RESEARCH PAPERS

Glutathione Status in the Roots of Tomato Plants Transgenic by Genes *psl* and *rapA1* in the Presence of *Rhizobium leguminosarum*

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Received January 29, 2021; revised February 26, 2021; accepted February 26, 2021

Abstract—The level of glutathione was investigated in the roots of tomato (*Solanum lycopersicum* L.) plants transgenic by genes *psl* and *rapA1* in the presence of a microsymbiont of leguminous plants *Rhizobium leguminosarum* VSy3. The plants transformed with gene *psl* showed a greater bacterial adhesion than the plants transformed with gene *rapA1*, which positively correlated with growth parameters of plants. Treatment with rhizobia elevated the content of glutathione in the roots of wild type plants three times, 4.7 times in the roots of plants transformed with gene *rapA1*, and more than five times in the plants transgenic by gene *psl*. The obtained results suggest that the level of glutathione in the roots may serve as a marker of efficiency of artificial symbiotic systems produced de novo.

Keywords: *Solanum lycopersicum*, *Rhizobium leguminosarum*, glutathione, *psl*, *rapA1*, artificial symbiotic systems

DOI: 10.1134/S1021443721050228

INTRODUCTION

Rhizobia, known as nodule bacteria of legumes, may act as associative microsymbionts stimulating growth in a number of nonlegume crops. Successful colonization of root hairs depends on the high competitive ability of rhizobial strains necessary to rival numerous microorganisms residing in rhizosphere and on some mechanisms ensuring effective suppression or overcoming of plant immune system. In the course of a classic symbiotic interaction between leguminous plant and rhizobia, nodules are formed as a result of coordinated multistage differentiation of plant cells and bacteria. Analysis of these intricate processes has revealed a positive correlation between the content of glutathione (reduced glutathione, GSH) and ascorbate, activity of the enzymes participating in ascorbate-glutathione cycle, and efficiency of nitrogen fixation in the nodules, which made it possible to assume that these antioxidants play an important role in nitrogen-fixing symbiosis [1]. The data reported in one of the first papers dealing with the role of glutathione in realization of symbiotic interactions suggest that glutathione is not only imperative for bacterial growth and reproduction but it is also a limitative factor in formation of associative symbioses and symbioses producing new cellular compartments (nodules) [2]. For instance, in bacteria *Sinorhizobium meliloti*, mutant strain SmgshA defective in gene *gshA* that

encodes the enzyme of the first stage of GSH biosynthesis was incapable of growing what ruled out formation of nodules on the surface of alfalfa roots; mutant strain SmgshB defective in the enzyme of the second stage of GSH synthesis was able to grow but produced abnormal nodules with a low efficiency of nitrogen fixation as compared with wild type. Both strains showed an elevated catalase activity, which points to the development of oxidative stress therein and corroborates the participation of glutathione in the protection of a nitrogen-fixing complex against toxic forms of oxygen arising as a result of active metabolism in the nodules [2]. It was shown earlier that GSH not only plays an important role in bacterial growth and reproduction but it directly participates in formation of infection threads and regulates expression of bacterial symbiotic genes [3, 4].

On the other hand, glutathione plays an important role in regulation of plant growth and development during ontogenesis since the presence of sulfhydryl group (SH) makes GSH a powerful reducing agent in the cells. At the same time, between glutamine and cysteine there exists a typical γ -peptide bond that can protect GSH from breakdown by peptidases, thus ensuring its considerable stability. Such chemical structure enables GSH to perform numerous physiological functions in a plant. Glutathione participates in regulation of cell divisions and death, development of embryo and meristem, germination of pollen and growth of pollen tubes, regulates the content of ascorbate and hydrogen peroxide. In addition to physiolog-

Abbreviations: GSH—glutathione reduced; GSSH—glutathione oxidized; MDA—malonic dialdehyde.

ical functions, glutathione was shown to play a role in the realization of plant resistance to abiotic and biotic stress factors. For instance, plant treatment with exogenous glutathione acting in this case as a mimetic of elicitors brought about an activation of defense genes, including PR1 genes. Moreover, accumulation of GSH also accompanies plant infection with pathogens. That points to participation of glutathione in regulation of redox status of the cell and its involvement in signaling of different phytohormones in the course of exposure to a biotic stress. At the same time, glutathione is necessary for detoxification of xenobiotics and heavy metals as well as assimilation, translocation, and storage of sulfur [5].

Presence of rhizobia and penetration of bacteria into plant cells within a framework of symbiosis modify polarization of the plasma membrane, cytoskeleton of root hairs, metabolism of auxins, and accumulation of reactive oxygen species [6]. In plant cells involved in formation of nodules, glutathione and its analog characteristic of the family Fabaceae (homoglutathione) are accumulated. That shows a great importance of this antioxidant in the formation of legume-rhizobial symbiosis; moreover, accumulation of GSH in plant cells in early stages of plant interaction with rhizobia may be a marker of the beginning of symbiotic process [7].

Development of symbiosis and formation of nodules on the roots of macrosymbiont plants are specific processes, whereas plant susceptibility to certain microsymbiont bacteria and successful interaction with them depend on the synthesis of signal molecules produced by both bacteria and plants. These signals are actual markers of symbiosis or suggest a possibility of its formation. Bacterial surface polysaccharides and adhesins, plant lectins and flavonoids act as intermediary molecules in early stages of development of symbioses between microorganisms and plants. These substances are undoubtedly promising instruments for modification of existent and production of new systems of associative symbiosis. Therefore, it is currently important to look into the ways of improving the competitive ability of rhizobia (including the methods of plant modification) so that plants would support on the surface of their roots only specific microsymbionts. The most promising for production of novel symbiotic systems are the genes whose products directly participate in the formation of plant-microbial interactions. Earlier, we worked out a system of agrobacterial transformation of tomato (*Solanum lycopersicum* L.), commercial cv. Gruntovyi Gribovskii 1180, with a garden pea lectin gene *psl*, which made it possible to obtain stable root associations of this important agricultural crop with rhizobia protecting it from pathogenic fungi [8]. Moreover, transformation of tomato with the gene of rhizobial agglutinin *rapA1* made it possible to produce efficient symbiotic systems for phytoremediation of soils polluted with cadmium [9]. The obtained transgenic plants of tomato that do not belong to legumes successfully interacted

with rhizobia on the surface of their roots. Such an interaction must undoubtedly affect redox status of tomato roots characterized by the content of glutathione and the content of malonic dialdehyde (MDA): a final product of lipid peroxidation in membrane cellular structures, which is a marker of the level of oxidative stress and survival potential of plant organism, including its symbiotic interactions with microorganisms [10, 11]. There are a number of reports dealing with the formation of plant-microbial symbioses; however, the roles of GSH and MDA in realization of adaptation processes occurring in the course of interaction of transgenic plants with mutualistic microflora in man-made symbiotic systems remain essentially unexplored.

The aim of this work was to look into the redox status of glutathione and estimate the level of MDA in different artificial symbiotic systems on the roots of tomato plants transformed with the gene of garden pea lectin *psl* and the gene of bacterial agglutinin *rapA1* in the presence of bacterium *Rhizobium leguminosarum* VSy3.

MATERIALS AND METHODS

Test subjects and materials. As microsymbionts, we used plants of tomato (*Solanum lycopersicum* L.), cv. Gruntovyi Gribovskii 1180, transformed with genes *psl* and *rapA1* [8, 9]. The experiments were conducted with a line of transgenic plants, which showed in the second generation a steady expression of genes *psl* and *rapA1* and the presence of respective proteins on the root surface confirmed by means of fluoroimmunoassay.

As a microsymbiont, we used *R. leguminosarum*, strain VSy3, isolated from the nodules of wild wood vetch (*Vicia sylvatica* L.) growing in the South Urals and showing a growth stimulating activity [12]. In order to visualize symbiotic interactions, rhizobia were marked with fluorescent protein TurboGFP [13]. Vector constructs were transferred to bacteria by means of electroporation. As a selective antibiotic, we used gentamicin (50 mg/mL).

Bacterial colonization of plant roots. Before the experiments, the seeds were surface-sterilized for 1 min in 70% alcohol followed by 20 min in 1% sodium hypochlorite supplemented with several drops of Tween-20. After fivefold washing in sterile water, the seeds were cultured on MS medium [14] during 3 weeks at a temperature of 25°C and 16-h light period in a KBW 400 controlled-climate chamber (Binder, Germany).

Bacteria designed for plant inoculation were grown at 28°C on a shaker (150 rpm) during 48 h in TY medium (mass % in aqueous solution: bacto-tryptone 0.3%, yeast extract 0.2%, and CaCl₂ 0.1%) bringing up the concentration to 10⁸ CFU/mL. Bacterial suspension was diluted to 10⁵ CFU/mL with sterile liquid TY medium, the roots were inoculated therein for 2 min, and the seedlings were transferred to MS medium for

cocultivation during 48 h. Then three 1-cm-long root fragments were taken from every plant, three times rinsed in sterile water for 5 min on a microshaker, and homogenized in 50 μ L of LB medium (mass % in aqueous solution: bacto-tryptone 1%, yeast extract 0.5%, and NaCl 0.5%).

The obtained volume was diluted 1000 times and 50 μ L of this suspension was transferred to agar TY medium with gentamicin (50 mg/mL) and cultured in a thermostat at 28°C for 48 h. The quantity of adhered bacteria was determined by the number of produced colonies and expressed in colony-forming units CFU/g dry root biomass.

In some plants, the roots were excised, weighed, frozen, and used for determining GSH, GSSG, and MDA. Individual root fragments were used for visual estimation of bacterial colonization of the surface of root hairs using an Axio Imager M1 fluorescence microscope (Carl Zeiss, Germany).

Some plants inoculated with bacteria were left for a week-long coculturing on MS medium, then photographed, and dry biomass of the roots was determined.

Assay of glutathione. Content of reduced (GSH) and oxidized (GSSG) forms of glutathione from the same plant sample was determined using a spectrofluorimetric method based on the formation of a fluorescent product *o*-phthalaldehyde (Sigma, Australia) dependent on pH of the medium. Root sample (0.5 g) was homogenized in 4 mL of the mixture containing 0.1 M potassium phosphate buffer (pH 8.0) and 25% metaphosphoric acid at a ratio of 3.75 : 1 (by volume) according to Hissin and Hilf [15]. Homogenate was centrifuged during 10 min at 8000 *g*, and then supernatant was repeatedly centrifuged for 5 min at 13000 *g*. GSH and GSSG in the obtained supernatant were quantified using reagents described in detail by Maslennikova et al. [16]. In order to determine the content of GSH and GSSG, we monitored kinetics of the fluorescence strength of the formed complexes at a temperature of 25°C using an EnSpire Model 2300 Multilabel Microplate Reader (PerkinElmer, United States) at 420 nm (excitation wavelength of 350 nm). Protein was assayed according to Bradford [17]. The content of GSH and GSSG was expressed in μ mol/mg protein.

Determining endogenous MDA. Content of MDA was determined by means of a color reaction with thiobarbituric acid [16]. For this purpose, a sample of plant material (0.5 g) was homogenized in 5 mL of 10% trichloroacetic acid with subsequent centrifugation of homogenate for 15 min at 13000 *g*. Supernatant was supplemented with equal volume of 0.5% thiobarbituric acid in 20% trichloroacetic acid. The obtained mixture was incubated on a water bath for 30 min at 100°C, then cooled and centrifuged for 15 min at 13000 *g*. Optical density of supernatant was determined at 532 nm (peak of MDA light absorption) and 600 nm (correction for nonspecific light absorption)

Fig. 1. Colonization of plant roots with *R. leguminosarum*, strain VSy3, (CFU/g dry root biomass); (1) control plant; (2) plant transformed with *psl*; (3) plant transformed with *rapA1*.

using a SmartSpec Plus spectrophotometer (BioRad, United States). MDA concentration was calculated using coefficient of molar extinction at 155 $M^{-1} cm^{-1}$ and expressed in mmol/g fr wt of the roots.

Statistical treatment. Experiments were repeated four times and each was independently reproduced at least three times. Figures show the means and their standard errors. Reliability of differences at $P \leq 0.05$ was determined by means of variance analysis.

RESULTS

In 2 days after inoculation of control and transgenic tomato plants with *R. leguminosarum*, strain VSy3 (GFP), it was found that the quantity of bacteria in the rhizosphere in terms of dry biomass of the roots was 193.5 ± 38.7 CFU/g $\times 10^6$ for plants transgenic by gene *psl* and 69.1 ± 13.82 CFU/g $\times 10^6$ for plants transformed with gene *rapA1*. This figure was 21.6 ± 4.32 CFU/g $\times 10^6$ on the roots of control plants (Fig. 1).

On the roots of tomato plants transformed with gene of bacterial agglutinin *rapA1*, microscopic examination showed formation of microcolonies (Fig. 2a). In plants transgenic by gene *psl* treated with bacteria, numerous bent root hairs were observed (Fig. 2b) and structures similar to infection threads filled with bacteria were found (Fig. 2c).

One-week-long coculturing with rhizobia of control conventional plants brought about a rise in dry biomass of the roots by 15%. This parameter in plants transgenic by gene *psl* was 50 and 35% on average in plants transformed with gene *rapA1* (Fig. 3a). This also told on appearance of the seedlings (Fig. 3b).

Determining GSH and GSSG in the roots of control and transgenic plants in the presence of rhizobia has shown a threefold elevation of GSH content in wild type plants; it rose 4.7 times in the roots of plants transformed with gene *rapA1* and more than five times in plants transgenic by gene *psl* (Fig. 4a). It is interesting that bacteria did not affect the content of GSSG in

Fig. 2. Microscopic images of the roots colonized with *R. leguminosarum*, strain VSy3: (a) visual comparison of the quantity of bacteria anchored on the roots of control and transgenic plants (scale bar of 1 mm); (b) bent root hairs on the plants transformed with *psl* (scale bar of 0.01 mm); (c) infection threads within root hairs on the plants transformed with *psl* (scale bar of 0.01 mm).

both types of transgenic plants examined in this work and its level remained the same as in control plants untreated with rhizobia (Fig. 4b). Figure 5 shows that the level of MDA was the same in all the types of treatment.

DISCUSSION

Treatment of agricultural crops with microorganisms promoting plant growth is currently looked upon as a safe alternative to chemical fertilizers. It was shown earlier that the strains of *Rhizobium* can colonize the roots of tomato and pepper plants facilitating their growth in different stages of plant development, elevating the yield and improving the quality of seedlings and fruit [18].

Plants' ability to retain useful bacteria (specifically, rhizobia) on the surface of their roots is very important for estimating prospects of formation and development of the symbiotic complex. Comparative analysis of the number of bacteria on the root surface of control and transformed plants has shown that the plants transformed with gene *rapA1* had lower adhesion of bacteria than those transformed with the gene of plant

agglutinin (lectin) *psl* whose product can recognize and selectively bind polysaccharides on the cell walls of only certain strains of rhizobia. At the same time, transformation of plants with the gene of bacterial agglutinin *rapA1* whose product directly participates in rhizobial biofilm formation promoted efficient production of microcolonies on plant roots, which may improve competitive ability of nodule bacteria in the rhizosphere (Fig. 2a). These facts fully agree with the earlier obtained data and corroborate the interaction of rhizobia with lectin PSL and agglutinin RapA1 on the surface of transgenic roots [8, 9]. Moreover, the plants transgenic by *psl* and treated with bacteria showed numerous bent root hairs characteristic of initial stages of legume-rhizobial symbiosis (Fig. 2b). We also observed structures similar to infection threads filled with bacteria, which were lacking on the roots of plants transformed with gene *rapA1* (Fig. 2c).

Such nonspecific symbiotic interactions were earlier discovered in the roots of sea buckthorn transformed with the gene *psl*. We investigated the effect of expression of lectin gene on the symbiotic interaction of sea buckthorn with *R. leguminosarum* (a symbiont of garden pea) and actinomycete from the genus *Frankia*

Fig. 3. Effect of *R. leguminosarum*, strain VSy3, on growth of control and transgenic plants 1 week after inoculation: (a) dry biomass of roots (100% corresponds to control plants untreated with bacteria); (b) control and transgenic plants (scale bar of 1 cm); (1) control plant; (2) control plant treated with *R. leguminosarum* VSy3; (3) plant transformed with *psl*; (4) plant transformed with *psl* and treated with *R. leguminosarum* VSy3; (5) plant transformed with *rapA1*; (6) plant transformed with *rapA1* and treated with *R. leguminosarum* VSy3.

(a symbiont of sea buckthorn). In the seedlings whose roots were jointly treated with these microsymbionts, we detected not only common actinorhizal nodules but also nodule-like structures uncharacteristic of sea buckthorn. RAPD analysis of bacteria isolated from these structures showed the presence of rhizobia *R. leguminosarum* and the absence of actinomycete from the genus *Frankia* [19]. Bent root hairs as an early symbiotic response were also detected in composite plants of tomato, rape, and tobacco whose roots were transformed with the gene *psl* and subsequently treated with *R. leguminosarum*. We did not observe such nonspecific reactions after plant treatment with eastern galega rhizobia *R. galegae* strain that does not recognize lectin PSL on the root surface [20]. However, it is possible that binding of only their own microsymbionts is not characteristic of all the lectins of legumes [21].

Determining root dry biomass that reflects plant growth and physiological state showed that the rhizobial strain used in this work stimulates growth in control untransformed tomato plants. The presence of bacteria on the root surface of transgenic plants brought about a considerable accumulation of root dry

Fig. 4. Content of (a) GSH and (b) GSSG in the roots of plants in the presence of bacteria. The same letters designate values with differences unreliable at $P \leq 0.05$; (1) control plant; (2) control plant treated with *R. leguminosarum* VSy3; (3) plant transformed with *psl*; (4) plant transformed with *psl* and treated with *R. leguminosarum* VSy3; (5) plant transformed with *rapA1*; (6) plant transformed with *rapA1* and treated with *R. leguminosarum* VSy3.

biomass in plants transgenic by gene *psl* and much lower accumulation in plants transformed with gene *rapA1* (Fig. 3a); this is evident from the appearance of intact plants (Fig. 3b).

It was shown earlier that *Pseudomonas* sp. 102 stimulates growth of tomato plants, including those exposed to a toxic effect of Cd^{2+} . This was most pronounced in plants transgenic by gene *rapA1* and associated with a more efficient bacterial colonization of the root surface. In spite of the fact that RapA1 was detected only in several rhizobial species, this protein is not strictly specific and can promote agglutination of other bacteria different from rhizobia, in particular, of some strains of *Pseudomonas* [22].

The obtained results have shown that plants transgenic by gene *psl* are more receptive to the presence of rhizobia when bacteria penetrate into the cells. A detected higher level of adhesion (nine times greater than in wild type under the same conditions) and dry weight of the roots of these tomato plants suggest that these plants positively respond to the presence of bacteria triggering a cascade of different reactions affecting plant metabolism, including synthesis of glutathione that characterizes the physiological state of a plant cell. Plants transformed with gene *rapA1* were less receptive and examined characteristics therein were lower than in plants transformed with gene *psl*. The

Fig. 5. Content of MDA in tomato roots. The same letter designates values with differences unreliable at $P \leq 0.05$; (1) control plant; (2) control plant treated with *R. leguminosarum* VSy3; (3) plant transformed with *psl*; (4) plant transformed with *psl* and treated with *R. leguminosarum* VSy3; (5) plant transformed with *rapA1*; (6) plant transformed with *rapA1* and treated with *R. leguminosarum* VSy3.

obtained results suggest that plant-microbial associations produced de novo in this work are hyperorganismic systems with unique properties, which require further investigation.

There are several reports indicating that glutathione participates in the induction of defense genes in plants, and a rise in GSH level and/or GSH-associated enzymes correlates with resistance to various biotic stresses, including viral, bacterial, and fungal attacks. In addition, some papers showed that a decrease in GSH level may induce development of symptoms caused by a pathogen in sensitive plants [23]. Response of antioxidant systems to pseudomonade infection was earlier investigated in two cultivars of tomato. In inoculated cultivar susceptible to disease, the content of GSH considerably decreased and the accumulation of GSSG rose. On the contrary, in the case of the resistant cultivar of tomato, the content of GSH essentially did not decrease [1]. These results indicate that the maintenance of glutathione homeostasis apparently contributes to inaccessibility of plant tissues to bacterial attack and the content of glutathione is an important indicator of biotic stress in plants during pathogen attack and upon interaction with bacteria stimulating growth.

In this work, we observed a distinct pattern: the more GSH was produced by plants within a framework of the examined symbiotic interactions (Fig. 4a), the more bacteria adhered to the root surface and penetrated into the tissues (this was mediated by the presence of genes *psl* and *RapA1*) (Figs. 1, 2).

Glutathione is known to be necessary for cell divisions in the root apical meristem and its quantity regulates the transition of the cells from G_1 phase to S phase of the cell cycle. Arrival of GSH in the nucleus in the G_1 phase greatly affects the redox state of the cytoplasm and the expression of redox-sensitive genes. Subsequent filling of the total cell pool of GSH above the level observed in the G_1 phase is necessary for cell

progression to the S phase of the cell cycle [24]. Therefore, one may think that accumulated GSH in the examined plants contributes to growth stimulation induced by bacteria, which shows in the figures of their roots' biomass (Fig. 3a). There exists a distinct direct relation between GSH content (Fig. 4a) and root biomass of tomato plants (Fig. 3a); this also positively correlates with the values of bacterial adhesion (Fig. 1).

Although members of the family Solanaceae do not form nitrogen-fixing symbiosis with rhizobia, a rise in the content of GSH observed in our work upon inoculation with rhizobia of tomato plants transgenic by genes *psl* and *rapA1* is probably accounted for by the presence in tomato of receptors essentially identical to legume kinases containing LysM motifs in extracellular domains. These receptors bind Nod factors of rhizobia produced in response to the synthesis of flavonoids by plants and activate symbiotic responses via signal pathways analogous to formation of arbuscular mycorrhizal symbiosis produced by tomato with *glomus* fungi [25]. Moreover, substrate specificity of tomato chitinases in respect to Nod factors of rhizobia (usually characteristic of the legumes) was shown earlier [26]. This fact suggests that specificity of symbiosis may partially depend on activity of plant chitinases [27].

It was also shown that GSH in rhizobia participates in adaptation to different stresses both in the initial stages of symbiotic interactions and during differentiation to bacteroids. This is related to the fact that nodule bacteria meet with reactive oxygen species and active forms of nitrogen produced by a host plant in all the stages of the symbiotic process [28]. GSH is very important for the competitive ability and the symbiotic efficiency of rhizobia. Strain 3841 of *R. leguminosarum* bv. *viciae* mutant in glutathione synthetase (*gshB*) not only weakly colonized the plant rhizosphere but also showed a reduction in plant dry biomass by 50% owing to a decrease in the efficiency of nitrogen fixation [29]. Therefore, changes in GSH metabolism undoubtedly directly affect symbiotic interactions between bacteria and plants.

It is worth noting that bacteria did not influence the content of GSSG in either types of transgenic plants examined in this paper; this suggests that the presence of bacteria on the surface of these tomato plants is not perceived by them as a damaging factor (Fig. 4b). MDA content in the roots is additional evidence that rhizobia do not have a negative effect on the integrity of membrane structures (Fig. 5). This fact once again confirms the beneficial effect of bacteria on physiological state of the examined tomato plants.

To conclude, the obtained results show prospects of investigation of the redox state of glutathione and the level of MDA in plants for estimating the efficiency of artificial symbiotic systems. Special attention should be paid to a more pronounced response of plants transformed with gene *psl* as compared with plants expressing gene *rapA1* to the presence in the rhizosphere of rhi-

zobia recognizing the products of these genes. The role of glutathione in the interactions between plants and microbes was predominantly investigated earlier within the framework of legume-rhizobial and mycorrhizal symbioses without paying attention to man-made symbiotic systems. The obtained data extend our knowledge about the role of glutathione in the realization of a possible symbiotic interaction between transgenic plants and microsymbionts. We believe that the proposed tool may turn out to be efficient for analysis of artificial symbiotic systems upon toxic effect of heavy metals or exposure to plant pathogens.

ACKNOWLEDGMENTS

This work was performed using equipment belonging to Agidel' Shared Instrumentation Center, USI Kodink Ufa Federal Research Center, Russian Academy of Sciences, within the framework of State Assignment no. AAAA-A21-121011990120-7, and supported by the Russian Foundation for Basic Research, project no. 18-34-20004.

COMPLIANCE WITH ETHICAL STANDARDS

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

Statement on the welfare of humans or animals. This article does not contain any studies involving humans or animals performed by any of the authors.

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Translated by N. Balakshina