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The *Arabidopsis* glutathione transferases, AtGSTF8 and AtGSTU19 are involved in the maintenance of root redox homeostasis affecting meristem size and salt stress sensitivity

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Highlights

- *Atgst* mutants had elevated superoxide radical levels in root apical meristem
- AtGSTF8 and AtGSTU19 are involved in fine-tuning of redox homeostasis
- Mutation of AtGSTF8 and AtGSTU19 genes altered the redox potential (E_{GSH}) of root tips
- More oxidized redox status in mutants resulted in smaller shift in E_{GSH} upon salt stress

Abstract

The tau (U) and phi (F) classes of glutathione transferase (GST) enzymes reduce the glutathione (GSH) pool using GSH as a co-substrate, thus influence numerous redox-dependent processes including hormonal and stress responses. We performed detailed analysis of the redox potential and reactive oxygen species levels in longitudinal zones of 7-day-old roots of *Arabidopsis thaliana* L. Col-0 wild type and *Atgstf8* and *Atgstu19* insertional mutants. Using redox-sensitive cytosolic green fluorescent protein (roGFP2) the redox status of the meristematic, transition, and elongation zones was determined under control and salt stress (3-hour of 75 or 150 mM NaCl treatment) conditions. The *Atgstu19* mutant had the most oxidized redox status in all root zones throughout the experiments. Using fluorescent dyes significantly higher superoxide radical ($O_2^{\cdot-}$) levels was detected in both *Atgst* mutants than in the Col-0 control. Salt treatment resulted in the highest $O_2^{\cdot-}$ increase in the *Atgstf8* root, while the amount of H_2O_2 elevated most in the case of *Atgstu19*. Moreover, vitality decreased in *Atgstu19* roots more than in wild type under salt stress. Our results indicate that AtGSTF8 and especially the AtGSTU19 proteins **function in the root** fine-tuning the redox homeostasis both under control and salt stress conditions.

Keywords: antioxidative mechanisms; *Arabidopsis thaliana*; glutathione transferases; reactive oxygen species; redox homeostasis; **roGFP2**

1 Introduction

Glutathione transferases (GSTs, EC 2.5.1.18) constitute a very ancient protein superfamily that participate in a broad network of catalytic and regulatory functions. Their most known role is the detoxification of exogenous and endogenous harmful compounds, including herbicides, xenobiotics and endogenous stress metabolites. They are also involved in numerous redox, hormone and stress responses. Mediating cross-talks between these signaling pathways, they have important roles in various developmental processes such as apoptosis or growth regulation [[1], [2], [3], [4]]. Plant GSTs are grouped into ten different classes, among them the tau (GSTU), phi (GSTF), lambda (GSTL) and dehydroascorbate reductase (DHAR) are specific to plants. The tau and phi classes are largely responsible for catalyzing conjugation of reduced glutathione (GSH; γ -glu-cys-gly) with wide range of electrophyl substrates [[5], [6]]. That is why their catalytic activity may reduce the GSH pool. These isoenzymes and a significant portion of other GSTs also has glutathione peroxidase (GPX) activity and can convert lipid peroxides and other peroxides to less harmful compounds [[7]]. DHAR and GSTL enzymes catalyse redox reactions, even deglutathionylation, and participate in the recycling of antioxidants, such as ascorbate (ASC) and flavonols [[8], [9]].

The total amounts of non-enzymatic antioxidants (e. g. GSH, ASC and flavonoids) and their reduced-oxidized status are essential elements of the redox homeostasis of cells [[10]]. They are linked with the production or enhanced availability of reactive oxygen species (ROS), among them superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^{\bullet}). ROS generation accompanies the normal aerobic metabolism, but their level typically increases in plants exposed to different stresses [[11]]. ROS production induces detrimental oxidation of macromolecules including DNA, proteins, and lipids. On the other hand, endogenous change in oxidant levels can fulfill signaling functions and play a positive role in adaptation to the changed environmental conditions [[12], [13], [14]]. In addition, they regulate many developmental processes [[15], [16]]. The amount and distribution of ROS have been shown to be crucial in maintaining the root meristem size, together with the antagonistic cytokinin/auxin interaction [[17]].

Plants maintain a high cellular ratio of GSH to its oxidized glutathione disulfide (GSSG) form, but GSH reacts with oxidants during environmental stress and becomes converted into GSSG. Shifts in the cellular glutathione redox status may reversibly modify redox-sensitive thiol groups in target proteins either through glutathionylation or formation of cysteine crossbridges. Many reports indicate that the [GSH]:[GSSG] ratio and the glutathione half-cell reduction potential ($E_{GSSG/2GSH}$), which depends on the absolute glutathione concentration and the ratio of GSH and GSSG, can be effective markers of the overall redox homeostasis [[18], [19], [20], [21], [22]].

A non-destructive technology to detect changes in redox potential has been developed by the introduction of redox-sensitive green fluorescent protein (roGFP) imaging [[23], [24], [21], [25], [26]]. Engineering of two surface-exposed cysteines into the GFP allows reversible disulfide formation. The thiol-disulfide status of the roGFP can be equilibrated with that of glutathione in the cells. The fluorescence image can be obtained by confocal microscope. Determination of the fluorescent intensity of the fully reduced and fully oxidized form of the probe enables quantitative monitoring of E_{GSH} without destroying the cell [[21], [25]].

Different roGFPs (roGFP1-4, roGFP-iX) have been used as ratiometric redox sensors [[27], [28], [29], [30], [31], [32]]. Jiang et al. [[33]] analysed the redox potential profile of the primary root tip of 5-day-old *Arabidopsis* seedlings applying roGFP1 as redox sensor and demonstrated that *E* values may differ according to root zones. They found that in seedlings grown on agar the most reduced redox status is at, or nearly at, the quiescent center (QC), and moving to proximal direction the redox status becomes more oxidized. The terminal 300 μm including the root cap initials, the QC and the most distal portion of the proximal meristem (PM) exhibited the highest redox difference of 5-10 mV under different growth condition. Treating roots with 50, 100 or 150 mM NaCl resulted in marked changes in root meristem structure and development, and also the redox profile [[33]].

It was reported that glutathione is specifically required to activate and maintain the cell division cycle in the root apical cells [[34], [35], [36], [37]]. Severe GSH depletion specifically inhibited root meristem development, while low root GSH levels decreased lateral root densities [[37]]. Low level of GSH significantly increased the redox potentials and caused arrest of the cell cycle in roots but not shoots. Applying the GSH-synthesis-inhibitor buthionine sulphoximine (BSO) resulted in significantly more oxidized redox potential in the root tip cells (the values were ca. 50 mV less negative both in the cytosol and nuclei). Vernoux and his colleagues performed a transcript profiling analysis of an *Arabidopsis thaliana* rootmeristemless1 (*rml1*) mutant, which is severely limited in GSH synthesis capacity. Expression of several hundred genes encoding transcription factors and proteins particularly involved in hormone-dependent regulation of plant growth and development had changed [[35]]. Although low GSH level affects mostly the hormonal homeostasis of plants modulating developmental responses, several results showed that it is also linked to stress responses [[22]]. Among the genes regulated by low GSH, numerous redox-related proteins were found, such as glutaredoxins (GRXs), h-type thioredoxins (TRXs), glutathione peroxidases (GPXs), DHARs and GSTs [[37]].

In *A. thaliana* the tau and phi GSTs are the two most numerous GST classes: they have 28 and 13 members, respectively [[38]]. Comprehensive expression analysis indicated overlapping and specific roles of GST genes during development and stress responses [[39]]. Overexpression of several GSTs have been reported to increase the chilling, osmotic stress, salinity and/or herbicide tolerance, and in most cases even the growth of transgenic plants were altered [[40], [41], [42], [43], [44], [45], [46], [47], [48], [49]]. Recently, Bielach et al. [[50]] suggested that the auxin-inducible *AtGSTU5* may represent a general component of the environmental stress response, because it was upregulated by both auxin and cytokinin hormones and all the investigated abiotic stresses.

Analysis of the expression pattern of *Arabidopsis* GST-coding genes using the Geneinvestigator online tool revealed that *AtGSTF8* and *AtGSTU19* express at a very high level especially in roots [[51]]. The expression of *AtGSTF8* was induced by short-term salicylic acid treatment [[52], [53]], ethylene, and H_2O_2 [[54]], while that of *AtGSTU19* was induced by biotic stress [[54]], salicylic acid (SA) and H_2O_2 [[55]]. Xu et al. [[45]] demonstrated that the *AtGSTU19*-overexpressing plants showed not only enhanced tolerance to different abiotic stresses, increased percentage of seed germination and cotyledon emergence, but also the expression levels of several stress-regulated genes were altered. The *AtGSTU19* overexpressing plants exhibited increased activities of antioxidant enzymes and had enhanced amount of proline along with decreased malondialdehyde level under stress conditions [[45]]. Interestingly, although the catalytic activities of GST proteins may reduce the GSH pool by using reduced glutathione as a co-substrate, it was also suggested that GST enzymes may participate in the

maintenance of the redox status of cells [[8], [9]]. For instance, in our earlier investigations we found that *Atgstf9* mutants accumulated more ASC and GSH than the wild type (WT) plants, and had altered redox homeostasis [[56]].

Here we report detailed analysis of the redox status across longitudinal zones of roots in one-week-old Col-0 and *Atgstf8*, *Atgstu19* insertional mutant plants expressing the GRX1-roGFP2 fluorescent protein. Our main aim was the comparison of the redox state and ROS levels in different zones of roots under control conditions and after applying salt stress. According to our results, the redox status of un-treated *Atgstu19* roots was more oxidized, than that of the Col-0 or *Atgstf8*, and the size of the mutant's meristem proved to be shorter compared to the wild type. The redox potential showed the biggest differences in the proximal meristem of the roots. Treatment with 75 or 150 mM NaCl for 3 hours resulted in more oxidized redox state generally in all studied zones of the roots of the investigated genotypes, but the highest redox potential values (most oxidized redox status) were detected in the transition zone of the *Atgstu19* mutant.

2 Materials and methods

2.1 Plant material and growth conditions

Arabidopsis thaliana (L.) ecotype Columbia (Col-0) as a wild-type control and the mutants, *Atgstf8* and *Atgstu19*, related to the At2g47730 and At1g78380 genes, respectively, were used in all experiments. The T-DNA insertional lines, *Atgstf8* (N859808) and *Atgstu19* (N541942) were obtained from the NASC [[57]], and tested for homogeneity using gene-specific PCR primers (Fig. S1, Table S1). The seedlings were grown *in vitro* at a photon flux density of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (12/12 day/night period), at a relative humidity of 70 % and 21 °C. Stress treatments were carried out on 7-day-old plants germinated on solid half strength Murashige and Skoog ($\frac{1}{2}$ MS, Duchefa Biochemie; [[58]]) and placed into liquid $\frac{1}{2}$ MS medium supplemented with 75 mM or 150 mM NaCl. Fluorescent and confocal microscopic (CM) analyses were performed after 3 h NaCl treatment [[33]]. The experiments were repeated at least twice, the measurements were performed in ten replicates (n=10) unless indicated otherwise.

2.2 Identification of the root zones and investigation of the root anatomy

Root zonation was determined according to Jiang et al. [[33]]. For the identification of the main regions, the landmarks of the cortical files were taken into consideration [[59]]. The meristem (proximal meristem) [[60]] includes cells between the quiescent center (QC) and the first isodiametric cortical cell. The transition zone (TZ) region includes all isodiametric cortical cells, elongation zone (EZ) is defined as originating at the site at which cells lose their isodiametric shape and begin to elongate [[33]] (**Figs. S2 and S3**). The length of the zones was determined using CM images of propidium iodide- (PI) stained roots as described earlier [[61]] and measured using the ImageJ software [[62]].

2.3 Detection of the cell viability, superoxide radical and H₂O₂ levels in roots

Fluorescein diacetate (FDA) was used to determine cell vitality [[56]]. *Arabidopsis* seedlings (7-day-old) were incubated in 2 mL of 10 μM FDA staining solution (prepared in 10/50 mM MES/KCl buffer, pH 6.15) for 15 min, then washed four times with MES/KCl.

Dihydroethidium (DHE) in Tris-HCl (10 mM, pH 7.4) buffer was used to visualise superoxide ($\text{O}_2^{\bullet-}$) radical in plants. Seedlings were incubated at 37 °C in darkness for 30 min

with 10 μM DHE and then the samples were washed twice in the same buffer for 15 min [[63]].

For hydrogen peroxide (H_2O_2) detection, 10-acetyl-3,7-dihydroxyphenoxazine (ADHP, Amplex Red or resorufin) fluorescent dye was used. Seedlings were incubated in 50 μM ADHP/Amplex Red dye solution (prepared in 50 mM sodium phosphate buffer, pH 7.5) for 30 min and washed twice with the buffer [[63]].

Zeiss Axiowert 200M microscope (Carl Zeiss, Jena, Germany) equipped with a high resolution digital camera (Axiocam HR, HQ CCD, Carl Zeiss, Jena, Germany) and filter set 10 (excitation 535–585 nm, emission 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 to detect fluorescence in the roots.

The intensity of fluorescence was quantified on digital images using Axiovision Rel. 4.8 software in the proximal meristem (PM), transition- and elongation zones, in a circle with 50 μm radius. The measurements were performed in two independent experiments ($n=10$) with the same microscopic settings.

2.4 Introducing GRX1-roGFP2 fluorescent marker into the wild type and mutant plants

To determine the glutathione redox potential in roots, we expressed the cytoplasmic GRX1-roGFP2 redox sensor protein in Col-0, *Atgstf8* and *Atgstu19* mutants. The vector construction expressing the human glutaredoxin1 (GRX1) and roGFP2 fusion protein was used [[30]]. Binary vectors were introduced into *Agrobacterium* GV3101 carrying pMP90 Ti-helper plasmid as described by Koncz et al. [[64]]. Stable transformation of wild type (Col-0) and *Atgstf8* and *Atgstu19* mutant *Arabidopsis* plants was achieved by the floral dip method [[65]]. As preculture, the *Agrobacterium* cells were incubated in 30 mL selective lysogeny broth (LB) medium (50 $\mu\text{g mL}^{-1}$ rifampicin and 50 $\mu\text{g mL}^{-1}$ kanamycin) at 28 °C and 180 rpm for 24 h to reach an OD_{600} of ~ 1.0 . Subsequently, 300 mL of selective LB medium was inoculated with this preculture and incubated for a further 24 h under the same conditions. The cells were harvested by centrifugation at 5000 g for 10 min at 4 °C and resuspended in the floral-dip medium containing 5 % (w/v) sucrose and 0.02 % (v/v) Silwet L-77 to OD_{600} of 1-1.5. The floral plant parts were dipped into the suspension for 5 s. The dipping procedure was repeated after one week to increase the transformation rate [[66]]. To identify the transformants UV stereomicroscope (Olympus SZX12, Hamburg, Germany) was used to visualize the expression of the roGFP2 protein (GFP filter was set at 470 ± 40 nm excitation and at 525 ± 50 nm emission).

2.5 Analysis of the redox potential by ratiometric measurements of the fluorescent probe

Fluorescence measurements were performed with a confocal laser scanning microscope (Olympus Fluoview FV1000, Olympus Life Science Europe GmbH, Hamburg, Germany). Excitation wavelengths were set at 405 nm and 485 nm, and fluorescence values were detected between the 505-530 nm emission wavelength.

The redox potential was calculated using the formula of Schwarzländer et al. [[25]]. The degree of oxidation (OxD) of the roGFP2 was:

$$\text{OxD}_{\text{roGFP2}} = \frac{R - R_{\text{red}}}{\left(\frac{I_{488\text{red}}}{I_{488\text{ox}}}\right) * (R_{\text{ox}} - R) + (R - R_{\text{red}})}$$

where: R is the ratio of excitation at 405/485 nm; R_{red} : the ratio of fully reduced form, using 10 mM dithiothreitol; R_{ox} : the ratio of the fully oxidized form, using 5 mM AldrithiolTM-4; $I_{485\text{ox}}$: the intensity at 485 nm for the fully oxidized form; $I_{485\text{red}}$: the intensity at 485 nm for the fully reduced form.

The redox potential was determined:

$$E_{GSH} = E_{roGFP2}^0 - \left(\frac{2,303 * R * T}{z * F} \right) * \log_{10} \frac{1 - OxD_{roGFP2}}{OxD_{roGFP2}}$$

where: E_{roGFP2}^0 , the midpoint potential of roGFP2 is -272 mV at 30°C pH 7; R : the gas constant (8.315 J K⁻¹ mol⁻¹); T : the absolute temperature (298.15 K); z : the number of transferred electrons (2); F : the Faraday constant (96485 C mol⁻¹).

Redox potential of the roots was calculated in the PM, TZ and EZ. Sites of analysis are shown in Fig. S2.

2.6 *In silico* analysis of *cis*-regulatory sequences in the 5' regulatory regions of *AtGST* genes

Sequences related to the 5' region of the *AtGSTF8* and *AtGSTU19* were retrieved from The Arabidopsis Information Resource (TAIR, <http://www.arabidopsis.org>) database and the putative *cis*-acting regulatory elements were identified in the 1.5 kb upstream region from the translational start site (ATG) using the PlantCARE database (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>).

2.7 Statistical analysis

Statistical analysis was carried out using SigmaPlot 11.0 software (SigmaPlot, Milano, Italy) by Duncan's test and differences were considered at significant difference with 5% confidence ($p \leq 0.05$). Values presented here represent mean with standard error (\pm SE), $n=10$ roots, unless indicated otherwise.

3 Results

3.1 *AtGSTF8* and *AtGSTU19* differently determines ROS levels and redox status

Our aim was to investigate the involvement of *AtGSTF8* and *AtGSTU19* in the control of the redox status and ROS homeostasis in the root meristem. For this, the redox-dependent fluorescence of GRX1-roGFP2-expressing roots of the *Atgstf8* and *Atgstu19* mutants was monitored and compared to that of the Col-0 control. The redox potential values (E_{GSH}) were calculated in the proximal meristem (PM), transition zone (TZ) and elongation zone (EZ). The most reduced, -301.02 mV, redox potential was detected in the proximal meristem of the Col-0, while in the TZ and the EZ we measured -300.66, and -295.25 mV, respectively. The redox potential of the *Atgstf8* roots increased by 5.41, 5.35 and 3.07 mV in the PM, TZ and EZ regions, respectively, when compared to the control, indicating more oxidized redox status in the whole root. In the *Atgstu19* roots, the E_{GSH} values shifted up by 12.19, 18.0 and 12.57 mV comparing to the respective regions of Col-0, showing an even higher oxidized redox environment in this mutant (Fig. 1A).

Applying different fluorescent dyes, ROS levels were measured in Col-0 and both mutants. In general, comparing the ROS level in roots, we found the same tendency in the investigated

lines (Fig. 1B, C); the highest ROS level was measured in the proximal meristem, after which it decreased towards the elongation zone. However, as Figure 1B shows, the measured DHE fluorescence values were significantly higher in the mutant lines when compared the relevant zones in the control. Interestingly, the difference was more pronounced in the *Atgstf8* line, indicating elevated $O_2^{\cdot-}$ levels in the studied regions of the mutant roots. The proximal meristem of both mutants had 20-25 % higher superoxide radical level than that of the Col-0 (Fig. 1B). The level of the resorufin fluorescence was comparable in the mutant, *Atgstf8* or *Atgstu19* and wild type roots, indicating similar H_2O_2 content in the primary roots of the investigated lines (Fig. 1C).

3.2 The redox status, metabolic activity and ROS levels of roots show region-specific changes in response to salt treatment

To compare salt stress sensitivity of Col-0, *Atgstf8* and *Atgstu19* roots, we used the transgenic lines, Col-0, *Atgstf8* and *Atgstu19* carrying cytosolic roGFP2 protein. After three hours treatment of 7 days-old seedlings with 75 and 150 mM NaCl, we studied the redox status, the metabolic activity (vitality) and ROS levels in primary roots. Representative images upon treatment are shown in Fig. 2. The fluorescent analysis (taking the ratio of the fluorescence intensity at 405 and 488 nm, Olympus Fluoview FV1000) revealed an overall higher oxidized status in all treated genotypes compared to non-treated controls. The vitality, superoxide radical and H_2O_2 levels analysed using fluorescent dyes (FDA, DHE, resorufin, respectively) by Zeiss Axiowert 200M microscope changed differently along the roots in the three genotypes upon salt stress.

3.3 Salt treatments affected the redox status of the proximal meristem in all investigated lines dependent on their original redox potentials

Treatment with 75 or 150 mM NaCl shifted the redox potential of the proximal meristem in all three investigated lines (Fig. 3A). However, no significant change was detected between the two treatments. In Col-0, the redox potential decreased by 9 - 11 mV reaching -291.32 and -289.85 mV (Fig. 3A). The changes in the mutant *Atgstf8* were comparable to the control, though as the non-treated control had a higher oxidized status, the relative changes were smaller. In contrast, in the *Atgstu19*, which had already under control conditions higher redox potential (-288.83 mV) in the PM, only the treatment upon 150 mM NaCl resulted in a significant change (-283.93 mV).

Under normal growth conditions, the vitality of control and mutant roots in the PMs showed no difference (Fig. 3B). In Col-0, low (75 mM) concentration of salt has not, while high (150 mM) significantly reduced the FDA fluorescence in the PM. In contrast, both, the lack of AtGSTF8 and AtGSTU19 led to decrease in vitality already at low concentration of NaCl, with a stronger response in the case of AtGSTU19. We detected 20 % reduction in *Atgstf8* and 55 % in *Atgstu19* upon 75 mM, while 60 % and 70 %, in response to 150 mM NaCl, respectively (Fig. 3B).

Salt treatments **did** not effect the level of superoxide radical in the wild type PM but drastically altered **it** in the mutants. The already elevated level of $O_2^{\cdot-}$ in the mutant PMs further increased due to NaCl treatments (Fig. 3C), and reached almost 2-fold higher $O_2^{\cdot-}$ level in the *Atgstf8* meristem (150 mM NaCl) compared to the Col-0 control (Fig. 3C). The analysis of H_2O_2 levels showed a different pattern (Fig 3D). Under normal growth conditions, the mutant *Atgstu19* had higher H_2O_2 level, both compared to Col-0 and *Atgstf8*. In the wild type roots, the H_2O_2 level increased only upon 150 mM, while in *Atgstf8*, only at 75 mM

NaCl. In contrast, in the meristem of *Atgstu19*, the H₂O₂ level decreased upon both NaCl treatments (Fig 3D).

3.4 The TZ of *Atgstu19* had the highest redox potential and the lowest vitality but its ROS levels hardly changed in response to salt

The redox potential in the transition zone (TZ) changed in the salt-treated roots similarly to the PM, although the level of oxidization was lower (Fig. 3A vs 3E). In the mutants, the TZ had a higher oxidized status than in Col-0, and upon salt treatment comparable changes were detected in Col-0 and *Atgstf8* (Fig. 3E). The *Atgstu19* roots, however showed an even higher oxidized redox status under control conditions, that further decreased only in response to high (150 mM) NaCl concentration (Fig. 3E). The metabolic activity (vitality) was effected stronger and decreased more by the salt stress in this zone than in the PM in any of the investigated lines (Fig. 3B vs 3F). The O₂^{•-} level was generally higher in the TZ of the mutant roots when compared to the wild type, especially in the case of *Atgstf8* (Fig. 3G). This level increased further upon salt treatment but only in the case of *Atgstf8*. Changes in the H₂O₂ level resulted in great variability among the studied lines (Fig. 3H), it increased in the Col-0, but in *Atgstf8* similar values were detected even upon low NaCl concentration. In *Atgstu19*, the H₂O₂ level was as high already under control conditions as in the salt-treated roots of the Col-0 (150 mM) and *Atgstf8* (75 or 150 mM). In this mutant, salt did not influence further the H₂O₂ level.

3.5 The salt treatments increased the E_{GSH} in the EZ of Col-0, but the already elevated values of the mutants were not altered

The redox potential of the EZ of the Col-0 increased by 5.28 and 6.25 mV, reaching a higher oxidized state, upon 75 and 150 mM NaCl, respectively. As in the mutants, the E' values were already less negative, salt treatments did not cause significant changes (Fig. 3I). The relatively low metabolic activity of cells in this root region decreased further due to salt stress; the FDA fluorescence indicated the lowest vitality in the EZ of *Atgstu19* roots (Fig. 3J). The used salt treatments did not increase the O₂^{•-} level in the EZ of wild type roots. In the *Atgstf8* mutant, where about 2-fold greater DHE fluorescence intensity was detected in the EZ of non-treated root than in the wild type, the 150 mM NaCl could further increase the superoxide level. The treatments caused less change in the O₂^{•-} level in the EZ of *Atgstu19* (Fig. 3K). Based on the detection of resorufin fluorescence, the H₂O₂ level was increased after the 150 mM NaCl treatment in the EZ of the roots of all genotypes (Fig. 3L).

3.6 The size of the proximal meristem was smaller in the 7-day-old mutants' roots than in the wild type

Identification of the root zones and determination of their size in one-week-old seedlings revealed that the total length of short epidermal cells and their number in the PM of mutants was lower compared to the wild type (Table 1). Determination of these parameters in the cortex cells of the Col-0 and mutant roots did not show differences in total length between the Col-0 and *Atgstf8* roots, however the number of cells was a little less in the PM of *Atgstu19* compared to other genotypes (Table 1). Identification of the two cell lines belonging to different zones are shown on Fig. S3.

3.7 *In silico* analysis of the 5' regulatory regions of *AtGSTF8* and *AtGSTU19* indicates the presence of both stress- and hormone-responsive elements

To predict *cis*-acting regulatory sequences in the 5' promoter regions of the selected *AtGST* genes we performed *in silico* analysis. The list and description of the main nucleotide motifs discovered in the 5' regulatory regions of the investigated genes are shown in Table S2. Beside some common regulatory elements involved in defense and abiotic/biotic stress responses (e.g. ARE, TC-rich repeats, MBS motif, ABRE elements), motifs involved in hormone regulation (auxin, gibberellin, methyl jasmonate, ethylene, salicylic acid) were identified in the investigated regions (1.5 kb). For example, the auxin-responsive TGA-element (AACGAC) can be found in the 5' regulatory region of *AtGSTU19*, while TGA-box (TGACGTGGC) sequences are present in both genes. Interestingly, numerous other *cis*-acting regulatory sequences associated with light responsiveness, the control of leaf morphology and seed development were also identified in the 5' upstream regions of one or both genes indicating the complex interaction between different signaling inputs controlling their expression.

4 Discussion

4.1 The *AtGSTF8* and *AtGSTU19* enzymes are differentially involved in the maintenance of the redox homeostasis of root meristem zones

ROS modulate cell division, differentiation, and expansion via functioning as second messengers and/or affecting the cell wall structure [[67], [68], [69], [16]]. The responses of cells to cellular oxidation due to abiotic stress or the action of defense phytohormones depend on cell identity [[70], [71]]. Jiang et al. [[33]] demonstrated using the roGFP1 probe that the redox potentials of various regions of 3-9 day-old *Arabidopsis* roots differ significantly. **In accordance with the above reports, we found that the redox potential of the Col-0 plant's proximal meristem (cell division) and the elongation zone (cell differentiation) differed by circa 6 mV.**

Hundreds of genes, including several ones coding for *AtGSTUs*, are differentially expressed in radial and/or longitudinal zones of the *Arabidopsis* root [[72]]. Dixon et al. [[51]] reported that the relative expression of *AtGSTU19* and *AtGSTF8* genes is higher in roots than in shoots especially in specific parts and tissues of the root, such as the stele, endodermis, epidermal atrichoblasts and the lateral root cap or root hair zone. Our investigations of the meristematic region of the roots of the *Atgstf8* and *Atgstu19* mutants revealed elevated $O_2^{\cdot-}$ level in comparison to the control. This indicates that both GSTs have important role in the ROS processing systems even in the root meristem.

Among the different redox active compounds determining the cellular redox status of plant cells [[73]], especially the ascorbate and GSH were reported to determine growth and development of plants by modulating processes, such as mitosis, cell elongation, senescence, cell death and stress responses [[50]]. The key role of the GSH redox potential in determining the "biological activity" of cells was suggested by Schafer and Buettner [[18]]. By analysis of the cellular redox environment through the life of different cell lines they reported that the proliferating state was connected with the calculated E_{GSH} ranging -260 mV to -210 mV, but the differentiation was associated with a more positive, -210 to -180 mV potential [[18]]. Although the exact E_{GSH} values of plant cells proved to be lower by using redox active fluorescent probes even by 50-60 mV [[21]], the correlation between the GSH redox status and different physiological processes in plants was proved [[74]]. **Detection of a more**

oxidized redox status across longitudinal zones of mutants compared to wild type suggests that both isoenzymes may be involved in the maintenance of the redox homeostasis. The shift in the redox potential of the PM, TZ and EZ regions in *Atgstu19* was 12-18 mV; 2-4 fold higher than in *Atgstf8*. According to these results, AtGSTU19 has more important role controlling the redox homeostasis in the investigated root zones than AtGSTF8.

Characterization of *Arabidopsis rml* mutants revealed that glutathione is specifically required to activate and maintain the cell division cycle in the root apical cells [[34], [35], [37]] however, it was also suggested that redox status by itself does not determine the cellular proliferation. Berndt et al. [[75]] proposed the concept that redox regulation by glutathione needs enzymes. In the *rml1* mutants, the post-embryonic root meristem does not form because of the defects of GSH biosynthesis and depletion of important antioxidants [[35]]. In these mutants, several transcripts encoding proteins involved in oxidative stress responses were decreased, indicating that low GSH is accompanied by a decrease in oxidative stress signaling. **In contrast, overexpression of *BcGSTU* in *A. thaliana* resulted in higher glutathione level and the promotion of root and shoot growth as well as better performance of the plants under abiotic and biotic stresses [[4]]. In our study, the requirement of AtGSTF8 and AtGSTU19 for proper root meristem organisation and function was revealed. It was found that the size of the PM and the TZ as well as the number of epidermal and cortical cells was decreased in the mutants. This showed correlation with the decreased redox potential in the meristem.**

4.2 The more positive redox potential of the *Atgstf8*- and *Atgstu19* mutants influences the salt response of their roots

Noctor et al. suggested that the same signaling pathways are involved in ROS-mediated acclimation in response to stress than in the control of normal growth and development [[76]]. Therefore, the potential involvement of AtGSTF8 and AtGSTU19 in the salt stress response of the root was tested. The vitality, redox potential, and ROS level of wild type and mutant seedling roots were compared after 3 hours treatments with 75 or 150 mM NaCl. Root cells of the *Atgstf8* mutant exhibited similar FDA fluorescence (vitality) than that of the wild type. In contrast, 75 mM NaCl resulted in significantly increased salt sensitivity in all root zones of the *Atgstu19* mutant in comparison to those of the wild type. Redox potential changes were in agreement with this difference. In the meristematic zones (PM and TZ), the redox potential of the *Atgstf8* mutant increased in a similar manner in response to salt than that of the wild type although the values, including that of the control sample, were somewhat more positive in the mutant. The meristematic regions of the *Atgstu19* mutant exhibited a similar redox potential under control conditions than that of the salt-treated *Atgstf8* and Col-0 roots. It was not increased further by the 75 mM only the 150 mM salt concentration in this mutant. In the elongation zone, both mutants had more positive redox potential than the wild type and this value could not be increased further by salt. The *Atgstu19* mutant exhibited a significantly higher redox value in this region as well as in the transition zone in comparison to the other two lines.

In general, one can state that the relative redox potential changes in the mutant roots were lower than in the wild type likely due to their already more positive value under control conditions. Their increased salt sensitivity can be associated with this decreased redox potential response. It is supported by the observation that the highest sensitivity towards 75

mM salt was shown by the *Atgstu19* mutant exhibiting no redox potential change in response to this salt concentration.

4.3 The determined ROS levels are not directly linked to the observed redox potential and vitality differences

Enhanced ROS production is suggested to temporarily shift the redox potential to more oxidized values that will alter the operational controls of many redox-sensitive proteins [[69], [77]]. A relative small global shift in the E_{GSH} to values of about -260 mV is associated with a very large change in gene expression and plant development [[32], [37]]. Our data did not reveal direct **correlation** between the E_{GSH} values and the level of investigated ROS or between the E_{GSH} and cell vitality. However, it is possible, that the mutations in these *AtGST* genes triggered changes in those mechanisms which are common in salt stress-induced responses. For example, activation of different enzymes with peroxidase activity (such as Class III peroxidases and GST isoenzymes) may account for the smaller increase of the H_2O_2 level observed in *Atgstf8/ut19* roots compared to the wild type after applying salt stress (Fig. 3). This may result in the mutant roots in a different $O_2^{\cdot-}/H_2O_2$ ratio than in the control. Tsukagoshi and his co-workers [[78]] reported that the *Arabidopsis* UPBEAT1 (UPB1) transcription factor directly regulates the expression of root peroxidases that modulate the ratio between the $O_2^{\cdot-}$ and H_2O_2 levels [[78]]. Moreover, the ability of cells to switch between ROS-processing pathways may restrict ROS-accumulation and detection [[76]]. **Using the membrane-anchored HyPer genetically encoded H_2O_2 sensor indicated the existence of discrete ROS maxima and a limited ROS diffusion distance [[79]]. Such spatial distribution of ROS might be explained by local downregulation of cellular antioxidant mechanisms that enables controlled accumulation of ROS, triggering downstream signaling processes [[80]].**

4.4 AtGSTF8 and AtGSTU19 may integrate redox regulation with stress and hormonal responses

It was suggested that the function of certain antioxidative enzymes is not only to keep ROS levels low, but also to allow the cells to sense and signal ROS availability and redox perturbations [[76]]. Because several GSH-related enzymes were reported playing role in root architecture and lateral root development, they can be among the components linking redox signaling to growth [[81], [50]].

Detailed investigations of the *Arabidopsis* GSTU17 revealed that it has a strong impact on GSH:GSSG ratio and thus on redox status of the cells, moreover its expression pattern appeared to be associated with auxin and ABA signaling [[82]]. The *AtGSTU5* expression was induced also by several different stressors and by auxin [[50]]. It was hypothesized that these genes represent general components of the environmental stress response. They could constitute central components of the auxin-cytokinin regulatory transcriptional network, functioning as regulatory hubs between growth and stress-tolerance pathways. Jiang et al. already demonstrated that salt-induced changes in redox status in roots can influence root meristem maintenance, at least in part, by auxin transport [[33]]. **The importance of the GSH/GSSG ratio and the expression of GSTs were also indicated in biotic stress responses [[83]]. For example, they were suggested as early indicators of the esca disease in grapevine [[84], [85]]. *AtGSTF8* was proposed to be a marker gene for early stress and defense responses too, because its expression was induced rapidly by diverse biotic and abiotic stresses including pathogen attack, phytohormones, herbicides, heat and high-light [[86]].**

Our analysis on possible regulatory sequences in the 5' regulatory regions of AtGSTF8 and AtGSTU19 also suggests that these isoenzymes can be part of a complex signaling network. Regulatory elements involved in defense, abiotic/biotic stress responses, hormone (auxin, gibberellin, methyl jasmonate, ethylene, salicylic acid) and light responsiveness, as well as in developmental pathways were identified in the investigated promoter regions of their genes. Both proteins might represent ROS-processing enzymes fine-tuning and maintaining redox homeostasis under plant development especially under stress conditions.

5 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6 Author Contributions

JC contributed in planning the experiments, wrote and edited the manuscript. EH designed and coordinated the study, analyzed the data, **wrote and edited the manuscript**. KB, BH, RR, ÁH, ÁBH and EH performed the experiments. AF, KB and ÁG discussed the results and contributed to the writing of the manuscript.

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Figure legends

Fig. 1 Calculated redox potential values of roots expressing cytosolic GRX1-roGFP2 (A), the pixel intensity of dihydroethidium (DHE) indicating the superoxide level (B), and that of 10-acetyl-3,7-dihydroxyphenoxazine (resorufin fluorescence) indicating the hydrogen peroxide level (C) of primary roots of 7-day-old *Arabidopsis thaliana* Col-0 and *Atgstf8*, *Atgstu19* insertional mutants. Scale bar = 100 μm . 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.

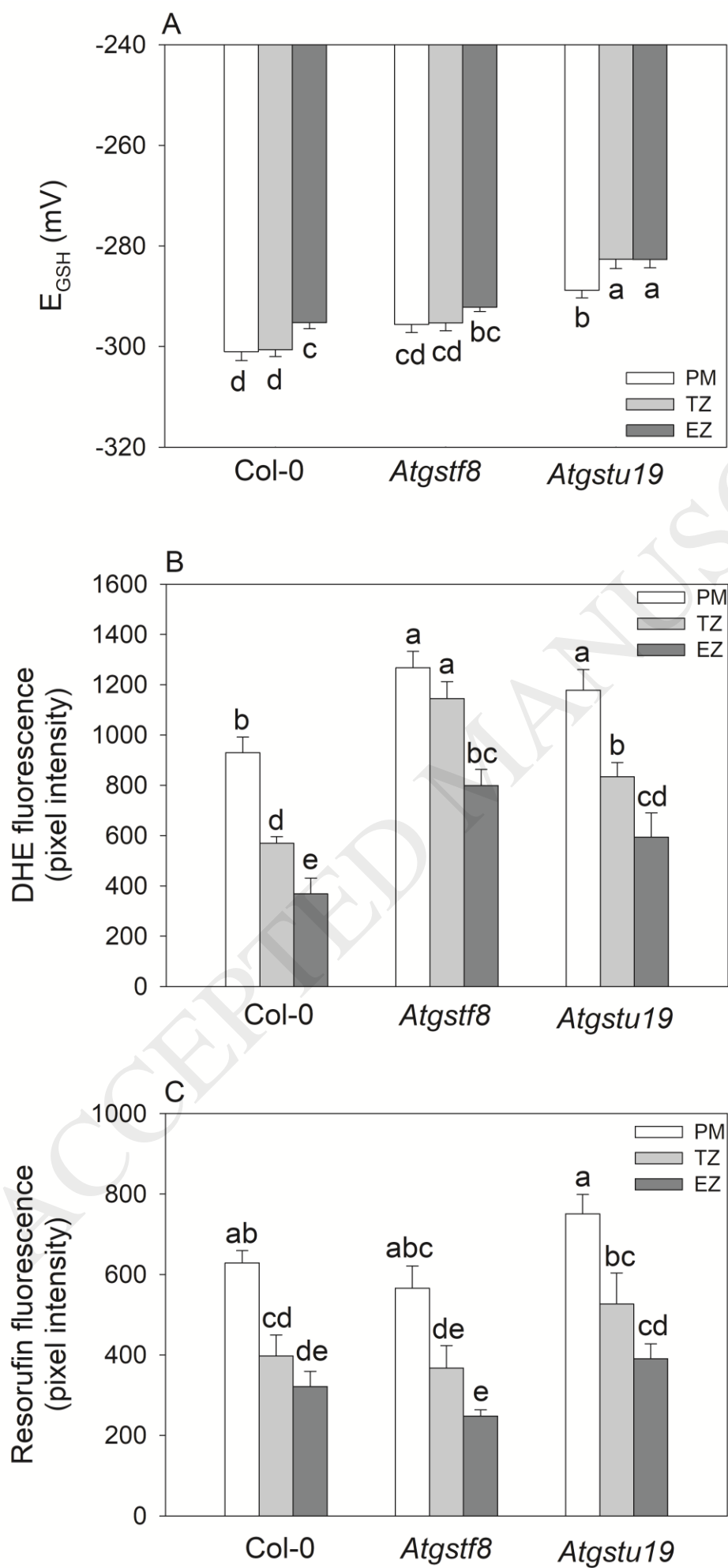


Fig. 2 Representative images of the fluorescent analysis of the redox status, the vitality, superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) levels in the 7-day-old *Arabidopsis thaliana* Col-0 and *Atgstf8*, *Atgstu19* insertional mutants after three hours of treatment with 75 and 150 mM NaCl. (See more details in Materials and methods). Scale bars = 100 μ m.

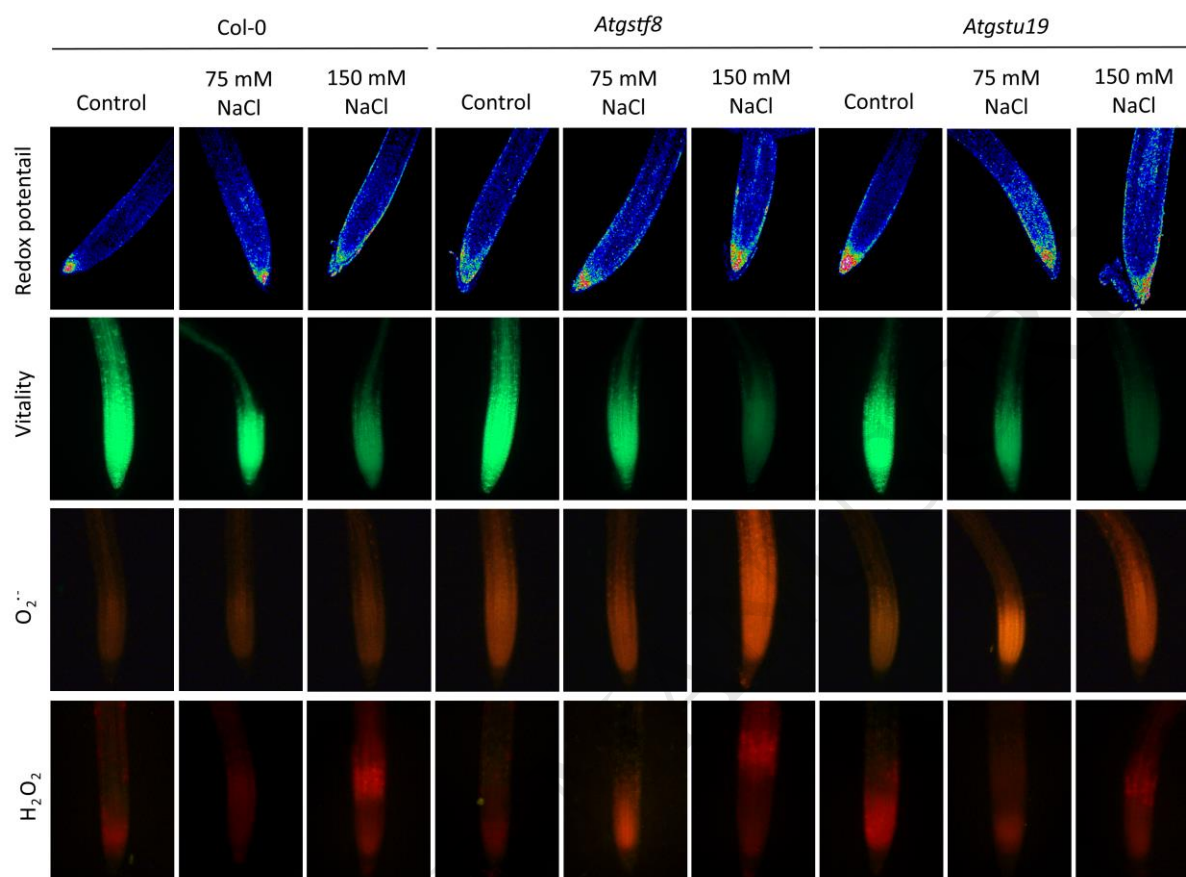


Fig. 3 The redox status (A, E, I), vitality (B, F, J), superoxide radical (DHE fluorescence, C, G, K) and hydrogen peroxide (resorufin fluorescence, D, H, L) levels in the proximal meristem (A-D), transition- (E-H) and elongation zones (I-L) of 7-day-old *Arabidopsis thaliana* Col-0 and *Atgstf8*, *Atgstu19* insertional mutants after three hours of treatment with 75 or 150 mM NaCl. EGSH: glutathione redox potential; FDA: fluorescein diacetate; DHE: dihydroethidium; Resorufin: 10-acetyl-3,7-dihydroxyphenoxazine. Data are means \pm SE, n=10. Columns with different letters are significantly different at $p\leq 0.05$, determined by Duncan's test.

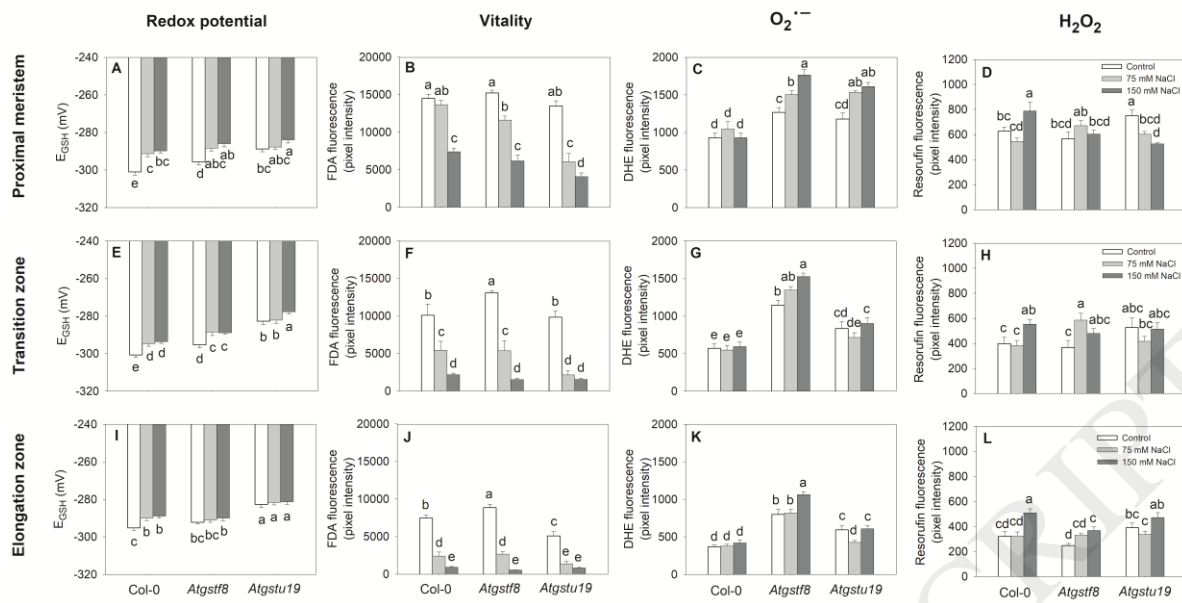


Table 1 The total length of epidermis and cortex cells in the proximal meristem and transitional zone (isodiametric cells, ID) in the one-week-old *Arabidopsis thaliana* Col-0 and *Atgstf8*, *Atgstu19* insertional mutants. Data are means \pm SE, n=10. Data with different letters are significantly different at $p\leq 0.05$, determined by Duncan's test. ns= not significant

	Plant type	Proximal meristem (till the first ID cell)	Transitional zone (ID cells)
Total length of epidermal cells (μm)	Col-0	231.30 \pm 3.96 ^a	57.69 \pm 1.57 ^{ns}
	<i>Atgstf8</i>	215.42 \pm 5.95 ^b	57.65 \pm 1.59 ^{ns}
	<i>Atgstu19</i>	214.85 \pm 5.49 ^b	54.67 \pm 2.56 ^{ns}
Number of epidermal cells (pieces)	Col-0	23.05 \pm 0.89 ^a	5.59 \pm 0.32 ^{ns}
	<i>Atgstf8</i>	19.20 \pm 0.69 ^b	5.56 \pm 0.31 ^{ns}
	<i>Atgstu19</i>	18.76 \pm 0.63 ^b	5.64 \pm 0.39 ^{ns}
Total length of cortex cells (μm)	Col-0	261.93 \pm 6.43 ^{ns}	63.75 \pm 1.18 ^{ns}
	<i>Atgstf8</i>	257.48 \pm 3.32 ^{ns}	66.48 \pm 4.08 ^{ns}
	<i>Atgstu19</i>	268.54 \pm 6.49 ^{ns}	67.99 \pm 1.85 ^{ns}
Number of cortex cells (pieces)	Col-0	24.95 \pm 0.87 ^a	5.81 \pm 0.34 ^{ns}
	<i>Atgstf8</i>	24.13 \pm 0.71 ^{ab}	5.56 \pm 0.32 ^{ns}
	<i>Atgstu19</i>	22.00 \pm 0.87 ^b	5.59 \pm 0.43 ^{ns}