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Auxin-dependent regulation of growth *via rol*B-induced modulation of the ROS metabolism in the long-term cultivated pRiA4-transformed *Rubia cordifolia* L. calli

Galina N. Veremeichik *, Tatiana Y. Gorpenchenko, Tatiana V. Rusapetova, Evgenia V. Brodovskaya, Galina K. Tchernoded, Dmitry V. Bulgakov, Yurii N. Shkryl, Victor P. Bulgakov

Federal Scientific Center of the East Asia Terrestrial Biodiversity of the Far East Branch of the Russian Academy of Sciences, Vladivostok, 690022, Russia

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ABSTRACT

Gene transfer from *Agrobacterium* to plants is the best studied example of horizontal gene transfer (HGT) between prokaryotes and eukaryotes. The *rol* genes of *A. rhizogenes* (*Rhizobium rhizogenes*) provide uncontrolled root growth, or "hairy root" syndrome, the main diagnostic feature. In the present study, we investigated the stable pRiA4-transformed callus culture of *Rubia cordifolia* L. While untransformed callus cultures need PGRs (plant growth regulators) as an obligatory supplement, pRiA4 calli is able to achieve long-term PGR-free cultivation. For the first time, we described the pRiA4-transformed callus cultures' PGR-dependent ROS status, growth, and specialized metabolism. As we have shown, expression of the *rol*A and *rol*B but not the *rol*C genes is contradictory in a PGR-dependent manner. Moreover, a PGR-free pRiA4 transformed cell line is characterised as more anthraquinone (AQ) productive than an untransformed cell culture. These findings pertain to actual plant biotechnology: it could be the solution to troubles in choosing the best PGR combination for the cultivation of some rare, medicinal, and woody plants; wild-type Ri-plants and tissue cultures may become freed from legal controls on genetically modified organisms in the future. We propose possible PGR-dependent relationships between *rol*A and *rol*B as well as ROS signalling targets. The present study highlighted the high importance of the *rol*A gene in the regulation of combined *rol* gene effects and the large knowledge gap in *rol*A action.

1. Introduction

Prokaryotes use horizontal gene transfer for biodiversity. The advancement of molecular analysis technologies has enabled the discovery of numerous examples of horizontal gene transfer between prokaryotes and eukaryotes. The best studied example is the DNA transfer from *Agrobacterium* to plants (Lacroix and Citovsky, 2018). *Agrobacterium* species can transfer and permanently integrate their own genes into the nuclear genomes of higher plants. The machinery of *Agrobacterium*-mediated gene transfers revolutionized molecular genetics, plant biotechnology, and breeding (Guo et al., 2019). The *Agrobacterium*-induced neoplastic diseases are determined by the transferred oncogenes.

A. rhizogenes, or *Rhizobium rhizogenes,* is 0.6–1 μm by 1.5–3 μm rod-shaped, a non-spore-forming, Gram-negative bacterium. The whole genome analysis of some *A. rhizogenes* strains (Flores-Felix et al., 2020; Hooykaas and Hooykaas, 2021) showed that the genome includes of a circular chromosome, a large virulence plasmid, and several smaller plasmids (Veena and Taylor, 2007; Vladimirov et al., 2015). The transferred DNA (T-DNA) of the virulence plasmids consist of opine catabolism genes and genes inducing neoplastic growth of host cells (Veremeichik et al., 2023). Hairy root syndrome is induced by oncogenes from the T-DNA of the virulence Ri-plasmid (root induced plasmid) of *A. rhizogenes*. As a result, pRi-transformed cells of the host plants supply opines to the bacteria (Veena and Taylor, 2007). Genes inducing neoplastic uncontrolled root growth are designated *root oncogene loci*, or *rol* genes (Ozyigit et al., 2013). The specific opine biosynthesis genes in the T-DNA determine the classification of Ri plasmids: mannopine (TR7, 8196, TR101), agropine (A4, 15,834, LBA9402, 1855), mikimopine (1724), and cucumopine (2659) (Vladimirov et al.,

* Corresponding author. Federal Scientific Centre of the East Asia Terrestrial Biodiversity FEB RAS, Vladivostok, 690022, Russia.
E-mail address: gala-vera@mail.ru (G.N. Veremeichik).

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2015). Agropine-type *A. rhizogenes* strains are more studied and considered to be more virulent; they are often used for establishing transformed plants and cultures (Sevon and Oksman-Caldentey, 2002; Desmet et al., 2019). Agropine Ri plasmids have a split T-DNA: the left TL-DNA and the right TR-DNA. The TR-DNA contains genes for mannopine and agropine biosynthesis, while *rol* genes (*rol*A, *rol*B, *rol*C, and *rol*D) are located in the TL-DNA (Otten, 2018; Veena and Taylor, 2007).

Wild-type pRi-expressed plants showed such features as dark-green, wrinkled leaves; reduced plant apical dominance, height, and root length; an increased number of shoots, nodes, and root branching; increased specialized metabolites and fragrance; and changes in essential oil composition (Christensen and Müller, 2009). Some of these features are important for crop selection. Producing callus and root cultures of medicinal plants as alternative sources of specialized pharmacological metabolites is an important application of wild *A. rhizogenes*-mediated transformation. (Sevon and Oksman-Caldentey, 2002). A detailed study of the effects of individual and joint expression of *rol* genes showed their great biotechnological significance as activators of specialized metabolism as well as their important role in the regulation of the tolerance of transgenic plants to stress (Guo et al., 2019). It is critical to understand the biological roles of each of the *rol* genes in this coordinated process. Transformation with wild-type *A. rhizogenes* can positively modulate the activity of the antioxidant system of plants (Matvieieva et al., 2022) and plant cell culture (Shkryl et al., 2010). Previously, it was shown that individual expression of the A4-*rol*B and A4-*rol*C genes led to antagonistic modulation of the ROS metabolism system. While *rol*B gene expression actively stimulates ROS generation and detoxification (Bulgakov et al., 2012; Veremeichik et al., 2016), expression of the *rol*C gene led to a decrease in the expression of genes in both systems (Shkryl et al., 2022). As a result, in both cases, we observed a decrease in intracellular ROS accumulation (Bulgakov et al., 2008, 2012). However, the decrease in ROS in *rol*B-expressed cells was accompanied by growth inhibition and activation of phytoalexin biosynthesis, while *rol*C-cells, in contrast, were well-grown and highly resistant to abiotic stress (Shkryl et al., 2022). The presence of *rol* genes in the genomes of some plant species, as well as detailed analysis of their roles, have suggested that *rol* genes can improve some physiological processes: metabolism, abnormal growth, and redirecting of growth (Otten, 2018; Desmet et al., 2020a, 2021). As a result, Japan and some other countries exempt wild-type Ri-plants from legal controls on genetically modified organisms (GMOs) (Mishiba et al., 2006; Christensen and Müller, 2009). Thus, clarification of the biological role of the *rol* genes is an important direction for improving of quality and quantity of crop and non-crop plants.

In the present study, we analysed such important biochemical and physiological indicators as growth, phytoalexin production, and ROS metabolic systems in pRiA4-transformed callus cultures of *Rubia cordifolia* L (Rubiaceae), which adapted to growth in the presence and absence of plant growth regulators. The data obtained for the first time demonstrated the role of PGR (auxin) in the combined effects of *rol* gene expression on the shift of ROS-dependent plant defence systems.

2. Materials and methods

2.1. Callus cultivation and abiotic stress treatments

Previously, an untransformed control cell line of *R. cordifolia* L. (designated as R) was obtained from leaf explants using W medium supplemented with 0.5 mg/l 6-benzylaminopurine and 2 mg/l α-naphthylacetic acid (Bulgakov et al., 1998). In the present work, this hormone-contained medium is designated as W/BA. The *R. cordifolia* pRiA4-transgenic callus lines were obtained with the wild-type agropine strain of *A. rhizogenes* A4 (Slightom et al., 1986) as described (Bulgakov et al., 2002). Whereas wild-type pRiA4 is free of any selectable antibiotic resistance genes, transformed callus lines were selected using hormone-free medium (designated as W0) due to the inability of the untransformed calli to grow in the absence of the plant growth regulators. Three independent callus lines with the same morphological and biochemical features were obtained and characterised as the RA4 callus line using semiquantitative PCR for growth, anthraquinonens (AQ) content and production, and *rol* gene expression (Shkryl et al., 2008). In the present study, these pRiA4-transformed callus lines were designated as RA4-I. Also, these pRiA4-transformed callus lines were adapted to W/BA medium and designated as RA4-II. Cell cultures were cultivated using agarised W/BA and W0 medium in the dark at 24 °C with 30-day subculture intervals.

The effect of salinity, cold, and heat stress on the biomass accumulation of the control R and transgenic callus lines was investigated using the following conditions: salinity (60 mM NaCl), cold (12 °C) and heat (28 °C) during the 30-day period of cultivation. The inoculum biomass for stress treatment was 200 mg (20 g/l) (each callus was weighted using an electronic balance). The treated calluses were weighed again after 30 days. All experiments were performed using W/BA and W0 medium in the dark three times with 20–30 replicas.

2.2. Estimation of the anthraquinones content

The qualitative composition of anthraquinone pigments in *R. cordifolia* calli was previously described in detail (Shkryl et al., 2008). To estimate quantitative changes in the total anthraquinone content induced by the *A. rhizogenes* A4 strain, UV–visible spectrophotometry was used according to the previously described method (Mischenko et al., 1999). Total anthraquinone content is presented as the sum of munjistin and purpurin (% of dry callus weight). Anthraquinone production was calculated as follows:

$$\text{Productivity (mg / l)} = \text{Content} \times \text{DW},$$

Where: Content, content of anthraquinones in callus culture (mg/g DW); DW, dry weight of the *R. cordifolia* callus culture per litre of medium (g/l).

2.3. Laser confocal imaging of intracellular ROS in living cells

The measurement of the intracellular ROS abundance was performed as described previously (Bulgakov et al., 2012). The ability of plant cells to oxidise fluorogenic dyes to their corresponding fluorescent analogues is the basis of the experiments. 30-day-old callus cultures (R, RA4-I, and RA4-II) that were stable or once (one passage, 30 days) growing on agarised W/BA or W0 were stained with 2,7-dichlorodihydrofluorescein diacetate (H2DCF-DA, Molecular Probes, Eugene, OR, USA) at the final concentration of 50 μg/ml in liquid W/BA or W0 medium for 10 min at 25 °C in the dark. Then, cells were washed twice, and the DCF fluorescence levels inside cells were immediately measured with an Axiovert 200M LSM510 META confocal microscope (Zeiss, Germany) using an argon laser (λex = 488 nm; emission Ch3-LP filter 505 nm). Time-series files were acquired and recorded on the computer's hard drive using LSM 510 software release 4.2 (Zeiss, Germany). Data were presented as the means of six separate experiments (at least 30–40 cells were analysed in each experiment).

2.4. RNA isolation, cDNA synthesis and real-time PCR

To analyse *rol*A, *rol*B, and *rol*C genes and ROS-generated or scavenged gene expression, samples of 30-day-old R, RA4-I, and RA4-II cell cultures were harvested, weighed, and used for RNA extraction and RT-PCR. In this work, we used two independent lines of control cell culture (R) and each type of transgenic callus line (RA4-I and RA4-II). For all RT-analysis, we used three independent RNA extractions. Isolation of total RNA and first-strand cDNA synthesis were carried out as described previously (Shkryl et al., 2008). For quantitative real-time PCR (qPCR) analysis, a CFX96 (Bio-Rad Laboratories, Inc., USA) was used with 2.5 × SYBR Green PCR Master Mix containing ROX as a passive reference dye

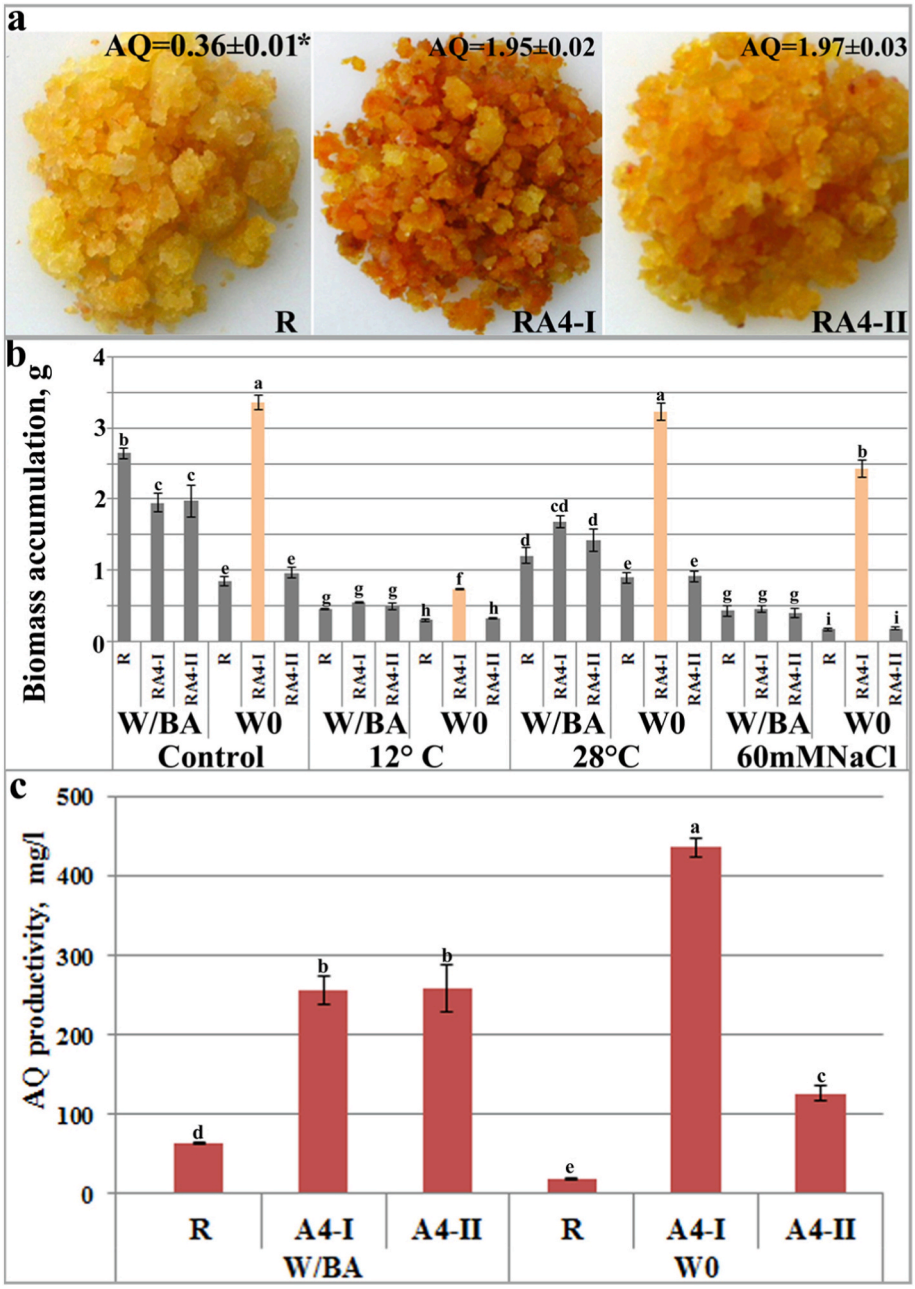


Fig. 1. Phenotypes and total anthraquinone content of *R. cordifolia* callus cultures (**a**); tolerability to temperatures and salt stress (**b**); and anthraquinone productivity during a 30-day cultivation period (**c**). Control (R) and RiA4-transformed (RA4-I and RA4-II) cell cultures grown on W/BA, W0, and W/BA medium, respectively. AQ, total anthraquinone content (mg/g DW, dry weight). Stress treatment experiments were performed in triplicate for 30 days on W0 and W/BA medium under control conditions (24 °C), at a low (12 °C) and high (28 °C) temperature, and in the presence of NaCl (60 mM). Data are presented as the mean ± SE from four subcultures (biological replicates) with two technical replicates for each experiment. Different letters above the bars indicate statistically significant differences ($P < 0.05$, Fisher's LSD).

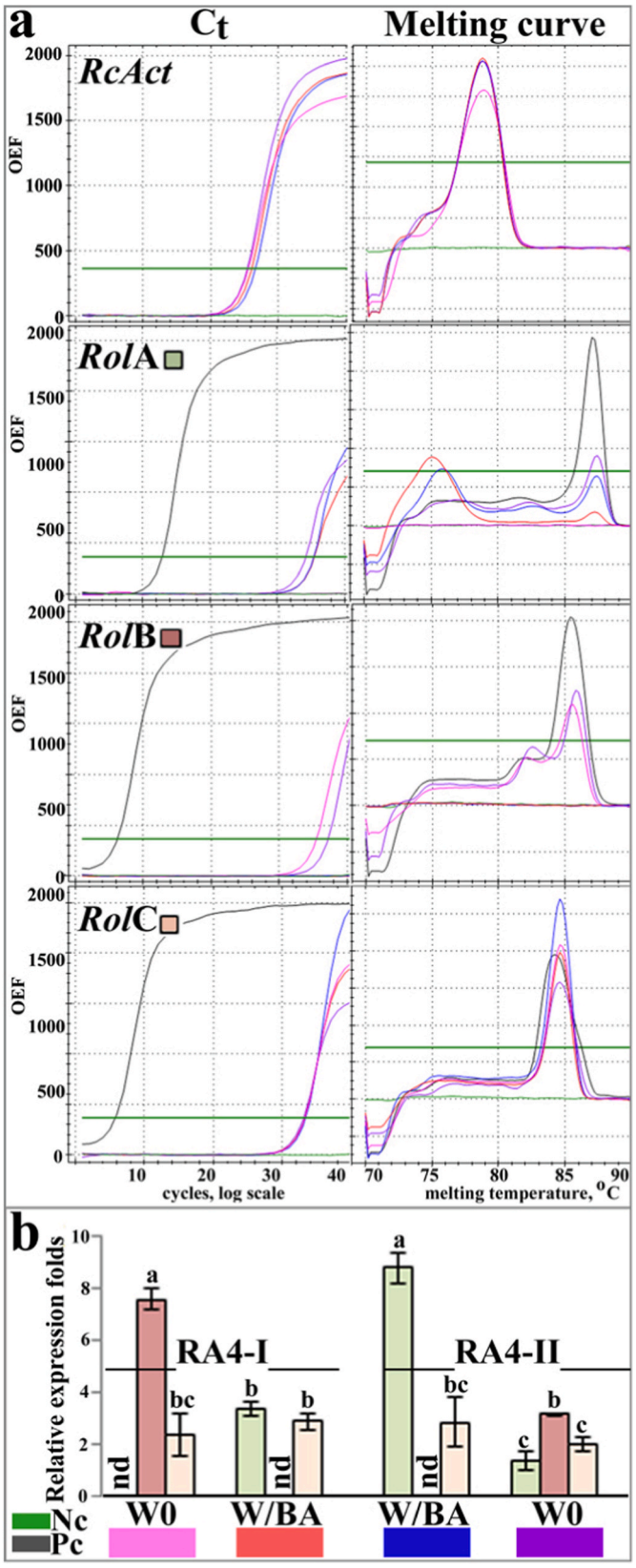
(Syntol, Russia).

Gene-specific primer pairs described previously were used for studying the expression by real-time PCR: transgenes (*rol*A, *rol*B, and *rol*C, Shkryl et al., 2008); genes of the ROS-generating enzymes (NADPH-oxidases, *RcRboh1* and *RcRboh3*, Veremeichik et al., 2016); genes of the enzymes for ROS-detoxification (catalase, *RcCat*; superoxide dismutases, *RcCSD1-3*; ascorbate peroxidises, *RcApx1-3*, Shkryl et al., 2010); and the housekeeping reference genes such as *Actin* and *18s* (Shkryl et al., 2011).

For analysis, we used three biological replicates and three technical replicates from three different RNA extractions. Reactions were done in a 10-μl volume containing 250 nM of each primer, 1 μl of the diluted cDNA sample, and 2 mM $MgCl_2$. The following were the PCR reaction conditions: 5 min at 96 °C, followed by 35 cycles of 15 s at 95 °C and 30 s at 60 °C in a 96-well reaction plate. Melting curve analysis and product visualization with electrophoresis on a 1% agarose gel stained with ethidium bromide were used to confirm the absence of non-specific products or primer–dimer artefacts in the samples. No-template controls and RNA-RT controls were included in the qPCR analysis to verify the absence of contamination. Data were analysed using CFX Manager Software (Version 1.5; Bio-Rad Laboratories Inc., Hercules, CA). The lower-expressing sample was assigned the value 1 in the relative mRNA calculation using the formula $2^{-\Delta\Delta Ct}$. Data were analysed using CFX Manager Software (Version 1.5; Bio-Rad Laboratories, Inc.).

2.5. Protein extraction procedure and peroxidase activity assay

Protein extraction (three technical replicates derived from three independent protein isolations) from the 30-day-old R, RA4-I, and RA4-II cultures (three biological replicates, separate cell passages), analysis of the peroxidase isoenzymes by isoelectric focusing (IEF), and determination of peroxidase-specific activities were carried out as described previously (Veremeichik et al., 2012). The proteins in the supernatant fractions of crude callus extracts in 100 mM Tris-acetate buffer (pH 5.0)



(caption on next column)

Fig. 2. Transgene expression in RA4-I and RA4-II cell lines during short- and long-term cultivation on the W0 and W/BA medium. cDNA samples were obtained from 30-day-old cell cultures of RA4-I and RA4-II, and the expression of the *rol*A, *rolB*, and *rol*C genes was analysed using real-time PCR (**a**, left panel) and melting curve analysis (**a**, right panel). Data of the relative expression folds are presented as the mean ± SE from four subcultures (biological replicates) with two technical replicates for each experiment. The coloured blocks below the diagram correspond to real-time curves, where green is the negative control sample (Nc); black is the positive control sample (Pc, pRiA4); pink and red are the RA4-I callus line stable growing on the W0 and growing for 30 days on the W/BA, respectively; blue and purple are the RA4-II callus line stable growing on the W/BA and growing for 30 days on the W0, respectively. Data are presented as the mean ± SE from four subcultures (biological replicates) with two technical replicates for each experiment. Different letters above the bars indicate statistically significant differences ($P < 0.05$, Fisher's LSD).

were precipitated in ice-cold acetone. The peroxidase isoenzymes separation by IEF and the total soluble peroxidase-specific activities of each callus culture extract were measured in the reaction with 1 mM 4-methoxy-α-naphthalol as the substrate and 0.33 mM H_2O_2 in a 100 mM Tris-acetate buffer (pH 5.0) at 593 nm using a Benchmark Plus microplate spectrophotometer (Bio-Rad Laboratories) (Ferrer et al., 1990). The peroxidase activity was expressed in nkat, using $\varepsilon_{593} = 2.1 \times 10^4$ nkat M^{-1} cm^{-1} for the dye product.

2.6. Statistical analysis

All values are expressed as the mean ± SE using Statistica 10.0 (StatSoft Inc., USA). A difference of $P < 0.05$ was considered significant. Two independent categories were compared using the Student's *t*-test, while comparisons among multiple groups were achieved by ANOVA followed by a multiple comparison protocol. Fisher's protected least significant difference (PLSD) *post-hoc* test was employed for the intergroup comparison.

3. Results

3.1. Anthraquinone productivity in untransformed and pRiA4-transformed callus lines

The untransformed control cell lines of ***R. cordifolia*** L. (R) had been obtained previously (Mischenko et al., 1999). The ***R. cordifolia*** pRiA4-transgenic callus lines were obtained with the wild-type agropine strain of ***A. rhizogenes*** A4 (Bulgakov et al., 2002) using hormone-free medium W0 due to the inability of the untransformed calli to grow in the absence of the plant growth regulators. Stable growing on the W0 medium, three independent, pRiA4-transformed callus lines were designated as RA4-I. Adapted to W/BA medium, pRiA4-transformed callus lines were designated as RA4-II.

The morphology of the control and transgenic calli is similar, friable, and homogeneous (Fig. 1, A–C). There is only a difference in color: R calli is deep yellow, while RA4-I and RA4-II lines are dark orange. This difference is due to the different content of *R. cordifolia*'s main specialized metabolites, anthraquinones (AQ). In both RA4-I and RA4-II callus cultures, the total AQ content was higher than in R by more than 5 times (Fig. 1, a). Analysis of the growth parameters of the callus lines R, RA4-I, and RA4-II growing on the W/BA and W0 showed significant differences (Fig. 1, b). The biomass accumulation of the R calli stable growing in the presence of PGR (W/BA medium) was 2.5 times higher after one passage on the W0 medium. The second passage of the R calli on the W0 medium led to the full death of the cells. pRiA4-transformed calli RA4-I, stable and growing on the W0 medium, showed a 1.5-fold increase in growth compared to the control R line, growing on the W/BA medium. Growth of the pRiA4-transformed calli RA4-II adapted to W/BA medium was slightly decreased compared to R calli. Change of the comfortable medium for one passage (30 days) led to a significant

decrease in growth of both pRiA4-transformed calli RA4-I and RA4-II, followed by stabilisation of growth after the second passage (Fig. 1, b).

Due to differences in growth parameters between the cell lines RA4-I and RA4-II, AQ productivity was also different for these cell lines despite equal content (Fig. 1, a, c). Stable growth of the RA4-I on the W0 medium led to an increase in AQ productivity up to 400 mg/l, which is 10 times more than in R cell lines that grew on the more comfortable W/BA medium. When compared to the RA4-I on the W0 medium, adapting the RA4-II to the W/BA medium resulted in a nearly twofold decrease in AQ productivity. A one-time shift of medium for 30 days led to almost a twofold decrease in AQ productivity for all established callus cultures (Fig. 1, c).

3.2. Characterization of the growth of untransformed and pRiA4-transformed cell cultures under stress conditions

Next, the effect of the PGR supplementation on the growth of untransformed and pRiA4-transformed cell cultures under stress conditions was investigated (Fig. 1, b). The effect of salinity, cold, and heat stress on growth of the control R and transgenic callus lines was investigated using the following conditions: salinity (60 mM NaCl), cold (12 °C), and heat (28 °C) using W/BA and W0 medium in the dark during the 30-day period of cultivation. As we have shown previously, these stress treatments are optimal for inhibiting the growth of control R cell cultures (Veremeichik et al., 2021).

Earlier, we described the stress tolerance of the control R and transgenic RA4-II cell lines (Shkryl et al., 2010). For that work, we have used pRiA4-transformed cell lines adapted to W/BA. In the present work, we analysed the effect of moderate salinity and temperature stress for all possible variants: R, RA4-I, and RA4-II stables growing on comfortable medium (W/BA, W0, and W/BA, respectively) and growing for 30 days on uncomfortable medium (W0, WBA, and W0, respectively). As expected, calli tolerance to stresses on the control lines R was significantly higher when grown on comfortable W/BA medium (Fig. 1, b). Growth of the pRiA4-transformed calli RA4-I on the W0 medium under salinity and heat treatments was almost not affected, while the calli RA4-II adapted to PGR was more sensitive to the stress treatments. However, tolerance to stress of RA4-II calli was higher than R due to lower biomass accumulation in control conditions, as described in our previous work (Shkryl et al., 2010).

3.3. PGR-dependent regulation of the rolA, rolB, and rolC genes expression in pRiA4-transformed callus lines

To clarify the individual roles of *rol* genes in the PGR-dependent growth alterations, we have analysed the expression level of *rol*A, *rolB,* and *rol*C genes using real-time PCR (Fig. 2, a, left panel) with melting curve analysis (**a**, right panel). Transgenes' expression was measured in cDNA samples obtained from 30-day-old RA4-I and RA4-II cell lines growing during once- and long-term cultivation on the W0 and W/BA medium. As shown in Fig. 1, the consistent absence of PGR in the medium resulted in complete inhibition of rolA gene expression (green bar, Fig. 2, b), accompanied by high rolB gene expression. Stable cultivation of the PGR-adapted RA4-II calli on the PGR-contained W/BA medium had the opposite effect: strong *rol*A expression and full inhibition of the *rol*B gene expression. Short, 30-day-long cultivation of the RA4-I calli in the presence of PGR (W/BA medium) led to the induction of the *rol*A gene expression and immediate inhibition of the *rol*B gene expression, while 30-day-long cultivation of the PGR-adapted RA4-II calli in the absence of PGR (W0 medium) only strongly reduced the *rol*A gene expression and slightly induced the *rol*B gene expression. At the same time, the presence or absence of the PGR in medium had no effect on the expression of the *rol*C gene.

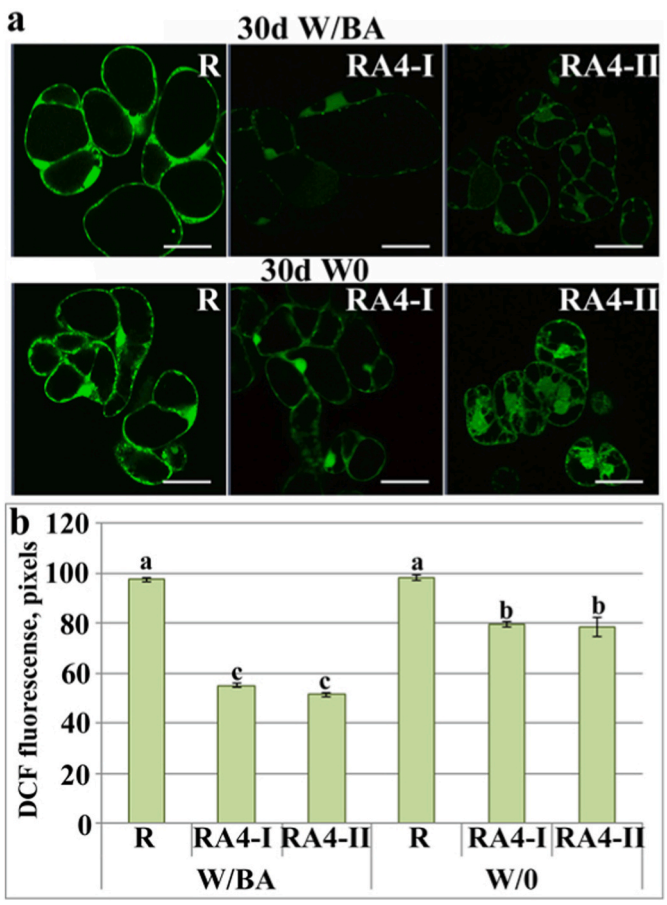


Fig. 3. ROS accumulation in *R. cordifolia cells* was measured by confocal microscopy (**a**) as DCF fluorescence (**b**). The abundance of intracellular reactive oxygen species (ROS) in control (R) and RiA4-transformed (RA4-I and RA4-II) cell cultures grown on W/BA, W0, and W/BA medium, respectively. ROS levels are presented as the mean ± standard error from six independent experiments at the end of the growth (30 days). Different letters above the bars indicate statistically significant differences ($P < 0.05$, Fisher's LSD).

3.4. ROS accumulation in pRiA4-transformed callus lines

Reactive oxygen species (ROS) are one of the major mediators of growth, development, stress responses, and many other processes in plant cells. Basal ROS levels are necessary for these cellular processes; an increase in ROS levels turns on stress responses, including the biosynthesis of specialized metabolites, while excess ROS has a cytotoxic effect as oxidative stress (Mittler, 2017). Previously, it was shown that individual expression of the *rol*B and *rol*C can modulate ROS accumulation, expression of the ROS generative system, and ROS scavengers (Bulgakov et al., 2008, 2012, 2013; Shkryl et al., 2022; Veremeichik et al., 2016). As we have shown in the present study, PGR-dependent modulation of the *rol*B gene expression in pRiA4-transformed cells makes it interesting to monitor intracellular ROS levels and the expression of genes involved in ROS metabolism in the presence and absence of PGR.

Previously, we have shown that intracellular ROS accumulation was significantly lower in pRiA4-transformed cell culture compared to control, untransformed cell culture (Shkryl et al., 2010). In that investigation, we used pRiA4-transformed calli adapted to the PGR-contained medium W/BA for the validity of the experiments and comparability with control calli R. In the present study, we used and compared all possible variants of the 30-day-old callus lines: R stable growing on W/BA and growing on W0 for 30 days; RA4-I stable growing on W0 and growing on W/BA for 30 days; and PGR-adapted RA4-II stable growing on W/BA and growing on W0 for 30 days. Fluorescence in single living

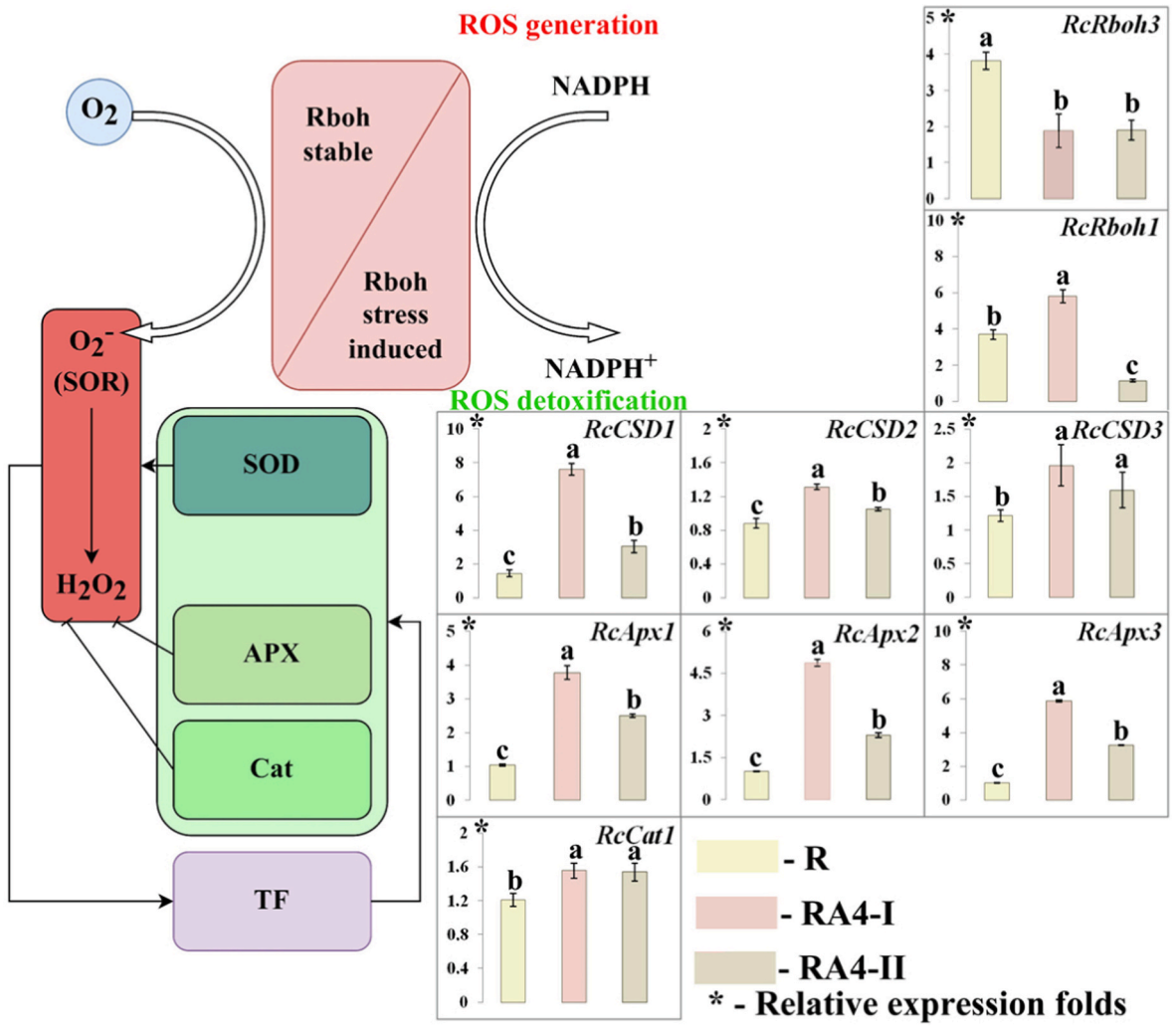


Fig. 4. Expression of ROS-metabolism genes in *R. cordifolia* control cells (R) and pRiA4-transformed (RA4-I and RA4-II) cell cultures. The left block of the figure shows the scheme presented (Mittler, 2017) for the stable and stress-induced generation of singlet oxygen radical (SOR) by Rboh enzymes and, below, the ROS detoxification system, including ROS scavenging enzymes (SOD, superoxide dismutase; APX, ascorbate peroxidase; Cat, catalases) and their ROS-activated transcription factors (TF). The right block presents the relative expression of the Rboh and antioxidant enzyme genes. mRNA levels measured by real-time RT-PCR Analysis was repeated three times; 30-day-old cell cultures were used. R, RA4-I, and RA4-II cell cultures were grown on W0 and W/BA medium, respectively. The data are presented as the mean ± standard error. Different letters above the bars indicate significantly different means ($p < 0.05$; Fisher's LSD).

cells loaded with H_2DCF-DA was measured by confocal microscopy. Monitoring intracellular ROS accumulation in R cells revealed that R cells grown on W/BA and W0 had the same ROS content (Fig. 3 a, b). The decrease by 40% in intracellular ROS was detected in both RA4-I and RA4-II calli growing in the presence of PGR compared to control R calli. In general, ROS accumulation was equal in both RA4-I and RA4-II cells and equally decreased in the presence of PGR. The growth of RA4-I and RA4-II callus lines on the PGR-free medium W0 led to an increase in ROS content of up to 80 percent compared to R calli (Fig. 3a and b).

3.5. *Expression and activity of the ROS metabolism genes in pRiA4-transformed callus lines*

The Rbohs proteins are responsible for the constitutive and stress-induced generation of singlet oxygen radicals (SOR) (Fig. 4, left panel; Mittler, 2017). Previously, we have shown that expression of *RcRboh1* is stress-induced, while *RcRboh3* is stable (Veremeichik et al., 2016). In addition, it was shown that overexpression of the *rol*B gene led to an increase in *Rboh*'s gene expression (Veremeichik et al., 2016), while *rol*C, in contrast, strongly affected it (Shkryl et al., 2022).

We compared the expression of the two Rbohs genes in pRiA4-transformed calli of *R. cordifolia* adapted to growing in the presence (RA4-II) and absence (RA4-I) of PGR to the control calli R, grown on PGR-containing W/BA medium for 30 days. In the R control cells, expression of the stable *RcRboh1* isoform was almost 2-fold higher than in both pRiA4-transformed lines, RA4-I and RA4-II (Fig. 4, right panel). Simultaneously, expression of the stress-induced *RcRboh3* gene was different: it was significantly higher in RA4-I calli, while it was significantly lower in RA4-II calli, more than four times lower than in control R calli.

In addition, at the end of growth, expression patterns of the ROS scavenging enzymes (*RcCSDs*, *RcApx*s, and *RcCat*, Fig. 4, left panel; Mittler, 2017) were measured. Among the three *RcCSD* genes, only the expression of *RcCSD1* was different in RA4-I and RA4-II calli; it was more than 8 times and 2 times higher than in R calli, respectively. Expression of the other two *RcCSDs* genes was the same in both pRiA4-transformed calli and was slightly higher than in R calli (Fig. 4, right panel). The expression of three RcApxs gene isoforms was higher in pRiA4-transformed calli than in R control cells. However, it was shown that stable growth of RA4-II cell culture in the presence of PGR led to a significant reduction in *Apx*s gene expression compared to RA4-I cell culture (Fig. 4, right panel). Expression of the *RcCat1* gene was similar in

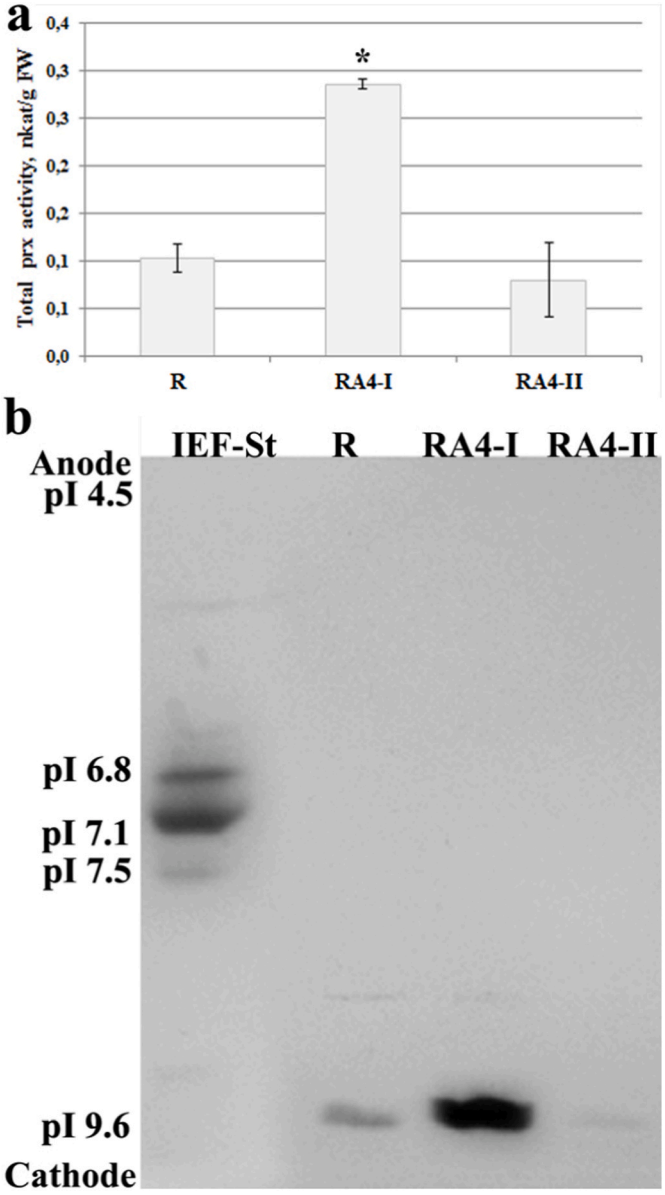


Fig. 5. Activity assay (**a**) and native IEF electrophoresis (**b**) of peroxidases in *R. cordifolia* control cells (R) and pRiA4-transformed cell cultures (RA4-I and RA4-II). Analysis was repeated three times; 30-day-old cell cultures were used. R, RA4-I, and RA4-II cell cultures were grown on W0 and W/BA medium, respectively. The data are presented as the mean ± standard error. Different letters above the bars indicate significantly different means ($p < 0.05$; Fisher's LSD).

both pRiA4-transformed callus lines and was slightly higher than in control R cells (Fig. 4, right panel).

The brightest effect on ROS metabolism was observed for plants' class III peroxidases, Prx. We have compared the activity and isozyme composition of Prx in pRiA4-transformed calli adapted to growing in the presence (RA4-II) and absence (RA4-I) of PGR compared to the control calli R, grown on PGR-containing W/BA medium for 30 days. We have shown that the activity (Fig. 5 a) and isozyme composition (Fig. 5 b) of Prxs in RA4-II calli adapted to growing in the presence of PGR (W/BA medium) were similar to control calli R. Stable growth of the RA4-I callus line in the absence of PGR (W0 medium) led to an increase in Prxs activity more than three times without changes in isozyme composition (Fig. 5).

4. Discussion

The main diagnostic feature of wild-type transformation is hairy root syndrome. It is also possible to regenerate a whole transgenic plant (Velazquez et al., 2005). However, cases of the formation of a stable transgenic callus culture have been recorded. It was shown that some agropine strains can induce tumor proliferation after infection in such plants as *Chrysanthemum indicum* L. and *Nicotiana tabacum* L. (van Wordragen et al., 1992), *Lycopersicon esculentum* L. (Velazquez et al., 2001), and *R. cordifolia* (Shkryl et al., 2008). While untransformed callus cultures need PGR as obligate supplementation (Phillips and Garda, 2019), pRi-induced calluses are independent of PGR for induction and long-term cultivation (van Wordragen et al., 1992; present study). We have shown that the pRA4-transformed cell culture of *R. cordifolia* is capable of long-term cultivation. Moreover, a PGR-free RA4 cell line is characterised as more AQ productive than an untransformed cell culture. This finding is significant and actual for plant biotechnology for two reasons. In plant biotechnology, one of the main problems is finding the best PGR combination to induce and long-term cultivate the cell cultures. It is actually true for some rare and medicinal plants, specifically woody plants. In second, in some countries, Ri-plants and tissue cultures derived from the transformation of wild-type *A. rhizogenes* are free from legal controls on genetically modified organisms (GMOs) (Christensen and Müller, 2009; Mishiba et al., 2006).

In the present study, we described for the first time the PGR-dependent ROS status, growth, and specialized metabolism of the pRiA4-transformed cells. In addition, we analysed individual expression of the agropine *rol*A, *rol*B, and *rol*C genes under long and short treatments of PGR. Our findings show that the expression of the *rol*A and *rol*B genes is contradictory in a PGR-dependent manner: in the presence of PGR, we detected *rol*A expression, whereas in the absence of PGR, we detected *rol*B expression. Short-term cultivation of RA4 calli in the presence of PGR resulted in a strong inhibition of the *rol*B gene expression. The absence of PGR resulted in a slight decrease in *rol*A expression in PGR-adapted calli. At the same time, no effect of PGR on *rol*C gene expression was detected. We can suggest that the antagonism between *rol*A and *rol*B can be regulated through auxin signaling. The data corresponds to the previous hypothesis. Transformed phenotypes (T) and super-transformed phenotypes (T′) are two categories of pRi-morphology; plants of the T-phenotype had reduced pRi-induced symptoms (Tepfer, 1984). It was proposed that the general reason for the smoothing of the pRi-phenotype is antagonism between *rol*A and *rol*B genes. Also, it was proposed that one of *rol*A's primary roles is to assist in the control of *rol*B expression. The discovery of additional *rol*A transcripts, measuring between 2.1 and 2.8 kb, that would span the entire *rol*B sequence and provide an antisense message is a good illustration (Durand-Tardif et al., 1985). There appears to be a mechanism that inhibits *rol*A and *rol*B from being expressed together (Capone et al., 1989; Veena and Taylor, 2007). Moreover, the *rol*A promoter's activity is distinct from that of the *rol*B and *rol*C promoters (Schmulling et al., 1989). The auxin-dependent control of *rol*A's promotors (Maurel et al., 1990), which prevents robust *rol*B-induced auxin sensitivity (Maurel et al., 1991) for balancing cell state. It is likely the cause of the antagonistic relationship between *rol*A and *rol*B (Costantino et al., 1994). Therefore, it may be hypothesized that pRi-transgenic plants only display all of the hairy root syndrome's symptoms when all three *rol* genes are expressed. The *rol* genes perform biological tasks in concert (Spena et al., 1987; Desmet et al., 2020b).

In fact, there are a lot of publications about the effects of wild-type pRi transformations in plants. Authors provided examples such as transgenic plants, hairy root cultures, and, less frequently, callus cultures. We have analysed available information about individual *rol* genes' expression in the different *A. rhizogenes*-transformed plant systems. In the majority of cases, only *rolB* gene expression was shown (Qin et al., 2022; Irigoyen et al., 2020; Xu et al., 2020). Individual expression of the three *rol* genes was established in the study Basu et al. (2015). The

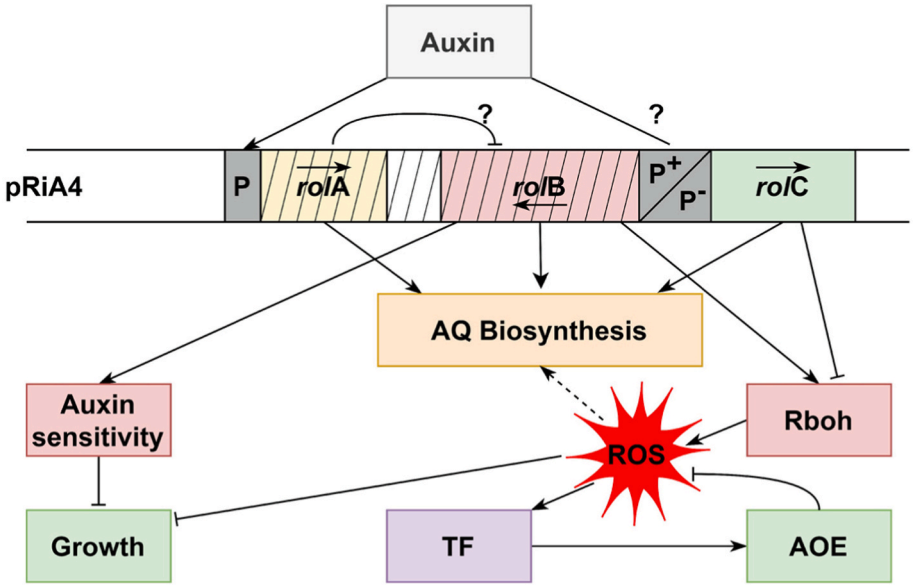


Fig. 6. A simplified model suggesting the possible auxin-dependent mechanism of the combined Rol action on ROS-regulated processes. First, this scheme demonstrates that the promoter (P, dark grey block) of the *rol*A gene can be auxin-regulated, as shown in the present study, due to the presence of DUE-NDE elements in the auxin-regulated genes at positions −595 and −525 (Carneiro and Vilaine, 1993). The presence of the anti-sense chain of the *rol*B gene in the *rol*A transcript is shown by hatching. Auxin regulation of the bidirectional promoter (Schmulling et al., 1989) of *rol*B and *rol*C genes (P^+/P^-, dark grey block) has not been shown reliably in auxin-treated pRiA4-expressed plant cells for a long time. The present model is based on our present and previous studies, which showed that individual expression of *rol*A, *rol*B, and *rol*C genes led to increases in AQ biosynthesis (Shkryl et al., 2008), and *rol*B expression affected growth. In addition, we have demonstrated that while *rol*B expression led to an increase in *Rboh* gene expression (Veremeichik et al., 2016), *rol*C expression led to a significant decrease in *Rboh* gene expression (Shkryl et al., 2022). Individual expression of *rol*B and *rol*C genes has a similar contradictory effect on the ROS scavengers' expression, antioxidant enzymes (AOE) (Bulgakov et al., 2012; Shkryl et al., 2022). The regulatory relationships between ROS, growth, TF (transcriptional factors), and the ROS scavengers' enzymes are represented accordingly to Mittler (2017). RolB-induced auxin sensitivity (Maurel et al., 1994), accompanied with necrosis and growth inhibition, are represented accordingly to Schmulling et al., 1988, and (1985).

authors obtained several hairy root lines of *Plumbago zeylanica* and showed the absence of the *rol*A gene expression in half of them. However, it was connected with the lack of some ORFs, including ORF10 (*rol*A), in the genomes of these hairy root lines (Basu et al., 2015). Several studies have found a lack of ORFs from T-DNA during the cultivation of pRi-transformed plants from hairy root cultures (Jouanin et al., 1987; Taneja et al., 2010; Ray et al., 2014). Only the *rol*B gene is strongly integrated into the plant genome. The *rol*B gene is often used for the determination of transformation or for comparison of the expression level in transformed lines or plants. Another study looked at the expression of three *rol* genes in *Ginkgo biloba* hairy root cultures. The absence of *rol*B gene expression was shown for mature roots and the presence of all three genes for root tips and meristems (Ayadi and Trémouillaux-Guiller, 2003). Anyway, there is not enough information about differential gene expression in pRi-transformed plant systems to compare with our results. Our previous study was performed with the line RA4-I. Using the method of semi-quantitative PCR, we have not shown significant differences in *rol* gene expression (Shkryl et al., 2008). Unfortunately, the method used in the work did not allow a reliable comparison of the level of expression of the *rol* genes with each other. Unfortunately, at that time, the PGR content and duration for RA4 cultivation were not strictly fixed. Only when analyzing class III Prx activity (Veremeichik et al., 2012) was a pattern revealed between the presence of PGR in the medium and Prx activity in the RA4 line. After that, a thorough study of the effects of HRR combinations and the duration of their action was carried out.

Possible PGR-dependent relationships between *rol*A and *rol*B, their possible targets, and their physiological effects are shown in Fig. 6. First, this scheme demonstrates that the promoter (P, dark grey block) of the *rol*A gene can be auxin-regulated, as shown in the present study, due to the presence of DUE-NDE elements in the auxin-regulated genes at positions −595 and −525 (Carneiro and Vilaine, 1993). However, there is no additional information about the *rol*A gene's cis-regulatory apparatus. Auxin regulation of the bidirectional promoter (Schmulling et al., 1989) of *rol*B and *rol*C genes (P^+/P^-, dark grey block) has not been shown reliably in auxin-treated pRiA4-expressed plant cells for a long time. Moreover, it was shown that *rol*B gene expression is strongly regulated through the ACTTTA motif with specifically binding Dof domain-containing transcription factors such as NtBBF1 (Baumann et al., 1999). Expression of this TF is not auxin-dependent, while expression of the *rol*B gene is correlated with auxin presence for a 48-h period in a doze-independent manner (Baumann et al., 1999). One more possible variant of regulation of the *rol*A and *rol*B genes' expression is miRNA machinery. It was shown that *rol*B can modulate the expression of genes encoding core and accessory proteins (DCL1, AGO1, and AGO4) of the microRNA processing machinery (Bulgakov et al., 2015). Later, analysis of small RNAs derived from the T-DNA of *A. rhizogenes* in the hairy roots of *P. vulgaris* showed that rolA is a major source of small RNAs, while no ArT-sRNAs were derived from the *rol*C gene (Pelaez et al., 2017). Moreover, it was shown that MiR393 is absent in the hairy root while present in pRi-transformed calli. It was suggested that MiR393 targets the main auxin receptors and can play an important role during the hairy root disease in calli formation and organ induction (Pelaez et al., 2017).

The supposed simplified model is based on the present and previous studies, which showed that individual expression of *rol*A, *rol*B, and *rol*C genes led to increases in AQ biosynthesis (Shkryl et al., 2008), and *rol*B expression affected growth. A model suggested the possible auxin-dependent mechanism of the combined Rol action on ROS-regulated processes. In addition, we have demonstrated that while *rol*B expression led to an increase in *Rboh* gene expression (Veremeichik et al., 2016), *rol*C expression led to a significant decrease in *Rboh* gene expression (Shkryl et al., 2022). Individual expression of *rol*B and *rol*C genes has a similar contradictory effect on the ROS scavengers' expression (Bulgakov et al., 2012; Shkryl et al., 2022). The upregulation of the expression and activity of Prxs, one of the plant's peroxidases III classes, is one of the most striking effects of *rol*B gene expression (Veremeichik et al., 2012; Shkryl et al., 2013). In this study, we discovered that Prx activity is strongly dependent on PGR-dependent *rol*B expression in RA4 calli. Mittler (2017) depicted the regulatory relationships between ROS, growth, TF (transcriptional factors), and ROS scavenger enzymes. It is known that RolB induces sensitivity to exogenous auxin (Maurel et al., 1994), accompanied by necrosis and growth inhibition (Schmulling et al., 1988; White et al., 1985), while expression of the *rol*A gene affects endogenous IAA (Bettini et al., 2016; Dehio et al., 1993).

The main conclusion of the obtained and discussed data of the

combined Rol action is the large knowledge gap in *rol*A action. There is a blind spot about *rol*A gene expression regulation and physiological effects (Veremeichik et al., 2023). However, the present study highlighted the high importance of the *rol*A gene in the regulation of the combined *rol* genes' effects.

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Code availability

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Ethics approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

The authors of this work agree to be accountable for all aspects of the work, ensuring that questions related to the accuracy and integrity of any part of the work are appropriately investigated and resolved.

Author contributions section

Galina N. Veremeichik, Conceptualization, Supervision, Investigations, Writing – original draft, Visualization.

Tatiana Y. Gorpenchenko, Evgenia V. Brodovskaya, Tatiana V. Rusapetova, Galina K. Tchernoded, Yurii N. Shkryl, Investigations.

Dmitry V. Bulgakov Visualization, Funding.

Victor P. Bulgakov Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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