Planta (2003) 217: 349–355
DOI 10.1007/s00425-003-0996-5

ORIGINAL ARTICLE

V.P. Bulgakov · G.K. Tchernoded
N.P. Mischenko · Y.N. Shkryl · V.P. Glazunov
S.A. Fedoreyev · Y.N. Zhuravlev

Effects of Ca^{2+} channel blockers and protein kinase/phosphatase inhibitors on growth and anthraquinone production in *Rubia cordifolia* callus cultures transformed by the *rolB* and *rolC* genes

Received: 10 September 2002 / Accepted: 18 January 2003 / Published online: 18 February 2003

Abstract The transformation of *Rubia cordifolia* L. cells by the 35S-*rolB* and 35S-*rolC* genes of *Agrobacterium rhizogenes* caused a growth inhibition of the resulting cultures and an induction of the biosynthesis of anthraquinone-type phytoalexins. Inhibitor studies revealed a striking difference between the *rolC*- and *rolB*-gene-transformed cultures in their sensitivity to verapamil, an L-type Ca^{2+} channel blocker. The *rolC* culture possessed a 2-fold lowered resistance to the inhibitor than the normal culture, while the *rolB* culture was 4-fold more resistant to the treatment. Additionally, growth of the *rolC* culture was totally inhibited when the culture was grown in Ca^{2+}-free medium, whereas growth of the *rolB* culture was reduced by less than half. We interpreted these results as evidence for a lack of calcium homeostasis in both transgenic cultures. Anthraquinone (AQ) production was not inhibited in the normal or transformed cultures by the Ca^{2+} channel blockers verapamil and $LaCl_3$, or by diphenylene iodonium, an inhibitor of NADPH oxidase, or by the protein kinase inhibitor staurosporine. These results indicate that the induction of AQ production in non-transgenic and transgenic cultures does not proceed through the activation of the common Ca^{2+}-dependent NADPH oxidase pathway that mediates signal transduction between an elicitor–receptor complex via transcriptional activation of defense genes. Okadaic acid and cantharidin, inhibitors of protein phosphatases 1 and 2A, caused an increase in AQ production in transgenic cultures. Okadaic acid stimulated AQ accumulation in the non-transformed culture, whereas cantharidin had no effect. These results show that different phosphatases are involved in AQ synthesis in normal and transgenic cultures of *R. cordifolia*.

Keywords Anthraquinone · Callus culture · *rolB* and *rolC* genes · *Rubia* · Signal transduction

Abbreviations AQ: anthraquinone · DPI: diphenylene iodonium · OA: okadaic acid

Introduction

Several studies have revealed an interesting ability of the *rolC* and *rolB* oncogenes of *A. rhizogenes* to stimulate secondary metabolite production in transgenic root (Bulgakov et al. 1998; Palazón et al. 1998a, 1998b; Bonhomme et al. 2000) and callus cultures (Bulgakov et al. 2002). When individually inserted in plants, the *rol* genes significantly affect plant growth and development (Spena et al. 1987; Schmülling et al. 1988; Nilsson and Olsson 1997; Holefors et al. 1998). The alteration to secondary metabolism is thought to be a side effect of more common mechanisms by which the oncogenes perturb normal cell growth.

In a recent publication, we have shown that anthraquinone (AQ) production is greatly increased in *rolC*- and *rolB*-transformed *R. cordifolia* cultures compared with the non-transformed culture (Bulgakov et al. 2002). Ethephon (a substance which is readily converted to ethylene in the cultural media) had no effect on AQ production in the control and transgenic cultures. Methyl jasmonate and salicylic acid strongly increased AQ accumulation in the transgenic and non-transgenic calluses in the same way. Treatment of the cultures with cantharidin resulted in a massive induction of AQ accumulation only in the transgenic cultures, which indicated the involvement of a cantharidin-sensitive

V.P. Bulgakov (✉) · G.K. Tchernoded · Y.N. Zhuravlev
Institute of Biology and Soil Science,
Far East Branch of the Russian Academy of Sciences,
159 Stoletija Str., 690022 Vladivostok, Russia
E-mail: bulgakov@ibss.dvo.ru
Fax: +7-4232-310193

N.P. Mischenko · V.P. Glazunov · S.A. Fedoreyev
Pacific Institute of Bioorganic Chemistry,
Far East Branch of the Russian Academy of Sciences,
690022 Vladivostok, Russia

Y.N. Shkryl
Far East State University, 690600 Vladivostok, Russia

protein phosphatase(s) in AQ biosynthesis in transgenic cultures (Bulgakov et al. 2002).

How and why do the *rol* genes activate secondary metabolism in plant cells? It is known that numerous phytoalexin types, such as terpenoids, flavonoids, anthraquinones, as well as different alkaloid classes, are synthesized as a consequence of plant–pathogen interaction (Hammond-Kosack and Jones 1996). Because synthesis of phytoalexin-type secondary metabolites is a part of the defense reaction of plants, the question arises whether or not *rol*-gene signals interfere with general plant defense pathways. In order to address this question, we began evaluating the roles of some key processes of defense reactions, such as Ca^{2+} signaling, phosphorylation and dephosphorylation events, on the growth and AQ production in transgenic cultures. In this stage of the investigation, we used the pharmacological approach, which provides information for the initial assessment of signal transduction. The difference in responses between a non-transformed and a transformed culture, caused by elicitor/inhibitor treatments, might indicate the site of the transgene action. Indeed, substantial differences between responses of transformed and non-transformed cultures were found.

Materials and methods

Plant culture

Transgenic cultures Rc-rolC3 and Rc-rolB were established from leaves of a 3-week-old axenic *Rubia cordifolia* L. plant (Bulgakov et al. 2002). Plasmid vectors pPCV002-CaMVBT and pPCV002-CaMVC (Spena et al. 1987) were used to obtain the transformed callus cultures. The non-transformed Rc callus culture was established from the leaves of the same plant and cultivated under identical conditions as the transformed cultures. All cultures were cultivated in 100-ml Erlenmeyer flasks on $W_{B/A}$ solid (Mischenko et al. 1999) or in $W_{B/A}$ liquid medium, supplemented with 0.5 mg l^{-1} 6-benzylaminopurine and 2.0 mg l^{-1} α-naphthaleneacetic acid, in the dark at 25 °C with 30-day subculture intervals.

Effector and inhibitor treatments

Reagents for tissue culture were obtained from Sigma, the others were from ICN Pharmaceuticals. Sterile solutions of verapamil and $LaCl_3$ (both as aqueous solutions), cantharidin, okadaic acid, staurosporine and diphenylene iodonium (DPI) (all as dimethyl sulfoxide solutions) were added to the autoclaved media aseptically in the desired concentrations. Equal volumes of dimethyl sulfoxide were added to the control flasks.

Estimation of the AQ content

Purpurin and munjistin were previously isolated from *R. cordifolia* calluses and analysed as described by Mischenko et al. (1999). Anthraquinone content was determined photometrically in 30-day cultures (Mischenko et al. 1999). Briefly, 100 mg of the dried powdered calluses with 0.3 ml 5 N HCl added was extracted with 3 ml ethanol at room temperature and absorption spectra were recorded. The purpurin content of the ethanol extract was determined by absorption at 515 nm. The content of munjistin was quantified by absorption at 421 nm, subtracting the absorption due to purpurin at this wavelength.

Results

Phenotypic aspects of the transformed and the non-transformed callus cultures

The normal Rc cultures, as well as Rc-rolC3- and Rc-rolB-transformed cultures, established from leaves of a single plant, were stable during long-term cultivation and exhibited quite different phenotypes (Fig. 1). Yellow-colored Rc calluses grew vigorously, whereas the Rc-rolC3 and Rc-rolB cultures showed slower growth (Bulgakov et al. 2002). All cultures produced only two major AQs, with munjistin and purpurin representing 90% of the total AQ yield. The transformed calluses could easily be distinguished from non-transgenic ones by their orange/orange-red pigmentation. As shown previously (Bulgakov et al. 2002), the Rc-rolC3 and Rc-rolB callus lines contained AQ concentrations that exceeded 1.9- to 3.2-fold those of the non-transformed culture. To determine whether this pattern of AQ accumulation is stable between subcultures, we monitored AQ levels in all lines for a long time (over 3 years). The results showed that differences in AQ accumulation within the cultures remained constant during this time (data not shown). The AQ levels in Rc cultures ranged from 0.4 to 1.2% DW, whereas those of the Rc-rolC3 and Rc-rolB cultures were 1.4–2.3 and 1.9–3.2% DW, respectively.

Different growth of the cultures in calcium-free medium

When callus aggregates of the Rc, Rc-rolB and Rc-rolC3 lines were cultivated in calcium-free liquid medium, a

Fig. 1 Phenotypes of the untransformed (*top*), Rc-rolC3 (*left*) and Rc-rolB (*right*) callus cultures of *Rubia cordifolia*. Cultures were grown for 3 weeks on $W_{B/A}$ medium

remarkable difference in their growth responses was detected. When grown in Ca^{2+}-free medium, the Rc culture accumulated $14.5 \pm 6.5\%$ (mean ± SE) fresh biomass, compared with the culture grown in the Ca^{2+}-supplied (3 mM) medium. Growth of the Rc-rolC culture was totally suppressed. The Rc-rolB culture accumulated $59.5 \pm 12.5\%$ fresh biomass, compared with values for the culture grown in standard medium.

We have shown, in previous experiments, that the significant growth inhibition of non-transformed *R. cordifolia* cultures resulted in total inhibition of AQ synthesis (Mischenko et al. 1999). Therefore, in this experiment, measurements of AQ content in Rc and Rc-rolC3 cultures were not conclusive. In contrast to these cultures, the *rolB* culture grew almost normally, allowing us to determine AQ content. The Rc-rolB culture produced high levels of AQs both in Ca^{2+}-supplied and Ca^{2+}-free media (2.72 ± 0.18 and $2.42 \pm 0.20\%$ DW, respectively).

Different sensitivities of the cultures to Ca^{2+} channel blockers

Verapamil and La^{3+} are known to block Ca^{2+} channels localized in the plasma membrane (Knight et al. 1992; Pineros and Tester 1997). To study whether Ca^{2+} influx in *R. cordifolia* calluses would have an effect on growth and AQ content, verapamil, an inhibitor of L-type calcium channels, was added at concentrations of 0.1 and 0.5 mM to the culture medium lacking calcium chloride. The treatment at 0.5 mM caused growth inhibition of the non-transformed culture and a cessation of growth of the *rolC* culture followed by a strong necrotic response. Surprisingly, we found that the growth of the *rolB* culture was unchanged by verapamil treatment (Fig. 2A).

When *R. cordifolia* calluses were cultivated on the Ca^{2+}-free medium, verapamil inhibited growth of the Rc, Rc-rolC3 and Rc-rolB lines with an $ID_{50} \approx 0.5$, 0.3 and 2.1 mM, respectively. The same cultures, grown on the standard medium containing 3 mM Ca^{2+}, possessed 2-fold higher resistance to verapamil and their ID_{50} values were calculated to be ≈1.2, 0.6 and 4.0 mM, respectively (data not shown). Thus, the Rc-rolC3 culture was almost 7-fold more sensitive to verapamil than the Rc-rolB culture.

In *Arabidopsis* plants with cell-specific expression of green fluorescent protein, a hyperpolarization-activated calcium current was irreversibly inhibited by 100 μM verapamil (Kiegle et al. 2000). Concentrations as low as 0.5 μM verapamil induced a blockade of the inward Ca^{2+} current through a wheat root plasma membrane Ca^{2+}-selective channel (Pineros and Tester 1997). In our experiments, the growth inhibition was observed at much higher concentrations of verapamil (Fig. 2A). The increased ID_{50} values for the cultures growing in the presence of Ca^{2+} indicate that verapamil did not block Ca^{2+} channels completely.

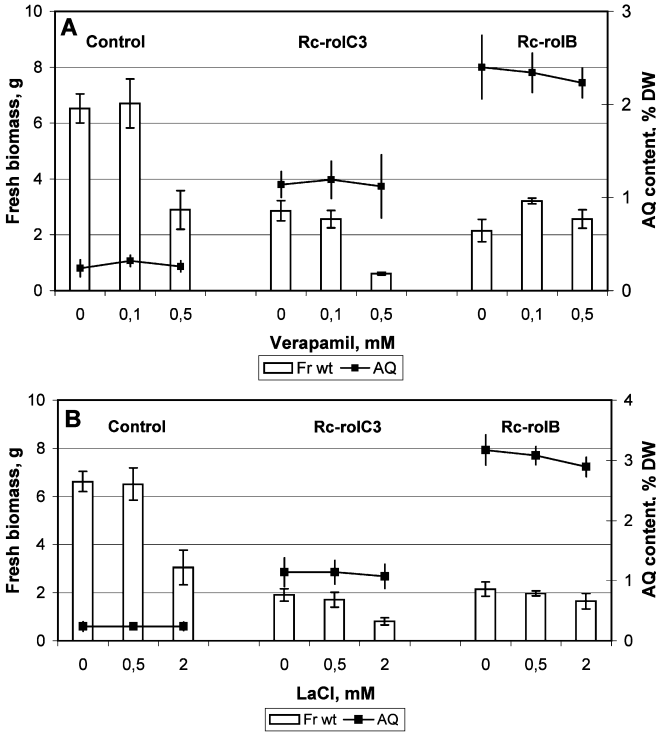

Fig. 2 Effect of verapamil (**A**) and $LaCl_3$ (**B**) on fresh biomass accumulation (g) after 4 weeks of culture and on AQ content (% DW) in *R. cordifolia* non-transformed (*Rc*) and transformed (*Rc-rolC3* and *Rc-rolB*) callus cultures. Values are means ± SE from 2 experiments with 10 replicates each

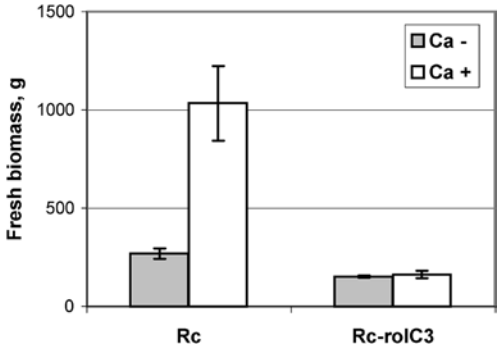

Fig. 3 Effect of low Ca^{2+} concentration (0.33 mM) on growth of Rc and Rc-rolC3 calluses of *R. cordifolia* in the presence of 0.5 mM of verapamil. Values are means ± SE from a single experiment with 10 replicates each

Pineros and Tester (1997) have shown that verapamil caused the blockage of Ca^{2+}-selective and voltage-dependent plant channels. The inhibition was due to a reduction in the maximum open-state probability of the channels. The blockage of the Ca^{2+} channels by verapamil caused the calcium deficiency that, in turn, led to cell growth inhibition. We suggested that the severe inhibition of Rc-rolC3 callus growth by verapamil was due to a limitation of availability of exogenous calcium for the cells. As shown in Fig. 3, a small amount of Ca^{2+} added to the culture medium was able to

Table 1 Effect of cantharidin (5 μM) on cell growth and AQ content in *Rubia cordifolia* cultures growing in the presence (3 mM) and absence of Ca^{2+}.The values are represented as a percentage of the values for the cultures growing on cantharidin-free medium. Values are means ± SE from 2 experiments with 10 replicates each

Callus culture	$+Ca^{2+}$		$-Ca^{2+}$	
	Growth	AQ content	Growth	AQ content
Rc	45 ± 8*	101 ± 8	7 ± 2*	N.d.[a]
Rc-rolC3	42 ± 12*	218 ± 12*	6 ± 2*	122 ± 18
Rc-rolB	98 ± 6	196 ± 20*	94 ± 9	85 ± 12

[a]Not detected

*$P < 0.05$ vs. cantharidin-free medium, Student's *t*-test

overcome the toxic effect of verapamil on growth of the non-transformed culture, but did not affect the growth of the Rc-rolC3 culture. This result seems to be most consistent with the presumption that expression of the *rolC* gene permanently blocks Ca^{2+}-permeable channels in the *rolC* culture.

We also tested the sensitivity of the cells to $LaCl_3$, a known general inhibitor of Ca^{2+} channels (Knight et al. 1992). Using this inhibitor, we were able to confirm the results obtained with verapamil: La^{3+} inhibited growth of Rc and Rc-rolC3 cultures and had no effect on the growth of the Rc-rolB culture (Fig. 2B).

Interestingly, when employed at 0.1 mM, verapamil increased fresh biomass accumulation of the *rolB* culture by 54% ($P < 0.05$, Fig. 2A), compared with growth on verapamil-free medium. Similarly, the *rolB* calli grew better in the presence of verapamil on the calcium-supplemented standard medium. Their growth was 57% higher ($P < 0.05$) in the presence of 0.5 mM (but not 0.1 mM) verapamil than in the absence of the inhibitor. It is plausible that growth of the *rolB* culture was improved owing to inhibition of Ca^{2+} overload by verapamil. $LaCl_3$ did not produce such effect.

Although verapamil and La^{3+} have been shown to block Ca^{2+} channels, they had in the same cases different effects on cytosolic Ca^{2+} concentration. Jones et al. (1998) have shown that La^{3+} induces a rapid decrease in cytosolic Ca^{2+} concentration in tobacco BY-2 cells but verapamil does not. Studying the external Ca^{2+} influx and the internal Ca^{2+} release in aequorin-transformed *Nicotiana tabacum* cells, Cessna and Low (2001) also showed that $LaCl_3$ inhibited both responses but verapamil had no effect. In general, the molecular identity of plant Ca^{2+}-permeable channels is poorly investigated (Sanders et al. 1999). Although L-type-like Ca^{2+} channels have been found in plants (Huang et al. 1994), the function of these channels in plants is not clear.

AQ accumulation was unchanged in either non-transformed or transformed cultures by verapamil and La^{3+} treatments (Fig. 2). This means that inhibition of extracellular Ca^{2+} entry does not block AQ synthesis in non-transformed cultures of *R. cordifolia*. The high level of AQs in transformed cultures seems not to be affected by inhibition of external Ca^{2+} fluxes.

Ca^{2+} deficiency completely abolished the stimulatory effect of cantharidin on AQ production

Cantharidin caused significant stimulatory effects on AQ formation in the *R. cordifolia* transgenic cells (Bulgakov et al. 2002). Since PP1- and PP2A-dependent phosphorylation plays an important role in regulation of the activity of L-type Ca^{2+} channels (Haby et al. 1994; Chik et al. 1999), it was of interest to determine the effect that cantharidin would have on calcium-depleted calluses.

No AQs were found when the Rc culture was grown in the presence of cantharidin on calcium-free medium. The removal of Ca^{+2} from the cultural medium prevented the cantharidin-mediated increase of AQs in both the *rolC* and *rolB* cultures (Table 1). The Rc and Rc-rolC cultures, cultivated on calcium-free medium, became highly sensitive to cantharidin: their growth was almost totally suppressed in the presence of 5 μM of the inhibitor (Table 1). In contrast, the growth of the Rc-rolB culture remained unchanged. Moreover, the culture possessed normal growth even in the presence of simultaneously added verapamil and cantharidin (data not shown).

Effects of okadaic acid and staurosporine

Okadaic acid (OA), at submicromolar concentrations, is an inhibitor of serine/threonine protein phosphatases 1 and 2A (MacKintosh and Cohen 1989). OA inhibited growth of the control and transgenic *R. cordifolia* cultures with similar dynamics (Fig. 4). The ID_{50} of OA was calculated to be ≈10 nM for all cultures. These data imply a similar sensitivity of the cultures to OA that is in contrast with cantharidin treatments, which revealed different sensitivity of the cultures to the treatment.

OA activated AQ production in the transgenic cultures between 10 and 100 nM in the *rolC* culture and between 1 and 100 nM in the *rolB* culture. Surprisingly, OA (10 nM) also caused elevation of AQs in the non-transformed culture. Concentrations of OA (100 nM) that stimulate the induction of AQs in transgenic cultures fail to stimulate AQ accumulation in the Rc culture. These results suggest that different OA-sensitive phosphatases are involved in the regulation of anthraquinone synthesis in the control and transgenic cultures. An alternative explanation is that the same phosphatase

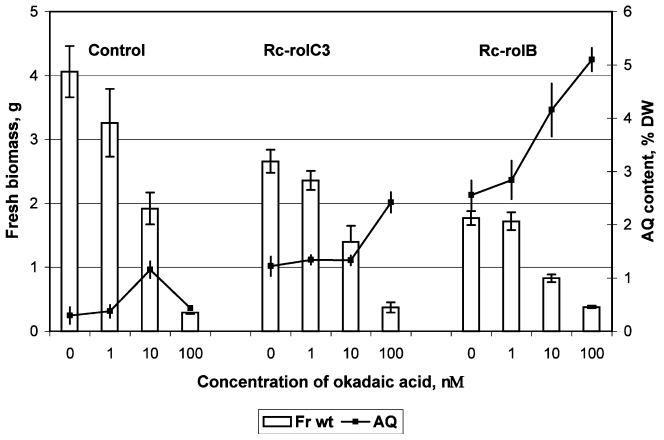

Fig. 4 Effect of okadaic acid (1, 10 and 100 nM) on fresh biomass accumulation (g) after 4 weeks of culture and on AQ content (% DW) in *R. cordifolia* non-transformed (*Rc*) and transformed (*Rc-rolC3* and *Rc-rolB*) callus cultures. Values are means ± SE from 2 experiments with 10 replicates each

is involved in the regulation of AQ synthesis, but that non-transformed and transformed cultures do not have the same sensibility to OA.

Staurosporine, a general inhibitor of protein kinases, at 1 μM inhibited elicitor- and methyl jasmonate-induced expression of terpenoid indole alkaloid biosynthetic genes (Menke et al. 1999), as well as mitogen-activated protein (MAP) kinase kinase-activated expression of pathogenesis-related and wound-inducible genes (Xing et al. 2001a). Staurosporine (4 μM) also diminished elicitor-stimulated H_2O_2 production (Cessna and Low 2001). We cultivated *R. cordifolia* cultures in the presence of 0.5, 1.0 and 5.0 μM staurosporine and showed that the growth of the Rc-rolC culture was inhibited at 5 μM, while growth of other cultures was unchanged by the inhibitor treatment. Neither of these concentrations was able to inhibit AQ production in all cultures tested (Table 2).

AQ production in transgenic and non-transgenic cultures is not affected by DPI

DPI, an inhibitor of NADPH oxidase, is often used to distinguish between pathways leading to phytoalexin formation that are activated via the NADPH-generated oxidative burst and those which are not regulated by the burst (Jabs et al. 1997; Guo et al. 1998). The ID_{50} concentrations required to half-maximally inhibit phytoalexin accumulation in cultured parsley cells were 1.0–2.1 μM DPI (Jabs et al. 1997). DPI at 10 μM inhibited growth of Rc and Rc-rolC3 cultures (Table 2). The inhibitor did not block AQ production in either of the cultures tested (Table 2).

Table 2 Effect of staurosporine and DPI on fresh biomass accumulation and AQ content of *R. cordifolia* cultures. Mean values ± SE based on 10 replicate samples obtained in a single experiment

Callus culture	Treatment (μm)	Fresh biomass (%)	AQ content (% DW)
	Staurosporine		
Rc	0	100 ± 9	0.32 ± 0.04
	0.5	101 ± 7	0.25 ± 0.07
	1	97 ± 9	0.24 ± 0.10
	5	105 ± 15	0.60 ± 0.22
Rc-rolC3	0	100 ± 12	0.68 ± 0.13
	0.5	133 ± 12	0.76 ± 0.08
	1	101 ± 16	0.91 ± 0.24
	5	36 ± 1*	1.19 ± 0.16
Rc-rolB	0	100 ± 11	2.60 ± 0.23
	0.5	107 ± 9	2.43 ± 0.25
	1	130 ± 10	2.38 ± 0.18
	5	122 ± 24	3.02 ± 0.45
	DPI		
Rc	0	100 ± 4	0.29 ± 0.03
	10	71 ± 8*	0.27 ± 0.05
Rc-rolC3	0	100 ± 8	2.38 ± 0.38
	10	73 ± 11*	1.94 ± 0.45
Rc-rolB	0	100 ± 7	2.69 ± 0.06
	10	102 ± 8	3.17 ± 0.19

*$P < 0.05$ vs. values obtained in the absence of the inhibitor, Student's *t*-test

Discussion

By investigating the responses of *R. cordifolia* cultures in the presence of various pharmacological inhibitors, we could show significant differences between the growth responses of the cultures transformed by the *rolC* and *rolB* genes. Compared with the Rc non-transformed culture, the Rc-rolC3 culture showed an abnormally high sensitivity to Ca^{2+}-deficiency in nutrient media and to verapamil treatment (Figs. 2A, 3). In contrast, the Rc-*rolB* culture was much more resistant to these treatments than the normal culture. The idea emerging from our results is that the *rolB* gene, being expressed from the strong viral promoter, maintains higher than normal activity of the Ca^{2+} channels. This leads to the inhibition of growth by disruption of Ca^{2+} homeostasis and allows the *rolB* culture to grow in the presence of verapamil. The *rolC* gene, on the contrary, decreases activity of Ca^{2+} channels, which causes, nevertheless, the same effect as *rolB*, i.e. growth inhibition. Correspondingly, the *rolC* culture is highly sensitive to verapamil and calcium deficiency.

These results are interesting in the light of the data (Röder et al. 1994) that constitutive expression of *rolB* apparently induced cellular death (necrosis) both in callus and leaves of young plants. Steady-state *rolB* mRNA levels were at least 10-fold lower in 35S-*rolB* transgenic plants than in plants that express the *rolC* gene under the transcriptional control of the same promoter. Moreover, higher *rolB* expression levels were obtained only in plants that express the *rolB* and *rolC* genes simultaneously (Röder et al. 1994), indicating that the *rolC* gene could eliminate the toxic effect of the *rolB* gene. Capone et al. (1989) reported that the sole *rolB* gene confers hairy root traits to transformed roots while its association with the *rolC* gene leads to a more normal phenotype. The authors hypothesized that "*rolC* have

an antagonizing effect to *rolB*". It seems to be plausible that the antagonistic effect of the *rolC* gene may be realised at the calcium signaling level.

The RolB protein was shown to be a membrane-associated protein possessing tyrosine phosphatase activity (Philippini et al. 1996). In animals, tyrosine phosphorylation plays a significant role in regulation of Ca^{2+} channels (Pafford et al. 1995; Arnoult et al. 1997). Less information is available from plant studies. The phosphorylation of tyrosine residues, as a direct regulatory mechanism for plant Ca^{2+} channels, has not been reported. Although a crucial role of tyrosine phosphorylation in some important regulatory processes, such as activation of plasma membrane H^+-ATPase and MAP kinases, which may indirectly affect activity of Ca^{2+} channels, is well established (Romeis et al. 1999; Olivari et al. 2000). It will be interesting for future experiments to test whether the RolB tyrosine phosphatase activity is involved in regulation of the phosphorylation level of Ca^{2+} channels in plant cells transformed by the *rolB* gene.

OA caused growth inhibition of all *R. cordifolia* cultures in a similar manner (Fig. 4). However, we have never been able to detect any growth retardation for the *rolB* culture in the presence of 5 μM of cantharidin (Table 1), although growth of both *rolC* and normal cultures was strongly inhibited by this treatment. One possible interpretation is that a cantharidin-sensitive (but OA insensitive) phosphatase(s) is/are highly active in the *rolB* culture. Although OA and cantharidin have often been described as inhibitors possessing very similar activity, some recent data have revealed they have different cellular targets (Knapp et al. 1999).

The signal that activates synthesis of secondary metabolites in transformed cultures is not obvious from our results. It is now generally accepted that after the perception of elicitors by receptors and the transduction by G proteins, the signal is further amplified via increased intracellular Ca^{2+} due to the opening of Ca^{2+} channels (Xing et al. 1997). The increase in intracellular Ca^{2+} concentration activates a Ca^{2+}-dependent protein kinase(s), which, in turn, activates NADPH oxidase (Xing et al. 1997, 2001b; Romeis et al. 2000). The involvement of the oxidative burst generated by NADPH oxidase in the process of phytoalexin stimulation is well known (Jabs et al. 1997; Guo et al. 1998), although there are several examples of Ca^{2+}-dependent regulation of defense genes, where the NADPH oxidase pathway is not involved (Romeis et al. 2000; Sasabe et al. 2000). Staurosporine was shown to block both Ca^{2+}-dependent protein kinase activity and the oxidative burst generated by NADPH oxidase (Xing et al. 1997; Romeis et al. 2000; Cessna and Low 2001).

In our experiments, the blockers of Ca^{2+} channels did not affect AQ production in either transgenic or non-transgenic cultures (Fig. 2). The finding that AQ production in the transgenic cultures is not inhibited by DPI (Table 2) indicates a signaling sequence that does not involve NADPH oxidase. In the experiments with staurosporine (Table 2), it was confirmed that the induction of AQ production in transgenic cultures did not involve either Ca^{2+}-dependent protein kinase or NADPH oxidase pathways. Collectively, these data indicate that the induction of AQ production in the transgenic cultures does not involve the activation of a Ca^{2+}-dependent NADPH oxidase pathway. It is possible that the *rol* genes perturb the secondary metabolism in an unusual way that is not ubiquitous to plants.

The only difference in AQ induction was detected between the transgenic and normal cultures. The transgenic cultures strongly responded to the treatment with protein phosphatase 1 and 2A inhibitors (cantharidin and OA) by induction of AQs (Table 1, Fig. 4). These data suggest the existence of a particular set of 1 and 2A types of Ser/Thr phosphatases that regulate synthesis of AQs in the transgenic *R. cordifolia* cells. Unfortunately, the mechanism by which Ser/Thr phosphatases regulate the processes of phytoalexin formation in plant cells is still unknown. Further investigations of plant cultures transformed by *rol* genes may be useful to unravel this mechanism.

Acknowledgements The authors express their thanks to Mr. Christopher Adams for helpful comments on the manuscript. This work was supported by the Russian Foundation for Fundamental Research. The research described in this publication was made possible in part by Award 99T-03 of the U.S. Civilian Research and Development Foundation For the Independent States of the Former Soviet Union (CRDF).

References

Arnoult C, Lemos JR, Florman HM (1997) Voltage-dependent modulation of T-type calcium channels by protein tyrosine phosphorylation. EMBO J 16:1593–1599

Bonhomme V, Laurain Mattar D, Fliniaux MA (2000) Effects of the *rolC* gene on hairy root: induction development and tropane alkaloid production by *Atropa belladonna*. J Nat Prod 63:1249–1252

Bulgakov VP, Khodakovskaya MV, Labetskaya NV, Tchernoded GK, Zhuravlev YN (1998) The impact of plant *rolC* oncogene on ginsenoside production by ginseng hairy root cultures. Phytochemistry 49:1929–1934

Bulgakov VP, Tchernoded GK, Mischenko NP, Khodakovskaya MV, Glazunov VP, Zvereva EV, Fedoreyev SA, Zhuravlev YN (2002) Effect of salicylic acid, methyl jasmonate, ethephon and cantharidin on anthraquinone production by *Rubia cordifolia* callus cultures transformed with the *rolB* and *rolC* genes. J Biotechnol 97:213–221

Capone I, Spanò L, Cardarelli M, Bellincampi D, Petit A, Costantino P (1989) Induction and growth properties of carrot roots with different complements of *Agrobacterium rhizogenes* T-DNA. Plant Mol Biol 13:43–52

Cessna SG, Low PS (2001) Activation of the oxidative burst in aequorin-transformed *Nicotiana tabacum* cells is mediated by protein kinase- and anion channel-dependent release of Ca^{2+} from internal stores. Planta 214:126–134

Chik CL, Li B, Karpinski E, Ho AK (1999) Regulation of the L-type Ca^{2+} channel current in rat pinealocytes: role of basal phosphorylation. J Neurochem 72:73–80

Guo Z-J, Lamb C, Dixon RA (1998) Potentiation of the oxidative burst and isoflavonoid phytoalexin accumulation by serine protease inhibitors. Plant Physiol 118:1487–1494

Haby C, Larsson O, Islam MS, Aunis D, Berggren PO, Zwiller J (1994) Inhibition of serine/threonine protein phosphatases promotes opening of voltage-activated L-type Ca^{2+} channels in insulin-secreting cells. Biochem J 298:341–346

Hammond-Kosack KE, Jones JDG (1996) Resistance gene-dependent plant defense responses. Plant Cell 8:1773–1791

Holefors A, Xue ZT, Welander M (1998) Transformation of the apple rootstock M26 with the *rolA* gene and its influence on growth. Plant Sci 136:69–78

Huang JW, Grunes DL, Kochian LV (1994) Voltage-dependent Ca^{2+} influx into right-side-out plasma membrane vesicles isolated from wheat roots: characterization of a putative Ca^{2+} channel. Proc Natl Acad Sci USA 91:3473–3477

Jabs T, Tschöpe M, Colling C, Hahlbrock K, Scheel D (1997) Elicitor-stimulated ion fluxes and O_2^- from the oxidative burst are essential components in triggering defense gene activation and phytoalexin synthesis in parsley. Proc Natl Acad Sci USA 94:4800–4805

Jones DL, Kochian LV, Gilroy S (1998) Aluminium induces a decrease in cytosolic calcium concentration in BY-2 tobacco cell cultures. Plant Physiol 116:81–89

Kiegle E, Gilliham M, Haseloff J, Tester M (2000) Hyperpolarisation-activated calcium currents found only in cells from the elongation zone of *Arabidopsis thaliana* roots. Plant J 21:225–229

Knapp J, Boknik P, Luss I, Huke S, Linck B, Luss H, Muller FU, Muller T, Nacke P, Noll T, Piper HM, Schmitz W, Vahlensieck U, Neumann J (1999) The protein phosphatase inhibitor cantharidin alters vascular endothelial cell permeability. J Pharmacol Exp Ther 289:1480–1486

Knight MR, Smith SM, Trewavas AJ (1992) Wind-induced plant motion immediately increases cytosolic calcium. Proc Natl Acad Sci USA 89:4967–4971

MacKintosh C, Cohen P (1989) Identification of high levels of type 1 and type 2A protein phosphatases in higher plants. Biochem J 262:335–339

Menke FLH, Parchmann S, Mueller MJ, Kijne JW, Memelink J (1999) Involvement of the octadecanoid pathway and protein phosphorylation in fungal elicitor-induced expression of terpenoid indole alkaloid biosynthetic genes in *Catharanthus roseus*. Plant Physiol 119:1289–1296

Mischenko NP, Fedoreyev SA, Glazunov VP, Tchernoded GK, Bulgakov VP, Zhuravlev YN (1999) Anthraquinone production by callus cultures of *Rubia cordifolia*. Fitoterapia 70:552–557

Nilsson O, Olsson O (1997) Getting to the root: the role of the *Agrobacterium rhizogenes rol* genes in the formation of hairy roots. Physiol Plant 100:463–473

Olivari C, Albumi C, Pugliarello CM, Michelis MID (2000) Phenylarsine oxide inhibits the fusicoccin-induced activation of plasma membrane H^+-ATPase. Plant Physiol 122:463–470

Pafford CM, Simples JE, Strong JA (1995) Effects of the protein tyrosine phosphatase inhibitor phenylarsine oxide on excision-activated calcium channels in *Lymnaea* neurons. Cell Calcium 18:400–410

Palazón J, Cusidó RM, Roig C, Piñol MT (1998a) Expression of the *rolC* gene and nicotine production in transgenic roots and their regenerated plants. Plant Cell Rep 17:384–390

Palazón J, Cusidó RM, Gonzalo J, Bonfill M, Morales S, Piñol MT (1998b) Relation between the amount the *rolC* gene product and indole alkaloid accumulation in *Catharanthus roseus* transformed root cultures. J Plant Physiol 153:712–718

Philippini F, Rossi V, Marin O, Trovato M, Costantino P, Downey PM, Schiavo F, Terzi M (1996) A plant oncogene as a phosphatase. Nature 379:499–500

Pineros M, Tester M (1997) Characterization of the high-affinity verapamil binding site in a plant plasma membrane Ca^{2+}-selective channel. J Membr Biol 157:139–145

Röder FT, Schmülling T, Gatz C (1994) Efficiency of the tetracycline-dependent gene expression system: complete suppression and efficient induction of the *rolB* phenotype in transgenic plants. Mol Gen Genet 243:32–38

Romeis T, Piedras P, Zhang S, Klessig DF, Hirt H, Jones JDG (1999) Rapid Avr9- and Cf-9-dependent activation of MAP kinases in tobacco cell cultures and leaves: convergence of resistance gene, elicitor, wound, and salicylate responses. Plant Cell 11:273–287

Romeis T, Piedras P, Jones JDG (2000) Resistance gene-dependent activation of a calcium-dependent protein kinase in the plant defense response. Plant Cell 12:803–815

Sanders D, Brownlee C, Harper JF (1999) Communicating with calcium. Plant Cell 11:691–706

Sasabe M, Takeuchi K, Kamoun S, Ichinose Y, Govers F, Toyoda K, Shiraishi T, Yamada T (2000) Independent pathways leading to apoptotic cell death, oxidative burst and defense gene expression in response to elicitin in tobacco cell suspension culture. Eur J Biochem 267:5005–5013

Schmülling T, Schell J, Spena A. (1988) Single genes from *Agrobacterium rhizogenes* influence plant development. EMBO J 7:2621–2629

Spena A, Schmülling T, Koncz C, Schell JS (1987) Independent and synergistic activity of *rolA*, *B* and *C* loci in stimulating abnormal growth in plants. EMBO J 6:3891–3899

Xing T, Higgins VJ, Blumwald E (1997) Race-specific elicitors of *Cladosporium fulvum* promote translocation of cytosolic components of NADPH oxidase to the plasma membrane of tomato cells. Plant Cell 9:249–259

Xing T, Malik K, Martin T, Miki BL (2001a) Activation of tomato PR and wound-related genes by a mutagenized tomato MAP kinase kinase through divergent pathways. Plant Mol Biol 46:109–120

Xing T, Wang X-J, Malik K, Miki BL (2001b) Ectopic expression of an *Arabidopsis* calmodulin-like domain protein kinase-enhanced NADPH oxidase activity and oxidative burst in tomato protoplasts. MPMI 14:1261–1264