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Participation of Glutathione in The Formation of the Associative Symbiosis of Trancsgenic Tomato Plants with *R. leguminosarum*

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Abstract: Rhizobia can serve as beneficial associative bacteria for many crops, including tomato. There are a number of studies to improve this associative interaction by transforming plants with various genes, in particular, the genes of plant lectins and bacterial agglutinins. It is known that glutathione (GSH) plays an important role in the formation of associative symbiosis between plants and bacteria. The aim of this work was to study the redox state of glutathione in various artificial symbiotic systems on the roots of tomato plants, transformed with the lectin gene *psl* and the bacterial agglutinin gene *rapA1* after treatment of rhizobia. Evaluation of the glutathione content in the roots of transgenic plants with *psl* gene showed a significant increase in the GSH content and the GSH/GSSG ratio was 4.6, while the adsorption of bacteria on the roots of these plants increased 9 times. In the transgenic roots with the *rapA1* gene bacteria were sorbed three times more in comparison with the control plants, and the GSH/GSSG ratio was 4.1. The results obtained demonstrate the promise of studying the redox state of glutathione for assessing the effectiveness of artificial symbiotic systems.

Keywords: rhizobia; glutathione; endosymbiont; lectin; agglutinin; tomato; *rapA1*; *psl*; transgenic plant

1. Introduction

Rhizobia, known as legume endosymbionts, can serve as associative bacteria for many non-leguminous crops, including tomatoes. There are a number of studies to improve these associations by transforming plants with various genes, the products of which are involved in the interaction with microsymbionts. For example, tomatoes were obtained that produce N-acyl-homoserine lactone, which is a regulator of the expression of quorum sensing genes [1]. We also obtained tomato plants transformed with the lectin gene *psl*, which improved the colonization of these plants with growth-stimulating bacteria *R. leguminosarum* [2,3]. Bacterial agglutinins, in particular the RapA1 protein, isolated from some rhizobia strains, can also serve as a component that improves associative interactions [4]. This protein is not strictly specific and also can contribute to the agglutination of non rhizobia strains on plant roots [5,6]. The studies of the last decade showed that glutathione (GSH) plays an important role in the formation of associative symbiosis between plants and bacteria [7]. It is known that GSH is the most important antioxidant, which is involved in maintaining the redox homeostsis in plants [8]. GSH is also involved in regulation of expression of symbiotic genes and processes of cell division during symbiotic interactions [9]. However, changes in the redox status of glutathione during the formation of associative interactions are not well defined.

The aim of this work was to study the redox status of glutathione in various artificial symbiotic systems on the roots of tomato plants transformed with the lectin gene *psl* and the bacterial agglutinin gene *rapA1* after the inoculation of the *R. leguminosarum* Vsy12.

2. Experiments

2.1. Transgenic Plants and Bacteria

Tomato plants of the cultivar Gruntovy Gribovsky 1180 transformed with the *psl* and *rapA1* genes were used as macrosymbionts [3,6]. For the experiments, the seeds were superficially sterilized for 1 min in 70% ethanol and then 20 min in 1% sodium hypochlorite solution with the addition of a few drops of Tween-20. After rinsing five times with sterile water, the seeds were cultured in MS medium [10] for 3 weeks at 25 °C and 16 h of light in a KBW 400 climatic chamber (Binder). As a microsymbiont, we used the *Rhizobium leguminosarum* VSy12 strain, isolated from *Pisum sativum* L. nodules and possessing growth-stimulating activity. To visualize symbiotic interactions, rhizobia were labeled with the fluorescent protein TurboGFP [11]. To inoculate plants, bacteria were grown at 28 °C on a shaker (150 rpm) for two days in TY medium (bacto-tryptone 0.3%, yeast extract 0.2%, CaCl2 0.1%) to a concentration of 10^8 CFU/mL. The bacterial suspension was diluted to 10^5 CFU/mL, and the roots were inoculated in it for 2 min, and the seedlings were transferred to a solid MS medium for co-cultivation for 2 days. After that, 3 root fragments, 1 cm long, were taken from each plant, washed three times with sterile water for 5 min on a microshaker and homogenized in 50 μL of LB medium. The resulting volume was diluted 1000-fold and 50 μL of this suspension was plated onto TY agar medium with gentamicin (50 mg/mL) and grown in a thermostat at 28 °C for two days. The number of adhered bacteria was determined by the number of grown colonies. Visual observation of labeled bacteria on roots was performed using an Axio Imager M1 fluorescence microscope (Carl Zeiss, Germany).

Glutathione Determination

The content of reduced (GSH) and oxidized (GSSG) forms of glutathione from one plant sample was determined using the spectrofluoromeric method based on obtaining a fluorescent product of O-phthalaldehyde (OPT) (Sigma, Australia) depending on the pH of the medium were recorded at pH 8.0 and 12.0, respectively [12]. 0.5 g fresh roots were ground with 4ml of a mixture consisting of 0.1 M potassium phosphate buffer (pH 8.0) and a 25% metaphosphoric acid solution in a ratio of 3.75: 1 (by volume) as recommended by Hissin and Hilf [1976], then centrifuged at 12 000× *g* for 20 min at 4 °C. For the measurement of GSH, 0.5 mL of the supernatant was mixed with 4.5 mL of 0.1 M phosphate buffer (pH 8.0, including 5 mM EDTA), then take about 0.1 mL from this mixture and 1.8 mL of 0.1 M phosphate buffer (pH 8.0) and 0.1 mL of OPT stock (1 mg mL^{-1} in ethanol) added. For GSSG, 0.5 mL of supernatant was initially mixed with 200 μL of 0.04 M N-ethylmaleimide for 20–30 min and subsequently added to 4.3 mL of 0.1 M NaOH then take about 0.1 mL from this mixture and 1.8 mL of 0.1 M NaOH and 0.1 mL of OPT stock (1 mg mL^{-1} in ethanol) added, fluorescencewas read at an excitation and emission wavelength of 350 and 420 nm using a Perkin Elmer LS 55 Luminescence Spectrometr Cell (USA).

3. Results and Discussion

3.1. Subsection

3.1.1. Amount of Bacteria Adhered to the Surface of the Roots

To assess the amount of bacteria adhered to the surface of the roots, plants were inoculated with the R. leguminosarum VSy12 (GFP) strain for 2 days, after which they were microscoped. It was shown that after treatment on the roots of transgenic plants, an increase in the number of adsorbed bacterial cells and an increase in the formation of biofilms are observed in comparison with the roots of control nontransformed plants. Plants transformed with the rapA1 gene showed lower results in bacterial adhesion than in the case of psl gene, the product of which is able to recognize and selectively bind polysaccharides on the cell walls of strictly defined rhizobia strains. Thus, three times more R. leguminosarum bacteria were sorbed on the roots transgenic for the rapA1 gene as compared to control plants, and in the case of psl gene, 9 times more (Figure 1).

However, the transformation of plants with the rapA1, the product of which is directly involved in the biofilm formation of rhizobia, promoted the effective formation of biofilms on plant roots, which may in the future increase the competitiveness of nodule bacteria in the rhizosphere. This fact fully agrees with the data obtained earlier [3,6] and confirms the interaction of rhizobia with lectin PSL and RapA1 agglutinin on the surface of transgenic roots.

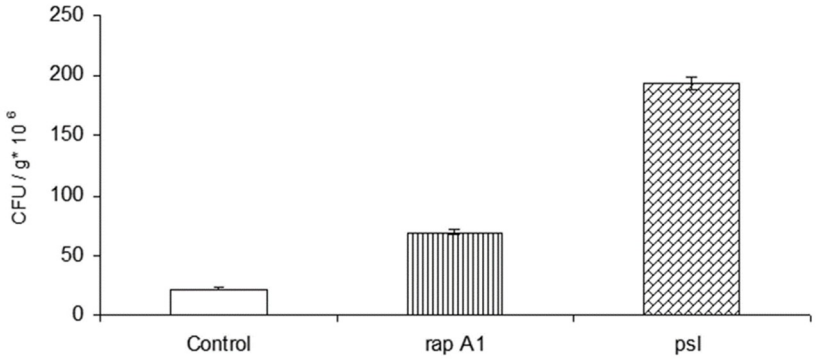

Figure 1. The number of adherent bacteria on the root surface. Y-axis—number of bacterial colonies per root weight. *Control*—non-transgenic plants; *rapA1*—transgenic plants with *rapA1* gene; *psl*—transgenic plants with *psl* gene. Different letters indicate a significant difference between the means at the probability level of $p < 0.05$.

3.1.2. The Redox State of Glutathione in the Roots of Tomato

It was found that in the roots of wild-type tomato, treatment with the bacteria caused the accumulation of GSH, without affecting its oxidized form-GSSG and the indicator of the GSH/GSSG ratio was 3.2. Evaluation of the glutathione content in the roots of transgenic plants with *psl* gene showed a significant increase in the GSH content and the GSH/GSSG ratio was 4.6, while the adsorption of bacteria on the roots of these plants increased 9 times (Figure 2).

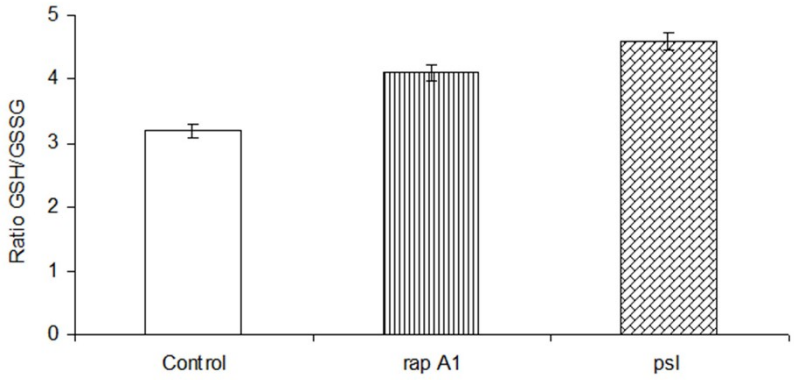

Figure 2. Effect of *R. leguminosarum* VSy12 on ratio GSH/GSSG in roots of tomato plants. *Control*—non-transgenic plants; *rapA1*—transgenic plants with *rapA1* gene; *psl*—transgenic plants with *psl* gene. Different letters indicate a significant difference between the means at the probability level of $p < 0.05$.

In the transgenic roots with the *rapA1* gene bacteria were sorbed three times more in comparison with the control plants, and the GSH/GSSG ratio was 4.1. It should be noted that in both variants of the studied transgenic plants, bacteria did not affect the GSSG content and its value remained at the level of untreated plants, which indicates the absence of a negative effect of rhizobia on tomatoes.

4. Conclusions

Previously, the role of glutathione in interactions between plants and microbes was best studied in legume-rhizobial and mycorrhizal symbioses. Undoubtedly, this substance plays an important role both in the initial stages of symbiosis and in the formation of new structures, in particular nodules. The results obtained demonstrate the promise of studying the redox state of glutathione for assessing the effectiveness of artificial symbiotic systems.

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Conflicts of Interest: The authors declare no conflict of interest.

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