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The *rolC* gene increases caffeoylquinic acid production in transformed artichoke cells

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Abstract Caffeoylquinic acids are found in artichokes, and they are currently considered important therapeutic or preventive agents for treating Alzheimer's disease and diabetes. We transformed artichoke [the cultivated cardoon or *Cynara cardunculus* var. *altilis* DC (*Asteraceae*)] with the *rolC* gene, which is a known inducer of secondary metabolism. High-performance liquid chromatography with UV and high-resolution mass spectrometry (HPLC-UV-HRMS) revealed that the predominant metabolites synthesized in the transgenic calli were 1,5-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid, and chlorogenic acid. The *rolC*-transformed calli contained 1.5 % caffeoylquinic acids by dry weight. The overall production of these metabolites was three times higher than that of the corresponding control calli. The enhancing effect of *rolC* remained stable over long-term cultivation.

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Introduction

Cynara cardunculus L. is a diploid, mostly cross-pollinated species belonging to the *Asteraceae* family. The wild perennial taxon [var. *sylvestris* (Lamk) Fiori] has been recognized as the ancestor of both the globe artichoke [var. *sativa* Moris, var. *scolymus* (L.) Fiori, ssp. *scolymus* (L.) Hegi] and the leafy or cultivated cardoon (var. *altilis* DC) (Sonnante et al. 2007). Previous classifications designated the cultivated artichoke as a separate species, namely, *C. scolymus* L. However, the current classification includes the cultivated artichoke, leafy cardoon, and wild cardoon in a single species known as *C. cardunculus* L. (Sonnante et al. 2007).

Pharmaceutical interest in the artichoke is related to its polyphenolic compounds, such as caffeoylquinic acids (CQA). The most abundant polyphenols in the artichoke are 5-caffeoylquinic acid, also known as chlorogenic acid (CGA), and dicaffeoylquinic acids. Dicaffeoylquinic acids are present in artichokes as different isomers; the primary isomer is 1,5-dicaffeoylquinic acid, followed by the 3,4-, 3,5-, and 4,5-isomers (Moglia et al. 2008). Another widely known substance found in artichoke extracts is 1,3-dicaffeoylquinic acid (cynarin), which is a product of the isomerization of 1,5-dicaffeoylquinic acid in warm aqueous media (Slanina et al. 2001). Slanina and colleagues (2001) reported that cynarin is an artifact that can be created from 1,5-dicaffeoylquinic acid via intramolecular transesterification during the solvent extraction of plant material. However, it is likely that artichokes can synthesize this metabolite in small quantities because cynarin was recently found in globe artichoke (*C. cardunculus* ssp. *scolymus*) (Shen et al. 2010; Negro et al. 2012).

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A summarized fraction of artichoke caffeoylquinic acids can be used to treat lead toxicity, liver diseases, hypercholesterolemia, and glycemia (Heidarian and Rafieian-Kopaei 2013; Rondanelli et al. 2013, 2014). Individual caffeoylquinic acids that are isolated from different sources possess different significant activities. For example, cynarin is a potential mild immunosuppressant that can be used to treat autoimmune diseases (Dong et al. 2009). 1,5-Dicaffeoylquinic acid has been shown to protect astrocytes from cell death (Cao et al. 2010) and prevents neuron apoptosis in Alzheimer's disease (Xiao et al. 2011). 3,4-Dicaffeoylquinic possesses a novel and unique mechanism of anti-influenza viral activity (Takemura et al. 2012).

The biotechnological production of high-value metabolites by plant in vitro systems is considered to be an attractive alternative to classical technologies (Georgiev et al. 2013). An artichoke callus culture (*C. cardunculus* var. *scolymus*) was established by Trajtemberg and co-workers (2006). This culture produced a total caffeoylquinic acid level of 0.49 % (dry wt.). Further progress in the biotechnology of artichoke metabolites was likely decelerated because this plant is recalcitrant to transformation (Sonnante et al. 2010). The first successful artichoke transformation was only recently performed with an *Agrobacterium tumefaciens* Agl0 01-124 strain harboring the binary vector pCAMBIA 2301 (Menin et al. 2012).

It is known that some transfer DNA (T-DNA) oncogenes, such as the *rolB* and *rolC* genes of *A. rhizogenes* and the *6b* gene of *A. tumefaciens*, affect the biosynthesis of secondary metabolites in transformed plant cells (Gális et al. 2004; Bulgakov 2008). Evidence indicates that the *rolC* gene mediates uncommon signal transduction pathways in plants. It acts on production of secondary metabolites independently of plant defense hormones and the calcium-dependent NADPH oxidase pathway (Bulgakov 2008). Transformation with the *rolC* gene is especially useful in those cases where different methods commonly used to increase secondary metabolite production (cell selection, elicitor treatments, and addition of a biosynthetic precursor) only slightly enhance cell productivity. The *rolC* gene is capable of stimulating the production of 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 (Shkryl et al. 2008) in transformed plants and plant cell cultures. The production of polyphenols by *rolC*-transformed cells is less studied in comparison with alkaloids and anthraquinones. The *rolC* gene initially inhibited the biosynthesis of caffeic acid metabolites, such as rabdosiin and rosmarinic acid in *Eritrichium sericeum* and *Lithospermum erythrorhizon* root and callus cultures (Bulgakov et al. 2005). However, after several years of cultivation, a stimulated biosynthesis of these metabolites was observed because of a progressive transcriptional activation of *CYP98A* genes encoding cytochrome P450-containing monooxygenases (Inyushkina et al. 2009). The content of polyphenols such as isoflavones and pterocarpans was not significantly affected by *rolC* in transformed *Maackia amurensis* calli, but their total production was enhanced because the gene stimulated the callus growth (Grischenko et al. 2013).

Therefore, it was interesting to determine whether *rolC* would stimulate production of other groups of polyphenols. In this study, we showed that *rolC* stimulated the production of both chlorogenic acid and dicaffeoylquinic acids in transformed artichoke callus cultures.

Materials and methods

Agrobacterium strains

For the genetic transformation, we used *A. tumefaciens* strain GV3101/pMP90RK (Koncz and Schell 1986), which is available at http://www.dna-cloning.com. This plant transformation vector contained the empty plasmid pPCV002 or pPCV002-CaMVC with the *rolC* gene under 35S promoter control (Spena et al. 1987). These constructs were provided by Angelo Spena (Max-Planck-Institut für Züchtungsforschung, Köln, Germany).

Plant material and callus cultures

Samples of cultivated cardoon [*C. cardunculus* var. *altilis* DC (*Asteraceae*)] were collected from the experimental field station at the Tay Nguyen Institute of Scientific Research (Dalat, Vietnam). Transformed artichoke callus cultures were established from the young apical shoots of cultivated plants as previously described (Bulgakov et al. 2010). A callus line transformed by the empty vector plasmid pPCV002 was designated Cyn-V and used as a control. A callus line that was transformed with the pPCV002-CaMVC plasmid was designated Cyn-C (the *rolC*-transformed culture). The control and *rolC* transgenic calli were cultivated in 100-ml Erlenmeyer flasks containing 40 ml of $W_{B/A}$ medium (Bulgakov et al. 2010) supplemented with the following components (mg/l): thiamine HCl (0.2), nicotinic acid (0.5), pyridoxine HCl (0.5), mesoinositol (100), peptone (100), sucrose (25,000), agar (6,000), 6-benzyladenine (BA, 0.5), and α-naphthaleneacetic acid (NAA, 2.0). Calli were grown at 25 °C in the dark at 30-day subculture intervals. Their transformation was confirmed by PCR with specific primers for the *npt-II* and *rolC* genes, as previously described (Bulgakov et al. 2010). Additionally, a non-transformed callus culture was established from the same explants. This culture possessed essentially the same properties as the vector-transformed culture with respect to the morphology, growth, and biosynthetic parameters. All obtained callus cultures are maintained by subculturing in the

Collection of Plant Cell Cultures at the Institute of Biology and Soil Science (Vladivostok, Russia).

Additionally, we used the high-*rolC*-expressing (RCH) callus culture of *Rubia cordifolia* (Shkryl et al. 2008) for the comparative investigation of *rolC* expression in transgenic artichoke calli. The RCH culture was cultivated under the same conditions as the artichoke calli.

Chemicals

Reagents for the plant tissue culture and all analytical standards (chlorogenic acid, 1,3-dicaffeoylquinic acid and 1,5-dicaffeoylquinic acid) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All solvents were of high-performance liquid chromatography (HPLC) grade.

RNA isolation, cDNA synthesis, and quantitative real-time PCR

Total RNA isolation and first-strand complementary DNA (cDNA) synthesis were performed as previously described (Bulgakov et al. 2010). The RNA concentrations and 28S/18S ratios were determined by using an RNA StdSens LabChip kit and an Experion Automated Electrophoresis Station (Bio-Rad Laboratories, Hercules, CA) with the Experion Software System Operation and Data Analysis Tools (version 3.0) by following the manufacturer's protocol and recommendations. Quantitative real-time PCR (qPCR) analysis was performed with a Bio-Rad CFX96 Real-Time System (Bio-Rad Laboratories) with a 2.5× SYBR green PCR master mix containing ROX as a passive reference dye. The qPCR reactions were performed under previously described conditions (Bulgakov et al. 2010). Two replicates from two independent RNA extractions were used for the analysis.

The *rolC* gene expression in *C. cardunculus* (Cyn-C) and *R. cordifolia* (RCH) *rolC*-transformed calli was investigated by qRT-PCR using RNA samples isolated from callus cultures during the linear growth phase (20–22 days). The primers 5′ CTCCTCACCAACCTTCCCCC and 5′GCTTGATGACAC GCCCAG were used for the analysis of *rolC* expression. A primer efficiency of >95 % was confirmed with a standard curve spanning 7 orders of magnitude. The data were analyzed with CFX Manager Software (version 1.5) (Bio-Rad Laboratories). Two RNA samples were analyzed with two analytical repetitions.

RT-PCR analysis

A reverse transcription (RT)-PCR analysis of the *rolC* gene (GenBank accession no. X03433.1) was performed as previously described (Shkryl et al. 2008). The total RNA was isolated from the callus cultures by using a LiCl method to avoid contaminating the sample with polyphenols. A solution containing 2.5 μg of mRNA and 2.5 μM oligo $d(T)_{16}$ (Syntol, Moscow, Russia) was preheated (5 min at 72 °C) and cooled on ice. Reverse transcription was performed in a 50-μl volume containing Moloney murine leukemia virus (M-MLV) buffer, 0.2 mM of each dNTP, and M-MLV reverse transcriptase. The primers 5′CTCCTCACCAACCTTCCCCC and 5′GCTTGA TGACACGCCCAG were used for amplification of a 151 bp *rolC* transcript. The RNA expression profiles were normalized by using the expression of the *C. cardunculus* var. *altilis 18S* rRNA gene (GenBank accession no. AM269939.1; the primer set: 5′CGCCCCAGACCACGACTCCC and 5′CTTTTTAG GCCAACCGCACC). The identities of the RT-PCR products were confirmed by DNA sequence analysis. The RT-PCR products were then subcloned into a pTZ57R/T plasmid by using an InsT/Aclone PCR Product Cloning Kit (Fermentas, Vilnius, Lithuania) and sequenced with a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) by following the manufacturer's protocol and recommendations. After an ethanol purification step, the sequences were identified with an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, USA).

Sample preparation for analytical chromatography

The oven-dried (at 50 °C to a constant weight) and powdered callus samples (200 mg) were extracted twice with 3 ml of 70 %*v*/*v* ethanol for 2 h at 40 °C. The extracts were combined and cleared with a 0.45-μm membrane (Millipore, Bedford, MA, USA), and 2-μl aliquots were then used for HPLC analysis.

Analytical chromatography

Analytical high-performance liquid chromatography with UV and high-resolution mass spectrometry (HPLC-UV-HRMS) was performed on the callus extracts by using a Shimadzu LCMS-IT-TOF (Shimadzu EUROPA GmbH, Duisburg, Germany) system equipped with an LC-20A Prominence chromatograph, an SPD-M20A photodiode array detector, and an ion-trap/time-of-flight mass spectrometer. The extracts were analyzed by using an Ascentis C18 column (100 mm, 2.1-mm i.d., 3-μm part size, Supelco, Bellefonte, Pennsylvania, USA) at 40 °C. The mobile phase consisted of 0.1 % aqueous acetic acid (A) and acetonitrile (B). The following elution gradient was used, with a flow rate of 0.2 ml/min: eluent A–eluent B for 35 min and then eluent B for 50 min.

The mass spectral (MS) parameters included electrospray ionization (ESI) and negative ion detection with a resolution of 12,000. The following settings were used: the range of detection was 200–1,000*m*/*z*, the drying gas (N_2) pressure was 195 kPa, the nebulizer gas flow rate was 1.5 l/min, the ion source potential was 4.5 kV, and the interface temperature

was 200 °C. All MS^2 experiments were run by adjusting the collision energy to the point where the precursor ion had an abundance of 50 % relative to the precollision intensity. The concentration of the collision gas (Ar) was 50 %. The range of detection for the precursor ion fragments was 100–550 *m*/*z*. UV-Vis spectra were recorded in the 190–800-nm range, and chromatograms were acquired at 321, 325, and 328 nm.

Polyphenols were identified on the basis of their retention times and MS and UV spectral data, with reference to the values of their respective standards and data from the literature (Clifford et al. 2005; Chen et al. 2013; Shen et al. 2010). The polyphenol compounds were quantified by HPLC-UV at the wavelength of the maximum UV-Vis absorbance by using four-point regression curves that were built with the standards. The wavelengths were set at 321 nm for cynarin, 325 nm for monocaffeoylquinic acids, and 328 nm for dicaffeoylquinic acids.

Results

The *rolC*-expressing artichoke callus culture

Primary vector-transformed (control) and *rolC*-transformed callus cultures of *C. cardunculus* var. *altilis* were obtained from the apical shoots of cultivated plants in 2010, as described in the "Materials and methods" section. After their induction (4–5 weeks), these calli were separated from the rest of the explants and placed in fresh selection medium. To select the transformed aggregates, we used a kanamycin concentration of up to 25 mg/l for the second subculture and up to 50 mg/l for the third and subsequent subcultures. Cefotaxime (250 mg/l) was applied to four subcultures to eliminate *Agrobacterium* cells. We selected the *rolC*-transformed and empty vector-transformed aggregates that were actively growing in the presence of 50 mg/l kanamycin for several months. Cell aggregates were cultivated at 4-week intervals on antibiotic-free medium for 1 year by selecting fast-growing aggregates. As a result, several stable homogenous callus lines were established. The Cyn-V (the control culture) and Cyn-C (the *rolC*-transformed culture) lines, which exhibited the highest growth, were chosen for further analysis.

Our RT-PCR analysis revealed that *rolC* expression in the Cyn-C culture is high (Fig. 1). To evaluate the relative strength of *rolC* expression, we performed a qPCR analysis by using *rolC*-transformed *R. cordifolia* callus culture as a reference. This analysis confirmed the RT-PCR data and revealed that the *rolC* expression in Cyn-V was four times higher than it was in the high-*rolC*-expressing RCH calli (0.85±0.15 and 0.20±0.03 relative fold expression, respectively). This result indicates that the *rolC* gene is actively transcribed in Cyn-V calli. The high expression of *rolC* remained stable for the 3-year cultivation period.

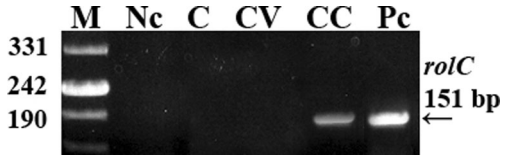

Fig. 1 An RT-PCR analysis of *rolC* expression in the Cyn-C culture. Total RNA was isolated from the 30-day callus cultures. *M* DNA marker, *Nc* negative control (PCR mixture without plant cDNA), *C* (untransformed callus line), *CV* (vector-transformed calli Cyn-V) and *CC* (*rolC*-transformed calli Cyn-C), *Pc* positive control (pPCV002-*rolABC* for the *rolC* gene)

Both callus cultures (Cyn-V and Cyn-C) are white yellow in color and are characterized by vigorous growth. The Cyn-C calli have a more compact structure in comparison with the friable Cyn-V calli. The often-mentioned growth stimulation caused by *rolC* (Bulgakov et al. 2013) was not observed in this system, likely because of the high *rolC* expression (*rolC* stimulates growth only at low doses). The growth dynamics of the Cyn-V and Cyn-C cultures are similar. Both of their growth patterns can be described by a standard sigmoid curve with an exponential growth phase (5–12 days of cultivation), a linear phase (13–23 days), and a stationary phase (24–35 days). After 35 days of cultivation, the fresh callus weights began to decline. Because the *rolC* gene is capable of triggering root formation (Spena et al. 1987), we tested whether the Cyn-C calli was able to produce transgenic roots. However, neither the Cyn-V nor the Cyn-C calli produced roots during the entire observation period.

Analysis of caffeoylquinic acids

An HPLC analysis of the Cyn-V and Cyn-C callus extracts revealed that both extracts exhibited similar chromatographic profiles (Fig. 2). Six peaks were detected in each analysis. Monocaffeoylquinic acids (1, 2) and dicaffeoylquinic acids (3, 4, 5, 6) were identified by comparing their retention times (t_R=5.7, 6.9, 8.3, 11.7, 12.0, and 12.7 min, respectively), UV absorption spectra, and ESI-MS data with standards and data from the literature (Clifford et al. 2005; Chen et al. 2013; Shen et al. 2010). The chromatographic and spectral characteristics of these metabolites are presented in Table 1 and in the Supplementary Material (Supplemental Figs. S1, S2, S3, S4, S5, and S6).

Peaks 1 and 2 corresponded to the isomers of monocaffeoylquinic acid. The value of the monoisotopic molecular mass for both of these components was found to be 354.0972 Da (calculated as 354.0951 Da), and the absorbance maximum was 325 nm. The retention time for peak 2 corresponded to that of the chlorogenic acid standard. The MS^2 pattern of this peak (Supplemental Fig. S2) also corresponded to chlorogenic acid, and the MS^2 pattern of peak 1 (Supplemental Fig. S1) corresponded to 3-caffeoylquinic acid, as described earlier (Clifford et al. 2005; Chen et al.

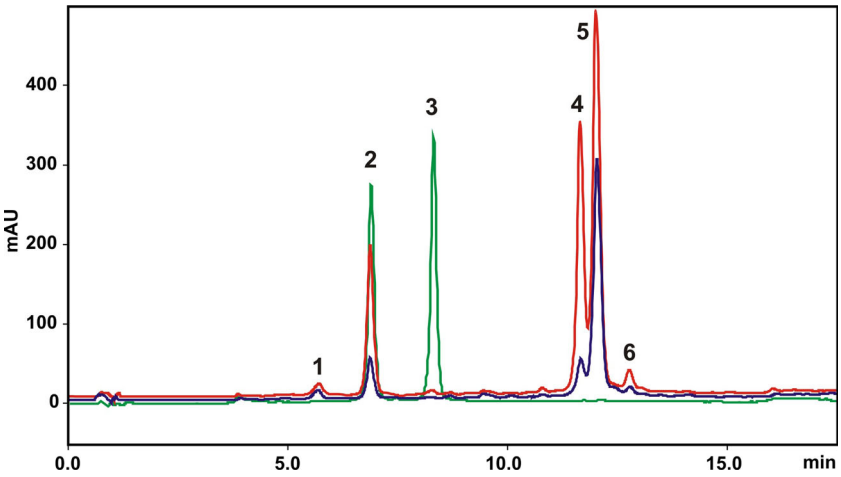

Fig. 2 A representative HPLC-UV profile for the extracts obtained from callus cultures of *C. cardunculus* (recorded at 325 nm). Cyn-V (*blue line*), Cyn-C (*red line*), and standards (*green line*). 3-caffeoylquinic acid (*1*), chlorogenic acid (*2*), cynarin (*3*), 3,4-dicaffeoylquinic acid (*4*), 1,5-dicaffeoylquinic acid (*5*), and 4,5-dicaffeoylquinic acid (*6*)

2013). Therefore, we assigned the structures of these components to the structures of 3-caffeoylquinic acid (1) and chlorogenic acid (2). Peak 3 corresponded to cynarin, which was determined by its retention time, its monoisotopic molecular mass of 516.1271 Da (calculated as 516.1268 Da) and its MS^2 and UV (λ_{max} 321 nm) data (Table 1; Supplemental Fig. S3). The ESI-MS of this component contained only two primary ions with the compositions $[M–H]^-$ and $[M–H–C_9H_6O_3]^-$ (Table 1; Supplemental Fig. S3), and it was similar to the spectrum of the cynarin standard. The values of the monoisotopic molecular masses for peaks 4, 5, and 6 were very similar (516.1281±0.0005 Da) and corresponded to the values for dicaffeoylquinic acid (calculated at 516.1268 Da). The ESI-MS results of all these compounds exhibited similar patterns and contained three primary ions each (Supplemental Figs. S4, S5, and S6). These three ions included a deprotonated quasi-molecular ion $[M–H]^-$ and ions with the compositions $[M–H–C_9H_6O_3]^-$ and $[M–H–C_9H_8O_4]^-$, which are formed by the loss of caffeoyl keten and caffeic acid fragments, respectively (Table 1). The absorbance maximum was 328 nm for all these compounds. The MS^2 patterns of these components (Table 1; Supplemental Figs. S4, S5, and S6) corresponded to 3,4-diCQA (4), 1,5-diCQA (5), and 4,5-diCQA (6), according to the data in the literature (Miketova et al. 1999; Schram et al. 2004; Clifford et al. 2005; Shen et al. 2010; Chen et al. 2013).

A quantitative HPLC analysis of the polyphenols in the artichoke callus cultures revealed 2.4-fold higher levels of total CQAs in the *rolC*-transformed calli in comparison with the vector culture (Table 2). In the vector culture, we found 0.11 and 0.54 % (dry wt.) monocaffeoylquinic acids and dicaffeoylquinic acids, respectively (Table 2). However, the monocaffeoylquinic acid level in the Cyn-C culture was 2.6-fold higher, and the dicaffeoylquinic acid level was 2.3-fold higher than the levels in the control calli. The production of these metabolites was correspondingly increased 3-fold (Table 3). These production parameters were stable over time (for 2 years of observation of callus cultures).

Discussion

The interest in the *rolC* gene stems from the well-known fact that hairy-root cultures, derived from various plants species, stably produce high amounts of secondary metabolites. The *rolC* gene is the most interesting gene for biotechnological studies because it is capable of stimulating both the growth of

Table 1 The HPLC-UV-ESI-MS characteristics of caffeoylquinic acids from *C. cardunculus* var. *altilis* callus cultures

Peak no.*	t_R (min)	λ_{max} (nm)	$[M–H]^-$ *m/z*	Ion formula	MS^2 (parent ion $[M–H]^-$) *m/z* (% base peak)	Assignment
1	5.7	325	353.0899	$C_{16}H_{17}O_9^-$	191.0593 (100), 179.0360 (43), 135.0467 (7)	3-CQA
2	6.9	325	353.0899	$C_{16}H_{17}O_9^-$	191.0595 (100), 179.0389 (3)	Chlorogenic acid
3	8.3	321	515.1198	$C_{25}H_{23}O_{12}^-$	353.0883 (100), 335.0786 (26), 191.0586 (5), 179.0358 (16)	Cynarin
4	11.7	328	515.1207	$C_{25}H_{23}O_{12}^-$	353.0885 (100), 335.0793 (15), 299.0563 (3), 203.0352 (4), 179.0355 (10), 173.0486 (12)	3,4-diCQA
5	12.0	328	515.1205	$C_{25}H_{23}O_{12}^-$	353.0887 (100), 335.0791 (3), 191.0600 (20)	1,5-diCQA
6	12.7	328	515.1212	$C_{25}H_{23}O_{12}^-$	353.0884 (100), 335.0785 (2), 317.0645 (4), 299.0544 (11), 255.0667 (6), 203.0349 (15), 179.0357 (8), 173.0476 (9)	4,5-diCQA

*The peaks are numbered as shown in Fig. 2

Table 2 Caffeoylquinic acid contents (% dry wt.) in callus cultures of *C. cardunculus* var. *altilis*

Metabolite	Cyn-V	Cyn-C
Monocaffeoylquinic acids	0.11±0.01	0.29±0.02*
3-CQA (1)	0.02	0.03
5-CQA (CGA, 2)	0.09	0.26
Dicaffeoylquinic acids	0.54±0.01	1.25±0.04*
1,3-diCQA (cynarin, 3)	tr.	0.01
3,4-diCQA (4)	0.08	0.44
1,5-diCQA (5)	0.45	0.77
4,5-diCQA (6)	0.01	0.03
Total caffeoylquinic acids	0.65±0.02	1.54±0.05*

The experiments were performed by using three biological samples from different passages with three analytical replicates each

tr: trace amounts (calculated as ~0.001 % dry wt.)

*$p<0.05$ versus values from the control (Cyn-V) culture, Student's *t* test

transformed cells and the biosynthesis of secondary metabolites (Bulgakov et al. 2013). There is information that the RolC protein activates secondary metabolism through the up-regulation of key genes of secondary metabolism (Shkryl et al. 2008), but the mechanism of activation is unknown. Transformation of plant cells with the *rolC* gene provoked a biphasic effect with an initial suppression and the subsequent activation of the production of rosmarinic acid (Bulgakov et al. 2005; Inyushkina et al. 2009). Biogenetically, artichoke polyphenols (chlorogenic acid and its related metabolites) are closely related to rosmarinic acid, since the biosynthetic pathways of rosmarinic acid and chlorogenic acid are similar and evolutionarily related (Petersen et al. 2009).

Therefore, it was unclear whether *rolC* could activate the production of caffeoylquinic acids in artichoke. Here, we presented evidence indicating the increased production of chlorogenic acid and its related metabolites in artichoke calli transformed with the *rolC* gene. The callus culture of artichoke Cyn-C grows stably and produces high amounts of caffeoylquinic acids. The levels of dicaffeoylquinic acids, the primary products of value in artichokes (Cao et al. 2010; Xiao et al. 2011; Takemura et al. 2012; Heidarian and Rafieian-Kopaei 2013; Rondanelli et al. 2013, 2014), were increased substantially in *rolC*-transformed calli (Table 2). Likewise, the production of caffeoylquinic acids and dicaffeoylquinic acids was increased 3-fold and 2.7-fold, respectively, relative to the control culture (Table 3). Thus, artichoke callus cultures can be used as an appropriate source of caffeoylquinic acids.

For some time, studies of the biochemical functions of *Agrobacterium rhizogenes* and *A. tumefaciens* T-DNA oncogenes expanded in nearly independent ways. This divergence was due to the concept that these pathogens use conceptually different ways to affect plant physiology (Nilsson and Olsson 1997). It was generally accepted that *A. tumefaciens* uses the widely known hormone biosynthesizing genes (*iaaM*, *iaaH*, and *ipt*) as the main pathogenic determinants and that *A. rhizogenes* uses the *rol* oncogenes. In this way, both pathogens disturb hormone metabolism or hormone perception pathways and attain their aim of successful pathogenesis.

Recent studies indicate involvement of *A. rhizogenes rol* oncogenes and *A. tumefaciens 6b* oncogene in plant defense reactions (Wang et al. 2011; Bulgakov et al. 2013). On this basis, studies of both pathogens acquire a common direction. It is therefore important to find the similarities and differences in the actions of *A. rhizogenes* and *A. tumefaciens* T-DNA oncogenes on secondary metabolism.

The results presented in this investigation are the first to indicate that the actions of *A. rhizogenes* and *A. tumefaciens* oncogenes on secondary metabolism may be similar. The RolC protein of *A. rhizogenes* is known to be most closely related to the 6b protein of *A. tumefaciens* (Helfer et al. 2002). A polyphenol analysis in C58-*6b*, 35S-AK-*6b*, and dex-AK-*6b*-transformed tobacco plants revealed increased levels of chlorogenic acid in transformed plants in comparison with control plants (Gális et al. 2002, 2004; Kakiuchi et al. 2006). Clément and colleagues (2007) also found an over-accumulation of chlorogenic acid in *6b*-expressing tissues of tobacco. Thus, agrobacterial T-DNA genes (such as *rolC* and *6b*) cause a similar effect on levels of chlorogenic acid in transformed cells of different plants.

According to the emerging role of the 6b oncoprotein as modulator of RNA silencing (Wang et al. 2011), a mechanism may be proposed by which T-DNA oncoproteins (such as RolC or 6b) interfere with RNA silencing to derepress genes

Table 3 The biomass accumulation and production of caffeoylquinic acids in *C. cardunculus* var. *altilis* callus cultures

Callus line	Fresh biomass[a] (g/l)	Dry biomass[a] (g/l)	Production (mg/l)		
			Monocaffeoylquinic acids (1, 2)	Dicaffeoylquinic acids (3, 4, 5, 6)	Total
Cyn-V	530±43	16.8±1.5	17	91	108
Cyn-C	500±75	19.5±2.1	56	244	300

The inoculum masses for Cyn-V and Cyn-C calli were approximately 25 g/l

[a] Mean values±SE based on ten replicate samples obtained during 2-year cultivation of the cultures (2012–2013)

of secondary metabolism normally repressed through miRNA. There may be two functions of the T-DNA oncogenes. The first is the direct transcriptional activation of key genes of secondary metabolism; the second function is the derepression of silencing genes. The study of these mechanisms is the subject of our current research.

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