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ORIGINAL ARTICLE

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Inhibitory effect of the *Agrobacterium rhizogenes rolC* gene on rabdosiin and rosmarinic acid production in *Eritrichium sericeum* and *Lithospermum erythrorhizon* transformed cell cultures

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Abstract Rabdosiin and related caffeic acid metabolites have been proposed as active pharmacological agents demonstrating potent anti-HIV and antiallergic activities. We transformed *Eritrichium sericeum* and *Lithospermum erythrorhizon* seedlings by the *rolC* gene, which has been recently described as an activator of plant secondary metabolism. Surprisingly, the *rolC*-transformed cell cultures of both plants yielded two- to threefold less levels of rabdosiin and rosmarinic acid (RA) than respective control cultures. This result establishes an interesting precedent when the secondary metabolites are differently regulated by a single gene. We show that the *rolC* gene affects production of rabdosiin and RA irrespective of the methyl jasmonate (MeJA)-mediated and the Ca^{2+}-dependent NADPH oxidase pathways. Cantharidin, an inhibitor of serine/threonine phosphatases, partly diminishes the *rolC*-gene inhibitory effect that indicates involvement of the *rolC*-gene-mediated signal in plant regulatory controls, mediated by protein phosphatases. We also show that the control MeJA-stimulated *E. sericeum* root culture produces (−)-rabdosiin up to 3.41% dry weight, representing the highest level of this substance for plant cell cultures reported so far.

Keywords Cantharidin · *Eritrichium sericeum* · *Lithospermum erythrorhizon* · Methyl jasmonate · *rolC* gene · Rabdosiin · Rosmarinic acid

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Abbreviations CAM: Caffeic acid metabolites · DPI: Diphenylene iodonium · MeJA: Methyl jasmonate · RA: Rosmarinic acid · ROS: Reactive oxygen species

Introduction

The *rolC* gene of *Agrobacterium rhizogenes* T-DNA plays an essential role in the development of hairy root disease and its expression in plants causes substantial morphological and biochemical alterations (Spena et al. 1987; Nilsson et al. 1993, 1996). The ability of the *rolC* gene to alter secondary metabolism in hairy root cultures, callus cultures and transgenic plants has recently been revealed (Palazón et al. 1998a, b; Bulgakov et al. 1998, 2002, 2003; Bonhomme et al. 2000). The *rolC*-stimulatory effect on secondary metabolism was demonstrated by investigations with different groups of secondary metabolites, such as tropane alkaloids (Bonhomme et al. 2000), pyridine alkaloids (Palazón et al. 1998a), indole alkaloids (Palazón et al. 1998b), ginsenosides (Bulgakov et al. 1998) and anthraquinones (Bulgakov et al. 2002, 2003). Some data, however, indicate decreased levels of tropane alkaloids in *Hyoscyamus muticus* plants and *Duboisia* hybrid plants transformed by wild-type *A. rhizogenes* strains containing the *rolC* among other T-DNA genes (Sevon et al. 1997; Roig Celma et al. 2001).

Such peculiarities of the *rolC* gene action stimulated investigations aimed to understand how the gene could affect secondary metabolism. The assessment of signal transduction pathways affected by the gene showed that the *rolC*-gene-mediated signal did not interfere with general plant defense pathways leading to synthesis of phytoalexin-type secondary metabolites. In particular, the Ca^{2+}-dependent NADPH oxidase pathway as well as salicylic-acid-mediated and octadecanoid pathways

have not been affected in *rolC*-transformed cells of *Rubia cordifolia* (Bulgakov et al. 2002, 2003, 2004).

A few years ago, rabdosiin and related caffeic acid metabolites (CAM) were proposed as active pharmacological agents demonstrating potent anti-HIV (Kashiwada et al. 1995; Abd-Elasem et al. 2002; Tewtrakul et al. 2003) and antiallergic activities (Ito et al. 1998). Rabdosiin, an ester of caffeic acid with a lignan skeleton, was isolated firstly from stems of *Rabdosia japonica* (Agata et al. 1988). Later, two enantiomers, (+)-rabdosiin and (−)-rabdosiin, were found in *Macrotomia euchroma* roots (Nishizawa et al. 1990). It was recently reported that *Lithospermum erythrorhizon* cell suspension cultures, which were initially established for the production of red naphthoquinone pigment shikonin, synthesized four CAM: rosmarinic acid (RA), lithospermic acid B, a monoglucoside of lithospermic acid B and (+)-rabdosiin (Yamamoto et al. 2000a, b).

We initiated research of plant cell cultures possessing increased ability to synthesize these substances. *Eritrichium sericeum* (Boraginaceae) callus and root cultures were established and analyzed for caffeic acid metabolite production. Two substances, (−)-rabdosiin and RA were identified as predominant CAM produced by these cultures (Fedoreyev et al., in press). In the present investigation, we transformed *E. sericeum* cells by the *rolC* gene to increase levels of these valuable products in cells. Surprisingly, the CAM content was clearly reduced in transformed tissues. This result, although ineffectual in practical aspects, establishes an interesting precedent when the shikimate-derived metabolites, such as anthraquinones and CAM, are differently regulated by a single gene.

Materials and methods

Plant material

Plants and seeds of *E. sericeum* (Lehm.) DC and *L. erythrorhizon* Sieb. et Zucc. (Boraginaceae) were collected in the southern regions of the Primorsky Territory and Kamchatka (Russian Far East), respectively, and determined in the Botany Department of the Institute of Biology and Soil Science.

Genetic transformation of *E. sericeum*

Plasmids pPCV002 (vector) and pPCV002-CaMVC (Spena et al. 1987) were transferred to the *A. tumefaciens* strain GV3101/pMP90RK as described (Bulgakov et al. 1998). pPCV002-CaMVC contains the *rolC* gene under cauliflower mosaic virus (CaMV) 35S promoter control (Spena et al. 1987). Both constructions also carried a gene for kanamycin resistance (*nptII*) under the control of nopaline synthase promoter. *Agrobacterium* strains were grown on the Tryptose agar (Ferak, Germany) containing 50 mg l^{-1} kanamycin sulfate and 100 mg l^{-1} carbenicillin at 28°C for 1 day before transformation.

Seeds of *E. sericeum* were sterilized and germinated in vitro, then seedlings were transformed by *A. tumefaciens* GV3101/pPCV002 (empty vector, control) or *A. tumefaciens* GV3101/pPCV002-CaMVC (*rolC* gene) as described (Bulgakov et al. 2002). Explants were cultivated on $W_{B/A}$ medium (Bulgakov et al. 2002) supplemented with 250 mg l^{-1} cefotaxim and 100 mg l^{-1} kanamycin sulfate at 25°C in the dark at 30-day subculture intervals. Well-growing aggregates were observed on the explants and selected to produce lines of kanamycin-resistant calluses. Calluses were cultivated in 100 ml Erlenmeyer flasks on $W_{B/A}$ medium in the dark at 25°C with 30-day subculture intervals. The primary *E. sericeum* calluses transformed with vector plasmid pPCV002 as well as with pPCV002-CaMVC spontaneously formed adventitious roots. Several root cultures of *E. sericeum* were established by placing root tips, isolated from the adventitious roots, into the liquid W_{IBA} medium (Bulgakov et al. 1998), supplemented with 1.0 mg l^{-1} indole-3-butyric acid. These cultures were further subcultured at 28-day intervals in the dark at 25°C in 500-ml Erlenmeyer flasks in an orbital shaker (100 rpm). Additionally, a nontransformed *E. sericeum* callus culture was obtained and cultivated under the same conditions as the transformed calli. All callus cultures used in this work were grown on solid agar media, whereas all root cultures were cultivated in liquid media.

Genetic transformation of *L. erythrorhizon*

The *rolC* gene-transformed culture of *L. erythrorhizon* (Le-rolC) was obtained by transformation of seedlings with *A. tumefaciens* GV3101/pPCV002-CaMVC as described above. Primary kanamycin-resistant Le-rolC calluses formed small roots, which grew in liquid media as 8- to 10-mm star-shaped aggregates. Because these root aggregates grew very slowly, we used the well-growing Le-rolC callus culture. The control nontransformed culture Le was established as described (Bulgakov et al. 2001). The Le and Le-rolC callus cultures were cultivated on the $W_{K/IAA}$ medium (Bulgakov et al. 2001), supplemented with 2.0 mg l^{-1} kinetin and 0.2 mg l^{-1} indole-3-acetic acid in the dark at 25°C with 30-day subculture intervals.

DNA and RNA analysis

DNA samples were isolated from 21-day plant cell cultures as described (Bulgakov et al. 2002). The primer set 5′-TAA CAT GGC TGA AGA CGA CCT GT-3′ and 5′-TGC AAA CTT GCA CTC GCC ATG CC-3′ allowed the amplification of a 537-bp *rolC* gene fragment. The *nptII* gene 700-bp fragment was amplified with primers 5′-GAG GCT ATT CGG CTA TGA CTG-3′ and 5′-ATC GGG AGC GGC GAT ACC

GTA-3′. Amplification reactions were performed in volumes of 25 μl containing 10 mM Tris–HCl (pH 8.5), 50 mM KCl, 2.0 mM $MgCl_2$, 0.01% gelatin, 0.1 mM Triton X-100, 0.2 mM of each dNTP, 0.2 μM of each oligonucleotide primer and 1.0 unit of *Taq* DNA polymerase. DNA (50–100 ng) was used as a template. Analysis was performed in a UNO Thermocycler (Biometra, Gottingen, Germany) programmed for an initial denaturation step of 3 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 62°C, 1 min at 72°C, and a last cycle of 72°C for 3 min, using the fastest available transitions between each temperature.

Total RNA was isolated from the Es-rolC culture by YellowSolve (Clonogen, St. Petersburg, Russia) extraction. Extracted RNA was treated with DNAase (Amresco, Solon, OH, USA) and purified with BlueSorb (Clonogen). Complementary DNAs were synthesized using 2–8 μg RNA by RNA PCR Kit (Silex M, Moscow, Russia). The reactions were performed in volumes of 50 μl containing RT buffer, 0.2 mM of each dNTP, 0.2 μM of oligo-$(dT)_{15}$ primer and 200 units of M-MLV polymerase at 37°C for 1 h. The 10-μl samples of reverse transcription products were then amplified by PCR with the primers 5′-GCG TAA ACC CTT GAT CGA GC-3′ and 5′-TTC GAA CCT AAG CTG GGT GC-3′ designed to flank 400-bp fragment of the *rolC* gene. Amplification reactions were performed as indicated above. The PCR products were separated by electrophoresis in 1.5% agarose gel.

Sequence analysis

The *rolC* gene 400-bp RT-PCR product was sequenced with the same primers and Big Dye Terminator Cycle Sequencing Kit (Perkin-Elmer Biosystems, Forster City, CA, USA) following the manufacturer protocol and recommendations. After purification with ethanol the sequences were identified on an ABI 310 Genetic Analyser (Perkin-Elmer Biosystems).

Effector and inhibitor treatments

Sterile solutions of methyl jasmonate (MeJA), verapamil, diphenylene iodonium (DPI) and cantharidin were added to 5-day *E. sericeum root* cultures aseptically in desired concentrations as described (Bulgakov et al. 2002, 2003). The cultures were further grown for 25 days. The chemicals were obtained from Sigma and ICN Pharmaceuticals.

Analysis of CAM and shikonin derivatives

Rabdosiin and RA were isolated from the callus and root cultures and analyzed by ^{1}H, ^{13}C NMR, FAB-MS, UV, IR and CD methods (Fedoreyev et al., in press). Quantitative high performance liquid chromatography (HPLC) determinations of rabdosiin and RA were performed using caffeic acid as an internal standard (Fedoreyev et al., in press). Shikonin derivatives were assayed by HPLC and ^{1}H NMR analysis (Bulgakov et al. 2001).

Results

Genetic transformation and analysis

The transformation experiments with *E. sericeum* plants and seedlings, carried out several times in 2001–2002, have shown that *E. sericeum* represents an example of a "difficult-to-transform" plant. The transgenic cultures were obtained only from seedlings. In our laboratory, the transformation of numerous plant species, based on such a convenient and powerful transforming system as GV3101/pMP90RK, has usually proceeded during a single experiment, and besides *Eritrichium*, only *Taxus* species have been regarded as recalcitrant to transformation (data not shown). Legumes are another example of plants that are difficult to transform (Somers et al. 2003). As all of these plants contain a large quantity of antimicrobial polyphenols, it is possible that this is a reason for the transformation problems.

Eritrichium sericeum seedlings were transformed as described in Materials and methods. Two root lines, transformed with *A. tumefaciens* GV3101/pMP90RK-pPCV002 and *A. tumefaciens* GV3101/pMP90RK-pPCV002-CaMVC constructs and designated as Es-vector and Es-rolC, respectively, were chosen for further analysis. These root cultures show an unusual dark-brown (Es-vector) or brown (Es-rolC) pigmentation (Fig. 1a, b). Phenotypically, the Es-rolC roots do not express characteristic hairy root traits, such as rapid growth, increased lateral branching and plagiotropic growth; they rather resemble normal *E. sericeum* roots (Fedoreyev et al., in press) or Es-vector roots, although they grow slightly faster than the latter (Table 1). The red *L. erythrorhizon* Le culture differed from the yellow Le-rolC culture owing to shikonin pigmentation (Fig. 1c, d).

Plant DNA samples were isolated from the cultures, which had been cultured for a long time (over 1.5 years). Gene-specific PCR analysis indicated that DNA from the Es-rolC roots contained the *rolC* and *nptII* gene sequences (Fig. 2a, b); DNA of the Es-vector culture contained the *nptII* gene sequence (Fig. 2b), thus confirming transformation. The RT-PCR analysis showed that the *rolC* gene was expressed in the Es-rolC root culture (Fig. 2c). We sequenced the 400-bp cDNA fragment of the *rolC* gene that was obtained after the reverse transcription reaction. Our sequence was identical to this fragment in the *A. rhizogenes* pRi_{A4}-*rolC* gene (GenBank accession no. K03313). The presence of the *rolC* gene sequences in the Le-rolC culture is also shown (Fig. 2d).

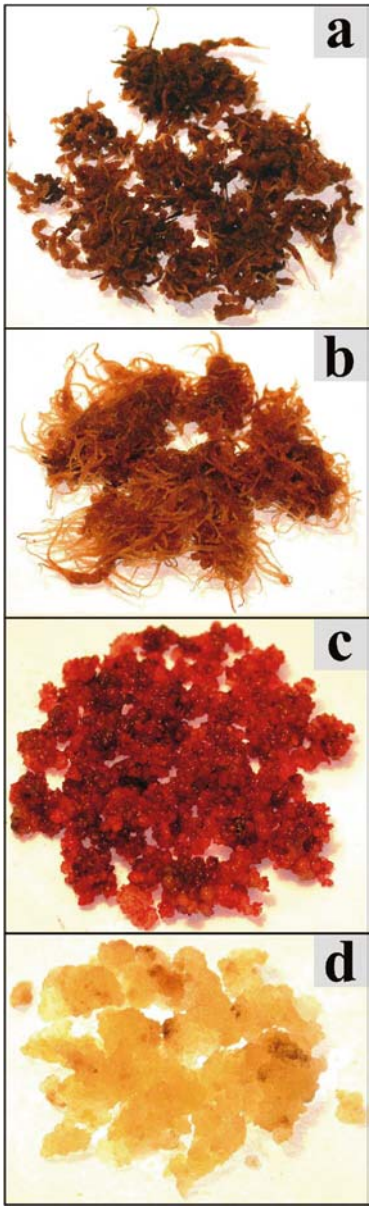

Fig. 1 Phenotypic changes in *Eritrichium sericeum* root and *Lithospermum erythrorhizon* callus cultures caused by the *rolC* gene transformation. Less colored Es-rolC root culture (**b**) compared with the control Es-vector root culture (**a**); absence of red shikonin pigmentation in the Le-rolC callus culture (**d**) compared with the red-colored Le callus culture (**c**). Cultures were grown for 4 weeks in the liquid W_{IBA} medium (*E. sericeum*) or on the solid $W_{K/IAA}$ medium (*L. erythrorhizon*)

Production of CAM

HPLC analysis of rabdosiin and RA content in Es-vector and Es-rolC callus cultures showed threefold reduced levels of these substances in the *rolC*-transformed calluses compared to that in the vector culture (Table 1). Because *E. sericeum* calluses produced less as well as less stable levels CAM than roots, our studies were focused further on the transformed roots. In the control *E. sericeum* roots (Es-vector), the HPLC determinations yielded rabdosiin and RA values 1.66% and 4.56% dry weight (DW), respectively (Table 1). The Es-rolC root culture produced twofold less rabdosiin and 2.3-fold less RA than the Es-vector root culture (Table 1). Rabdosiin production, although clearly reduced, was more or less constant in the Es-rolC root culture within subcultures whereas RA production varied more significantly, from 0.76% to 3.6% DW. As a rule, a strong decrease of RA content did not coincide with a corresponding reduction of rabdosiin content. A weak correlation was revealed between rabdosiin and RA accumulation in the Es-rolC culture ($r = 0.10$, correlation analysis). One possible interpretation of this fact is that the *rolC* gene, being expressed in *Eritrichium* cells, predominantly inhibits RA synthesis, thereby reducing the pool of metabolites for rabdosiin synthesis.

Analysis of CAM production in nontransformed (Le) and transformed (Le-rolC) *Lithospermum* callus cultures showed the presence of rabdosiin and RA (Table 1). In contrast to Japanese *L. erythrorhizon* cell cultures (Yamamoto et al. 2000a, b), our cultures did not produce lithospermic acid B. Rabdosiin in the Le and Le-rolC callus cultures was produced exclusively as (+)-enantiomer but in both *Eritrichium* cultures as (−)-enantiomer. Transformed by the *rolC* gene *L. erythrorhizon* calluses showed the same phenomenon as *Eritrichium* cultures, namely, significantly decreased CAM content (Table 1). Fresh biomass accumulation was increased in *rolC*-transgenic calluses of both Boraginaceae plants (Table 1). Interestingly, the hairy root cultures of *L. erythrorhizon*, established by Yamamoto et al. (2000a) by transformation with wild-type *A. rhizogenes* also contained significantly reduced RA levels, in comparison with nontransformed cells.

The Le-rolC culture was unable to produce shikonin derivatives as well. Shikonin molecule is derived from shikimate and mevalonate biosynthetic pathways and availability of shikimate precursors is most critical for shikonin biosynthesis (Bulgakov et al. 2001). We tried to initiate shikonin biosynthesis in this culture by different treatments, which were effective for shikonin-producing normal calluses, i.e., by addition of Cu^{2+} and reduction of ammonium content in the culture medium (Bulgakov et al. 2001). Pink colored aggregates were observed within cell clusters for 2 weeks of cultivation of the treated calluses, and at this time the cells produced small amounts of shikonin derivatives, up to 0.03% DW. Further, during the late exponential and then stationary phases of growth, the cells lost the ability to synthesize shikonin and grew vigorously as uncolored aggregates. Presumably, shikonin would eventually cease to be synthesized when a certain level of the transgene expression was attained. At the same conditions, the control Le culture synthesized large amounts of shikonin derivatives, up to 6% DW.

In summary, the *rolC* gene integration in cells of two Boraginaceae plant species ultimately results in repression of shikimate-derived secondary metabolism.

Table 1 Growth and biosynthetic activity of *Eritrichium sericeum* and *Lithospermum erythrorhizon* root and callus cultures

Cell culture	Fresh biomass (g l^{-1})	Dry biomass (g l^{-1})	Percentage of dry biomass	Rabdosiin (% DW)	Rosmarinic acid (% DW)	Total CAM (% DW)	CAM production (mg l^{-1})
E. sericeum							
Es-vector calli	190 ± 30	9.7 ± 0.8	5.1	0.90 ± 0.35	3.32 ± 1.30	4.22 ± 1.22	409
Es-rolC calli	316 ± 43*	12.9 ± 1.2	4.1	0.59 ± 0.32	0.81 ± 0.48*	1.40 ± 0.40*	181
Es-vector roots	169 ± 19	12.0 ± 0.8	7.1	1.66 ± 0.10	4.56 ± 0.50	6.22 ± 0.43	746
Es-rolC roots	211 ± 17	14.6 ± 0.9	6.9	0.84 ± 0.07*	2.00 ± 0.30*	2.84 ± 0.25*	415
L. erythrorhizon							
Le callus	290 ± 30	18.9 ± 1.3	6.5	0.82 ± 0.08	1.10 ± 0.15	1.92 ± 0.12	363
Le-rolC callus	488 ± 23*	15.5 ± 0.9	3.2	0.27 ± 0.05*	0.64 ± 0.27*	0.91 ± 0.32*	141

*$P < 0.05$ versus values of the control (Es-vector and Le) cultures, Student's *t*-test. Mean values ± SE based on ten determinations obtained during 2-year cultivation of the cultures

CAM production in the Es-vector and Es-rolC root cultures is similarly regulated by MeJA

Several reports indicate that MeJA strongly induces activity of enzymes involved in the synthesis of RA (Mizukami et al. 1993; Szabo et al. 1999), thereby increasing production of this substance by plant cell cultures. Therefore, it was not surprising that MeJA substantially increased RA and rabdosiin production in our cultures (Table 2). Both cultures were highly responsive to MeJA treatment: the concentrations of the effector higher than 5 μM (10 μM and 100 μM) strongly inhibited growth and CAM production. MeJA completely compensated the inhibitory effect of the *rolC* gene on CAM production, although the CAM content in the MeJA-treated *rolC* roots was less than that of the MeJA-treated control roots (Table 2). It is likely that the *rolC* gene inhibits CAM production irrespective of the MeJA pathway. This result provides additional evidence supporting the observation that the *rolC* gene affects secondary metabolism independently of the MeJA-mediated signaling pathway (Bulgakov et al. 2004).

It is worth noting that rabdosiin content was determined in the Es-vector culture as high as 3.41% DW (2.78 mg/g FW). To our knowledge, this is the highest level of this substance for plant cell cultures as well as natural sources reported so far.

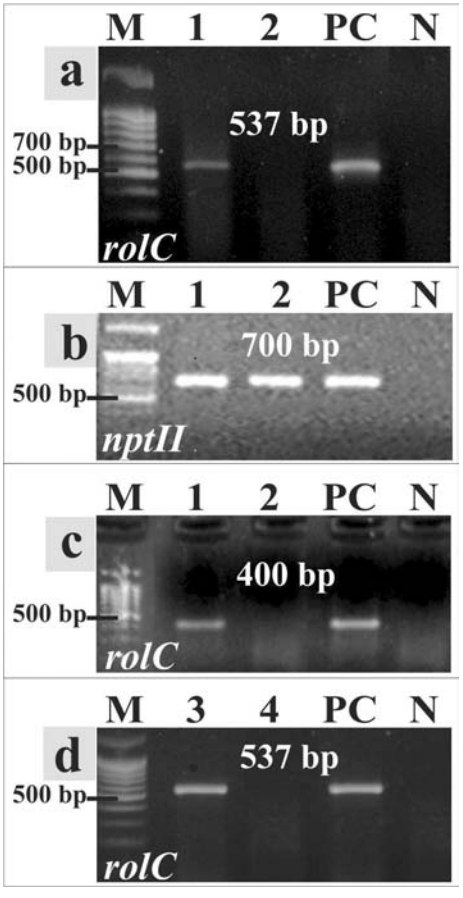

Fig. 2 Molecular analysis of *E. sericeum* and *L. erythrorhizon* cultures. PCR analysis of the *rolC* (**a**) and *nptII* (**b**) genes in DNA samples of *E. sericeum* transformed roots: *M* synthetic marker, *1* Es-rolC culture, *2* Es-vector culture, *PC* positive control (pPCV002-*rolABC*), *N* negative control (DNA of nontransformed *E. sericeum* calluses). **c** RT-PCR analysis of the *rolC* gene in the Es-rolC root culture (for designations see above). **d** PCR analysis of the *L. erythrorhizon* Le-rolC callus culture: *3* Le-rolC culture, *4* nontransformed Le culture, *PC* positive control (pPCV002-*rolABC*), *N* negative control (PCR mixture without plant DNA)

CAM production in the Es-vector and Es-rolC root cultures is similarly regulated by the Ca^{2+}-dependent NADPH oxidase pathway

It is now generally accepted that after the perception of elicitors by receptors the signal is further amplified via

Table 2 Rabdosiin and RA content (% DW) in MeJA-treated Es-vector and Es-rolC roots

MeJA conc. (μM)	Fresh biomass (g)	Rabdosiin	Rosmarinic acid	Total
Es-vector				
0	7.38 ± 1.12	2.06 ± 0.40	5.53 ± 0.27	7.59
0.5	7.33 ± 0.28	2.63 ± 0.47	7.31 ± 0.52	9.94
1.0	7.24 ± 0.19	3.41 ± 0.32	6.92 ± 0.42	10.33
5.0	5.39 ± 0.78	3.05 ± 0.27	4.40 ± 0.32	7.45
Es-rolC				
0	10.44 ± 0.18	0.95 ± 0.11	3.62 ± 0.02	4.57
0.5	9.41 ± 1.60	1.23 ± 0.14	4.71 ± 0.19	5.94
1.0	9.00 ± 1.11	1.30 ± 0.18	5.39 ± 0.20	6.69
5.0	7.61 ± 0.72	2.27 ± 0.29	5.73 ± 0.42	8.00

Values are presented as mean ± SE obtained in two separate experiments with three replicates each

Table 3 Production of CAM (rabdosiin and RA, % DW) by *E. sericeum* roots in the presence of effector (Ca^{2+}) and inhibitors (verapamil and DPI) of the calcium-dependent NADPH oxidase pathway

Treatment	Es-vector	Es-rolC
Ca^{2+}		
0	1.87	0.75
3.0 mM	2.79	1.13
Verapamil		
0	5.38	2.34
0.1 mM	5.15	1.86
0.5 mM	3.94	1.56
DPI		
0	6.12	2.44
10 μM	2.00	0.79

In the experiments with verapamil and DPI, the cultures were cultivated in the medium containing standard 3.0 mM Ca^{2+} concentrations. Mean values based on five replicate samples obtained in a single experiment

increased intracellular Ca^{2+} due to the opening of Ca^{2+} channels and the activation of NADPH oxidase (Xing et al. 1997) that cause oxidative burst and activation of synthesis of phytoalexin-type secondary metabolites. To compare the responses of the vector and *rolC*-transformed cultures on effectors of the pathway, we cultivated *E. sericeum* roots in the presence or absence of the exogenously added calcium, in the presence of the calcium channel blocker verapamil and NADPH oxidase inhibitor DPI. Table 3 presents results showing that all of these treatments similarly affected CAM production in both cultures. As the Ca^{2+} depletion, inhibition of calcium influx and inhibition of NADPH oxidase result in reduction of CAM content, we concluded that the Ca^{2+}-dependent NADPH oxidase pathway is involved in regulation of CAM synthesis in the *E. sericeum* cultures.

Effect of cantharidin

It has been shown previously that in *R. cordifolia* cells transformed with the *rolC* gene a treatment of the cultures by cantharidin (an inhibitor of Ser/Thr protein phosphatases) resulted in a massive induction of anthraquinone accumulation, whereas the control culture did not respond to this treatment (Bulgakov et al. 2002). Cantharidin showed a remarkably high selectivity in the process of anthraquinone synthesis in *rolC*-transgenic *R. cordifolia* calluses, whereas okadaic acid, another phosphatase inhibitor, possessed less specificity. The existence of a particular set of Ser/Thr phosphatases that regulate synthesis of anthraquinones in the *rolC*-transgenic *R. cordifolia* cells has been suggested (Bulgakov et al. 2003). Therefore, it was of interest to determine what effect cantharidin would have on CAM production in *E. sericeum* cultures. The inhibitor concentrations 1 μM and 5 μM, effective in *Rubia* cells (Bulgakov et al. 2002), and used for inhibitor studies by other authors (Shirasu et al. 1997; Romeis et al. 1999; Grant et al. 2000), caused the strong growth inhibition of the Es-vector and Es-rolC roots. Decreasing cantharidin concentration stepwise, we found an effective concentration of the inhibitor for *Eritrichium* root cultures (0.01 μM). At this dose, cantharidin inhibited CAM production in the Es-vector culture but stimulated that in the Es-rolC culture (Fig. 3). Cantharidin at 0.1 μM almost totally inhibited growth of both cultures, thereby suppressing the biosynthetic activity of the cells (Fig. 3). Lower doses of cantharidin did not affect growth and CAM production (data not shown). Thus, the *rolC*-transgenic and control cultures demonstrated the same phenomenon as found earlier for *R. cordifolia* cells, namely, the different responsiveness to the protein phosphatase inhibitor.

Discussion

Recently, the strong stimulatory effect of the *rolC* gene on synthesis of shikimate-derived anthraquinone phytoalexins in the *R. cordifolia* transgenic callus cultures has been shown (Bulgakov et al. 2002, 2003). Taking this result into account, as well as the data of other laboratories describing activator function of the *rolC* gene on secondary metabolism (Bonhomme et al. 2000; Palazón et al. 1998a, b), it was surprising to find that the gene inhibited CAM production in the studied Boraginaceae callus and root cultures. Why does such controversial activity of the *rolC* gene occur? As stated for many plant-microbe systems, plants responded on pathogen's invasion by synthesis of defense metabolites via the calcium-dependent NADPH oxidase pathway (Xing et al. 1997; Romeis et al. 2000). The mode of CAM activation in *Eritrichium* cultures fits this scheme, because calcium depletion, blocking of calcium channels by verapamil and inhibition of NADPH oxidase by DPI ultimately results in the decrease of CAM production (Table 3). An opposite situation was observed in

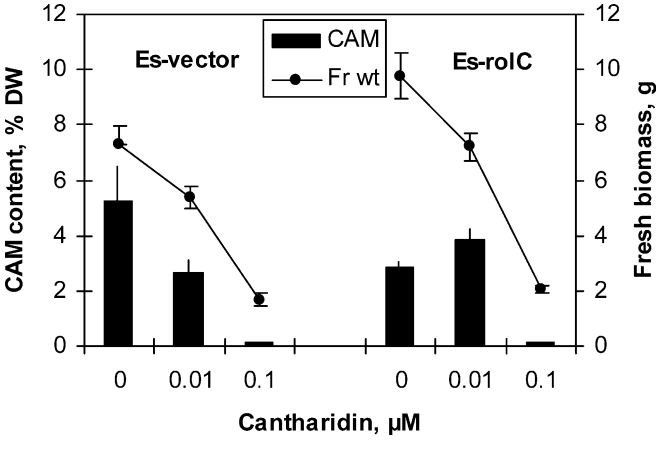

Fig. 3 Effect of cantharidin on growth and CAM production in *E. sericeum* root cultures. Values are presented as mean ± SE obtained in three separate experiments with three replicates each

R. cordifolia cells, where these treatments were shown to be ineffective (Bulgakov et al. 2003). Thus, although caffeic acid metabolites are derived from the shikimate pathway, they are regulated in plant cells in an essentially different way than anthraquinones. Therefore, a possible interpretation of the controversial activity of the *rolC* gene on secondary metabolism is that the *rolC*-mediated signal interferes with different regulatory backgrounds existing in plants. However, it is unknown which chains of regulatory pathways the gene affects.

A possible target of the *rolC* gene is protein phosphatases (Bulgakov et al. 2003). In *R. cordifolia rolC*-transgenic cultures cantharidin, an inhibitor of Ser/Thr protein phosphatases, induced anthraquinone synthesis whereas the control nontransformed culture did not respond to this treatment (Bulgakov et al. 2002). Moreover, cantharidin was the only inhibitor that had different effects on secondary metabolism in the normal and *rolC*-transgenic *R. cordifolia* cultures; other inhibitors and effectors of general signaling pathways (such as the Ca^{2+}-dependent NADPH oxidase pathway, salicylic acid-mediated and octadecanoid pathways) acted in this system in the same way. A similar result was obtained with *E. sericeum* cultures, where the dynamics of CAM accumulation in the *rolC*-culture paralleled that in the control culture (Tables 2, 3). We performed extensive studies with cantharidin to examine whether this pharmacological agent could induce changes in CAM production in the *rolC*-expressing *Eritrichium* root culture. The Es-vector and Es-rolC cultures have shown unexpectedly high responsiveness to cantharidin (Fig. 3), because the effective concentration of the inhibitor, 0.01 μM, was 1–3 orders of magnitude less than those used in plant (1–5 μM) (Shirasu et al. 1997; Romeis et al. 1999; Grant et al. 2000) and animal studies (0.1–100 μM) (Knapp et al. 1999). Such increased susceptibility to cantharidin resembles the situation with the transgenic tobacco plants lacking the alternative oxidase function, where cantharidin causes programmed cell death due to the inability of the plants to prevent chronic oxidative stress (Robson and Vanlerberghe 2002). Cantharidin is able to trigger ROS synthesis, thereby inducing plant defense responses in the absence of pathogens (Levine et al. 1994; Piedras et al. 1998; Romeis et al. 1999), although it can activate expression of defense genes independently of redox cues (Grant et al. 2000). When employed with an appropriate agonist such as salicylic acid, cantharidin dramatically increased ROS production (Shirasu et al. 1997). The *rolC*-gene-mediated signal seemingly plays the role of such an ''agonist'' in our test systems, because cantharidin activates secondary metabolism in the *rolC*-transgenic cultures while the control cultures do not respond to the inhibitor (*R. cordifolia*) or even respond conversely (*E. sericeum*, Fig. 3).

These correlations point toward a signaling sequence in which the *rolC*-gene-mediated signal could override plant regulatory controls, mediated by phosphatases. A hypothetical mechanism of the *rolC* gene action on CAM production in *Eritrichium* roots may be proposed. Protein phosphatases are thought to participate in regulation of CAM synthesis. Protein phosphatases act as positive regulators of CAM synthesis in the Es-vector culture. Cantharidin inhibits the protein phosphatase activity, thereby decreasing CAM content. The signal, initiated by the *rolC* gene product, modulates the activity of existing phosphatases, or activates another set of nonexpressing protein phosphatases under normal physiological conditions, thereby inhibiting CAM production. Cantharidin partly diminishes this inhibitory effect, thus restoring biosynthetic activity of the *rolC*-transformed *Eritrichium* root cultures.

As cantharidin might act on CAM production in *Eritrichium* root cultures by activation of ROS synthesis (a process regulated by protein phosphatases; Levine et al. 1994) or independently of ROS (Grant et al. 2000) presumably by modulation of the phosphorylated status of transcription factors (Broun 2004), it would be of interest to discriminate between these possibilities. For this, additional experiments are needed.

The physiological relevance of such perturbations caused by the *rolC* gene on secondary metabolism is not clear yet. In principle, it is not even known whether the *rolC*-gene-mediated effects have a physiological importance for bacteria or whether they are simply a side effect of the mechanism by which the oncogene perturbs normal cell growth. Recently, an interesting finding has been published to show that *A. tumefaciens*, the species closely related to *A. rhizogenes* , has developed several strategies to overcome induced plant defense mechanisms (Escobar and Dandekar 2003). It is, however, unlikely that the *rolC* gene can serve as a bacterial defender, because numerous investigations with wild-type *A. rhizogenes* transformed hairy roots have conclusively shown increased levels of secondary metabolites in transformed tissues (Sevon and Oksman-Caldentey 2002).

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