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Natural transgene trolC as a potential candidate for increasing plant productivity and stress tolerance

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Abstract

Homologues of the *rol* genes from *Agrobacterium rhizogenes* have become established in the genome of *Nicotiana tabacum* L. as a result of horizontal gene transfer under natural conditions. It is known that one of these plant homologues, namely the *trolC* gene, is expressed in young tissues, and, according to some data, is capable of influencing plant growth and stress resistance. However, the question of the mechanisms by which *trolC* gene expression affects plant resilience and productivity remains open. Therefore, the aim of this study was to investigate the role of the *trolC* gene in the regulation of plant growth and stress tolerance. To this end, modelling of the tRolC protein structure, molecular docking, as well as morphometric and biochemical analysis of *N. tabacum* transgenic plants with constitutive expression of a *trol*C gene fragment in antisense orientation. The proposed model of the tRolC protein shows a high structural similarity to the *Agrobacterium vitis* 6b protein and likely belongs to the NAD(+)-ADP-ribosyltransferase family. Suppression of *trolC* expression under salinity stress was accompanied by a decrease in the activity of catalase, guaiacol peroxidase, glutathione-S-transferase and superoxide dismutase, compared with the wild-type. Under cadmium stress conditions, transgenic plants exhibited reduced antioxidant enzyme activity and proline content, whilst total soluble protein, water-soluble sugars and total antioxidant capacity were increased compared to the wild-type. The results suggest that the *trolC* gene product is involved in the regulation and maintenance of root growth under normal conditions, as well as in response to cadmium and hypothermia.

1. Introduction

When plants are infected by Agrobacteria, T-DNA is not usually passed on to subsequent generations; however, the existence of naturally transgenic plants whose genomes contain sequences homologous to the T-DNA of *Agrobacterium* spp. (Pavlova et al., 2013; Matveeva, 2018) indicates the possibility of such inheritance on an evolutionary scale. To date, sequencing and bioinformatics screening have identified around 40 genera of naturally transgenic plants (Matveeva, 2021). The origin of these plants is likely due to the regeneration of plant organs under natural conditions from cells genetically transformed by Agrobacterium. Furthermore, the possibility of Agrobacterium-mediated transformation of generative tissues in ancestral forms cannot be ruled out (Matveeva and Sokornova, 2017). The preservation of Agrobacterium transgenes in a largely unchanged form over the course of long-term plant evolution suggests that these genes perform important adaptive functions and are subject to stabilising selection (Khafizova and Matveeva, 2021). The best-known natural plant transgenes are the *rol* genes (from «root locus»), the functions of which have not yet been fully elucidated; therefore, a key focus for further research should be to determine the role of their expression in regulating morphogenesis and conferring resistance to abiotic and biotic stresses. There is a hypothesis that Agrobacterium genes contributed to the adaptation of naturally transgenic plants during evolution, namely, the acquisition of competitive advantages over parental organisms and survival under adverse conditions (Enikeev, 2018). If *rol* genes did not perform any biological functions, mutations would accumulate in them at the same rate as in the intergenic regions of cell T-DNA (cT-DNA) (Pavlova et al., 2013).

Of all known naturally transgenic plants, those belonging to the genus *Nicotiana* L. have been the most extensively studied (Khafizova and Matveeva, 2021). In the genome of the model plant *Nicotiana tabacum* L., for example, there are homologues of the T-DNA genes of *Agrobacterium rhizogenes* (Otten, 2018). The most famous of these, the *trolC* gene, belongs to the *plast*-gene family; it was incorporated into the *N. tabacum* genome, has become evolutionarily established over several generations, and is likely involved in the regulation of growth and stress tolerance. It is known that *trolC*, like its Agrobacterium predecessor, is conserved, and its expression is detected in young tobacco tissues (Meyer et al., 1995). Furthermore, it is suggested that the role of the *rolC* gene in naturally transgenic plants is likely linked to three main areas: the regulation of regeneration processes, the enhancement of adaptive potential, and the development of resistance to phytopathogens (Chen and Otten, 2017). It has been established that cT-DNA genes are indeed capable of activating secondary metabolism as well as plant defence mechanisms. It has been found that the levels of major fractions of secondary metabolites in naturally transgenic plants exceed those in their non-transformed relatives (Matveeva et al., 2014; Matveeva and Sokornova, 2016). Previously, using *N. tabacum* plants, we demonstrated that the expression of a *trolC* gene fragment in antisense orientation under the control of the 35S CaMV promoter leads to a reduction in plant resistance to cold and to the action of cadmium acetate (Kuluev et al., 2021). Furthermore, increased expression of the *trolC* gene in *N. tabacum*, as well as of *rolC* genes of various origins in transgenic lines, correlates positively with increased glucose concentration and total sugar content (Matveeva et al., 2020). Despite this, the question of the mechanisms by which *trolC* gene expression affects plant growth and stress tolerance remains open. Therefore, the aim of this study was to investigate the role of the *trolC* gene in the regulation of plant growth and stress tolerance.

2. Materials and methods

2.1 Modeling of the tRolC Protein Structure and Molecular Docking

To predict gene function using bioinformatics methods, we investigated the putative tRolC protein. The NCBI Entrez (National Center for Biotechnology Information 2026) and NCBI BLAST (National Center for Biotechnology Information 2025). Multiple sequence alignment was performed using the EMBL-EBI Clustal Omega service (EMBL-EBI 2025). Jalview 2.11.4.1 (Waterhouse et al., 2009) was used to construct phylogenetic trees using the Neighbour-Joining method, and MEGA 11 (Tamura et al., 2021) was used for their visualisation. The I-TASSER service (Zhang 2026) and AlphaFold 3 (Google DeepMind and Isomorphic Labs 2024) were used to predict protein structure and function. Maestro 14.2 (Schrödinger 2026) was used to visualise and analyse spatial structures. The SwissDock online service (Swiss Institute of Bioinformatics 2025) was used for docking.

2.2 Morphometric Analysis of Roots of Transgenic Tobacco Plants Exposed to Salinity, Hypothermia, and Cadmium Acetate

In this study, we utilised four lines of transgenic Petit Havana tobacco plants previously generated by us (Kuluev et al., 2021), which exhibit constitutive expression of a *trolC* gene fragment in antisense orientation under the control of the 35S CaMV promoter.

Quantification of *trolC* mRNA levels was performed by real-time quantitative RT-PCR in the presence of the intercalating dye SYBR Green I in a Rotor-GeneTM 6000 thermal cycler («CorbettResearch», Australia) (Fig. S1). The primer sequences for RT-PCR were initially selected using the PrimerSelect program (DNAStar, USA): trolCRTF 5'-CGGGGGCCAAACGACTACTG-3' and 5'-AGGGCTACGTGCATAAAGACTGAA-3' (Evrogen, Russia). The size of the PCR product was 320 bp. The mRNA of the elongation factor EF-1α gene (AF120093.1) was used as a standard, with its expression level set at 100%. For RT-PCR of the EF-1α gene, the primers 5'-GAATTGGTACTGTCCCTGTT-3' and 5'-TTGCCAATCTGTCCTGAAT-3' (Evrogen, Russia) were used.

Non-transgenic tobacco plants of the *Petit Havana* variety, SR1 line (wild type – WT), were selected as controls. A sample of 60 plants was taken for each line (n = 60). Seeds from the transgenic lines (3, 6, 16, 17) were pre-sterilised using a 20% bleach solution (Ekol LLC, Russia), rinsed five times with sterile distilled water, sown on MS solid medium supplemented with hygromycin (10 mg/ml) and grown in Binder growth chambers (Germany) at a temperature of + 25°C, under light intensity of approximately 140 μmol/(m²·s) and a 16/8-hour photoperiod (light/dark) (normal conditions). Ten-day-old seedlings with roots of equal length were transferred to vertically oriented Petri dishes. After 10 days, to assess root growth characteristics, morphometric analysis was carried out under normal conditions, and under the influence of NaCl (50 mM and 100 mM), hypothermia (+ 12°C) and cadmium (100, 200 and 400 μM CdAc). Solutions of NaCl and CdAc were added to the medium prior to autoclaving. To determine the optimal concentration of NaCl, the plants were first transferred to MS medium supplemented with the following concentrations of this salt: 25, 50, 100 and 200 mM. For subsequent experiments, two concentrations were selected: 50 mM and 100 mM, at which growth improvement and growth retardation were observed, respectively. Cadmium acetate concentrations were selected in a similar manner using the following concentrations: 50, 100, 200 and 400 μM. It was found that a concentration of 50 μM had no significant effect on root growth compared with the control. Consequently, three concentrations of cadmium acetate were selected for further work: 100, 200 and 400 μM, which had a negative effect on plant growth.

2.3 Analysis of the Antioxidant System of Transgenic Tobacco Plants in Response to NaCl and Cadmium Acetate

10-day-old seedlings of transgenic *N. tabacum* plants from lines 3, 6, 16 and 17, expressing a fragment of the *trolC* gene in antisense orientation under the control of the 35S CaMV promoter, grown in Petri dishes on solid MS medium supplemented with hygromycin (10 mg/ml) and exhibiting roots of similar size, were transferred to vertically oriented Petri dishes containing MS medium supplemented with 50 mM NaCl and 100 μM CdAc.

To determine the activity of superoxide dismutase (SOD) in the roots, a method based on the ability of SOD to compete with nitroblue tetrazolium for superoxide anions was used (Chevari et al., 1995). Ascorbate peroxidase (APX) activity was determined using a method based on measuring the rate of hydrogen peroxide decomposition by ascorbate peroxidase, yielding water and dehydroascorbate (Verma et al., 2003). Guaiacol peroxidase (GPOX) activity was determined by the polymerisation of guaiacol to tetraguaiacol (Ermakov et al., 1987). Catalase (CAT) activity was assayed by the rate of degradation of hydrogen peroxide molecules (Panchuck et al., 2002). The malondialdehyde (MDA) content in the sample was determined using thiobarbituric acid (Taylor et al., 2007). The method for proline determination was based on the work of Khedr et al. (2003). The content of water-soluble sugars was determined by the method described in the work of Dubois et al. (1956). The total antioxidant capacity (TAC) was assessed in methanol (80%) extracts based on the reduction of Mo(VI) to Mo(V) at acidic pH (Boestfleisch et al., 2014). Glutathione S-transferase (GST) activity was determined by the rate of conjugate formation between reduced glutathione and 1-chloro-2,4-dinitrobenzene (Habig et al., 1984). The content of oxidised and reduced glutathione was determined by the method of Hissin et al. (1976). The total soluble protein content was determined by the Bradford method. The hydrogen peroxide content was determined using the method described in the works of Chaouch et al. (2010) and Bindschedler et al. (2001). The activity of all enzymes in the roots was recalculated per mg of total soluble protein. All biochemical studies were performed in 10 replicates (n = 10).

The results of all studies are presented as histograms showing the mean values of the sample. The bars represent the standard error of the mean (M ± SEM). Statistical significance was assessed in all experiments using the Mann–Whitney U test ($p < 0.05$).

2.4 Determination of Cadmium Content in Plant Roots and Leaves

Cadmium content was determined by inversion polarography using an «Ecotest-VA» voltammetric analyser with a programmable robotic autosampler AS-01/3D and a «three-in-one» carbon electrode (Ekonyks-Expert Research and Production Company, Russia). Initially, 2-month-old *N. tabacum* plants of lines 3, 6, 16 and 17 with reduced expression of the *trolC* gene, grown in universal potting soil, were watered twice a week with 50 ml of a 100 μM CdAc solution for 14 days. The leaves and roots (200 mg sample) were then dried to constant weight at 90°C in an oven and ground in a porcelain mortar. The resulting powder was ashed in a muffle furnace at 500°C for 4 hours. The ash residue was dissolved in 1 ml of 1 M HNO_3, transferred to 100 ml volumetric flasks and made up to the mark with 0.01 M HNO_3 solution. The cadmium content was then measured in accordance with the instructions for use of the voltammetric analyser.

2.5 Measurement of Growth Parameters in Transgenic Plants Exposed to Salinity, Drought, and Cold Stress

Twenty-day-old transgenic seedlings, grown on MS medium supplemented with hygromycin (10 mg/ml), and wild-type seedlings were transplanted into growth vessels (450 ml) containing universal potting

compost. To determine salt tolerance, transgenic and control plants were grown for 20 days, then watered with 50 ml of a 2% NaCl solution twice a week for 14 days; control plants were watered with an equivalent volume of distilled water. Growth parameters were assessed 30 days after the start of the salt application experiment. To determine cold tolerance, plants were grown in soil for 12 days in 250 ml growing vessels under normal conditions; subsequently, some of the plants were transferred to a Sanyo MIR 154 climate chamber (Japan) at a temperature of + 12°C. All plants were watered with 20 ml of distilled water three times a week, and measurements of the fresh/dry weight of the aboveground parts were taken 20 days later. To determine drought tolerance, experimental and control plants were grown in 450 ml growth vessels under standard conditions for 22 days, after which watering was carried out according to the following schedule: moderate drought – once a week (50 ml), normal conditions – three times a week (50 ml). After 30 days of cultivation, the fresh and dry weights of the aboveground parts were determined. To assess plant growth parameters during the flowering period, five plants were selected from each of the control and drought groups; for these, the length and area of the leaves, the length of the stem and five flowers, and the fresh and dry weight of the above-ground parts were measured.

3. Results

3.1 Modeling of the tRolC Protein Structure and Molecular Docking

The nucleotide and amino acid sequences listed in Table S1 were used for the phylogenetic analysis. In addition to *rolC*-like genes and proteins, the sequences of *Agrobacterium vitis* protein 6b – the closest structural homologue of tRolC (Tsvetkova et al., 2024) – were also analysed.

Fig. S2 shows phylogenetic trees of the similarity of the studied nucleotide and amino acid sequences. For example, for the cT-DNA of plants of the genus *Linaria*, the closest matches were strains *A4, 15834* and *NCPPB1855* of *A. rhizogenes*.

Despite significant differences in the amino acid sequence of protein 6b compared to RolC-like proteins, its nucleotide sequence placed it in the same clade as homologues of the *rolC* gene from *N. glauca* and one of the sequences from *N. tomentosiformis*.

To predict the three-dimensional structure of the protein under investigation, we utilised the AlphaFold 3 (Abramson et al., 2024) and I-TASSER (Xu et al., 2024) services (Fig. 1a). The latter is also capable of predicting enzymatic activity and identifying the closest structural homologues.

We previously found that the closest homologues of tRolC are bacterial exotoxins exhibiting ADP-ribosyltransferase activity (6b, exotoxin A, cholera toxin) (Tsvetkova et al., 2024). In this study, we conducted more detailed investigations into the molecular docking of the predicted tRolC protein.

The root mean square deviation (RMSD) was calculated for the structures under investigation using the Maestro programme. For the I-TASSER and AlphaFold structures, it was 3.226 Å; for I-TASSER and 6b, it

was 2.228 Å; and for AlphaFold and 6b, it was 3.248 Å.

In the tRolC structure predicted using AlphaFold, similar to protein 6b, there are two antiparallel β-strands with a loop situated between them, which exhibits a high structural similarity to the ARTT motif (ADP-ribosylating toxin turn–turn motif) (Deng and Barbieri, 2008). Furthermore, the predicted model contains a structure similar to the protein-binding loop of 6b.

Fig. 1b illustrates the hydrophobic interactions between nicotinamide and certain residues of the tRolC protein. It has been found that LEU 109, TYR 118 and ARG 123 are most likely involved in the stabilisation of nicotinamide.

Using the SwissDock online service, the interaction between nicotinamide and the tRolC amino acids was predicted at a distance of no more than 4 Å. Hydrophobic interactions form on the nicotinamide ring, whilst ARG 123 exerts the greatest influence on its OH group (Fig. 1c).

Like tRolC's closest structural homologue, protein 6b, the hydrophobic environment created by aromatic amino acids appears to be involved in the binding of the nicotinamide ring of NAD+ (Wang et al., 2011).

3.2 Morphometric Analysis of Roots of Transgenic Tobacco Plants Exposed to Salinity, Hypothermia, and Cadmium Acetate

Under normal conditions (+25°C), two lines of transgenic tobacco plants expressing the *trolC* gene in antisense orientation showed a significant increase in root length compared with the control (Fig. 2a). The degree of growth increases averaged 43% for plants of lines 6 and 17 compared with the control. Under the influence of NaCl at concentrations of 50 mM and 100 mM, no significant differences in root growth were found between the control and the transgenic plants (Fig. 2b, c). Under hypothermic conditions, three lines (6, 16, 17) of transgenic plants showed a deterioration in root growth parameters compared with the WT. The greatest reduction in root length was observed in tobacco plants of line 6 – almost 10-fold compared with the WT. For the roots of lines 16 and 17 under hypothermic conditions, the reduction in root length compared with the WT averaged 35% (Fig. 2d).

Under cadmium stress conditions at a CdAc concentration of 100 μM, a reduction in root length was observed in lines 6, 16 and 17, on average by a factor of 8.5 (Fig. 3b). Under the influence of cadmium acetate at a concentration of 200 μM, a reduction in root growth rate was observed in lines 6, 16 and 17, on average by a factor of 4.8 compared to the WT (Fig. 3c). In all the transgenic lines studied, under the action of cadmium acetate at a concentration of 400 μM, root growth decreased on average by a factor of 4.3 compared with the WT (Fig. 3d).

3.2. Analysis of the Antioxidant System of Transgenic Tobacco Plants under Salinity and Cadmium Acetate

To investigate the effect of the *trolC* gene on components of the antioxidant system under salinity, a minimum concentration of 50 mM NaCl was selected, as this concentration promotes improved root

growth in tobacco through the activation of adaptive mechanisms (Kuluev et al., 2019).

Under normal conditions, APX activity in the roots (Fig. 4a) was significantly reduced by an average factor of 1.4-fold in plants of lines 3 and 6, and increased by an average factor of 1.7-fold in line 16 compared to WT plants. Under salinity, APX activity in the roots of WT plants increased and was on average 5.9 times higher than in the roots of WT lines. In transgenic plants with reduced *trolC* gene expression, APX activity under salinity remained at the same level.

Under normal conditions, catalase activity (Fig. 4b) in transgenic plants was on average 2.1 times lower than in WT. Under salinity conditions, there was no significant difference in catalase activity between the WT and the transgenic plants; however, a slight decrease was observed compared with normal conditions.

The proline content (Fig. 4c) in the roots of transgenic plants under normal conditions and under salinity stress did not differ significantly from that of the WT. Under salinity stress, the proline content in transgenic plants and the WT increased by an average of 3.9-fold and 2.9-fold, respectively, compared with normal conditions.

GPOX activity (Fig. 4d) n the roots of all transgenic lines under normal conditions was, on average, 2.8 times higher than in WT plants. Under saline conditions, no significant differences were observed compared with WT.

The amount of hydrogen peroxide in the roots of transgenic plants and WT under normal conditions and under salinity differed only slightly (Fig. 4e). Line 6 was characterised by a higher hydrogen peroxide content compared to the control, both under normal conditions and under salinity stress.

Under normal conditions, the content of water-soluble sugars in the roots of transgenic plants of lines 3 and 16 (Fig. 4f) was significantly higher than that of the control, on average 1.8 times higher. Under salinity stress, the amount of water-soluble sugars in the roots of all the lines studied did not differ significantly from the WT, nor from the values obtained under optimal cultivation conditions. Line 17, on the other hand, was characterised by a lower content of water-soluble sugars.

In the roots of transgenic plants from lines 3 and 16, the MDA content (Fig. 5a) was significantly higher than that of the WT, both under normal conditions and under salinity stress. Moreover, the difference in MDA content under salinity stress was, on average, 2.1 times greater than that of the WT.

SOD activity (Fig. 5b) in the roots of the three transgenic plant lines analysed under normal conditions was, on average, 2.1-fold lower than in the WT. In response to NaCl treatment, SOD activity did not differ significantly from that of the WT, but decreased slightly relative to normal conditions in both the control and the transgenic plants.

Under normal conditions, TAC in the roots (Fig. 5c) of transgenic plants of lines 3 and 17 was on average 1.5 times higher than in the WT. However, under salinity stress, TAC in the roots of transgenic plants of

line 6 was higher, and in line 17 lower, than in the WT.

Under normal conditions, GST activity (Fig. 5d) in all the transgenic plant lines analysed was on average 65.4% lower than in the WT. Under salinity conditions, GST activity decreased by an average of 1.7-fold compared with normal conditions. No significant difference in GST activity was detected between the control and the transgenic plants under salinity conditions.

Under normal conditions, GSH levels (Fig. 5e) did not differ significantly from those of WT. In response to NaCl GSH levels in the roots of the transgenic plants increased by an average of 1.5-fold. Thus, the roots of all transgenic plants with reduced *trolC* gene expression contained higher levels of GSH than WT under saline conditions.

Under normal conditions, the GSSG content (Fig. 5f) in the roots of transgenic plants differed significantly from that of WT only in line 6. Under saline conditions, the oxidized glutathione content increased by an average of 1.4-fold compared with normal conditions, exceeding WT value by an average of 2.7-fold in transgenic plants. Only line 17 did not differ significantly from WT under salinity conditions.

The results of the morphometric analysis of roots exposed to cadmium acetate at a concentration of 100 μM showed a reduction in root length in three lines of transgenic plants by an average of 8.5-fold compared with WT, therefore, we decided to analyse the antioxidant system in response to cadmium stress using precisely this concentration of CdAc.

Under cadmium stress, APX activity (Fig. 6a) decreased on average by a factor of 3.4 relative to WT and by a factor of 8.6 compared with normal culture conditions.

In response to cadmium acetate treatment, catalase activity (Fig. 6b) did not differ significantly from WT in lines 3 and 17, but decreased on average by a factor of 2.8 compared with the control. In transgenic plants of lines 6 and 16, catalase activity was lower than in WT by an average of 2.5 times.

GPOX activity (Fig. 6c) in the transgenic plants was only slightly reduced compared with WT.

The hydrogen peroxide content (Fig. 6d) in the transgenic plants under cadmium stress was 43.2% lower on average compared with WT.

In response to cadmium acetate at a concentration of 100 μM, protein levels (Fig. 7a) increased on average 2.5-fold compared with WT and 3.7-fold compared with normal growing conditions.

Under cadmium stress conditions, the proline content in the roots of transgenic plants (Fig. 7b) was on average 2-fold lower than in WT.

In response to CdAc, the amount of water-soluble sugars (Fig. 7c) increased 1.8-fold, exceeding WT value in transgenic plants by an average of 1.2-fold.

Under cadmium acetate treatment, the TAC of transgenic plants (Fig. 7d) increased by a factor of 1.5-fold compared with normal conditions, exceeding the WT value by an average of 1.3-fold.

In the roots of transgenic plant lines 3 and 17, a 1.3-fold decrease was observed, while in line 6, MDA content (Fig. 7e) increased 1.2-fold compared to WT under cadmium exposure.

SOD activity (Fig. 7f) under cadmium stress in transgenic plants was on average 1.8 times lower compared to WT.

GST activity (Fig. 8a) in the roots of all the transgenic plant lines analysed was significantly lower on average by a factor of 4.5 following exposure to CdAc compared with WT. In response to cadmium acetate, GST activity (Fig. 8a) in transgenic plants decreased on average by a factor of 2 compared with normal conditions.

The levels of GSH (Fig. 8b) and GSSG (Fig. 8c) in the roots of transgenic plants in response to cadmium acetate did not differ from WT, but were elevated relative to the control by an average of 2.3 and 2.6-fold, respectively.

3.3. Measurement of Growth Parameters in Plants Grown under Normal, Salt, Drought, and Cold Stress Conditions

In terms of changes in fresh weight (Fig. 9a) and dry weight (Fig. 9b) of the aboveground parts under drought conditions, the transgenic plants of all the studied lines showed no statistically significant differences from WT plants. In response to hypothermia, plant growth was reduced, as evidenced by the fact that the fresh weight of the aboveground parts of the transgenic plants was on average 2.8 times lower (Fig. 9c), and the dry weight 2.9 times lower (Fig. 9d) compared with WT.

When grown in soil under saline conditions, no significant differences were observed in stem height (Fig. 10a), shoot dry/fresh weight (Fig. 10b, c), as well as leaf length (Fig. 10d) and area (Fig. 10e) in transgenic plants with reduced expression of the *trolC* gene and WT. Under saline conditions, growth parameters were reduced in the same way as in WT.

During the flowering period, no significant differences were observed between the transgenic plants and WT grown under normal conditions in terms of stem height (Fig. 11a), whilst leaf area (Fig. 11b) was on average 20.6% lower. A significant reduction in dry (Fig. 11c) and fresh (Fig. 11d) weight was characteristic of lines 6, 16 and 17, amounting to an average of 18.8% and 13.3%, respectively. In terms of leaf length (Fig. 11e), some lines of transgenic plants were characterised by a significant reduction of 12.5% on average. There was no significant difference in flower length between WT and transgenic plants.

3.4. Determination of Cadmium Content in Roots and Leaves

Under cadmium stress, the concentration of Cd^{2+} in the leaves of transgenic plants was found to be 1.9 times higher on average than in WT (Fig. 12). In the leaves, the cadmium ion content exceeded that in the roots by 2.1 times in transgenic plants and by 3.6 times on average in WT. In terms of cadmium content in the roots, the transgenic plants differed less from WT; only in line 16 was a significant excess recorded.

4. Discussion

Today, soil salinisation, drought and heavy metal contamination are among the most significant challenges faced in crop cultivation, with these stress factors having a particularly detrimental effect on root growth. Therefore, the search for new genes that confer increased resistance and productivity in plants under the influence of these stress factors is a promising avenue of research.

Alignment of nucleotide sequences revealed that in *N. tomentosiformis, L. creticola* and *L. vulgaris* possess two *rolC*-like genes that differ in structure (Fig. S2), which appears to be linked to different times of T-DNA horizontal transfer (Chen et al., 2014).

It should be noted that the similarity in the amino acid sequences of the tRolC protein in *N. tabacum* and *N. tomentosiformis* is likely due to their common ancestry. It is believed that the species *N. tabacum* was formed as a result of hybridisation between *N. tomentosiformis* and *N. sylvestris*; however, during the course of evolution, the cT-DNA underwent significant changes, which explains the accumulated differences in their structure (Matveeva and Sokornova, 2017). The structural similarity we have identified between RolC-like proteins and protein 6b is likely related to their biological functions. It is known that expression of the *6b* gene, like that of *trolC*, leads to abnormal morphology of roots and shoots (Wang et al., 2011). The predicted tRolC model contains a structure similar to the protein-binding loop of 6b, which may play a role in both self-association and interaction with other proteins (Wang et al., 2011). In line with our previous findings (Tsvetkova et al., 2024), tRolC most likely belongs to the NAD(+)-ADP-ribosyltransferase family (2.4.2.30). Enzymes of this class are capable of transferring their ADP-ribosyl group to an acceptor molecule-nucleic acid or protein thereby influencing the regulation of gene expression through interaction with transcription factors, affecting RNA interference, and possibly through the manifestation of histone-like activity (Tsvetkova et al., 2024).

It is interesting to note that nicotinamide, a by-product of ADP-ribosyltransferase activity, can be converted into nicotine within tobacco cells; it is precisely this nicotine that accumulates in the plant's tissues (Frost et al., 1967; Amini et al., 2020). There is also evidence that increased expression of the *rolC* and *trolC* genes in tobacco leads to higher nicotine content (Palazon et al., 1998; Khafizova and Matveeva, 2021), which may be linked to the NAD(+)-ADP-ribosyltransferase activity of these proteins.

The study found that the *trolC* gene product is involved in regulating and supporting root growth under cadmium and hypothermic conditions. It should be noted that hypothermia is a significant constraint on growth and water uptake in tobacco, as this species is a thermophilic crop (Kuluev et al., 2019). Furthermore, cis-regulatory elements induced by low temperatures have been identified in the promoter

region of the *rolC* oncogene (Hu et al., 2003), which provides further support for the proposed role of the *trolC* gene in the response to cold stress. Our results of morphometric analysis of root length in vertically oriented Petri dishes are corroborated by a reduction in dry/wet weight when grown in growth vessels.

A reduction in the expression level of the tobacco *trolC* gene leads to a decrease in the activity of catalase, GPOX, GST and SOD, as well as an increase in the levels of MDA and GSSG under normal conditions and under saline stress, which is due to the gene's pronounced effect on the components of the root antioxidant system. This hypothesis is supported by a study by Xu et al. (2024), which found that the expression of the *rolC* gene in *Atropa belladonna* plants contributes to drought tolerance by increasing the activity of SOD, peroxidase and catalase, increasing proline content and reducing MDA levels under stress conditions. In response to cadmium stress, a reduction in the expression level of the *trolC* gene contributed to a decrease in the activity of APX, GPOX, catalase, GST and SOD, a decrease in proline and H_2O_2 content, as well as an increase in TAC and an increase in the cellular content of total soluble protein and water-soluble sugars under normal conditions and, in some cases, under the influence of cadmium acetate. Once cadmium enters a plant, it stimulates the formation of reactive oxygen species (ROS), leading to lipid peroxidation, the accumulation of MDA, damage to cell membranes and the oxidative modification of proteins. In a study by Mahmoudi et al. (2024), exposure of tobacco plants to cadmium chloride resulted in increased levels of proline, MDA, H_2O_2, and antioxidant capacity. In the plants we studied, gene expression is regulated at the post-transcriptional level via RNA silencing; consequently, the reduction in expression appears to be compensated for by an increase in TAC, total soluble protein and water-soluble sugars.

Analysis of cadmium content demonstrated that a reduction in *trolC* expression leads to increased accumulation of Cd^{2+} in the leaves compared with the control. It is known that tobacco is able to accumulate cadmium to a greater extent in the roots, as the root system acts as a barrier that limits its transport to the stems and leaves. As Cd2 + concentration increases, its content in leaves rises (Mahmoudi et al. 2024), predominantly in older leaves (Yang et al., 2017). In a study by Zheng et al. (2024), it was found that, under cadmium exposure, the expression of cell wall biosynthesis proteins increases in root cells, leading to cell wall thickening and a restriction of Cd^{2+} transporter protein activity, thereby isolating these ions in the apoplast. We previously found that the tRolC protein is likely capable of interacting with the cell wall biosynthesis proteins GT14 and GALT29A (Tsvetkova et al., 2024), which are involved in the assembly of arabinogalactan proteins (AGPs). Given that the cell wall is the first barrier to cadmium ions, it can be suggested that the correct assembly of AGPs under the control of these genes influences the cell wall's ability to bind and retain cadmium, preventing it from penetrating into the cell. Consequently, it is likely that reduced expression of the *trolC* gene disrupts these defence mechanisms, facilitating cadmium uptake into the roots and its transport to the leaves and shoots.

Thus, the putative model of the tRolC protein, predicted using AlphaFold, contains a protein-binding loop that mediates interactions with other proteins and an ARTT loop, and also exhibits a high structural similarity in the amino acid residues of the active site and the binding site with the *A. vitis* protein 6b. Molecular docking has established that the hydrophobic environment created by aromatic amino acids is

involved in the binding of the nicotinamide ring, confirming the putative NAD(+)-ADP-ribosyltransferase activity of the tRolC protein. The activity of this protein at the physiological level manifests itself through its influence on growth and stress resistance, as well as on the biosynthesis of secondary metabolites. The product of the *trolC* gene is likely to be involved in the regulation and maintenance of root growth under normal conditions, as well as in response to cadmium and hypothermia. Suppression of *trolC* expression under salinity is accompanied by a decrease in the activity of key enzymes of the antioxidant system: catalase, GPOX, GST and SOD, as well as the accumulation of oxidative stress markers: MDA and GSSG. Under cadmium stress, alongside a reduction in the activity of antioxidant enzymes and proline content, an increase in total soluble protein, water-soluble sugars and TAC is observed, which may be compensatory in nature due to post-transcriptional silencing of the *trolC* gene. The obtained results indicate the importance of the *trolC* gene for the growth and adaptation of tobacco under changing environmental conditions, as well as the potential for using this gene to develop new plant varieties and lines with increased productivity and stress tolerance.

Declarations

Author Contributions: DS and KhM: biochemical analysis of the antioxidant system of transgenic plants. ATs: tRolC protein structure modeling and molecular docking. VF: determination of cadmium content in roots and leaves. EV: real-time quantitative reverse transcription PCR. ZB: morphometric analysis of transgenic plants. KB, DS, ATs: study design, data analysis, article writing and editing. All authors took part in editing the article and agreed to the published version of the manuscript.

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Figures

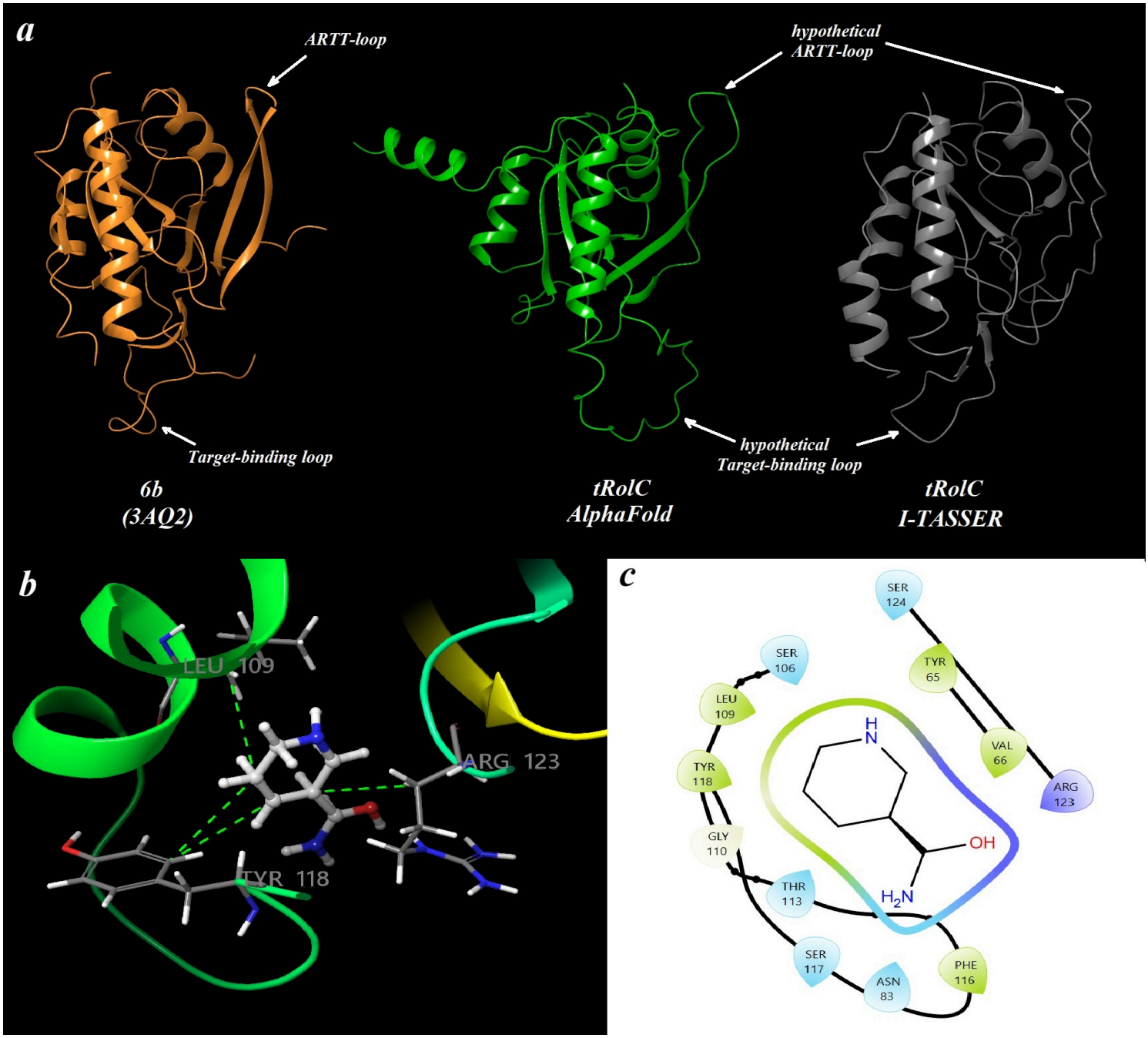


Figure 1

a – structures of predicted proteins 6b and tRolC using AlphaFold and I-TASSER. b – docking of the predicted tRolC protein structure with nicotinamide: hydrophobic interactions are shown by a green dotted line. c – schematic representation of ligand interactions with certain amino acids (blue – positively charged, light blue – polar, green – hydrophobic, grey – non-specific).

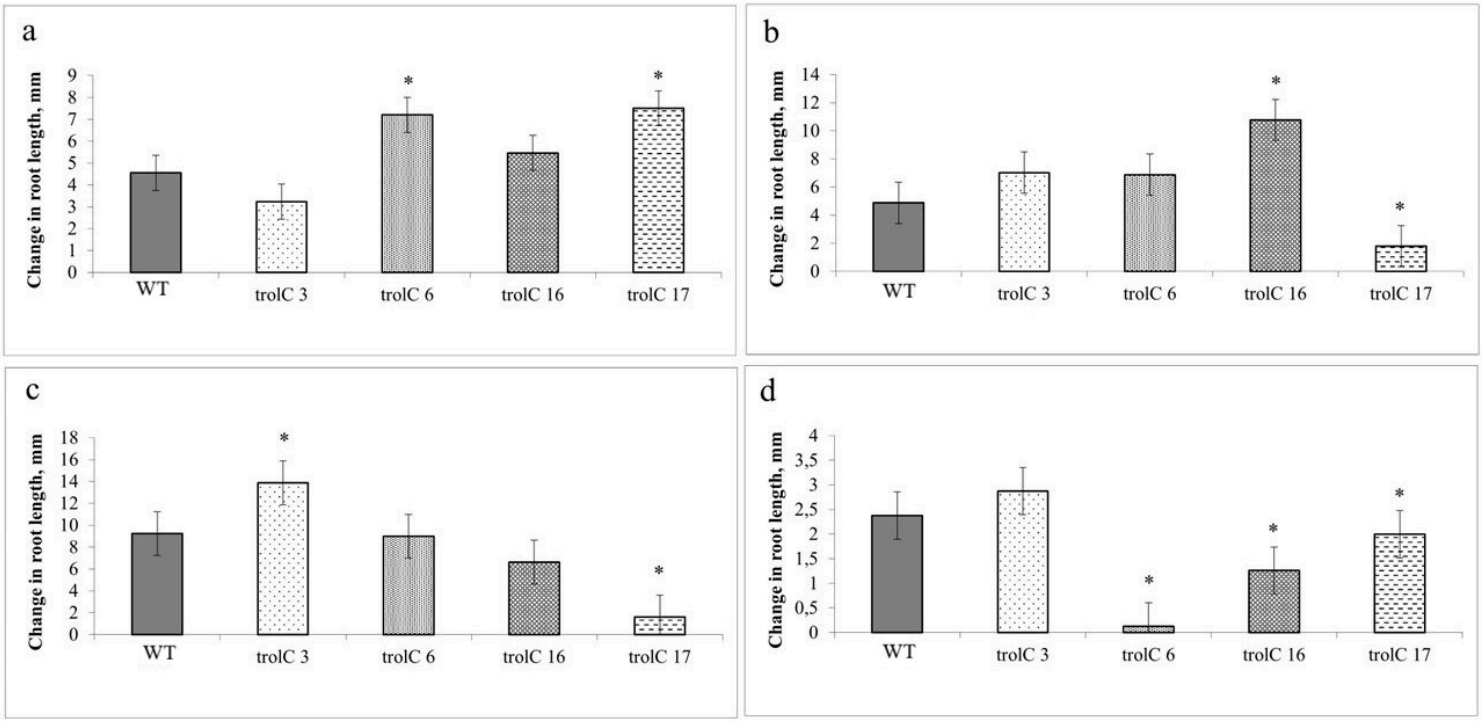


Figure 2

Analysis of changes in tobacco root length: a – under normal conditions; under the influence of stress factors: b – 50 mM NaCl; c – 100 mM NaCl; d – +12°C (d). trolC3-trolC17 – transgenic plant lines. Asterisks (*) indicate statistically significant differences in root length between WT and transgenic plants (p≤0.05).

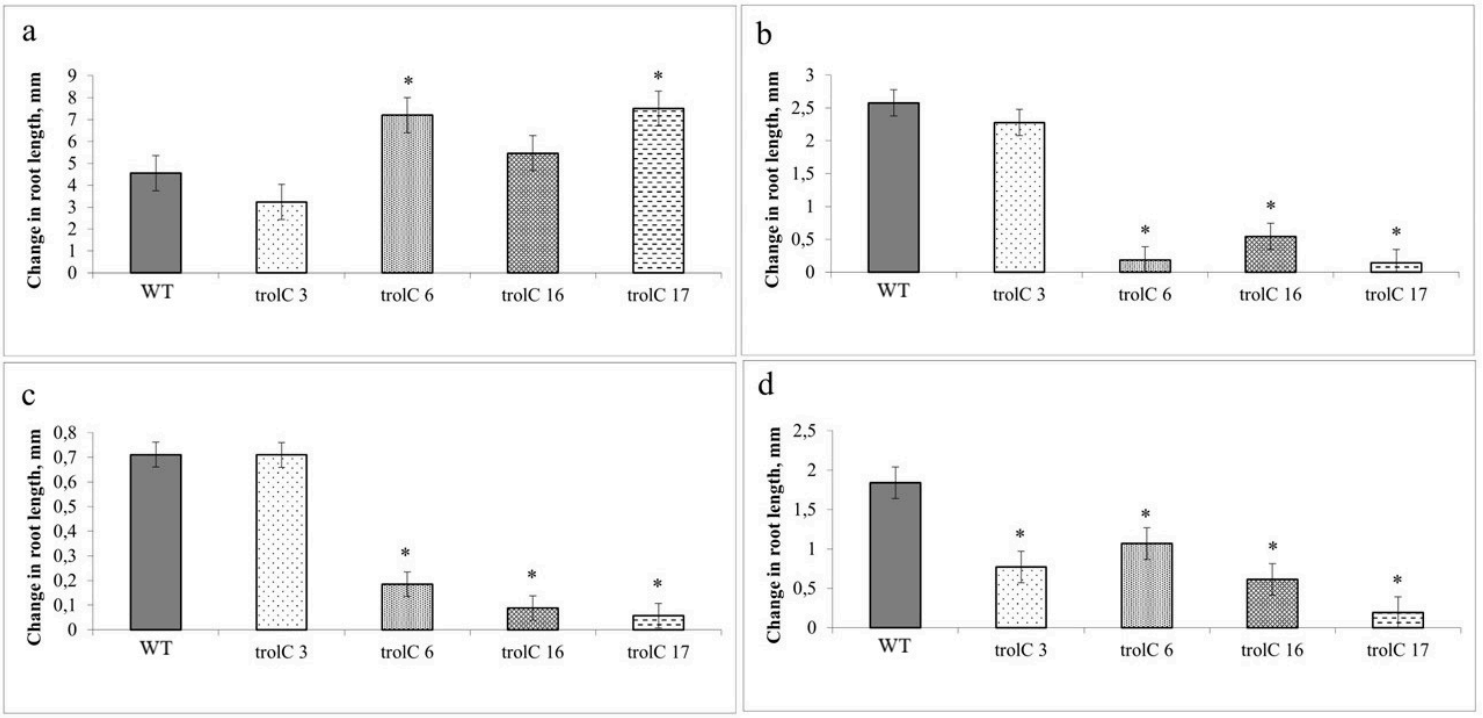


Figure 3

Analysis of changes in tobacco root length: a – under normal conditions, following cadmium treatment: b – 100 μM CdAc; c – 200 μM CdAc; d – 400 μM CdAc. trolC3-trolC17 – transgenic plant lines. Asterisks (*) indicate statistically significant differences in root length between WT and transgenic plants (p≤0.05).

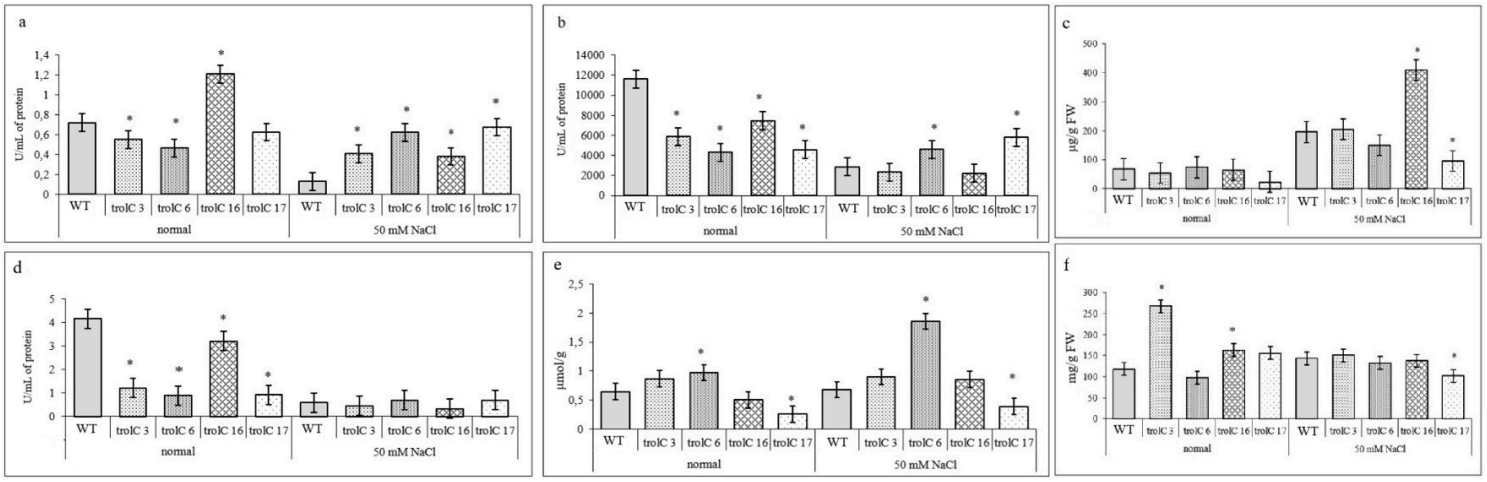


Figure 4

Analysis of the antioxidant system in the roots of transgenic tobacco plants under NaCl stress: a – ascorbate peroxidase activity. b – catalase activity. c – proline content. d – guaiacol peroxidase activity. e – hydrogen peroxide content. f – water-soluble sugar content. trolC3-trolC17 – transgenic plant lines. Asterisks (*) indicate statistically significant differences between WT and transgenic plants (p≤0.05).

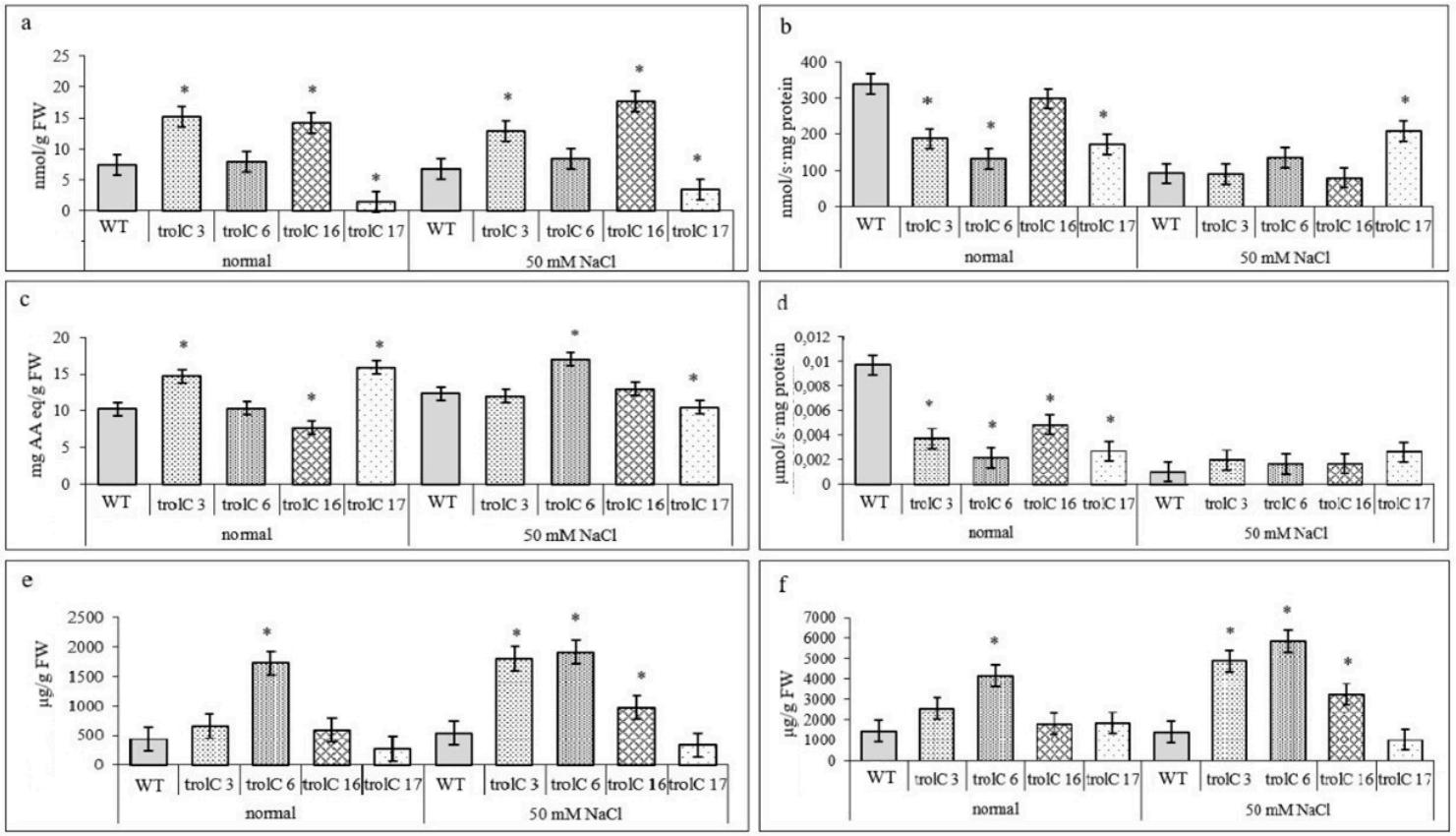


Figure 5

Analysis of the antioxidant system in the roots of transgenic tobacco plants exposed to NaCl: a – malondialdehyde content. b – superoxide dismutase activity. c – total antioxidant capacity. d – glutathione-S-transferase activity. e – reduced glutathione content. f – oxidized glutathione content. trolC3-trolC17 – transgenic plant lines. Asterisks (*) indicate statistically significant differences between WT and transgenic plants (p≤0.05).

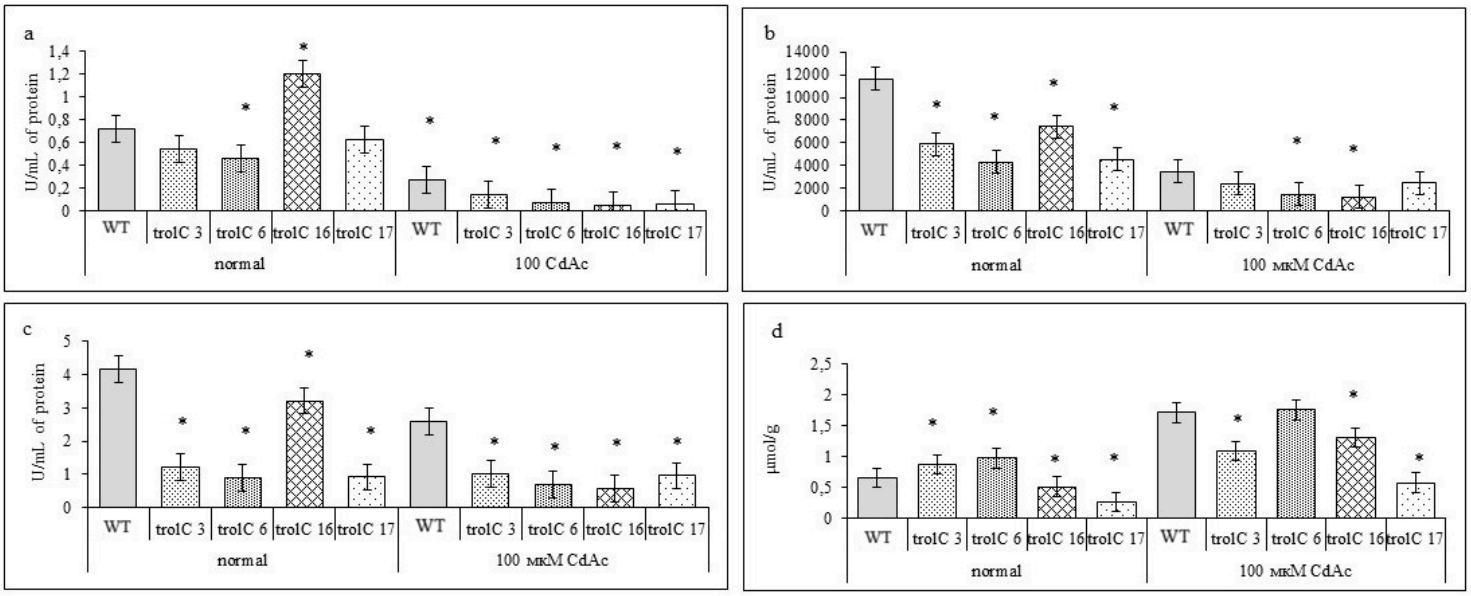


Figure 6

Analysis of the antioxidant system in the roots of transgenic tobacco plants exposed to CdAc: a – ascorbate peroxidase activity; b – catalase activity; c – guaiacol peroxidase activity; d – hydrogen peroxide content. trolC3-trolC17 – transgenic plant lines. Asterisks (*) indicate statistically significant differences between WT and transgenic plants (p≤0.05).

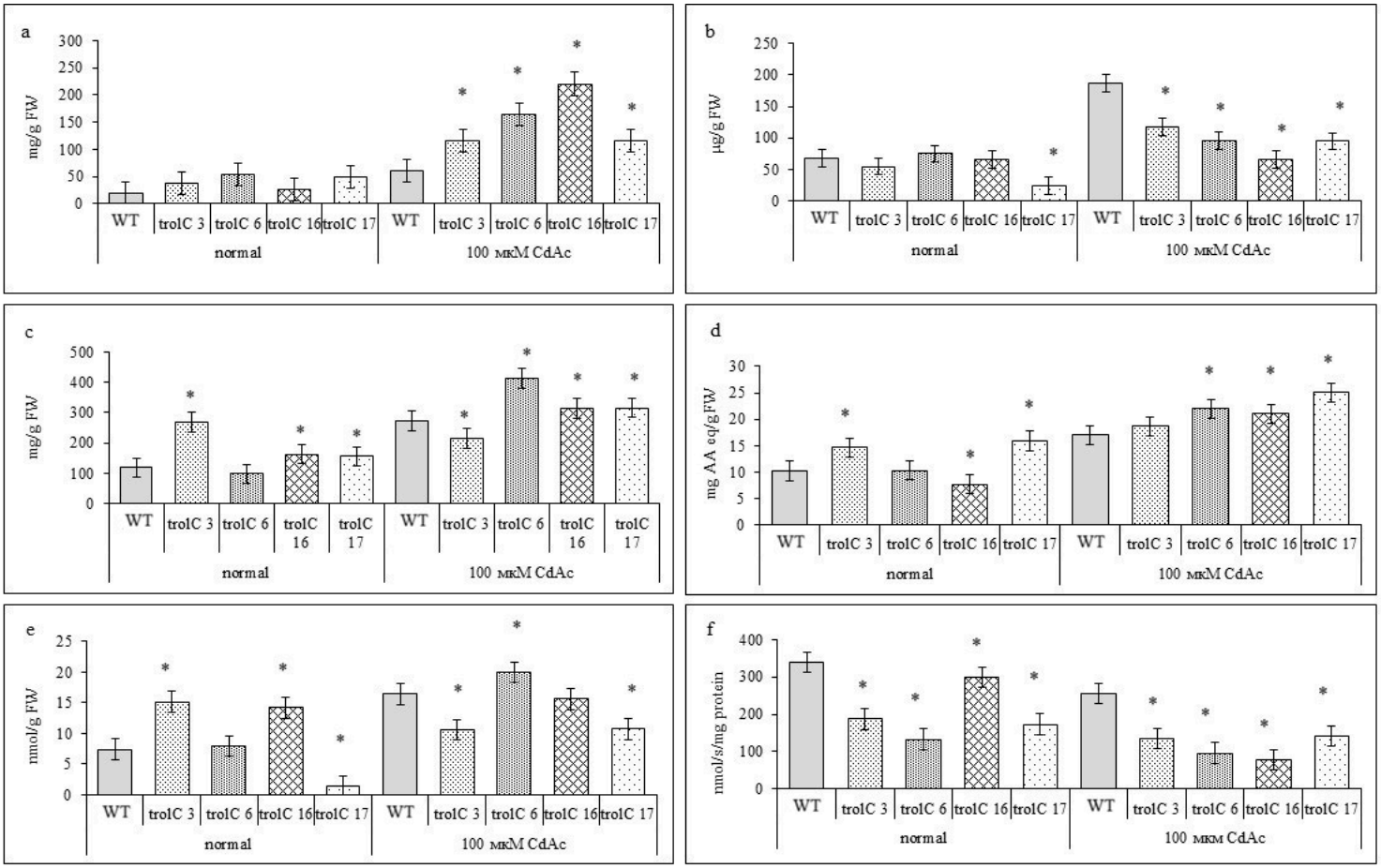


Figure 7

Analysis of the antioxidant system in the roots of transgenic tobacco plants exposed to CdAc: a – total soluble protein content; b – proline content; c – water-soluble sugar content; d – total antioxidant capacity; e – malondialdehyde content; f – superoxide dismutase activity. trolC3-trolC17 – transgenic plant lines. Asterisks (*) indicate statistically significant differences between WT and transgenic plants ($p \leq 0.05$).

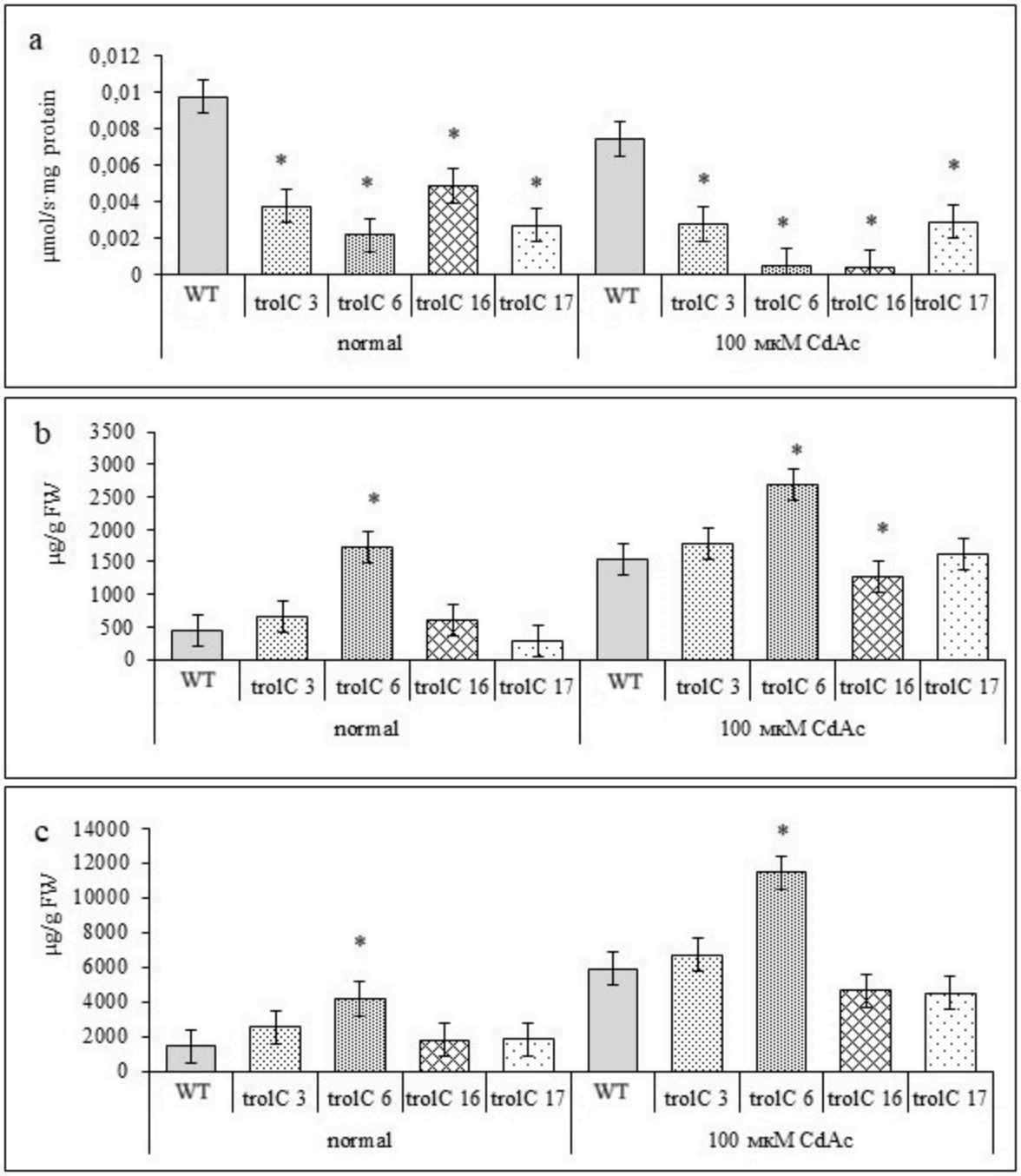


Figure 8

Analysis of the antioxidant system in the roots of transgenic tobacco plants exposed to CdAc: a – glutathione S-transferase activity; b – reduced glutathione content; c – oxidized glutathione content. trolC3-trolC17 – transgenic plant lines. Asterisks (*) indicate statistically significant differences between WT and transgenic plants ($p \leq 0.05$).

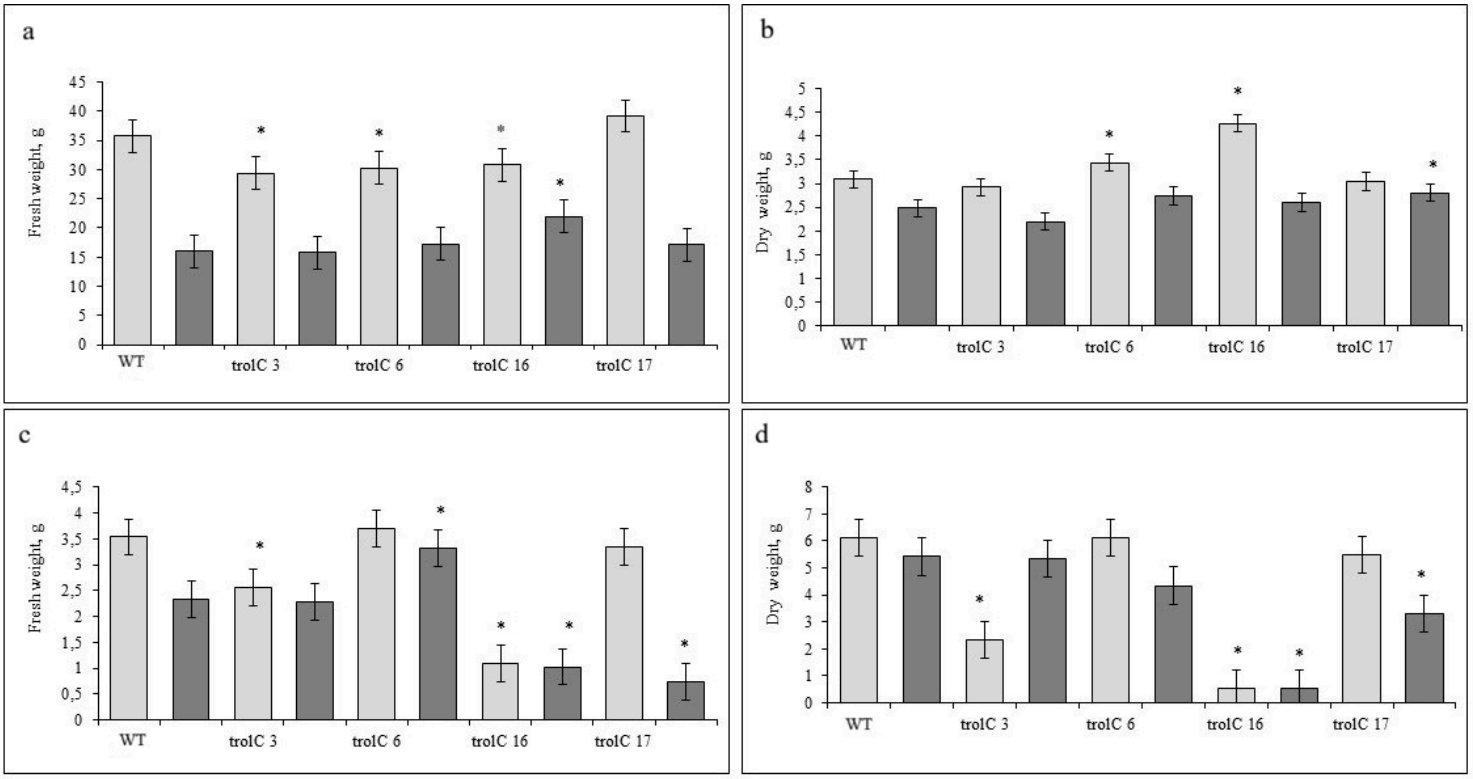


Figure 9

Morphometric parameters of transgenic tobacco plants with reduced expression of the *trolC*gene under stress conditions: a – fresh weight of the aboveground biomass under drought conditions; b – dry weight of the aboveground biomass under drought conditions; c – fresh weight of the aboveground biomass under cold conditions; d – dry weight of the aboveground part under cold stress. The light grey bar indicates morphometric parameters under normal conditions and the dark grey bar indicates morphometric parameters under drought stress. Plant age under drought stress was 70 days, and under cold stress was 52 days. Asterisks (*) indicate statistically significant differences between WT and transgenic plants (p≤0.05).

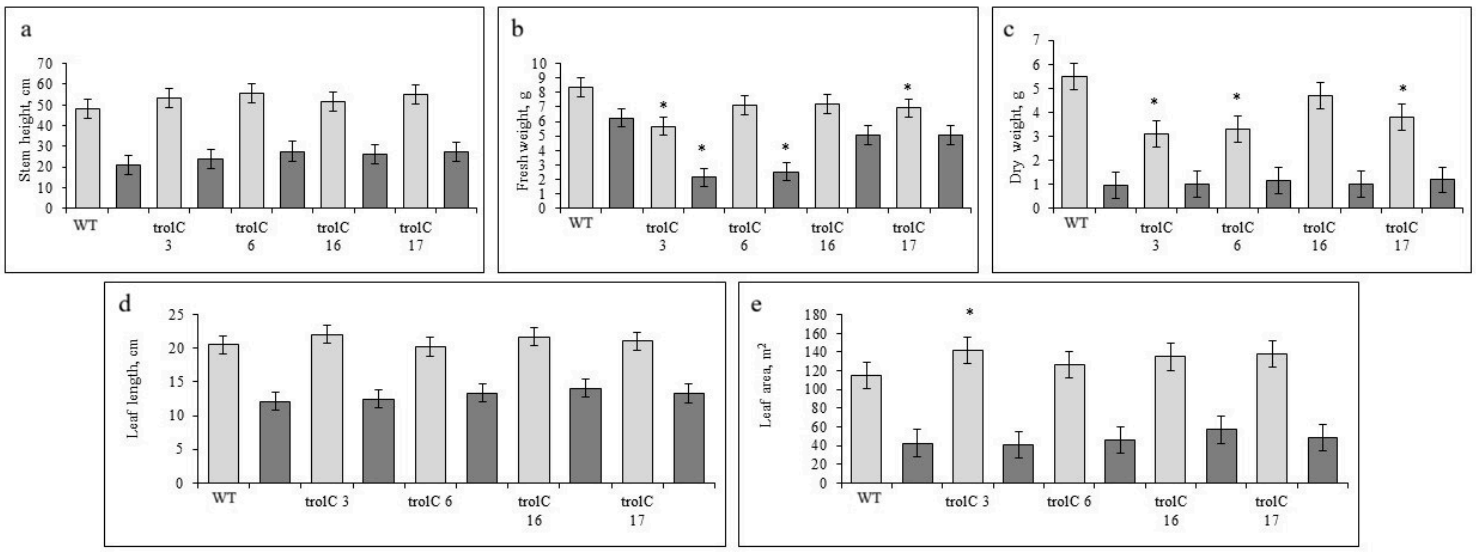


Figure 10

Morphometric parameters of transgenic tobacco plants with reduced expression of the *trolC*gene under salinity stress: a – stem height; b – fresh weight of the aboveground parts; c – dry weight of the aboveground parts; d – leaf length; e – leaf area. Plant age – 72 days. Asterisks (*) indicate statistically significant differences between the control and transgenic plants (p≤0.05).

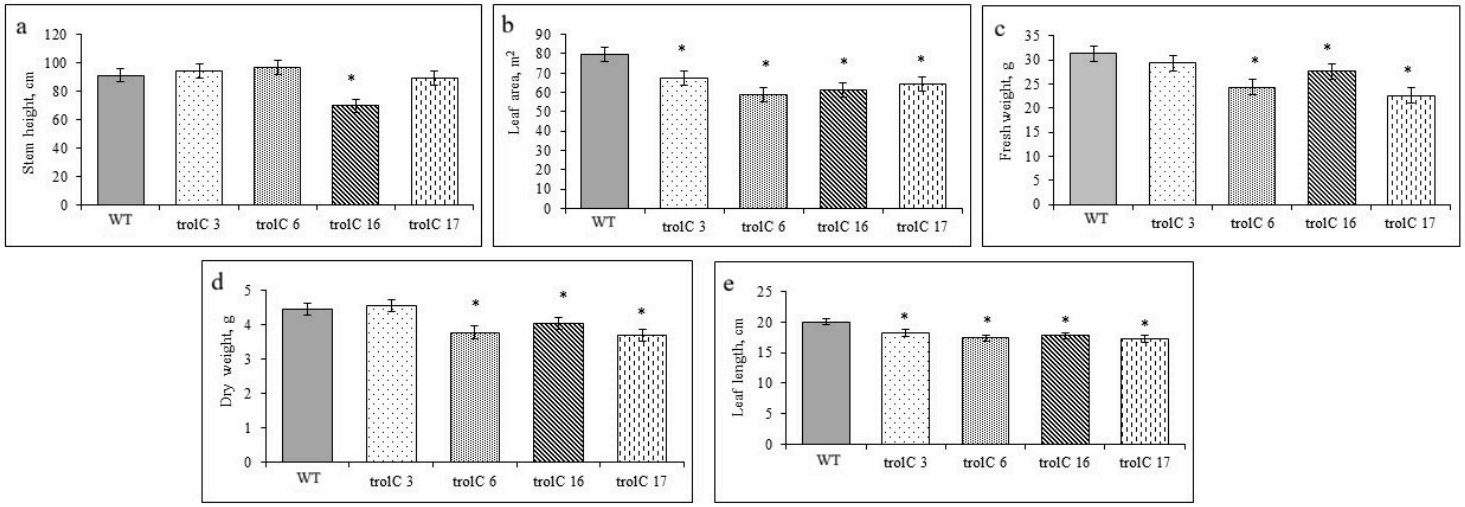


Figure 11

Morphometric parameters of transgenic tobacco plants with reduced expression of the *trolC* gene under normal growing conditions, grown to the flowering stage: a – stem height; b – leaf area; c – fresh weight of the aboveground parts; d – dry weight of the aboveground parts; e – leaf length. Asterisks (*) indicate statistically significant differences between WT and transgenic plants (p≤0.05).

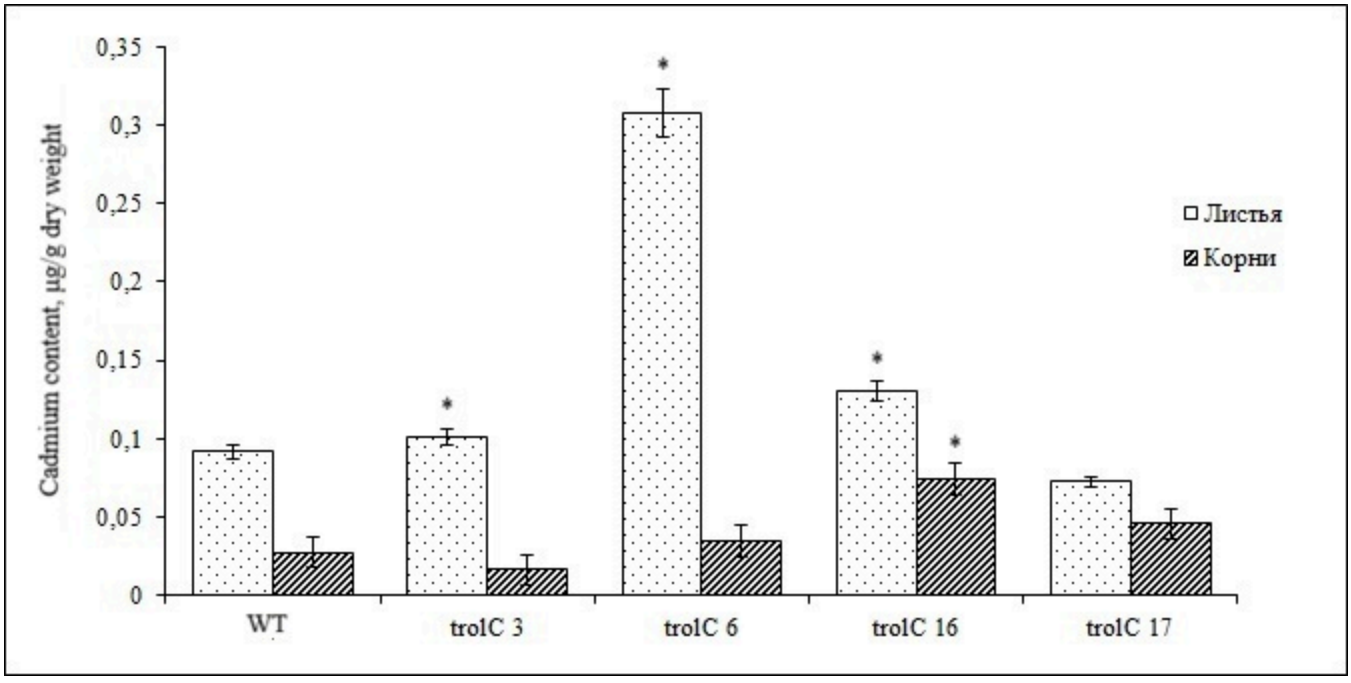


Figure 12

Cadmium ion content in the leaves and roots of wild-type tobacco plants and transgenic plants with reduced expression of the *trolC* gene following treatment with cadmium acetate

Supplementary Files

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