ROS suppression by the rolB oncogene

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Plants Interacting with Other Organisms
The rolB gene suppresses reactive oxygen species in transformed plant cells through the sustained activation of antioxidant defense

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Abstract

The rolB oncogene has previously been identified as a key player in the formation of hairy roots during the plant-Agrobacterium rhizogenes interaction. In this study, using single-cell assays based on confocal microscopy, we demonstrated reduced levels of reactive oxygen species (ROS) in rolB-expressing Rubia cordifolia, Panax ginseng and Arabidopsis thaliana cells. The expression of rolB was sufficient to inhibit excessive elevations of ROS induced by paraquat, menadione and light stress and prevent cell death induced by chronic oxidative stress. In rolB-expressing cells, we detected the enhanced expression of antioxidant genes encoding cytosolic ascorbate peroxidase, catalase and superoxide dismutase. We conclude that, similar to pathogenic determinants in other pathogenic bacteria, rolB suppresses ROS and plays a role not only in cell differentiation but also in ROS metabolism.

Keywords - Agrobacterium rhizogenes · rolB oncogene · defense reactions · paraquat · reactive oxygen species · stress resistance

Abbreviations - AQ: anthraquinone, H2DCF-DA: 2,7-dichlorofluorescein diacetate, H2R123: dihydrorhodamine 123, ROS: reactive oxygen species
Introduction

During agrobacterial infection, the rolA, rolB and rolC genes of the plant pathogen Agrobacterium rhizogenes are transferred into the plant genome, causing tumor formation and hairy root disease (reviewed by Nilsson and Olsson, 1997). The expression of the rol genes, and, most importantly, the rolB gene, is critical for hairy root production (Nilsson and Olsson, 1997). The function of rolB is not restricted to root formation; the gene promotes the de novo formation of floral and shoot meristems (Altamura et al., 1994; Koltunov et al., 2001), induces parthenocarpy (Carmi et al., 2003), causes a delay in pistil and anther development (Cecchetti et al., 2004) and modifies the balance between the proliferation of procambial cells and xylem differentiation during stamen development (Cecchetti et al., 2007). The mechanism by which the RolB oncoprotein exerts such different morphological alterations remains unknown. RolB was shown to exhibit tyrosine phosphatase activity (Filippini et al., 1996) and to interact with 14-3-3 proteins (Moriuchi et al., 2004). RolB has no homology to any prokaryotic or eukaryotic proteins except the RolB (PLAST) family of oncoproteins in Agrobacterium species (Levesque et al., 1988; Otten and Schmidt, 1998). These RolB-related oncoproteins have been proposed to alter the developmental plasticity of transformed plants (Levesque et al., 1988; Moriuchi et al., 2004).

A new function for the rol genes in plant-Agrobacterium interactions was revealed with the discovery that these genes are potential activators of secondary metabolism in transformed cells from different plant families (Bulgakov, 2008). An investigation of the rolA-, rolB-, rolC-, rolABC- and pRiA4- (wild type A. rhizogenes, strain A4) transformed cells of Rubia cordifolia revealed that each of the rol genes appears to have its own individual mechanism of secondary metabolism activation (Shkryl et al., 2008).

Recently, we performed experiments to understand the relationship between the activation of secondary metabolism and the production of reactive oxygen species (ROS) in R. cordifolia cells transformed with A. rhizogenes pRiA4 and the rolC gene (Bulgakov et al., 2008; Shkryl et al., 2010). Single-cell assays based on confocal microscopy showed that rolC significantly lowers intracellular ROS levels, thus acting as a powerful suppressor of ROS. The transformation of R. cordifolia calli with the wild-type A. rhizogenes A4 strain resulted in the decrease of ROS levels in pRiA4-transformed cells. However, this effect was weaker than that observed with the expression of the single rolC gene (Shkryl et al., 2010). The suppression of ROS in pRiA4-cells was accompanied by
the enhanced expression of several genes encoding ROS-detoxifying enzymes (Shkryl et al., 2010).

The effect of the rolB gene on ROS metabolism in transformed cells has not been studied. As far as the rolB and rolC genes act together in the process of neoplastic transformation, it is reasonable to expect that rolB would act in concert with rolC to decrease ROS levels. However, the participation of rolB in the induction of cellular death (necrosis) in the callus and leaves of transformed plants (Schmülling et al., 1988) and activation of secondary metabolism (Bulgakov, 2008), i.e., the processes which are often associated with the increased production of ROS, would indicate increased ROS levels in transformed tissues. The aim of the present investigation was to discriminate between these possibilities.

RESULTS

Steady-state ROS Levels in rolB-transformed Cells

Three cell lines, RB-L (low rolB expression), RB-M (moderate rolB expression) and RB-H (high rolB expression), were established several years ago and recently re-examined in terms of their gene expression, growth and anthraquinone production (Shkryl et al., 2008). In these cell lines, rolB is expressed at a ratio of 1:4:10, respectively. The stability of gene expression was controlled during this work. The RB-L, RB-M and RB-H cultures consisted of cell aggregates with yellow, deep-yellow and orange-red colors, respectively. The deep-colored RB-H culture occasionally forms small black zones of necrotic cells and represents a culture with the maximum possible rolB transcript abundance; the increased expression of rolB in these cells induces cell death.

H2DCF-DA is currently the most widely used fluorogenic probe for real-time ROS imaging in plants (Swanson et al., 2011). Subsequent to the cleavage of the diacetate ester by intracellular esterase, this dye reacts with reactive oxygen species, such as hydrogen peroxide, peroxyl radicals and peroxynitrite (Crow 1997). When H2DCF-DA was used as a fluorogenic dye, the rolB-expressing lines showed a highly reproducible decrease of the steady-state levels of ROS (Figure 1).

These results were confirmed using another fluorescent probe, dihydrorhodamine 123 (H2R123). The specificity of H2R123 and H2DCF-DA for ROS is similar (Crow 1997; Abele et al. 2002). H2R123 has less molar fluorescence than H2DCF-DA, but the former
penetrates the mitochondrial membrane and thereby reflects the total cytosolic and mitochondrial ROS levels (Hempel et al., 1999). The level of ROS in the RB-L and RB-M cultures was 81-83% of that in the R culture, similar to the measurements obtained with H$_2$DCF-DA (80-83%, Figure 1A). Low levels of ROS were detected in the RB-H line (Figure 1). The cells were analyzed during the exponential growth phase (4-5 days of cultivation). An analogous result was obtained when we analyzed the cells during the linear growth phase (day 7) and in the stationary growth phase (11-12 days). These results were confirmed using callus cultures (Figure 1B).

To test whether this effect was species-specific, we included other model systems in our investigation, i.e., the long-standing test system based on Panax ginseng transformed cell cultures (Bulgakov et al., 1998) and the recently transformed Arabidopsis thaliana cells. Measurements of ROS in these cultures revealed that ROS inhibition by rolB was also observed in these systems (Table 1).

ROS measurements were also performed on the callus extracts of all species studied using an independent method, luminol-based luminometric determination. In this assay, the ROS were determined as a sum of hydrogen peroxide, superoxide anion and hydroxyl radicals (Komrskova et al., 2006). The values obtained from these measurements were consistent with confocal microscopy data and revealed that ROS concentration in rolB-transformed calli from all species were 10-30% lower than in the control calli (Table 2).

Taken together, our results indicate that rolB expression reproducibly decreases the steady-state ROS level in transformed plant cells.

**ROS Accumulation in Stressed Cells**

Different treatments were applied to trigger ROS production in rolB-transformed cell cultures of *R. cordifolia*. For this purpose, we used paraquat, menadione and light stress.

To study the effect of paraquat on ROS levels, moderate treatment conditions (10 μM paraquat and 1 h light incubation) were used, in which paraquat did not cause cell death.

As shown in Figure 2, the measurements performed with H$_2$DCF-DA and H$_2$R123 revealed lower ROS levels in the paraquat-treated rolB-transformed cells than in the control cells. From these results, we concluded that rolB effectively prevented the excessive increase in ROS levels induced by paraquat. In the R culture, R123 fluorescence revealed ROS localization at the periphery of the cells, around the nuclear envelope and in the cytosol.
(Figure 2). In the RB-H culture, R123 fluorescence showed that ROS localized inside the cells, with almost no ROS in the plasma membrane or nucleus (Figure 2). This result indicates that rolB mainly suppresses intracellular ROS related to the plasma membrane and nuclear regions.

The experiments using menadione and H$_2$DCF-DA as a probe showed similar results. While rolB-expressing cells responded to paraquat treatment with a slight but noticeable ROS induction, these cells were almost insensitive to menadione, showing no ROS elevation compared with the control cells (Table 3). Menadione produces superoxide radicals and H$_2$O$_2$ at the plasma membrane by the single-electron reduction of O$_2$ in a reaction catalyzed by NAD(P)H:quinone-acceptor oxidoreductase (Schopfer et al., 2008). Paraquat has another mechanism of ROS generation: it acts as a terminal oxidant of photosystem I. In light, it reduces oxygen to a superoxide radical, which subsequently dismutates to H$_2$O$_2$ (Mehler 1951). In our experiments, paraquat caused the rapid elevation of ROS production after 1 h of treatment; menadione caused a gradual elevation of ROS levels over a more prolonged period (20 h).

To test the viability of the cells during prolonged cultivation, the cells were stained with propidium iodide after 24 and 48 h of cultivation with 0-500 μM of menadione. Propidium iodide can only enter cells with damaged membranes, whereupon it intercalates into double-stranded nucleic acids, resulting in a bright red fluorescence in non-viable cells, particularly in the nucleus (Figure 3). From these experiments, the IC$_{50}$ values (the concentration of menadione that decreases cell viability by 50%) were calculated. At 24 h of cultivation, the IC$_{50}$ values were 100 μM and 250 μM for the R and RB-H cultures, respectively. At 48 h of cultivation, the same level of resistance in the rolB-transformed cells to menadione was observed (a 2.5-fold difference); the IC$_{50}$ values were 70 μM and 180 μM, respectively. The rolB-transformed cells were viable even in the presence of 500 μM menadione (Figure 3). This is probably the highest level of resistance to the inhibitor reported for plant cells.

The results of these experiments suggest that rolB-expressing cells could sustain a permanently active mechanism suitable for ROS detoxification.

In the subsequent experiment, the R and RB cells were subjected to light stress by Ar laser illumination (488 nm) for 16 min. In the control R culture, there was a 1.7-fold increase in the ROS level during this time (Table 3). In the low rolB-expressing cells, the increase in ROS levels was less significant (1.45-fold). The RB-H culture showed no elevation of ROS levels. Because the rolB-transformed cells initially contained less ROS,
the light-induced ROS levels were similar to those observed in the control cells before light treatment (Table 3). Thus, rolB prevented excessive ROS accumulation during light-induced stress.

The rolB Gene Prevents Cell Death During Long-term Application of Paraquat

In the experiments described above, the cell cultures were subjected to the acute action of ROS-inducing stimuli. We were interested in examining the effect of rolB on long-term ROS stress. For this experiment, we added paraquat (100 μM) to actively growing four-day-old R. cordifolia cell suspension cultures. These cultures were subsequently cultivated for four days. To assess the effect on ROS, half of the culture vessels were incubated in the dark and the other half in the light (paraquat induces ROS only under light conditions). Subsequently, the cells were stained with propidium iodide to determine the percentage of non-viable cells. In the presence of paraquat, 11% and 85% of the cells in the R culture were non-viable under dark and light conditions, respectively (Figure 4). In contrast, no dead cells were detected for the rolB culture under dark conditions, and only 16% of the cells were damaged under light conditions. This result indicates that rolB expression strongly protects cells against ROS-induced cell death.

For comparison, we included rolC-expressing cells in this experiment. Interestingly, rolC did not prevent cell death because 92% of the rolC cells were damaged. This damage resulted in distinct phenotypical effects: the R and RC-H cultures demonstrated a dying phenotype, whereas the RB-H culture was viable (Figure 4).

In an additional experiment, we applied H₂O₂ exogenously to suspension cultures of R. cordifolia and measured their growth for 6 days. At the 2 mM concentration, hydrogen peroxide inhibited the growth of the R- and rolC-transformed cultures but not that of the RB-H culture. This culture was viable even under treatment with H₂O₂ at concentrations as high as 10 mM (data not shown).

Expression of Genes Participating in ROS Detoxification in rolB Cells

It is known that the mechanism of ROS detoxification in plants involves the enhanced expression of genes encoding antioxidant enzymes, such as superoxide dismutase, ascorbate peroxidase, catalase, glutathione peroxidase and other enzymes (reviewed by Mittler et al. 2004).
We studied whether the expression of genes encoding antioxidant enzymes was changed in rolB-transformed cells as compared with the control cells. The expression of *A. thaliana* genes encoding ascorbate peroxidase (EC 1.11.1.11), superoxide dismutase (EC 1.15.1.1) and catalase (EC 1.11.1.6) and the corresponding *R. cordifolia* genes, described previously (Shkryl et al., 2010), was studied using quantitative real-time RT-PCR (Table 4). In this table, we also show the expression of the rolB gene measured in parallel with that of the antioxidant genes (upper panel of Table 4).

The expression of the *AtCat1* gene (GenBank accession no. NP_564121.1) and the orthologous *R. cordifolia* catalase *RcCat1* gene (GQ380493) (sharing 85 % identity at the amino acid level, Table 4) showed a 2-3 fold increase in rolB-expressing cells. This effect was dependent on the strength of rolB expression. These genes were previously shown to be the main catalase genes participating in ROS detoxification in Arabidopsis (Frugoli et al., 1996) and *R. cordifolia* (Shkryl et al., 2010).

The expression of Arabidopsis ascorbate peroxidase genes *AtApx1* (GenBank accession number AT1G07890), *AtApx2* (AT3G09640) and *AtApx3* (NP_195226) was compared with that of the orthologous genes *RcApx1* (GQ380494), *RcApx2* (GU949549) and *RcApx3* (GU949550). According to the literature, *AtApx1* and *AtApx2* of Arabidopsis (Panchuk et al., 2002; Davletova et al., 2005), and *RcApx1* and *RcApx2* of *R. cordifolia* (Shkryl et al., 2010) are cytosolic isoforms of ascorbate peroxidases that play a pivotal role in ROS degradation. *AtApx3* and *RcApx3* are peroxisomal membrane-bound isoforms. We found that all these forms were upregulated in rolB-transformed cells as compared with normal cells, but this upregulation was observed only in cultures with a low and moderate expression of rolB (Table 4). In cells with high rolB expression (RB-H line), the expression of the *Apx* genes was similar to that in the control. Expression of the rolB gene in this line was 11 times higher than that of the RB-L line, i.e., the rolB gene was strongly overexpressed (Table 4). We could not select Arabidopsis cells with an analogous high expression of rolB because such cells were not viable. Thus, in our test systems we observed the rolB-dose-dependent process of Apx gene regulation. The behavior of Apx genes can be explained by a phenomenon known as the “mystery of APX silencing during excessive stress” (Foyer and Shigeoka, 2011), where the inactivation of APX is associated with the increased expression of catalase.

Three Arabidopsis Cu/Zn superoxide dismutase genes and the corresponding genes of *R. cordifolia* were also studied. Arabidopsis contains three Cu/Zn superoxide dismutases, cytosolic *AtCSD1* (AT1G08830), chloroplastic *AtCSD2* (AT2G28190) and
peroxisomal AtCSD3 (AT5G18100) (Kliebenstein et al., 1998). The AtCSD2 and AtCSD3 genes, like the orthologous genes of *R. cordifolia* (RcCSD2, GU949547 and RcCSD3, GU949548), showed no upregulation in *rolB*-expressing cells. In contrast, AtCSD1 and RcCSD1 (GQ380492) were upregulated.

**Effect of *rolB* on GSH/GSSG Ratio**

Permanent transcriptional activation of antioxidant genes in *rolB*-expressing cells are expected to cause perturbations of redox homeostasis in cells. Here, we provide a short overview on the redox balance to estimate the degree to which *rolB* expression and antioxidant activation modify the redox balance of transformed cells.

The balance between the reduced and oxidized glutathione (GSH and GSSG) is a central factor in maintaining the cellular redox state (Foyer and Noctor, 2005). It has been reported that when the intensity of a stress increases, GSH concentrations decline, and the redox state becomes more oxidized, leading to the deterioration of the system. An elevated GSH concentration is correlated with the ability of plants to withstand induced oxidative stress.

The contents of GSH and GSSG in plant and callus tissues of *R. cordifolia* were measured by mass spectrometry, and the data are presented in Table 5. The concentration ranges of GSH and GSSG in leaves of *R. cordifolia* were 156 nmol g\(^{-1}\) FW (GSH) and 44 nmol g\(^{-1}\) FW (GSSG), which are consistent with the values reported for other plant species (Rellán-Álvarez et al., 2006). The GSH/GSSG ratio was 3.6. In the control R calli, we detected the decreased concentration of GSH and a corresponding decreased GSH/GSSG ratio (2.2). In the *rolB*-transformed cells, the total pool of glutathione (GSH+GSSG) and the GSH/GSSG ratio was slightly higher than the corresponding values in the normal cells.

According to the literature data, Arabidopsis leaves in normal physiological conditions contain 152-263 nmol g\(^{-1}\) FW of GSH and 21-75 nmol g\(^{-1}\) FW of GSSG, thus maintaining the GSH/GSSG ratio in the range of 2.0-12.5 (Vanhouwt et al., 2011). In transformed *A. thaliana* cells, *rolB* caused a moderate increase of the GSH content and GSH/GSSG ratio (compared with vector control cells), but these values remained within the normal physiological parameters (Table 5). Thus, non-stressed *rolB*-transformed cells maintain the normal redox balance.

**Tolerance to Salt**
ROS contributes to stress damage, as evidenced by observations that transgenic plants overexpressing ROS scavengers show increased tolerance to environmental stresses (Xiong et al. 2002). An example of this effect is the increased resistance of rolC-expressing cells to salt stress (Bulgakov et al., 2008). The R, RB-L and RB-H suspension cultures were grown in the presence of varying NaCl concentrations. The IC$_{50}$ of the R culture was 16 mM. The RB-L and RB-H cells were more tolerant to NaCl than the control culture, with the IC$_{50}$ values of 21 and 25 mM, respectively (the difference is statistically significant; $P < 0.05$ vs. value of the R culture, Student’s $t$-test.). Notably, the rolC gene alone was capable of increasing the IC$_{50}$ to 70 mM (Bulgakov et al., 2008). The IC$_{50}$ values for the RABC and RA4 cultures were 45 and 41 mM, respectively (Shkryl et al., 2010). Therefore, the effects of the rol genes on salt tolerance were not additive.

**Discussion**

**The rolB Gene as a ROS Suppressor**

We have previously reported that rolB-transformed cells of *R. cordifolia* contain a large amount of anthraquinones, especially in those cells where the gene is highly transcribed (Shkryl et al., 2008). This effect is combined with the necrotizing effect of rolB. As these features are associated with a high ROS level (Bulgakov et al., 2011; Shkryl et al., 2011), one would expect that the gene could induce ROS production. Our results, however, contradict this hypothesis. The rolB gene suppresses ROS in resting plant cells (Figure 1, Tables 1, 2) and prevents or attenuates the elevation of intracellular ROS levels caused by external stimuli (Figures 2-4, Table 3). For this reason, rolB-cells have enhanced resistance to salt, paraquat, menadione, light stress and external H$_2$O$_2$.

The mechanism by which RolB permanently supports an active anti-oxidative status of transformed cells is probably the upregulation of antioxidant genes. The majority of the antioxidant genes studied, including those encoding the Cu/Zn superoxide dismutases, catalases and ascorbate peroxidases, were upregulated in rolB-expressing cells (Table 4). The activation was dependent on the strength of rolB expression and, in particular, on the cell line and type of antioxidant gene. For example, low doses of rolB failed to activate the expression of the Cu/Zn superoxide dismutase genes *AtCSD1, AtCSD2, AtCSD3, RcCSD1, RcCSD2 and RcCSD3*. High doses of rolB failed to activate
the expression of the ascorbate peroxidase genes *AtApx1*, *AtApx2*, *AtApx3*, *RcApx1*, *RcApx2* and *RcApx3*. RolB activated the complete set of the ascorbate peroxidase genes but did not activate all of the Cu/Zn superoxide dismutase genes (Table 4). Such patterns of antioxidant gene expression in transformed cells may reflect a non-specific (secondary) effect of *rolB* on ROS metabolism.

Therefore, we suggest that the activation of antioxidant genes is not a consequence of a direct action of *rolB*. Alternatively, one can propose a scenario in which transformed cells receive an unknown deleterious signal from the RolB protein. The cells try to compensate for this effect by cellular compensatory mechanisms, adjusting available antioxidant systems at the right place and time. In many cases, the compensation is successful, and cells maintain almost normal redox balance (Table 5). In cases, where it is not possible because of excessive *rolB* expression, cells die by necrosis.

From the physiological point of view, the effect of *rolB* is similar to the phenomenon known as stress acclimation or, more specifically, systemic acquired acclimation (Mullineaux *et al.*, 2000; Gechev *et al.*, 2006). During ROS-induced stress acclimation, plants produce catalases, ascorbate peroxidases and other ROS-detoxifying enzymes to protect their cells against new stresses (Gechev *et al.*, 2006). This leads to sustained antioxidant defenses and the protection of the plants from subsequent stresses.

**Similarity and Dissimilarity Between RolB- and *Pseudomonas syringae* HopAO1-mediated Effects**

An interesting analogy between the effects mediated by RolB of *A. rhizogenes* and some type III proteins of *P. syringae* emerges from our results. The effector HopAO1 (HopPtoD2) protein of *P. syringae* is injected from bacterial to plant cells to promote bacterial growth through suppression of the innate immunity of host cells. It was shown that HopAO1 possesses protein tyrosine phosphatase activity (Espinosa *et al.*, 2003; Bretz *et al.*, 2003) and suppresses induced ROS in plants (Bretz *et al.*, 2003). The observation that the *rolB* gene causes ROS inhibition in plant cells indicates a functional analogy between the RolB and HopAO1 tyrosine phosphatases. *Pseudomonas* and *Agrobacterium* use different mechanisms to deliver pathogenic determinants to plant cells, using type III and IV secretion systems, respectively. However, a strategy aimed at the suppression of plant defense responses seems to be logical for both pathogens.
One could propose that HopAO1 and RolB are related proteins that originate from lateral gene transfer between Agrobacterium and Pseudomonas because it is known that both microorganisms are amenable to such genetic innovation (Kado, 2009). However, our comparison of the HopAO1 and RolB amino acid sequences showed only limited amino acid similarity (24% amino acid identity). The localization of these proteins in plant cells is also different. HopAO1 is localized to the soluble fraction of protein extracts (Underwood et al., 2007), whereas RolB is localized in the plasma membrane (Filippini et al., 1996) or in the nucleus (Moriuchi et al., 2004). Therefore, these proteins have probably evolved independently.

**Combined Effect of the rolB and rolC Genes**

Although the rolB and C genes promote root formation synergistically, an antagonistic effect of the rol genes has been demonstrated at different levels. The stimulatory effect of the rolB gene on AQ formation was weaker when this gene was combined with rolC (Shkryl et al., 2008). Constitutive rolB expression suppressed the growth of tobacco cells, and the rolC gene was able to attenuate this growth inhibition (Schmülling et al., 1988). Likewise, rolC diminished the rolB-induced high sensitivity to auxin in transformed cells (Maurel et al., 1991) and the severity of rolB-induced phenotypes (Capone et al., 1989; Vanaltvorst et al., 1992). Recently, a contrasting difference between the action of rolB and rolC on class III peroxidase gene expression has been demonstrated (Veremeichik et al., 2012). While overexpression of a single rolB gene caused dramatic upregulation of R. cordifolia class III peroxidase genes, the effect of the rolC gene on peroxidase transcript abundance was minimal. Interestingly, the effect of the rolB gene was almost totally suppressed in pRiA4 calli, where rolB and rolC were expressed simultaneously.

It has been shown that the combined actions of the rolB and C genes do not cause significant ROS suppression (Shkryl et al., 2010). If it were otherwise, the combined effect of the rol genes could cause totally disturbed ROS homeostasis and cell death. However, the strategy of the phytopathogen A. rhizogenes is not to kill cells. Instead, the bacteria, acting via the transferred genes, render cells to be more tolerant of environmental stresses and increase their defense potential. In many cases, the rol genes ensure a high growth rate of transformed cells and their hormonal independence. In this context, the actions of each of the rol genes seem to be in tune with the actions of the other, providing physiological conditions for better cell fitness in the face of changing environmental conditions. Perhaps
this is the main effect of the rol genes as members of the RolB (plast) gene family. The
rol-induced perturbations are beneficial to transformed cells, but not to the whole
organism, as in the case with animal tumors. A. rhizogenes-infected plants have abnormal
metabolism and produce large amounts of opines, which are necessary for bacterial
growth, but cannot be utilized by plants. An interesting question arises: what kind of cells
can be active producers of opines? It is logical to propose that transformed plant cells with
increased growth and viability fit this criterion.

The Interplay between ROS Production and Morphogenetic Responses

Although some biochemical perturbations caused by the rolC and rolB genes can be
explained, the root-forming activity of these genes and the phenotypical abnormalities
caused by them are more difficult to understand. It is known that ROS control cell
expansion and root elongation (reviewed by Gapper and Dolan, 2006). Recently published
data suggest a complex and dynamic role for ROS in stress-induced morphogenetic
responses (SIMR), indicating the involvement of ROS in cell developmental programs
(Tsukagoshi et al., 2010; Blomster et al., 2011). Transgenic plants with reduced ROS
levels showed a reduced apical dominance, enhanced branching, decreased chlorophyll
content, abnormal flower development (abnormal petal number, fasciated styles and
ovaries), parthenocarpy, reduced leaf lobing and curled leaflets (Sagi et al., 2004).

Most of these traits, excluding the epinastic curling in the leaf margin, are similar to
those described previously for pRiA4-, rolABC-transformed plants or plants transformed
with single rol genes (reviewed by Nilsson and Olsson, 1997). The most typical effects of
rolB on plant development are heterostylly, altered leaf and flower morphology, and the
increased formation of adventitious roots on stems (Schmülling et al., 1988). RolB
promotes de novo meristem formation in cultured tissues (Altamura et al., 1994) and plants
(Koltunov et al., 2001). The type of organ that is formed from these meristems (roots,
shoots, vegetative rosettes or capitula) depends on the developmental and hormonal
context. Furthermore, RolB perturbs the growth of plant reproductive organs by altering
the developmental potential and reproductive fate of the ovule and affecting the processes
of anther dehiscence and style elongation (Koltunov et al., 2001; Carmi et al., 2003;
Cecchetti et al., 2004). RolB is thought to be involved in auxin signal
perception/transduction pathways (Cecchetti et al., 2004). Because recent data indicate a
clear interplay between auxin and ROS level in altering the leaf developmental program
(Blomster et al., 2011), the interaction between auxin signaling and ROS metabolism in rolB-induced morphological responses is especially interesting in our case. There may be a link between the morphological responses and ROS level perturbations induced by rolB.

MATERIALS AND METHODS

Plant Cell Cultures

All the cell cultures described in the present work were established in 2000 using R. cordifolia L. (Rubiaceae) clonally cultivated plantlets (Bulgakov et al., 2002). The plantlets were transformed with A. tumefaciens strain GV3101 harboring the pPCV002-CaMVBT construct (rolB under the 35S CaMV promoter) (Spena et al., 1987). The control non-transformed culture (R) was established from the same plantlets and cultivated under the same conditions as the transformed ones. Independently transformed lines with low, moderate and high expression of the rolB gene (RB-L, RB-M and RB-H callus cultures, respectively) were obtained by selection of homogenous yellow, deep-yellow and orange-red colors, respectively. These lines have been previously characterized to have stable morphology, growth, biosynthetic parameters and levels of rolB expression (Shkryl et al., 2008). Cell suspensions were cultivated using W_{BA} liquid medium (Bulgakov et al., 1998) supplemented with 0.5 mg l^{-1} 6-benzylaminopurine and 2.0 mg l^{-1} \alpha-naphthaleneacetic acid in the dark (excluding experiments with paraquat, where cells were cultivated in the light; see below) at 24°C with 12-day subculture intervals.

The Panax ginseng GV (vector control) and rolB-transformed callus cultures were cultivated using W_{4CPA} medium supplemented with 0.4 mg l^{-1} 4-chlorophenoxyacetic acid in the dark as described previously (Bulgakov et al., 1998). The suspension variants of these cultures were grown in W_{4CPA} liquid medium.

The Arabidopsis thaliana vector control and rolB-transgenic callus cultures were obtained from Columbia (Col-0) seedlings using the pPCV002-CaMVBT construct as described previously (Bulgakov et al., 2010). The calli were cultivated using W_{2,4-D} medium (Bulgakov et al., 1998) supplemented with 0.4 mg l^{-1} 2,4-dichlorophenoxyacetic acid in the dark at 24°C with 30-day subculture intervals. Two rolB-expressing callus lines (AtB-1 and AtB-2) were selected from these primary calli as described previously (Bulgakov et al., 2010) to obtain lines with different strengths of rolB expression. The AtB-2 line showed a four-fold higher expression of rolB in comparison with AtB-1. These
cultures were one year of age. The cell suspension cultures AtB-1 and AtB-2 were cultivated using W2,4-D liquid medium (Bulgakov et al., 1998) in the dark at 24°C with 14-day subculture intervals.

**Laser Confocal Imaging of Intracellular ROS**

Measurements of intracellular ROS were performed as described previously (Bulgakov et al., 2008). The experiments were based on the ability of plant cells to oxidize fluorogenic dyes to their corresponding fluorescent analogues that allowed ROS determination in living cells. Suspensions of plant cells were grown in liquid nutrient medium for 4-12 days and filtrated through 100 μm of mesh nylon to separate cell clusters. Single cells and 10-20 cell aggregates were gently centrifuged and resuspended in liquid W_B/A medium containing 50 μM 2,7-dichlorodihydrofluorescein diacetate (H2DCF-DA, Molecular Probes, Eugene, OR) or 10 μM dihydrorhodamine 123 (H2R123, Molecular Probes) and incubated at 25 ± 1°C in the dark. Cells were incubated with H2DCF-DA and H2R123 for 10 min. Slices from calli were prepared by using a vibratome HM650V (Microm, Germany). Dye-loaded cells were washed in the medium and re-suspended. 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, Germany) equipped with an argon laser with an effective power of 30 mW. The intensity of the Ar laser was 5.9 % of the maximal value for H2DCF-DA and 10 % for H2R123. 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 the above described settings and analyzed with LSM 510 Release 3.5 software (Carl Zeiss, Germany). Data were presented as the mean from several separate experiments (at least 30-40 cells were analyzed in each experiment).

**Luminometric Determination of ROS**

The production of ROS in callus cultures was measured by the luminol-based luminometric method according to Piedras et al. (1998). The control and rolB-transformed calli were harvested at 21 and 28 days of cultivation and analyzed using a RF-1501 instrument (Shimadzu EUROPA GmbH). The following settings were used: excitation 355 nm; emission 420 nm; response: auto; number of iterations: 3; reaction 8 sec, analysis 5 sec.
The calibration curve used was linear in concentrations from 50 to 800 \( \mu \text{mol l}^{-1} \) of H\(_2\)O\(_2\). Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) was obtained from ICN Pharmaceuticals.

**Cell Viability**

The viability of cells was tested by the addition of propidium iodide (Sigma, 0.3 mg ml\(^{-1}\), final conc. in water) to cell suspension cultures. Confocal images were obtained after excitation at 536 nm and emission at 617 nm (laser wave 543, intensity 20\% and filter LP 560).

**Paraquat, Menadione and Light Treatments**

Suspension-cultivated *R. cordifolia* cells were grown for 4 days in the dark and treated with paraquat (Aldrich, 10 \( \mu \)M, final conc.) for 1 h under continuous light exposure (200 \( \mu \)mol m\(^{-2}\) s\(^{-1}\) radiation). Menadione (Sigma, 100 \( \mu \)M, final conc.) was added to 4-day suspension cultures, which were subsequently cultivated for 20 h in the dark. Light stress was caused by continuous illumination of cells with the LSM 510 META Ar laser (effective power 30 mW, 5.9\% maximal laser intensity) at 488 nm.

**Tolerance to Salt**

Resistance of the *R. cordifolia* transformed cell cultures to salt stress was determined as described previously (Bulgakov *et al.*, 2008). Cell suspensions were cultivated in the presence of NaCl (0, 30, 60 and 120 mM) for ten days. Data were obtained from two separate experiments consisting of 10 replicates each.

**Real-time RT-PCR**

Quantitative real-time RT-PCR (qRT-PCR) was performed as described previously (Skryl *et al.*, 2010). RNA concentration and 28S/18S ratios were determined using an RNA StdSens LabChip® kit and Experion™ Automated Electrophoresis Station (Bio-Rad Laboratories, Inc., USA) with Experion™ Software System Operation and Data Analysis Tools (version 3.0) following the manufacturer’s protocol and recommendations.
The qRT-PCR analysis was performed using the Bio-Rad CFX96 Real-Time System (Bio-Rad Laboratories, Inc., USA) with a 2.5x SYBR green PCR master mix containing ROX as a passive reference dye (Syntol, Moscow, Russia). Two biological replicates, resulting from two different RNA extractions, were used for analysis, and three technical replicates were analyzed for each biological replicate. No-template controls and RNA-RT controls were included in the analysis to verify the absence of contamination. The absence of non-specific products or primer-dimer artefacts in the samples was confirmed by melting curve analysis at the end of each run and by product visualization using electrophoresis. Primer efficiency of > 95% was confirmed with a standard curve spanning seven orders of magnitude. Data were analyzed using CFX Manager Software (Version 1.5) (Bio-Rad Laboratories).

Analysis of GSH and GSSG by Mass Spectrometry

The reduced and oxidized forms of glutathione (GSH and GSSG) were extracted from *R. cordifolia* cells quantitatively as described by Rellán-Álvarez *et al*. (2006) and analyzed according to the recommendations of these authors in the Instrumental Centre for Biotechnology and Gene Engineering at the Institute of Biology and Soil Science using a HCTultra PTM Discovery System (Bruker Daltonik GmbH, Germany). The HCTultra is equipped with a high-capacity ion trap that enables the acquisition of MS/MS data on low-abundance precursor ions and is designed to determine low-weight peptides. Cell extracts or solutions of commercial GSH or GSSG (Sigma) were directly injected in the spectrometer, outfitted with an electrospray ion source, at a flow rate of 120 µl/h (mass range mode: ultra scan, ion polarity: positive or negative, ion source type: ESI, scan mode: standard-normal). The identity of GSH was confirmed by analysis of the masses of the deprotonated molecules of GSH [M-H]− with m/z 306.0 and GSH [M+H]+ with m/z 308.0 as well as product ions with m/z 161.9, 179.0, 233.0 and 290.1 specific for GSH (Suppl. Fig. 1). The identity of GSSG was confirmed by analysis of the masses of GSSG [M-H]− with m/z 611.1 and [M+H]+ with m/z 613.1 as well as product ions with m/z 355.1 and 484.1 (Suppl. Fig. 1). The analysis parameters (in MS and MS/MS modes) were optimized for the production of characteristic precursor and product ions in the positive ionization mode. GSH and GSSG levels were determined based on a comparison of the averaged peak heights of [M+H]+ ions in concentrations of 0.1-5 µM (GSH) and 0.05-0.5 µM (GSSG). GSH and GSSG solutions and extracts were measured using identical conditions.
**Statistical Analysis**

In statistical evaluation, the Student’s t test was used for the comparison between 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. A difference of $P < 0.05$ was considered significant.
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stress. Plant Cell 14: 165-183


Table 1. Intracellular ROS levels in *P. ginseng* and *A. thaliana* control and rolB-expressing cell cultures measured by laser confocal microscopy

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Number of cells analyzed</th>
<th>DCF fluorescence, pixels</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. ginseng</em> calli (vector control)</td>
<td>58</td>
<td>65.4 ± 3.9</td>
</tr>
<tr>
<td><em>P. ginseng</em> rolB-expressing calli</td>
<td>79</td>
<td>48.2 ± 2.4 *</td>
</tr>
<tr>
<td><em>P. ginseng</em> cell suspension (vector control)</td>
<td>80</td>
<td>60.0 ± 4.5</td>
</tr>
<tr>
<td><em>P. ginseng</em> rolB-expressing cell suspension</td>
<td>80</td>
<td>48.5 ± 2.0 *</td>
</tr>
<tr>
<td><em>A. thaliana</em> calli (vector control)</td>
<td>100</td>
<td>75 ± 3.5</td>
</tr>
<tr>
<td><em>A. thaliana</em> low-rolB-expressing calli AtB-1</td>
<td>100</td>
<td>60 ± 2.5 *</td>
</tr>
<tr>
<td><em>A. thaliana</em> high-rolB-expressing calli AtB-2</td>
<td>100</td>
<td>47.5 ± 1.9 *</td>
</tr>
<tr>
<td><em>A. thaliana</em> cell suspension (vector control)</td>
<td>80</td>
<td>86.1 ± 5.0</td>
</tr>
<tr>
<td><em>A. thaliana</em> low-rolB-expressing cell suspension AtB-1</td>
<td>80</td>
<td>66.4 ± 4.5 *</td>
</tr>
<tr>
<td><em>A. thaliana</em> high-rolB-expressing cell suspension AtB-2</td>
<td>80</td>
<td>56.5 ± 4.2 *</td>
</tr>
</tbody>
</table>

*P. ginseng* and *A. thaliana* calli or cell suspensions were cultivated for 21 days or 5 days, respectively and analyzed by confocal microscopy. ROS levels are presented as mean ± SE from a single experiment. *P* < 0.05 vs. values of the corresponding controls, Student's *t*-test.
Table 2. ROS levels in extracts of *rolB*-expressing callus cultures measured by the luminol-based luminometric determination

<table>
<thead>
<tr>
<th>Callus line</th>
<th>H₂O₂ content, % of the control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>21 days of cultivation (the linear phase of growth)</td>
</tr>
<tr>
<td><em>R. cordifolia, R</em> (control)</td>
<td>100</td>
</tr>
<tr>
<td><em>R. cordifolia, RB-L</em></td>
<td>92</td>
</tr>
<tr>
<td><em>R. cordifolia, RB-M</em></td>
<td>80 *</td>
</tr>
<tr>
<td><em>R. cordifolia, RB-H</em></td>
<td>68 *</td>
</tr>
<tr>
<td><em>P. ginseng</em> (vector control)</td>
<td>100</td>
</tr>
<tr>
<td><em>P. ginseng</em> <em>rolB</em>-expressing</td>
<td>74 *</td>
</tr>
<tr>
<td><em>A. thaliana</em> (vector control)</td>
<td>100</td>
</tr>
<tr>
<td><em>A. thaliana</em> low-<em>rolB</em>-expressing AtB-1</td>
<td>83 *</td>
</tr>
<tr>
<td><em>A. thaliana</em> high-<em>rolB</em>-expressing AtB-2</td>
<td>70 *</td>
</tr>
</tbody>
</table>

The data are presented as mean from 3 independent experiments. * P < 0.05 vs. values of the control cultures, ANOVA followed by Fisher's PLSD test.
Table 3. Suppression of ROS elevations induced by menadione and light stress in *R. cordifolia* rolB-expressing cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DCF fluorescence, pixels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R (control)</td>
</tr>
<tr>
<td><strong>Menadione, μM</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>82.0 ± 4.3</td>
</tr>
<tr>
<td>100</td>
<td>123.5 ± 5.0</td>
</tr>
<tr>
<td><strong>Light (time of illumination, seconds)</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>77.0 ± 4.0</td>
</tr>
<tr>
<td>600</td>
<td>113.9 ± 5.3</td>
</tr>
<tr>
<td>1000</td>
<td>130.7 ± 6.4</td>
</tr>
</tbody>
</table>

<sup>a</sup> Menadione was added to 4-day suspension cultures. After 20 h of incubation, cells were analyzed by laser confocal microscopy using H<sub>2</sub>DCF-DA as a probe.

<sup>b</sup> The data was obtained by confocal imaging of ROS in cells subjected to illumination from an argon laser (excitation at 488 nm, intensity of the laser 5.9% of maximal). Images of single cells were captured and video files of the images were analyzed.
Table 4. Expression of genes encoding antioxidant enzymes in non-transformed and rolB-transformed cells

<table>
<thead>
<tr>
<th>Identity of At/Rc proteins (%)</th>
<th>Cell cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Arabidopsis thaliana</td>
</tr>
<tr>
<td>At</td>
<td>AtB-1</td>
</tr>
<tr>
<td>AtB-1</td>
<td>0.054±0.013</td>
</tr>
<tr>
<td>Expression of antioxidant genes</td>
<td></td>
</tr>
<tr>
<td>Catalase</td>
<td></td>
</tr>
<tr>
<td>AtCat1</td>
<td>0.278±0.014</td>
</tr>
<tr>
<td>RcCat1</td>
<td>0.511±0.004*</td>
</tr>
<tr>
<td>Ascorbate peroxidase</td>
<td></td>
</tr>
<tr>
<td>AtApx1</td>
<td>0.288±0.019</td>
</tr>
<tr>
<td>RcApx1</td>
<td>0.324±0.003</td>
</tr>
<tr>
<td>Cu/Zn superoxide dismutase</td>
<td></td>
</tr>
<tr>
<td>AtApx2</td>
<td>0.341±0.041</td>
</tr>
<tr>
<td></td>
<td>AtCSD1</td>
</tr>
<tr>
<td>---</td>
<td>----------------</td>
</tr>
<tr>
<td>68</td>
<td>0.496±0.005</td>
</tr>
<tr>
<td></td>
<td>0.966±0.033*</td>
</tr>
<tr>
<td></td>
<td>0.498±0.025</td>
</tr>
<tr>
<td>83</td>
<td>0.882±0.013</td>
</tr>
<tr>
<td></td>
<td>0.915±0.086</td>
</tr>
<tr>
<td></td>
<td>0.571±0.054</td>
</tr>
<tr>
<td>72</td>
<td>0.577±0.027</td>
</tr>
<tr>
<td></td>
<td>0.597±0.017</td>
</tr>
<tr>
<td></td>
<td>0.621±0.023</td>
</tr>
<tr>
<td></td>
<td>0.761±0.006</td>
</tr>
</tbody>
</table>

RNA samples were isolated from callus cultures during the linear phase of growth (20-22 days). Two RNA samples were analyzed with three analytical repetitions. The data are presented as mean ± SE. *P < 0.05 vs. values of the control cultures, ANOVA followed by Fisher's PLSD test.
Table 5. Content of GSH and GSSG, and the GSH/GSSG ratio in leaves and callus cultures of *R. cordifolia* and *A. thaliana*

<table>
<thead>
<tr>
<th>Plant samples and callus cultures</th>
<th>GSH, nmol g⁻¹ FW</th>
<th>GSSG, nmol g⁻¹ FW</th>
<th>GSH/GSSG</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>R. cordifolia</em> leaves</td>
<td>156 ± 31</td>
<td>44 ± 15</td>
<td>3.6 ± 1.1</td>
</tr>
<tr>
<td>R</td>
<td>118 ± 21</td>
<td>53 ± 0.3</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>RB-L</td>
<td>114 ± 10</td>
<td>53 ± 1</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>RB-H</td>
<td>128 ± 12</td>
<td>51 ± 2</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td><em>A. thaliana</em> (vector control)</td>
<td>78 ± 4.5</td>
<td>15 ± 3</td>
<td>5.2 ± 1.1</td>
</tr>
<tr>
<td><em>A. thaliana</em> low-<em>rolB</em>-expressing AtB-1</td>
<td>80 ± 12</td>
<td>13 ± 5</td>
<td>6.2 ± 1.6</td>
</tr>
<tr>
<td><em>A. thaliana</em> high-<em>rolB</em>-expressing AtB-2</td>
<td>97 ± 10*</td>
<td>11 ± 3</td>
<td>8.8 ± 2.1*</td>
</tr>
</tbody>
</table>

Measurements were performed in 3 independent samples. Callus cultures were analyzed at the linear (20-22 days) phase of growth with three biological replicates. Averaged data (mean ± SE) are presented. *P < 0.05 vs. values of control calli, ANOVA followed by Fisher's PLSD test.
Legends to the figures

**Figure 1.** Steady-state ROS levels in control (R) and *rolB*-expressing (RB-L, RB-M and RC-H) *R. cordifolia* cell suspension (A) and callus (B) cultures. The cells cultivated for 5 days (A) or 21 days (B) were loaded with H$_2$DCF-DA or H$_2$R123, and fluorescence of DCF or R123 was visualized by laser-scanning confocal microscopy. Fluorescence of DCF and R123 reflects intracellular ROS abundance. The results are presented as mean ± SE from six independent experiments. *P* < 0.05 vs. values of the R culture, ANOVA followed by Fisher's PLSD test.

**Figure 2.** Effects caused by paraquat (Prq). Intracellular ROS abundance in control (R) and *rolB*-expressing *R. cordifolia* cell suspension cultures was determined by laser-scanning confocal microscopy. The cultures were cultivated in the dark for 4 days and treated with paraquat (10 µM) for 1 h under continuous light exposure. A diagram in the lower panel represents ROS levels obtained from two independent experiments (mean ± SE). Scale bars, 20 µm.

**Figure 3.** Viability of *R. cordifolia* cells (R and RB-H lines) in the presence of a high concentration of menadione. Confocal imaging of intact cells (A, C) and cells treated with menadione (500 µM) (B, D) for 24 h. Living cells (green) were visualized by fluorescein diacetate, and dead cells (red) with collapsed nuclei were visualized with propidium iodide. Scale bars, 50 µm.
**Figure 4.** The rolB gene prevents cell death induced by paraquat. Phenotypes of the cultures (A) and cell count (B). The control cells (R line), high-rolB-expressing cells (RB-H line) and high-rolC-expressing cells (RC-H line) were cultivated in the presence of 100 μM paraquat for eight days in the dark or in the light (200 μmol m$^{-2}$ s$^{-1}$ radiation). Then the cells were stained with propidium iodide and analyzed by confocal microscopy. 40-50 cells were counted in each variant and the percentage of non-viable cells was calculated. The data are presented as mean ± SE from a single experiment with 3 replicates.