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RESEARCH PAPERS

Effect of *rol* Genes of the A4, 15834, and K599 Strains of *Agrobacterium rhizogenes* on Root Growth and States of the Antioxidant Systems of Transgenic Tobacco Plants Subjected to Abiotic Stress

D. Yu. Shvets[a, b, *], Z. A. Berezhneva[a], Kh. G. Musin[a], and B. R. Kuluev[a, c]

[a] *Institute of Biochemistry and Genetics, Ufa Federal Research Center, Russian Academy of Sciences, Ufa, 450054 Russia*

[b] *Bashkir State Medical University, Ufa, 450008 Russia*

[c] *Ufa University of Science and Technology, Ufa, 450076 Russia*

**e-mail: shvetsdasha99@yandex.ru*

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Abstract—An attempt is made to create transgenic plants using *rol* genes from A4, 15834, and K599 strains of *A. rhizogenes*. Strains A4 and 15834 transformed tobacco plants are found to display better root growth than their wild types under normal conditions and at elevated concentrations of sodium chloride or cadmium acetate. At the same time, the *rol* genes acquired from the K599 strain negatively affect root growth under both normal and stressful conditions (e.g., salinization, hypothermia, or excess cadmium acetate). The levels of total protein, proline, total glutathione, and superoxide dismutase activity are higher in the roots of the transgenic plants with *rol* genes from the A4 and 15834 strains than in nontransgenic control plants under both optimum conditions and salinization. When using *rol* transgenes from the K599 strain, the activities of superoxide dismutase, guaiacol peroxidase, and glutathione-S-transferase are enhanced in the roots subjected to salinization. The positive effect *rol* genes have on the root growth of transgenic plants could therefore be explained by their influence on components of the antioxidant system. Results suggest that using *rol* genes from the A4 and 15834 strains of *A. rhizogenes* are promising for breeding plant cultivars and liness with improved parameters of root growth and stress tolerance. At the same time, *rol* genes from the K599 strain appear to be inadequate for this task.

Keywords: *Agrobacterium rhizogenes*, *Nicotiana tabacum*, agrobacterial transformation, antioxidant system, stress tolerance, *rol* genes

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INTRODUCTION

Genetic transformation caused by the *rol* genes of *Agrobacterium rhizogenes* results in numerous phenotypical changes that include the enhanced stress tolerance of transgenic plants. Mechanisms of the stress tolerance mediated by these transgenes remain obscure. In addition, phenotypical effects of the corresponding transgenes depend on the agrobacterial strains of their origin.

The aim of this work was to create transgenic tobacco (*Nicotiana tabacum* L.) plants bearing *rol* genes from the A4, 15834, and K599 strains of *A. rhizogenes* and study their stress tolerance and the states of their antioxidant systems. During genetic transformation, bacteria *Agrobacterium rhizogenes* (*Rhizobium rhizogenes*) [1, 2] transfer certain genes in T-DNA to the plant genome. These are so-called root locus genes *rolA, rolB, rolC*, and *rolD*, which perform a variety of biological functions. The genes alter hormonal system of the plant, disturbing normal vital functions and eventually resulting in the formation of hairy roots [3–5]. It is known that expression of any *rol* genes, either separately or collectively, can have numerous effects on morphogenesis, antioxidant systems, stress tolerance, and secondary metabolisms. We considered these aspects in [6].

Data is being compiled on the positive effects some *rol* genes have on stress tolerance through components of the antioxidant system and hormonal status of a plant. For example, *rolB* genes can activate the protective systems of a plant in response to reactive oxygen species by preventing the death of necrotic cells, and reduce symptoms of apoptosis [7]. *Rubia cordifolia* L. plants bearing *rolC* genes inactivate ROS more efficiently than their wild types [8]. Myb-response elements and C-related elements, which control the synthesis of secondary metabolites are activated in *rolC*-transformed plants, reducing the level of ROS [3, 9]. These *rolC*-transformants also display 2–3 times higher tolerance to stress from salt and low and high

temperatures [8]. In *Centella asiatica* L. plants transformed with the A4 strain of *A. rhizogenes*, each *rol* gene elevated the content of abscisic acid [10].

These results fail to explain how the growth and stress tolerance of a plant can be changed by inserting all *rol* genes from different agrobacterial strains into its genome. It seems that no comparative studies have been performed to distinguish between the effects caused by specific *rol* transgenes taken from different strains of agrobacteria on the stress tolerance and antioxidant systems of transgenic plants. The aim of this study was to create three variants of transgenic tobacco containing *rol* genes from different strains of *A. rhizogenes* (A4, 15834, and K599) to compare the stress tolerance and states of the antioxidant systems of these plants.

Strains of *A. rhizogenes* are classified according to the main opines (agropine, mannopine, cucumopine, and mikimopine) used for their nutrition [11–13]. The best known strains of agropines are 15834, A4, and LBA9402. These are the ones most often used to transform the genes of plants to produce hairy roots. Cucumopine strains include the less studied K599 (NCPPB2659) strain, which has been used to transform plants from the Fabaceae family [14, 15]. It has been suggested that *rol* genes taken from strains of agropine and cucumopine would influence the growth, stress tolerance, and components of antioxidant systems differently in transformed plants.

EXPERIMENTAL

Creating Transgenic Tobacco Plants with rol Genes from the A4, 15834, and K599 Strains of A. rhizogenes.

In this study, we used the cucumopine K599 (NCPPB2659) strain of *A. rhizogenes* with the pRi2659 plasmid, along with the agropine A4 and 15834 strains containing the pRi15834 and pRiA4 plasmids. Transgenic tobacco plants (*Nicotiana tabacum* L., cv. Petit Havana, line SR1) were obtained via the agrobacterium-mediated transformation of leaf discs with the corresponding agrobacterial strains. Two-month-old tobacco plants grown *ex vitro* were subjected to transformation. An agrobacterial culture was maintained on a Lysogeny Broth (LB) liquid medium adjusted with 100 mg/L of rifampicin (Farmasintez, Russia). Bacterial cells were precipitated via 5 min of centrifugation at 4000 RPM. Each pellet was dissolved in 5 mL of a minA medium (1.37% K_2HPO_4, 0.45% KH_2PO_4, 0.1% $(NH)_4SO_4$, 0.05% sodium citrate, 0.025% $MgSO_4 \cdot 7H_2O$, and 0.2% sucrose, pH 5.4) with 100 μM acetosyringone (Sigma, United States). The bacterial suspension was agitated in an Innova 40 orbital shaker (Germany) for 2 h at 180 RPM and 25°C. The leaf explants were sterilized with 70% ethanol for nearly 1 min and 10% sodium hypochlorite for nearly 10 min, followed by washing five times with sterile distilled water. The leaf explants were inoculated with the agrobacterial suspension for 30 min. They were then dried on sterile filter paper and transferred to a solid MS medium for co-cultivation at 25°C. After two days, the leaf material was transferred to a solid MS medium with 100 mg/L cefotaxime (Biokhimik, Russia) to eliminate agrobacteria.

Emerging hairy roots as long as 1.5–2 cm were placed into separate Petri plates with an MS medium containing 100 mg/L cefotaxime to incubate them at 24 ± 1°C in darkness. To regenerate shoots, the hairy roots were transferred to a Binder growth chamber (Germany) illuminated at 140 μmol/(m^2 s). The roots were then grown on an MS medium at pH 5.7–5.8 [16], with 1 mg/L 6-benzylaminopurine (BAP) and 0.5 mg/L naphthaleneacetic acid. DNA was isolated from the shoots using the standard CTAB procedure [17]. The presence of the transgenes in the induced shoots was verified via PCR using primers RolAB1F 5'-AATTGCTACGAGGGGACGCTTTGT-3', RolAB1R 5'-ACGCTCCGCCGGTGGTCATACTTA-3', RolAB2F 5'-TCGGCGGGCTAAGGTCAAGAA-3', and RolAB2R 5'-CTCGCGAGAAGATGCAGAAAGTA-3'. The primers were selected earlier from different *A. rhizogenes* strains for the simultaneous detection of *rolA* and *rolB* genes. In our PCR analysis for the *rolC* gene, we used primers rolC1F 5'-GGCGCACTCCTCACCAACCTTC-3' and rolC1R 5'-CTCGCCATGCCTCACCAACTCA-3' that were preliminarily tested toward different strains of *A. rhizogenes* [18]. It should be noted that these primers do not yield PCR-specific products on wild tobacco, even though this species of plant is naturally transgenic and contains homologs of *rol* genes [4]. The roots of the regenerants were purified of agar to acclimate them to the conditions of the soil. They were then planted in the soil's substrate and grown under a film (to conserve humidity) at 26 ± 1°C with 16-h daily illumination at 35 μmol/(m^2 s). The film was removed after 1 week of acclimation, and the plants were grown up to the stage of ripe seeds. PCR-positive transgenic tobacco plants A4 (numbered 16, 17, and 19), 15834 (5 and 7), and K599 (14 and 24) were randomly selected for subsequent reproduction. The seeds harvested from these seven transgenic plants became ancestors of the corresponding lines used in this study. PCR was also used to select transgenic forms of tobacco that would produce *rolA*, *rolB*, and *rolC* genes in a number of generations.

Morphometrical Analysis of Roots of Tobacco Transgenic Plants Subjected to Salinization, Hypothermia, and Cadmium Acetate

Seeds of transgenic *N. tabacum* plants of the T_2 generation of the A4 (16, 17, and 19), 15834 (5 and 7), and K599 (14 and 24) lines were planted in a firm MS medium. The plants were cultivated under normal conditions in a Binder growth chamber at 25°C with 16 h of daily illumination at approximately 140 μmol/(m^2 s). Seedlings aged 10 days with roots of uniform length

were transplanted into vertically-positioned Petri plates with subsequent alternative cultivation under normal conditions, the effects of salinization (at 50 or 100 mM NaCl) or cadmium acetate (at 100 or 200 μM), and hypothermia (12°C). Solutions of NaCl or CdAc were added to the media before autoclaving. Optimum NaCl concentrations were determined earlier using WT tobacco exposed to 25, 50, 100, or 200 mM of salt. A concentration of 25 mM only briefly changed the length of roots relative to normal conditions. At 200 mM, the growth was the same as at 100 mM. Two concentrations were chosen in subsequent experiments: one that improved root growth (50 mM) and one that retarded it (100 mM). Similar estimates of the concentration dependences were made using WT plants with 50, 100, 200, and 400 μM CdAc. At 50 μM, growth was the same as under normal conditions; at 200 and 400 μM, it was similar but smaller than without the additions. We therefore chose 100 and 200 μM CdAc as our minimum growth-retarding concentrations. After 10 days of cultivation under alternative conditions, we measured the growth of root length. The initial WT plants of tobacco cv. Petit Havana, line SR1 were used as non-transgenic controls. Each line was represented by 60 plants.

Analyzing the Antioxidant Systems and Total Soluble Protein in Transgenic Tobacco Plants Subjected to Salinization

Seedlings of *N. tabacum* from the T_2 generation of the A4 (16, 17, and 19), 15834 (5 and 7), and K599 (14 and 24) lines were grown over ten days on a firm MS medium. The seedlings, which had roots of uniform length, were transplanted to vertically-positioned Petri plates containing an MS medium with 50 mM NaCl.

The activity of superoxide dismutase (SOD) was measured in the roots using the competition between SOD and nitroblue tetrazolium for superoxide anions [19]. The activity of ascorbate peroxidase (APX) was determined from the rate of hydrogen peroxide decomposition using this enzyme, which yielded water and dehydroascorbate [20]. The activity of guaiacol peroxidase (GPX) was estimated from the polymerization of guaiacol to tetraguaiacol [21]. The activity of catalase (CAT) was determined from the rate hydrogen peroxide decomposition [22]. The content of malondialdehyde was measured with thiobarbituric acid [23]. Proline was assayed according to Khedr et al. [24]. The content of water-soluble sugars was estimated by to Dubois et al. in [25]. The Total Antioxidant Capacity (TAC) was measured in methanol (80%) extracts through the reduction of Mo (VI) to Mo (V) at acid pH values [26]. The activity of glutathione-S-transferase (GST) was determined from the rate of conjugate formation using glutathione and 2,4-dinitrochlorobenzene [27]. The concentrations of oxidized and reduced glutathione were measured by Hissin and Hilf in [28].

The activity of each enzyme was normalized to the one milligram of total soluble protein determined by Bradford. All biochemical experiments were repeated ten times ($n = 10$).

Statistics

Results from all measurements are presented as histograms showing their means and SEs. The significance of differences was estimated using the Mann–Whitney U test ($P < 0.05$).

RESULTS AND DISCUSSION

Creating Transgenic Tobacco Plants with the rol Genes of Strains A4, 15834, and K599

Due to the agrobacterium-mediated transformation of tobacco of the K599 strain, sites of regeneration emerged on the surfaces of leaf explants after seven days post-inoculation (Fig. 1a). The sites produced shoots instead of roots after seven more days (Fig. 1b). We then isolated the shoots from the mother explants and implanted them in an MS medium without hormones (Figs. 1d, 1e). It should be noted that typical hairy roots 1.5–2 cm long also arose on the surfaces of explants on post-inoculation day 25 (Fig. 1c). The roots were transplanted to a fresh MS medium with 1 mg/L BAP and 0.5 mg/L NAA to induce shoot regeneration. The resulting shoots (eight of which formed spontaneously and ten were induced from hairy roots) were used to isolate genomic DNA for subsequent PCR tests to identify *rolA, rolB,* and *rolC* genes. A total of 16 transgenic plants were selected, 14 of which successfully acclimated to soil conditions (Fig. 1f).

With the A4 and 15834 strains, hairy roots were induced on the surfaces of explants kept on a hormone-free nutrient medium for 7–10 days after their transformation (Fig. 2a). In contrast to the K599 strain, no shoots arose without exogenous hormones. Isolated hairy roots started to form shoots after being transplanted to a medium containing 1 mg/L BAP and 0.5 mg/L NAA (Fig. 2b). The shoots were transplanted to a fresh medium with the same content every two weeks (Figs. 2c, 2d). PCR analysis revealed the presence of the *rolA, rolB,* and *rolC* genes in all of the examined regenerants. As a result, we obtained 15 transgenic tobacco plants with strain A4 and 12 plants with strain 15834.

To measure the root growth and activities of the components of antioxidant system, seven plants (Figs. 3a–3d) were randomly selected from *rol*-transformants A4 (16, 17, and 19), 15834 (5 and 7), and K599 (14 and 24). Transgenic plants of generation T_1 were chosen, and only those containing agrobacterial *rol* genes (verified by PCR) were left. Their seeds were also used to produce the plants of generation T_2 that were also analyzed for *rol* genes by PCR.

Fig. 1. Visual results from the transformation of tobacco leaf explants by *rol* genes from the K599 strain of *A. rhizogenes*: (a) emergence of sites of regeneration on an explant's surface on post-inoculation day 7; (b) regeneration of shoots on post-inoculation day 14; (c) formation of hair roots (shown by arrow) on post-inoculation day 25; (d, e) regeneration of roots from shoots on a hormone-free medium; (f) regenerant acclimation to soil conditions.

Morphometry of Roots of Tobacco Transgenic Plants Grown under Normal and Stressful Conditions

Under normal conditions (25°C), the transgenic tobacco plants bearing *rol* genes from the A4 and 15834 strains displayed a considerable increase in root growth, relative to the WT. In contrast, root growth fell in the lines of the K599 strain (Fig. 4a). The average degrees of stimulated growth were 35 and 57% for all lines of the A4 and 15834 strains, respectively, while the degree of growth retardation was 86% for the K599 strain.

With 50 mM of NaCl, root growth rose considerably in the A4 (16 and 19) and 15834 (5) lines, but fell in those of K599 (14 and 24), relative to the WT (Fig. 4b). Similar relationships were observed with 100 mM NaCl: an increase in root growth in the A4 (19) and 15834 (5) lines, and a drop in the K599 (14 and 24) lines. The average degree of root growth stimulation was 52 and 38% for lines 19 and 5, respectively, and that of the growth retardation was 88% for lines 14 and 24 (Fig. 4c).

Hypothermia lowered the parameters of root growth more in the transgenic plants than in the WT. Under these conditions, growth was retarded by 86%, relative to the WT in the lines transformed with the K599 strain. This index was 23 and 26% for the roots of the A4 and 15834 lines, respectively (Fig. 4d).

The stress caused by 100 μM of cadmium acetate had a positive influence on root elongation in the A4 (19) and 15834 (5 and 7) lines. Elongation fell in the A4 (16) line and in both of the tested K599 lines, relative to the WT (Fig. 4e). Cadmium stimulated the growth of the A4 (17 and 19) and 15834 (5 and 7) lines better at a higher concentration (200 μM) (Fig. 4f). The average increases in root growth against the WT were 23 and 50% for the A4 and 15834 lines, respectively, at 100 μM CdAc, and 69 and 82% at 200 μM CdAc. Growth was retarded by 47% in the lines transformed by the K599 strain, relative to WT at 100 μM CdAc (Fig. 4e).

States of Antioxidant Systems and Contents of Total Soluble Proteins in Tobacco Transgenic Plants under Conditions of Salinization

Along with stress factors, salinization has a negative effect on plants by intensifying the formation of ROS and the resulting oxidative stress. Since sodium

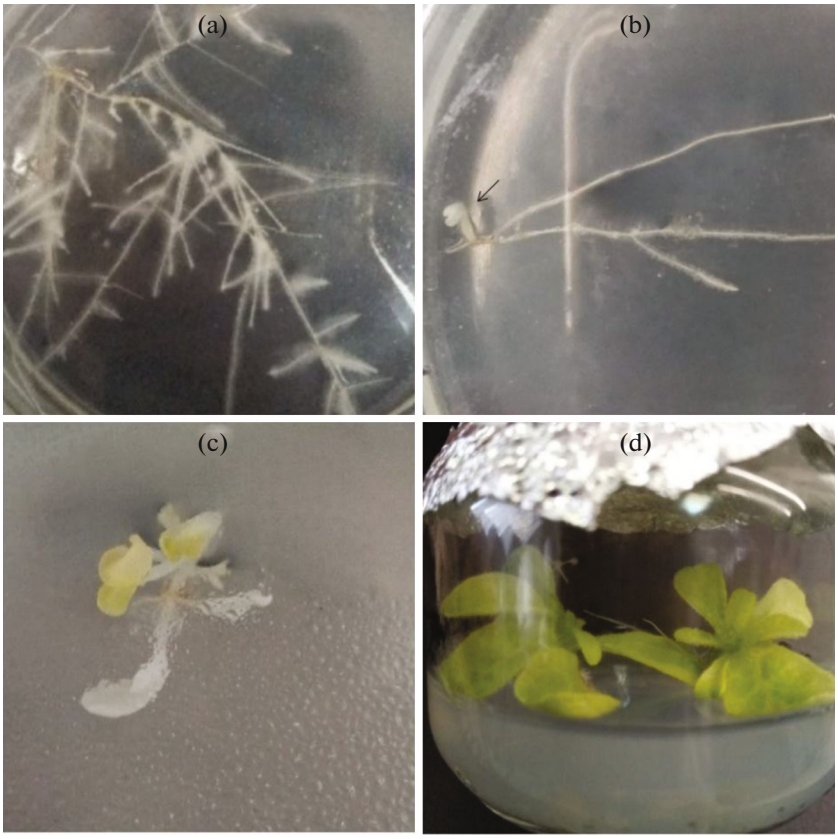

Fig. 2. Visual results from the transformation of tobacco leaf explants with *rol* genes from the A4 strain of *A. rhizogenes*: (a) exterior view of hairy roots on day 14 of their cultivation on a hormone-free medium; (b, c) regeneration of a shoot from a hairy root on a medium with BAP and NAA; (d) regenerants cultured on a medium with BAP and NAA.

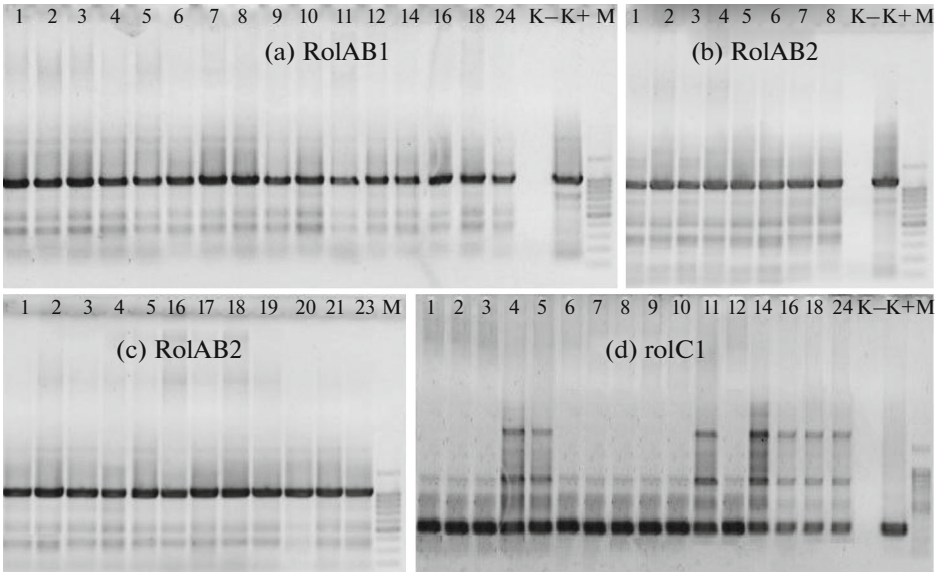

Fig. 3. Electrophoregrams from our PCR analysis of regenerated shoots with RolAB1, RolAB2, and rolC1 primers: (a, d) K599; (b) 15834; (c) A4. M = marker of DNA length 1kb DNA Ladder; K− = negative PCR control; K+ = positive PCR control. Sizes of PCR products: 1112 bp (RolAB1), 1127 bp (RolAB2), and 267 bp (rolC1).

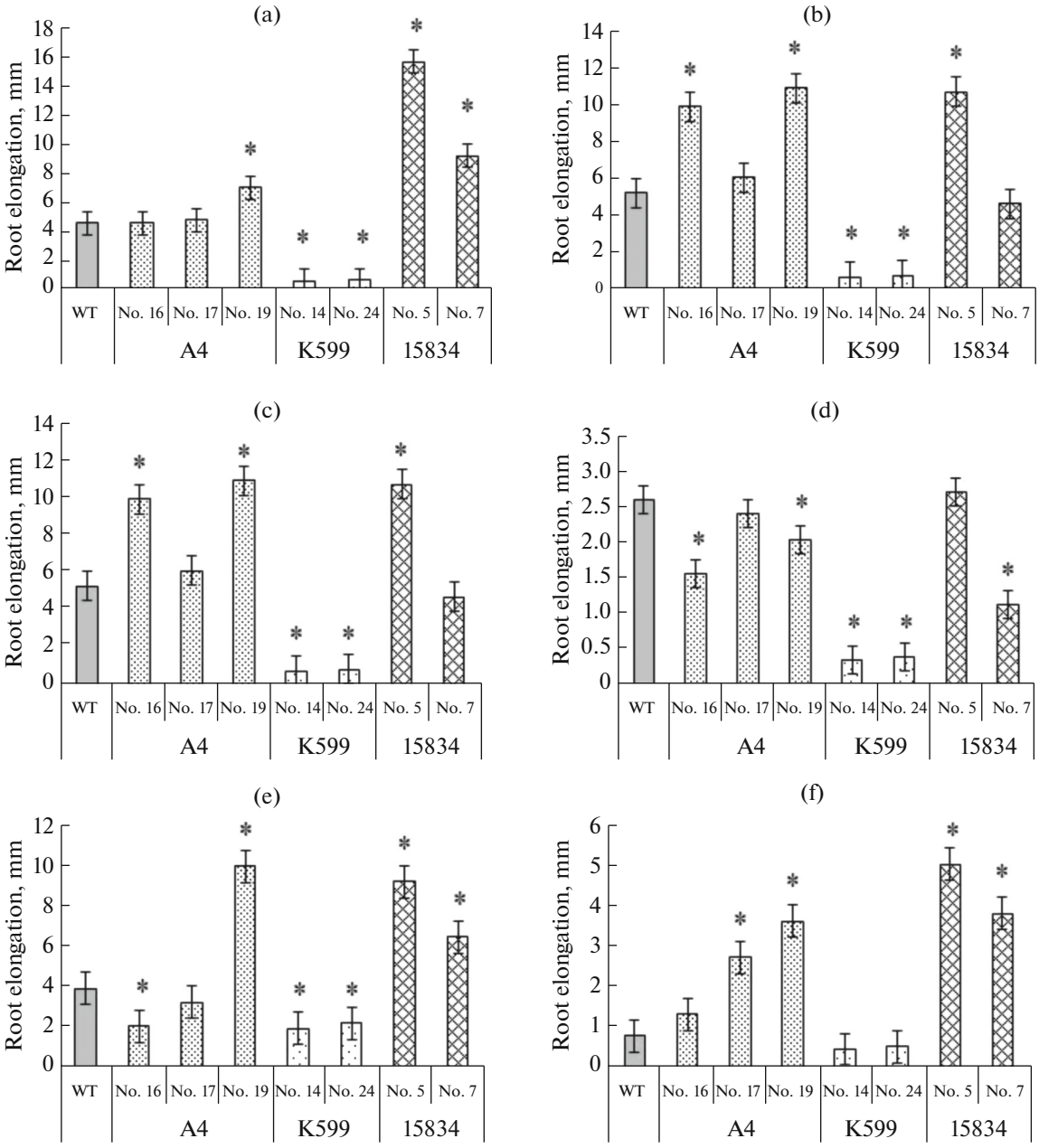

Fig. 4. Augmentation of the root length of transgenic tobacco plants grown in vertically positioned Petri plates: (a) normal conditions; (b) 50 mM NaCl; (c) 100 mM NaCl; (d) +12°C; (e) 100 μM CdAc; (f) 200 μM CdAc. Means of transgenic plants that differ significantly from those of WT (at $P \leq 0.05$) are marked with asterisks.

chloride is a component of soil, the primary target of this stress factor is roots [29], the organs in which *rol* genes are expressed [6]. Because our morphometric analysis revealed the strong effect 50 mM of NaCl has on the root growth of all transgenic plants, the effect salinization has on an antioxidant system was also tested at the same concentration of salt.

The content of water-soluble sugars in the roots of the transgenic plants of the A4 and 15834 lines was lower than in the WT under normal conditions (Fig. 5a). Salinization diminished this index significantly in the WT and did not change it in either transgenic line, both of which became richer in sugars than the WT. Under normal conditions, the level of water-soluble sugars was considerably lower in the K599 line than in the WT, but it grew 1.8 times upon salinization to a value higher than the one in the WT.

Under normal conditions, the concentration of proline in the roots of the A4 and 15834 transgenic plants was 4 and 4.5 times higher than in the WT, respectively (Fig. 5b). Upon salinization, the concentration of proline grew by an average of 1.5 times in the A4 and 15834 lines, and by 3 times in the K599 lines, relative to normal conditions. This index exceeded the

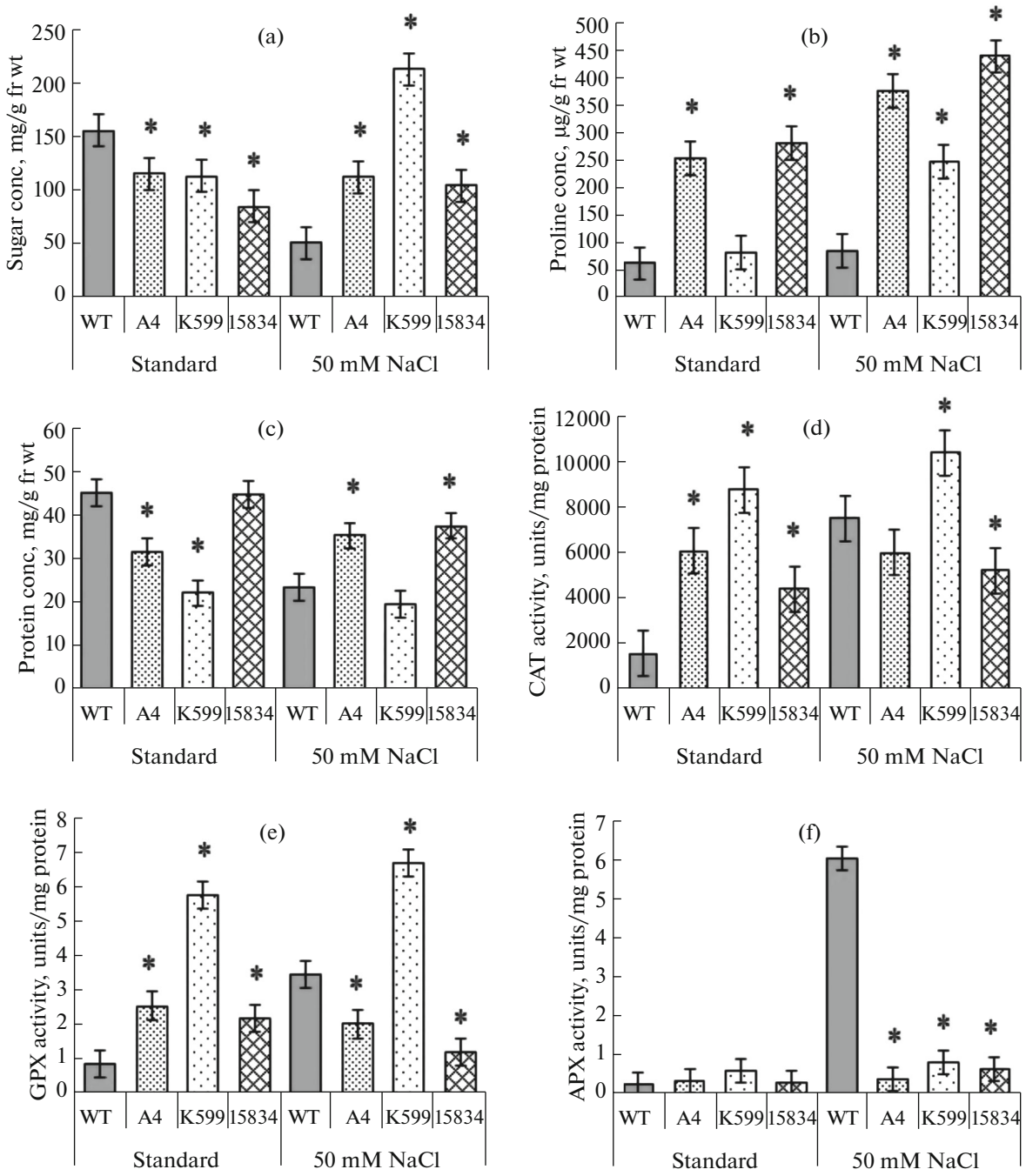

Fig. 5. States of antioxidant systems and contents of total soluble protein in WT tobacco and transgenic plants grown under normal conditions or upon salinization at 50 mM NaCl: (a) content of water-soluble sugars; (b) content of proline; (c) content of total soluble protein; (d) catalase activity; (e) guaiacol peroxidase activity; (f) ascorbate peroxidase activity. Means of transgenic plants that differ significantly from those of WT (at $P \leq 0.05$) are marked with asterisks.

level of the WT by 3.5 and 3 times, respectively. The K599 plants therefore contained the same amount of proline as the WT under normal conditions and a larger amount after salinization.

The content of all soluble proteins under normal conditions in the roots of the 15834 transgenic lines was the same as in the WT. This index was 30 and 51% lower in the A4 and K599 lines than in the WT, respectively (Fig. 5c). In the plants subjected to salinization, the level of soluble proteins fell in the K599 line but did not differ appreciably from that of the WT. In contrast, the salinity enriched the roots of the A4 and 15834 lines with proteins so that the corresponding values exceeded those of the WT by 51 and 60%, respectively.

Under normal conditions, the catalase activity of the transgenic plants of the A4, 15834, and K599 lines exceeded the level of the WT by 3.8, 2.8, and 5.5 times, respectively (Fig. 5d). Exogenous 50 mM NaCl enhanced the activity by 4.8 times in the WT roots. In the K599 plants, this treatment raised the CAT activity by 1.4 times, above the level of the WT. However, this increase was small in comparison to normal conditions. The impact of salt did not change the activities in the A4 and 15834 lines, which did not differ from the corresponding value of the WT.

Under normal conditions, the activity of guaiacol peroxidase was higher in all transgenic than in the WT plants: by 2.9, 2.5, and 6.5 times in the A4, 15834, and K599 lines, respectively (Fig. 5e). Upon salinization, the GPX activity fell by 1.3 and 1.8 times in the A4 and 15834 lines, respectively, down to levels 1.7 and 2.8 times lower than in the WT roots. At the same time, the activity in the K599 lines grew by 1.2 times, exceeding the WT value by 1.9 times.

Under normal conditions, the transgenic and WT plants did not differ from one an other in the activity of ascorbate peroxidase (Fig. 5f). Upon salinization, the activity increased in the WT roots and exceeded the values of the A4, 15834, and K599 lines by 14.4, 7.3, and 9.3 times, respectively; in these transgenic plants, the APX activity remained at the same level.

The MDA level in the roots of all transgenic lines was significantly lower than in the WT under both normal conditions and salinization (Fig. 6a). In the last case, the difference between the WT and transgenic plants was 1.4 times for A4 and 15834 and 3.4 times for K599.

Under normal conditions, the activity of superoxide dismutase in all tested transgenic lines was significantly higher than in the WT, namely, by 2.8, 2, and 3.6 times in the A4, 15834, and K599 lines, respectively (Fig. 6b). In the salt-treated counterparts, the activity increased by 1.2 times as compared with normal conditions and doubled the WT level. In the roots of the A4 and 15834 lines, the SOD activity did not change and did not exceed the WT value.

Under normal conditions, the general antioxidant activity in the A4, 15834, and K599 transgenic lines was higher than in the WT plants by 2.9, 1.8, and 2 times, respectively (Fig. 6c). However, upon salinization, the roots of these lines accumulated lesser amounts of ascorbic acid equivalents by 21, 35, and 25%, respectively, than the WT roots did.

The activity of glutathione-S-transferase was higher in the plants of the K599 line than in the WT in both normal conditions and salinization (Fig. 6d). In the presence of NaCl, this difference was as high as 2.7 times.

Under normal conditions, the content of reduced glutathione was larger by 41, 26.8, and 65.5 times in the roots of the transgenic A4, 15834, and K599 plants than in the WT ones (Fig. 6e). Salinization enhanced the GSH level by 43.6 times in WT and, additionally, by 1.7, 2.8, and 1.2 times in the A4, 15834, and K599 lines, respectively. Therefore, upon salinization, all the examined plants with *rol* transgenes accumulated more GSH than WT did.

The content of oxidized glutathione was also larger in the roots of transgenic plants than in those of the WT under normal conditions (Fig. 6f). This difference was 78.7, 29.6, and 40 times, respectively in the A4, 15834, and K599 lines. In the presence of exogenous NaCl, the level of GSSG grew by 30 times in the WT, relative to normal conditions, and by 1.4 and 2 times in the K599 and 15834 lines, respectively. In contrast, this index fell by 11% in the A4 line. Nevertheless, all of the studied plants bearing *rol* genes accumulated much more GSSG than the WT upon salinization.

RESULTS AND DISCUSSION

Plant tissue transformation performed with different strains of *A. rhizogenes* to obtain hairy root cultures that produce desirable secondary metabolites is widely used in plant biotechnology [13, 30]. Transgenes from the T-DNA of agrobacteria can modify the growth and stress tolerance of transformed plants, but this remains poorly studied [10, 31–33, 36]. In this study, we used the A4, 15834, and K599 strains of *A. rhizogenes* to create tobacco transgenic plants bearing the *rolA, rolB, rolC,* and *rolD* genes with their native promoters. These transgenic lines were used as model objects to reveal the roles of these genes in regulating plant growth and stress tolerance. We earlier reported the agrobacterium-mediated transformation of leaf explants of the K599 strain where hairy roots arose and there was intense direct induction of shoots [18]. We also used agropine strains to transform leaf explants and obtained cultures of hairy roots that grew better visually than a culture obtained with the K599 strain. It should be noted that there was no spontaneous formation of shoots on the surfaces of explants transformed with the A4 or 15834 strains (in contrast to the K599 strain) on a hormone-free nutrient medium. Shoot regeneration was induced from roots only by using 1 mg/L BAP and 0.5 mg/L NAA. Because the three strains differed in their capacity to initiate the formation of shoots and roots, we suggested that the corresponding transgenic plants would also differ in their parameters of growth and stress tolerance. Since *rol* genes are expressed mainly in roots, it was logical to analyze the root growth of transgenic plants under both normal and stressful conditions.

Our morphometrical data proved the *rol* genes of the A4 and 15834 strains participate in regulating and sustaining root growth under both normal conditions and at increased concentrations of sodium chloride or cadmium acetate. It seems that no other results associated with the *rol* genes with native promoters of the A4 and 15834 strains have so far been reported. Data obtained for the K599 transformed tobacco plants prove the *rol* genes of this strain negatively influence

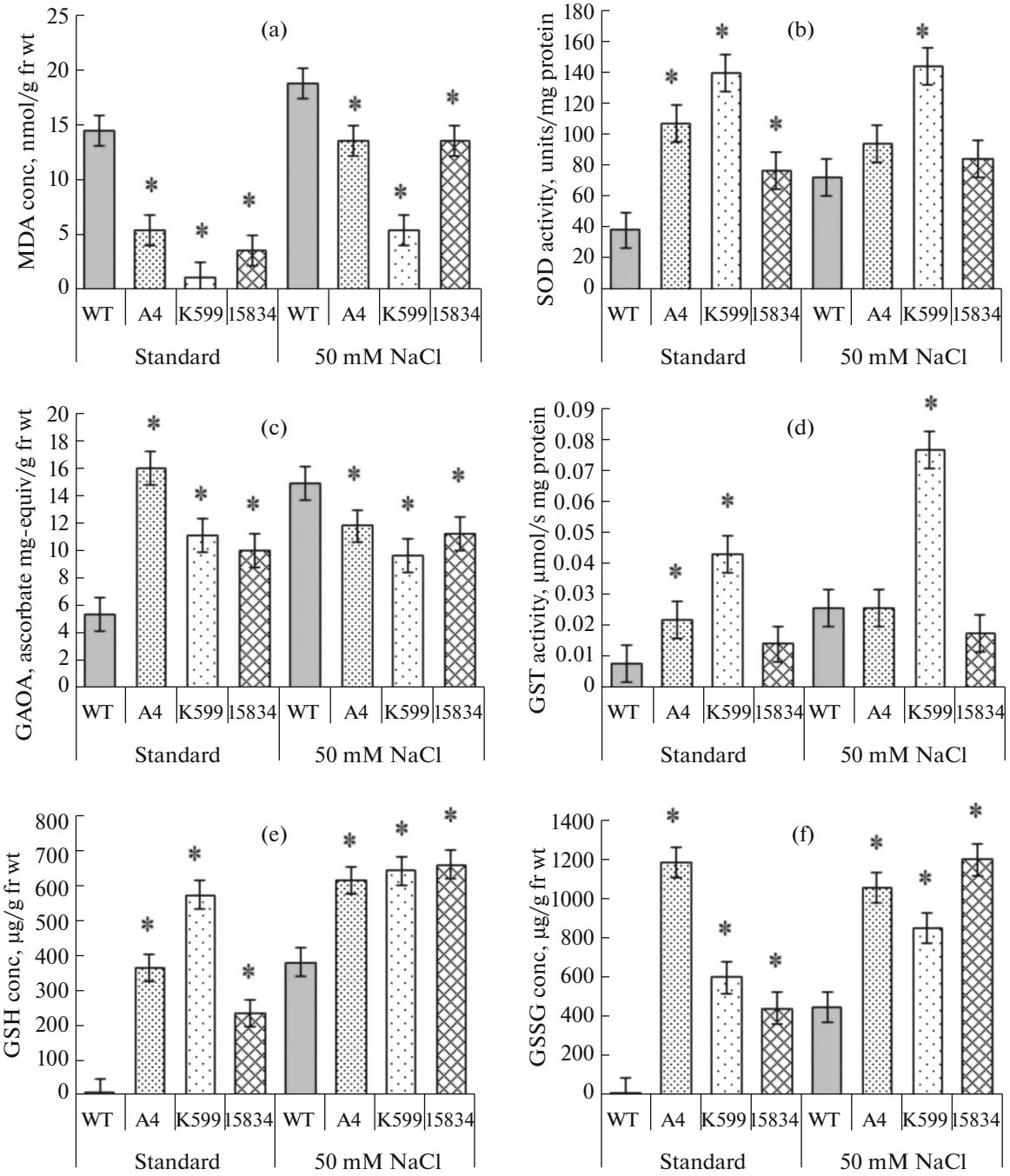

Fig. 6. States of antioxidant systems in WT tobacco and transgenic plants grown under normal conditions or upon salinization at 50 mM NaCl: (a) content of MDA; (b) superoxide dismutase activity; (c) general antioxidant activity; (d) glutathione-S-transferase activity; (e) content of GSH; (f) content of GSSG. Means of transgenic plants that differ significantly from those of WT (at $P \leq 0.05$) are marked with an asterisk.

tobacco root growth under both normal and stressful conditions.

Several studies now confirm importance of certain *rol* genes in controlling the growth and stress tolerance of plants exposed to salinization, drought, and temperatures that are too low or too high [10, 31]. The *rolC* callus cultures of *R. cordifolia* and *Arabidopsis thaliana* are thus more tolerant to abiotic impacts than their controls, due to the ability of the *rolC* genes to specifically regulate gene expressions of NADPH oxidase, CAT, SOD, and APX, which suppresses ROS generation [31]. Transformation of *R. cordifolia* by the A4 strain of *A. rhizogenes* lowers the ROS level, accompanied by increased expression of certain genes

encoding the ROS-detoxifying enzymes [32]. Genes that code cytosolic APX, CAT, and SOD are overexpressed in the *rolB*-transformants of *R. cordifolia, A. thaliana*, and *Panax ginseng* C.A. Mey, which in turn inhibits surplus ROS [33].

Our data on antioxidant systems show that transgenic tobacco plants bearing *rol* genes of the A4 and 15834 strains have elevated amounts of water-soluble sugars and proteins (Figs. 5a, 5c) and reduced MDA levels (Fig., 6a) upon salinization. The levels of proline (Fig. 5b) and glutathione, both reduced and oxidized (Figs. 6e, 6f), were also higher in the A4 and 15834 transformants than in the WT under normal conditions or salinization. Our observations agree with data that stressed plant cells accumulate a variety of osmo- and cryoprotectors that includes proline, a high concentration of which could play an adaptive role in *rolD*-transformed plants, probably because this gene encodes the enzyme ornithine cyclodeaminase that converts L-ornithine into L-proline [34].

We observed considerable augmentation in the glutathione pool (GSH + GSSG) in the roots of A4 and 15834 transgenic plants normally cultivated or salt-stressed (Figs. 6e, 6f). Bulgakov et al. [33] also found that the total glutathione pool and the GSH/GSSG ratio are larger in *rolB*-transformed *R. cordifolia* cells than in an untransformed control. These relations could be explained as the GSH/GSSG ratio being a pivot that maintains the cellular redox balance. Here the elevated concentration of reduced glutathione correlates with the plants' potential to cope with induced oxidative stress.

Higher amounts of proline, water-soluble sugars, and the total glutathione pool, relative to the WT, were observed in the roots of the salt-stressed transgenic plants transformed with the K599 strain, as were the considerably more active levels of GPX (Fig. 5e), SOD, and GST (Figs. 6b, 6d), and the reduced content of MDA (Fig. 6a). It is important that the levels of water-soluble sugars in the roots of the K599 transgenic tobacco considerably exceeded the corresponding values associated with the agropine strains upon salinization (Fig. 5a). Accumulation of water-soluble sugars in the roots is known to be an effective mechanism of salt stress tolerance in response to surplus monovalent ions in cell vacuoles [35]. The elevated activities of SOD, GPX, and GST antioxidant enzymes in the roots of the transgenic tobacco plants of the K599 line were presumably due to the capability of *rol* genes to activate plant protective systems in response to contact with ROS. Bulgakov et al. [36] reported the capacity of *rolB*-transformed *R. cordifolia* cells for high tolerance to superhigh ROS doses, due to the synthesis of enzymes that provide stable antioxidant protection from such stresses. Positive changes (relative to the WT) in the antioxidant complex were much more prominent in the roots of the K599 plants than those of the A4 and 15834 lines. However, the K599 roots grew more slowly under both normal and salt-stressed conditions, relative to the WT, A4, and 15834 plants. This means the parameters of growth, stress tolerance, and the state of components of an antioxidant system do not always depend directly on one another. In the K599 plants, positive changes of the antioxidant apparatus slowed growth and lowered stress tolerance. This assertion is supported by the work of Katsuhara et al. [37], where the overexpression of GST did not have any marked effect on the root growth retardation caused by 100 mM NaCl, since ROS detoxication alone is inadequate to restore osmotic misbalance caused by salt stress. However, in the study by Bernstein et al. [38], the activation of SOD and APX in growing leaves can inhibit the growth of plant cells upon salinization, since the ROS eliminated by these enzymes is needed for normal growth.

CONCLUSIONS

Having *rol* transgenes from the A4 and 15834 strains of *A. rhizogenes* in tobacco plants stimulates the growth of their roots under normal conditions and in contact with high concentrations of sodium chloride or cadmium acetate. In contrast, the expression of *rol* genes from the K599 strain suppresses root growth under both normal and stressful conditions. Upon salinization, the *rol* genes from agropine strains promote the accumulation of proline and both oxidized and reduced forms of glutathione in normal and salt-stressed plants. At the same time, *rol* genes from the K599 strain enhance the activities of superoxide dismutase, guaiacol peroxidase, and glutathione-S-transferase. Our results suggest that using *rol* genes from the A4 and 15834 strains of *A. rhizogenes* are promising for breeding plant cultivars and lines with improved traits of root growth and stress tolerance. However, *rol* genes from the K599 strain do not appear to be of help for this purpose.

ABBREVIATIONS AND NOTATION

APX	ascorbate peroxidase
BAP	benzylaminopurine
CAT	catalase
TAC	total antioxidant capacity
GPX	guaiacol peroxidase
GST	glutathione-S-transferase
SOD	superoxide dismutase

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This work did not include any studies involving human or animal subjects.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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