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GLUTATHIONE REDUCTASE SIGNIFICANCE FOR *IN VITRO* SOMATIC EMBRYOGENESIS AND SALT STRESS RESPONSE OF *DACTYLIS GLOMERATA* L.

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Abstract: The non-protein thiol tripeptide glutathione and its corresponding disulfide represent a redox couple, which is essential for the maintenance of the intracellular redox state. It is involved in numerous physiological and developmental processes in plants, including somatic embryogenesis and abiotic stress response. Glutathione reductases, the enzymes, responsible for reduction of glutathione disulfide are crucial to sustain favorable intracellular redox environment and therefore, play a key role in the above-mentioned processes. The activity of glutathione reductase was investigated on different stages of somatic embryogenesis and different salt treatments (enhancing – 0.085 M and inhibitory – 0.17 M NaCl for its concentrations) in *in vitro* cultures of *Dactylis glomerata* L. To evaluate their role in the control of developmental processes and abiotic stress response, thiol disulfide reducing enzymes were investigated in assay, following the oxidation of NADPH and isoforms were determined after electrophoretic separation. The highest activity of glutathione reductase was detected on the 10th day of cultivation when pro-embryogenic masses were formed. Activity in high salt treated cultures was low during the whole cultivation period. Zymographic analysis revealed three different glutathione reductase isoforms that could use both NADPH and NADH and a single NADH-dependent isoform. Glutathione reductase was shown to be important for the process of somatic embryogenesis, probably by controlling the low molecular weight thiol redox status on different developmental stages.

INTRODUCTION

The non-protein tripeptide γ-L-Glutamyl-L-cysteinyl glycine or glutathione (GSH) is a major antioxidant with a variety of functions in abiotic stress response. In plants, it participates in free radicals scavenging, directly or through enzyme-mediated reactions of the ascorbate-glutathione cycle. It also participates in sequestration of heavy metals and xenobiotics, in protein thiols defense and regulation of protein functions (Noctor et al., 2012; Zagorchev et al., 2013). Glutathione, along with its corresponding disulfide (GSSG), forms a redox couple which abundance establishes it as the major intracellular redox buffer (Schafer and Buettner, 2001). Apart from the stress responsive functions, GSH appeared to be also an important regulator of plant growth and development. The discovery of a nuclear GSH pool suggested its role in regulation of genes expression at different developmental stages (Vivancos et al., 2010). Also, a clear correlation between the half-cell redox potential of GSSG/2 GSH (EGSSG/2 GSH) couple and embryogenesis was reported (Stasolla, 2010; Zagorchev et al., 2012).

Glutathione reductase (EC 1.6.4.2, GR), the enzyme, responsible for the reduction of GSSG receives major attention in studies, related to abiotic stress response and tolerance (Mudalkar et al., 2017). Different GR isoforms with localization in the cytoplasm, chloroplasts and other organelles adjust the redox state of the GSSG/2 GSH couple and reduced the oxidized GSSG, resulting from free radicals scavenging and deglutathionylation of protein thiols (Zagorchev et al., 2016). Thus, GR is suggested to be involved in developmental processes and abiotic stress response.

Somatic embryogenesis (SE) is a remarkable plant feature, during which somatic cells (or competent differentiated cells) dedifferentiate and undergo formation of somatic embryos either directly (e.g., direct SE) or indirectly (or indirect SE), through stages of embryogenic callus and proembryogenic masses (PEM) (Fehér, 2015). The potential of somatic embryos to regenerate into a genetically identical, fully functional plants lay in the basis of micropropagation (Raju et al., 2015), genetic transformation (Ravanfar et al., 2017) and plant biotechnologies in general (Shahzad et al., 2017). Both external and internal factors, affecting SE were widely studied throughout the years (Fehér, 2015), but still many molecular and metabolic aspects of this process remain elusive.

Dactylis glomerata L. (Orchardgrass) is widely spread in temperate climate perennial grass. It is also an important forage species (Madesis et al., 2014). The highly embryogenic genotype (Conger and Hanning, 1991) used in the present study was previously established as a suitable system to study somatic embryogenesis in monocotyledonous (Krishnaraj and Vasil, 1995). Mild salt treatment (Odjakova et al., 2001) was shown to enhance SE in this genotype. Correlation between the GSH/GSSG, thiol/disulfide half-cell redox potential and the developmental stage of SE was also shown (Zagorchev et al., 2012). The activity of enzymes, controlling the intracellular redox status such as glutathione reductase, however, was not studied.

MATERIALS AND METHODS

Plant material and in vitro cultivation

In vitro cultures of *Dactylis glomerata* L. from the highly embryogenic genotype Embryogen P (Conger and Hanning, 1991) were used as plant material. They were initiated from the meristem tissue of the first leaf on solid SH media (Schenk and Hildebrandt, 1972), supplemented with 30 μM Dicamba (Duchefa) and grown in the dark at 25°C until somatic embryos were formed (around the 30th day). In vitro suspension cultures were initiated from somatic embryos in liquid SH media, supplemented with 30 μM Dicamba and grown at 25°C in the dark on a rotary shaker (New Brunswick) at 105 rpm for 1, 5, 10 and 15 days. Sodium chloride at 0.085 and 0.17 M final concentration was added for salt treatment (Odjakova et al., 2001).

Protein isolation

Cells were collected from the growth medium on nylon filters, washed three times with deionized water, briefly soaked on filter paper and frozen in liquid nitrogen. Afterwards cells were ground in liquid nitrogen. The resulting powder was resuspended in phosphate buffered saline (PBS), pH 7.2, centrifuged at 15 000 x g at 4°C for 15 min and the supernatant, containing soluble intracellular proteins was used for further analysis (Odjakova et al., 2001). The protein concentration was estimated by BCA kit (ThermoScientific) according to producer's manual.

Glutathione reductases assay

Glutathione reductase activity was estimated by following the rate of NADPH oxidation, resulting in decrease in absorbance at 340 nm (Bailly and Kranner, 2011). The assay mixture contained 10 mM GSSG, 3 mM $MgCl_2$ and appropriate amount of protein extract, giving sufficient NADPH oxidation rate in 0.1 mM potassium phosphate buffer, pH 7.8 in final volume of 0.5 ml. The decrease in absorbance was followed on UV/Vis Spectrophotometer Jenway after the addition of NADPH (0.5 mM final concentration) for 10 min. Glutathione reductase activity was calculated as nmoles oxidized NADPH per min per mg protein using the molar absorbance coefficient of NADPH ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$).

Glutathione reductase zymogram analysis

Glutathione reductase isoforms were visualized after electrophoretic separation both on native and denaturing (SDS) PAGE in 10% T gels. Equal amount of protein (15 μg) was loaded for each sample. Electrophoreses were conducted according to (Laemmli, 1970). For native conditions SDS was omitted from the gels, sample buffer and electrode buffer. For renaturation after SDS PAGE, 30 min incubation in 2.5% Triton X 100, followed by extensive washing with deionized water was performed. Afterwards the gels were overlaid with 1% agarose in 0.15 M TRIS.HCl pH 8, supplemented with GSSG at 1.5 mg ml^{-1} and NADPH (NADH) at 0.5 mg ml^{-1} final concentrations,

incubated at 37°C in the dark for varying time (from 15 to 60 min depending on the velocity of the reaction) and pictures were taken on UV transilluminator. Activity was visualized as dark bands on light background.

Western Blot

Western blot was performed with polyclonal anti-GR antibody (Agrisera), developed against maize plastidial GR isoform in rabbit as first antibody (1:5000) and alkaline phosphatase conjugated goat anti-rabbit as secondary antibody (1:10 000, Sigma). First, proteins, separated on SDS PAGE were transferred to nitrocellulose membrane, 0.22 um on Hoeffer Semi-dry Blot equipment in Towbin's transfer buffer (Towbin et al., 1979) according to producer's manual. Then membranes were immediately transferred in PBS buffer, containing 1% BSA and blocked overnight at 4°C. Incubation with both antibodies was performed for 1 h at room temperature on a shaker and triple washing with PBS containing 0.1% Tween 20 was applied between every step. Membranes were developed with Sigma Fast BCIP/NBT substrate tablets until clear dark-pink bands appeared. Molecular mass after SDS PAGE was calculated according to Rainbow Clear Molecular mass protein standards (GE Healthcare) on Gene Tools (Syngene) software.

Statistical analysis

All activity assays were performed in triplicates of two independent *in vitro* suspension cultures. Significant values were calculated by Student's T-test on GraphPad Prism software. Zymogram analysis was performed in triplicates both after native and SDS PAGE.

RESULTS AND DISCUSSION

Glutathione reductase activity in the time course of cultivation

The activity of GR in the time course of cultivation is shown on Fig. 1. The activity was measured on the 1st, 5th, 10th and 15th day when different stages of somatic embryos formation were observed. The 5th day is characteristic for fresh mass accumulation and single cells in the growth medium. Then PEMs were formed around the 10th day. Mature somatic embryos were observed at the 15th day. In confirmation of the results of Odjakova et al. (2001), somatic embryogenesis was suppressed in cultures, treated with 0.17 M NaCl. Concentrations of 0.085 M NaCl had a positive effect on somatic embryogenesis at lower fresh mass accumulation compared to controls (Odjakova et al., 2001). The highest GR activity was measured at the 10th day in controls and mild-stressed cultures. The activity on the 5th and 15th day is lower and similar in all cultures. In highly-stressed cultures there is no significant change of GR activity by days.

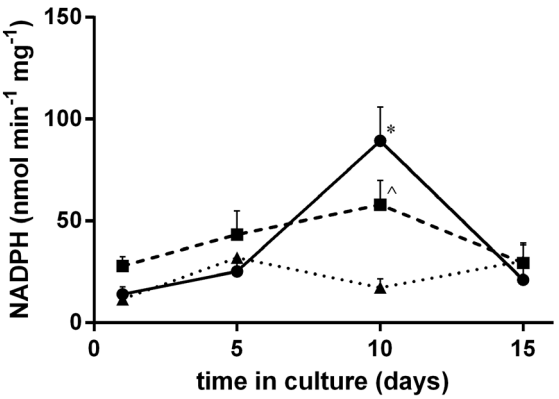

Fig. 1. The effect of different NaCl concentrations on glutathione reductase activity by days. GR activity is expressed in nmol oxidized NADPH for 1 min by 1 mg of total intracellular protein. Activity in controls (dots) and cultures, treated with 0.085 M (squares) and 0.17 M (triangles) NaCl was measured. Bars indicate ± SD (n = 6). Asterisk and caret denote significantly differing values at $P < 0.05$, Student's T-test.

Glutathione reductase activity regulates different developmental stages

Glutathione reductase is known as an important stress responsive enzyme, crucially involved in the maintenance of low GSSG concentrations and high GSH to GSSG ratio (Gill et al., 2013). Thus, it is a regulator of the intracellular redox environment as the GSH/GSSG is the most abundant redox pair (Schafer and Buettner, 2001). Increase of GR activity is often observed in stressed plants and correlated to stress tolerance (Gill et al., 2013). Similarly, in the present experiment, cultures, treated with 0.085 M NaCl showed higher activity than controls (Fig. 1). The activity pattern seems to be more characteristic for the developmental stage rather than the salt treatment. The highest activity in controls and mild-salt treated cultures was detected on the 10^{th} day when the highest GSH concentration was also observed (Zagorchev et al., 2012) and decreased accordingly. The stably low GR activity (Fig. 1) in high-salt treated cultures could explain the inability of these cultures to further regulate their GSH/GSSG ratio (Zagorchev et al., 2012). The transition state from microclusters to PEMs may require higher GSH concentration, maintaining also more reductive environment. Further in the development, the differentiation to mature somatic embryos would require more oxidizing environment thus explaining the decrease in GR activity. The slightly higher GR activity in mild-stressed cultures would be necessary to overcome the stress conditions and keep the cellular redox state to an optimal value as there were no apparent differences in $E_{GSSG/2\ GSH}$ between

controls and mild-stressed cultures (Zagorchev et al., 2012). Such SE stage-specific changes in GR activity are in agreement with findings in *Eleutherococcus senticosus* (Shohael et al., 2007), but not reported in other plant species such as saffron where no significant differences were observed (Blazquez et al., 2009). In the higher salt concentration, the GR activity was severely inhibited at the 10th day. In response to salinity, GR activity may decrease (Hossain et al., 2017) or increase, especially in salt tolerant genotypes (Vighi et al., 2017). The most pronounced difference on the 10th day would explain the suppressed somatic embryogenesis at this salt concentration.

From one side the cellular redox potential itself is a major regulator of developmental processes and a decisive marker for the further development (Schafer and Buettner, 2001; Stasolla, 2010). On the other side, GR is also involved in the activity of the glutaredoxin system, thus regulating the protective and regulatory mechanism of protein glutathionylation/deglutathionylation (Colville and Kranner, 2010). As the glutaredoxin activity was inhibited at this salt concentration (Zagorchev et al., 2014b), this may be also related to the lower GR activity reported here. In summary the formation of somatic embryos (around the 15th day of culturing) requires more oxidizing cellular environment (thus higher $E_{GSSG/2\ GSH}$ (Zagorchev et al., 2012) and higher glutaredoxin activity (Zagorchev et al., 2014b), but the redox control would occur during the PEMs stage (on the 10th day) and GR is an important player in this event. The results presented also confirmed that GR is not a crucial stress responsive enzyme at least in some monocots as GR activity also decreased in *Dactylis glomerata in vitro* regenerants and in seedling of salt-tolerant barley cultivars (Zagorchev et al., 2014a).

Glutathione reductase zymograms and immunoblot detection

Zymographic analysis was used for visualization of different GR isoforms (Fig. 2 and Fig. 3) on the 10th day of culturing. Both NADPH (Fig. 2) and NADH (Fig. 3) were used as electron donors for reduction of GSSG. In the NADPH-dependent reaction three isoforms were detected in controls and salt-treated *in vitro* suspension cultures. The intensity of bands diminished accordingly to the levels of salt stress. Two additional isoforms were detected in roots and leaves of *in vitro* regenerated plants (Fig. 2).

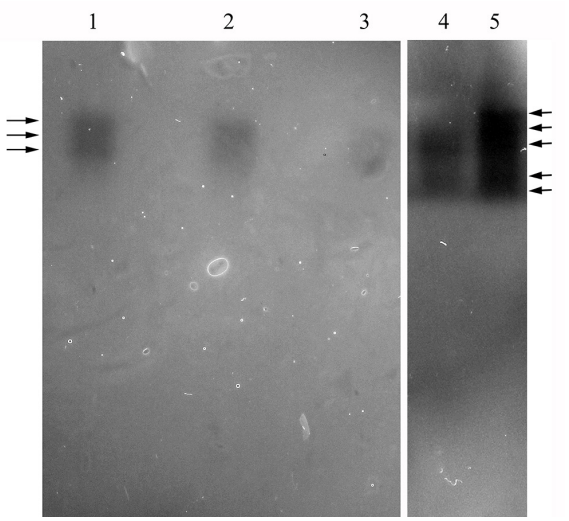

Fig. 2. Glutathione reductase zymograms after native PAGE separation of intracellular proteins. Dark bands appeared in the places of NADPH oxidation. Suspension cultures on 10th day of incubation (a) and roots and leaves of in vitro regenerated plants (b) were tested. Different isoforms are indicated by arrows.

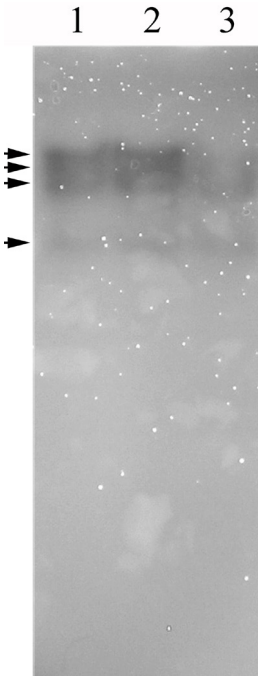

Fig. 3. Glutathione reductase zymograms after native PAGE separation of intracellular proteins. Dark bands appeared in the places of NADH oxidation. Suspension cultures on 10th day of incubation were studied. Different isoforms are indicated by arrows.

Both analyses were performed after protein separation in native conditions. Additionally, the same procedure was used for proteins, separated on SDS PAGE following renaturation by Triton X-100, but no bands were visualized. In NADH-dependent reduction of GSSG the same three bands were visualized. An additional, NADH-dependent but NADPH-independent GR isoform was also detected (Fig. 3). The incubation time at 37°C was however longer for the NADH-dependent reaction – approximately 60 minutes compared to 15 minutes for the NADPH-dependent reaction.

Immunoblot analysis with anti-GR polyclonal antibody showed recognition of a single fraction after separation on native (data not shown) and denaturing PAGE (Fig. 4). The apparent molecular weight of the detected fraction was 60 kDa, like the 54 kDa expected (Agrisera product specification).

Glutathione reductase could utilize NADH as an alternative reductive donor

Numerous reports support the view that GR are NADPH-dependent oxidoreductases. However, it was shown in few studies that at least several GR isoforms could also utilize NADH though with lower efficiency (Boggaram et al., 1978) and was reported for other reductases (Giberti et al., 2014). The results presented showed that all three isoforms as detected by zymographic analysis,

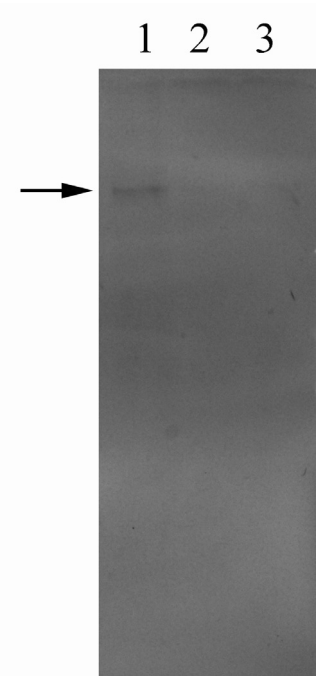

Fig. 4. Detection of GR on Western blot after 12.5% T SDS-PAGE by anti-GR antibody. Secondary antibody is alkaline phosphatase conjugated.

could also benefit of the NADH. Moreover, an additional isoform was detected that could use only NADH but did not show activity to NADPH. This might be of crucial importance for *in vitro* non-photosynthesizing tissues where the main source of reduced NADP is cut. Thus, the GSSG reducing capacity could be maintained either by NADPH from photo-independent reactions or by NADH from catabolic reactions. This defines the independence of this highly important oxido-reductive system from the metabolic needs of the cells. Glutathione reductase would function either when the carbon source is used mainly for energy production (glycolysis and cycle of tricarboxylic acids) or for metabolic precursors (pentose-phosphate pathway).

It is also noteworthy that the GR isoforms are fewer in *in vitro* cultures compared to leaves and roots. This is not unexpected as in non-photosynthesizing tissues, e.g., in suspension cultures, grown in the dark some of the plastidial isoforms of the enzymes may diminish (Ishikawa and Shigeoka, 2008). Only one of the GR isoforms was recognized by polyclonal antibody, selected against GR from *Zea mays*, suggesting substantial differences in the epitopes of the others.

CONCLUSION

The variety of glutathione reductase isoforms in plants are involved in the developmental control by adjusting the redox potential of the GSH pool. Although *in vitro* embryogenic cultures expressed fewer isoforms, their controlled activity on different stages and the flexibility to use both NADPH and NADH as electron donors are important for the ability to keep optimal redox environment. The somatic embryogenesis inhibiting effect of the higher salt concentration may be partially caused by the decreased glutathione reductase activity and the resulting increase of the $E_{GSSG/2\ GSH}$ toward more oxidizing values. The results obtained further elucidated and are complement to the established significance of GSH/GSSG redox state during the developmental stages of somatic embryogenesis in *Dactylis glomerata*.

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