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Increase in Anthraquinone Content in *Rubia cordifolia* Cells Transformed by *rol* Genes Does Not Involve Activation of the NADPH Oxidase Signaling Pathway

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Abstract—It has been reported that *rol* plant oncogenes located in Ri-plasmids of *Agrobacterium rhizogenes* activated synthesis of secondary metabolites in the transformed plant cells. The activator mechanism is still unknown. In this work, we studied whether the NADPH oxidase-signaling pathway, which regulates the synthesis of defense metabolites in plants, is involved in the activator function of the *rol* genes. It was demonstrated that the transformation of *Rubia cordifolia* cells by the *rolB* and *rolC* genes caused an induction of biosynthesis of anthraquinone-type phytoalexins. Inhibition studies revealed a striking difference between the *rolC* and *rolB* transformed cultures in their sensitivity to Ca^{2+} channel blockers and calcium deficiency. The *rolC* culture displayed lowered resistance to the inhibitors compared to the non-transformed culture, while the *rolB* culture was more resistant to the treatment. The assumption was made that the oncogenic potential of *rol* genes is realized through the alteration of calcium balance in the plant cells. Anthraquinone production was not inhibited in the non-transformed and transformed cultures by Ca^{2+} channel blockers, as well as by diphenylene iodonium, an inhibitor of NADPH oxidase, and by the protein kinase inhibitor staurosporine. These results indicate that the induction of anthraquinone production in transgenic cultures does not involve the activation of Ca^{2+}-dependent NADPH oxidase pathway.

Key words: *rolB* and *rolC* genes, *Agrobacterium rhizogenes*, Ca^{2+}, NADPH oxidase, *Rubia cordifolia*, anthraquinone

During the interaction between *Agrobacterium rhizogenes* and plants, partial transfer of the agrobacterial plasmid DNA (T-DNA) into the plant genome occurs. T-DNA contains the *rolB* and *rolC* genes, each of which is able to induce a neoplastic transformation of plant cells [1].

An interesting ability of plant cell cultures transformed by *rol* genes was recently established, the ability of increased synthesis of secondary metabolites [2-4]. The activation mechanism of secondary metabolite synthesis in the transformed cultures has not been investigated. However, the understanding of this mechanism is important for two reasons. First, it allows obtaining new data about plant oncogenesis processes; second, it provides an opportunity to extend knowledge about general regulation principles of synthesis of plant secondary metabolites.

In spite of intensive research, the primary targets of oncogenes inducing the neoplastic transformation process are yet not identified. Estruch et al. [5, 6] reported that *rol* genes influence physiologic processes in plant cells by affecting their hormone balance. It was believed that *rolB* gene encodes an enzyme that cleaves glycosides of indoleacetic acid [5], while the *rolC* gene encodes glycosidase of bound forms of cytokinins [6]. Recently, however, it was established that levels of free and bound indoleacetic acid do not change in *rolB* gene expressing plants [7]. The protein encoded by the *rolC* gene did not cleave cytokinin glycosides *in planta* [8, 9]. The most intriguing discovery within the recent years is the establishment of the fact that RolB protein displays tyrosine phosphatase activity [10]. It is assumed that RolB can modify the signaling pathways

Abbreviations: CDPK) calcium-dependent protein kinase; DPI) diphenylene iodonium; MAP-kinase) mitogen-activated protein kinase.
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of indoleacetic acid [11]. However, it is also possible, that the modification of auxin signals along with an impact on secondary metabolism processes are secondary effects of the gene. It was of interest to determine the primary target of oncogenes and also to study the changes affecting different signaling pathways in the transformed cells.

The content of secondary metabolites carrying out protective functions (phytoalexins) increases when the plant is damaged by pathogens. The most general and well-studied pathway of the activation of plant defense mechanisms consists in the: 1) detection of pathogen signal; 2) activation of H^+-ATPase; 3) increase in calcium influx from an intercellular space into the cells; 4) activation of calcium-dependant protein kinase (CDPK), and 5) activation of NADPH-oxidase. Active oxygen radicals generated by NADPH-oxidase participate in the activation of MAP-kinases, which leads to the increased expression level of certain protective genes, including the genes of biosynthesis of secondary metabolites (Fig. 1).

An important role in the stimulation of secondary metabolite synthesis is also played by the jasmonic acid signaling pathway [19], and in some cases by signaling pathways of salicylic acid and ethylene [20]. In our recently published work, we have illustrated that *rolB* and *rolC* genes in *Rubia cordifolia* strains activate the synthesis of anthraquinones, typical plant phytoalexins [21]. Ethylene did not affect anthraquinone synthesis either in the transgenic or in non-transformed strains. Jasmonic and salicylic acids increased the content of anthraquinones in both transgenic and normal strains with very similar dynamics. These data indicate that pathways of ethylene, jasmonic, and salicylic acid are not involved in the activator function of *rol* genes [21].

Signaling pathway inhibitors are used for the primary estimation of the participation of these pathways in various cellular functions. We utilized this technique in order to clarify whether the growth and biosynthetic parameters of transgenic and reference calluses change when affected by inhibitors. Difference in these parameters could indicate the transgenic “application point”. Indeed, significant growth differences between transgenic and reference cultures were noticed in the experiments with calcium

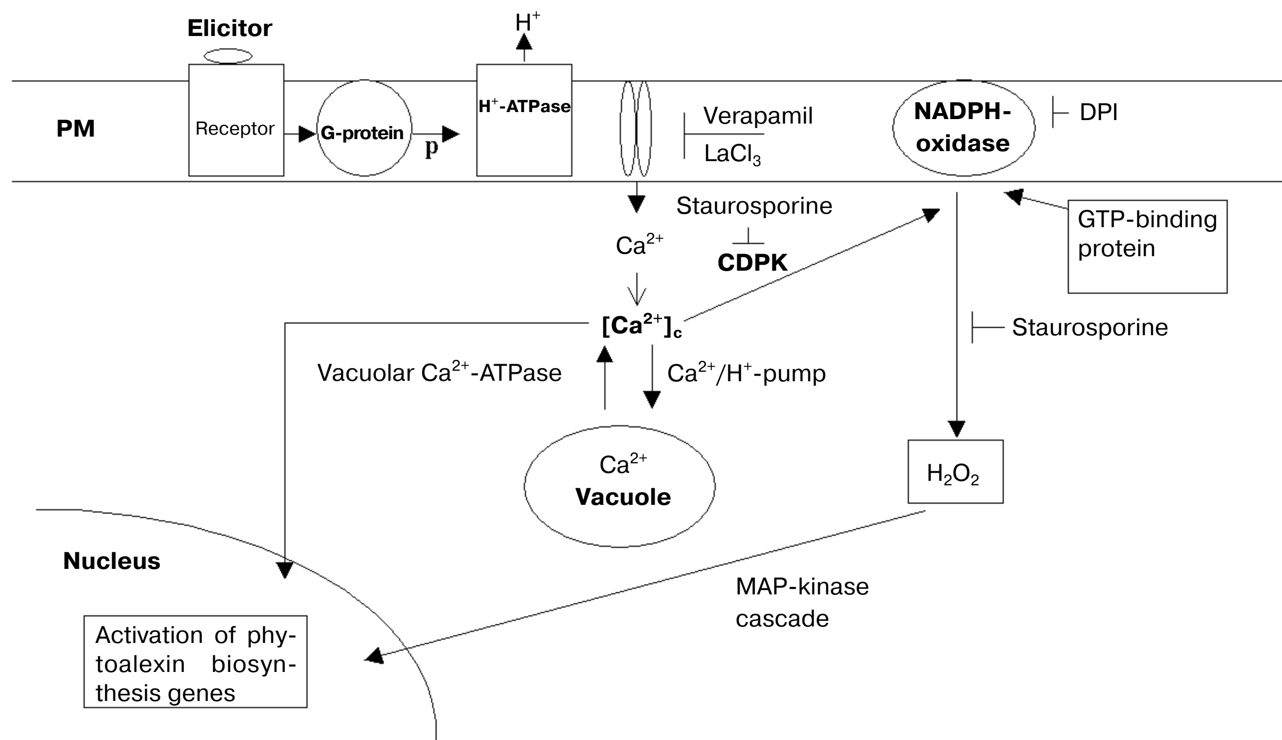

Fig. 1. Diagram of early signaling pathways resulting in the activation of synthesis of plant defense compounds [12-18]. Localized in the plasma membrane (PM), the receptors receive elicitor signal activating H^+-ATPase using G-protein and phosphatase (**p**). Hyperpolarization of the membrane leads to activation of calcium channels and to the increase in cytoplasmic calcium content. Homeostasis of calcium is maintained by the coordinated work of calcium channels and intracellular calcium pumps, regulating calcium exchange between vacuoles and cytoplasm. The increase in cytoplasmic calcium concentration leads to CDPK kinase activation that, in turn, regulates the “assembling” of NADPH-oxidase complex. Active oxygen radicals and also H_2O_2 generated by NADPH-oxidase activate MAP-kinases that phosphorylate the transcription factors and increase expression levels of the defense genes. Activation of defense genes can also take place with the participation of calcium via various regulation mechanisms, bypassing the NADPH-oxidase pathway. |—, site of inhibitor action.

channel inhibitors. At the same time, inhibitors of calcium channels along with inhibitors of CDPK and NADPH-oxidase did not inhibit the synthesis of anthraquinones in all investigated lines. This result suggests that signals from *rolB* and *rolC* genes activating anthraquinone synthesis are not regulated by the CDPK-dependant NADPH-oxidase pathway.

MATERIALS AND METHODS

Non-transformed and transformed cultures. Callus cultures were obtained from leaves of three-week-old sterile *Rubia cordifolia* L. microplants [21]. Untransformed culture used as a reference was labeled Rc. Transgenic cultures of Rc-rolB and Rc-rolC3 were obtained from the leaves of the same plant using transformation by agrobacterial vector GV3101 containing either *rolB* gene controlled by 35S-promoter of cauliflower mosaic virus (plasmid pPCV002-CaMVBT [1]), or *rolC* gene controlled by the same promoter (pPCV002-CaMVC [1]). Transgenic calluses Rc-rolC3 and Rc-rolB were obtained from primary transformed cultures by selecting aggregates not containing roots [21]. Cultivation conditions and media composition were identical for both non-transformed and transgenic cultures: calluses were grown in 100 ml Erlenmeyer flasks at 25°C in darkness on $W_{B/A}$ medium containing 6-benzylaminopurine (0.5 mg/liter) and α-naphthylacetic acid (2.0 mg/liter) [22]. Calluses were subcultivated with 30-day period. To grow the suspension culture, 250 ml Erlenmeyer flasks were used. Flasks with the culture were placed at an orbital shaker and grown at 100 rpm in darkness at 25°C for 14 days.

Experiments with inhibitors. Tissue culture reagents and dimethyl sulfoxide were obtained from Sigma (USA) and inhibitors from ICN Biomedicals (USA). Sterile aqueous solutions of verapamil and $LaCl_3$ and also solutions of staurosporine and DPI in dimethyl sulfoxide were aseptically added to autoclaved medium at required concentrations. Equivalent amounts of dimethyl sulfoxide were added to the reference flasks.

Determination of anthraquinone content. Purpurin and munjistin comprising 80% of *R. cordifolia* anthraquinones were initially extracted and characterized by physicochemical methods [22]. Anthraquinones were determined spectrophotometrically as the total amount of purpurin and munjistin [22]. For this procedure, 100 mg of dry callus tissue was treated with 0.3 ml of 5 M HCl solution and extracted with 3 ml of ethanol at room temperature. The absorption spectrum in visible range was recorded for each measurement. Content of purpurin in the extract was determined at 515 nm, and content of munjistin at 421 nm with subtraction of purpurin absorption at this wavelength.

The results were statistically processed using STATISTICA WINDOWS 98 software.

RESULTS

Growth of transgenic cultures under calcium deficiency conditions. Figure 1 presents a diagram over early signaling pathways participating in the activation of the synthesis of phytoalexins. Investigation of the role of these pathways in the process of anthraquinone biosynthesis activation in *R. cordifolia* transgenic cells was started with a study of the influence of calcium on the growth and production of characteristics of plant cultures.

Callus aggregates of Rc, Rc-rolC3, and Rc-rolB lines were grown in liquid calcium chloride-free media and also in standard medium with 3 mM Ca^{2+}. Decreased growth and appearance of necrotic aggregates were observed for the non-transformed culture in the absence of calcium (Fig. 2). Under these conditions, Rc-rolC3 line was affected by necrosis and did not grow. Compared to these cultures, Rc-rolB culture did not change morphologically, and its growth decreased by less than half (Fig. 2). The average content of anthraquinones in Rc-rolB culture was 2.72 or 2.42% of dry cell weight during the cultivation in medium with and without calcium, respectively. The 11% difference reflects usually observed variability in the content of anthraquinones between particular calluses. Hence, the absence of calcium in the cultural medium did not lower the content of anthraquinones in *rolB* culture of *R. cordifolia*. Further experiments were performed on callus cultures grown on agarized medium. The preliminary trials revealed that the cultivation on agarized calcium chloride-free medium does not result in significant inhibition of culture growth, apparently because of the presence of a small amount of calcium in agar (results not shown).

Impact of calcium channel inhibitors on growth and content of anthraquinones in *R. cordifolia*. An obvious explanation of the observed differences in *rolC* and *rolB* culture growth under calcium deficiency conditions (Fig.

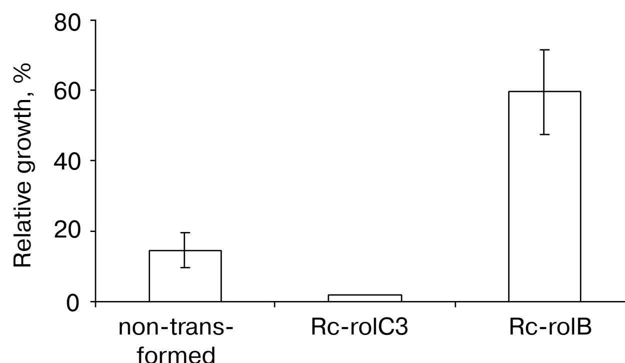

Fig. 2. Growth of *R. cordifolia* culture (% with respect to the growth of the same cultures in the presence of 3 mM $CaCl_2$) in the absence of calcium in liquid medium $W_{B/A}$.

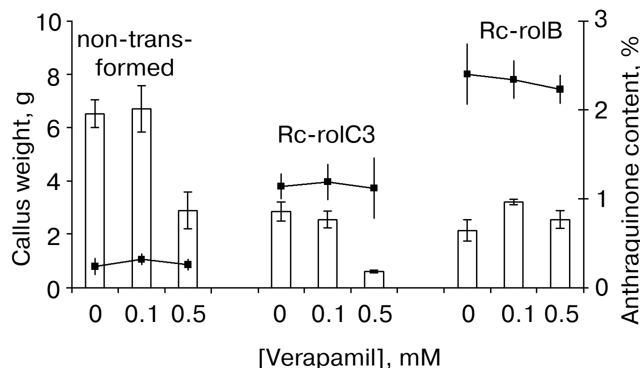

Fig. 3. Influence of verapamil on callus weight (rectangles) and anthraquinone content (curves) in dry tissue. Calluses were cultivated on agarized calcium chloride-free medium $W_{B/A}$.

2) could be a different permeability of the calcium channels in transgenic cultures. While planning the experiments with channel inhibitors, we expected to see a difference in growth between the transgenic cultures. An inhibitor of calcium channels (verapamil) was added to calcium chloride-free media. Calluses were grown on these media and the accumulation of the biomass and the content of anthraquinones were determined after the completed cultivation cycle. Indeed, verapamil at the concentration of 0.5 mM induced various effects: the inhibition of non-transformed culture growth, necrotic response, and complete termination of *rolC* culture growth, with no impact on *rolB* culture growth (Fig. 3).

To confirm this observation, we performed a similar experiment on another model system, including three cultures of *Papaver alboroseum* cells transformed by a vector system with *rolC* and *rolB* genes (manuscript in preparation). This experiment confirmed that the features of verapamil action were not a reflection of any specific properties of the *R. cordifolia* culture (Table 1).

Table 1. Relative growth of *R. cordifolia* and *P. alboroseum* cultures transformed by *rol* genes compared to the non-transformed culture (%)

Material	*R. cordifolia*[a]	*P. alboroseum*[b]
Non-transformed culture	59*	75*
rolB	100.5	98
rolC	22.5*	40*

Note: Cultures were grown on agarized, calcium chloride-free medium $W_{B/A}$. Verapamil concentration was 0.5 mM.
* $p < 0.05$ (compared to the parameters of culture cultivated on medium without the inhibitor).
[a] Mean values from three experiments; each experiment included 10 repetitions.
[b] Mean values from one experiments with 10 repetitions.

Using experimental data presented in Fig. 3, the concentrations of verapamil inhibiting the callus growth by 50% (ID_{50}) were determined. For Rc, Rc-rolC3, and Rc-rolB cultures, the values were 0.5, 0.3, and 2.1 mM, respectively. In a particular experiment, verapamil was introduced into the full composition nutritious media containing 3 mM $CaCl_2$. Values of ID_{50} in this experiment were ~2 times higher compared to the experiment when calcium was excluded from the media composition; 1.2, 0.6, and 4.0 mM for cultures enumerated in the same order. Hence, the sensitivity of *rolC* and *rolB* cultures to verapamil was approximately sevenfold in both experiments. It is known that verapamil blocks Ca^{2+}-selective channels in plants causing calcium deficit and, therefore, the decline of cell growth [23]. Rc-rolC3 calluses have lower growth than the normal calluses. This fact can be accounted for by the decrease in intracellular calcium content due to the blocking of verapamil-sensitive calcium channels by the *rolC* gene product.

Lanthanum chloride, a commonly used inhibitor of Ca^{2+}-channels, introduced into the culture media at 0.5 and 2.0 mM concentrations provided a differential action on the culture growth analogous to verapamil. Similarly to verapamil, La^{3+} did not lower the content of anthraquinones either in the non-transformed or in transgenic cultures (data not presented).

Effects of staurosporine and DPI. Staurosporine is a well-known protein kinase inhibitor and is widely used for elucidating the role of kinases in the regulation of activity of various signaling pathways in plant cells. For example, 1 μM staurosporine inhibited elicitor- and methyl jasmonate-induced expression of terpenoid indole alkaloid biosynthetic genes [24], as well as MAP-kinase-activated expression of pathogenesis-related proteins [25]. Staurosporine completely blocked CDPK activity [13, 15].

R. cordifolia calluses were cultivated on media containing staurosporine at the concentrations of 0.5, 1.0, and 5.0 μM. It was found that staurosporine did not decrease the content of anthraquinones either in non-transformed or in transgenic cultures over all tested concentrations (Table 2). The growth of Rc and Rc-rolB cultures was not affected, while using staurosporine at 5 μM concentration approximately 2-fold growth inhibition of Rc-rolC3 culture was observed (Table 2).

Being an inhibitor of NADPH-oxidase, DPI is often used while determining the role of this enzyme in plants defense reactions [14, 26]. DPI inhibited the accumulation of phytoalexins in cultivated parsley cells already at concentrations of 1.1-2.1 μM [14]. In our experiments, 10 μM DPI did not affect anthraquinone content in all tested cultures (Table 2). The inhibitor suppressed the

Table 2. Influence of staurosporine and DPI on *R. cordifolia* callus weight and anthraquinone content[a]

Callus culture[b]	Callus weight compared to reference, %	Content of anthraquinones, % of dry weight
Staurosporin, μM		
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, μM		
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

[a] Mean values (± standard error) calculated based on 10 independent samples obtained in one experiment.
[b] Numbers show inhibitor concentrations.
* $p < 0.05$ (compared to the parameters of culture cultivated on medium without the inhibitor).

growth of Rc and Rc-rolC3 cultures and did not affect the growth of *rolB* culture (Table 2).

DISCUSSION

The regulation of secondary metabolites synthesis in general and phytoalexins in particular in plant cells under normal conditions and as affected by the environment is not yet sufficiently investigated. Most available information relates to the action of jasmonic acid, probably the key inductor of secondary metabolites synthesis. A certain role may also be played by salicylic acid and ethylene. Independent from these three inducers is a generation pathway by NADPH-oxidase of active oxygen radicals [15], which are required (but not sufficient) for phytoalexins biosynthesis gene activation [14, 26]. Transgenic plants were recently obtained where the constitutive expression of a gene activating NADPH-oxidase (minor GTP-binding protein) or a gene imitating the oxidase action (MAP-kinase gene) resulted in the development of abiotic and biotic stress stability of the plants, and also in the increased accumulation of phytoalexins [16, 17]. These facts indicate that the regulating pathway of NADPH-oxidase is an important component in the regulation cascade, providing increased synthesis of defense compounds in plants.

To evaluate the influence of *rol* genes on secondary metabolite synthesis, a model system was created consisting of undifferentiated non-transformed and transgenic culture cells of *R. cordifolia*.

During the cultivation of *R. cordifolia* calluses in the standard and calcium-free media, it was established that *rolC*- and *rolB*-calluses significantly differ in growth from each other and compared to the non-transformed culture (Fig. 2). Experiments with calcium channel inhibitor (verapamil) confirmed the presence of these differences (Fig. 3). We propose a working hypothesis describing the function of the *rolB* gene. Phosphatase (labeled as **p** in Fig. 1) participates in plant H^+-ATPase activation, thus leading to the stimulation of calcium channels and an influx of Ca^{2+} from the extra-cellular space. It was recently revealed that tyrosine phosphatase might also participate in this reaction [27]. It can be assumed that the *rolB* gene product, i.e., tyrosine phosphatase localized in the plasma membrane, promotes the proton pump activity and thus maintains constantly high activity of calcium channels. This hypothesis describes well our experimental observations: the stability of *rolB*-calluses towards verapamil (Fig. 3), stability to the lack of calcium in the cultural medium (Fig. 2), and also decreased growth compared to the normal culture. Culture of *rolB* grows ~2 times less actively than the untransformed calluses [21], which might be caused by the disruption of calcium balance due to its excessive accumulation inside the cell

[18]. Indeed, verapamil at 0.1 mM concentration increased the growth of Rc-rolB culture by 54% ($p < 0.05$, Fig. 3), probably by preventing excessive calcium accumulation. However, there is another possible gene functioning mechanism implying the direct action of RolB on the calcium channels. The phosphorylation of tyrosine residues is a widespread mechanism of animal calcium channel activity [28, 29]. For plants, this mechanism has not yet been described.

Calcium channel activity is apparently diminished in *rolC* culture compared to the normal value. Let us focus closely on certain oncogene effects that were observed earlier but not explained. It is known that an active expression of *rolB* gene leads to cell death [30]. While obtaining *rolB*-transgenic plant cultures, selection aimed to weaken the transgene expression in plants is taking place. Tobacco culture transformed by *rolB* gene grows normally if the gene expression level is low, approximately 10 times lower than *rolC* gene expression level [30]. The higher expression of *rolB* gene could only be reached when the plants contained both *rolB* and *rolC* genes at the same time [30]. Capone et al. [31] noted that "... *rolC* gene exhibits an antagonistic effect towards *rolB*". Enlightened by the data obtained by us, it becomes clear how the antagonistic effect of the oncogenes can be implemented, based on the level of calcium signal transduction. Obviously, this assumption needs to be tested by direct experimental techniques.

Results illustrating that staurosporine and DPI do not diminish the content of anthraquinones in the transgenic cultures (Table 2) show that CDPK and NADPH-oxidase do not participate in the transduction of *rol* genes activator signal. This result is new and interesting, but not unexpected in terms of discussion about *rol* gene action mechanism towards secondary metabolites. Reports about the realization of plant defense reactions that bypass the oxidative burst and H_2O_2 generation pathways have been frequently published in recent years. For instance, Romeis et al. [20] present examples of calcium-dependent, but independent from NADPH-oxidase defense reactions of plants, regulated by kinase–phosphatase cascades. It was established that the activation of certain genes of the phenylpropanoid biosynthetic pathway does not depend on the oxidative burst caused by the elicitors [32].

An interesting fact is that verapamil and La^{3+} did not decrease anthraquinone content in the transgenic cultures (Fig. 3). This means that the decrease in intracellular calcium content does not inhibit anthraquinone synthesis. In other words, the decrease in calcium influx does not influence in the behavior of the activator function of *rol* genes. Data about the absence of an effect of calcium channel inhibitors on anthraquinone content in the transgenic cultures together with experiments using staurosporine and DPI are rather unexpected. They indicate that an unusual for plant–microbe interactions type of regulation of phytoalexin biosynthesis may be present in the transgenic cultures. This theory will be tested with other groups of secondary metabolites synthesized along with different biosynthetic pathways.

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