COMMUNICATION TO THE EDITOR

BIOTECHNOLOGY and BIOENGINEERING

Induction of Anthraquinone Biosynthesis in *Rubia cordifolia* Cells by Heterologous Expression of a Calcium-Dependent Protein Kinase Gene

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Received 8 September 2010; revision received 24 December 2010; accepted 18 January 2011
Published online 15 February 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/bit.23077

ABSTRACT: Calcium-dependent protein kinases (CDPKs) play an important role in plant cell responses to stress and pathogenic attack. In this study, we investigated the effect of heterologous expression of the Arabidopsis CDPK gene, *AtCPK1*, on anthraquinone production in transgenic *Rubia cordifolia* cells. *AtCPK1* variants (a constitutively active, Ca^{2+}-independent form and a non-active form used as a negative control) were transferred to callus cells by agrobacterial transformation. Overexpression of the constitutively active, Ca^{2+}-independent form in *R. cordifolia* cells caused a 10-fold increase in anthraquinone content compared with non-transformed control cells, while the non-active form of *AtCPK1* had no effect on anthraquinone production. Real-time PCR measurements showed that the activation of anthraquinone biosynthesis in transgenic calli correlated with the activation of isochorismate synthase gene expression. The activator effect of *AtCPK1* was stable during prolonged periods of transgenic cell cultivation (more than 3 years) and the transgenic cultures exhibited high growth. Our results provide the first evidence that a *CDPK* gene can be used for the engineering of secondary metabolism in plant cells.

Biotechnol. Bioeng. 2011;108: 1734–1738.

KEYWORDS: calcium-dependent protein kinase; *Rubia cordifolia*; anthraquinones; isochorismate synthase; callus culture; metabolic engineering

Calcium-dependent protein kinases (CDPKs) represent a class of a multigene family that is ubiquitous in the plant kingdom and serves as one of the components in a complex calcium decoding system (Harper et al., 2004). CDPKs participate in the regulation of a variety of cell functions, and play an important role in biotic and abiotic plant stress responses (reviewed in Cheng et al., 2002; Ludwig et al., 2004).

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Contract grant sponsor: Russian Foundation for Basic Research
Contract grant sponsor: Far East Branch of the Russian Academy of Sciences
Additional Supporting Information may be found in the online version of this article.

The idea that CDPKs could be involved in the regulation of secondary metabolite biosynthesis was initially developed by the use of common protein kinase inhibitors and CDPK antagonists that decreased accumulation of phytoalexins in different plants (Choi and Bostock, 1994; Vitrac et al., 2000). Identification of specific CDPK isoforms in French bean and Arabidopsis that could phosphorylate phenylalanine ammonia lyase (PAL), a key enzyme in defense responses, provided an apparent link between secondary metabolism and stress perception via CDPK (Allwood et al., 1999, 2002; Cheng et al., 2001). To our knowledge, the effect of heterologous expression of *CDPK* genes on secondary metabolism in plants or in plant cell cultures has not yet been studied.

AtCPK1 is an intensively studied member of the Arabidopsis CDPK gene family. Expression of *AtCPK1* in tomato protoplasts enhanced the activity of NADPH oxidase and led to the oxidative burst (Xing et al., 2001). Native AtCPK1 is localized in the membrane of peroxisomes and may play a role in the regulation of oxidative stress (Dammann et al., 2003). The PAL enzyme was identified as one of the possible substrates for AtCPK1 phosphorylation, thus providing a link between CDPK and defense responses in plants (Cheng et al., 2001).

In the present study, we investigated the effect of expression of the Arabidopsis *AtCPK1* gene on growth and the production of secondary metabolites in *Rubia cordifolia* cell cultures. *R. cordifolia* calli, which produce colored phytoalexins (anthraquinones), represent a well-known model for studies of effects of transgenes on secondary metabolism (Bulgakov et al., 2010; Shkryl et al., 2008).

Transgenic calli R-Ca (callus line transformed with AtCPK1-Ca, a constitutively active, Ca^{2+}-independent form) and R-Na (callus line transformed with AtCPK1-Na, a non-active form) were obtained via Agrobacterium-mediated transformation of *R. cordifolia* suspension culture R as described in the Materials and Methods Section. Selection of transgenic callus lines was achieved through five passages on the medium supplemented with kanamycin.

After 2–3 weeks of selection, the AtCPK1-Ca transformed culture (R-Ca) began to form red-colored callus aggregates, whereas the AtCPK-Na transformed culture (R-Na) formed yellow-colored tissues.

cDNA samples were prepared from control and transgenic cultures and RT-PCR amplification with primers corresponding to *RcActin* was performed to verify that the cDNA was native and free of genomic DNA (Fig. 1A). Gene-specific RT-PCR analysis showed that *R. cordifolia* calli, transformed with AtCPK1-Ca and AtCPK-Na, expressed both *NPTII* and *AtCPK1* gene sequences (Fig. 1B and C). The type of mutation of the *AtCPK1* gene in corresponding callus cultures was confirmed by sequencing.

All callus cultures grew vigorously as friable tissues, differing in color. The R and R-Na cell lines were yellow-orange while the R-Ca culture was orange-red (Fig. 2). Investigation of growth parameters of these cultures showed that expression of either the constitutively active, Ca^{2+}-independent form or the non-active form of *AtCPK1* did not significantly affect callus growth (Table I). Indeed, all of these cultures possessed a high growth: growth indexes were calculated to be 18.0, 16.6, and 17.5 for the R, R-Ca, and R-Na lines, respectively. Dynamics of callus growth of non-transgenic and transgenic cultures was similar and could be described by standard sigmoid curves.

All cultures produced two major AQ, with munjistin and purpurin representing 90% of the total anthraquinone yield. Minor AQs were presented by ruberritrinic acid, alizarin, xanthopurpurin, and trace amounts of metoxymunjistin and ethoxyxanthopurpurin (data not shown). When the calli were analyzed at the exponential phase of growth (5–12 days of cultivation) munjistin content was low and stable. When the calli entered into the linear phase of growth (13–23 days), munjistin content began to rise rapidly and continued to grow at the stationary phase of growth (24–32 days). Purpurin content was nearly stable during the entire growth cycle. After 35 days of cultivation, callus growth ceased and AQ content decreased.

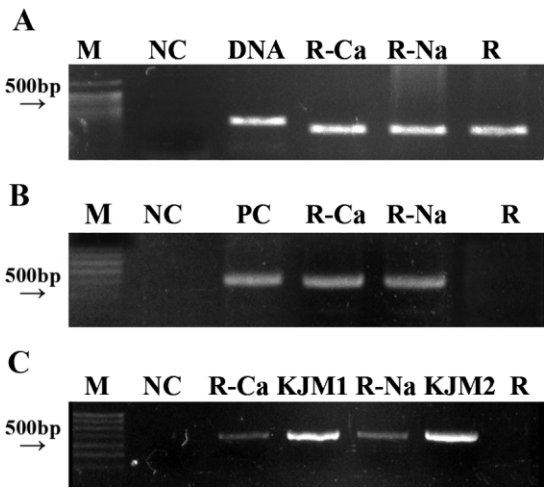

Figure 1. Agarose gel electrophoresis of RT-PCR products. **A**: RT-PCR products of *RcActin* generated on cDNA from R-Ca, R-Na, and R callus cultures. DNA—PCR products generated on DNA isolated from *Rubia cordifolia* plants. **B**: RT-PCR products of *NPTII* generated on cDNA from R-Ca, R-Na, and R callus cultures. PC—positive PCR control (pART27 DNA). **C**: RT-PCR products of *AtCPK1* generated on cDNA from R-Ca, R-Na, and R callus cultures. KJM1—PCR products generated on pART27/AtCPK1-Ca DNA. KJM2—PCR products generated on pART27/AtCPK1-Na DNA. NC—negative controls (no RT-enzyme added). M—DNA markers (100 bp + 1.5 kb ladder, SibEnzyme, Novosibirsk, Russia).

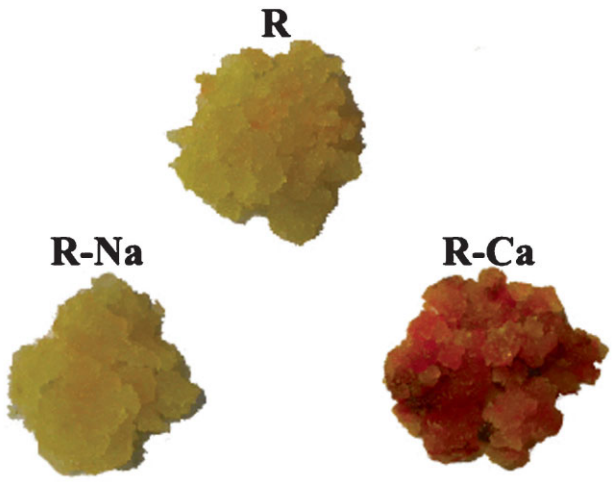

Figure 2. Phenotypes of the *Rubia cordifolia* callus cultures. R, untransformed callus line; R-Na, callus line transformed with non-active *AtCPK1*; R-Ca, callus line transformed with constitutively active *AtCPK1*. Cultures were grown for 3 weeks on $W_{B/A}$ medium. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/bit]

As shown in Table I, transformation with the non-active *AtCPK1* gene did not cause changes in AQ content in transgenic calli compared to non-transformed culture. The level of AQ was approximately 0.4% in both cultures. On the contrary, the constitutively active form of *AtCPK1* caused a significant activation of AQ biosynthesis. AQ accumulation in R-Ca cells was 4.36% of callus dry weight, 10 times higher as compared with R-Na and non-transformed *R. cordifolia* cells (Table I).

Isochorismate synthase (ICS) plays a key role in anthraquinone biosynthesis (Han et al., 2001). The *RcICS* gene was previously isolated from *R. cordifolia* calli and its expression was shown to be tightly connected with AQ accumulation (Bulgakov et al., 2008; Shkryl et al., 2008). To estimate the effect of heterologous *CDPK* expression on the *RcICS* gene expression, we performed quantitative real-time RT-PCR analysis. Our results show an eightfold increase in *RcICS* expression in R-Ca cell culture (Table I). A high positive correlation dependence was revealed between total AQ content and *RcICS* expression in R and R-Ca calli using Pearson analysis ($r = 0.98$, $P < 0.01$).

Thus, we demonstrated that engineering of plant secondary metabolism can be achieved by using the heterologous expression of a CDPK gene. The gene encoding

Table I. Biomass accumulation, anthraquinone content, and expression of the *RcICS* gene in non-transgenic and *AtCPK1*-transgenic callus cultures of *R. cordifolia*.

Callus line	Fresh biomass (g/L)[a]	Purpurin (% dry wt)[a]	Munjistin (% dry wt)[a]	Total (% dry wt)[a]	AQ production (mg/L)[a]	Expression of the *RcICS* gene (relative fold expression)[b]
R (non-transformed culture)	380 ± 32	0.12 ± 0.03	0.32 ± 0.07	0.44 ± 0.06	62 ± 8	0.12 ± 0.01
R-Ca (constitutively active *AtCPK1*)	348 ± 25	$0.48 \pm 0.04^*$	$3.88 \pm 0.16^*$	$4.36 \pm 0.08^*$	$523 \pm 36^*$	$1.00 \pm 0.03^*$
R-Na (non-active *AtCPK1*)	377 ± 31	0.10 ± 0.02	0.32 ± 0.09	0.42 ± 0.23	55 ± 6	0.09 ± 0.01

[a]Mean values ± SE. The experiments were repeated four times (with 10 replicates each) during 2-year cultivation of the cultures (2009–2010).
[b]Data are expressed as mean ± SE from two biological replicates, resulting from two different RNA extractions, with three technical replicates for each biological replicate.
*Statistically significant differences at $P < 0.05$, Fisher's LSD.

the constitutively active, Ca^{2+}-independent kinase was used as a dominant, positive transgene. Expression of this gene in transgenic *R. cordifolia* calli caused a substantial increase of AQ content. Expression of the negative transgene, a non-active *AtCPK1*, in which kinase activity was hampered by mutagenesis, failed to promote AQ accumulation. This result suggests that the effect of activation was caused by the kinase activity of CDPK, and not by other factors, such as T-DNA insertional mutagenesis.

It is interesting to compare the effect of different transgenes on AQ content in *R. cordifolia* cells. Such pronounced activation of AQ biosynthesis in cells, as described here, was reported previously only for the *rolB* gene of *Agrobacterium rhizogenes*, which caused a 14-fold increase of AQ content, corresponding to ~5.85% of the dry weight (Shkryl et al., 2008). However, this effect was accompanied by a substantial loss of cell biomass. The present investigation indicates that the *AtCPK1*-mediated activation of AQ biosynthesis do not suppress cell growth. These results allow us to suggest CDPKs as a new instrument for secondary metabolite manipulation in cultured plant cells.

Materials and Methods

Callus Cultivation

The untransformed callus line of *R. cordifolia* L. (designated in this work as R) was cultivated in 100 mL Erlenmeyer flasks on $W_{B/A}$ medium supplemented with 0.5 mg/L of 6-benzylaminopurine and 2.0 mg/L of α-naphthaleneacetic in the dark at 25°C with 30-day subculture intervals as described (Bulgakov et al., 2002). Transgenic callus lines were cultivated under the same conditions. Inoculum biomass was 200 mg (each callus was weighted using an electronic balance). Samples were harvested from 30 days cultures, weighted and used for RT-PCR. Then the calli were dried under hot air flow (60°C for 5 h) and used for determination of anthraquinones. Growth indexes were calculated as follows: $(W - W_0)/W_0$, where W_0 is callus weight at the beginning of cultivation and W is resulting callus weight after 30 days of cultivation.

Vector Construction

The KJM1 and KJM2 junction domain mutants of the *Arabidopsis thaliana* calcium-dependent protein kinase gene CPK1 (AK1 isoform; GenBank accession no. L14771) were engineered by Harper et al. (1994). The KJM1 mutation (designated in this work as AtCPK1-Ca) converted wild-type AtCPK1 to the constitutively active, Ca^{2+}-independent form (Harper et al., 1994). The KJM2 mutation (designated in this work as AtCPK1-Na) converted wild-type AtCPK1 to the non-active form (Huang et al., 1996). The mutated CDPK genes subcloned into the plant cassette vector pMON999 under the control of the cauliflower mosaic virus (CaMV) 35S promoter were a gift kindly provided by Professor Tim Xing.

We cloned a 3-kb Not I fragment containing the 35S-CDPK-nos-3′ into the Not I site of plant binary vector pART27 (Gleave, 1992). The resulting constructs, pART27/AtCPK1-Ca and pART27/AtCPK1-Na, also included a gene for kanamycin resistance (*NPTII*) under the control of eukaryotic sequences. Transformation of *Agrobacterium tumefaciens* strain EHA105/pTiBo542 with these vectors was performed by electroporation (BioRad Gene Pulser, 0.1 cm cuvettes, 25 μF, >2.5 kV) in accordance with the manufacturer's protocol.

Plant Transformation and Cell Line Selection

The transformation and selection of *R. cordifolia* cells were performed as described previously (Bulgakov et al., 2002). For transformation, we used a suspension variant of the R culture (Bulgakov et al., 2008). Cell cultures of *R. cordifolia* at the exponential phase of growth (6 days old) were inoculated with *A. tumefaciens* containing either pART27/AtCPK1-Ca or pART27/AtCPK1-Na plasmids. Well-growing aggregates were selected in the presence of kanamycin to produce R-Ca and R-Na lines of kanamycin-resistant calli.

Gene Expression

Isolation and analysis of total RNA from *R. cordifolia* cultures, cDNA synthesis and quantitative real-time PCR

Table II. Sequences of gene-specific primers used in this study.

Target genes	Forward primer (5′-3′)	Reverse primer (5′-3′)	Expected size (bp)	Calculated efficiency (E) for qPCR reactions and correlation coefficients (r^2) for calibration curves
Primers for RT-PCR analysis				
RcAct	GAGCACGGTATTGTTAGCAATTG	GATCAAGACGGAGAATGGCATGT	320	—
NPT II	GAGGCTATTCGGCTATGACTG	ATCGGGAGCGGCGATACCGTA	697	—
CDPK	TGTCTGGAGTGCTGGAGTGATTGTG	TAGTCTATTCGCCCGTCATTGTCTT	741	—
Primers for qPCR				
RcAct	GATTGAGCACGGTATTGTTAG	ACACCATCACCAGAATCCAAC	266	$E = 101.2\%$, $r^2 = 0.974$
18S	GGAGAGGGAGCCTGAGAAAC	GATTTAGATTGTACTCATTCC	146	$E = 95.7\%$, $r^2 = 0.970$
RcICS	TATCGTGCGAGAGAACATCA	GAATTCTGGCCAGTTTGCGA	94	$E = 100.5\%$, $r^2 = 0.995$

(qPCR) analysis were performed as described previously (Shkryl et al., 2008, 2010). A detailed description of RT-PCR analysis is presented in Supplementary Online Material. The gene-specific primers used in qPCR and RT-PCR analyses are listed in Table II. As a reference gene for the normalization of expression data in qPCR, we used *RcActin* (Shkryl et al., 2008). Stability of the *RcActin* gene expression was confirmed using the *18S* gene expression. Primer efficiency of >95% was confirmed with a standard curve spanning seven orders of magnitude (Table II). Data were analyzed using CFX Manager Software (Version 1.5; Bio-Rad Laboratories Inc., Hercules, CA).

AQ Content

Purpurin and munjistin were previously identified in *R. cordifolia* calli as major anthraquinones (Mischenko et al., 1999). The content of these anthraquinones was determined in 30 days cultures photometrically with a UV–Vis Spectrophotometer 1240 (Shimadzu Corp., Kyoto, Japan) as described (Bulgakov et al., 2010).

Statistical Analysis

All values are expressed as the mean ± SE. For comparison among multiple data, analysis of variance (ANOVA) followed by a multiple comparison procedure 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.

This work was supported by a grant from the Russian Foundation for Basic Research, by the Grant Program "Molecular and Cell Biology" of the Russian Academy of Sciences and by grants from the Far East Branch of the Russian Academy of Sciences. We would like to express our gratitude to Professor Tim Xing for kindly providing plasmid clones. We also thank Dr. Sergey Titov and Dr. Alexey Kochetov for useful comments on molecular cloning.

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