



Glutathione Transferases are Involved in Salicylic Acid-Induced Transcriptional Reprogramming

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Abstract

Salicylic acid (SA) plays a crucial role not only in defence against pathogen attacks, but also in abiotic stress responses. Recently, some key steps of SA signalling outlined the importance of redox state-dependent processes. This study explores the role of glutathione transferases (GSTs) in the transcriptional reprogramming of redox status-related genes in seven-day-old wild type and *Atgst* mutant *Arabidopsis thaliana* plants. The timing of redox changes, detected by the redox-sensitive green fluorescent protein (roGFP2), differed in wild type roots treated with 10 μ M or 100 μ M SA. Our results verified how the applied SA concentrations had different effect on the expression of oxidative stress- and redox-related genes, among them on the expression of *AtGSTF8* and *AtGSTU19* genes. Lower vitality and less negative E_{GSH} values were specific characteristics of the *Atgst* mutants compared to the wild type plants throughout the experiment. Changes in the redox potential were only modest in the mutants after SA treatments. A slightly modified gene expression pattern was observed in control conditions and after 1 h of SA treatments in *Atgst* mutants compared to Col-0 roots. These data originating from the whole roots provide indirect evidence for the role of the investigated AtGSTF8 and AtGSTU19 isoenzymes in the transduction of the redox signal. Our results demonstrate that the investigated *Arabidopsis* GSTs have a role in maintaining the levels of reactive oxygen species- and redox homeostasis and are involved in transcriptional reprogramming in the roots.

Keywords *Arabidopsis thaliana* · Gene expression · Glutathione transferase · Reactive oxygen species · Redox state · Salicylic acid

Introduction

The phenolic compound salicylic acid (SA) is a plant hormone, which is involved in the complex network of growth responses, affected by developmental and environmental signals, that also regulate various physiological and biochemical processes in plants (Rivas-San Vicente and Plasencia 2011). One of its well-known effects is the induction of pathogenesis-related proteins and systemic acquired resistance (Uknes et al. 1992; Vlot et al. 2009). Immune reactions mediated by SA are regulated by nonexpressors of pathogenesis-related genes (*NPR1-4*) in *Arabidopsis* (Ding and Ding 2020). Exploring the NPR1 signalling mechanisms revealed its action requires upstream regulatory events, such as the changes in cellular redox status (Pokotylo et al. 2019). Normally, NPR1 is in an inactive state and forms oxidized oligomeric complexes in the cytosol via intermolecular disulphide bonds. Infection initiates a rapid change in the ion flux and in the levels of reactive oxygen species (ROS),

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leading to redox-dependent monomerization and translocation of NPR1 to the nucleus (Mou et al. 2003; Tada et al. 2008). NPRs and other transcription factors, such as TGA (TGACG motif-binding protein) or TGA box- and as-1-like elements containing proteins involved in SA signal transduction, are considered redox sensors for temporal control of gene expression (Blanco et al. 2009).

SA contributes to both plant protection against pathogen attack, and SA-mediated abiotic stress responses. Furthermore, its developmental role under different stresses and normal conditions has been recently highlighted (Miura and Tada 2014; Pasternak et al. 2019). Low concentrations of exogenous SA promote plant growth, but high concentrations of exogenous and endogenous SA have an inhibitory effect. However, the boundaries of these concentrations differ among plant species (Jirage et al. 2001; Tari et al. 2002; Horváth et al. 2015a; Pasternak et al. 2019). SA also influences root meristem structure and root system architecture in *Arabidopsis* in a concentration-dependent manner (Pasternak et al. 2005, 2019; Armengot et al. 2014; Horváth et al. 2015a; Zhao et al. 2015). Although the function of plant hormones and several transcription factors in the formation of root meristem has previously been described, the regulatory roles of ROS and redox status have only emerged in recent years. The central role of ROS in the gene regulatory network in root development was demonstrated by Mase and Tsukagoshi (2021). Plants are constantly subjected to different stresses that significantly increase ROS levels, leading to redox imbalance (Hasanuzzaman et al. 2020).

Jiang and coworkers explored the redox profile of *Arabidopsis* root using cytoplasmic redox-sensing green fluorescent protein (c-roGFP1). They revealed a shift in redox potential in the longitudinal zones of root tips (Jiang et al. 2016). More oxidizing redox status and higher ROS levels were detected in the roots of *Arabidopsis glutathione transferase* mutants (*Atgstf8* and *Atgstu19*) compared to the wild types, while the size of the mutants' meristem was shorter (Horváth et al. 2019).

In higher plants, glutathione transferases (GSTs) are grouped into ten classes, of which four (tau, phi, dehydroascorbate reductase, and lambda) are specific to plants. A widely accepted concept states that this protein superfamily evolved to mitigate the effects of oxidative stress, but distinct classes participate in partially specific reactions. Using glutathione (GSH) as a cosubstrate, specifically the most abundant tau (GSTU) and phi (GSTF) isoenzymes may diminish the GSH pool, thereby influencing various redox-dependent processes (Marrs 1996; Cummins et al. 2011). GSTs can also bind to different hormones such as auxins and cytokinins at their ligand-binding sites (Moons 2005). Moreover, several GSTs have been identified among SA-binding proteins (Tian et al. 2012; Manohar et al. 2015). SA alters the enzymatic activity

of *Arabidopsis* GSTF8, GSTF9, GSTF10, and GSTF11 (Tian et al. 2012). Since it was suggested that many of SA's effects are mediated through SA-binding proteins whose enzymatic activities are altered by the binding of SA (Manohar et al. 2015), these proteins may deserve special attention. The expression of *AtGSTF8* (At2g47730) is induced by short-term treatments with SA (Sappl et al. 2004; Uquillas et al. 2004), ethylene, and H₂O₂ (Wagner et al. 2002) and was proposed to be a marker gene for early stress and defence responses (Thatcher et al. 2015). AtGSTF8 isoenzyme is mainly localized in the cytosol (the other splice variant contains a chloroplast-targeting peptide signal) and, similarly to AtGSTU19, can use a wide range of substrates during GSH conjugation activity (Thatcher et al. 2007; Dixon et al. 2009; Krajewski et al. 2013). Under control conditions, *AtGSTF8* and *AtGSTU19* (At1g78380) are expressed almost in the whole *Arabidopsis* plant, but especially in specific parts and tissues of the root, such as the stele, endodermis, epidermal atrichoblasts, lateral root cap, and root hair zone (Dixon et al. 2010). The cytosolic AtGSTU19 is one of the most abundant GST isoenzymes involved in the stress- and hormonal responses of *Arabidopsis*. Its accumulation can be detected after SA treatment together with AtGSTF8, AtGSTF7, and AtGSTU24 proteins (Wagner et al. 2002; Sappl et al. 2004, 2009; Dixon et al. 2009; Xu et al. 2016). *AtGSTU19* expression is induced by biotic stress (Wagner et al. 2002; Otulak-Kozziel et al. 2022), NaCl, SA, and H₂O₂ as well (Sappl et al. 2009; Horváth et al. 2015a, 2019, 2020). The overexpression of the *AtGSTU19* gene increased abiotic stress tolerance by strengthening ROS scavenging activity or maintaining ROS homeostasis by the elevation of antioxidant enzyme activities (Xu et al. 2016). Due to their GSH-conjugating activity, GSTs are pivotal in the cellular detoxification processes, and via their glutathione peroxidase or dehydroascorbate reductase (DHAR) activities, they can influence the redox status of GSH and ascorbic acid.

As an antioxidant, GSH is involved in ROS processing and limits the lifetime of oxidative signals (Diaz-Vivancos et al. 2015). GSH can also regulate the expression of genes by modulating the redox state of proteins and transcription factors, but this mechanism is yet poorly understood (Dietz 2014; He et al. 2018). Thiol redox biochemistry plays a crucial role in cellular processes, thus preserving the GSH pool, and the relatively high levels of reduced glutathione compared to the oxidized glutathione levels (GSH: GSSG ratio) are essential factors. The maintenance of a highly negative redox potential of glutathione (around—310 mV) is achieved through continuous reduction of glutathione disulphide by glutathione reductase (GR) (Aller et al. 2013). A useful tool for imaging thiol redox potential is a redox-sensitive green fluorescent protein (roGFP2) expressed in

plants, which allows monitoring the redox status in selected parts via microscopy (Meyer et al. 2007; Schwarzländer et al. 2008).

In previous experiments, we demonstrated that supplementing the root media of five-week-old *Arabidopsis* plants with 10^{-6} M and 10^{-5} M SA for two weeks alleviated the adverse effects of one-week-long NaCl stress (Horváth et al. 2015a). Interestingly, due to increased antioxidant activities, H_2O_2 and lipid peroxidation marker malondialdehyde levels were similar to those measured in untreated controls after three weeks of treatment. The activity of superoxide dismutase, guaiacol peroxidase, glutathione peroxidase, GST, and GR enzymes was elevated. Among the investigated GST-coding genes, the transcript levels of *AtGSTU19* and *AtGSTU24* increased most at the end of the long-term experiments (Horváth et al. 2015a). Further studies showed that 10^{-5} M SA treatment caused a temporary shift in redox potential towards a less negative redox state, but after two and three weeks, it was close to untreated controls (Csiszár et al. 2018).

It is well documented that high endogenous or exogenous SA concentrations induce ROS accumulation, but less information is available about the changes in the redox state during exogenously applied lower SA concentrations and its effects on plant redox status. In this paper, we applied the cytosolic roGFP2 redox probe to monitor the changes in different root regions after applying 10 and 100 μ M of SA. We aimed to investigate the relationships between ROS, redox status, and salicylic acid-induced transcriptional reprogramming. The potential roles of *AtGSTF8* and *AtGSTU19* as redox transducers in these processes were investigated by introducing *Atgstf8* and *Atgstu19* mutants into our experiments, which have elevated ROS levels and altered redox potentials in their roots compared to the wild type (Horváth et al. 2019).

Materials and Methods

Plant Material and Growth Conditions

We used the plant lines from our previous study (Horváth et al. 2019), namely the Columbia (Col-0) ecotype of *Arabidopsis thaliana* (L.) as a wild type control and *Atgstf8* (At2g47730) and *Atgstu19* (At1g78380) T-DNA insertional lines. The seedlings were grown under controlled conditions (100 μ mol m^{-2} s^{-1} photon flux density, 12/12 day/night period, 70% relative humidity, 21 °C) on solid half-strength Murashige and Skoog medium ($\frac{1}{2}$ MS, Duchefa Biochemie; Murashige and Skoog 1962). The seedlings were treated with SA in liquid medium, as described previously (Horváth et al. 2019). Briefly, seven-day-old *Arabidopsis* plants were placed into a liquid medium containing 10 μ M

or 100 μ M of SA. The treatment solutions were uniformly adjusted to a pH of 5.6 before use. Although some changes were observed in ROS levels and in root vitality even after 30 min (data not shown), but fluorescent microscopic and gene expression analyses were performed after 1, 3, and 24 h long treatments. The experiments were performed in at least two repetitions and ten replicates ($n = 10$). The statistical analysis was performed as described previously (Horváth et al. 2019).

Detection of Cell Vitality, Superoxide Radical, and H_2O_2 Levels in Roots Using Fluorescent Microscopy

Fluorescent staining of roots was performed as described previously (Horváth et al. 2019). For cell vitality determination, seedlings were placed in fluorescein diacetate (FDA) solution (10 μ M FDA in 10 mM/50 mM MES/KCl buffer, pH 6.15) for 15 min and washed with buffer. FDA is a cell membrane permeable substrate for non-specific esterases, widely used as a probe for vitality. By using FDA, the intracellular esterase activity which is required to activate its fluorescence and membrane integrity to retain the fluorescent probe inside the cell, was measured according to McCabe and Leaver (2000) and Feigl et al. (2019). By using FDA and propidium iodide (PI) double staining (Ambastha et al. 2020), we demonstrated that similar conclusion could be drawn related to the vitality of the cells from the two methods (data are not shown). Superoxide radical ($O_2^{\bullet-}$) was visualized with dihydroethidium (DHE) by placing the seedlings in DHE solution (10 μ M DHE in 10 mM, Tris-HCl buffer, pH 7.4) for 30 min at 37 °C. Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) dye was used for hydrogen peroxide (H_2O_2) detection since it reacts with H_2O_2 in the presence of horseradish peroxidase to produce the fluorescent compound resorufin (reviewed in Gomes et al. 2005). Seedlings were incubated in Amplex Red solution (50 μ M Amplex Red prepared in 50 mM sodium phosphate buffer, pH 7.5) for 30 min. Measurements were performed with the same microscope and settings as in our previous study (Horváth et al. 2019). Microscopic studies were carried out using a Zeiss Axiowert 200 M microscope (Carl Zeiss, Jena, Germany) equipped with a high-resolution digital camera (Axiocam HR, HQ CCD, Carl Zeiss, Jena, Germany) and filter set 10 (exc.: 535–585 nm, em.: 600–655 nm) for FDA, filter set 9 (exc.: 450–490 nm, em.: 515– ∞ nm) for DHE, or filter set 20HE (exc.: 546/12, em.: 607/80) for Amplex Red was used. The intensity of fluorescence was quantified using Axiovision Rel. 4.8 software in the proximal meristem (PM), transitional, and elongation zones (TZ and EZ, respectively) in a circle with 50 μ m radius. The zones were specified according to Jiang et al. (2016). PM was defined as cells between the quiescent centre and the first isodiametric

cortical cell. Isodiametric cortical cells constitute the TZ, and EZ is originated from cells which lose their isodiametric shape and elongate (Verbelen et al. 2006; Perilli and Sabatini 2010; Jiang et al. 2016). Measurement sites of fluorescent staining are shown in Supplementary Figure S1.

Redox Potential Determination by Ratiometric Measurements of GRX1-roGFP2

Fluorescence measurements were performed as described in Horváth et al. (2019), using a confocal laser scanning microscope (Olympus Fluoview FV1000, Olympus Life Science Europe GmbH, Hamburg, Germany) with 405 and 485 nm excitation wavelengths. Fluorescence was detected between 505 and 530 nm emission wavelengths. Each experiment included a calibration with 5 mM Aldrithiol™-4 and 10 mM dithiothreitol to fully oxidize and reduce the roGFP2, respectively. Redox potential was calculated as described by Schwarzländer et al. (2008):

$$E_{\text{GSH}} = E_{\text{roGFP2}}^0 - \left(\frac{2.303 * R * T}{z * F} \right) * \log_{10} \frac{1 - \text{OxD}_{\text{roGFP2}}}{\text{OxD}_{\text{roGFP2}}}$$

where the midpoint potential of roGFP2 (E_{roGFP2}^0) is -272 mV at 30 °C and pH 7; the gas constant (R) is $8.315 \text{ J K}^{-1} \text{ mol}^{-1}$; the absolute temperature (T) is 298.15 K ; the number of transferred electrons (z) is 2; the Faraday constant (F) is $96,485 \text{ C mol}^{-1}$. The degree of oxidation (OxD) of roGFP2 ($\text{OxD}_{\text{roGFP2}}$ values) and the equation for $\text{OxD}_{\text{roGFP2}}$ calculation can be found in Supplementary Table S1. The sites of redox potential analysis performed by the confocal laser scanning microscope are shown in Supplementary Figure S1.

High-Throughput Quantitative Real-Time Polymerase Chain Reaction (PCR) Measurements

High-throughput quantitative real-time PCR (HT-qPCR, Avidin Ltd.) assays were used to detect expression of the selected *Arabidopsis thaliana* *GST*- and other stress-related genes in the roots of seedlings. Roots were collected from approximately 120 seedlings using a scalpel to separate roots from shoots. RNA purification from 50 mg of root tissues was performed according to the manufacturer's instructions using Quick-RNA Miniprep Kit (Zymo Research). Additional DNase digestion (Thermo Scientific) and purification was applied (RNA Clean and Concentrator-25 Kit, Zymo Research), and cDNA was synthesized from $1 \mu\text{g}$ of total RNA using RevertAid reverse transcriptase (Thermo Scientific) and random hexamers. For HT-qPCR analysis, 22 gene sequences were selected. The selected genes for gene expression analysis and primer sequences are described in the Supplementary material (Supplementary Tables S2 and

S3). A single product-specific melting temperature peak was found in every case following melting curve analysis. Data were normalized to the expression levels of glyceraldehyde-3-phosphate dehydrogenase C2 (*GAPDH2*, *At1g13440*) and *monensin sensitivity1*, a SAND family protein (*MON1*, *At2g28390*) genes since these genes showed constant expression in our experiments (Czechowski et al. 2005; Papdi et al. 2008). The relative transcript levels in *Arabidopsis thaliana* wild type control root samples were taken as 1, and $2^{-\Delta\Delta\text{Ct}}$ values were presented in the diagrams.

Results

The Vitality of Root Zones Showed a Rapid, Transitional Decrease to SA Treatments

We investigated the vitality of one-week-old seedlings to analyse how exogenously applied SA at different doses (10 or $100 \mu\text{M}$ of SA) affects *Arabidopsis* roots. Fluorescence intensity measured in the proximal meristem (PM), transition zone (TZ), and elongation zone (EZ) by FDA staining revealed SA-induced concentration- and time-dependent changes in both wild type and mutant plants (Fig. 1).

In Col-0 plants, 1 h of lower, $10 \mu\text{M}$ of SA treatments slightly reduced vitality (Figs. 1 and 2). A further decrease in FDA fluorescence was measured in EZ after 24 h but not in PM and TZ. The $100 \mu\text{M}$ of SA treatment reduced vitality after 1 h, followed by an additional decrease after 3 h. Not only time-, but also root zone-dependent changes were observed. Wild type roots had higher FDA fluorescence in PM and TZ after 24 h of $100 \mu\text{M}$ of SA treatments, but EZ showed even lower vitality than after 3 h of treatments compared to the untreated root regions (Fig. 1).

The vitality of *Atgstf8* mutants was similar to Col-0 plants, but *Atgstu19* mutants had a lower FDA fluorescence under control conditions for almost all time periods and root zones throughout the experiments. Ten μM of SA decreased the vitality of EZ in *Atgstf8* and *Atgstu19* mutants after 3 h and in TZ and EZ after 24 h of treatment. All mutants showed lower vitality after 1 h of $100 \mu\text{M}$ of SA treatments compared to their own controls, and further reductions were measured after 3 h. Similarly to Col-0 plants, we observed recovered vitality after 24 h in the PM and TZ of *Atgstf8* roots but not in the *Atgstu19* mutants, which showed remarkably lower values (Fig. 1).

SA Treatments Caused Time- and Concentration-Dependent Changes in Redox Potential

An in vivo redox probe (roGFP) was applied to detect the redox state of different root apical meristem (RAM) zones.

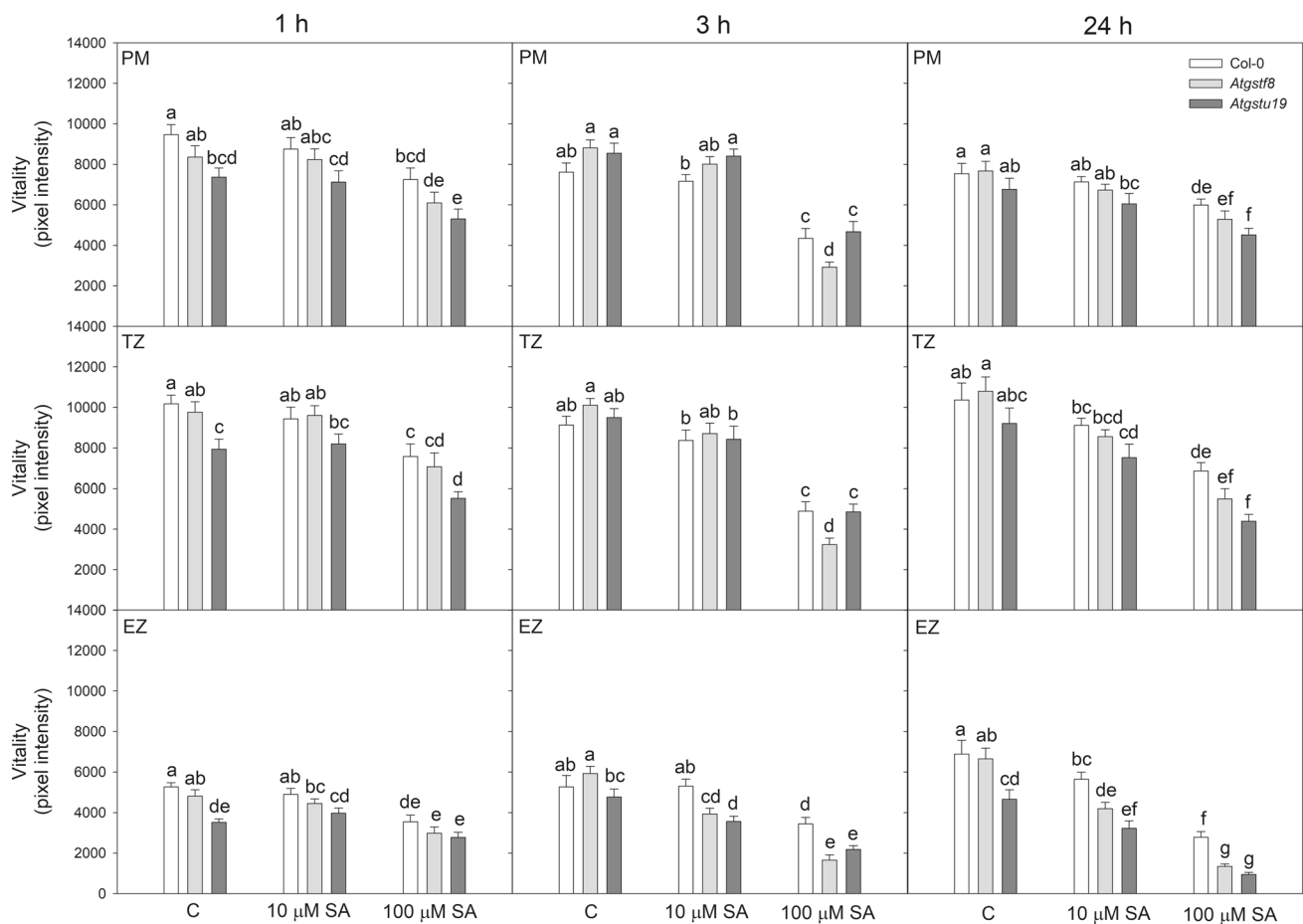


Fig. 1 Vitality in the proximal meristem (PM), transition- (TZ), and elongation zones (EZ) of seven-day-old *Arabidopsis thaliana* Col-0, *Atgstf8*, and *Atgstu19* mutants after 1, 3, and 24 h of treatment with

10 or 100 μM of salicylic acid. Data are means \pm SE, $n = 10$. Columns with different letters are significantly different at $p \leq 0.05$, determined by Duncan's test

The redox-dependent fluorescence signals from GRX1-roGFP2-expressing Col-0, *Atgstf8*, and *Atgstu19* mutants were monitored and compared.

The most negative redox potential (from -305 mV to -311 mV) was detected in control Col-0 plants (Figs. 2 and 3). The lower concentration of SA treatments (10 μM) caused a higher shift in the redox potential towards more oxidized values after 1 and 3 h of treatment in contrast with the effects of 100 μM of SA in wild type plants. However, glutathione redox potential (E_{GSH}) of Col-0 became less negative after 24 h of 100 μM SA treatment (by 13 mV [-292.44 mV] in PM and 8 mV [-303.41 mV] in EZ), in comparison to 10 μM of SA treatment, which caused 11 mV (-294.10 mV) and 6 mV (-304.81 mV) shifts in the PM and EZ, respectively.

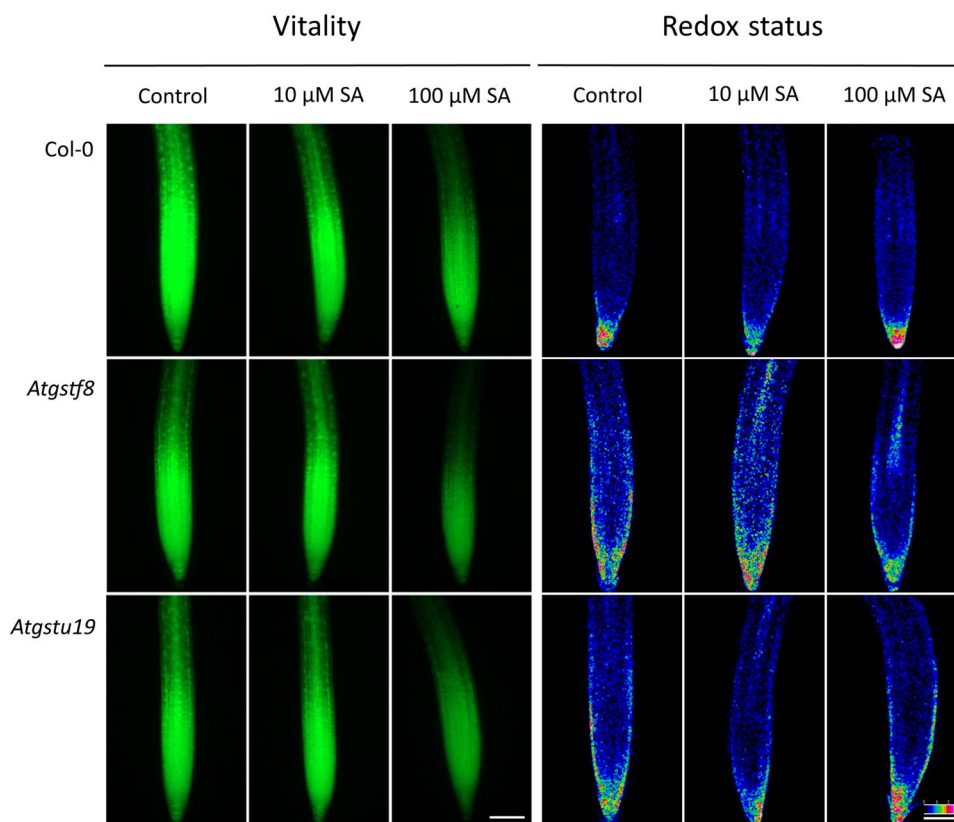
In general, *Atgst* mutants showed a less negative redox state in RAM than the untreated Col-0 plants under control conditions (by 7–30 mV) and after SA treatments (by 4–26 mV). In contrast to Col-0 plants, the redox potential was more negative in *Atgst* mutants after SA treatments

compared to their own untreated controls; moreover, they remained more oxidized over time (Figs. 2, 3). In *Atgstf8* mutants, 10 μM and 100 μM of SA-induced changes in E_{GSH} after 1 h of treatment. These changes were less definite after 3 h. However, the redox potential values of SA-treated roots were more negative in all root zones after 24 h of treatment (by 20–24 mV after 10 μM SA and 11–16 mV after 100 μM SA, respectively) in the *Atgstf8* mutant compared to their control. In *Atgstu19*, the most significant decrease in the redox potential was measured in the EZ after 3 h (by 6 mV and 5 mV after 10 μM and 100 μM SA, respectively) and in the PM after 24 h (by 6 mV and 11 mV after 10 μM and 100 μM SA, respectively).

Mutation in GSTs Alters the ROS Levels and Changes in Roots After SA Treatments

Since different changes were observed in the vitality and redox potential of wild type and *Atgst* mutants after SA treatment, a detailed analysis of ROS levels

Fig. 2 Representative images of fluorescent analysis of vitality and redox status in seven-day-old *Arabidopsis thaliana* Col-0, *Atgstf8*, and *Atgstu19* insertional mutants after 1 h of treatment with 10 and 100 μM of salicylic acid (see more details in Materials and methods). The redox status is represented as the ratio of fluorescence intensities (I_{405}/I_{488}) based on the corresponding colour scale, where black indicates a lower I_{405}/I_{488} ratio (reduced), and white colour indicates a higher I_{405}/I_{488} ratio (oxidized). I_{488} : the intensity at 488 nm; I_{405} : the intensity at 405 nm. Scale bar = 100 μm (Color figure online)



was performed. The levels of superoxide radical ($\text{O}_2^{\bullet-}$) dependent DHE fluorescence were slightly higher in *Atgst* mutants (especially in *Atgstf8* under control conditions) compared to the Col-0 plants. Superoxide radical levels remained unaltered in the roots of investigated plants after 1 h of treatment (Fig. 4; Supplementary Fig. S2). However, after SA treatments, significantly higher $\text{O}_2^{\bullet-}$ levels were detected in *Atgst* mutants compared to the Col-0 plants after 3 h (in TZ and EZ) and 24 h (in PM and TZ) of SA treatments (Fig. 4).

SA treatments induced time- and concentration-dependent changes in the H_2O_2 -dependent resorufin fluorescence intensities; however, some fluctuation in the H_2O_2 levels was observed over time. While the accumulation of H_2O_2 was detected in all investigated root zones of wild type plants after 1 and 24 h of SA treatment, minor deviations were detected in the SA-treated roots after 3 h. The higher (100 μM) SA treatment induced a more noticeable accumulation of H_2O_2 in all investigated root zones after 1 and 24 h than 10 μM of SA treatment, but the highest levels were detected in the TZ of Col-0 after 24 h. The changes detected in H_2O_2 levels were similar in *Atgst* mutants and wild type roots. However, the SA treatments resulted in even higher H_2O_2 levels in the roots of mutants than in wild type plants after 1 and 3 h (Fig. 5 and Supplementary Fig. S3).

HT-qPCR Analyses Revealed Modified Expression Levels of Certain Glutathione- and Stress-Related Genes After One Hour of SA Treatment

To investigate the effects of rapid changes in redox status and H_2O_2 levels within the roots, we analysed the expression patterns of some *GSTs* and other oxidative stress-inducible genes in wild type and *Atgst* mutants. The expression of 22 selected genes was investigated in whole roots after 1 h of 10 and 100 μM of SA treatment using high-throughput quantitative real-time PCR (HT-qPCR; Fig. 6 and Supplementary Fig. S4). More details of the selection of genes can be found in Supplementary Table S3. The expression of *GSTF8* and *GSTU19* was significantly lower in *Atgstf8* and *Atgstu19* mutants, respectively. Among the investigated genes, the highest induction was detected in *WRKY38* after 1 h of treatment, similarly affected by 10 and 100 μM of SA concentration (Fig. 6). In addition, some elevation was detected in the transcript levels of *WRKY46*, *GSTU7*, *GSH1*, *HSFA8*, *GSTF8*, *ZAT10*, *UPB1*, *NAC032*, and *OXII* after applying 10 μM SA on Col-0 plants. A partially different set of genes were induced in the presence of 100 μM of SA compared to 10 μM of SA treatments. Also, higher relative transcript levels were observed at *WRKY38*, *GSTU7*, *GSTU24*, *NAC032*, *GSTU19*, *AKR4C9*, *GSTU25*, *WRKY46*, *OXII*, *HSFA8*, *GPXL6*, *GSTF8*, *RRTF1*, *ZAT10*, and *GSH1* genes compared

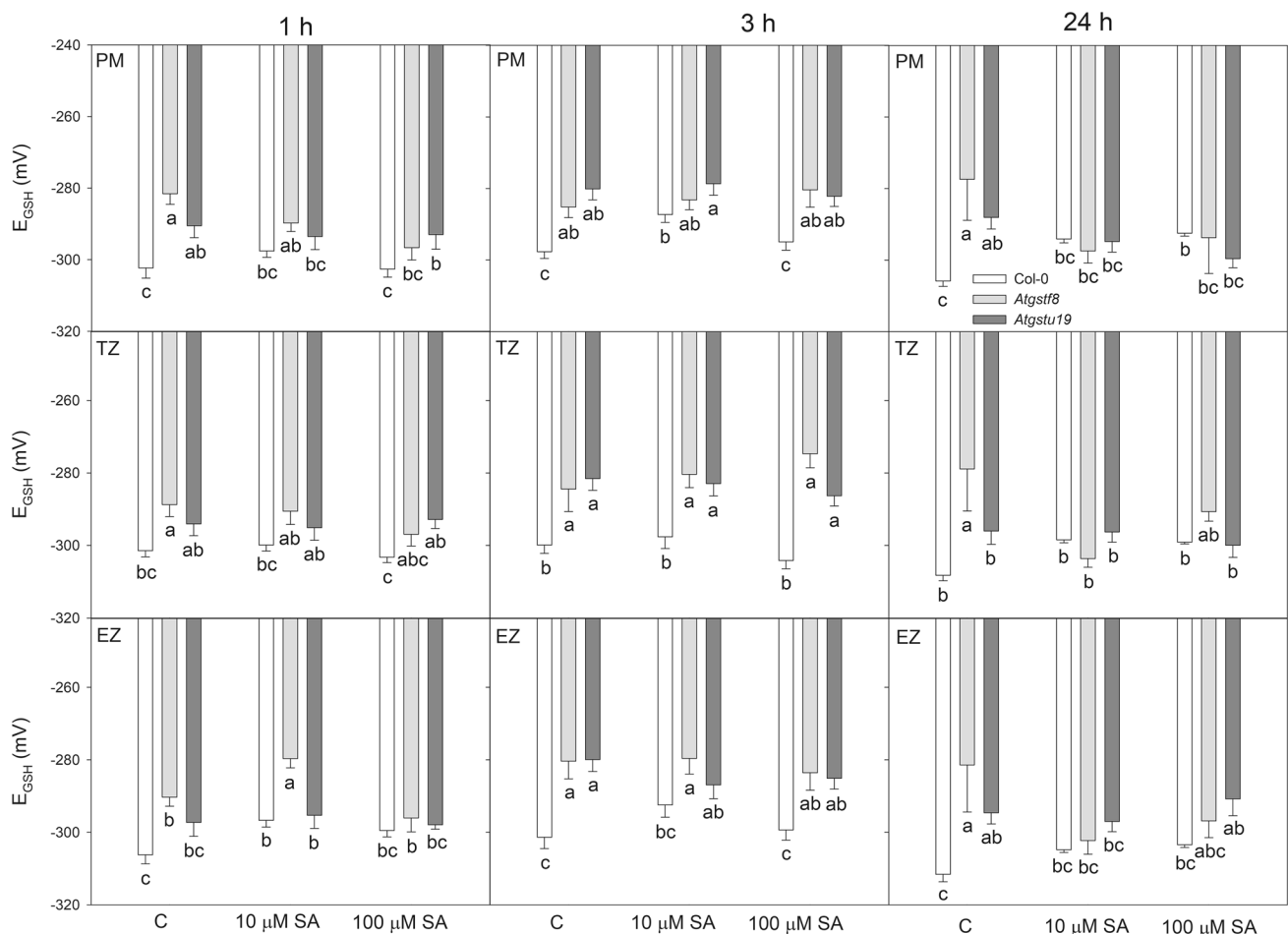


Fig. 3 Calculated redox potential values of roots expressing cytosolic GRX1-roGFP2 in primary roots of seven-day-old *Arabidopsis thaliana* Col-0, *Atgstf8*, and *Atgstu19* mutants. Data are means \pm SE,

$n=10$. Columns with different letters are significantly different at $p \leq 0.05$, determined by Duncan's test. PM proximal meristem, TZ transition zone, EZ elongation zone

to controls. Decreased expression was detected in the case of *WRKY28* and *MYC2* genes in 100 μ M of SA-treated roots (Fig. 6 and Supplementary Fig. S4).

Under control conditions, in *Atgstf8* mutants, *GSH1* showed slightly higher, while *WRKY38* showed lower expression in contrast to the Col-0 plants. The knockdown mutation of *GSTU19* resulted in moderately reduced transcript levels of *GRI*, *GSTU24*, *GSTU25*, and *AKR4C9* genes, where only *ZAT10* showed elevated expression compared to the wild type. After SA treatments, different changes were detected in the gene expression of *Atgstf8* and *Atgstu19* compared to Col-0 plants. The exogenous SA treatments, especially at 100 μ M concentration, slightly (*GSH1*, *GSTU7*, *HSAFA8*, and *GPXL6*) or significantly (*GSTU24* and *GSTU25*) elevated the transcription of investigated genes in both mutants compared to the Col-0 roots. Furthermore, *GSTU19*, *OXII*, and *WRKY38* genes in *Atgstf8* and *AKR4C9*, *ZAT10*, *WRKY28*, and *WRKY46* in *Atgstu19* showed significantly higher expression levels. Other sets of

genes demonstrated lower transcript amounts in mutants compared to wild type plants after SA treatments, including *WRKY46* and *NAC032* in *Atgstf8* and *GRI* and *GSTU7* in *Atgstu19* (Fig. 6).

Discussion

SA is a promising natural compound which can be applied exogenously to enhance stress tolerance in plants; however, several studies have confirmed concentration-dependent opposing effects (Tari et al. 2002; Horváth et al. 2007; Szepesi et al. 2009; Rivas-San Vicente and Plasencia 2011; Pál et al. 2013; Horváth et al. 2015a, b; Szalai et al. 2016). In our previous studies, we demonstrated that 10^{-4} M (100 μ M) SA treatment decreased the growth of five-week-old *Arabidopsis*, leading to the death of plants, but 10^{-5} M (10 μ M) SA pre-treatment promoted the growth and induced hardening, alleviating the effects of subsequently applied salt treatments

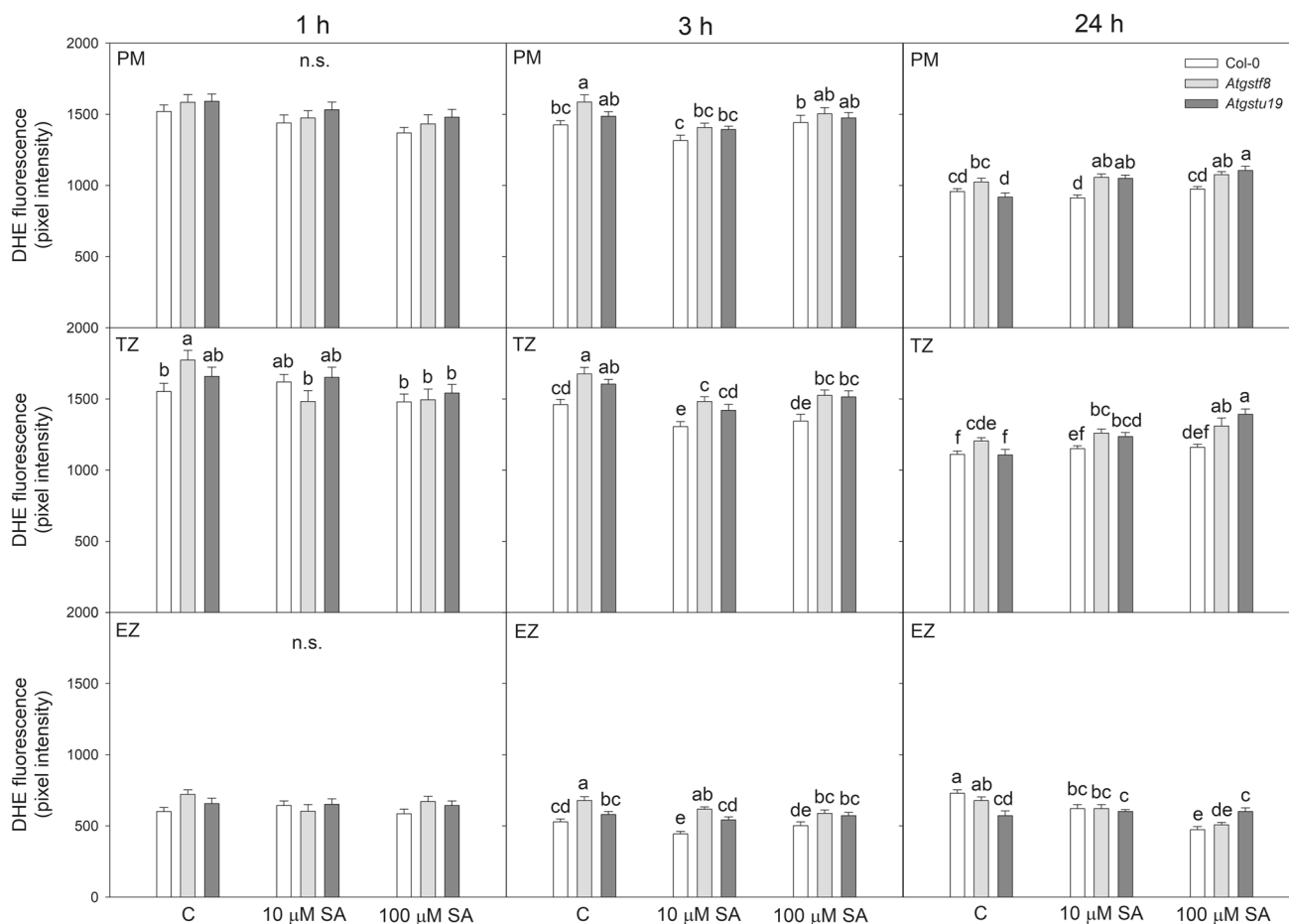


Fig. 4 The superoxide radical (dihydroethidium fluorescence) levels in the proximal meristem (PM), transition- (TZ), and elongation zones (EZ) of seven-day-old *Arabidopsis thaliana* Col-0, *Atgstf8*, and *Atgstu19* mutants after 1, 3, and 24 h of treatment with 10 or 100 μM

of salicylic acid. Data are means \pm SE, $n = 10$. Columns with different letters are significantly different at $p \leq 0.05$, determined by Duncan's test

(Horváth et al. 2015a). Earlier results suggested that concentrations lower than 50 μM of SA have regulatory roles in the development of *Arabidopsis* roots, while higher concentrations act as stress hormones (Pasternak et al. 2019). However, other components and molecular mechanisms are suspected to be involved in the complex SA signalling as well. The current short-term experiments verified that exogenously applied 100 μM SA treatments decreased the vitality of seven-day-old *Arabidopsis* wild type roots. At the same time, 10 μM SA induced only minor changes in all investigated root zones, where vitality of the roots did not differ significantly from controls after 1 and 3 h (Figs. 1, 2).

It was reported that SA directly affects NPR1 (SA receptor and transcriptional regulator) by binding and indirectly inducing redox changes in the cells, leading to NPR1 monomerization (Ding et al. 2018; He et al. 2018). Cysteine residues are sensitive targets of H_2O_2 , and diverse effects can be attributed to changes in their oxidized status (reviewed in He et al. 2018 and Foyer 2020). Recently, Wu et al. (2020)

identified the first known cell-surface H_2O_2 receptor hydrogen-peroxide-induced Ca^{2+} increase1 (HPCA1) in plants. Similarly, for NPR proteins and interacting TGA transcription factors, the redox conditions of HPCA1 are crucial for its activation. HPCA1 kinase is activated by oxidation of two special cysteine residues in the presence of extracellular H_2O_2 , which in turn induces Ca^{2+} influx in stomata and other cell types. This extracellular H_2O_2 can trigger Ca^{2+} influxes between cells and activate pathogen resistance or other stress acclimation processes in the entire plant (Evans et al. 2016). In the present study, elevated H_2O_2 levels were detected after 1 h of treatment with 100 μM SA in wild type roots; however, fluctuations were observed in all root zones over time, suggesting a tight control of H_2O_2 levels in SA response (Fig. 5). Using the roGFP2 redox probe to study the in vivo effects of SA treatments on redox status of GSH revealed that both SA concentrations resulted in a less negative redox potential in wild type *Arabidopsis* roots. However, the timing of these changes differed. The lower

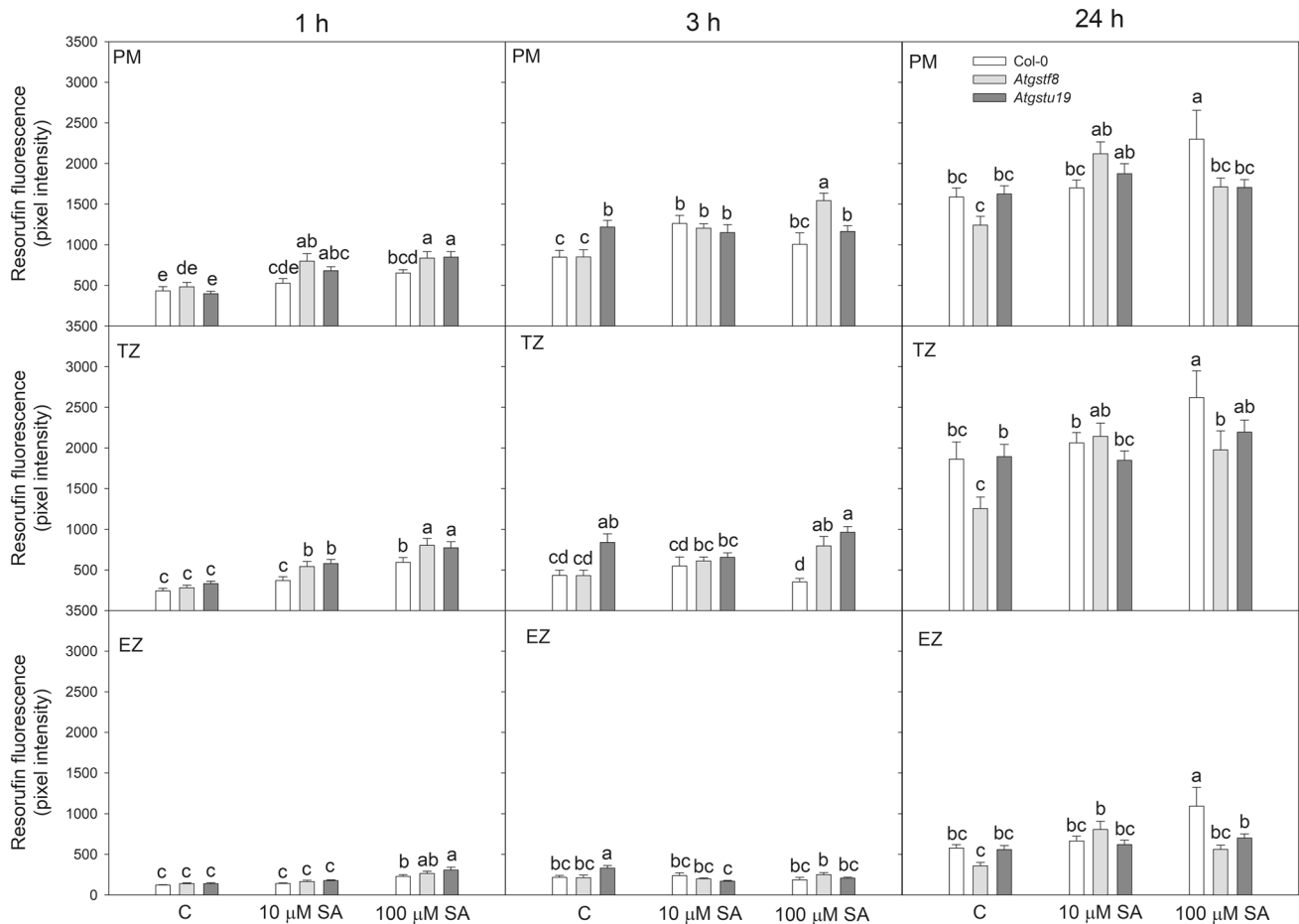


Fig. 5 The hydrogen peroxide-dependent fluorescence (resorufin fluorescence) in the proximal meristem (PM), transition- (TZ), and elongation zones (EZ) of seven-day-old *Arabidopsis thaliana* Col-0, *Atgstf8*, and *Atgstu19* mutants after 1 h of treatment with 10 or 100 μM

of salicylic acid. Data are means \pm SE, $n = 10$. Columns with different letters are significantly different at $p \leq 0.05$, determined by Duncan's test

concentrations of SA (10 μM) elevated E_{GSH} after 1 h of treatment in all root zones, but the redox potential remained more oxidized in the presence of 100 μM of SA compared to 10 μM SA. It showed further oxidation in TZ of Col-0 roots after 24 h (Figs. 2, 3). Early increments in H_2O_2 levels and/or redox potential values were detected after 1 h of treatment, which could cause rapid induction of *GSTs*, *GPXL6*, and oxidative stress-induced genes, such as *ANAC032*, *AKR4C9*, *HSA8*, and *OXII* in 100 μM SA-treated roots (Figs. 5, 6). H_2O_2 -induced expression of *AtANAC032* and *AtAKR4C9* in *Arabidopsis* seedlings has been previously reported by Claeys et al. (2014). The schematic model of SA-induced changes in the roots of Col-0 plants after 1 h of treatment, along with the supposed interaction between GSH redox state and H_2O_2 levels, supported by our results and by data found in the literature, are summarized in Fig. 7.

AtGSTF8 (earlier called *GST6*), together with *AtGSTU7* (*GST25*), were identified as early SA response genes and characterized as genes involved in detoxification or defence

against stress (Uquillas et al. 2004; Blanco et al. 2005). Moreover, the expression of *AtGSTF8* is induced by a wide range of stressors, thus its expression is considered as a marker gene for early stress and defence responses. *AtGSTF8* promoter fused to a luciferase reporter in forward genetic studies found mutants with altered SA responses (Sappl et al. 2009; Gleason et al. 2011). In our experiments, SA treatment induced the expression of the *AtGSTF8* gene in a concentration-dependent manner. According to our results, the SA-binding function of *AtGSTF8* in SA response is more important than its glutathione transferase enzyme activity.

In our previous experiments, the *Atgstu19* mutants were characterized by lower vitality, higher H_2O_2 levels, and less negative redox state in RAM compared to wild type plants under salt stress (Horváth et al. 2019). Furthermore, *Atgstu19* mutants showed lower GST activities than wild type plants. Despite this, *Atgstu19* mutants can compensate the effect of the mutations by elevating the non-enzymatic antioxidant levels, a modified GSH redox status, and elevated

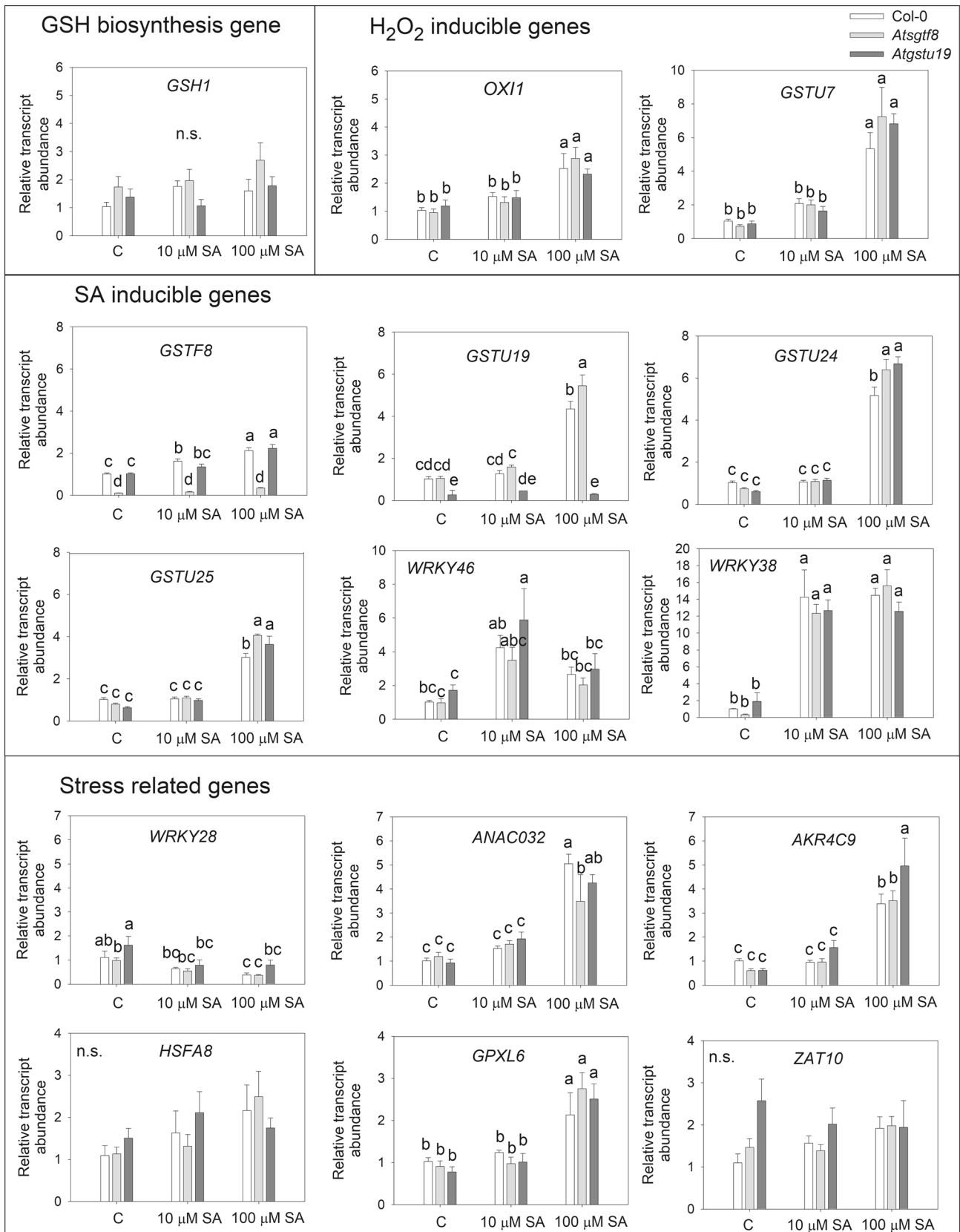


Fig. 6 The expression rate of *Arabidopsis thaliana* glutathione transferase and selected stress-related genes. Relative transcript amounts of genes were determined by high-throughput quantitative RT-PCR under control conditions (C) and after 1 h under 10 and 100 μM salicylic acid treatments in wild type (Col-0) and mutant (*Atgstf8* and *Atgstu19*) *Arabidopsis* roots. Expression of genes was normalized first by referencing the average transcript amount of glyceraldehyde-3-phosphate dehydrogenase C2 (*GAPDH2*, *At1g13440*) and *monensin sensitivity1* (*MON1*, *At2g28390*) genes, then by the average transcript amount of each gene in wild type control plants. $2^{-\Delta\Delta C_t}$ values are presented in the diagrams. Data are means \pm SE and the presented data are the average of two biological replicates. Columns with different letters are significantly different at $p \leq 0.05$, determined by Duncan's test. *n.s.* not significant

DHAR and GR enzyme activities (Horváth et al. 2020). In the current study, we estimated the role of AtGSTU19 in early SA responses and redox-related salicylic acid-induced transcriptional reprogramming in *Arabidopsis* roots. Several *cis*-acting regulatory sequences involved in stress responses or hormone regulation can be found in the 5' promoter region of the *AtGSTU19* gene (Horváth et al. 2019), which may explain the increased expression of the *AtGSTU19* gene in Col-0 plants after 1 h of SA treatment (Fig. 6). Besides the lower vitality and higher E_{GSH} values of *Atgstu19* mutants compared to wild type plants (Figs. 1, 3), several *AtGSTs* increased expression profiles were observed (Fig. 6), which corroborates with our earlier results about how plants compensate the effects of mutations in *AtGSTU19* by activating other processes (Horváth et al. 2020).

Our results supported the role of AtGSTU19, especially in early responses to above 50 μM of SA. While lower transcript levels were observed in several redox-related genes (*GRI*, *GSTU24*, *GSTU25*, and *AKR4C9*), the expression of the abiotic stress marker gene, *AtZAT10*, was elevated in

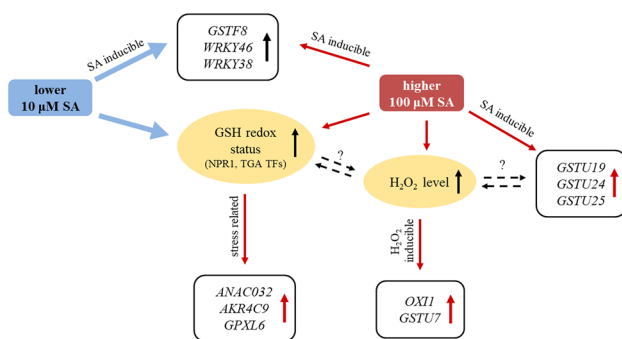


Fig. 7 Schematic model summarizing the results presented in this study. Gene expression changes after 1 h of salicylic acid (SA) treatment in Col-0 plants, and their supposed regulation by SA, glutathione (GSH) redox status, and H_2O_2 levels are depicted in this figure. (An upward arrow indicates increase; the thickness of the arrows refers to the extent of the changes; blue arrows: 10 μM SA-induced changes, red arrows: 100 μM SA-induced changes, black arrows: both 10 and 100 μM SA treatment-induced changes) (Color figure online)

the roots of untreated mutants, suggesting a less negative redox state affected cell processes in control roots (Fig. 6). Several studies have shown that *AtZAT10* overexpression, knockout, and RNAi mutants displayed enhanced resistance to drought-, osmotic-, and salt stresses, highlighting its dual role in response to environmental stresses (Sakamoto et al. 2004; Mittler et al. 2006). Recent studies found that *AtZAT10* is part of the regulatory network responsible for jasmonic acid-induced growth control by repressing auxin biosynthesis, transport, and signalling (Sakamoto et al. 2004; Zhang et al. 2020). Although the results from the current study do not confirm the direct role of AtGSTU19 in SA perception or signalling, the modified redox processes and gene expression patterns suggested its involvement in SA responses, which may exceed its antioxidant functions. However, estimating its role in SA signalling requires further investigations.

In conclusion, this study showed that during early SA-induced responses, the vitality of the root tips decreased; however, the regained FDA fluorescence indicated a recovered vitality after 24 h of treatment in wild type *Arabidopsis* plants. After 1 h of SA treatment, increased H_2O_2 levels and GSH-dependent redox potential values were measured, demonstrating a more oxidized redox environment in the cells. These changes led to altered gene expression in several cases. Investigating the role of AtGSTs in the SA response of the roots by studying roGFP2-expressing *Atgst* mutants revealed that lower vitality and higher E_{GSH} values characterized these mutants compared to wild type plants. Only modest changes were found in the redox potential values in mutants following SA treatments. We observed modified gene expression patterns in the *Atgst* mutants not just in control conditions, but even after applying 1 h of SA treatment compared to the Col-0 roots. Our results may verify the varying effects of applied SA concentrations on the expression of oxidative stress- and redox-related genes, among them the expression of *AtGSTF8* and *AtGSTU19* genes. The enhanced expression of redox-related genes in *Atgst* mutants may be the result of either direct antioxidant roles or their involvement in redox signalling. These data, originating from the whole roots, provided indirect evidence for the role of AtGSTF8 and AtGSTU19 isoenzymes within the transduction of the redox signal and strengthened the potential SA binding functions of AtGSTF8.

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Author Contributions EH: contributed to Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Writing—original draft, Writing—review & editing, Funding acquisition; KB: contributed to Conceptualization, Investigation, Writing—review & editing, Funding acquisition; KK: contributed to Investigation, Visualization; NF: Investigation; RR: Investigation, Writing—review & editing; ÁG: contributed to Conceptualization, Writing—review & editing; LGP: contributed to Conceptualization, Supervision; JC: contributed to Conceptualization, Methodology, Resources, Supervision, Writing—original draft, Writing—review & editing, Funding acquisition.

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Declarations

Conflict of interest The authors declare that they have no competing interests.

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