Suppression of Reactive Oxygen Species and Enhanced Stress Tolerance in *Rubia cordifolia* Cells Expressing the *rolC* Oncogene

Victor P. Bulgakov,¹ Dmitry L. Aminin,² Yuri N. Shkryl,¹ Tatiana Y. Gorpenchenko,¹ Galina N. Veremeichik,¹ Pavel S. Dmitrenok,² and Yuri N. Zhuravlev¹

¹Institute of Biology and Soil Science, and ²Pacific Institute of Bioorganic Chemistry, Far East Branch of the Russian Academy of Sciences, Vladivostok 690022, Russia

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It is known that expression of the *Agrobacterium rhizogenes* *rolC* gene in transformed plant cells causes defense-like reactions, such as increased phytoalexin production and expression of pathogenesis-related proteins. In the present study, we examined whether this phenomenon is associated with increased production of reactive oxygen species (ROS). Single-cell assays based on confocal microscopy and fluorogenic dyes (2,7-dichlorofluorescein diacetate and dihydrorhodamine 123) showed reduced steady-state levels of ROS in *rolC*-expressing *Rubia cordifolia* cells as compared with normal cells. Paraquat, a ROS inducer, caused significant ROS elevation in normal cells but had little effect on *rolC*-transformed cells. Likewise, ROS elevation triggered by a light stress was suppressed in transformed cells. Our results indicate that the *rolC* gene acts as a ROS suppressor in unstressed cells and its expression prevents stress-induced ROS elevations. We detected a two- to threefold increase in tolerance of *rolC*-transformed cells to salt, heat, and cold treatments. Simultaneously, *rolC*-transformed cells maintained permanently active defensive status, as found by measuring isochlorismate synthase gene expression and anthraquinone production. Thus, the oncogene provoked multiple effects in which ROS production and phytoalexin production were clearly dissociated.

Additional keyword: defense reactions.

Processes of reactive oxygen species (ROS) generation by mammalian oncogenes as well as the role of oxidative stress in many aspects of oncology are subjects of unending interest of numerous investigations (Kovacic and Jacintho 2001; Ramsey and Sharpless 2006). However, such a theme for plant oncogenes has never been considered. Involvement of important animal regulators of oncogenesis, such as Rac1 (Cool et al. 1998), v-Ha-ras (Yang et al. 2002), c-Myc (Vafa et al. 2002), and NF- κ B (Sakon et al. 2003) in intracellular ROS production is well documented. In normal human fibroblasts, c-Myc activation induced DNA damage that correlated with induction of ROS. The oncogene activation overrides damage controls, thereby accelerating tumor progression via genetic instability (Vafa et al. 2002). Increased expression of these animal proto-oncogenes causes ROS elevation, except for NF- κ B, which prevents accumulation of ROS (Sakon et al. 2003). Recent

data indicate a complex dynamic role of ROS in oncogenesis, pointing to involvement of ROS in cell senescence accompanied by tumor suppression (Ramsey and Sharpless 2006). Thus, the triangle combining the interplay between oncogene expression, ROS production, and cell senescence or apoptosis is a hot spot in animal cell biology.

The *rolC* gene is a plant oncogene that is contained in plasmids of the plant pathogen *Agrobacterium rhizogenes*. Following agrobacterial infection, the gene is transferred to the plant genome, causing tumor formation and hairy root disease. In transformed plants, *rolC* induces substantial morphological and biochemical alterations (Casanova et al. 2005; Faiss et al. 1996; Nilsson et al. 1996; Shmülling et al. 1988; Spena et al. 1987). Constitutive expression of *rolC* in cultured plant cells leads to alteration of hormone (auxin) sensitivity (Maurel et al. 1991), activates secondary metabolism (Bonhomme et al. 2000; Bulgakov et al. 1998, 2002; Palazón et al. 1998) and expression of pathogenesis-related (PR) proteins (Kiselev et al. 2006), and causes somatic-to-embryo transition (Gorpenchenko et al. 2006) and calcium imbalance (Bulgakov et al. 2003). These pleiotropic effects are not explained at present because the biochemical function of *rolC* remains unclear (Binns and Costantino 1998; Faiss et al. 1996; Nilsson et al. 1996). This might be due to the fact that *rolC* is a unique gene that has no significant sequence similarity with any other genes of prokaryotes or eukaryotes. The *rolC* protein is a member of the *rolB* family of eukaryotic regulators (Otten and Schmidt 1998). It has been proposed that proteins belonging to the family associate with plant proteins to alter developmental plasticity, as in the case of viral oncoproteins in animals (Moriuchi et al. 2004).

After the publication by the V. Citovsky group (Kunik et al. 2001) showing that *Agrobacterium* could transform HeLa cells, it was hypothesized that *Agrobacterium* spp. could transform any eukaryotic organisms (Lacroix et al. 2006). Recently, the *rolC* and *rolB* oncogenes have been shown to cause formation of teratoma-like structures in animal (sea urchin) embryos (Bulgakov et al. 2006). The ability of plant oncogenes to function in animals and the ability of some animal proto-oncogenes (such as members of the Bcl-2 family) to function in plants (Kawai-Yamada et al. 2004) suggests that processes of oncogenesis in plant and animals may have some common features.

ROS play a key role in plant defense reactions (Hammond-Kosack and Jones 1996). Avirulent plant pathogens elicit a biphasic ROS accumulation, with a low-amplitude transient first phase followed by a sustained phase of much higher magnitude that correlates with disease resistance (Torres et al. 2006). Virulent pathogens that avoid host recognition induce the transient,

Fig. 1. Dependence between *rolC* and *Rc-ICS* expression, growth, and anthraquinone (AQ) content in control (R) and *rolC*-expressing (RC-L and RC-H) *Rubia cordifolia* cell-suspension cultures. **A**, Growth of the cultures. **B**, Reverse transcription-polymerase chain reaction (PCR) analysis of *rolC* and *Rc-ICS* (*ICS*) transcripts. Total RNA was isolated from 7-day suspension cultures. NC = negative control (PCR mixture without plant cDNA), PC = positive control (pPCV002-*rolABC* for the *rolC* gene and PCR fragment of *Rc-ICS* cDNA for *Rc-ICS* and the actin cDNA for *Rc-actin*). **C**, Quantitative real-time PCR. **D**, AQ content in 14-day suspension cultures. Total AQ content (% dry cell weight) represents sum of munjistin, purpurin, and minor AQ (ruberritrinic acid, alizarin, xanthopurpurin, metoxymunjistin, and ethoxyxanthopurpurin). Data are presented as mean $\pm$ standard error from two separate experiments with three replicates each.

low-amplitude first phase. Fungal elicitors and other microbe-associated molecular patterns also trigger rapid ROS accumulation (Torres et al. 2006). As *rolC* gene expression leads to activation of secondary metabolites (Bonhomme et al. 2000; Bulgakov et al. 1998, 2002, 2004; Palazón et al. 1998) and PR proteins (Kiselev et al. 2006), one could expect the activation of ROS production by the oncogene. The aim of the present investigation was to determine whether expression of *rolC* affects ROS levels in transformed cells. We used a *Rubia cordifolia* model system in which several *rolC*-transformed cell lines with different expressions of the gene were selected (Bulgakov et al. 2002).

Our data indicate that *rolC* expression actively perturbs ROS levels in transformed cells of *R. cordifolia*. The gene does not merely trigger oxidative burst but significantly lowers intracellular ROS, thus acting as a powerful ROS suppressor. Production of anthraquinones (AQ) and production of ROS in *R. cordifolia* *rolC*-transformed cells represent two independent processes.

RESULTS

Line characterization.

The R (control), RC-L (low *rolC*-expressing), and RC-H (high *rolC*-expressing) cell lines of *rolC*-transformed *R. cordifolia* callus cultures were established seven years ago (Bulgakov et al. 2002). Recently, they were reexamined in detail (Shkryl et al. 2008) to confirm stability of transgene expression and to study combined effects of the *rolA*, *rolB*, and *rolC* genes. The study indicated that the *rolC*-transformed callus phenotypes were conserved and that there was stable expression of *rolC* and high AQ levels during long-term cultivation (Shkryl et al. 2008). However, a contrast was revealed in growth parameters. During long-term cultivation, the growth rate of *rolC*-transformed calli progressively increased, whereas the growth rate of control calli remained stable (Shkryl et al. 2008). As shown in Figure 1A, the growth of *rolC*-transformed suspension-cultivated cells was more active as compared with that of control cells. Reverse transcription-polymerase chain reaction (RT-PCR) analysis revealed that the *rolC* transcripts were three times more abundant in the RC-H culture compared with the RC-L culture (Fig. 1C).

R. cordifolia cultures produce easily detectable colored phytoalexins called AQ. In plants, AQ are biosynthesized by two pathways (polyketide and o-succinylbenzoic acid) and, in members of the Rubiaceae family, the second one, which involves the shikimic acid pathway and isochlorismate synthase (*ICS*), is used (Van Tegelen et al. 1999). Production of AQ and expression of a key gene of AQ biosynthesis, the *ICS* gene, were used as indicators of the defensive status of *R. cordifolia* cells. To analyze expression of *Rc-ICS* in *R. cordifolia* transformed cultures, we used RT-PCR and real-time PCR (Fig. 1B and C). A high positive correlation between the accumulation of *rolC* and *Rc-ICS* transcripts ($r = 0.846$; $P < 0.001$) was detected (Fig. 1C). AQ content was measured by high-pressure liquid chromatography (HPLC) as described below. AQ in the control R culture were presented by the following substances (% total AQ): munjistin (62%), purpurin (22%), ruberritrinic acid (7%), alizarin (6%), xanthopurpurin (1.2%), and trace amounts of metoxymunjistin and ethoxyxanthopurpurin. Composition of AQ was not changed significantly in either of the cultures of *rolC*-transformed calli. However, expression of *rolC* caused a significant increase of total AQ production. HPLC determinations revealed that AQ content was 2.1- and 4.4-fold higher in the RC-L and RC-H cultures, respectively, than in the nontransformed culture (Fig. 1D). Thus, dependence between *rolC* expression and defense-related responses of

cells was evident; the higher the expression of the *rolC* gene, the higher the observed expression of the *Rc-ICS* gene and accumulation of AQ (Fig. 1).

***RolC* expression does not lead to increased endogenous levels of salicylic acid (SA).**

One important issue regarding function of isochorismate synthases in plants is their involvement in biosynthesis of the plant defense hormone SA (Strawn et al. 2007; Wildermuth et al. 2001). Therefore, the increase of the *Rc-ICS* expression in *rolC*-transformed cells would lead to increased levels of endogenous SA that, in turn, would affect *rolC*-mediated defense reactions and, in particular, biosynthesis of AQ. We measured endogenous levels of SA in *R. cordifolia* calli by mass spectrometry (MS). SA levels were low both in the control and *rolC*-transformed cells and they did not exceed concentrations of 13 ng of fresh callus weight per gram (12 ± 2 , 9.0 ± 1.5 , and 13 ± 2 ng g⁻¹ for the R, RC-L, and RC-H cultures, respectively, $P > 0.05$). These concentrations of SA are similar to those reported for other plant species growing under noneliciting conditions (Pan et al. 2008; Segarra et al. 2006). According to these results, we suggest that the induced expression of the *Rc-ICS* gene does not entail increased SA biosynthesis.

Decreased ROS levels in *rolC*-transformed cells.

To test whether the expression of the *rolC* gene in transformed cells is accompanied by an oxidative burst, we monitored steady-state ROS levels in control and *rolC*-expressing live *R. cordifolia* cells, using the fluorescent dye H₂DCF-DA (2,7-dichlorofluorescein diacetate). This dye, after cleavage of diacetate ester by intracellular esterases, reacts with ROS such as hydrogen peroxide, peroxy radicals, and peroxynitrite (Crow 1997).

To exclude the possibility that *rolC*-transformed cells might have different permeability for the dye and different esterase activity compared with control cells, which potentially could confuse ROS measurements, the cells cultivated for 4 days were loaded with fluorescein diacetate, and fluorescence of free fluorescein was measured. Fluorescein diacetate (FDA) fluorescence of cells of R, RC-L, and RC-H cultures was 148.6 ± 4.1 , 158.7 ± 4.4 , and 159.6 ± 3.8 fluorescence units respectively, indicating no significant difference between these cultures ($P > 0.05$ for both RC cultures). Thus, transformed and control cells responded equally to this test, showing similar esterase activity inside cells and equal uptake of the dye.

Measurements with H₂DCF-DA performed with 4- and 7-day cultures showed differences between the R cells and the *rolC*-expressing cells. When the cells were analyzed at the exponential phase of growth (fourth day of cultivation; Fig. 1A) DCF fluorescence was 64 and 47% in RC-L and RC-H cells, respectively, of the DCF fluorescence level seen in R cells (Fig. 2A and C). After 7 days of cultivation, the R and RC-H cultures entered into the stationary phase of growth, whereas the RC-L culture continued to grow actively, being at the linear phase of growth (Fig. 2A). At this time, DCF fluorescence of the *rolC*-expressing cells was half that of the control cells (Fig. 2D). Because AQ are known to possess antioxidant activity (Yen et al. 2000) and, thus, would have an impact on ROS metabolism in *rolC*-transformed cells, we included in the investigation *Panax ginseng rolC*-transformed cultures (GV and 2c2), which contain no AQ (Bulgakov et al. 1998). Measurements of ROS in *P. ginseng* cells revealed that ROS inhibition was also observed in this system (Table 1).

Analogous results were obtained with another fluorescent probe, dihydrorhodamine 123 (H₂R123). ROS specificity of H₂R123 and H₂DCF-DA is similar (Abele et al. 2002; Crow 1997). However, in contrast to H₂DCF-DA, H₂R123 penetrates

Fig. 2. Intracellular reactive oxygen species (ROS) abundance in control (R) and *rolC*-expressing (RC-L and RC-H) *Rubia cordifolia* cell-suspension cultures. Laser-scanning confocal ROS imaging in 4-day resting cells visualized by **A**, 2,7-dichlorofluorescein and **B**, R123. **C**, ROS levels in 4-day and **D**, 7-day cultures are presented as mean $\pm$ standard error from two independent experiments. Asterisks (*) indicate $P < 0.05$ vs. values of the R culture, Student's *t*-test.

the mitochondrial membrane and thereby reflects summarized cytosolic and mitochondrial ROS levels (Abele et al. 2002). Under fluorescence microscopy, control cells were stained extensively with R123, whereas *rolC* cells were stained at a lower intensity (Fig. 2B). At a higher magnification, R123 fluorescence in cells was observed in numerous small particles, which indicated mitochondria. At the exponential phase of growth, the average fluorescence of the RC-L and RC-H

Fig. 3. Effects caused by paraquat (Prq). Intracellular reactive oxygen species (ROS) abundance in control (R) and *rolC*-expressing *Rubia cordifolia* cell-suspension cultures. The cultures were cultivated in the dark for 6 days and were treated with paraquat (10 μ M) for 1 h under continuous light exposure. **A**, Laser-scanning confocal ROS imaging visualized by 2,7-dichlorofluorescein fluorescence. **B**, ROS levels presented as mean $\pm$ standard error from two independent experiments. Asterisks (*) indicate $P < 0.05$ vs. values of the corresponding untreated cultures, Student's *t*-test.

cells was 78 and 46%, respectively, that of the control R cells (Fig. 2C). Measurements performed after 7 days of cultivation also showed decreased levels of ROS in *rolC*-expressing cells (Fig. 2D). R123 fluorescence of the R cells was 65 pixels at this timepoint, i.e., less than half that at day 4 of cultivation (Fig. 2C and D). This might be due to altered permeability of the dye at this stage of cell growth.

ROS measurements performed with callus extracts using an independent method (luminol-based fluorimetric detection) revealed decreased ROS levels in extracts of *rolC*-transformed calli throughout the growth cycle of the RC-L and RC-H cultures, as compared with the R culture (mean values were $68.5\% \pm 10\%$ and $52\% \pm 8\%$, respectively).

Thus, our experiments revealed decreased ROS levels in *rolC*-transformed resting cells.

Paraquat treatment.

The herbicide paraquat acts as a terminal oxidant of photosystem I. In light, it reduces oxygen to the superoxide radical, which subsequently dismutates to hydrogen peroxide (Mehler 1951). As a result, paraquat induces stable ROS elevation in treated plant tissues. In this experiment, moderate treatment conditions (10 μ M paraquat and 1 h light incubation) were used, in which paraquat caused a twofold increase of ROS in the control tissue but did not cause cell death.

As shown in Figure 3, ROS content increased in both the control and *rolC*-expressing cultures after paraquat treatment, suggesting that the *rolC* gene hardly affects the paraquat-mediated mechanism of ROS generation. At the same time, ROS levels in *rolC*-expressing cells remained low, even after application of such a powerful ROS-inducing substance as paraquat. This result means that expression of *rolC* in transformed cells prevented excessive ROS accumulation caused by paraquat.

Time series of ROS accumulation in cells subjected to light stress.

R. cordifolia R and RC-L cells were subjected to acute light stress by Ar laser illumination (488 nm) for 15.8 min. A massive oxidative burst was observed in the control R culture during this time (Fig. 4A). ROS accumulation was initially evident at the periphery of the cells and subsequently spread throughout, excluding vacuoles. No signs of oxidative burst were observed in *rolC*-expressing cells (Fig. 4B). A slight ROS accumulation was evident in the RC-L culture when the laser intensity was increased threefold (Fig. 4C). ROS accumulation reached a plateau after 600 s of illumination in the control cells and after 300 s of illumination in the *rolC*-expressing cells. After this time, ROS accumulation in *rolC*-expressing cells stopped (Fig. 4C, bottom panel). Essentially the same result was obtained when we analyzed RC-H cells (data not shown). Additionally, we performed an analogous experiment with H₂R123. The experiment yielded similar results, i.e., absence of ROS accumulation in *rolC*-expressing cells at the low laser intensity. These peculiarities of ROS accumulation indicate that ROS production is attenuated in the *rolC*-expressing

Table 1. Intracellular reactive oxygen species (ROS) levels in *Panax ginseng* control and *rolC*-expressing cells^a

Cell line	No. of cells analyzed	DCF fluorescence ^b
GV (vector control)	58	65.4 $\pm$ 3.9
2c2 (<i>rolC</i> -expressing)	82	37.0 $\pm$ 1.8 *

^a Seven-day-old cell-suspension cultures of *P. ginseng* were analyzed.

^b DCF = 2,7-dichlorofluorescein, measured in pixels. ROS levels presented as mean $\pm$ standard error from two independent experiments. * $P < 0.01$, Student's *t*-test.

Fig. 4. Confocal imaging of reactive oxygen species (ROS) during light-induced oxidative burst. **A**, Time series of ROS production in control (R) cells subjected to illumination from an argon laser (excitation at 488 nm). The intensity of the laser was 5.9% maximal. **B**, ROS in the low *rolC*-expressing (RC-L) cells at laser intensity 5.9%. **C**, ROS in the RC-L cells at laser intensity 17.7%. Images of single cells were captured as indicated in the top panel, video files of the images were analyzed using LSM 510 Release 3.5 software and are presented in the bottom panel. Cells on the images were marked with different colored regions of interest. Different colored curves on the graphs at the bottom indicate individual changes in 2,7-dichlorofluorescein fluorescence intensity of corresponding cells over time.

cells. In other words, high ROS levels cannot be produced in *rolC*-expressing cells under different treatments (similar to results from the experiment with paraquat).

Increased salt, cold, and heat tolerance of transformed cells.

It is known that ROS contribute to stress damage, as evidenced by observations that transgenic plants overexpressing ROS scavengers show increased tolerance to environmental stresses (Xiong et al. 2002). We hypothesized that *rolC*-expressing cells could be more tolerant to abiotic stresses than normal cells owing to their ability to prevent oxidative burst. To test the sensitivity of *rolC*-transformed cells to salt stress, the R, RC-L, and RC-H callus cultures were grown in the presence of varying NaCl concentrations. As shown in Figure 5A, the *rolC*-expressing cultures were more resistant to salt stress than the corresponding control culture. The concentration of NaCl that decreases callus growth by 50% (IC₅₀) was approximately 15 mM, 35 mM, and 60 mM for the R, RC-L, and RC-H callus lines, respectively. The difference between the R and *rolC*-expressing cultures (RC-L and RC-H) was significant (analysis of variance [ANOVA] followed by Fisher's protected least significant difference [PLSD] test). Thus, the *rolC* gene conferred a two- to fourfold increase in tolerance of transformed cells to salt treatment. Both *rolC*-expressing cultures could grow in the presence of a NaCl concentration as high as 200 mM, whereas growth of the control culture was completely inhibited at the same concentration. To test ROS levels in salt-stressed *rolC* cells, we performed additional ROS measurements in suspension-cultivated cell cultures. The data presented in Table 2 indicate that the cells incubated in the presence of NaCl (150 mM for 30 min) accumulated higher levels of ROS. However, in the presence of 150 mM of salt, ROS levels in *rolC*-expressing cells were below the level observed in the control nonstressed cells (Table 2). Therefore, the *rolC*-medi-

ated tolerance to salt stress can be explained by the fact that the *rolC*-transformed cells produced low levels of ROS.

Cultivation of *R. cordifolia* calli under conditions of cold (8°C) and heat (28°C) demonstrated increased resistance of the *rolC* culture as compared with the control culture (Fig. 5B). Differences between the R and RC-L cultures, as well as between the R and RC-H cultures, were statistically significant for both cold and heat treatments ($P < 0.01$, ANOVA). Experiments performed under more severe conditions, such as cultivation at 4 or 37°C, caused total growth inhibition of both control and *rolC* cultures.

DISCUSSION

AQ and ROS production.

Because a clear negative correlation between ROS levels and AQ content was observed (Figs. 1 and 2), an important question arose whether AQ could have an impact on ROS in *R. cordifolia* cells. It is known that AQ possess a potent antioxidant activity in vitro (Yen et al. 2000). *R. cordifolia* AQ have an in vitro antioxidative effect (Tripathi et al. 1997), which was confirmed by our measurements as well (data not shown). However, in live organisms (animals and plants), AQ often show prooxidative effects (Babu et al. 2005; Verma 2006) because of the ability of 1,4-naphthoquinones to accept electrons to form the corresponding radical anion species that yields ROS (Verma 2006). By this reason, many chemotherapeutic strategies use natural AQ as ROS generators (Verma 2006; Yang et al. 2004). Our results also showed that AQ isolated from the R callus culture did not possess a direct neutralizing action on free radical products in in vivo experiments and suppressed a total antioxidant activity in rat's blood (Mischenko et al. 2007). While effect of exogenously added AQ on ROS content is well studied in many models, none is known about the effect of endogenous AQ on ROS metabolism in plant cells. We have some indirect evidence suggesting that endogenous AQ have a small effect on cell oxidative status. Our measurements, performed with *rolB*-transformed cells of *R. cordifolia*, showed that *rolB*, in contrast to *rolC*, has some influence on ROS content in resting cells (V. Bulgakov, D. Aminin, T. Gorpenchenko, and Y. Shkryl unpublished data), although these cells contain much more AQ than *rolC*-producing cells (Shkryl et al. 2008). ROS suppression was observed also in *rolC*-transformed cells of *Panax ginseng*, which do not produce AQ (Table 1). These results suggest that ROS suppression was caused by the *rolC*-transformation but not by increased levels of AQ.

ROS inhibition in *rolC*-transformed cells.

In this investigation, we revealed a strong inhibitory effect of the *rolC* plant oncogene on ROS levels in transformed cells of *R. cordifolia*. The constitutive expression of the oncogene led to decreased steady-state levels of ROS in the cells and

Fig. 5. Resistance of the *Rubia cordifolia* *rolC*-transformed callus cultures to **A**, salt and **B**, temperature stresses. The control (R) and *rolC*-transformed calli were cultivated in the presence of different concentrations of NaCl (0, 20, 60, and 200 mM) or at low (8°C) or high (28°C) temperatures for one month. Normal cultivation temperature was 24°C. Values are presented as mean $\pm$ standard error obtained in three separate experiments consisting of 10 replicates each.

Table 2. Reactive oxygen species (ROS) levels^a

Treatment	2,7-Dichlorofluorescein fluorescence ^b		
	R	RC-L	RC-H
NaCl, 0 mM	72.8 $\pm$ 4.2	46.6 $\pm$ 0.9	43.5 $\pm$ 0.3
NaCl, 150 mM	100.9 $\pm$ 3.6	69.7 $\pm$ 5.7	52.0 $\pm$ 3.6

^a Measured by confocal microscopy in *Rubia cordifolia* cells treated with 150 mM NaCl for 30 min. Five-day-old cell-suspension cultures were used for these determinations, and 30 cells were analyzed in each case. The data were obtained in a single experiment and are presented as mean $\pm$ standard error.

^b Measurement given in pixels. R, RC-L, and RC-H indicate control and low and high *rolC*-expressing lines, respectively.

prevented ROS accumulation after different treatments (Figs. 2 through 4). This prevention seems to be efficient in establishing an enhanced resistance of *rolC*-transformed cells to salt and temperature stresses (Fig. 5). Acting as a ROS suppressor in transformed cells, the *rolC* gene probably could prevent oxidative damage, which is the main destructive factor of abiotic stresses (Xiong et al. 2002).

It was suggested that *A. rhizogenes* could manipulate defense pathways in transformed plant cells via expression of T-DNA oncogenes (Bulgakov 2008). This proposition was recently supported by experiments showing that *rol* genes are main players in the phenomenon of increased phytoalexin production caused by *A. rhizogenes* T-DNA integration to the plant genome (Shkryl et al. 2008). Although effects generated by *rolC* in transformed cells, such as increased phytoalexin production (Bulgakov 2008) and enhanced expression of PR proteins (Kiselev et al. 2006), resemble those caused by fungal elicitors, it is evident that the *rolC* gene activates defense reactions by a different mechanism. The main difference is that *rolC* expression does not lead to an increased ROS level in transformed cells and actively suppresses the oxidative burst. The combination of defense effects coupled with the effect of ROS suppression represents a unique case of a single transgene action.

In principle, the effect of ROS inhibition by a phytopathogen is commonly observed in many plant-microbe interactions (Van Sluys et al. 2002). *A. tumefaciens*, which is closely related to the *A. rhizogenes* species, has developed several unique strategies to overcome induced plant defense mechanisms (Escobar and Dandekar 2003). The *A. tumefaciens* catalase KatA, which converts hydrogen peroxide into oxygen and water, is required for virulence on kalanchoe (Xu and Pan 2000). This strategy of phytopathogens is aimed at inhibition of host defense reactions. However, such a defense response as phytoalexin production in *rolC*-transformed *R. cordifolia* cells is not inhibited. It is possible that situations occur where *rolC* performs its own critical function to regulate secondary metabolism by bypassing upstream plant control mechanisms and directing defense reactions via a "short cut." Pharmacological experiments suggest that the *rolC* gene acts on phytoalexin production independently of plant defense hormones and the calcium-dependent NADPH oxidase pathway (Bulgakov et al. 2002, 2003, 2004, 2005). The signal network that functions independently of ethylene, SA, and methyl jasmonate was reported for *Arabidopsis* plants infected with avirulent strains of *Pseudomonas syringae* (Grant et al. 2000). However, in that particular case, plant defense was based on sustained production of ROS and subsequent activation of mitogen-activated protein kinases. Another example in which common plant defense hormones and ROS signals were not required for plant defense responses was reported by Singh and Roberts (2004). The number of such examples is very limited, and they are an exception to the general rule postulating involvement of ROS and defense hormones in plant defense reactions (Hammond-Kosack and Jones 1996). Considering the natural history of *A. rhizogenes*-plant interactions, when ancestral T-DNA genes participated in ancient infection processes and played a role in the early events of plant species differentiation (Aoki and Syono 1999; Intrieri and Buiatti 2001), one can speculate that *rolC* influences primitive plant regulatory networks and does not affect the more recent flexible instruments developed by plants through ROS and defense hormone signaling.

In contrast to several animal proto-oncogenes (*rac1*, Cool et al. 1998; *c-myc*, Vafa et al. 2002; *v-Ha-ras*, Yang et al. 2002) that accelerate tumor progression via enhanced ROS production, *rolC* probably does not act in a similar way, because expression of the gene does not trigger oxidative burst. It is inter-

esting that despite the active influence of *rolC* on many cellular processes, its expression does not deter growth of *R. cordifolia*-transformed cells (Fig. 1A). During long-term cultivation, the growth of the *rolC*-transformed calli progressively increased, whereas the growth of the control calli remained stable (Shkryl et al. 2008). The highest growth rate was observed in the *rolC*-low expressing callus line. A similar observation was also made for *rolC*-low expressing *P. ginseng* calli (2c2 line), which were characterized by a remarkably stable and vigorous pattern of growth over a long period (over 10 years) without any selection (Gorpenchenko et al. 2006). This effect was also observed for *Eritrichium sericeum* and *Lithospermum erythrorhizon* cells (Bulgakov et al. 2005). It looks like that there is an optimal level of ROS removal in *rolC*-transformed cells and that excessive ROS suppression is negative for cell homeostasis. The ROS-suppressing function of *rolC* requires further careful examination to evaluate whether or not *rolC* has a function similar to those animal proto-oncogenes (such as NF- κ B) that repress apoptosis by ROS suppression, thereby ensuring tumor survival.

MATERIALS AND METHODS

Plant cell cultures.

The control nontransformed callus culture (R) and the independently transformed lines with low and high expression of the *rolC* gene (RC-L and RC-H callus cultures, respectively) were obtained from a single clonally cultivated plantlet of *Rubia cordifolia* L. (Rubiaceae) as described previously (Bulgakov et al. 2002). In both cultures, the *rolC* gene was expressed under the control of the *Cauliflower mosaic virus* 35S promoter (Bulgakov et al. 2002). The nontransformed and *rolC*-transformed calli were cultivated using identical conditions in 100-ml Erlenmeyer flasks on W_{B/A} medium (supplemented with 0.5 mg of 6-benzylaminopurine and 2.0 mg of α -naphthaleneacetic acid per liter) in the dark at 25°C with 30-day subculture intervals. Cell suspensions were cultivated using W_{B/A} liquid medium in the dark at 25°C with 14-day subculture intervals. The *Panax ginseng* GV (control) and 2c2 (*rolC*-transformed) cell cultures (Gorpenchenko et al. 2006) were cultivated using W_{4CPA} liquid medium (supplemented with 0.4 mg of 4-chlorophenoxyacetic acid per liter) in the dark as described previously (Bulgakov et al. 1998).

Laser confocal imaging of intracellular ROS.

Measurements of intracellular ROS were based on the ability of plant cells to oxidize fluorogenic dyes to their corresponding fluorescent analogs that allowed ROS determination in living cells (Allan and Fluhr 1997; Yamamoto et al. 2002). *R. cordifolia* cell suspensions were grown in liquid W_{B/A} medium for 4 or 7 days and were filtrated through 100- μ m mesh nylon to separate cell clusters. Single cells and 10- to 20-celled aggregates were gently centrifuged and resuspended in liquid W_{B/A} medium containing 50 μ M H₂DCF-DA (Molecular Probes, Eugene, OR, U.S.A.), 10 μ M H₂R123 (Molecular Probes), or 100 μ M FDA (Molecular Probes) and were incubated at 25 $\pm$ 1°C in the dark. Cells were incubated with H₂DCF-DA and H₂R123 for 10 min and with FDA for 15 min. Dye-loaded cells were washed in W_{B/A} medium and resuspended. Intracellular oxidation of H₂DCF-DA and H₂R123 yielded DCF and R123 that were detected by microscopy. Examination of fluorescence in single living cells was performed with a LSM 510 META confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) equipped with an argon laser with effective power 30 mW. For FDA and DCF measurements, the following settings were used: scan mode: plane, time series, 1,000 frames, 8 bit; stack size: 512 $\times$ 512 $\times$ 1;

pixel time: 1.6 μ s; objective: Plan-Neofluar 40 $\times$ 1.3 Oil DIC; beam splitter: HFT 488; NDD MBS: none; filter: LP 505; pin-hole: 66 μ m; detector gain: 712; amplifier offset: 0.034; amplifier gain: 1. R123 was measured using similar settings with a slight modification (detector gain: 774; amplifier offset: 0.051). Confocal images were obtained after excitation at 488 nm and emission at 522 nm. The intensity of the Ar laser was 5.9% of maximal for FDA and H₂DCF-DA and 10% for H₂R123, if not indicated otherwise (Fig. 4C). These settings were constant for every set of experiments. All confocal images were recorded as 40-s time series at intervals of 0.5 ms. Video files of the captured images were recorded using settings described above and were analyzed with LSM 510 Release 3.5 software. Data were presented as the mean of two or three separate experiments (at least 30 to 40 cells were analyzed in each experiment).

Fluorimetric determination of ROS.

The production of ROS in callus cultures was measured by the luminol-based fluorimetric method according to Piedras and associates (1998). The control and *rolC*-transformed calli were harvested at 14, 21, and 28 days of cultivation and were analyzed. The results of ROS measurements were averaged.

Paraquat and light treatments.

Suspension-cultivated *R. cordifolia* cells were grown for 6 days in the dark and were treated with paraquat (10 μ M, final concentration) for 1 h under continuous light exposure (200 μ mol m⁻² s⁻¹ radiation). Light stress was caused by continuous illumination of cells with the LSM 510 META-Ar laser (effective power 30 mW, 5.9 or 17.7% maximal laser intensity) at 488 nm.

RT-PCR and real-time PCR analyses.

RNA isolation and RT-PCR analysis of the *rolC* gene and *R. cordifolia* genes (*Rc-actin*, GenBank accession number DQ531565, and *Rc-ICS*, GenBank accession number EF090619) were performed as described previously (Shkryl et al. 2008). Total RNA was isolated from suspension cultures by a LiCl method, to avoid sample contamination with AQ. RNA expression profiles were normalized using the expression of the *Rc-actin* gene. The identities of all RT-PCR products were confirmed by DNA sequence analysis. RT-PCR products were subcloned into a pTZ57R/T plasmid using an InsT/Aclone PCR product cloning kit (FERMENTAS, Vilnius, Lithuania) and were sequenced using a Big Dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, U.S.A.), following the manufacturer's protocol and recommendations. After an ethanol purification step, the sequences were identified on an ABI 310 Genetic Analyser (Applied Biosystems).

Quantitative real-time was performed according to Giulietti and associates (2001). The gene-specific primer pairs were: for *rolC*, 5'-CACGGCTGCTGTACCTCTA and 5'-GGAAGGTTGGTGAGGAGTGC (*TaqMan* probe 5'-TCGACTGCCCGACGATGATGCTCTGCTTCT); for *Rc-ICS*, 5'-TATCGTGCGAGAGAATCA and 5'-GAATTCTGGCCAGTTTGCGA (*TaqMan* probe 5'-CACGAACGTGCAAGCTAATCCGGAGAAGGT); for *Rc-actin*, 5'-TCAATCCAAACGAGGTATCTT and 5'-TGTAGAAAGTGATGCCAAAT (*TaqMan* probe 5'-TGAGCAGGTATTGTTAGCAATTGGGATGA). The suitability of the primers' sequences in terms of efficiency of annealing was evaluated using the Primer Premier 5.0 program. For real-time quantitative PCR, cDNAs were amplified by using a Real-time PCR kit (Syntol, Moscow) with an iQ5 thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.) supplied with Optical system software v.2.0.

Estimation of the AQ content.

The analytical HPLC was carried out using an Agilent Technologies 1100 Series HPLC system equipped with a VWD detector (Agilent Technologies, Inc., Santa Clara, CA, U.S.A.). Extracts were prepared and analyzed using a HYPERSIL BDS-C-18 column (5 μ m, 250 $\times$ 5 mm) as described previously (Mischenko et al. 2007). The data were analyzed with the ChemStation program var. 09 (Agilent Technologies, Waldbronn, Germany). AQ were identified by ¹H and ¹³C nuclear magnetic resonance (NMR) using a Bruker NMR Avance DPX-500 instrument as described previously (Mischenko et al. 2007).

Analysis of SA by MS.

SA was extracted from *R. cordifolia* cells quantitatively as described by Segarra and associates (2006) and was analyzed according to Pan and associates (2008) in the Far Eastern Instrumental Center for Biotechnology and Gene Engineering at the Institute of Biology and Soil Science, Far East Branch of the Russian Academy of Sciences (Vladivostok, Russia) using a hybrid quadrupole time of flight mass spectrometer (Agilent 6510 Q-TOF LC/MS, Agilent Technologies, Inc.). Cell extracts or methanol solutions of commercial SA (Sigma, St. Louis) were directly injected in the spectrometer outfitted with an electrospray ion source at a flow rate of 0.3 ml/h. The identity of SA was confirmed by analysis of exact masses of the deprotonated molecules of SA [M-H]⁻ with m/z 137.0244 and product ion with m/z 93.0346 specific for SA and representing a major fragmentation path. The analysis parameters (in MS and MS/MS modes) were optimized for the production of characteristic precursor and product ions in negative ionization mode. SA levels were determined based on comparison of the averaged peak heights of [M-H]⁻ ions in concentrations of 3 to 3,000 ng/ml. SA solutions and extracts were measured using identical conditions.

Statistical analysis.

In statistical evaluation, a Student's *t* test was used for comparison between two independent groups. For comparison among multiple data, ANOVA was followed by a multiple comparison procedure. A Fisher's PLSD post hoc test was employed for the intergroup comparison. A difference of *P* < 0.05 was considered significant.

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