Plant Cell Rep
DOI 10.1007/s00299-016-2005-z

ORIGINAL ARTICLE

Increase of anthraquinone content in *Rubia cordifolia* cells transformed by native and constitutively active forms of the *AtCPK1* gene

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Received: 22 March 2016 / Accepted: 25 May 2016

Abstract

Key message **Overexpression of both native and mutant forms of *AtCPK1* in *Rubia cordifolia* cells increased anthraquinone production and transcript abundance of the *RcIPPI*, *RcOSBL*, *RcOSBS*, and *RcICS* genes to different extents.**

Abstract Calcium-dependent protein kinases (CDPKs) are involved in various cell processes and are regulated by a calcium signal system. CDPKs also function in plant defense against stress factors such as pathogens, temperature, and salinity. In this study, we compared the effect of heterologous expression of two forms of the *Arabidopsis AtCPK1* gene, native and constitutively active (Ca^{2+}-independent), on anthraquinone production in transgenic *Rubia cordifolia* cells. Significant qualitative and quantitative differences were found in the content of anthraquinone derivatives in control and *AtCPK1*-transgenic calli. Expression of the *AtCPK1* gene increased anthraquinone production by 3 and 12 times for native and constitutively active forms, respectively, compared with control cells. In addition, we identified and quantified the expression of genes encoding key enzymes of the anthraquinone biosynthesis pathway, including isochorismate synthase (ICS), o-succinylbenzoate synthase (OSBS), o-succinylbenzoate ligase (OSBL), and isopentenyl diphosphate isomerase (IPPi). In all *AtCPK1*-transgenic cell lines, expression of *ICS*, *OSBS*, *OSBL,* and *IPPi* increased considerably at 14–15 days of subculture and decreased at the end of cultivation (30 days). The results suggest that both native and constitutively active *AtCPK1* forms induced anthraquinone accumulation at the logarithmic growth stage via enhancement of expression of genes involved in the metabolism of anthraquinones or their regulatory mechanisms.

Keywords AtCPK1 · *Rubia cordifolia* · Anthraquinones · Plant cell cultures

Abbreviations

AQs	Anthraquinones
CDPKs	Calcium-dependent protein kinases
ROS	Reactive oxygen species

Introduction

Calcium-dependent protein kinases (CDPKs) are members of a large multigene family, acting as Ca^{2+} sensors in plants. These enzymes have been implicated in responses to biotic (Sudha and Ravishankar 2002; Boudsocq et al. 2010; Coca and San Segundo 2010) and abiotic (Asano et al. 2012) stresses. CDPKs are also known to be involved in growth (Matschi et al. 2013), development (Jain et al.

Communicated by L. Tripathi.

Electronic supplementary material The online version of this article (doi:10.1007/s00299-016-2005-z) contains supplementary material, which is available to authorized users.

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Published online: 01 June 2016

2011), cell differentiation (Schulz et al. 2013), and plant–microbe interactions (Veremeichik et al. 2014). 6-Dimethylaminopurine and other commonly used kinase inhibitors may decrease the accumulation of phytoalexins through a decline of CDPK activity in a variety of plants (Choi and Bostock 1994; Vitrac et al. 2000). In Arabidopsis and French bean particular CDPK proteins may phosphorylate phenylalanine ammonia lyase (PAL) which is involved in the biosynthesis of the polyphenols. Thus, stress perception via CDPK and phosphorylation of PAL enzyme provides a certain connection between defense reactions and regulation of secondary metabolism in plant cells (Allwood et al. 1999, 2002; Cheng et al. 2001).

The first experimental evidence for the influence of CDPK on secondary metabolism was demonstrated using mutant forms of the *AtCPK1* gene from *Arabidopsis thaliana*. The *Rubia cordifolia* cell line transformed with mutant constitutively active form of *AtCPK1* was found to be 10-times more active in anthraquinone (AQ) biosynthesis than untransformed culture (Shkryl et al. 2011). The activation of AQ metabolism was associated with the rise in the intracellular level of reactive oxygen species (ROS) (Bulgakov et al. 2011). Thereby, expression of the constitutively active form *AtCPK1* may mimics the effects of elicitors, causing an accumulation of anthraquinones by elevation of ROS content.

AQs are known phytoalexins of *Rubia* species which possess antitumor (Lajkó et al. 2015), antifungal (Kang et al. 2010), antioxidant (Zengin et al. 2015), and moderate cytotoxic (Mishchenko et al. 2014) activities. AQs are also considered useful for the disintegration and elimination of urinary stones (Singh and Chauhan 2004). In the biosynthetic pathway of *Rubia*-type AQs, shikimic acid, α-ketoglutarate via o-succinylbenzoate leads to the production of ring A and B. In contrast, ring C is produced from isopentenyl diphosphate, a common precursor for the synthesis of isoprenoids. Several key enzymes implicated in AQ metabolism have been identified. Isochorismate synthase (ICS) converts chorismate, from the shikimate pathway, to isochorismate; o-succinylbenzoate synthase (OSBS) catalyzes the conversion of isochorismate to o-succinyl benzoic acid (OSB) in the presence of α-ketoglutarate and thiamine diphosphate; o-succinylbenzoate ligase (OSBL) forms CoA-linked o-succinylbenzoate; isopentenyl diphosphate isomerase (IPPi) catalyzes the conversion of isopentenyl pyrophosphate (IPP) to more-reactive dimethylallyl pyrophosphate (DMAPP) (Han et al. 2001).

The aims of this study were to compare the effects of overexpression of native and constitutively active forms of the *AtCPK1* gene on AQ biosynthesis in *R. cordifolia* cell lines and to evaluate the changes in expression profiles of genes involved in the AQ metabolic pathway.

Materials and methods

Callus cultures

The untransformed callus line of *R. cordifolia* (designated in this work as R) and the transgenic callus line, transformed with the mutant, constitutively active form of *AtCPK1* (designated in this work as R-Ca), used in this study have been described previously (Shkryl et al. 2011). The *R. cordifolia* transgenic callus line overexpressing the native form of the *AtCPK1* gene (designated in this work as R-Ak) was created via *Agrobacterium*-mediated transformation of the R callus line.

To amplify the full-length sequence of the *AtCPK1* gene (GenBank accession no. NM_120569), we used amplified cDNA of *Arabidopsis thaliana* Col-0 and the following gene specific primers: forward 5′-ACA AAG ATC TTT ATG GGT AAT ACT TGT GTT G-3′ (the *Bgl* II restriction site is underlined) and reverse 5′-GAC TGG GCC CCT AGA GTT TAA GAG CAA TGC-3′ (the *Apa* I restriction site is underlined). PCR was performed using these primers and conditions described previously (Kozhemyako et al. 2010) and resulted in an 1855-bp-long sequence. Plasmid vector pSAT6-MCS (Tzfira et al. 2005), which contained the tandem cauliflower mosaic virus (CaMV) 35S promoter, tobacco etch virus (TEV) leader, and the CaMV 35S terminator, was used for the construction of the plant expression vector. *AtCPK1* was subcloned as a *Bgl*II-*Apa*I fragment into the same sites of linearized plasmid. The obtained constructions, pSAT6-AtCPK1, were checked for the absence of mutations by DNA sequencing as described previously (Shkryl et al. 2010) at the Instrumental Centre of Biotechnology and Gene Engineering of IBSS FEB RAS using an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The newly constructed expression cassette was excised as a PI-PspI-fragment and subcloned into the binary vector pPZP-RCS2-nptII (Goderis et al. 2002; Tzfira et al. 2005), containing left and right border regions of the Ti plasmid and a gene for kanamycin resistance (nptII) under the control of the octopine synthase (ocs) promoter and terminator sequences. The final construction (Fig. 1), pPZP-RCS2-nptII-AtCPK1, was transferred into *Agrobacterium tumefaciens* strain EHA105/pTiBo542 (Hood et al. 1993) by electroporation (BioRad Gene Pulser, 0.1-cm cuvettes, 25 mF, >2.5 kV), following the manufacturer's protocol. Individual colonies were selected on LB medium containing 150 mg/l rifampicin, 300 mg/l spectinomycin, and 200 mg/l streptomycin at 28 °C.

The R-Ak callus line was obtained via *Agrobacterium*-mediated transformation of *R. cordifolia* suspension culture R as described previously (Shkryl et al. 2011). Selection of

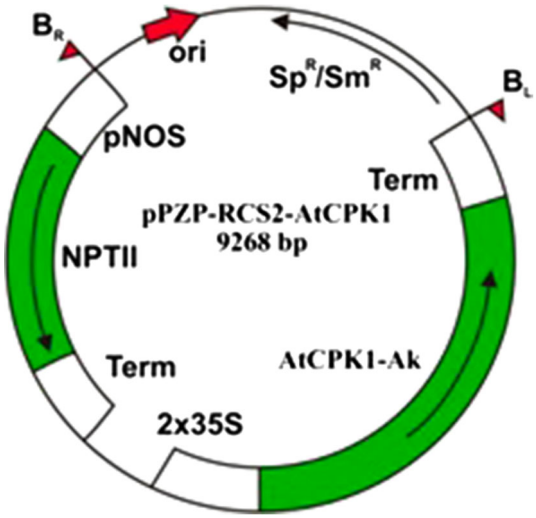

Fig. 1 Physical map of pPZP-RCS2-AtCPK1 binary vector. B_R and B_L—right border and left border, respectively; 2 × 35S, double 35S promoter; AtCPK1-Ak, native, protein coding sequence of *A. thaliana* CPK1 gene; Term, terminator; pNOS, promoter of the nopaline synthase gene; NPTII, neomycin phosphotransferase II (kanamicine resistance); ori, origin of replication; Sp^R/Sm^R, resistance to spectinomycin and streptomycin, respectively

transgenic calli was achieved through five passages on the medium, supplemented with kanamycin (50 mg/l).

R, R-Ca, and R-Ak callus lines were cultivated in 100 ml Erlenmeyer flasks on "W" agarized medium (Bulgakov et al. 2011), supplemented with 0.5 mg/l 6-benzylaminopurine and 2.0 mg/l α-naphthaleneacetic acid, in the dark at 25 °C with 30-day subculture intervals. Calli were dried under hot air flow (60 °C for 5 h) and used for determination of composition and quantity of anthraquinones.

Growth indices were calculated as follows: final fresh weight of tissues—initial fresh weight of tissues/initial dry weight of tissues.

RNA isolation, cDNA synthesis and PCR reactions

The isolation of total RNA and first-strand cDNA synthesis were carried out as described previously (Shkryl et al. 2008, 2010).

PCRs were performed in 50-ml reaction volumes containing 1 μl of RT reaction product, 1 × PCR buffer with 2.5 mM $MgCl_2$, 200 μM of each deoxynucleotide, 1 μM of the specific primer pair and 1 U ColoredTaq polymerase (Sileks, Russia). PCRs were performed in a iCycler (Bio-Rad Laboratories) and cycle conditions were as follows: 96 °C for 10 s, 55 °C for 30 s, 72 °C for 1 min.

For testing the integrity of the cDNA preparation primers used for the *R. cordifolia* actin gene (GenBank accession no. DQ531565) were applied. The primer set 5′-TCC AAA CGA GGT ATC TTA AC-3′ and 5′-GCA AGA TCA AGA CGG AGA AT-3′ spans a 91-bp intron sequence that allowed for testing for the absence of DNA contamination in the sample.

The primers 5′-AGC CTG AAA CGC TAG AGG AG-3′ and 5′-CTA GTG AGC TCA GCC GCT TT-3′ were used for amplification of a 517 bp *AtCPK1* transcript.

To amplify sequences corresponding to *OSBS*, *OSBL* and *IPPi* genes, degenerate primers were designed according to the GenBank conserved amino acid sequences of the corresponding enzymes from different plant species.

The primer set forward 5′-GTN GCN CAY GGN YTN GGN AC-3 and reverse 5′-TCY TTN ARN CCN GGN SWN CC-3′ correspond to the conserved amino acid sequences VAHGLGTY and SGSPGLKD of OSBS proteins. These conserved amino acid segments were chosen based on the alignment of known plant OSBS genes from *Arabidopsis lyrata* (GenBank accession no. XP_002887212), *Ricinus communis* (GenBank accession no. XP_002514555), *Vitis vinifera* (GenBank accession no. XP_002268761), *V. vinifera* (GenBank accession no. CBI27421), *V. vinifera* (GenBank accession no. CAN71091), *Populus trichocarpa* (GenBank accession no. XP_002314907).

The primer set forward 5′-CCN YTN TGY CAY ATH GGN GGN A-3, reverse 5′-CAN GCY TCN GTC ATN CCR TAN GC-3′ corresponding to *OSBL* gene were designed using the amino acid sequences PLVHIGG and YGMTEA. These conserved amino acid segments were chosen based on the alignment of known plant *OSBL* genes from *Physcomitrella patens* (GenBank accession no. XP_001761932), *Zea mays* (GenBank accession no. NP_001143117), *Sorghum bicolor* (GenBank accession no. XP_002444999), *P. trichocarpa* (GenBank accession no. XP_006377959), *R. communis* (GenBank accession no. XP_002528558).

The primer set forward 5′-CAY MGN GCN TTY WSN GTN TTY YT-3, reverse 5′-TAR TCN ARY TCR TGY TCN CCC CA-3′ corresponding to *IPPi* gene were designed using the amino acid sequences HRAFSVF and WGEHEVD These conserved amino acid segments were chosen based on the alignment of known plant *IPPi* genes from *A. thaliana* (GenBank accession no. NP_197148 и NP_186927), *A. lyrata* (GenBank accession no. XP_002884339), *Brassica oleracea* (GenBank accession no. AAF36996), *V. vinifera* (GenBank accession no. XP_002277935).

Using PCR with these primers and total RNA from *R. cordifolia* cells, cDNA fragments of predicted lengths were amplified. These fragments were isolated from gels with a Glass Milk Kit (Sileks, Russia) and sequenced as described earlier (Shkryl et al. 2008) at the Instrumental Centre of Biotechnology and Gene Engineering of IBSS FEB RAS

using an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City CA, USA).

Rapid amplification of cDNA ends

To obtain full-length sequences of *R. cordifolia OSBL* and *IPPi* genes, we performed Rapid Amplification of cDNA Ends (RACE), using step-out PCR technology (Matz et al. 1999) with modifications (see below). cDNA was synthesized from total RNA isolated from the *R. cordifolia* callus culture and subjected to 25 cycles of amplification, using the SMART cDNA Amplification Kit, according to the manufacturer's instructions (Clontech, CA, USA). In our modified protocol of RACE, we used the degenerate primers as described in previously in the first round of PCR instead of gene-specific primers. The use of these primers allowed for the simultaneous amplification of all expressed isoforms of *RcOSBL* and *RcIPPi*. The gene-specific primers (Supplemental Table 1) were employed in the second round of RACE to obtain individual amplicons for each isoform of *RcOSBL* and *RcIPPi* (Veremeichik et al. 2012). The fragments obtained by RACE were cloned into the plasmid pJET1.2 (Fermentas, Lithuania) and sequenced.

Real-time PCR

The quantitative real-time PCR (qPCR) analysis was performed using a CFX96 (Bio-Rad Laboratories, Inc., USA) with 2.5× SYBR green PCR master mix containing ROX as a passive reference dye (Syntol, Russia).

Gene-specific primers were used for qPCR as follows: *RcICS* (GenBank accession no. EF090619) forward 5′-TAT CGT GCG AGA GAA CAT CA-3′ and reverse 5′-GAA TTC TGG CCA GTT TGC GA-3′; *RcOSBL* (GenBank accession no. KT893312) forward 5′-TAC GGA GAT GGT GGT TGC-3′ and reverse 5′-ATC CGT CAC CCA TGC CAT-3′; *RcOSBS* (GenBank accession no. KU725713) forward 5′-AAG TGG CTC AAA GAA GAT G-3′ and reverse 5′-CAC ATT TTG ATT GAT TTG AA-3′; *RcIPPi* (GenBank accession no. KT222914) forward 5′-GTT GGT CTG GAC AAA TAC CGG-3′ and reverse 5′-GTG AAC TGA TCA ACT GGA AC-3′.

Reactions were done in a 12.5 µl volume containing 300 nM of each primer, 1 µl of the diluted cDNA sample and 2.5 mM $MgCl_2$. All PCR reactions were performed under the following conditions: 5 min at 95 °C, followed by 35 cycles of 10 s at 95 °C and 30 s at 60 °C in a 96-well reaction plate. Two biological replicates, resulting from two different RNA extractions, were used for analysis, and three technical replicates were analysed for each biological replicate. No-template controls and RNA-RT controls were included in the qPCR analysis to verify the absence of contamination. The absence of non-specific products or primer-dimer artefacts in the samples was confirmed by melting curve analysis at the end of each run and by product visualization using electrophoresis on a 1 % agarose gel stained with ethidium bromide.

The actin gene of *R. cordifolia* was used for normalization of the qPCR data. The stability of the *RcActin* gene expression was previously confirmed using 18S gene expression (Shkryl et al. 2011). Data were analyzed using CFX Manager Software (Version 1.5; Bio-Rad Laboratories Inc., Hercules, CA, USA).

Determination of composition and quantity of AQ pigments

The composition and quantity of AQ pigments were determined photometrically in 15- and 30-day cultures with a UV-mini 1240 (Shimadzu, Japan) as described previously (Mischenko et al. 1999). HPLC–DAD-MS was performed on a Shimadzu LCMS-2020 chromatograph (Shimadzu Corp., Japan) with a diode-matrix and mass-spectrometric detectors as described previously (Bulgakov et al. 2011). The separation was carried out over a Discovery HS C18 column (150 × 2.1 mm, 3 µm particle size, Sigma-Aldrich) using a binary gradient of HOAc (0.1 %): MeCN from 10 to 100 % MeCN over 10 min at flow rate 0.2 ml/min and column temperature 40 °C. Mass spectra were taken in electrospray ionization (ESI) mode at atmospheric pressure, recording negative ions (1.50 kV) in the *m/z* range 100–800.

Statistical analysis

All values are expressed as the mean ± SE. For comparison among multiple data, analysis of variance (ANOVA) followed by a multiple comparisons procedures was employed. Fisher's protected least significant difference (PLSD) post hoc test was employed for the inter-group comparison. Pearson correlation analysis was used to reveal relationships between two variables.

Results

Growth parameters of *R. cordifolia* transformed callus cultures

Full-length sequence of the *AtCPK1* (GenBank № NM_120569) gene was obtained from amplified cDNA of *Arabidopsis thaliana* Col-0 using PCR and cloned into pPZP-RCS2 binary vector. Transgenic callus line overexpressing native form of the *AtCPK1* gene was obtained via *Agrobacterium*-mediated transformation of *R. cordifolia* suspension culture R as described in the Materials and

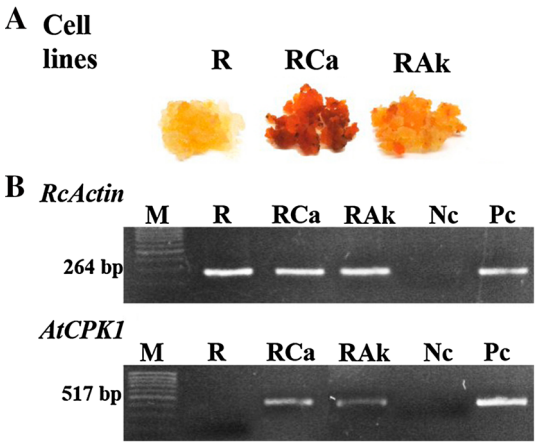

Fig. 2 **a** Phenotypes of the *Rubia cordifolia* callus cultures. R, untransformed callus line; R-Ca, callus line transformed with constitutively active, calcium-independent form of *AtCPK1*; R-Ak, callus line transformed with native form of *AtCPK1*. Cultures were grown for 3 weeks on $W_{B/A}$ medium. **b** PCR products of *RcActin* and *AtCPK1* from cDNA of R, R-Ca, R-Ak, and callus cultures. PC, positive PCR control (pPZP-RCS2-AtCPK1 DNA); NC, negative control (no cDNA added), M, DNA markers (100 bp + 1.5 kb ladder, SybEnzyme, Russia)

Methods Section. After 2–3 weeks of selection, the *AtCPK1*-transformed culture (R-Ak) began to form yellow-orange, vigorously growing callus aggregates. This cell line had a stronger yellow color than the control, non-transgenic cell culture, but was less intensely colored than the callus line transformed with the constitutively active form of the *AtCPK1* gene (R-Ca) (Fig. 2a). The transgenic cell culture R-Ak, generated for this study, had stable growth characteristics and morphological homogeneity for at least 12 months of observation.

To determinate heterologous expression of the *AtCPK1* gene in *R. cordifolia* transformed calli, we performed PCR with gene-specific primers. *RcActin* gene expression was used to evaluate the integrity of the cDNA obtained (Fig. 2b). *AtCPK1* transcripts were detected in R-Ca and R-Ak transgenic cultures while no PCR products were observed in the control culture. All cultures grew vigorously with growth indices between 9.3 and 12.5.

Effect of *AtCPK1* gene on biosynthesis of anthraquinones

To estimate the effect of *AtCPK1* overexpression on secondary metabolism of transgenic *R. cordifolia* cells, composition and quantity of AQ pigments of R, R-Ca, and R-Ak callus lines were examined at exponential (15 days) and stationary phases of growth (30 days) by HPLC–DAD-MS and UV-spectrophotometry.

HPLC–DAD–MS analysis of the R, R-Ak and R-Ca ethanol extracts allowed the identification of 10 main AQ derivatives (Fig. 3): pseudopurpurin primveroside (**1**), pseudopurpurin glucoside (**2**), munjistin glucoside (**3**), ruberitrinic acid (**4**), munjistin primveroside (**5**), lucidin primveroside (**6**), pseudopurpurin (**7**), alizarin (**8**), purpurin (**9**) and munjistin (**10**). The major component produced by R callus culture was AQ **10**; other compounds were produced at levels 2–5 times lower than AQ **10**. All specific AQ derivatives found in the control cells were also present in *AtCPK1*-transgenic calli but in substantially greater quantities. *AtCPK1* also changed which compounds were produced in R-Ak and R-Ca callus lines. The main AQ pigments found in R-Ak cells were compounds **4** and **8**, while compound **10**, the major component found in the R

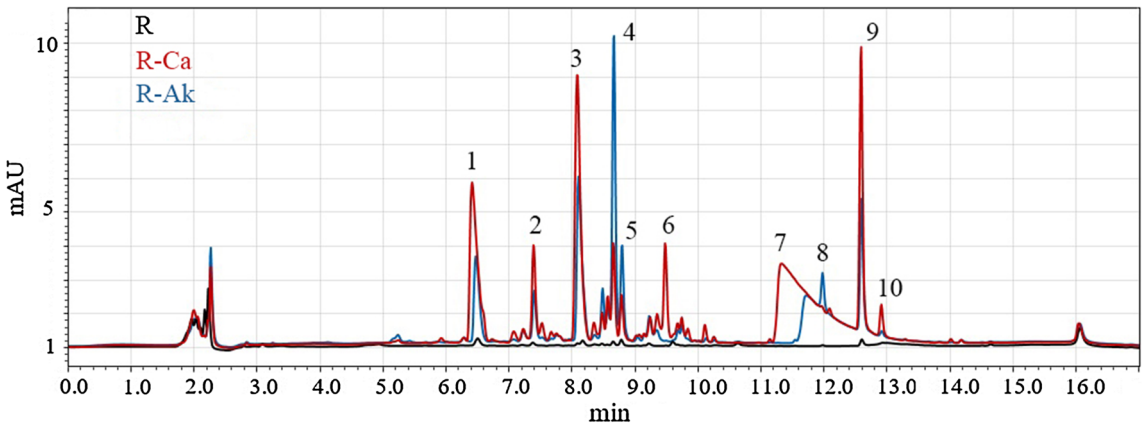

Fig. 3 HPLC separation of the AQ pigment extracts obtained from R, R-Ak, and R-Ca calli. Discovery HS C18 column, binary gradient elution with 0.1 % acetic acid-acetonitrile, flow rate 0.2 ml/min, detection at 254 nm. AQ derivatives: pseudopurpurin primveroside (**1**), pseudopurpurin glucoside (**2**), munjistin glucoside (**3**), ruberitrinic acid (**4**), munjistin primveroside (**5**), lucidin primveroside (**6**), pseudopurpurin (**7**), alizarin (**8**), purpurin (**9**) and munjistin (**10**) (identification see in Table 1)

Table 1 List of the retention times, MS data of pseudomolecular ions ([M−H]-), UV–Vis absorption data and structural elucidation for AQs identified from *R. cordifolia AtCPK1*-transformed and untransformed cultures using HPLC–DAD-MS

No.	RT (min)	[M-H]-	Molecular formula	λ max (nm)	Compound	Content of anthraquinones, %		
						R	R-Ak	R-Ca
1	6.42	593	C26H26O16	253, 288, 432	Pseudopurpurin primveroside	8.86	8.55	13.71
2	7.39	461	C21H18O12	249, 313, 350, 425	Pseudopurpurin glucoside	4.46	3.25	3.79
3	8.09	445	C21O18H11	247, 271, 346, 408	Munjistin glucoside	6.57	14.18	15.74
4	8.66	533	C25H26O13	260, 331, 416	Ruberitrinic acid	3.25	18.25	3.31
5	8.79	577	C26H26O15	258, 335, 407	Munjistin primveroside	4.92	5.51	1.92
6	9.48	563	C26H28O14	277, 338, 419	Lucidin primveroside	–	–	3.58
7	11.33	299	C15H8O7	258, 290, 330, 497	Pseudopurpurin	–	10.71	24.25
8	11.98	239	C14H8O4	248, 279, 438	Alizarin	0.56	14.09	–
9	12.59	255	C14H8O5	255, 293, 481	Purpurin	4.68	12.09	11.11
10	12.91	283	C15H8O6	249, 278, 438	Munjistin	15.87	–	2.02

Table 2 Biomass accumulation, anthraquinone (AQ) quantity and AQ production in untransformed and *AtCPK1*-transgenic callus cultures of *R. cordifolia* at 15 and 30 days of culture

Callus line	Fresh biomass (g/l)	Total AQ quantity (% dry wt)	AQ production (mg/l)
15 days			
R (untransformed culture)	99.78 ± 3.70^{A}	0.25 ± 0.01^{C}	10.28 ± 0.38^{C}
R-Ca (constitutively active *AtCPK1*)	70.21 ± 7.08^{B}	4.64 ± 0.18^{A}	188.72 ± 14.52^{A}
R-Ak (native *AtCPK1*)	97.30 ± 3.60^{A}	1.13 ± 0.12^{B}	53.05 ± 1.97^{B}
30 days			
R (untransformed culture)	399.07 ± 20.90^{A}	0.29 ± 0.04^{C}	51.46 ± 2.70^{C}
R-Ca (constitutively active *AtCPK1*)	339.95 ± 42.67^{B}	3.56 ± 0.24^{A}	635.65 ± 77.52^{A}
R-Ak (native *AtCPK1*)	389.22 ± 21.48^{A}	1.01 ± 0.08^{B}	152.44 ± 8.41^{B}

The data presented are mean values ± SE. Different superscript letters indicate statistically significant differences of means ($p \leq 0.05$) in each column, Fisher's LSD

culture, was present only in its glycosylated form, **3**. Major components found in R-Ca cells were AQs **1**, **3**, and **7**. In addition, R-Ca calli began to produce compound **6**, which was not found in other cultures, but stopped biosynthesizing compound **8**. The retention times, MS and UV–Vis absorption data of the identified compounds are given in Table 1.

AQ quantity in control and *AtCPK1*-transgenic calli was estimated by UV–visible spectrophotometry (Table 2). AQ accumulation in the transgenic cell line expressing the native form of *AtCPK1* (R-Ak) showed a 4.5-fold increase on day 15 and 3.5-fold increase on 30 day of culture ($p \leq 0.01$) (Table 2). AQ accumulation in the transgenic cell line, expressing the constitutively active form of *AtCPK1* (R-Ca), showed an 18.6-fold increase on day 15 and 12.3-fold increase on 30 day of culture ($p \leq 0.01$) (Table 2). The production of AQs rose with biomass accumulation and reached maximal values at the end of passage (Table 2).

Identification and analysis of *R. cordifolia* genes involved in anthraquinones metabolism

To identify genes encoding key enzymes of anthraquinone biosynthesis, including *OSBL*, *OSBS*, and *IPPi*, we designed sets of degenerative primers for each gene. These primers were used in PCR with cDNA templates obtained from cell cultures, R, R-Ca, and R-Ak. Fragments of the predicted lengths were obtained and sequenced, and partial sequences of *R. cordifolia OSBL*, *OSBS*, and *IPPi* genes were identified. To obtain full-length sequences, a modified protocol of RACE was performed using degenerate primers in the first round of amplification for all genes studied. Full-length sequences of *RcOSBL* and *RcIPPi* transcripts and the partial sequence of *RcOSBS* transcripts were identified and submitted to GenBank under the accession numbers KT893312, KT222914, and KU725713, respectively.

High levels of amino acid identity were found between the deduced *R. cordifolia* RcOSBL, RcIPPI, and RcOSBS

proteins and known enzymes from other plants, using the BLAST algorithm (Supplemental Table 2).

Sequence analysis of the *RcOSBL* and *RcIPPI* genes indicated that the full-length cDNA contains the 5′-UTR, the complete open reading frame (ORF) and 3′-UTR. The BLASTp search utility identified the ATG translation start in both genes. *RcOSBL* and *RcIPPI* have 2040 and 976 bp ORFs, encoding proteins of 559 and 237 amino acids, respectively. Sequence analysis of *RcOSBS* indicated that the sequence contains part of the ORF and has 250 bp.

Analysis of *ICS*, *OSBL*, *OSBS,* and *IPPi* gene expression

The expression profiles of *RcICS*, *RcOSBL*, *RcOSBS*, and *RcIPPi* genes in non-transformed and *AtCPK1*-transgenic calli were examined, using cDNA samples isolated from R, R-Ak, and R-Ca callus cultures at 12, 13, 14, 15 and 30 days of growth (Fig. 4). The *AtCPK1* gene increased the expression levels of all genes studied. Notably, the effect of the constitutively active form of AtCPK1 was 1.2–3.2 times greater than that of the native form. In the untransformed callus line, *RcICS*, *RcOSBL*, *RcOSBS*, and *RcIPPi* transcript abundance decreased during the growth cycle and reached minimal values at the end of cultivation. In both R-Ak and R-Ca transgenic cells, the expression of *RcICS*, *RcOSBL*, *RcOSBS*, and *RcIPPi* transcripts peaked at day 14 and 15 (i.e., the exponential phase). Thus, at 14 days of culture, the expression of *RcICS*, *RcOSBS*, *RcOSBL*, and *RcIPPi* in R-Ak and R-Ca calli was 3–10 and 7–23 times higher than in R cells, respectively. The expression levels of *RcOSBS*, *RcOSBL*, and *RcIPPi* at the end of cultivation period (30 days) was lower than in all other time points studied.

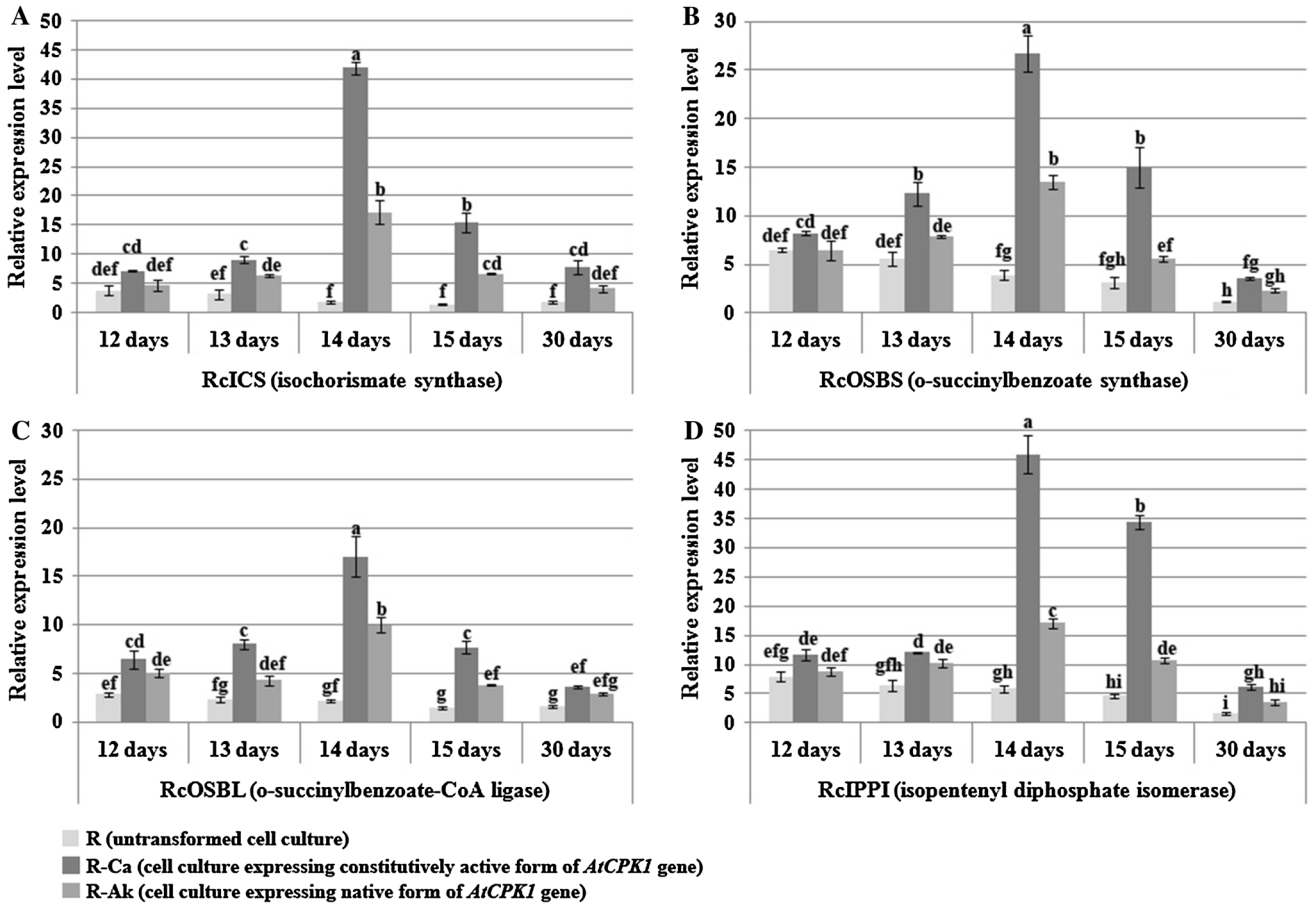

Fig. 4 Expression patterns of the *R.cordifolia* genes involved in anthraquinone biosynthesis during exponential (12–15 days) and stationary phases (30 days): **a** isochorismate synthase; **b** o-succinylbenzoate synthase; **c** o-succinylbenzoate-CoA ligase; **d** isopentenyl diphosphate isomerase. Data (mean ± standard error) represent measurements of three independent replicates from two different RNA isolations and are presented as relative expression levels normalized to the expression of the *R. cordifolia* actin gene. *Different letters above bars* indicate statistically significant differences of means ($p \leq 0.05$), Fisher's LSD

Discussion

The biotechnological production of secondary metabolites on an industrial scale is not well developed with rare exceptions. Thus, improving the methods of industrial cultivation of plant cells (Weathers et al. 2010) and creating new generations of plant cell cultures with improved characteristics (Wang 2008; Nascimento and Fett-Neto 2010) are active areas of research. Overexpression of a key enzyme, regulating the rate-limiting step in a biosynthetic pathway, is widely used as a biotechnological approach to increase metabolite production (Farré et al. 2014). However, this strategy is not always successful. For example, expression of bacterial isochorismate synthase in transgenic root cultures of *Rubia peregrina* resulted in only 20 % higher levels of total AQs after 30 days in culture (Lodhi et al. 1996). Similarly, transgenic cell lines of *Morinda citrifolia* overexpressing 1-deoxy-D-xylulose-5-phosphate synthase showed a 21 % increase of anthraquinone quantity on day 3, a 24 % increase on day 6 and an 18 % increase on day 9 of culture (Quevedo et al. 2010). The use of regulatory genes for the improvement of plant cell cultures is a promising approach in biotechnology (Verpoorte and Memelink 2002).

Calcium and calcium sensor protein serine/threonine kinase signaling networks participate in elicitor-induced accumulation of plant secondary metabolites (Smith 1994; Zhao et al. 2005). In particular, chitosan-induced AQ accumulation in *Rubia tinctorum* involves increase in intracellular calcium ions, which regulate protein kinases participating in elicitation (Vasconsuelo et al. 2005). Different CPDKs could potentially regulate phytoalexins production via ROS signaling (Xing et al. 2001) or direct phosphorylation of key enzymes in biosynthetic pathways (Allwood et al. 1999, 2002; Cheng et al. 2001).

We have previously demonstrated that heterologous expression of a mutant, Ca^{2+}-independent (constitutively active) form of the *Arabidopsis AtCPK1* gene caused a significant increase in AQ quantity in transformed *R. cordifolia* cells. In contrast, overexpression of the non-active form of *AtCPK1* had no effect on anthraquinone production. In the work presented here, we obtained transgenic *R. cordifolia* cells expressing the native form of *AtCPK1* and compared its action with a constitutively active variant of the gene. Both forms of the *AtCPK1* gene triggered significant activation of AQ biosynthesis but to different extents. Accumulation of AQs in the R-Ak cell line showed a 4.5-fold increase on day 15 and 3.5-fold increase on day 30 of culture. AQ quantity in the R-Ca calli showed an 18.6-fold increase on day 15 and 12.3-fold increase on 30 day of culture (Table 2). *AtCPK1* gene expression also led to significant changes in composition of AQ pigments in transgenic calli. R-Ak and R-Ca cells began to produce pseudopurpurin as one of the major compounds, but stopped biosynthesizing munjistin. AQ accumulation in the *AtCPK1*-transgenic cell lines was likely caused by *de novo* biosynthesis of AQs, because the rise in AQ quantity coincided with the activation of expression of the key genes for AQ biosynthesis, including *RcICS*, *RcOSBS*, *RcOSBL*, and *RcIPPi* (Fig. 4). Interestingly, in both, R-Ak and R-Ca transgenic cells, the expression of genes involved in metabolism of anthraquinones peaked at the exponential phase of cell growth and significantly decreased at the end of cultivation.

The mechanism of AQ activation by CDPK is presently unknown. The mechanism might involve direct activation of the enzymes involved in the AQ metabolic pathway, analogous to the process described for *PAL* genes (Allwood et al. 1999, 2002; Cheng et al. 2001). However, in silico analysis using the KinasePhos online tool (Huang et al., 2005) did not reveal any putative sites for phosphorylation by AtCPK1 in ICS, OSBL, OSBS, and IPPi enzymes of *R. cordifolia*. It seems more likely that AtCPK1 is involved in indirect activation of *ICS* expression via transcription factors and/or regulatory proteins. For example, CDPKs could elevate *ICS* transcriptional levels via phosphorylation of WRKY transcription factors, which directly interact with the *ICS* promoter (van Verk et al. 2011; Seyfferth and Tsuda 2014). Recently, the 14-3-3 isoforms χ and ε were identified as potential substrates for AtCPK1 (Swatek et al. 2014). In soybean, 14-3-3 proteins interact with GmMYB62 and GmMYB176 transcription factors, involved in isoflavonoid biosynthesis (Dhaubhadel and Li 2010; Li and Dhaubhadel 2012), and thus, may directly or indirectly play a role in secondary metabolism. The latter mechanism may be similar to the action of the *rolB* gene from *Agrobacterium rhizogenes*. We have previously shown that *rolB* and the constitutively active form of *AtCPK1* act on AQ production in very similar ways (Bulgakov et al. 2012). Although the precise mechanism of RolB function is unclear, it might be associated with 14-3-3 proteins to some degree (Moriuchi et al. 2004). Reactive oxygen species (ROS) generation, mediated by AtCPK1 (Xing et al. 2001), also could affect AQ biosynthesis. These mechanisms are not mutually exclusive, and could possibly interact to activate the AQ metabolic pathway.

Author contribution statement GV, DM and SS performed the experiments and carried out data analysis. NM and EV performed the determination of anthraquinones composition and quantity. YS, GV and DM wrote the paper. VB and SF revised the article critically and made contribution to interpretation of data. YS, GV and VB conceived of and supervised the study. All authors read and approved the final manuscript.

Acknowledgments The analyses described in this work were performed using equipment from the Instrumental Centre for Biotechnology and Gene Engineering at the Institute of Biology and Soil Science. Financial support was provided by the Russian Science Foundation (RSF No.14-14-00230) for the generation of transgenic cultures, cloning, sequencing, real-time detection of gene expression, bioinformatics analysis, and preparation of the article, and by Far Eastern Branch of the Russian Academy of Sciences (FEB RAS No.15-I-5-002) for determination of composition and quantity of AQ pigments.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Allwood EG, Davies DR, Gerrish C, Ellis BE, Bolwell GP (1999) Phosphorylation of phenylalanine ammonia-lyase: evidence for a novel protein kinase and identification of the phosphorylated residue. FEBS Lett 457:47–52. doi:10.1016/S0014-5793(99)00998-9

Allwood EG, Davies DR, Gerrish C, Bolwell GP (2002) Regulation of CDPKs, including identification of PAL kinase, in biotically stressed cells of French bean. Plant Mol Biol 49:533–544. doi:10.1023/A:1015502117870

Asano T, Hayashi N, Kikuchi S, Ohsugi R (2012) CDPK-mediated abiotic stress signaling. Plant Signal Behav 7:817–821. doi:10.4161/psb.20351

Boudsocq M, Willmann MR, McCormack M, Lee H, Shan L, He P, Bush J, Cheng S-H, Sheen J (2010) Differential innate immune signalling via Ca^{2+} sensor protein kinases. Nature 464:418–422. doi:10.1038/nature08794

Bulgakov VP, Gorpenchenko TY, Shkryl YN, Veremeichik GN, Mischenko NP, Avramenko TV, Fedoreyev SA, Zhuravlev YN (2011) CDPK-driven changes in the intracellular ROS level and plant secondary metabolism. Bioeng Bugs 2:327–330. doi:10.4161/bbug.2.6.16803

Cheng SH, Sheen J, Gerrish C, Bolwell GP (2001) Molecular identification of phenylalanine ammonia-lyase as a substrate of a specific constitutively active *Arabidopsis* CDPK expressed in maize protoplasts. FEBS Lett 503:185–188. doi:10.1016/S0014-5793(01)02732-6

Choi D, Bostock RM (1994) Involvement of de novo protein synthesis, protein kinase, extracellular Ca^{2+}, and lipoxygenase in arachidonic acid induction of 3-hydroxy-3-methylglutaryl coenzyme A reductase genes and isoprenoid accumulation in potato (*Solanum tuberosum* L.). Plant Physiol 104:1237–1244. doi:10.1104/pp.104.4.1237

Coca M, San Segundo B (2010) AtCPK1 calcium-dependent protein kinase mediates pathogen resistance in Arabidopsis. Plant J 63:526–540. doi:10.1111/j.1365-313X.2010.04255.x

Dhaubhadel S, Li X (2010) A new client for 14-3-3 proteins. GmMYB176, an R1 MYB transcription factor. Plant Signal Behav 5:921–923

Farré G, Blancquaert D, Capell T, Van Der Straeten D, Christou P, Zhu C (2014) Engineering complex metabolic pathways in plants. Ann Rev Plant Biol 65:187–223. doi:10.1146/annurev-arplant-050213-035825

Goderis IJ, De Bolle MF, François IE, Wouters PF, Broekaert WF, Cammue BP (2002) A set of modular plant transformation vectors allowing flexible insertion of up to six expression units. Plant Mol Biol 50:17–27. doi:10.1023/A:1016052416053

Han YS, Van der Heijden R, Verpoorte R (2001) Biosynthesis of anthraquinones in cell cultures of the Rubiaceae. Plant Cell Tiss Org 67:201–220. doi:10.1023/A:1012758922713

Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993) New *Agrobacterium* helper plasmids for gene transfer to plants. Transgenic Res 2:208–218. doi:10.1007/BF01977351

Huang HD, Lee TY, Tzeng SW, Horng JT (2005) KinasePhos: a web tool for identifying protein kinase-specific phosphorylation sites. Nucleic Acids Res 33:W226–W229. doi:10.1093/nar/gki471

Jain M, Pathak BP, Harmon AC, Tillman BL, Gallo M (2011) Calcium dependent protein kinase (CDPK) expression during fruit development in cultivated peanut (*Arachis hypogaea*) under Ca^{2+}-sufficient and-deficient growth regimens. J Plant Physiol 168:2272–2277. doi:10.1016/j.jplph.2011.07.005

Kang K, Fong W-P, Tsang PW-K (2010) Novel antifungal activity of purpurin against *Candida* species *in vitro*. Med Mycol 48:904–911. doi:10.3109/13693781003739351

Kozhemyako VB, Veremeichik GN, Shkryl YN, Kovalchuk SN, Krasokhin VB, Rasskazov VA, Zhuravlev YN, Bulgakov VP, Kulchin YN (2010) Silicatein genes in spicule-forming and nonspicule-forming Pacific demosponges. Mar Biotechnol 12:403–409. doi:10.1007/s10126-009-9225-y

Lajkó E, Bányai P, Zámbó Z, Kursinszki L, Szőke É, Kőhidai L (2015) Targeted tumor therapy by *Rubia tinctorum* L.: analytical characterization of hydroxyanthraquinones and investigation of their selective cytotoxic, adhesion and migration modulator effects on melanoma cell lines (A2058 and HT168-M1). Cancer Cell Int 15:1–15. doi:10.1186/s12935-015-0271-4

Li X, Dhaubhadel S (2012) 14-3-3 proteins act as scaffolds for GmMYB62 and GmMYB176 and regulate their intracellular localization in soybean. Plant Signal Behav 7:965–968. doi:10.4161/psb.20940

Lodhi A, Bongaerts R, Verpoorte R, Coomber S, Charlwood B (1996) Expression of bacterial isochorismate synthase (EC 5.4. 99.6) in transgenic root cultures of *Rubia peregrina*. Plant Cell Rep 16:54–57. doi:10.1007/BF01275449

Matschi S, Werner S, Schulze WX, Legen J, Hilger HH, Romeis T (2013) Function of calcium-dependent protein kinase CPK28 of *Arabidopsis thaliana* in plant stem elongation and vascular development. Plant J 73:883–896. doi:10.1111/tpj.12090

Matz M, Shagin D, Bogdanova E, Britanova O, Lukyanov S, Diatchenko L, Chenchik A (1999) Amplification of cDNA ends based on template-switching effect and step-out PCR. Nucleic Acids Res 27:1558–1560. doi:10.1093/nar/27.6.1558

Mischenko N, Fedoreyev S, Glazunov V, Chernoded G, Bulgakov V, Zhuravlev Y (1999) Anthraquinone production by callus cultures of *Rubia cordifolia*. Fitoterapia 70:552–557. doi:10.1016/S0367-326X(99)00085-4

Mishchenko N, Vasil'eva E E, Sholokh A, Dyshlovoi S, Fedoreev S (2014) Anthraquinones of *Rubia jesoensis* roots. Chem Nat Compd 50:349–351. doi:10.1007/s10600-014-0950-8

Moriuchi H, Okamoto C, Nishihama R, Yamashita I, Machida Y, Tanaka N (2004) Nuclear localization and interaction of RolB with plant 14-3-3 proteins correlates with induction of adventitious roots by the oncogene *rolB*. Plant J 38:260–275. doi:10.1111/j.1365-313X.2004.02041.x

Nascimento do NC, Fett-Neto AG (2010) Plant secondary metabolism and challenges in modifying its operation: an overview. In: Fett-Neto GA (ed) Plant secondary metabolism engineering: methods and applications. Humana Press, Totowa, pp 1–13

Quevedo C, Perassolo M, Alechine E, Corach D, Giulietti AM, Talou JR (2010) Increasing anthraquinone production by overexpression of 1-deoxy-D-xylulose-5-phosphate synthase in transgenic

cell suspension cultures of *Morinda citrifolia*. Biotechnol Lett 32:997–1003. doi:10.1007/s10529-010-0250-8

Schulz P, Herde M, Romeis T (2013) Calcium-dependent protein kinases: hubs in plant stress signaling and development. Plant Physiol 163:523–530. doi:10.1104/pp.113.222539

Seyfferth C, Tsuda K (2014) Salicylic acid signal transduction: the initiation of biosynthesis, perception and transcriptional reprogramming. Front Plant Sci 5:10–3389. doi:10.3389/fpls.2014.00697

Shkryl YN, Veremeichik GN, Bulgakov VP, Tchernoded GK, Mischenko NP, Fedoreyev SA, Zhuravlev YN (2008) Individual and combined effects of the *rolA*, *B*, and *C* genes on anthraquinone production in *Rubia cordifolia* transformed calli. Biotechnol Bioeng 100:118–125. doi:10.1002/bit.21727

Shkryl YN, Veremeichik GN, Bulgakov VP, Gorpenchenko TY, Aminin DL, Zhuravlev YN (2010) Decreased ROS level and activation of antioxidant gene expression in *Agrobacterium rhizogenes* pRiA4-transformed calli of *Rubia cordifolia*. Planta 232:1023–1032. doi:10.1007/s00425-010-1237-3

Shkryl YN, Veremeichik GN, Bulgakov VP, Zhuravlev YN (2011) Induction of anthraquinone biosynthesis in *Rubia cordifolia* cells by heterologous expression of a calcium-dependent protein kinase gene. Biotechnol Bioeng 108:1734–1738. doi:10.1002/bit.23077

Singh R, Chauhan S (2004) 9, 10-Anthraquinones and other biologically active compounds from the genus *Rubia*. Chem Biodivers 1:1241–1264. doi:10.1002/cbdv.200490088

Smith CJ (1994) Signal transduction in elicitation of phytoalexin synthesis. Biochem Soc T 22:414–420. doi:10.1042/bst0220414

Sudha G, Ravishankar G (2002) Involvement and interaction of various signaling compounds on the plant metabolic events during defense response, resistance to stress factors, formation of secondary metabolites and their molecular aspects. Plant Cell Tiss Org 71:181–212. doi:10.1023/A:1020336626361

Swatek KN, Wilson RS, Ahsan N, Tritz RL, Thelen JJ (2014) Multisite phosphorylation of 14-3-3 proteins by calcium-dependent protein kinases. Biochem J 459:15–25. doi:10.1042/BJ20130035

Tzfira T, Tian G-W, Vyas S, Li J, Leitner-Dagan Y, Krichevsky A, Taylor T, Vainstein A, Citovsky V (2005) pSAT vectors: a modular series of plasmids for autofluorescent protein tagging and expression of multiple genes in plants. Plant Mol Biol 57:503–516. doi:10.1007/s11103-005-0340-5

van Verk MC, Bol JF, Linthorst HJ (2011) WRKY transcription factors involved in activation of SA biosynthesis genes. BMC Plant Biol 11:1–12. doi:10.1186/1471-2229-11-89

Vasconsuelo A, Morelli S, Picotto G, Giulietti AM, Boland R (2005) Intracellular calcium mobilization: a key step for chitosan-induced anthraquinone production in *Rubia tinctorum* L. Plant Sci 169:712–720. doi:10.1016/j.plantsci.2005.05.022

Veremeichik GN, Shkryl YN, Bulgakov VP, Avramenko TV, Zhuravlev YN (2012) Molecular cloning and characterization of seven class III peroxidases induced by overexpression of the agrobacterial *rolB* gene in *Rubia cordifolia* transgenic callus cultures. Plant Cell Rep 31:1009–1019. doi:10.1007/s00299-011-1219-3

Veremeichik GN, Shkryl YN, Pinkus SA, Bulgakov VP (2014) Expression profiles of calcium-dependent protein kinase genes (*CDPK1–14*) in *Agrobacterium rhizogenes* pRiA4-transformed calli of *Rubia cordifolia* under temperature- and salt-induced stresses. J Plant Physiol 171:467–474. doi:10.1016/j.jplph.2013.12.010

Verpoorte R, Memelink J (2002) Engineering secondary metabolite production in plants. Curr Opin Biotech 13:181–187. doi:10.1016/S0958-1669(02)00308-7

Vitrac X, Larronde F, Krisa S, Decendit A, Deffieux G, Mérillon J-M (2000) Sugar sensing and Ca^{2+}–calmodulin requirement in *Vitis vinifera* cells producing anthocyanins. Phytochemistry 53:659–665. doi:10.1016/S0031-9422(99)00620-2

Wang Y (2008) Needs for new plant-derived pharmaceuticals in the post-genome era: an industrial view in drug research and development. Phytochem Rev 7:395–406. doi:10.1007/s11101-008-9092-6

Weathers PJ, Towler MJ, Xu J (2010) Bench to batch: advances in plant cell culture for producing useful products. Appl Microbiol Biot 85:1339–1351. doi:10.1007/s00253-009-2354-4

Xing T, Malik K, Martin T, Miki BL (2001) Activation of tomato PR and wound-related genes by a mutagenized tomato MAP kinase kinase through divergent pathways. Plant Mol Biol 46:109–120. doi:10.1023/A:1010633215445

Zengin G, Degirmenci N, Alpsoy L, Aktumsek A (2015) Evaluation of antioxidant, enzyme inhibition, and cytotoxic activity of three anthraquinones (alizarin, purpurin, and quinizarin). Hum Exp Toxicol. doi:10.1177/0960327115595687

Zhao J, Davis LC, Verpoorte R (2005) Elicitor signal transduction leading to production of plant secondary metabolites. Biotechnol Adv 23:283–333. doi:10.1016/j.biotechadv.2005.01.003