extraction, transcriptome sequencing, gene expression analysis, and functional annotation

Total RNA of each sample was extracted using a Quick RNA extraction kit (Bioteke Corporation, Beijing, China). RNA was purified and examined with a NanoDrop ND1000

spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The RIN (RNA integrity number) values (> 8.0) of these samples were assessed using an Agilent 2100 Bioanalyzer (Santa Clara, CA, USA). The construction of the libraries and the RNA-Seq were performed by the Biomarker Biotechnology Corporation (Beijing, China). The cDNA libraries were paired-end sequenced using an Illumina HiSeq™ 2000. The clean reads were mapped to the *Populus* genome database (Phytozome10, *Populus trichocarpa* v. 3.0) using TopHat2 (Kim et al. 2013). Transcript levels are presented as reads per kilobase of exon model per million mapped reads (RPKM) values (Florea et al. 2013). Differentially expressed genes (DEGs) were identified using the following criteria: Fold Change ≥ 2 and false discovery rate (FDR) ≤ 0.01, and DESeq was used to analyze the differential expression between sample groups (Anders and Huber, 2010). Pathway annotation of differentially expressed genes was based on the KEGG (Kyoto Encyclopedia of Genes and Genomes) databases (http://www.genome.jp/kegg/kegg2.html) (Gerlich and Neumann 2000).

Gene validation of expression analysis

The cDNA synthesis was obtained from total RNA using the SuperScript III First-Strand Synthesis System (Promega) according to the manufacturer's instructions. Control reactions without a template were performed for each primer pair. RT-q-PCR was performed using a LightCycler 480 instrument (Roche Applied Science, Indianapolis, IN, USA). At least two independent biological replicates and four technical replicates of each biological replicate for each sample were analyzed by q-PCR to ensure reproducibility and reliability. Normalization was performed using three stably expressed reference genes (UBQ-L, αTub, and ACT2). The relative quantification value for each target gene was compared to those three reference genes. Annotation of differentially expressed genes on the pathway of sulfur, glutathione, cysteine, and methionine metabolism was focused and subjected to real-time quantitative PCR (q-PCR) with specific primers identified by Primer3 (Supplementary Table S1).

Quantification of enzyme activities

Total protein was extracted from frozen leaves using the Plant Total Protein Extraction Kit (Sigma) and detected at a wavelength of 595 nm using a microplate ELISA reader (SpectraMax 190, Molecular Devices, USA). For calibration, different amounts of bovine serum albumin (Sigma-Aldrich) were measured as a standard.

SiR activity was determined using the method as described by Randewig et al. (2012) with some modification. For SiR activity measurement, 10 µL total protein was diluted in 90 mL Tris acetate buffer (pH 7.8) to a final volume of 100 µL. The obtained solution is enzyme solution, and 20 µL enzyme solution was added to 80 µL reaction buffer (25 mM HEPES pH 7.8; 1 mM Na_2SO_3; 5 mM OAS-HCl, 10 mM DTT, 30 mM $NaHCO_3$, 15 mM $Na_2S_2O_4$, and 5 mM reduced methylviologen). The enzyme reaction was started by pipetting 20 µL of diluted enzyme solution into the reaction buffer and was stopped at 15 min by adding 50 µL of 20% TCA. After adding 100 µL glacial acetic acid and 200 µL of ninhydrine reagent (250 mg ninhydrine, 6 mL concentrated acetic acid, and 4 mL concentrated HCl), the light absorption of the reaction solution was measured at a wavelength of 560 nm using a microplate ELISA reader. For calibration, different amounts of cysteine were measured as a standard.

O-acetylserine(thiol) lyase (OAS-TL) and serine acetyltransferase (SAT) activity was measured using the method as described by Hartmann et al. (2000) with some modification. Briefly, homogenized frozen leaf material (0.1 g) was added to 3 mL pre-cool extraction buffer (30 mM Tris–HCl with 10 mM DTT) and then centrifuged for 20 min at 14,000 g at 4 °C. The enzyme assay contained 0.1 mL of supernatant and 0.1 mL reaction buffer (50 mM K_2HPO_4-KH_2PO_4 (pH = 7.5), 5 mM DTT, 10 mM O- acetylserine, 2 mM Na_2S) for OAS-TL determination, or 0.1 mL reaction buffer (4 mM serine, 2 mM acetyl-CoA, 50 mM K_2HPO_4-KH_2PO_4 (pH = 7.5), 0.5 mM DTT, and 1 mM Na_2S) for SAT determination. The mixture was incubated at 25 °C for 10 min (for OAS-TL assay): and for 30 min (for SAT assay). Then, 50 µL 20% TCA was added to the reaction solution which then was centrifuged for 20 min at 14,000 g at 4 °C. The supernatant was transferred to the test tube, to which 100 µL glacial acetic acid and 200 µL of ninhydrine reagent (250 mg ninhydrine, 6 mL concentrated acetic acid, 4 mL concentrated HCl) were added. The mixture was boiled for 10 min, and then, 550 µL 95% ethyl alcohol was added. After quick-cooling, the light absorption of the reaction solution was measured at a wavelength of 560 nm using a microplate ELISA reader. For calibration, different amounts of cysteine were measured as a standard.

For APR activity measurements, frozen leaf material (100 mg) was homogenized in extraction buffer [100 mM mono-/dipotassium phosphate buffer (pH 7.7) including 10 mM Na_2SO_3, 0.5 mM AMP, 10 mM DTT, 5 mM sodium EDTA, 10 mM L-Cys, 1% Triton X-100, and 2% polyvinylpyrrolidone (PVP40)] to a final volume of 3 mL, and then centrifuged for 10 min at 12,000 g at 4 °C. APR activity measurement followed the method by Trüper and Rogers (1971). The reaction mixture consisted of aliquots of 1 mL extract with 3 mL reaction buffer [50 mM Tris–HCl buffer (pH = 7.2) including 1.2 µmol/L AMP, 1.5 µmol/L $K_3Fe(CN)_6$, 12 µmol/L Na_2SO_3 and 24 µmol/L EDTA]. Light absorption was measured at a wavelength of 420 nm using a microplate ELISA reader. For calibration, different

amounts of bovine serum albumin (Sigma-Aldrich) were measured as a standard.

SO activity was determined using the method by Randewig et al. (2014) with some modification. Frozen leaf material (1.8 g) was grinded in liquid nitrogen and mixed with extraction buffer (100 mM Tris acetate buffer pH 7.5, 10 mM KCl, 1 mM EGTA, 1 mM EDTA, 10 mL glycerin, freshly added: 2% v/v Triton X-100, 2% w/v PEG 1500, 2% v/v PMSF, 0.2% w/v ascorbic acid) to a final volume of 3 mL, and then centrifuged for 10 min at 12,000 g at 4 °C. Protein was re-dissolved in 0.6 mL modified resuspension buffer (50 mM HEPES pH 7.4, 1 mM EDTA, 6 mM DTT, 0.5 mM PMSF). Protein quantification was determined with the method of Bradford (1976), with Coomassie brilliant blue G-250 (mainly binds to basic or aromatic amino acid residues). Light absorption was measured at a wavelength of 595 nm using a microplate ELISA reader. For calibration, different amounts of bovine serum albumin (Sigma-Aldrich) were measured as a standard. For measuring SO activity, 200 µL of the protein extract was diluted in 200 µL Tris acetate buffer pH 7.25 to a final volume of 400 µL. Reaction was started by adding 100 µL of 0.5 mM sulfite. Quantification of sulfite reduction was measured using a solution containing formaldehyde and acid fuchsin for stopping the enzyme reaction and visualizing the color change by the formation of sulfate mediated by SO (Lang et al. 2007). The correlation between sulfite content (0–200 µM) and absorption values at 580 nm was determined for calculating SO activity (data for calibration curves not shown).

Metabolite determination

Quantification of thiols

The thiol content was determined by Agilent 1200 high-pressure liquid chromatography (HPLC) system (water 2695 HPLC, USA) (Ju et al. 2011). Homogenized frozen leaf material (200 mg) was extracted with extraction buffer (5 mM DTPA, 0.1% TFA, pH = 3.7) and then centrifuged for 10 min at 12,000 g at 4 °C. One mL supernatant was added to 2.6 mL HEPES buffer (5 mM DTPA, 200 mM HEPES, pH = 9) and 0.1 mL TCEP (20 mM with HEPES buffer) and incubated at 25 °C for 10 min in the dark, and then, 80 µL monobromobimane (50 mM with ACN) was added. The solution was derivatized at 25 °C for 30 min, and then, 400 mL MSA (1 mol·L^{-1}) was added for stopping reaction. The derivative samples were filtered through 0.22 µm Nylon filter membrane (Millipore Corp., USA) and transferred to Agilent brown head empty bottle at 4 °C. Determination of thiol content was performed by fluorometric analysis (excitation at 380 nm, emission at 470 nm) with an HPLC Monitor and accounted by external standard method according to the apex apices area. The moving phase A was 0.1% TFA, and the moving phase B was 100% acetonitrile. The procedure was: 0–20 min, 8%-26% B; 20–22 min, 26%-100% B; 22–24 min, 100% B; 24–28 min, 100%-8% B; 28–30 min, 8% B.

Quantification of sulfate

Leaf SO_4^{2-} content was quantified using anion exchange chromatograph (Dionex ICS-3000) (Brychkova et al. 2007). Homogenized oven-dried leaf material (0.1 g) was added to 1 mL deionized water containing 100 mg PVPP. The samples were shaken for 1 h at 4 °C, boiled for 15 min, and then centrifuged for 10 min at 15,000 g at 4 °C. The supernatant was centrifuged again for 5 min and diluted 20 times. Sulfate was quantified using a calibration curve of increasing sulfate concentration (50–800 µM).

Statistical analyses

Statistical analyses were performed using SAS 10.0 (SAS Institute Inc., Cary, NC, USA). Data were subjected with two-way ANOVA (with variety and treatment as factors) to detect differences between treatments (SO_2 exposure and controls) and between different poplar varieties. Before ANOVA, data were ln-transformed to meet the assumptions of homogeneity of variance and normality when necessary.

Results

Under ambient air condition, the difference of enzyme activities, metabolites, and gene expression between the two varieties

Under ambient air conditions, there were no significant differences in SO ($P = 0.052$), APR ($P = 0.537$), and OASTL ($P = 0.441$) activities (Fig. 2), and CYS ($P = 0.937$) and GSH ($P = 0.150$) contents (Fig. 3) in leaves between *P.* × *euramericana* cv. 'Purui' and *P.* × *euramericana* cv. '74/76'. While *P.* × *euramericana* cv. 'Purui' had higher SiR activity (Fig. 2c, $P = 0.009$) than *P.* × *euramericana* cv. '74/76', the latter had a significantly higher SAT activity (Fig. 2d) (1.2-fold, $P = 0.006$) compared with *P.* × *euramericana* cv. 'Purui'. *P.* × *euramericana* cv. 'Purui' had roughly 1.6-fold ($P = 0.017$) higher sulfate contents (Fig. 3a) in leaves compared with *P.* × *euramericana* cv. '74/76'.

For assimilation sulfate reduction, sulfate has to be activated by the ATP sulfurylase (ATPS) to form adenosine 5′-phosphosulfate (APS), which catalyzes the first step in this pathway. APS is converted into PAPS (3´-phosphoadenosine 5´-phosphosulfate) by 3'-phosphoadenosine 5'-phosphosulfate synthase (PAPSS), and the activated sulfate is partly converted into APS by PAPSS (Supplementary Fig.

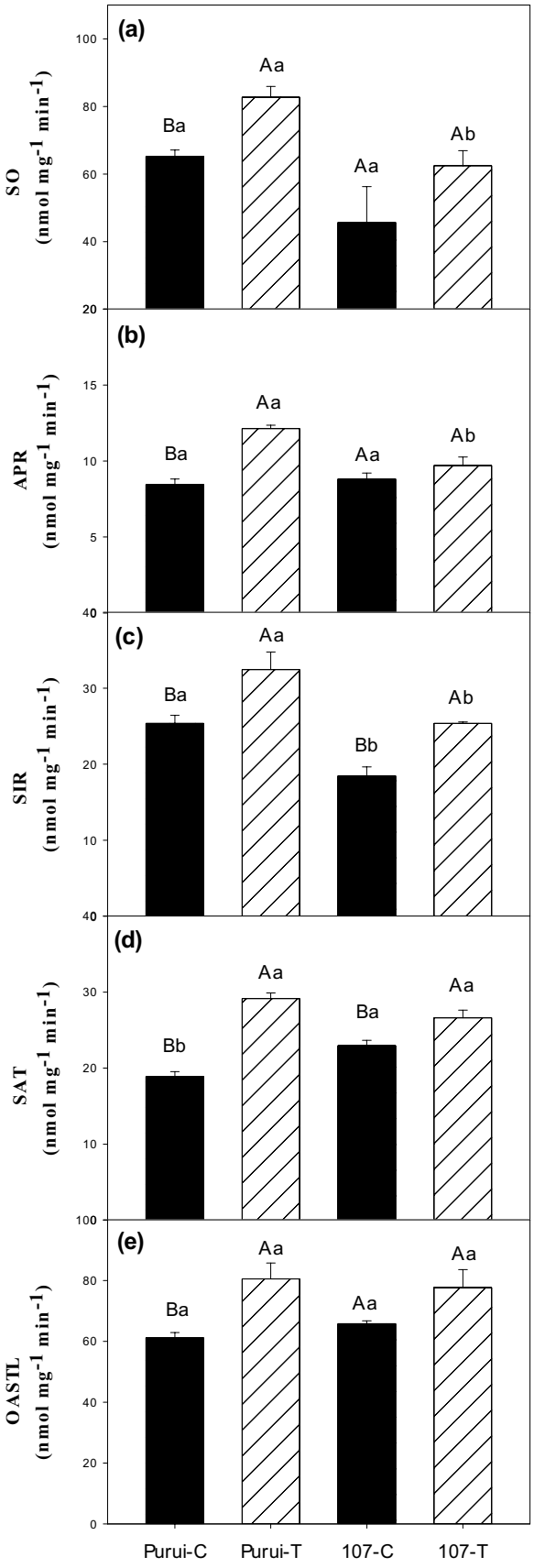


Fig. 2 Activities of important sulfur metabolism enzymes in ▶ *P.*×*euramericana* cv. 'Purui' and *P.*×*euramericana* cv. '74/76' in response to 1.4 μL L^{-1} SO_2 for 5 h. Means and standard errors ($n=3$) of enzyme activities are shown. Different capital letters indicate significant differences between the treatments within one poplar variety ($P<0.05$). Different lower case letters indicate significant differences between the poplar varieties within one treatment ($P<0.05$): **a** sulfite oxidase (SO), **b** adenosine 5′-phosphosulfate reductase (APR), **c** sulfite reductase (SIR), **d** serine acetyltransferase (SAT), and **e** O-acetylserine (thiol) lyase (OASTL). Purui-T is for SO_2 treatment of *P.*×*euramericana* cv. 'Purui'; Purui-C for control of *P.*×*euramericana* cv. 'Purui'; 107-T for SO_2 treatment of *P.*×*euramericana* cv. '74/76'; 107-C for control of *P.*×*euramericana* cv. '74/76'

f). PAPSS in *P.*×*euramericana* cv. '74/76' was higher than that in *P.*×*euramericana* cv. 'Purui' (Fig. 4a). For glutathione metabolism, glutathione (GSH) partly converted into L-cysteinyl-glycine by gamma-glutamyltranspeptidase (E2.3.2.2) or glutathione hydrolase (E3.4.19.13) (ggt) (Supplementary Fig. g). The ggt in *P.*×*euramericana* cv. '74/76' was lower than that in *P.*×*euramericana* cv. 'Purui'. The low sulfate may be attributed to the relatively higher PAPSS activity that consumes the sulfate. On the other hand, higher SiR activity in *P.*×*euramericana* cv. 'Purui' did not result in more CYS and GSH contents, probably because the higher ggt may keep the organic sulfur substances in balance.

After SO_2 exposure, the difference of enzyme activities, metabolites, and gene expression between the two varieties

After SO_2 exposure, SO, APR, and SiR enzyme activities of *P.*×*euramericana* cv. 'Purui' were higher (1.3-fold, $P=0.046$; 1.3-fold, $P=0.003$; 1.3-fold, $P=0.007$) than those of *P.*×*euramericana* cv. '74/76' (Fig. 2). There were no significant differences in SAT (Fig. 2d $P=0.053$) and OASTL (Fig. 2e $P=0.627$) activities between the two varieties. *P.*×*euramericana* cv. 'Purui' had roughly 2.0-fold ($P=0.002$) higher sulfate contents (Fig. 3a) and higher GSH contents (Fig. 3c) in leaves than *P.*×*euramericana* cv. '74/76'. There was no significant difference in CYS (Fig. 3b $P=0.357$) content between the two varieties.

For sulfate metabolism, there was no significant difference in the process between *P.*×*euramericana* cv. 'Purui' and *P.*×*euramericana* cv. '74/76' after exposure to SO_2 (Fig. 4b). For cysteine and methionine metabolism, L-homocysteine converted into L- methionine by homocysteine S-methyltransferase (mmuM or BHMT2) or 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (metE), L-methionine (Met) partly converted into S-Adenosyl- L-methionine (SAM) by S-adenosylmethionine synthetase (metK). SAM partly converted into S-adenosyl-L-homocysteine by DNA (cytosine-5)-methltransferase 1 (DNMT1 OR dcm). S-adenosyl- L-homocysteine converted into L- momcysteine by adenosylhomocysteinase (ahcY). On

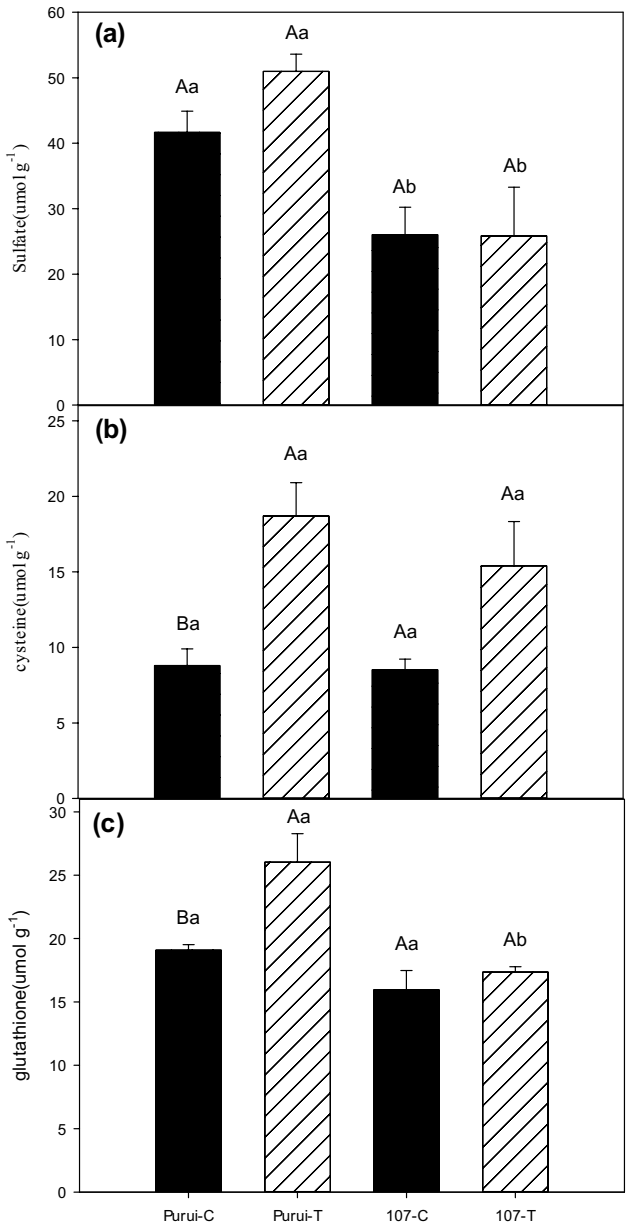


Fig. 3 Sulfur metabolites in controls and SO^2-exposed (1.4 μL L^{-1} SO_2 for 5 h) *P.*×*euramericana* cv. 'Purui' and *P.*×*euramericana* cv. '74/76' poplars. Means and standard errors ($n=3$) of sulfur metabolites are shown. The differences of sulfur metabolites were analyzed using two-way ANOVA (with variety and treatments as factors); **a** sulfate, **b** cysteine, and **c** glutathione. Different capital letters indicate significant differences between the treatments within one poplar variety ($P<0.05$). Different lower case letters indicate significant differences between the poplar varieties within one treatments ($P<0.05$). Purui-T is for SO_2 treatment of *P.*×*euramericana* cv. 'Purui'; Purui-C for control of *P.*×*euramericana* cv. 'Purui'; 107-T for SO_2 treatment of *P.*×*euramericana* cv. '74/76'; 107-C for control of *P.*×*euramericana* cv. '74/76'

the other hand, 4-methylthion-2-oxobutanoate converted into L-methionine by tyrosine aminotransferase (TAT) (Supplementary Fig. h). After SO_2 exposure, mmuM, metE, ahcY, and TAT were down-regulated in *P.*×*euramericana* cv. '74/76' compared with Purui poplar variety (Fig. 4b).

Effect of SO_2 on enzyme activities, metabolites, and gene expression in *P.*×*euramericana* cv. 'Purui'

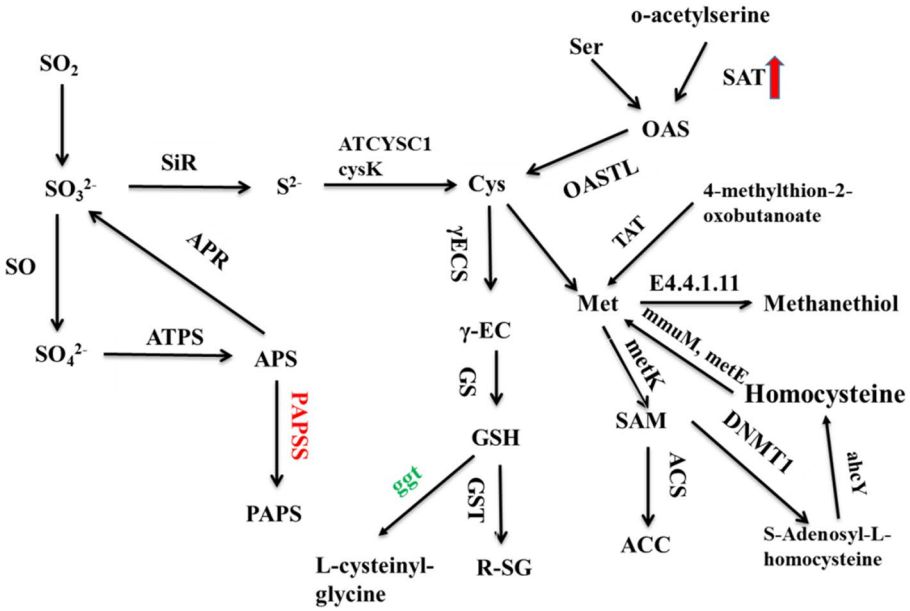
There were significant differences in the all five enzymes and two organic thiol compounds in leaves of *P.*×*euramericana* cv. 'Purui' between SO_2 exposure treatment and the control (exposure to ambient air). SO, APR, SiR, SAT, and OASTL activities increased in response to SO_2 by 27% ($P=0.045$), 44% ($P<0.001$), 28% ($P=0.007$), 54% ($P<0.001$), and 32% ($P=0.010$), respectively (Fig. 2). *P.*×*euramericana* cv. 'Purui' after exposure to SO_2 had a significantly higher CYS (1.4-fold, $P=0.019$) (Fig. 3b) and GSH contents (2.1-fold, $P=0.008$) (Fig. 3c) than that under ambient air condition. However, there was no significant difference in sulfate between SO_2 exposure and the control (Fig. 3a $P=0.112$).

For cysteine and methionine metabolism, sulfide and O-acetyl- L-serine together synthetize L-cysteine by L-3-cyanoalanine synthase (ATCYSC1) or cysteine synthase (cysK), which produces organic sulfocompounds from sulfide (Supplementary Fig. a, b). Under SO_2 fumigation, L-3-cyanoalanine synthase (ATCYSC1) in *P.*×*euramericana* cv. 'Purui' was down-regulated compared with that in ambient air condition (Fig. 4c). Met was partly converted into SAM by S-adenosylmethionine synthetase (metK), SAM was partly converted into 1-aminocyclopropane-1-carboxylate (ACC) by 1-aminocyclopropane-1-carboxylate synthase (ACS) (Supplementary Fig. b). MetK and ACS were up-regulated in Purui poplar variety by SO_2 fumigation (Fig. 4c). For glutathione metabolism, glutathione (GSH) together with RX partly converted into R-S-glutathione by glutathione S-transferase (GST) (Ye and Song 2005) (Supplementary Fig. c). GST was up-regulated in *P.*×*euramericana* cv. 'Purui' after SO_2 fumigation compared with that in ambient air condition.

Effect of SO_2 on enzyme activities, metabolites, and gene expression in *P.*×*euramericana* cv. '74/76'

SO, APR, SiR, SAT, and OASTL activities increased in response to SO_2 by 37% ($P=0.086$), 10% ($P=0.152$), 37% ($P=0.009$), 16% ($P=0.011$), and 18% ($P=0.073$), respectively (Fig. 2). SiR ($P=0.009$) and SAT ($P=0.011$) activities in *P.*×*euramericana* cv. '74/76' after SO_2 fumigation were higher than those under ambient air condition, while there were no significant differences in SO ($P=0.086$), APR ($P=0.152$), and OASTL ($P=0.073$) activities in *P.*×*euramericana* cv. '74/76' over the two different treatments (Fig. 2). Sulfate ($P=0.979$) levels, CYS ($P=0.076$), and GSH ($P=0.496$) contents of *P.*×*euramericana* cv. '74/76' were not changed by the SO_2 treatment (Fig. 3).

(a) *P.* × *euramericana* cv. 'Purui' vs *P.* × *euramericana* cv. '74/76', under ambient air condition



(b) *P.* × *euramericana* cv. 'Purui' vs *P.* × *euramericana* cv. '74/76', exposed to 1.4 µL L^{-1} SO_2 for 5 h

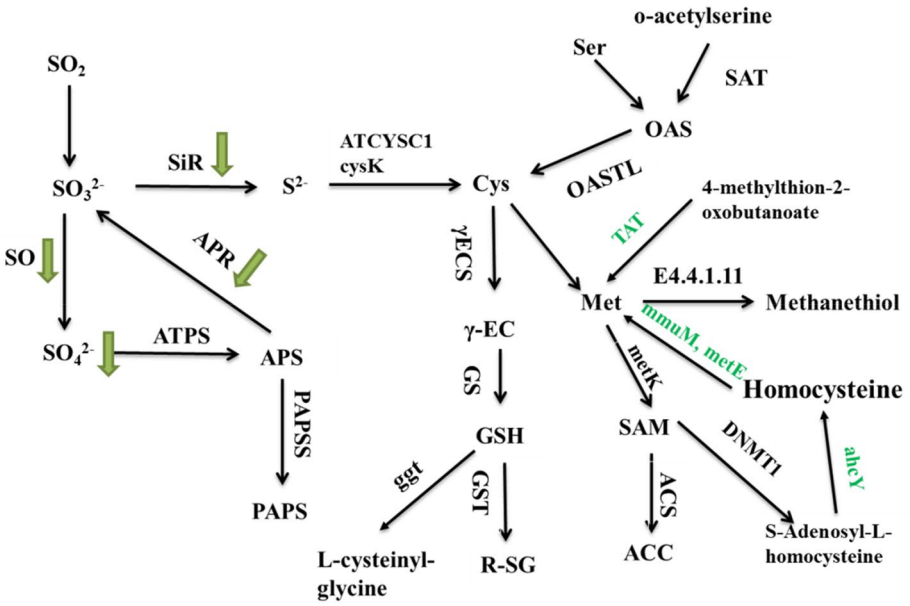
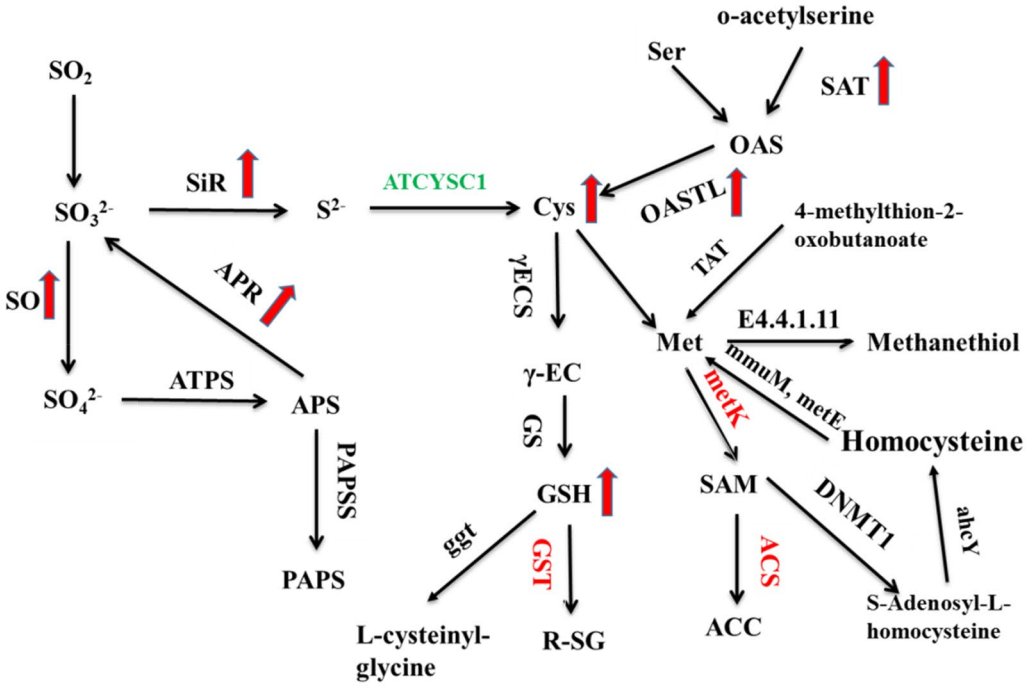


Fig. 4 Schematic views of enzyme activities and S metabolites between *P.*×*euramericana* cv. 'Purui' and *P.*×*euramericana* cv. '74/76' varieties in ambient air and SO_2-exposed conditions. **a** *P.*×*euramericana* cv. 'Purui' vs *P.*×*euramericana* cv. '74/76' in ambient air condition, **b** *P.*×*euramericana* cv. 'Purui' vs *P.*×*euramericana* cv. '74/76' in SO_2-exposed condition, **c** Control vs SO_2 treatment of *P.*×*euramericana* cv. 'Purui', **d** Control vs SO_2 treatment of *P.*×*euramericana* cv. '74/76'. Increasing metabolite levels and enzyme activities are marked with a thick red up arrow. A red word indicates that the enzyme is up-regulated by gene expression. A green word indicates that the enzyme is down-regulated by gene expression

(c) Control vs SO_2-treatment of *P.* × *euramericana* cv. 'Purui'



(d) Control vs SO_2-treatment of *P.* × *euramericana* cv. '74/76'

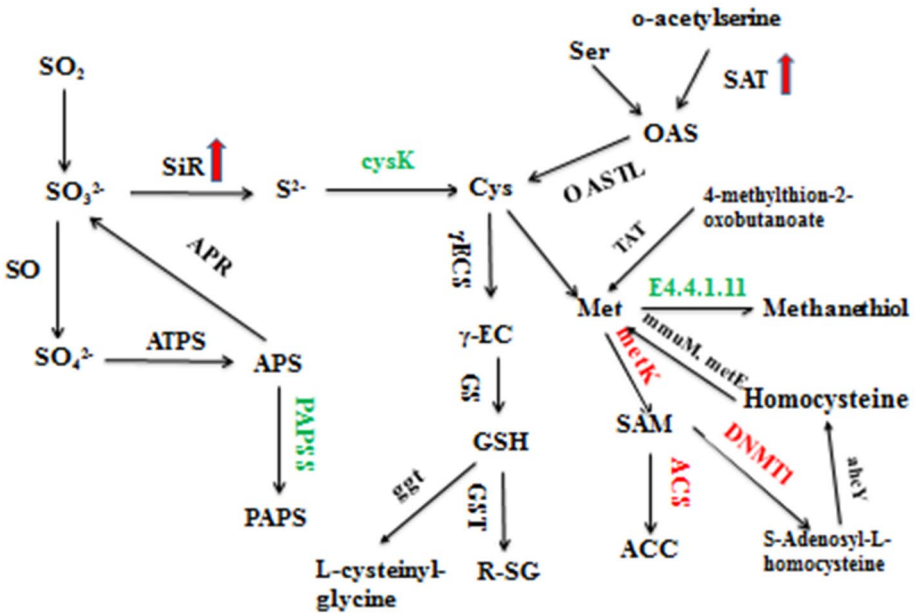


Fig. 4 (continued)

For sulfate metabolism, PAPSS in *P.*×*euramericana* cv. '74/76' was down-regulated by the SO_2 treatment (Fig. 4d). For cysteine and methionine metabolism, sulfide and O-acetyl-L-serine together synthetize L-cysteine by cysteine synthase (cysK), which produces organic sulfo-compounds from sulfide (Supplementary Fig. e). Under SO_2 fumigation, cysteine synthase (cysK) in *P.*×*euramericana* cv. '74/76' was down-regulated compared with that in

ambient air condition. Met partly converted into S-adenosyl- L-methionine (SAM) by S-adenosylmethionine synthetase (metK) and partly converted into methanethiol by methionine-gamma-lyase (E4.4.1.11). SAM partly converted into 1-aminocyclopropane-1-carboxylate (ACC) by 1-aminocyclopropane-1-carboxylate synthase (ACS) and partly converted into S-adenosyl-L-homocysteine by DNA (cytosine-5)-methltransferase 1 (DNMT1 or dcm) (Supplementary Fig. e). Methionine-gamma-lyase (E4.4.1.11) was down-regulated in *P.*×*euramericana* cv. '74/76' by SO_2 fumigation. MetK, DNMT1 and ACS were up-regulated in *P.*×*euramericana* cv. '74/76' by SO_2 fumigation (Fig. 4d).

Discussion

The different detoxification mechanisms in coping with SO_2 between the two varieties

Most poplar species are ranked as highly SO_2 sensitive up to intermediately tolerant (Kozlowski 1980), but clonal variations in pollution resistance are also well known for poplar species (Karnosky 1976, 1977). *Populus*×*euramericana* cv. 'purui' has strong survival ability in the high SO_2 pollution environment with some characteristics of SO_2 tolerance (Xu et al. 2011); however, the metabolite mechanisms of detoxification of sulfur dioxide toxic gases is not clear. Previous experiments with model plant *Arabidopsis thaliana* have identified the detoxification mechanisms of sulfur-containing gases, namely oxidative detoxification (sulfite is oxidized into sulfate) or reductive detoxification (sulfite is reduced to sulfide) (Van der Kooij et al. 1997; Lang et al. 2007; Brychkova et al. 2012, 2013; Hamisch et al. 2012; Randewig et al. 2012). The capacity of sulfite-to-sulfate oxidation is determined by measuring SO activity. To survive under high atmospheric SO_2 concentration, plants need the presence of effective SO, and it is one of the main mechanisms to remove excess sulfite in *Arabidopsis* (Lang et al. 2007; Randewig et al. 2012). In the present study, the SO activity in *P.*×*euramericana* cv. 'Purui' increased after SO_2 exposure, but not in *P.*×*euramericana* cv. '74/76' (Fig. 2a). On the other hand, SiR-defective mutants revealed higher sensitivity to SO_2, whereas SiR-overexpressing plants showed higher resistance (Brychkova et al. 2013; Yarmolinsky et al. 2013). In the present study, the accumulation of Cys and GSH resulted from a strong increase of the SO and SiR activities in *P.*×*euramericana* cv. 'Purui' after SO_2 exposure. However, the only SiR activity was increased in *P.*×*euramericana* cv. '74/76' and the Cys and GSH remained unchanged after SO_2 exposure (Fig. 2c). From the current results, it is evident that both sulfite oxidation and sulfite reduction as well as the assimilation contribute to SO_2 detoxification in Purui poplar variety, which is consistent to the finding with *Populus*×*canescens* (Randewig et al. 2014). The detoxification mechanism of *P.*×*euramericana* cv. '74/76' is only by reductive detoxification with SiR to copy with SO_2. By contrast, *P.*×*euramericana* cv. 'Purui' increases not only SO but also SiR activity, contributing to sulfite detoxification via the sulfite network, which realizes sufficient sulfite reduction for Cys synthesis on one hand and prevents cells from toxic sulfite levels on the other hand. This mechanism is consistent to these previous studies (Brychkova et al. 2013; Yarmolinsky et al. 2013).

APR is one of the main control enzymes in the sulfate assimilation pathway (Kopriva 2006; Khan et al. 2010). The activity of APR is controlled by the negative feedback of reduced sulfur compounds (Tsakraklides et al. 2002). It has been previously reported that exposure of plants to SO_2 can lead to such a down regulation (Randewig et al. 2014; Brunold et al. 1983; Tschanz et al. 1986). The experiment with *Populus*×*canescens* is conducted with different SO_2 concentrations (0.65, 0.8, 1.0, and 1.2 μL L^{-1}) exposed for approximately 3 days. The APR activity is down-regulated and thiol contents increased further with increasing SO_2 concentrations (up to 1.0 μL L^{-1} SO_2). However, the accumulation of Cys and GSH tends to decline after exposure to 1.2 μL L^{-1} SO_2. This indicates that sulfur assimilation may be disturbed during exposure to 1.2 μL L^{-1} SO_2 that is the critical level for *P.*×*canescens* (Randewig et al. 2014). In this study, the APR activity of *P.*×*euramericana* cv. '74/76' remained unchanged, while that of *P.*×*euramericana* cv. 'Purui' was increase after exposure to 1.4 μL L^{-1} SO_2 (Fig. 2b). The increased activity of the APR enzyme can lead to the production of more sulfite, SO_3^{2-}. However, when the activity of APR enzyme is decreased, the content of sulfate will increase. Although SO_4^{2-} is less toxic than SO_3^{2-}, it is associated with stomatal closure in SO_2 polluted environments (Robinson et al. 1998). The sulfate level in leaves of *P.*×*euramericana* cv. 'Purui' and *P.*×*euramericana* cv. '74/76' varieties remained unchanged after SO_2 fumigation (Fig. 3a). While the accumulation of Cys and GSH in leaves of *P.*×*euramericana* cv. 'Purui' tended to increase after exposure to 1.4 μL L^{-1} SO_2, the Cys and GSH levels in leaves of *P.*×*euramericana* cv. '74/76' remained unchanged after SO_2 fumigation (Fig. 3b, c). This might indicate that sulfur assimilation became obviously disturbed during exposure to 1.4 μL L^{-1} SO_2 for *P.*×*euramericana* cv. '74/76'. Cysteine and methionine are sulfur-containing amino acids. CYS is the first organic compound containing reduced sulfur synthesized by the plant (Takahashi et al. 2011). In plants, cysteine is converted by transferring hydrogen sulfide to serine (via acetylserine). The surplus sulfide might have been used as a macronutrient after assimilation into organic sulfur compounds, such as Cys, for growth and development in Purui poplar variety. On the other hand, the APR activity enhanced and hence improved reduction

capacities, which might contribute to maintain the sulfate stability of *P.*×*euramericana* cv. 'Purui'. In addition, GSH is an important antioxidant responsible for maintenance of the antioxidative machinery of the cells under stress (Nagalakshmi and Prasad 2001).

There is a regulatory association of SAT and SiR activities to promote the flux through the sulfate reduction to sulfide pathway (Berkowitz et al. 2002; Riemenschneider et al. 2005; Scheerer et al. 2010). Promotion of this flux appears appropriate to prevent enhanced sulfite levels during SO_2 fumigation in cooperation with enhanced availability of OASTL for cysteine synthesis. From the present experiment, OASTL activity significantly increased in *P.*×*euramericana* cv. 'Purui', but not in *P.*×*euramericana* cv. '74/76', although both *P.*×*euramericana* cv. 'Purui' and *P.*×*euramericana* cv. '74/76' increased SAT and SiR activities in response to SO_2 fumigation. An increased cysteine after SO_2 exposure in *P.*×*euramericana* cv. 'Purui', not in *P.*×*euramericana* cv. '74/76', is also evident.

The difference in transcriptional regulation of enzymes related to sulfur metabolism between the two varieties coping with SO_2

After SO_2 exposure, *P.*×*euramericana* cv. 'Purui' up-regulated mmuM, metE, ahcY, and TAT expression compared with *P.*×*euramericana* cv. '74/76' variety (Fig. 4b). At the same time, *P.*×*euramericana* cv. 'Purui' poplar also up-regulated SO and SiR activity, which can convert toxic sulfite into nontoxic sulfate and organic sulfur substances, such as GSH and CYS. The sulfate and GSH accumulation in *P.*×*euramericana* cv. 'Purui' is evident for that. On the other hand, the up-regulated expressions of some enzymes genes may promote the cysteine and methionine metabolism and cysteine conversion, which may be a reason why CYS did not accumulated.

For assimilation sulfate reduction, sulfate has to be activated to form APS by the ATP sulfurylase (ATPS) and APS is converted into PAPS (3´-phosphoadenosine 5´-phosphosulfate) by 3'-phosphoadenosine 5'-phosphosulfate synthase (PAPSS). On the other hand, the activated sulfate is partly converted into APS by PAPSS. In the current results, transcripts encoding PAPSS in *P.*×*euramericana* cv. '74/76' was down-regulated in response to SO_2 exposure, which could affect the generation of APS and PAPS, and the consumption of sulfite. Thus, the decrease of PAPSS is not conducive for *P.*×*euramericana* cv. '74/76' to detoxify sulfite.

Glutathione S-transferases (GST) were up-regulated in *P.*×*euramericana* cv. 'Purui' after SO_2 fumigation, indicating that a growing number of toxic intermediate metabolites are converted into innocuous substances through combining GSH (Gill and Tuteja 2010; Giraud et al. 2012). GSTs are ubiquitous proteins in plants that play important roles in stress tolerance and detoxification metabolism (Lan et al. 2009; Chan and Lam 2014). In addition, under SO_2 fumigation, L-3-cyanoalanine synthase (ATCYSC1) in *P.*×*euramericana* cv. 'Purui' and cysteine synthase (cysK) in *P.*×*euramericana* cv. '74/76' were down-regulated. It is estimated that SO_2 will affect the cysteine content of plants in the future.

It is also controversial over SO transcriptional regulation in response to SO_2 stress. Plants with over-expressed SO gene increase sulfite oxidation capacity and hence adapt to high concentrations of SO_2 (Brychkova et al. 2007). With Arabidopsis SO knockout lines, Hamisch et al. (2012) have found that the two splice variants of the SO gene are up-regulated under mild SO_2 stress, which provides evidence for the co-regulation between SO and APR at the mRNA level. However, Arabidopsis transcripts encoding SO were not significantly changed in the SO_2-fumigated plants (Zhao and Yi 2014). In this study, we found that transcripts encoding SO also were not significantly changed in both *P.*×*euramericana* cv. 'Purui' and *P.*×*euramericana* cv. '74/76' after SO_2 fumigation, although SO activity in *P.*×*euramericana* cv. 'Purui' was increased after SO_2 exposure. Transcripts encoding APR and sulfate transporter (SULTR) were down-regulated in the chloroplast upon SO_2 exposure (Hamisch et al. 2012). However, we found that transcripts encoding SO, APR, SULTR, and SiR were not significantly changed in *P.*×*euramericana* cv. 'Purui' and *P.*×*euramericana* cv. '74/76' after SO_2 fumigation.

Conclusion

SO_2 enters leaf cells through leaf stomata and combines with water to produce sulfite SO_3^{2-}, which is considered to be toxic to plants. *P.*×*euramericana* cv. 'Purui' can survive in the environment with high concentration of sulfur dioxide, and it has two ways of detoxification: (1) oxidation detoxification, oxidizing sulfite (SO_3^{2-}) to sulfate (SO_4^{2-}) by sulfite oxidase (SO); (2) reductive detoxification, SO_3^{2-} is reduced to S^{2-} by sulfite reductase (SiR). By contrast, *P.*×*euramericana* cv. '74/76" is sensitive to sulfur dioxide, and it detoxifies sulfite only through reduction. In this study, it was found that after SO_2 fumigation, the activity of APR enzyme increased and the content of SO_4^{2-} remained unchanged in *P.*×*euramericana* cv. 'Purui'. Thus, we speculate that *P.*×*euramericana* cv. 'Purui' could reduce and consume SO_4^{2-} produced by the oxidation pathway of SO with increasing the activity of APR enzyme, as to maintain the stability of SO_4^{2-} content in cells. In addition, the increased activity of sulfite reductase (SiR) will reduce SO_3^{2-} to S^{2-}, which is the donor of all sulfur-containing amino acids. This study also found increased OASTL and SAT activity, and increased levels

of cysteine (CYS) and glutathione (GSH) in *P.* × *euramericana* cv. 'Purui' in response to SO_2 fumigation. Cysteine is a precursor of methionine (MET), and the expression of the gene encoding s-adenosine methionine synthetase (metK) is up-regulated. We speculate that more methionine (MET) will be converted to s-adenosine-l-methionine (SAM). The up-regulated expression of genes encoding 1-amino-cyclopropane-1-carboxylate synthetase (ACS) suggests that more s-adenosine-l-methionine (SAM) will be converted into 1-amino-cyclopropane-1-carboxylate (ACC). This process makes more surplus cysteine (CYS) to be utilized. Under the concentration of 1.4 μL L^{-1} SO_2, the sulfur metabolism of *P.* × *euramericana* cv. 'Purui' is not much disturbed. The absorbed SO_2 can be detoxified through the metabolic pathway, and the metabolites generated can be used for the growth of *P.* × *euramericana* cv. 'Purui' in the polluted environment. This may be the reason that *P.* × *euramericana* cv. 'Purui' can survive in the environment polluted by high concentration of SO_2. For *P.* × *euramericana* cv. '74/76' with weak resistance to sulfur dioxide, it only detoxifies sulfite by increasing the activity of sulfite reductase (SiR), and down-regulates the expression of gene encoding 3'-phosphoadenosine 5'-phosphosulfate synthase (PAPSS) under SO_2 stress, which will affect the generation of 3´-phosphoadenosine 5´-phosphosulfate (PAPS), and the consumption of sulfite.

After SO_2 exposure, *P.* × *euramericana* cv. 'Purui' up-regulated mmuM, metE, ahcY, and TAT expression compared with *P.* × *euramericana* cv. '74/76' variety. The up-regulated expressions of those enzymes genes may promote the cysteine and methionine metabolism and GSH accumulation. In addition, glutathione S-transferases (GST) were up-regulated in *P.* × *euramericana* cv. 'Purui' after SO_2 fumigation, suggesting that a growing number of toxic intermediate metabolites are converted into innocuous substances through combining GSH. However, there is no strong correlation between the expression of some enzymatic genes in pathways of sulfur metabolism and the response to SO_2 stress, probably because the post-transcriptional processing of the genes may play a regulatory role.

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Author contribution statement Conceived and designed the experiments: JF and XW. Performed the experiments: JF, LW, WL, ZCh, and JZ. Analyzed the data: JF, LW, and XW. Wrote the paper: JF and XW.

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