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ORIGINAL PAPER

Transcriptional regulation of enzymes involved in ROS metabolism and abiotic stress resistance in *rolC*-transformed cell cultures

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Abstract
The *rolC* (root locus *C*) gene from *Agrobacterium rhizogenes* belongs to the family of *plast* oncogenes causing significant changes in plant growth and morphology. The overexpression of the *rolC* provokes the suppression of reactive oxygen species (ROS) generation and reinforces stress tolerance in transgenic cells. In this study, we tested whether this effect is associated with changes in the expression of oxidative and antioxidative enzymes such as NADPH oxidase (termed respiratory burst oxidase homologs; RBOH), superoxide dismutase, catalase, and ascorbate peroxidase. The expression of genes, encoding RcRBOHs and AtRBOHs, the major producers of ROS, was two times lower in both *Rubia cordifolia* and *Arabidopsis thaliana rolC*-expressing cultures. Temperature and salt stresses induced expression of *Rboh* genes, yet this effect was significantly less evident in transgenic calli. The mRNA transcript abundance of most of the antioxidant genes was strongly inhibited in *rolC*-expressing cultures under normal conditions. However, expression levels of antioxidant enzymes were 1.5 times higher in transgenic calli compared to the control under tested abiotic stresses. Our results indicate that RolC specifically regulates NADPH oxidase and antioxidant genes expression to suppress ROS production. In summary, these effects result in an increased tolerance of *rolC*-expressing cells to abiotic stress conditions.

Keywords *Arabidopsis thaliana* · *Rubia cordifolia* · *Agrobacterium rhizogenes* · *rolC* · NADPH oxidase · Antioxidant enzyme · Abiotic stress · Nucleoli

Introduction

Plant *rol* oncogenes located in T-DNA of *Agrobacterium rhizogenes* are known to be an effective tool for plant bioengineering, causing plural effects in transformed cells (Pavlova et al. 2014; Otten 2018). Among other *rol* group members, *rolB* and *rolC* are of special interest since they were found to function as strong activators of secondary metabolism in different plant species (Matveeva et al. 2015; Paolis et al. 2019). Using individual or combined action of *rol* genes, plant cell lines accumulating high amounts of terpenoids (Kiani et al. 2012; Dilshad et al. 2015), flavonoids (Dilshad et al. 2016), alkaloids (Verma et al. 2012), and phenolic compounds (Mardani-Nejad et al. 2016) have been obtained. Recent metabolomic analysis of *rolABC*- and *rolC*-transformed *Lactuca sativa* plants revealed a remarkably widespread and complicated impact on the plant's metabolism, displaying simultaneous activating and inhibitory effects on a variety of compounds, with several of them altered by 1,000% (Ismail et al. 2019a).

It was previously established that both *rolB* and *rolC* genes reduce intracellular reactive oxygen species (ROS) levels. In particular, the constitutive expression of *rolC* led to the suppression of steady-state level of ROS in transgenic calli (Bulgakov et al. 2008). Similar results were obtained in *rolB*-transformed cell cultures of *Arabidopsis thaliana*, *Rubia cordifolia*, and *Panax ginseng*, and a positive correlation between the level of transgene expression and ROS decrease was observed (Bulgakov et al. 2012). Both genes

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Main ConclusionThe RolC oncogene is localized in the nucleoli of transformed cells and downregulates Rboh gene expression in normal and stressed conditions.

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prevented paraquat-induced oxidative burst; however, only *rolB* was able to protect transgenic cells from excessive ROS accumulation, indicating that RolB and RolC might have different strategies in regulating ROS metabolism. The combined action of the *rol* genes (wild-type *A. rhizogenes* A4) resulted in a moderate decrease in ROS (Shkryl et al. 2010).

ROS are known to function as signal transmitters during various biological processes, including growth, development, and stress-related reactions (Ma et al. 2012; Baxter at al. 2014; Wang et al. 2018). Plants possess a broad set of ROS-generating enzymes, namely amine oxidases, class III peroxidases, lipoxygenases, quinone reductases, apoplastic oxalate oxidases, and plasma membrane NADPH oxidases (Mittler 2017; Marino et al. 2012). Among others, NADPH oxidases, designated as respiratory burst oxidase homologues (RBOHs), are considered to be the primary source of ROS (Suzuki et al. 2011). It is known that ROS overproduction or insufficient decomposition may cause toxic effects provoking cell damage and death (Choudhary et al., 2019). Particularly, Cu/Zn, Mn, and Fe superoxide dismutases (CSD, MSD, FSD), ascorbate peroxidase (Apx), catalase (Cat), and glutathione peroxidase (GPX) play pivotal roles in ROS detoxification (Filiz et al. 2019; Kapoor et al. 2019). Some plant pathogens have evolved specialized mechanisms of decreasing ROS levels aimed to inactivate immune responses. For example, in cauliflower mosaic virus-infected *Arabidopsis* plants, P6 protein acts as a suppressor of oxidative burst making them more susceptible to secondary invasions by *Pseudomonas syringae* (Zvereva et al. 2016). P6 activates target of-rapamycin (TOR) kinase, which links environmental signals and growth processes in plants (Rosenberger and Chen 2018), thus leading to ROS suppression and salicylic acid-dependent autophagy. *A. tumefaciens* contains two chromosomal catalase genes, i.e. *katA* and *catE*, for which expression levels are regulated independently (Prapagdee et al. 2004a, b). The *katA* gene-encoded bifunctional catalase-peroxidase catalyzes the decomposition of H_2O_2 and promotes bacterial ability to cause tumors on plants (Xu and Pan 2000; Eiamphungporn et al. 2003). *CatE* is less important in the protection of bacteria from toxic H_2O_2 molecules and acts synergistically with *katA* (Prapagdee et al. 2004b).

Our previous data suggest that *A. rhizogenes* also uses the strategy aimed at suppression of ROS production in host cells (Shkryl et al. 2010). *R. cordifolia* calli transformed with pRiA4 plasmid showed a decreased ROS level and enhanced expression of several genes encoding ROS scavenging enzymes. One of the pathogenic determinants of *A. rhizogenes* pRiA4, the *rolB* gene, upregulated expression levels of antioxidant genes, encoding the *CSD*, *Cat*, *Apx* and particular *Rboh* isoforms (Bulgakov et al. 2012; Veremeichik et al. 2016). In this study, it was attempted to reveal the effect of the *A. rhizogenes* pRiA4 *rolC* oncogene on the expressional pattern of NADPH oxidase and antioxidant enzyme genes in transgenic callus cultures of *A. thaliana* and *R. cordifolia* in normal conditions and exposed to abiotic stresses.

Materials and methods

Plant cell cultures and treatments

The control, non-transformed (R), and transformed callus cultures of *R. cordifolia* were established and characterized previously (Bulgakov et al. 2008). The transgenic callus line with a comparatively high expression level of the *rolC* gene (RCH) was employed in this study. Calli were cultivated on agarized W medium supplemented with 0.5 mg/L 6-benzylaminopurine and 2.0 mg/L α-naphthaleneacetic acid in 100 ml glass Erlenmeyer flasks.

A. thaliana non-transformed (At) and *rolC*-transgenic callus cultures were obtained as described (Bulgakov et al. 2012) from wild-type Columbia (Col-0) seedlings. For transformation, *A. tumefaciens* strain GV3101 harboring the pPCV002-CaMVC construct, carrying *rolC* under the control of 35 S CaMV promoter was used (Spena et al. 1987). Several *rolC*-transgenic lines were selected from primary transformed calli and one of them, having the transgene expression level comparable to that in RCH calli, was used in this study. In accordance with the previously adopted nomenclature, the transgenic callus line with a comparatively high level of the *rolC* gene expression was denoted as "ACH." *Arabidopsis* calli were cultivated using agarized W medium supplemented with 0.4 mg/L 2,4-dichlorophenoxyacetic acid in 50 ml glass Erlenmeyer flasks.

Routine cultivation of *R. cordifolia* and *A. thaliana* cell lines was conducted in the dark at 24°C with 30-day subculture intervals. For abiotic stress experiments, which consisted in temperature and salinity stress, 200 mg of calli were placed in 20 × 200 mm glass test tubes containing 15 ml of medium. A temperature stress was performed with *R. cordifolia* and *A. thaliana* calli cultivated at 12°C and 28°C for the entire subculture period in a KS-200 climatic test chamber (Smolensk SKTB SPU, Russia). A salinity stress treatment was performed by supplementation of the growth medium with varying concentrations (60 or 120 mM) of sodium chloride (NaCl). At the end of the experiments, samples were harvested, weighed, and used for real-time PCR analysis.

Isolation of cDNA and real-time PCR analysis

Isolation of total RNA and first-strand cDNA synthesis were carried out as described previously (Shkryl et al. 2010). A quantitative real-time PCR (qPCR) analysis was performed using the Bio-Rad CFX96 Real-Time System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with 2.5x SYBR green PCR master mix, containing ROX as a passive reference dye (Syntol, Moscow, Russia) as described by Veremeichik et al. (2014).

The gene-specific primer pairs used in the qPCR are listed in Supplementary Table 1. Actin genes of *R. cordifolia* and *A. thaliana* were used as reference genes. Primer pairs efficiency of > 95% was verified with a standard curve that was established using serial dilutions of the corresponding purified PCR products. Four biological replicates, resulting from four different RNA extractions, were used and three technical replicates were analyzed for each biological replicate. Data were analyzed using CFX Manager Software (Version 3.1; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Laser confocal imaging of intracellular ROS

Measurements of intracellular ROS were performed as described previously (Bulgakov et al. 2008; Shkryl et al. 2010). Plant cell suspensions were grown in liquid medium for 4–12 days and passed through 100 µm mesh nylon filter to remove large cell clusters. Single cells and 10–20 cell aggregates were gently centrifuged and resuspended in liquid W medium containing 50 µM 2,7-dichlorodihydrofluorescein diacetate (H_2DCF-DA, Molecular Probes, Inc., Eugene, OR, USA) and incubated at 25 ± 1°C in the dark for 10 min. Dye-loaded cells were washed in the medium and resuspended. Intracellular oxidation of H_2DCF-DA yielded DCF detected by the examination of fluorescence in single living cells using a LSM 510 META confocal laser scanning microscope (Carl Zeiss, Germany) at the Instrumental Centre of Biotechnology and Gene Engineering of FSCEATB FEB RAS. A microscope equipped with an argon laser with an effective power of 30 mW was used. For the analysis, the intensity of the Ar laser was set at 5.9% of the maximal value. All images were recorded as 40 s time series with intervals of 0.5 ms and analyzed with LSM 510 Release 3.5 software (Carl Zeiss, Germany). Data are presented as the mean from several replicated experiments in controlled conditions (at least 40–50 cells were analyzed in each experiment).

Construction of the *rolC-EGFP* chimeric gene and its transient expression in *R. cordifolia* protoplasts

To evaluate subcellular localization of RolC protein in plant cells, *rolC* gene was fused to the N-terminus of the *EGFP* gene. The full-length sequence of *rolC* (GenBank accession no. X03433) was amplified using pPCV002-CaMVC plasmid DNA and gene-specific primers: forward *rolC*-D 5'-TAC GAA TTC ATG GCT GAA GAC GAC CTG TG-3' (the *Eco*RI restriction site is underlined) and reverse *rolC*-R 5'-TAC GGG CCC GCC GAT TGC AAA CTT GCA CT-3' (the *Apa*I restriction site is underlined). PCR was performed using previously described conditions (Veremeichik et al. 2014), and 540-bp long fragment was obtained. pSAT6-EGFP-N1 plasmid (Tzfira et al. 2005), containing the tandem 35 S CaMV promoter, tobacco etch virus (TEV) leader, and 35 S CaMV terminator, was used as a plant expression vector. *RolC* was sub-cloned as an *Eco*RI-*Apa*I fragment into the same sites of linearized plasmid producing an in-frame fusion to N-terminal end of EGFP. Obtained construction pSAT6-rolC-EGFP was checked for the absence of mutations by DNA sequencing using an ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

The newly constructed expression cassette pSAT6-rolC-EGFP and original plasmid vector pSAT6-EGFP-N1 were used for the transformation of *R. cordifolia* protoplasts using PEG-mediated transfection. Protoplasts were isolated according to the protocol described previously (Yoo et al. 2007). In brief, enzyme hydrolysis of freshly collected leaf slices was performed for two hours at room temperature in a reaction mixture containing 1.5% cellulase, 0.4% macerozyme R-10, 0.4 M mannitol, 20 mM KCl, 20 mM MES-NaOH, pH 5.7, and 0.1% BSA. After incubation, protoplasts were washed twice in W5 solution containing 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, and 2 mM MES-NaOH, pH 5.7. Cells were spun down at 100 g for three minutes and the buffer was exchanged to MMG solution, containing 15 mM $MgCl_2$, 0.55 M mannitol, 4 mM MES-NaOH, pH 5.7. Protoplasts were incubated at room temperature for an additional 30 min prior to the transfection procedure. Equal amount of PEG buffer containing 40% PEG 4000, 0.2 M mannitol, 100 mM $CaCl_2$, and 30 µg of plasmid DNA were added into protoplasts followed by gentle agitation and incubation for 15 min in darkness at 25 °C. Afterward, protoplast were gently washed with W5 solution three times and left for an extra 16–18 h in darkness at 25 °C. In a typical experiment, 1×10^8 protoplasts per 10 µg of plasmid DNA were used for transfection.

Laser confocal imaging of RolC-EGFP fusion protein in living cells

The detection of the EGFP fluorescence in *EGFP*- and *rolC-EGFP*-transformed cells was performed using LSM 510 META and LSM 710 LIVE confocal laser scanning microscopes (Carl Zeiss, Germany) with an excitation wavelength of 488 nm and an emission filter of 505–530 nm. Non-transformed cells were used as a negative control. To monitor emission maximum EGFP, spectral imaging in the 505–720 nm range with a 10.4 nm detection bandwidth was performed in the all examined cell lines. Video files and single frames of the captured images were recorded and analyzed with LSM 510 Release version 4.2 and ZEN 2011 software.

Statistical Analysis

All values are expressed as the mean ± SE. For statistical evaluation, Student's *t* test was used to compare the two independent groups. For comparison among multiple data, analysis of variance (ANOVA) followed by a multiple comparison procedure was employed. Fisher's protected least significant difference (PLSD) *post-hoc* test was employed for the inter-group comparison. The level of statistical

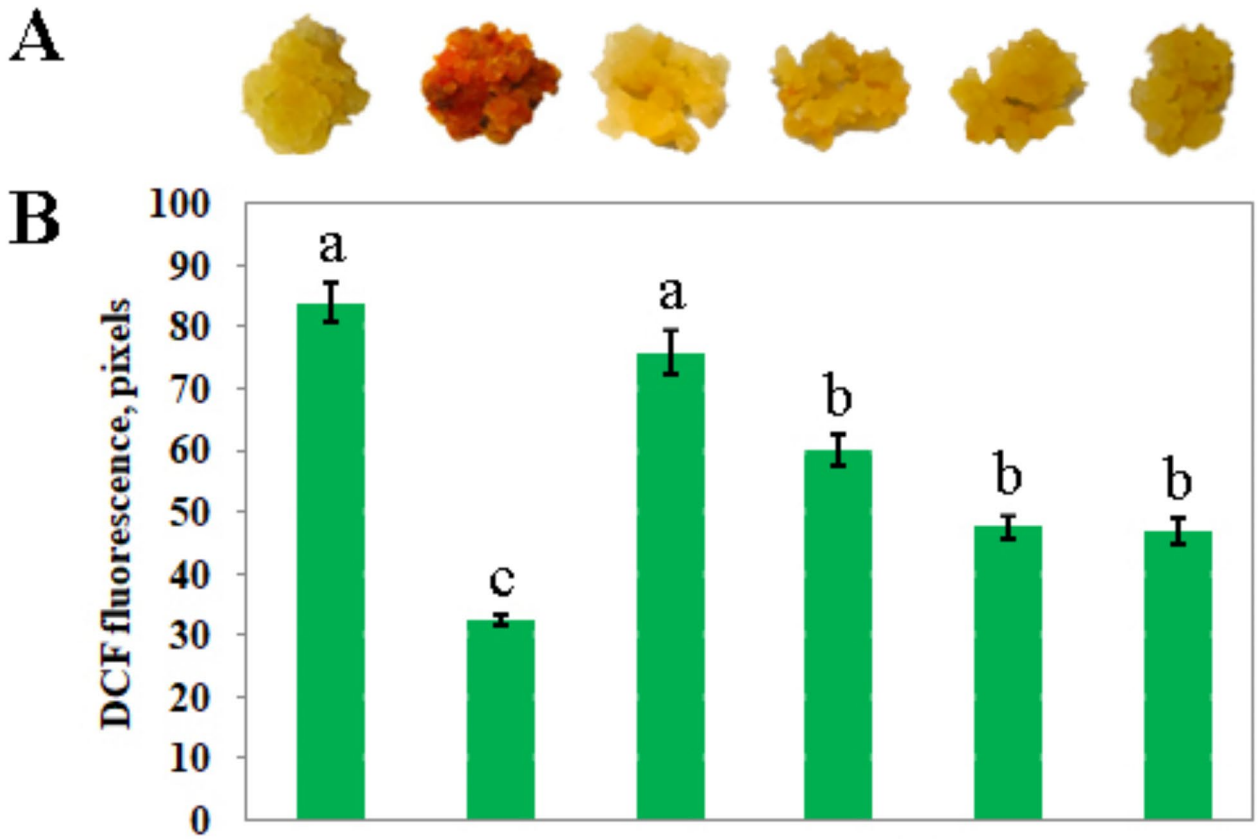

Fig. 1 The morphology of the *R. cordifolia* and *A. thaliana* callus lines used in experiments (A). Steady-state ROS levels in the *R. cordifolia* control (R) and *rolC*-expressing (RCH) callus line as well as in the *A. thaliana* control (At) and *rolC*-expressing (AtC1, AtC2, and AtC3) callus lines (B). The fluorescence of DCF reflects intracellular ROS abundance. The relative expression levels of the *rolC* gene calculated from the qPCR analysis are shown below the graph (C). Values are presented as means ± standard errors. Different letters indicate statistically significant differences of means ($P < 0.05$), Fisher's LSD

significance was set at $p < 0.05$. Pearson correlation analysis was used to reveal relationships between two variables.

Results

*Arabidopsis thaliana rolC-expressing*lines and their characterization

We first aimed to obtain *rolC*-expressing *A. thaliana* cell lines as a model system for the investigation of the oncogene effects. Three independent callus lines have been obtained

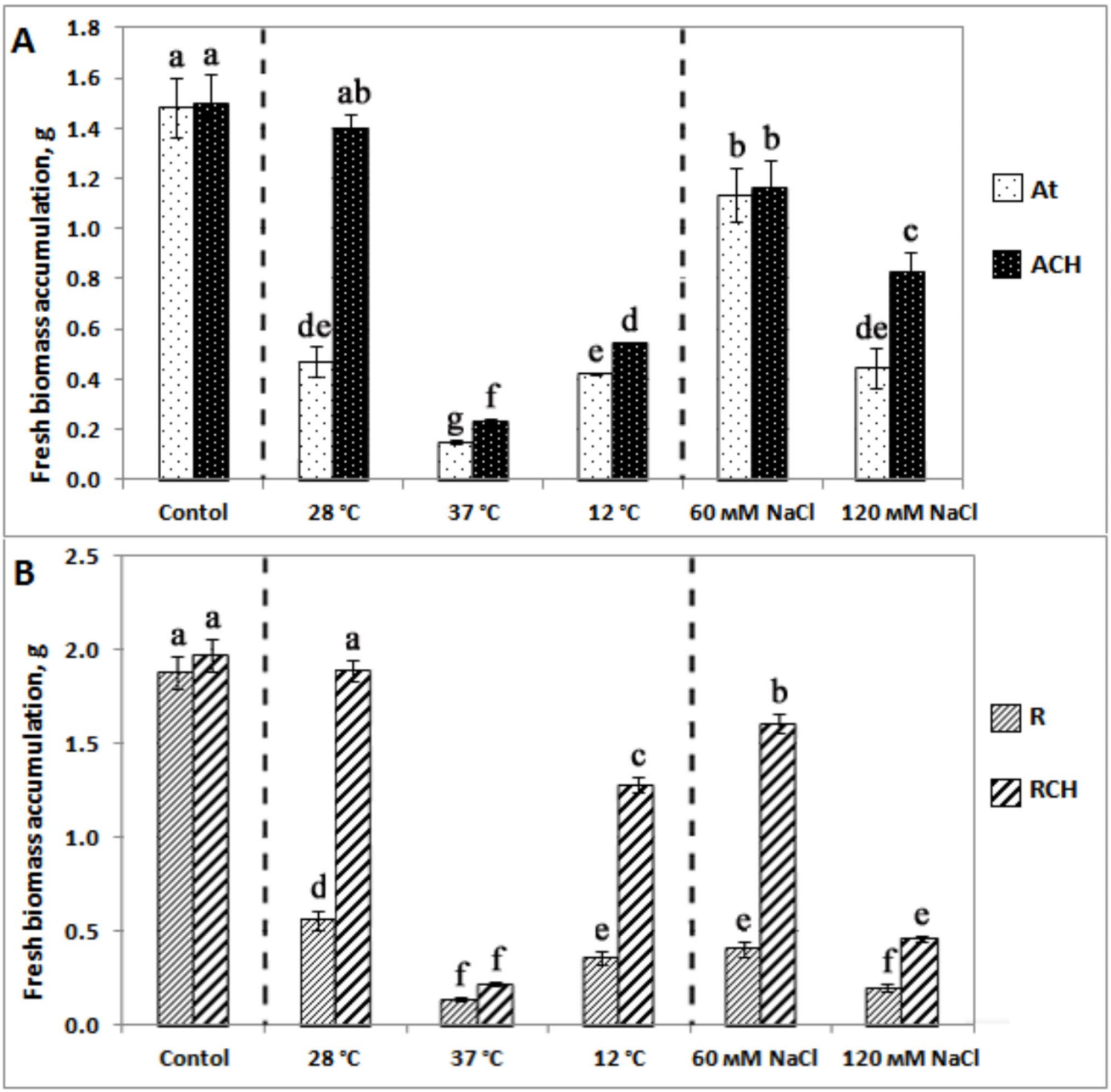

Fig. 2 The effect of different temperature regimes and NaCl concentrations on the growth of *A. thaliana* (A) and *R. cordifolia* (B) callus lines. The control (At and R) and *rolC*-expressing (ACH and RCH) calli were cultivated at high (28°C and 37°C), low (12°C) temperatures, and in the presence of different concentrations of NaCl (60 and 120 mM) for one month. The normal cultivation temperature was 24°C. Values are presented as means ± standard errors. Different letters indicate statistically significant differences of means ($P < 0.05$), Fisher's LSD

(namely AtC1, AtC2, and AtC3) and levels of intracellular ROS accumulation have been investigated in comparison with the high-level *rolC R. cordifolia* callus line – RCH (Fig. 1). Our measurements showed that DCF fluorescence levels in *A. thaliana rolC*-transgenic calli were 79%, 63%, and 62% in AtC1, AtC2, and AtC3, respectively, compared to At control cells. At the same time, DCF fluorescence of RCH cells was only 39% of the control R cells.

As most of *rol*-pronounced effects are gene-dose-dependent, we studied the expression levels of *rolC* gene in newly obtained cell lines of *A. thaliana* and *R. cordifolia* RCH line (Fig. 1). According to qPCR data, *rolC* mRNA transcript abundance in AtC1 and AtC2 cells was almost equal and showed approximately 1.6 times lower levels than in AtC3 cells. Moreover, *rolC* transgene expression in the AtC3 line was comparable to its level in RCH cells. Taking all of these factors into consideration, the AtC3 callus line was designated as ACH, i.e. the *A. thaliana* cell line with a comparatively high level of the *rolC* gene expression, and was used for further investigation.

Biomass accumulation of the *rolC*-expressing calli under salt and temperature stresses

To evaluate the differences of cells' sensitivity to temperature and salt stresses, we conducted the parallel cultivation of *A. thaliana* and *R. cordifolia* control (At and R, respectively) and *rolC*-expressing calli (RCH and ACH, respectively) under higher (28ºC and 37ºC) and lower (12ºC) temperatures (compared to normal conditions at 24ºC), as well as in the presence of different NaCl concentrations (60 and 120 mM).

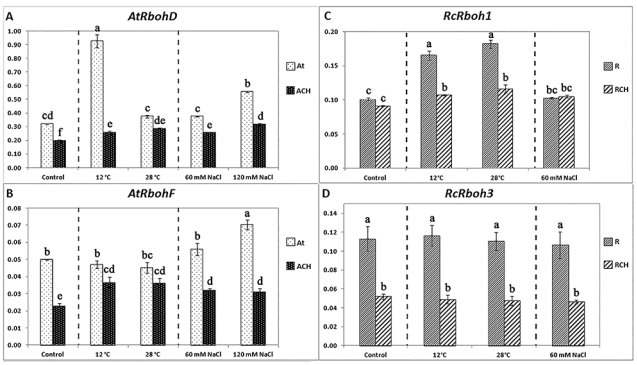

Fig. 3 Expression of *A. thaliana* and *R. cordifolia* NADPH oxidase genes in control callus cultures (At and R, respectively) and in *rolC*-expressing callus cultures (ACH and RCH, respectively) under high (28ºC), low (12ºC) temperatures, and in the presence of different NaCl concentrations (60 and 120 mM). A: expression levels of *AtRbohD* isoform, B: expression levels of *AtRbohF* isoform, C: expression levels of *RcRboh1* isoform, and D: expression levels of *RcRboh3* isoform. Calli were grown for 30 days before harvesting and analysis. Values are presented as means ± standard errors. Different letters above the bars indicate statistically significant differences of means ($P < 0.05$) for each of the *Rboh* isoform, Fisher's LSD

High temperature significantly inhibited growth of both, R and At, control cell lines (Fig. 2). A temperature rise of 4ºC over control conditions resulted in a more than three-fold decrease of R and At calli biomass accumulation. At the same time, growth of the *rolC*-expressing callus lines was not inhibited. A more significant temperature rise of 13ºC over control conditions almost completely abolished control calli growth, whereas ACH and RCH lines, although being inhibited by 85% and 88%, respectively, retained the ability for growth.

Cultivation of calli under conditions of cold (12 °C) demonstrated increased resistance of the *rolC*-expressing lines as compared with the control cultures (Fig. 2). Biomass accumulation of ACH was 36% of those under normal conditions, while the growth of At culture reached only 28%. Even more pronounced tolerance was observed in *R. cordifolia rolC*-transgenic calli. Cold inhibited the growth of the RCH culture by 35%, while the growth of the control callus line was inhibited by 81%.

Salt stress affected growth of *A. thaliana* and *R. cordifolia* callus lines differently (Fig. 2). In general, *A. thaliana* was more resistant to NaCl stress than *R. cordifolia*. At the concentration of 60 mM, At and ACH calli growth was similarly inhibited by 23%. A further increase of NaCl concentration revealed that *rolC*-expressing cells are more tolerant to salt stress. Biomass accumulation of ACH cultivated in the presence of 120 mM of NaCl was 55% of those under normal conditions, while the growth of At culture reached only 30%. Meanwhile, 60 mM of NaCl inhibited the growth of the *R. cordifolia* control callus line by 78%, while at 120 mM of NaCl its growth was almost completely abolished. The *rolC*-expressing *R. cordifolia* cells demonstrated a pronounced salt tolerance because the growth of RCH in the presence of 60 and 120 mM of NaCl was 82% and 24%, respectively, of the growth at normal conditions.

Analysis of NADPH oxidase gene expression in *rolC*-transformed callus cultures of *R. cordifolia* and *A. thaliana*

The expression levels of two NADPH oxidase, i.e. respiratory burst oxidase homolog (*Rboh*), genes in *A. thaliana*, *AtRbohD*, and *AtRbohF*, and its homologues in *R. cordifolia*, *RcRboh1*, and *RcRboh3*, respectively, were analyzed in the control and *rolC*-transgenic cell lines cultured under normal and stress conditions. *A. thaliana* cells cultured at 37ºC as well as *R. cordifolia* cells cultured at the same temperature and in the presence of 120 mM of NaCl were omitted from the analysis because corresponding control lines did not survive under such adverse conditions and were not suitable

for RNA isolation. As shown in Fig. 3, expression levels of *AtRbohD* and *AtRbohF* were approximately two times lower in the *rolC*-expressing *A. thaliana* line compared to the control. Under stress conditions, the mRNA transcript abundance of *AtRbohD* in the At callus line was upregulated three and two times under low-temperature cultivation and in the presence of 120 mM NaCl, respectively. Expression of the *AtRbohF* gene was 1.4 times higher in At cells subjected to 120 mM NaCl. Other stress conditions (i.e. high temperature and 60 mM of NaCl) had only barely detectable effects on the expression of these NADPH oxidase isoforms. On the contrary, the expression of both *AtRboh* genes in ACH calli increased by 1.3–1.6 times in response to all studied stress stimuli.

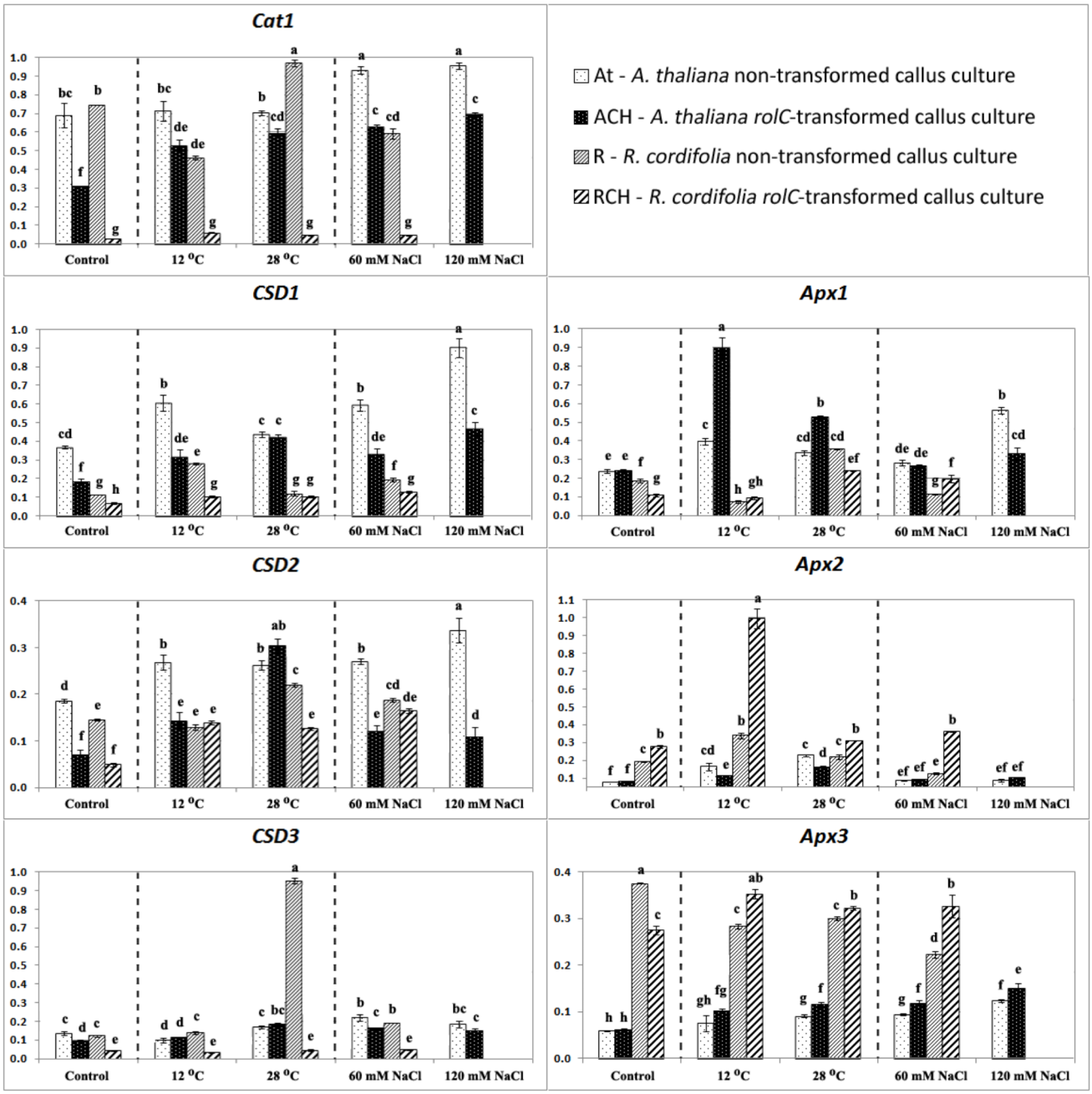

Fig. 4 Expression of *A. thaliana* and *R. cordifolia* antioxidant genes in control callus cultures (At and R, respectively) and in *rolC*-expressing callus cultures (ACH and RCH, respectively) under high (28ºC), low (12ºC) temperatures, and in the presence of different NaCl concentrations (60 and 120 mM). Calli were grown for 30 days before harvesting and analysis. Values are presented as means ± standard errors. Different letters above the bars indicate statistically significant differences of means ($P < 0.05$) for each single gene, Fisher's LSD

In *R. cordifolia*, the expression of *RcRboh1* was only insignificantly downregulated by the *rolC* gene transformation, while the *RcRboh3* mRNA transcript abundance was two times lower in RCH compared to the control cell line. When exposed to low and high temperatures, the mRNA transcript abundance of *RcRboh1* was upregulated 1.6 and 1.8 times, respectively, in R cells, while the addition of NaCl did not affect the expression of this NADPH oxidase isoform. Interestingly, temperature stress had a much less evident effect on *RcRboh1* expression levels in RCH calli. Transcription of the *RcRboh3* remained stable during all stress treatments in both the control and *rolC*-transgenic cultures of *R. cordifolia*.

Analysis of catalase gene*expression*in*rolC*-transformed callus cultures of*R. cordifolia*and*A. thaliana*

The pattern of the catalase gene expression levels in stress conditions was different in control cell cultures of *A. thaliana* and *R. cordifolia* (Fig. 4). Thus, in At cells, *AtCat1* mRNA transcription was upregulated 1.4 times in response to 60 and 120 mM NaCl stress conditions and was not influenced by temperature stresses. At the same time, expression levels of the homologues gene, *RcCat1*, in R cells was 1.6 and 1.3 times lower under cold and salt stresses, respectively, and 1.3 times upregulated under high temperature conditions. However, *rolC* transformation pronounced a similar effect on catalase gene expression in both plant species. The *Cat1* gene was strongly inhibited in *rolC*-expressing cultures of *A. thaliana* and *R. cordifolia* by 2.2 and 27.3 folds, respectively. Expression patterns of this gene under stress stimuli were also similar, i.e. 1.7–2.2 fold increase in *Cat1* gene expression in both, ACH and RCH, calli was observed in response to all studied stress stimuli.

Analysis of copper-*zinc*superoxide dismutase gene expression in*rolC*-transformed callus cultures of*R. cordifolia*and*A. thaliana*

The expression of three copper-zinc superoxide dismutase (*CSD*) isoforms was studied in *A. thaliana* and *R. cordifolia* callus lines grown under normal and stressed conditions (Fig. 4). Notably, the *rolC* transgene provoked a two to threefold decrease in transcriptional levels of all studied *CSD* genes in ACH and RCH cells. The pattern of the *AtCSD* and *RcCSD* isoforms expression levels under stress conditions in control cell cultures of *A. thaliana* and *R. cordifolia* was very similar. Both *AtCSD1* and *RcCSD1* were upregulated by lower temperature and salt stress. The expression of *CSD2* and *CSD3* genes in both, At and R, cells was mainly activated by heat and salt.

In contrast to control cells, the expression of *CSD1* and *CDS2* genes in *rolC*-transgenic calli of *A. thaliana* and *R. cordifolia* was activated 1.5–4.3 folds by all tested stress stimuli. Transcription of *AtCSD3* in ACH was two times upregulated under high temperature and NaCl treatment compared to normal conditions. The mRNA transcript abundance of *RcCSD3* in RCH calli was not affected by stress treatments.

Analysis of ascorbate*peroxidase*gene expression in*rolC*-transformed callus cultures of*R. cordifolia*and*A. thaliana*

Ascorbate peroxidases showed differential expression in non-transformed *A. thaliana* and *R. cordifolia* calli (Fig. 4). Specifically, in At cells, *AtApx1* was 1.2–2.4 fold increased by all tested stress stimuli. The expression of *AtApx2* was 4.1 and 6.3 times higher under low and high temperatures, respectively. The transcription of the *AtApx3* isoform was upregulated by 1.5–2.1 folds by high temperature and salt stress. An opposite effect of low temperature and NaCl supplementation was detected in the case of *RcApx1* expression, i.e. its levels were 2.5 and 1.6 times lower compared to normal conditions. However, *RcApx1* mRNA content increased 1.9 times with higher temperature. The expression levels of *RcApx2* under lower temperature provoked a twofold increase, while salt stress caused a twofold decrease in *RcApx2* mRNA abundance. All tested stress conditions resulted in a 1.3–1.7-fold decrease in the transcriptional level of the *RcApx3* isoform.

The *rolC* gene had no significant effect on the expression of *AtApx1*, *2*, and *3* in the *A. thaliana* transgenic cell line. At the same time, in *R. cordifolia rolC*-transgenic cells *RcApx1* and *RcApx3* genes showed a 1.7- and 1.4-fold decrease in expression levels, respectively, while *RcApx2* was upregulated 1.6 times. Changes in expression levels of *AtApx1*, *2*, and *3* genes in ACH calli in response to stress stimuli showed a similar pattern as in the control cells. However, the effect of temperature stresses was more pronounced and resulted in up to 4– and 6-fold increase in the transcriptional levels of *AtApx1* and *AtApx2* isoforms, respectively. In RCH callus line, temperature stresses induced expression of *RcApx1* and *RcApx2* by 2.2–4.2 times, compared with their levels under normal conditions. Cold stress promoted significant activation of *RcApx2* expression, while high temperature had no effect on its level. Salt stress caused an opposite effect on *RcApx2* mRNA abundance. Expression level of this gene was 1.9 times lower in R and 1.4 times higher in RCH cells under NaCl treatment compared to normal conditions. Expression pattern of *RcApx3* under temperature and salt stresses was similar, i.e. 1.3–1.7 fold decrease in R and

1.2–1.3 fold increase in RCH calli was observed in response to all studied stress stimuli.

Subcellular locations of RolC-EGFP*fusion*protein in*Rubia cordifolia*protoplasts

EGFP was used as a visual marker to follow the distribution of the chimeric protein RolC-EGFP in transiently transformed *R. cordifolia* protoplasts (Fig. 5). The full-length *rolC* gene sequence was engineered as the N-terminal fusion of *EGFP* and expressed under the control of a tandem 35 S CaMV promoter. Cells transformed with the *EGFP* gene alone as well as non-transformed cells were used as a control. The subcellular distribution of EGFP alone was visualized mainly in the cytosol and nucleus except vacuoles (Fig. 5B). The RolC-EGFP fusion in general showed similar subcellular distribution patterns (Fig. 5 A). The projections of images along the optical z-axis did not reveal a RolC-EGFP protein at the endoplasmic reticulum. However, further analysis of the nuclei of the *rolC-EGFP* transformed cells allowed us to identify small single spots inside the nucleoli (Fig. 5 C), suggesting that RolC may also localize in this subnuclear compartment.

Discussion

Agrobacterium species have developed molecular mechanisms aimed at reducing ROS level in transformed plant cells to increase the chance of successive invasion. The hairy root culture of *Nicotiana tabacum* as well as transgenic plants regenerated from hairy roots showed decreased H_2O_2 levels and increased production of antioxidant enzymes (Nikravesh et al. 2012a, b). However, another study with *N. tabacum* hairy roots showed a different effect of *rol* genes on ROS production. Both the *rolB*- and *rolC*-deficient mutants produced less ROS and expressed less ROS-related transcripts compared with pRiA4-transformed wild-type hairy roots (Wang et al. 2014). Individual overexpression of *A. rhizogenes* A4-derived oncogenes, such as *rolA, rolC* and *rolB*, also reduces the ROS level in transformed plant cells (Bulgakov et al. 2008, 2012). Inhibition of ROS is most strongly induced by *rolC*, followed by *rolB* and *rolA*. Surprisingly, *rolB*-caused inhibition of ROS production was not associated with the reduction of NADPH oxidase genes expression (Veremeichik et al. 2016). At the same time, a strong transcriptional induction of antioxidant enzymes was observed (Bulgakov et al. 2012; Shkryl et al. 2013). Thus, *rolB* seems to act as an activator of both systems, i.e. generation and detoxification of ROS. The way of the *rolC*-induced perturbations in ROS metabolism remained largely unknown.

In this investigation we have established three independent *rolC*-expressing *A. thaliana* cell lines (AtC1, 2, 3) and compared them with a previously characterized *R. cordifolia* RCH callus line with a high level of *rolC* expression (Shkryl et al., 2008). In accordance with previously obtained data (Bulgakov et al. 2008), *rolC*-transformed cultures of *A. thaliana* and *R. cordifolia* showed pronounced resistance to abiotic stress factors (Fig. 2). Both transgenic lines were 1.5–4 times more tolerant to temperature and salt stresses than the control calli. Enhanced cell growth and resistance to heat and salt stress in *rolC*-transformed lines of *L. sativa* has also been reported (Ismail et al. 2019b). The transformation of *N. langsdorffii* with the *rolC* gene enhanced free sugar and polyphenol concentration which contribute to water and heat resistance of transgenic plants (Ancillotti et al. 2015).

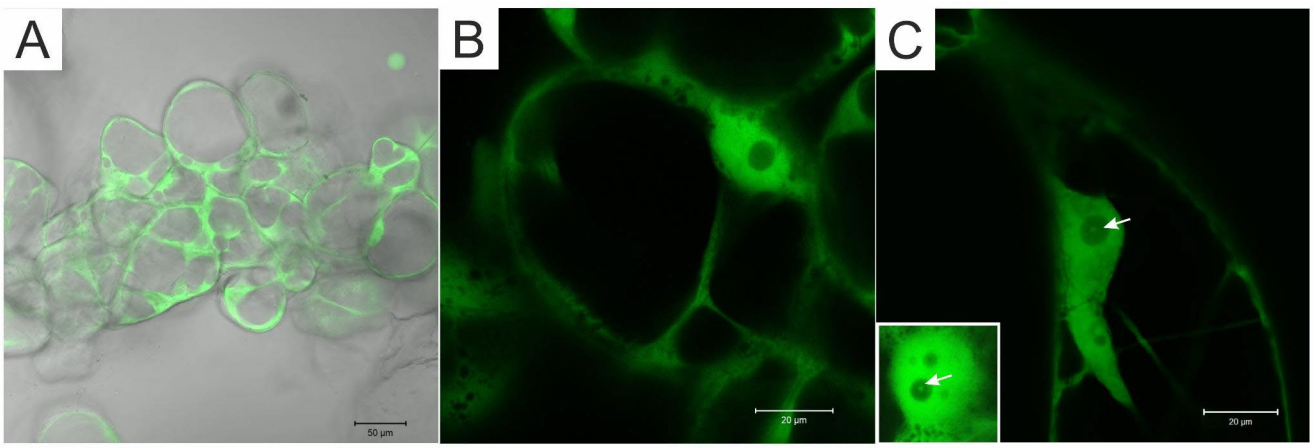

Fig. 5 Localization of the RolC-EGFP protein in transformed *R. cordifolia* cells. A: common view of *rolC-EGFP* transformed cells. B: cells, transformed with *EGFP* gene alone. C: nuclear localization of the RolC-EGFP protein (arrows indicate spot fluorescence of the RolC-EGFP fusion protein in nucleoli)

Rboh genes responded differently to stress conditions in our experimental settings (Fig. 3). In *Arabidopsis*, the *RbohD* isoform was primarily activated by cold, while *RbohF* showed a salt-induced pattern. In *R. cordifolia* cells, only the *Rboh1* isoform was temperature-regulated, while *Rboh3* showed constant expression under stress conditions. It was previously demonstrated that *Rboh* genes show different patterns of expression in normal conditions and in response to various environmental factors (Marino et al. 2012). In particular, *AtRbohD* and *AtRbohF* are involved in the coordination of ROS production during stress stimuli (Torres et al. 2002; Kwak et al. 2003; Chang et al. 2016). Strong upregulation of *AtRbohD* gene expression occurred during heat treatment, while low temperature caused its reduction (Suzuki et al. 2011; Chang et al. 2016). Both genes, *AtRbohD* and *AtRbohF*, were activated in response to NaCl treatment, although *RbohF* was found to be less sensitive to salt (Ma et al. 2012). In the control *A. thaliana* and *R. cordifolia* callus lines, the means of total *Rboh* isoforms transcript abundances were 1.7 and 1.6-fold higher than

Table 1 List of primer sequences used in this study

Gene (GenBank accession no.)	Forward (5′ to 3′)	Reverse (5′ to 3′)
qPCR of the *rolC* gene		
rolC (X03433)	CGACGATGATGCTCTGCTTC	GCCTGAGCCCTCTATTGACC
A. thaliana specific primers		
AtRbohD (NM_124165)	CAGTAACAACAATAGTAAAGGG	TACCCGACACAACATCCAC
AtRbohF (NM_105079)	GAAGAAGCCAAAAGCCCTC	CCCTCACCAAACCT-CACCTC
AtCat1 (NP_564121)	GCACAGGGATGAGGAGGTC	AGCACTTCTCACGATTTC-CAG
AtCSD1 (NM_100757)	TTGTTGGTAGGGCTGTTGTTG	TGCGTTTCCAGTAGCCAGG
AtCSD2 (NM_128379)	GTGGTGTGATTGGCTTGACGC	ATCAACACAGTAGA-CATAAG
AtCSD3 (NM_001125773)	GAGGCTGTCTTCAATTCGTA	GTCCAGTAGAGATG-CAGCCA
AtApx1 (At1G07890)	TGAGCGGAGAGAAGGAAGGC	CTTCATCAGCAGCG-TATTTCTCG
AtApx2 (AT3G09640)	CACAAGGAGCGTTCAGGATTC	CCTTCTTTCTCTCCGCTTAG
AtApx3 (NP_195226)	GAAAGGAGAATCAGAGGGCT	GAGTTTCTTGTGCGATTCCG
AtAct2 (AT3G18780)	ACCTTGCTGGACGTGACCTT	GTTGTCTCGTGGATTCCAGC
R. cordifolia specific primers		
RcRboh1 (KP987238)	GTTGGGGTGTTTTACTGTGGG	CAGGGGAGATGAGGTGGTC
RcRboh3 (KP987240)	GCCAAAGAGATAGGAAAACT	CATCCACCTCTCCTATTGT
RcCat1 (GQ380493)	TCCCAGTTTTCTTCATTCG	GGGATGATGCGAGAAGAAA
RcCSD1 (GQ380492)	ATCACGGTTCTCCCAAAG	CAATGATGGAATGTGGTC
RcCSD2 (GU949547)	GCCTCATTTCAACCCTAA	GGACCAATCAGAGGAATC
RcCSD3 (GU949548)	GCCTCATTTTAACCCACTG	CTGGGTGTCTTTGATTGAT
RcApx1 (GQ380494)	GAGTTCCCCATCCTTTCAC	CTCAACCTTATCCTCTCTTC
RcApx2 (GU949549)	GTTGGCGGGAGTTGTTGCTG	CCTTCCCAATGTATGAGCG
RcApx3 (GU949550)	AAAGGAGAAAGTGAAGGG	GGCGAAATTCAG-GATCTTCA
RcActin (DQ531565)	GATTGAGCACGGTATTGTTAG	ACACCATCACCAGAATC-CAAC

that in *rolC*-transgenic callus lines, respectively ($F = 5.84$, $P < 0.001$). Moreover, abiotic stresses elevated *Rboh* expression levels twofold in control cells, while *rolC*-expressing cells showed a maximum 1.4-fold increase. Thus, it is apparent that RolC downregulates *Rboh* gene expression in both normal and stressed conditions.

Considerable induction of catalase, superoxide dismutase, and ascorbate peroxidase genes was detected in *Arabidopsis* and *R. cordifolia* cells subjected to abiotic stresses (Fig. 4). Induction of antioxidant genes was previously detected in plants and cell cultures exposed to adverse conditions (Filiz et al. 2019; Singh et al. 2020). In the control *A. thaliana* and *R. cordifolia* callus lines, the means of total antioxidant genes transcript abundances were 1.7 and 3.3-fold higher than that in *rolC*-transgenic callus lines, respectively ($F = 9.10$, $P < 0.001$). However, abiotic stresses elevated their expression levels by a maximum of 1.5 times in control cells, while *rolC*-expressing cells showed at least a twofold increase. In summary, our data demonstrate that the expression of *Rboh* isoforms and antioxidant genes execute a complicated interplay in the regulation of ROS metabolism in *rolC*-expressing plant cells under both normal and abiotic stress conditions.

To evaluate the subcellular localization of RolC protein, we transformed *R. cordifolia* cells with pSAT6-rolC-EGFP fusion and pSAT6-EGFP control constructions (Fig. 4). RolC-EGFP localized predominantly in cytoplasm in a way similar to native EGFP. However, a portion of RolC-EGFP was also clearly detected in the nucleoli of transformed cells. As GFP protein without specialized NLS tags does not accumulate in nucleolus (Martin et al. 2015), we conclude that RolC promotes specific localization of the fusion protein. This result was unexpected because previously it was generally accepted that RolC has exclusively cytosolic patterning (Estruch et al. 1991; Oono et al. 1991). Interestingly, another *plast* member, *6b* oncogene from *A. tumefacines*, was also detected in the nucleolus (Kitakura et al. 2002). In this compartment, the 6b protein interacts with newly identified H3 histone NtSIP2 and serves as a histone chaperone, changing transcriptional levels of genes responsible for tumor formation and leading to the reprogramming of the plant cell's fate (Ishibashi et al. 2014). Identification of the RolC protein in the plant nucleolus may suggest its function in the epigenetic control of genome-wide gene expression via a similar yet unidentified mechanism. The *rolC*- and *6b*-induced morphogenetic activities in transformed plants are similar, potentially indicating the existence of a conserved molecular mechanism of action (Mohajjel-Shoja et al. 2011).

Together with previously reported data, results of our research demonstrate the opposing mechanism by which the *rolB* and *rolC* genes provoke a decrease of ROS. In particular, *rolB* gene upregulates expression of *Rboh* and antioxidant genes (Bulgakov et al. 2012; Shkryl et al. 2013), while the *rolC* gene suppresses their expression (Fig. 3; Table 1). *Rol* oncogenes are also implicated in modification of calcium-dependent protein kinases (CDPKs) expression, acting as negative regulators of isoforms, implicated in ROS production, and as positive regulators of isoforms, which might be engaged in ROS detoxification (Veremeichik et al. 2014). In nature, cooperative action of *plast* and apparently non-*plast* T-DNA genes can be important for suppression of the plant immune system for more successful colonization of host cells by *Agrobacterium*. It can be speculated that Rol proteins can orchestrate ROS metabolism by a different molecular mechanism with similar resulting effects to duplicate each other's functions. This assumption may partly explain the results obtained by Wang et al. (2014) in which inactivation of one of the individual *rol* genes in tobacco hairy roots did not lead to a rise in ROS content. According to the proposed hypothesis, in that case, ROS suppression could still be maintained by other T-DNA genes. In this regard, further investigations of *Agrobacterium* T-DNA genes effects on ROS metabolism are important to clarify the strategy of genetic colonization realized by this pathogen.

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Author contribution statement YS and GV conceived and designed the research. YS, GV, TG, TA, and GT performed the experiments. YS, GV, and VB analyzed the data. VB contributed reagents and analytical tools. YS wrote the manuscript. All authors approved its final version.

Data Availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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