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The role of Arabidopsis glutathione transferase F9 gene under oxidative stress in seedlings

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Running title: The role of AtGSTF9 in oxidative stress response

Abstract

Arabidopsis thaliana contains 54 soluble glutathione transferases (GSTs, EC 2.5.1.18), which are thought to play major roles in oxidative stress responses, but little is known about the function of individual isoenzymes. The role of AtGST phi 9 (GSTF9) in the salt- and salicylic acid response was investigated using 2-week-old *Atgstf9* and wild type (Wt) plants. *Atgstf9* mutants accumulated more ascorbic acid (AsA) and glutathione (GSH) and had decreased glutathione peroxidase (GPOX) activity under control conditions. Treatment of 2-week-old seedlings with 10^{-7} M salicylic acid (SA) for 48 h resulted in elevated H_2O_2 level and enhanced GST activity in *Atgstf9* plants, 10^{-5} M SA treatment enhanced the malondialdehyde and dehydroascorbate contents compared to Wt. 50 and 150 mM NaCl increased the GST activity, AsA and GSH accumulation in *Atgstf9* seedlings more pronounced than in Wt plants. We found that the *Atgstf9* mutants had altered redox homeostasis under control and stress conditions, in which elevated AsA and GSH levels and modified GST and GPOX activities may play significant role. The half-cell potential values calculated from the concentration of GSH and GSSG indicate that this GST isoenzyme has an important role in the salt stress response.

Key words: *Arabidopsis thaliana*; ascorbic acid; glutathione; glutathione transferase phi 9; glutathione peroxidase activity

INTRODUCTION

Plant glutathione transferases (GSTs, EC 2.5.1.18) are ubiquitous enzymes that catalyze the conjugation of the tripeptide glutathione (GSH; γ-Glu–Cys–Gly) to an electrophilic centre within

a small acceptor molecule. A key role of GSTs is their ability to inactivate toxic compounds and this is crucial to the survival of cells and organisms both under normal and different stress conditions [13]. All plants possess multiple cytosolic GSTs, each of which displays distinct catalytic as well as non-catalytic binding properties [11]. *Arabidopsis thaliana* contains 54 soluble GSTs which can be divided into seven distinct classes: tau, phi, theta, zeta, lambda, dehydroascorbate reductase (DHAR) and tetrachlorohydroquinone dehalogenase (TCHQD). Plant GSTs that belong to tau, phi and theta classes exhibit GSH-dependent peroxidase activity (GPOX, EC 1.11.1.9), reducing organic hydroperoxides, e.g. lipid peroxides, to monohydroxyalcohols. The phi GSTs (GSTFs) represent a large, plant-specific class of proteins with 13 members in *Arabidopsis* that are likely to be involved in conferring tolerance to oxidative stress [4]. Increased expression of phi class GSTs was provoked by ethylene, methyl jasmonate, H_2O_2 and salicylic acid (SA) treatments [28]. However, very little is known about the function of individual GSTFs because of their possible functional redundancy. *AtGSTF9* (At2g30860) is a highly abundant and constitutively expressed gene [28], which can be readily detected at the protein level [21]. Several GSTF enzymes (GSTs F6, F7, F9 and F10) were knocked down using RNAi which did not lead to phenotypic alterations, suggesting that individual GSTFs are non-essential for normal growth [22]. However, overexpression of *AtGSTF9* conferred dominant, estradiol-dependent salt tolerance to transgenic *Arabidopsis* [18].

Salt stress is one of the most important stress factors which negatively affect plant growth and development. The plant response to salinity can be separated into two phases that are characterized as osmotic and ionic stress [17], but as a secondary phenomenon most stress factors are usually associated with oxidative burst, leading to oxidative stress [19]. Salt stress

leads to the accumulation of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2) [17].

ROS are highly reactive and cytotoxic compounds in cells, therefore a tight control is needed to balance the individual ROS and reactive nitrogen species, ROS-producing enzymes, antioxidants and their oxidation/reduction states [5, 2]. All these contribute to the general redox homeostasis, which plays a central role in the mediation of plant responses to abiotic or biotic stresses [20]. Salicylic acid (SA) is a plant hormone with well-known effect on ROS-accumulation and influence on redox status of cells through regulation of ROS generating enzymes. ROS triggered changes have central role in GSH/GSSG and thiol-disulphide exchanges of non-expressor of PR protein 1 (NPR1) and in regulation the expression of pathogenesis-related (PR) proteins [16]. Monomerization of NPR1, changes in NPR1 thiol-disulphide status and ROS-induced increases in cytosolic calcium, which cause activation of kinase cascades are all important features of oxidative signalling [6].

Our experimental system was designed to investigate the particular role of AtGSTF9 in oxidative stress responses induced by NaCl or salicylic acid and we measured GST and GPOX activities, the H_2O_2 and malondialdehyde (MDA) contents, and the ascorbate and glutathione pool in the seedlings. The aim of our study was to gain deeper insights into the mechanisms which may be influenced by AtGSTF9 using a T-DNA insertion mutant. After 48 h salt- or SA treatments, *Atgstf9* mutants had elevated ascorbic acid (AsA) and GSH contents assuming that these plants had a modified redox homeostasis. Our results indicate that AtGSTF9 plays a role in the fine tuning of redox homeostasis under control and stress conditions.

MATERIALS AND METHODS

Plant material and growth conditions

14-day-old wild type (Col-0) and *Atgstf9* mutant *Arabidopsis thaliana* L. seedlings were used in all experiments. The knockout *Arabidopsis* line (SALK_148672C) containing a T-DNA insertion in GSTF9 (*At2g30860*) gene was obtained from the Salk Institute (http://signal.salk.edu/cgi-bin/tdnaexpress). The relative transcript amount of *AtGSTF9* was $2^{-\Delta\Delta Ct}$ = 0.065 in the *Atgstf*9 compared to the wild type under control conditions. Stress treatments were carried out on two-week-old *in vitro* grown plants by placing them onto agar-solidified culture medium supplemented with 50 and 150 mM NaCl or 10^{-7} and 10^{-5} M salicylic acid (SA). The Petri dishes were kept in growth chamber (Fitoclima S 600 PLH, Aralab, Portugal) at a photon flux density of 100 μmol m^{-2} s^{-1} (10/14 day/night period), at a relative humidity of 70 % and 21 °C. Samples were taken after 48 h NaCl or SA treatments. The experiments were repeated at least two times, the measurements were performed in three replicates unless indicated otherwise.

Investigation of viability using fluorescent microscopy

Fluorescein diacetate (FDA) was applied for the determination of cell viability according to Lehotai et al. [12]. Fluorescence intensity values were determined in 80 μm diameter circles, at 150 μm distance from the root tip in roots and 600 μm diameter circles in leaves by Zeiss Axiowert 200M microscope (Carl Zeiss, Jena, Germany) using Axiovision Rel. 4.8 software. The diameter of circles was not modified during the experiments. The measurements were performed in 10 replicates, mean ± SE are given on the figures.

Determination of H_2O_2 level

Hydrogen peroxide level was measured spectrophotometrically after homogenization of 200 mg shoot or root tissue on ice with 300 µl 0.1% trichloroacetic acid (TCA) as described earlier [8]. The amount of H_2O_2 was calculated using a standard curve prepared with 0.1-5 µmol ml^{-1} H_2O_2 concentrations.

Malondialdehyde determination

Malondialdehyde (MDA) formation was followed by using the thiobarbituric acid method [1]. 50 mg tissue was homogenized with 500 µl 0.1 % TCA and 50 µl 4% butylhydroxytoluene was added to avoid further lipid peroxidation. The absorbance was measured at 532 nm and adjusted for nonspecific absorbance at 600 nm. MDA concentration was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Determination of glutathione transferase and glutathione peroxidase enzyme activities

Glutathione transferase (GST, EC 2.5.1.18) activity was determined as published earlier [1] with some modifications. 200 mg tissue was homogenized on ice in 1 ml 100 mM phosphate buffer (pH 7.0) containing 1 mM phenylmethylsulfonyl fluoride and 1% polyvinyl-polypirrolidone. The homogenate was centrifuged for 20 min at 10 000 *g* at 4 °C and the supernatant was used for enzyme activity assays. GST activity was determined spectrophotometrically by using an artificial substrate, 1-chloro-2,4-dinitrobenzene (CDNB, Sigma-Aldrich). GST activity was given in nkat (nmol conjugated products sec^{-1}, ε_{340} = 9.6 mM^{-1} cm^{-1}). Glutathione peroxidase (GPOX, EC 1.11.1.9) activity was measured with cumene hydroperoxide (CHP; Sigma-Aldrich) as a substrate. The reaction mixture contained 4 mmol l^{-1} GSH, 0.2 mmol l^{-1} NADPH, 0.05 U of GR (from baker's yeast, Sigma-Aldrich), 100 µl enzyme extract, and 0.5 mmol l^{-1} substrate in

phosphate buffer (0.1 mol l^{-1}, pH 7.0) in a total volume of 1 ml. GPOX activity was given in nkat (nmol of converted NADPH sec^{-1}, ε_{340} = 6.22 mM^{-1} cm^{-1}).

Ascorbate and glutathione extraction and determination

Ascorbate and glutathione contents were determined according to Tari et al. [26]. Two-hundred mg of seedlings was homogenized with 0.8 ml of 5 % TCA. The homogenate was centrifuged at 10 000 *g* for 20 min at 4°C and the supernatant was used for further determinations. To assay total ascorbate (AsA), 100 µl of 10 mM dithiothreitol (DTT) was added to the extract and the excess of DTT was removed by adding 100 µl, 0.5 % (w:v) N-ethylmaleimid (NEM). Ascorbate concentrations were determined spectrophotometrically at 525 nm. Dehydroascorbate (DHA) content was calculated as the difference between the concentration of total and reduced ascorbate.

Total and oxidized glutathione concentrations were measured spectrophotometrically using an enzymatic assay. To mask reduced glutathione (GSH) 4-vinylpyridine was added to the extract and incubated for 60 min. The reaction mixture contained 0.1 M phosphate buffer pH 7.5, 1 mM 5,5'-dithio*bis*(2-nitrobenzoic acid) (DTNB), 1 mM NADPH, 1 U of glutathione reductase (GR baker's yeast, Sigma) and 20 µl of the tissue extract in 1 ml volume. GSH content was calculated from the difference between the concentration of total and oxidized glutathione. Standard curves were obtained for total glutathione and GSSG within the 0-2 µM range.

The reduction potential of the GSH/GSSG couple was determined with the Nernst equation using the form of Schafer and Buettner [23]: E_{hc}= -240 - (59.1/2) log($[GSH]^2$/[GSSG]) mV; where E_{hc} = $E_{GSSG/2GSH}$: the half-cell redox potential of glutathione, -240 mV: the standard reduction potential of glutathione on 25°C, pH=7.0.

Statistical analysis

Statistical analysis was carried out with SigmaPlot 11.0 software (SigmaPlot, Milano, Italy) by Duncan's test and differences were considered significant at $P \leq 0.05$. Data presented here are the means ± SD of at least 3 measurements unless indicated otherwise.

RESULTS

Different viability of wild type and Atgstf9 mutant plants under adverse conditions

The root lengths of 2-week-old *Atgstf9* seedlings was significantly decreased compared to wild type plants, but only slight differences were detected between the plants in the presence of 150 mM NaCl or 10^{-5} M (data not shown). The viability of wild type and *Atgstf9* mutant *Arabidopsis* was similar under control conditions. Treatment with 50 and 150 mM NaCl decreased the viability of wild type *Arabidopsis* leaves by 50% and 60%, while viability of *Atgstf9* mutants was reduced by 70% and 80%, respectively. Similar tendencies could be observed in roots of wild type and *Atgstf9* plants (Fig. 1). SA treatment decreased the viability of wild type seedlings only in leaves, but 10^{-7} M SA did not affected it in mutants (Fig. 1).

H_2O_2 accumulation and oxidative damage in SA- and salt-treated plants

H_2O_2 and MDA accumulation during the stress was detected using the whole seedlings. Under control conditions, the wild type and the mutant seedlings had similar H_2O_2 and MDA contents. Salt treatment induced H_2O_2 accumulation only in *Atgstf9* mutants when it was applied in 150 mM concentration but the MDA content was elevated both in wild type and mutant seedlings. 10^{-}

^{7} M SA triggered the most pronounced increase in the H_2O_2 content and the MDA level was also increased compared to the untreated plants (Fig. 2).

Glutathione transferase and glutathione peroxidase activity in wild type and mutant plants

150 mM NaCl or 10^{-5} M SA treatment increased the glutathione transferase activity in the wild type plants. The mutant seedlings had significantly higher GST activity after 50 or 150 mM NaCl treatment or after application of 10^{-7} M SA comparing to the wild type. GST activity in 10^{-5} M SA-treated *Atgstf9* plants declined compared to other treatments (Fig. 3). The GPOX activity was significantly reduced in *Atgstf9* mutant seedlings under control conditions. Salt treatment reduced the GPOX activity both in the wild type and in mutant plants, however in *Atgstf9* it was lower than in the wild type. SA elevated the GPOX activity both in wild type and mutant plants, and there were no significant difference between them after 48 h of treatments (Fig. 3).

Role of the ascorbate-glutathione pool in the NaCl and SA stress responses

The total ascorbate and glutathione contents of the *Atgstf9* seedling were higher compared to the wild type under control conditions. Salt treatment increased the total ascorbate content of seedlings after two days, and the *Atgstf9 Arabidopsis* accumulated more ascorbate. The proportion of the oxidised form of ascorbate (DHA), was not changed after 50mM NaCl, but was higher in plants treated with 150 mM NaCl, where the glutathione accumulation was also induced, and this accumulation was more pronounced in *Atgstf9* plants. SA treatments dramatically reduced AsA, while enhanced the DHA contents in both genotypes, and increased the accumulation of GSH in wild type and in *Atgstf9* plants. The amount of GSSG barely changed in these treatments and was similar in wild type and mutant plants (Fig 4). The used salt

treatments resulted in distinct $E_{GSSG/2GSH}$ levels, because the wild type and *Atgstf9* plants had different GSH and GSSG contents (Table 1).

DISCUSSION

The primary components of the system to combat excess ROS in plants are non-enzymatic antioxidants (such as ascorbate, glutathione, carotenoids, tocopherols) and enzymes which are involved in quenching of reactive oxygen species (superoxide dismutase, catalase, peroxidases), re-reduction of the members of ascorbate-glutathione cycle and/or in maintenance of cellular redox state (such as ascorbate peroxidase, glutathione reductase) and other proteins with general or specific roles in stress responses, e.g. GSTs [15, 9, 20]. Sappl et al. [21] reported that AtGSTF9 among a set of other GSTs (AtGSTF2, AtGSTF6/7, AtGSTF10, AtDHAR1, AtDHAR3, AtGSTU5 and AtGSTU19) is common to non-treated shoots and cell culture [21].

Despite the high abundance, the decreased expression of ATGSTF9 did not affect the GST activity measured with CDNB substrate in the mutants under control conditions. After applying NaCl or SA the total extractable GST activity was even higher supposedly because of activation of other GST isoenzymes and/or GST coding sequences. However, specific GPOX activity data revealed that AtGSTF9 has important role both under control and salt stress conditions of plants (Fig. 3). While the viability, level of H_2O_2 or the lipid peroxidation marker MDA showed similar values in the wild type and mutant seedlings under control conditions, the level of AsA and GSH was increased in the mutants. Ascorbate and glutathione are non-enzymatic antioxidants which may play roles in signal transduction and/or transmission during cell death and defence responses. Moreover, ascorbate was suggested to be the redox molecule which primarily

regulates plant development, and glutathione is also involved in this process, although it is mainly important for stress defence and signalling [20]. In accordance with these, in our experiments the role of increased AsA and GSH pools was more pronounced after applying NaCl or SA treatments for two days on the 2-week-old plants. While the salt stress affected mainly the size of the AsA pool, SA resulted in shift of AsA pool towards the DHA. Detoxification of H_2O_2 through the glutathione – ascorbate cycle leads to a transient change in the degree of oxidation of the cellular glutathione pool, and thus a change in the glutathione redox potential, which is not only an indicator of the general redox status, but it is part of the redox regulatory mechanism [14, 10, 24]. Changes in the concentration and in the reduction potential of the GSH/GSSG and other redox couples make possible the fine regulation of the cellular redox environment and consequently the growth of the plants.

Glutathione has pivotal role in oxidative stress defence, and depletion of the cytosolic GSH pool is associated with large changes in the abundance of transcripts encoding proteins that are involved in oxidative defence [7]. Mining of the proteome data for GSH-associated genes showed that disruption of the pathway for the synthesis and degradation of glutathione in the *Atggt1* knockout leaves was associated with the induction of genes encoding four GSTs in the phi class (*GSTF2*, *GSTF6*, *GSTF9*, and *GSTF10*), a GSH peroxidase (*GPX1*), and glyoxylase II [27]. The important role of the a high GSH/GSSG ratio, maintained by increased GSH synthesis and/or GSSG reduction, in the efficient protection of plants against abiotic stress-induced accumulation of ROS was indicated at several plant species [25].

Based on our findings, the elevated AsA and GSH content of *Atgstf9* mutants may be the result of a complex defence mechanism which helps to maintain the cellular homeostasis in the case of very low *AtGSTF9* expression. These results suggest a modified redox homeostasis which could

induce a redox signalling pathway resulting in even elevated GST activities under stress conditions.

CONCLUSION

The observed differences, induced by salt stress and SA in the viability, GPOX activity, AsA and GSH contents between *Atgstf9* and Col-Wt plants can be in connection with the different redox status measured in the seedlings. In our experiments, the E_{hc} values calculated from the concentration of GSH and GSSG indicate that AtGSTF9 isoenzyme has a positive role in the salt stress response of seedlings by modulating the short-term redox homeostasis of plants. The mutants had significantly lower GPOX activity than the wild type, however, we did not found a significant decrease in GST activity. This result can be explained by the high redundancy of GSTs, supposedly other isoenzymes were induced. The results of the present study clearly show that the lack of AtGSTF9 increased the AsA and GSH pools, affected the redox status, which might influence the long-term growth of plants.

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Figure legends

Fig. 1 Cell viability in leaves and root tips of Wt (Col-Wt) and *Atgstf9 Arabidopsis* treated with different NaCl (50 mM or 150 mM) or SA (10^{-7} M or 10^{-5} M) concentrations. Means denoted by different letters indicate a significant difference between the treatments at a level $P<0.05$ according to Duncan's test.

Fig. 2 H_2O_2 and malondialdehyde (MDA) contents of Wt (Col-Wt) and *Atgstf9 Arabidopsis* seedlings treated with different NaCl (50 mM or 150 mM) or SA (10^{-7} M or 10^{-5} M) concentrations. Means denoted by different letters indicate a significant difference between the treatments at a level $P<0.05$ according to Duncan's test.

Fig. 3 Glutathione transferase (GST) and glutathione peroxidase (GPOX) activities of Wt (Col-Wt) and *Atgstf9 Arabidopsis* seedlings treated with different NaCl (50 mM or 150 mM) or SA (10^{-7} M or 10^{-5} M) concentrations. Means denoted by different letters indicate a significant difference between the treatments at a level $P<0.05$ according to Duncan's test.

Fig. 4 Total, reduced and oxidized ascorbate (AsA and DHA) and glutathione (GSH and GSSG) contents of Wt (Col-Wt) and *Atgstf9 Arabidopsis* seedlings treated with different NaCl (50 mM or 150 mM) or SA (10^{-7} M or 10^{-5} M) concentrations. Means ± SD. Means denoted by different letters indicate a significant difference between the treatments at a level $P<0.05$ according to Duncan's test (Italic for AsA and GSH, normal letters for DHA and GSSG). * n.s. – not significant. There were no significant differences among GSSG contents.

Table 1 Half-cell reduction potential of the GSH/GSSG redox couple ($E_{GSSG/2GSH}$) in *Arabidopsis* seedlings following 48 h of NaCl or SA treatment

	$E_{GSSG/2GSH}$ (mV)	
	Col-Wt	*Atgstf9*
Control	-267.9	-259.5
50 mM NaCl	-239.7	-246.9
150 mM NaCl	-254.8	-265.3
10^{-7} M SA	-264.5	-264.2
10^{-5} M SA	-266.5	-264.5