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Effect of salicylic acid, methyl jasmonate, ethephon and cantharidin on anthraquinone production by *Rubia cordifolia* callus cultures transformed with the *rolB* and *rolC* genes

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Abstract

It has been suggested that the *rol* genes of *Agrobacterium rhizogenes* could play an essential role in the activation of secondary metabolite production in plant transformed cultures. This study investigated whether the content of anthraquinone phytoalexins was changed in callus cultures of *Rubia cordifolia* transgenic for the 35S-*rolB* and 35S-*rolC* genes in comparison with a non-transformed callus culture. The anthraquinone content was shown to be significantly increased in transgenic cultures, thus providing further evidence that the *rol*-gene transformation can be used for the activation of secondary metabolism in plant cells. Methyl jasmonate and salicylic acid strongly increased anthraquinone accumulation in both transgenic and non-transgenic *R. cordifolia* calluses, whereas ethephon did not. A treatment of the cultures by cantharidin, the protein phosphatase 2A inhibitor, resulted in massive induction of anthraquinone accumulation in the transgenic cultures only. We suggest the involvement of a cantharidin-sensitive protein phosphorylation mechanism in anthraquinone biosynthesis in transgenic cultures.

Keywords: *Rubia cordifolia*; Callus culture; *rolB* and *rolC* genes; Anthraquinones

1. Introduction

The *rolB* and *rolC* genes of the central part of Ri-plasmids of *A. rhizogenes* are known as plant oncogenes and have been extensively studied during recent years with respect to plant-microbe

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interaction (Faiss et al., 1996; Nilsson and Olsson, 1997; Baumann et al., 1999). Several studies revealed an interesting ability of the *rol* genes to stimulate secondary metabolite production in transgenic plant tissues, e.g. nicotine production in *Nicotiana tabacum* root cultures, indole alkaloid production in *Catharantus roseus* cultures (Palazón et al., 1998a,b), ginsenoside production in cultures of *Panax ginseng* (Bulgakov et al., 1998) and tropane alkaloid production in *Atropa belladonna* hairy root cultures (Bonhomme et al., 2000). However, *rolB*-transformed ginseng tissues showed decreased levels of ginsenosides, compared with the non-transformed culture (Bulgakov et al., 1998). Similarly, Sevon et al. (1997) reported that alkaloid production in *Hyoscyamus muticus* plants transgenic for the *rol* genes was clearly reduced. Apparently, activation of the secondary metabolite production in plant cells cannot be considered as a general phenomenon linked to the *rol* gene function and this phenomenon needs more careful examination.

In a previous publication, we have described the establishment of callus cultures of *Rubia cordifolia* and demonstrated that the cultures produced only two major anthraquinones (AQ), with munjistin and purpurin representing 90% of the total anthraquinone yield (Mischenko et al., 1999). A simple method for determination of these pigments was developed (Mischenko et al., 1999). AQ are known to be formed via the shikimate pathway (Van Tegelen et al., 1999). To evaluate effect of the *rolB* and *rolC* genes on the shikimate metabolite pathway, we have transformed plants of *R. cordifolia* by plant vectors containing these genes and shown increased levels of AQ in most of the resulting transgenic cultures. Additionally, experiments were undertaken to determine the effect that some inductors of secondary metabolism in plants would have on AQ production.

2. Material and methods

2.1. Plant material

A plant of *R. cordifolia* L. (Rubiaceae), collected from the southern Primorsky Region of the Russian Far East and identified in the Botany Department of the Institute of Biology and Soil Science, was used to obtain the sterile plantlets. Tops of the axillary shoots were excised and placed on hormone free W_0 agarized medium (Bulgakov et al., 1998) and resulting plantlets were grown with 1-month subculture intervals.

2.2. Transformation

Plasmid vectors pPCV002-CaMVBT and pPCV002-CaMVC (Spena et al., 1987) were transferred to the *A. tumefaciens* strain GV 3101/pMP90RK as described (Bulgakov et al., 1998). These plasmids contain the plant cassette vector pPCV002 containing the *rolB* and *rolC* genes under cauliflower mosaic virus (CaMV) 35S promoter control (Spena et al., 1987). Constructions also carried a gene for kanamycin resistance (NPT-II) under eukaryotic control sequences. GV 3101-derived 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.

Leaves and stems of a 3-week-old axenic plant were cut into 5–8 mm sections with a scalpel containing a small amount of bacteria harbouring either *rolB* or *rolC* gene. Explants were placed on $W_{B/A}$ medium (supplemented with 0.5 mg l^{-1} 6-benzylaminopurine and 2.0 mg l^{-1} α-naphthaleneacetic acid, Mischenko et al., 1999), and stored for 2 days at 20 °C in the dark. After this time, the explants were transferred to a fresh $W_{B/A}$ medium supplemented with 250 mg l^{-1} cefotaxim and 100 mg l^{-1} kanamycin sulfate and cultivated at 25 °C in the dark at 30-days subculture intervals. Well-growing aggregates were observed on leaf-derived explants only and selected to produce lines of kanamycin-resistant calluses. The control non-transformed culture was established from the leaves of the same plant and cultivated in the same conditions as the transformed cultures. Calluses were cultivated in 100 ml Erlenmeyer flasks on $W_{B/A}$ medium in the dark at 25 °C with 30-day subculture intervals.

2.3. Effector and inhibitor treatments

Sterile solutions of methyl jasmonate (MeJA,

Sigma), salicylic acid (SA, Serva), ethephon (Sigma) and cantharidin (ICN Pharmaceuticals) were added to the autoclaved media aseptically in desired concentrations. Stock solution of MeJA in DMSO was prepared at a concentration of 0.1 g ml^{-1} w/v (in the control flasks equal volumes of DMSO were added). SA was dissolved in water (1 mg ml^{-1}) and titrated with 5% KOH to pH 5.6. Aqueous solutions of ethephon (1 mg ml^{-1}) were prepared and used immediately before treatments. Ethephon was used because this substance is readily converted to ethylene in the cultural media (Cho et al., 1988).

Statistical analysis was performed using the STATISTICA (Windows 98) program package.

2.4. PCR-analysis

Plasmid DNA and DNA samples from pPCV002-CaMVBT and pPCV002-CaMVC-transformed as well as non-transformed *R. cordifolia* cultures were isolated as described (Bulgakov et al., 1998). Amplification of 900 bp *rolB* gene fragment was obtained with primers 5′-TCGTCG-ACATCCAACTCACATCACAATGG-3T′ and 5′-AAGGTACCCTACAACTCCCAAGGTTCT-GTG-3′. The primer set 5′-ATGGCTGAAGAC-GACCTGTT-3′ and 5′-TTAGCCGATTGAAA-ACTTGCAC-3′ allowed the amplification of a 540 bp *rolC* gene fragment. Amplification reactions were performed in volumes of 20 μl containing 1 × reaction buffer (GOS NII Genetica, Russia), 3.5 mM $MgCl_2$, 0.2 mM of each dNTP, 25 pmol of each oligonucleotide primer, and 2.0 units of *Taq* DNA polymerase. Approximately, 100 ng of DNA was used as a template. Analysis was performed in an UNO Thermoblock thermal cycler (‘Biometra’, Germany) programmed for an initial denaturation step of 3 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 58 °C, 1 min at 72 °C and a last cycle of 1 min 30 s at 72 °C. Amplified products were detected by ultraviolet light after electrophoresis in 1.0% agarose gels stained with ethidium bromide. Size of the fragments was estimated by comparing with *Hind*III digests of Lambda phage DNA.

2.5. Estimation of the anthraquinone content

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

3. Results

Leaves of a sterile plant of *R. cordifolia* were inoculated by *Agrobacterium* strains, bearing *rolB* or *rolC* genes. Microcalli, which have been formed at the sites of inoculation, were excised and cultivated for several subcultures on the $W_{B/A}$ medium containing kanamycin. Approximately, one third of the primary kanamycin-resistant calluses transformed by both the *rolB* and the *rolC* genes spontaneously formed small roots. We selected fast-growing aggregates of transformed calluses in the presence of kanamycin and established several kanamycin-resistant callus lines. The control non-transformed Rc callus culture was obtained simultaneously from the same plant which had been used for the transformation. Gene-specific PCR analysis revealed that DNA samples of *rolB* and *rolC* gene-treated calluses contained the *rol* gene sequences (Fig. 1), thus confirming that cells were transformed. The callus cultures established after treatment of *R. cordifolia* explants by bacteria harbouring pPCV002-CaMVC (*rolC* gene) and pPCV002-CaMVBT (*rolB* gene) plasmids were designated as Rc-rolC (lines 3, 7 and 8) and Rc-rolB, respectively.

The Rc callus culture grew vigorously as friable yellow–orange tissue. All transformed cultures were deeply colored; growing as orange or orange–red tissues on the same medium as the non-transformed culture. We found that the growth of the Rc-rolC7 and Rc-rolB cultures was

reduced almost two-fold compared with the control culture (Table 1). However, these cultures possessed a stable growth and minimal variability of biomass accumulation during long-term cultivation. The control Rc culture was more watery than the transgenic cultures. Correspondingly, percentage of dry biomass of the callus cultures varied in the range of 2.8–4.2, 3.5–5.4 and 4.5–6.0 for Rc, Rc-rolC and Rc-rolB calluses, respectively.

The examination of anthraquinone levels in the transformed and non-transformed lines was initiated after six subcultures of the calluses had been grown on the media without kanamycin, and showed that up to two times more of these substances were being accumulated in the Rc-rolC3, Rc-rolC7 and Rc-rolB lines, compared with the control culture (Table 1). The experiments were carried out several times during 2-year cultivation of the cultures to ensure statistical validity of the results. Callus cultures transgenic for the *rolC* gene differed in their growth and production parameters. A purpurin/munjistin ratio was essentially changed in Rc-rolC8 culture, thereby reflecting that a modification of the anthraquinone formation occurred in this culture (Table 1). Despite on decrease of fresh biomass accumulation of the transgenic cultures, total AQ production (mg l^{-1} of the medium) was higher in these cultures compared with non-transformed control cells (Table 1). The responses of *R. cordifolia* transgenic calluses resemble the response of *Cinchona robusta* cell suspension culture treated with a fungal elicitor. The treatment of the cells resulted in cessation of growth and a rapid induction of the biosynthesis of anthraquinone-type phytoalexins (Ramos-Valdivia et al., 1997).

3.1. Effect of SA, MeJA and ethephon on anthraquinone content

The *rolC* and *rolB*-gene signals that trigger alterations of secondary metabolite biosynthesis in transgenic cultures are still unknown. A great deal of evidence has been reported to show that salicylic acid, methyl jasmonate and ethylene play a key role in co-ordination of plant defence gene expression. Since AQ represent a class of phy-

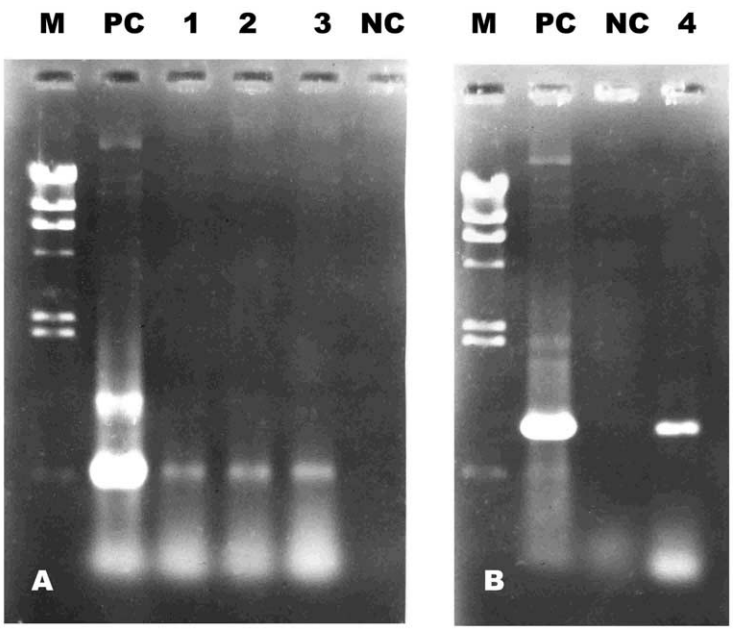

Fig. 1. Electrophoretic separation of the PCR products. M, λ/*Hind*III marker; PC, positive control; pPCV002-*rol*ABC (Spena et al., 1987); lines (1–3), Rc-rolC3, Rc-rolC7 and Rc-rolC8 cultures, respectively; line 4, Rc-rolB culture; NC, non-transformed culture.

Table 1
Biomass accumulation and anthraquinone content (% dry wt) of non-transgenic and transgenic callus cultures of *R. cordifolia*

Callus line	Fresh biomass ($g\ l^{-1}$)	Purpurin	Munjistin	Total	AQ production, ($mg\ l^{-1}$)	Purpurin/munjistin ratio
Rc (non-transformed culture)	402 ± 52	0.36 ± 0.03	0.72 ± 0.14	1.08 ± 0.16	162 ± 22	0.50 ± 0.03
Rc-rolC3	320 ± 30	0.59 ± 0.03*	1.24 ± 0.07*	1.83 ± 0.08*	293 ± 36*	0.48 ± 0.03
Rc-rolC7	224 ± 18*	0.58 ± 0.05*	1.39 ± 0.19*	1.94 ± 0.23*	217 ± 26	0.42 ± 0.04
Rc-rolC8	412 ± 88	0.55 ± 0.05*	0.76 ± 0.15	1.31 ± 0.19	214 ± 30	0.72 ± 0.06*
Rc-rolB	200 ± 12*	0.69 ± 0.08*	1.44 ± 0.20*	2.13 ± 0.25*	236 ± 18*	0.48 ± 0.14

Mean value $\pm$ S.E. The experiments were repeated eight times (with ten replicates each) during 2-year cultivation of the cultures. *, $P < 0.05$ vs. values of the non-transformed culture, Student's t-test.

toalexins involved in plant defence reactions, the question arose whether or not SA, MeJA and ethylene could interfere with a *rol*-gene-mediated pathway leading to activated anthraquinone synthesis. To address this question, we cultivated the control and transgenic cultures in the presence of SA, MeJA and ethephon.

The examination of growth and production parameters of the callus cultures growing in the presence of SA showed that SA inhibited growth of all cultures at 100 μM and stimulated anthraquinone production at all doses tested (Table 2). Subsequent increase of SA concentrations in the media strongly suppressed the growth of cultures (data not shown).

MeJA inhibited the growth of all *R. cordifolia* cultures in a dose-dependent manner (Table 2). However, both the *rolB* and *rolC* cultures were more resistant to increased levels of MeJA: these cultures were viable in the presence of 100 μM of MeJA, while growth of the control culture was almost totally inhibited under the same conditions (Table 2). The dose of MeJA, which decreases cell growth by 50% (ID_{50}), was found to be approximately 3 μM and 12 μM MeJA for non-transgenic and transgenic cultures, respectively. The treatment of MeJA also significantly affected AQ production (Table 2). MeJA at 1 μM increased AQ content in Rc and Rc-rolB cultures. When used at a concentration of 10 μM, MeJA increased the production of AQ in all cultures substantially (Table 2). The concentration of MeJA (100 μM), which stimulated the induction of AQ synthesis in transgenic cultures, failed to stimulate such a response in the control culture.

Correlation coefficients (r) between AQ content in Rc and transgenic cultures treated with SA and MeJA were calculated to evaluate similarity of their responses to the treatment. Correlation coefficients were calculated to be 0.95 and 0.97 (SA); 0.57 and 0.79 (MeJA) for Rc-rolC3 and Rc-rolB cultures, respectively.

Ethephon at concentrations which have been used in our experiments (10 and 100 μM) stimulated secondary metabolite production in different plant callus cultures (Cho et al., 1988; our unpublished observations). It was shown that higher, i.e. millimolar concentrations of ethephon, stimulated plant defence responses not only by ethylene liberation but also by phosphonic acid release (Lawton et al., 1994). Therefore, we did not used doses of ethephon higher than 100 μM in our experiment. Ethephon treatment did not significantly change growth and production parameters of the cultures (Table 2). It is very likely that ethylene is not involved in stimulation of AQ production either in the control or in transgenic cultures.

3.2. Effect of the protein phosphatase inhibitor cantharidin on anthraquinone accumulation

A significant body of evidence points to the involvement of a protein kinase/phosphatase cascade as part of the signal transduction pathway between molecular recognition of elicitors and activation of plant defence reactions (Shirasu et

al., 1997; Romeis et al., 1999; Menke et al., 1999). The fact that a specific protein phosphatase inhibitor stimulates a response means that protein phosphatase plays an important role in the stimulus–response system. Therefore, in order to gain more information about the factors involved in

Table 2
Effect of SA, MeJA and ethephon treatment on fresh biomass accumulation and AQ content of *R. cordifolia* non-transgenic and *rol*-transgenic cultures

Treatment and callus culture		Fresh biomass (g)[a]	AQ content (% DW)		
			Purpurin	Munjistin	Total (% of the control)
SA (μM)					
Rc	0	2.24 ± 0.21	0.29	0.94	1.23
	1	2.68 ± 0.26	0.34	1.03	1.37 (111)
	10	2.73 ± 0.11	0.39	1.45	1.84 (150)
	100	1.00 ± 0.10*	0.50	2.38	2.88 (234)
Rc-rolC3	0	1.13 ± 0.08	0.54	1.26	1.80
	1	1.08 ± 0.09	0.72	1.38	2.10 (117)
	10	1.15 ± 0.10	0.66	1.36	2.02 (112)
	100	0.51 ± 0.08*	0.78	2.32	3.10 (172)
Rc-rolB	0	0.80 ± 0.03	0.42	1.60	2.02
	1	0.87 ± 0.04	0.47	1.72	2.19 (108)
	10	0.63 ± 0.06	0.55	2.17	2.72 (135)
	100	0.23 ± 0.02*	0.63	2.55	3.17 (157)
MeJA (μM)					
Rc	0	1.41 ± 0.17	0.26	0.53	0.79
	1	0.92 ± 0.05*	0.39	1.09	1.48 (187)
	10	0.35 ± 0.05*	0.43	1.28	1.71 (216)
	100	0.13 ± 0.02*	0.16	0.53	0.69 (87)
Rc-rolC3	0	0.73 ± 0.07	0.63	1.66	2.29
	1	0.65 ± 0.07	0.68	1.62	2.30 (100)
	10	0.42 ± 0.04*	1.09	2.54	3.63 (159)
	100	0.27 ± 0.02*	0.91	1.77	2.68 (117)
Rc-rolB	0	0.74 ± 0.06	0.68	1.82	2.50
	1	0.53 ± 0.04	1.04	2.80	3.84 (154)
	10	0.45 ± 0.05*	1.38	2.95	4.33 (173)
	100	0.28 ± 0.02*	1.20	2.32	3.51 (140)
Ethephon (μM)					
Rc	0	1.66 ± 0.08	0.25	0.50	0.75
	1	1.89 ± 0.11	0.22	0.50	0.75 (100)
	10	2.01 ± 0.19	0.24	0.53	0.77 (103)
	100	1.49 ± 0.17	0.23	0.50	0.74 (100)
Rc-rolC3	0	0.98 ± 0.04	0.36	1.07	1.42
	1	1.00 ± 0.13	0.42	1.09	1.51 (106)
	10	1.00 ± 0.09	0.39	1.02	1.41 (100)
	100	0.92 ± 0.04	0.39	1.09	1.48 (104)
Rc-rolB	0	0.75 ± 0.09	0.70	1.84	2.54
	1	0.71 ± 0.03	0.60	1.85	2.45 (96)
	10	0.85 ± 0.08	0.59	1.49	2.08 (81)
	100	0.73 ± 0.03	0.73	2.12	2.85 (112)

*, $P<0.05$ vs. control culture, Student's t-test. For anthraquinone determination, the contents of the ten replicate flasks were sampled and the average value reported. Deviations of any of the flasks from the average value were less than 10%.

[a] Mean value ± S.E. based on ten replicate samples obtained in a single experiment.

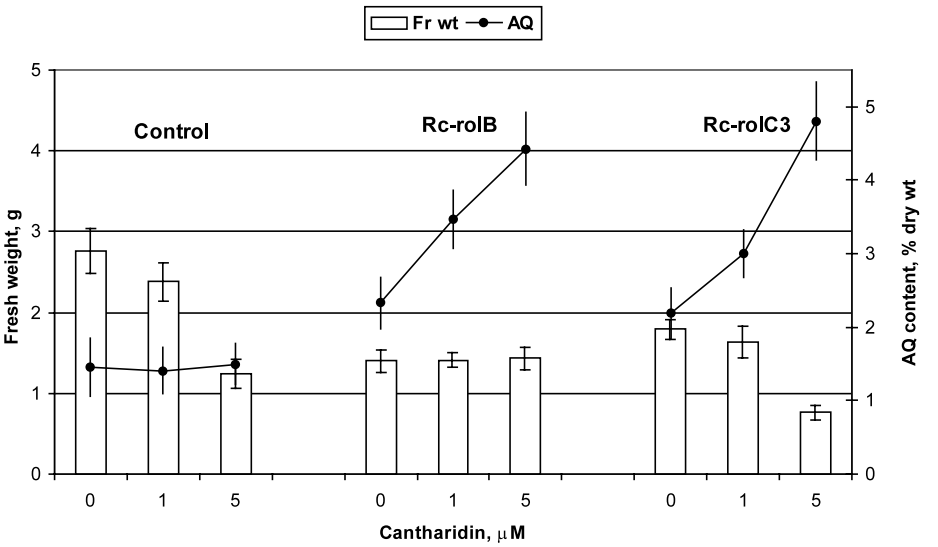

Fig. 2. Effect of cantharidin on fresh biomass accumulation and AQ content in *R. cordifolia* callus cultures. Values are means from two experiments with ten replicates each. Vertical bars represent standard errors.

the *rol* gene-mediated signal transduction pathway leading to induction of AQ accumulation, we have investigated the effect of cantharidin, an inhibitor of protein phosphatase 2A (Li and Casida, 1992).

The control and transgenic callus culture were grown on media containing cantharidin and analysed for AQ content. The non-transformed calluses were unresponsive to cantharidin treatment regarding AQ content. In contrast, significant and dose-dependent activation of AQ accumulation was registered in both transgenic cultures (Fig. 2). Cantharidin in a concentration of 5 μM inhibited growth of the control and *rolC* cultures. Interestingly, growth of the *rolB* culture was unchanged by the inhibitor treatment. Simultaneously added cantharidin and MeJA substantially reduced the growth of the non-transformed and Rc-rolC3 cultures and provoked a strong necrotic response. The Rc-rolB culture in the presence of the above-mentioned compounds showed a delay of growth without necrotic response. When cantharidin and MeJA were employed simultaneously, substantial increase of AQ content was detected in the transgenic cultures only (Fig. 3).

4. Discussion

Previous studies performed with *rolB* and *rolC* gene transformed plant cultures, although limited to particular plant species, established that the activation of secondary metabolism occurred in most but not all cultures. With this consideration in the mind, we started our work with *R. cordifolia* transgenic cultures. Studies were conducted over 2 years and showed that the *rolB* and *rolC* transgenic cultures contained about two times higher levels of AQ then the respective control. Thus, the stimulatory effect of transformation of plant cells by *rol* genes on secondary metabolite production was confirmed, at least for the shikimate-derived anthraquinone phytoalexins. The maximal yield of purpurin and munjistin of the transgenic cultures was as high as 4.8% dry wt; much more than the values reported for *R. cordifolia* wild-growing roots (0.2% dry wt.) and non-transformed callus cultures (0.62–1.22% dry wt.) (Mischenko et al., 1999). It is significant that neither of these pigments possess mutagenic activity (Mischenko et al., 1999).

We performed experiments with effectors (jasmonate, SA, ethephon) that mediate signal trans-

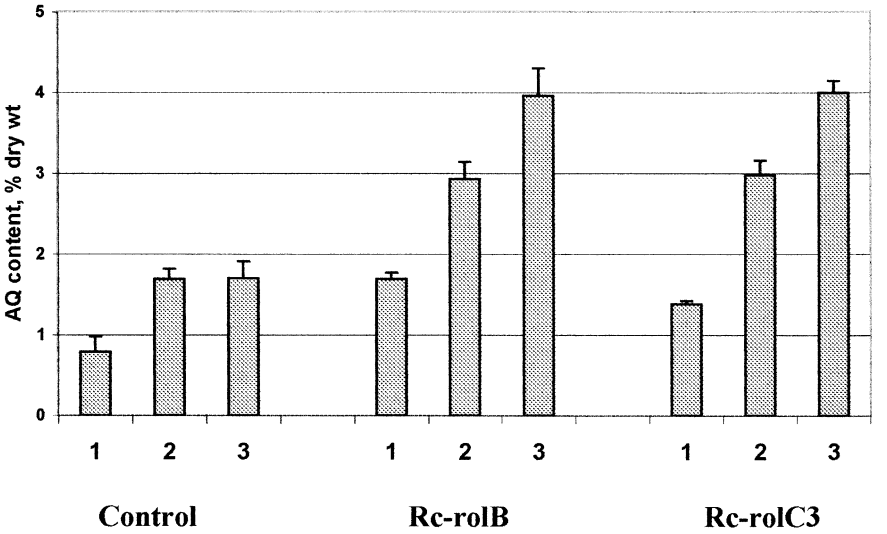

Fig. 3. AQ content in the cultures without treatment (1) and treated with MeJA, 10 μM (2), or MeJA 10 μM + cantharidin 5 μM (3). Values are means from two experiments with six replicates each. Vertical bars represent S.E.

duction between an elicitor–receptor complex via transcriptional activation of defense genes (Hammond-Kosack and Jones, 1996; Menke et al., 1999). Since MeJA and SA stimulate AQ accumulation in *R. cordifolia* cultures (Table 2), we assume the involvement of jasmonate and SA-mediated pathways in biosynthesis of AQ. The role of ethylene seems to be less prominent.

High correlation coefficients between values of AQ content in Rc and transgenic cultures growing in the presence of SA and MeJA mean that these effectors activate AQ synthesis similarly both in non-transgenic and transgenic cultures.

Additional information was obtained from inhibitor experiments. Concentrations of cantharidin (1 and 5 μM) that stimulate the induction of AQ *in R. cordifolia* transgenic cells failed to stimulate any response in the non-transformed culture (Fig. 2). Additionally, in experiments with methyl jasmonate and cantharidin, it was confirmed that cantharidin had the potential to activate the accumulation of AQ in the transgenic cultures (Fig. 3). AQ synthesis in the transgenic cultures appears to be regulated by a balance between phosphorylation and dephosphorylation of one or more components of the signal pathway. Cantharidin acting synergistically with an appropriate agonist, such as MeJA, thus switches this regulatory balance to pathway activation.

The AQ content was changed similarly upon different treatments both in the *rolB* and *rolC* cultures (Table 2; Figs. 2 and 3). However, a difference in growth response of cultures treated with cantharidin was repeatedly detected. Growth of the *rolB*-transformed culture was unchanged upon cantharidin treatment whereas the inhibitor strongly suppressed growth of the control and *rolC* cultures (Fig. 2). These data, together with previously reported information that the *rolB* protein possesses tyrosine phosphatase activity (Philippini et al., 1996), point toward involvement of the *rolB* gene product in a signalling sequence in which phosphorylation processes play an essential role.

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