Acta Societatis Botanicorum Poloniae

DOI: 10.5586/asbp.3513

Publication history
Received: 2015-12-08
Accepted: 2016-09-07
Published: 2016-09-30

Handling editor
Grzegorz Jackowski, Faculty of Biology, Adam Mickiewicz University, Poland

Authors' contributions
PS: study idea, design, and promoter cloning, in silico and microarray analysis; ES, RG: plant culture and transformation; MŻ, AJ, EB: RNA isolation and RT-PCR

Funding
Research supported by statutory funds of the following departments at Medical University of Łódź: Pharmaceutical Biotechnology (503/3-012-02/503-31-003); Pharmaceutical Biochemistry and Molecular Diagnostics, (503/3-015-02/503-01); Biology and Pharmaceutical Botany (503/3-012-01/503-01). Research was also supported by Dean Elżbieta Mikiciuk-Olasik, the Dean of the Faculty of Pharmacy, Medical University of Łódź (500/3-012-02/500-43-310).

Competing interests
No competing interests have been declared.

Citation
Szymczyk P, Skała E, Grąbkowska R, Jeleń A, Żebrowska M, Balcerczak E. Isolation and characterization of a copalyl diphosphate synthase gene promoter from *Salvia miltiorrhiza*. Acta Soc Bot Pol. 2016;85(3):3513. http://dx.doi.org/10.5586/asbp.3513

ORIGINAL RESEARCH PAPER

Isolation and characterization of a copalyl diphosphate synthase gene promoter from *Salvia miltiorrhiza*

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Abstract

The promoter, 5′UTR, and 34-nt 5′ fragments of protein encoding region of the *Salvia miltiorrhiza* copalyl diphosphate synthase gene were cloned and characterized. No tandem repeats, miRNA binding sites, or CpNpG islands were observed in the promoter, 5′UTR, or protein encoding fragments. The entire isolated promoter and 5′UTR is 2235 bp long and contains repetitions of many *cis*-active elements, recognized by homologous transcription factors, found in *Arabidopsis thaliana* and other plant species. A pyrimidine-rich fragment with only 6 non-pyrimidine bases was localized in the 33-nt stretch from nt 2185 to 2217 in the 5′UTR. The observed *cis*-active sequences are potential binding sites for *trans*-factors that could regulate spatio-temporal *CPS* gene expression in response to biotic and abiotic stress conditions. Obtained results are initially verified by in silico and co-expression studies based on *A. thaliana* microarray data.

The quantitative RT-PCR analysis confirmed that the entire 2269-bp copalyl diphosphate synthase gene fragment has the promoter activity.

Quantitative RT-PCR analysis was used to study changes in *CPS* promoter activity occurring in response to the application of four selected biotic and abiotic regulatory factors; auxin, gibberellin, salicylic acid, and high-salt concentration.

Keywords

promoter; cloning; *trans*-factor; bioinformatics; microarray data; RT-PCR

Introduction

Tanshinones are abietane-type norditerpenoid quinones found in the Chinese medicinal herb *Salvia miltiorrhiza* Bunge [1,2]. They are synthesized from a common precursor geranylgeranyl diphosphate (GGPS) in a sequential pair of cyclization reactions [1]. The first reaction, catalyzed by copalyl diphosphate synthase (EC 5.5.1.12), is based on carbon–carbon double-bond protonation that leads to the production of copalyl diphosphate (CPS). Subsequent cyclization and rearrangement reactions, catalyzed by kaurene-like synthase (KLS), result in the formation of a miltiradiene moiety, a key intermediate in tanshinone biosynthesis [3,4]. Both CPS and KSL enzymes were previously cloned in *S. miltiorrhiza*. Their cDNA, together with that of GGPS synthase and farnesyl synthase, was then incorporated in modular miltiradiene biosynthesis pathway in yeast (*Saccharomyces cerevisiae*). This approach allowed

miltiradiene concentrations 365 mg/L to be reached in a 15-L bioreactor [5]. CPS is also the first committed enzyme in plant hormone gibberellin biosynthesis [1].

Metabolic engineering approaches to increase the concentration of particular metabolite are generally based on the overexpression of crucial, rate-limiting enzymes in homologic or heterologic hosts [5]. Instead of using strong, constitutive promoters to achieve overexpression of crucial enzyme genes, the transcriptional regulation of particular pathway in native plant condition could be achieved by controlling the overexpression of particular *trans*-factors, which play a significant role in regulation of rate-limiting pathway enzymes [6].

Therefore, data related to promoter structure and distribution of *cis*-active elements are important and could be used to produce modified or synthetic promoters, that are active under particular environmental conditions or respond positively to the action of particular *trans*-factor. Such promoters could be used to regulate the expression of genes encoding rate-limiting enzymes of a particular secondary metabolite pathway in a concerted way. As a result, greater concentrations of a particular secondary metabolite, often of significant medical importance can be achieved [7].

However, the detailed promoter structure of *CPS* gene is as yet unknown. Available results of deletion mutagenesis of the *CPS* gene from *Arabidopsis thaliana* indicate that several promoter regions together with intron 1 and 2, are needed for efficient expression of the *gus* reporter gene. The *CPS* gene region localized at −997 to −726, containing the putative ACGT (−934 to −931) *cis*-active motif is responsible for seed specific gene expression [8,9].

In the present report, the isolation and in silico characterization of *CPS* promoter and 5′-untranslated region (5′UTR) from *S. miltiorrhiza* (GenBank accession number KF718290.2) is described. Moreover, both the activity of isolated fragment and its relative strength as compared to the CAMV35S promoter were revealed in the RT-PCR analysis of the transformed *S. miltiorrhiza* plants. The influence of auxin, gibberellin, salicylic acid, and high-salt concentration on promoter activity was assessed: *gus* gene expression being measured 0, 12, 24, 48, 72, and 96 hours after hormonal treatment, or during a 96-hour exposure to 100 mM NaCl.

Material and methods

Plant material

Salvia miltiorrhiza seeds were provided by the Medicinal Garden of the Department of Pharmacognosy at the Faculty of Pharmacy, Medical University of Łódź (Poland). Young plants up to 8 weeks old were cultivated at 26°C ±2°C and natural light in pots of 0.5 L (diameter 12 cm) containing composite soil.

Isolation of genomic DNA

The genomic DNA was isolated from fresh plant material according to Khan et al. [10]. The purity and concentration of DNA was assessed on the basis of A_{260}, $A_{260/280}$, and $A_{260/230}$ parameters using a P300 Nanophotometer (Implen, Germany).

Promoter isolation and in silico analysis

The putative promoter region of the *S. miltiorrhiza CPS* gene was isolated using Genome Walker™ Universal Kit (Clontech, USA) according to the manufacturer's instructions. A 5′-terminal fragment of cDNA sequence deposited in GenBank at accession number EU003997.1 was used as a target for two gene specific primers GSP1 and GSP2 presented in the Fig. 1. Amplified DNA fragments were TOPO-TA cloned into a pCR®II-TOPO® vector (Life Technologies, USA) and sequenced. The obtained sequence was searched for *cis*-active elements, tandem repeats, CpG/CpNpG islands and microRNA binding sites using PlantPAN and PlantPAN 2.0 [11,12]. TSSP software

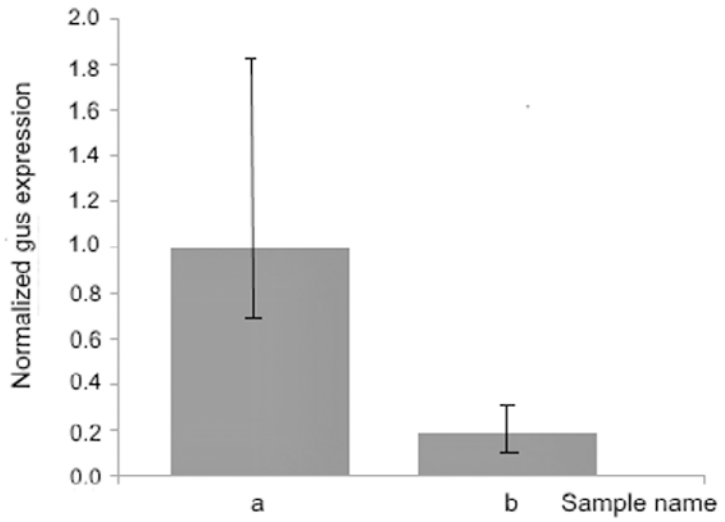

Fig. 1 Quantitative RT-PCR analysis of *GUS* expression, performed on *S. miltiorrhiza* plants transformed by pXK2FS7 (**a**) or CPS-pKGWFS7 (**b**) plasmids. Results presented as normalized *gus* expression.

and RegSite Plant DB, (Softberry Inc., USA) were employed to localize TATA-box and transcription initiation site (TIS).

The reliability of PlantPAN2.0 was ascertained as described by Chow et al. [12]. The *A. thaliana* transcription factor (TF) binding sites used in PlantPAN2.0 were experimentally verified by high-throughput, in vitro protein binding microarray technology. In total, 12 829 TFs and 615 position weight matrices were integrated into PlantPAN2.0. The IUPAC consensus motifs of plant TFs were extracted manually from the Uniprot database. These matrices were supplemented with 548 up-to-date matrices from JASPAR, TRANSFAC (public release 7.0), and PLACE. Finally, 19 960 TFs and 1143 matrices of TF binding sites from 76 plant species were introduced into PlantPAN2.0 [12]. Match™ software was used in PlantPAN2.0 to scan putative *cis*-acting sequences against its library of positional weight matrices. The cut-off profiles of TF-matrices and matrices without TF, curated in PlantPAN2.0 and TRANSFACR, were trained using the following essential parameters: minimize false negative matches (minFN), minimize false positive matches (minFP), and minimize the sum of both error rates (minSUM). To calculate the minFN, random sequences (100 000 sequences, 50 bp per sequence) were generated and scanned with TFBS weight matrices using Match™ without any cut-off threshold. To find the minFN cut-off, the core and matrix similar score were estimated by selecting a score recognizing at least 90% of the oligonucleotides. The minFN cut-off was used to scan the 30 000 reliable promoters of *A. thaliana*, *Oryza sativa*, and *Glycine max*. As a result, the minFP value was estimated as a score that recognized the best 1% of hits. As the trained minFP is set as the cut-off profile in PlantPAN2.0, strict scanning results are obtained [12].

Preparing the CPS-pKGWFS7 plasmid

The 2.3-kb *Sal*I/*Eco*RI fragment, encompassing the *CPS* promoter was cloned into the donor pENTR™ 4 Dual Selection plasmid. Finally, the *CPS* promoter was transferred into the acceptor Gateway™ vector pKGWFS7 through GATEWAY™ LR homology recombination among attL1/attL2 and attR1/attR2 sites, catalyzed by the Gateway® LR Clonase® II enzyme mix (Thermo Fisher Scientific, USA).

Plant transformation

The *Agrobacterium tumefaciens* GV2260 competent cells were transformed using heat shock protocol [13] by CPS-pKGWFS7 or pXK2FS7 plasmids. The *A. tumefaciens* GV2260 line was a kind gift from Prof. Dirk Inzé (Gent University, Belgium). The leaves of *S. miltiorrhiza* plants were used as explants and transformed according to Yan and Wang [14].

PCR analysis

Total genomic DNA was isolated from young leaves of putative transformants and untransformed (control) plants using the Isolate II Plant DNA kit (Bioline, Singapore). The purity and concentration of DNA was assessed on the basis of A_{260}, $A_{260/280}$, and $A_{260/230}$ parameters using a P300 Nanophotometer (Implen, Germany).

The *GUS* gene fragment (162 bp) was amplified using *GUS* F 5′-TCAGCGCGAAGTCTTTATAC-3′ and *GUS* R 5′-ATAACATACGGCGTGACATC-3′ primers.

The *EGFP* gene fragment (416 bp) was amplified by *EGFP* F 5′-AGTGCTTCAGCCGCTACCCCGACCACAT-3′ and *EGFP* R 5′-GATCGCGCTTCTCGTTGGGGTCTTTGCTCA-3′ primers.

The PCR reaction was performed in a volume of 25 µL. A 50-ng sample of freshly-prepared genomic DNA was used as a template. The primer concentration was 0.4 µM. The PCR reaction mixture contained also 2.5 mM Mg^{2+}, nucleotide triphosphates (0.25 mM each), 1 U of TaqNova DNA polymerase (DNA Gdańsk, Poland) and an appropriate, 1×-concentrated PCR buffer. The PCR reaction parameters were as follows: initial denaturation (95°C, 3 min), denaturation (95°C, 1 min), primer annealing (54°C for *gus* and 70°C for *egfp*, 1 min), extension (72°C, 1 min), and final extension (72°C, 3 min). In total, 40 PCR cycles were performed. The salt adjusted (50 mM Na^+) temperatures of primer melting (T_M) were computed by the OligoCalc on-line calculator [15]. The temperature of primer annealing was set 2–4°C below the calculated salt adjusted T_M. The PCR reaction was realized in the MyCycler™ Thermal Cycler (Biorad, USA).

Fluorescence analysis

The detection of EGFP fluorescence in leaf cross sections of transformed or control plants was carried out using a model Axio Scope A1, Carl Zeiss, Germany. An AxioCam MRm Rev. 3 FireWire camera was used to capture the fluorescence image. Observations and image captures were carried out at 150× magnification using EC Plan-Neofluar objectives. Blue light was provided by an LED 470 nm module. The 520-nm emission filter was used for observations under blue light.

The emission spectrum of chlorophyll *a*, produced by photosystems I and II is approximately in the range of 640–800 nm. As the excitation spectrum for EGFP is localized between 470 and 600 nm (emission max. at 509 nm), the 520-nm emission filter efficiently removes the chlorophyll *a* autofluorescence, allowing only the EGFP signal to pass [16].

Confirmation of plant transformation

The transformation of plant material was confirmed by PCR analysis of genomic DNA. The results of the ethidium-bromide agarose gel electrophoresis of PCR products specific for transformed plants are presented in Fig. S1. Plant transformation was confirmed through microscope fluorescence analysis (Fig. S2a–c).

The leaves of transformed plants leaves revealed strong EGFP-mediated fluorescence (Fig. S2a,b), while only unspecific fluorescence localized on vascular bundle elements was observed in control plants (Fig. S2c). This phenomenon is mediated by lignified secondary cell wall fluorescence in the green range with emission spectra of 440–540 nm, which overlaps with EGFP's emission spectra of 507 nm [17].

Biotic and abiotic treatments for transgenic *Salvia miltiorrhiza* plants

For the hormonal treatment, transgenic *S. miltiorrhiza* plants transformed by CPS-pKGWFS7 plasmid were sprayed with 2.85 µM (0.5 mg/L) indole-3-acetic acid (IAA) or 2.88 µM (1.0 mg/L) gibberellic acid (GA). The salicylic acid was used at concentration 0.2 mM (27.62 mg/L). A nonionic detergent (0.05% Tween 20) was used to efficiently damp the plant surface. Plants transformed by CPS-pKGWFS7 treated with sterile water, containing 0.05% Tween 20 were used as controls.

High-salt conditions were obtained by adding 100 mM NaCl (5.84 g/L) to MS solid medium containing 1.5% of cell culture grade agar (Sigma Aldrich, Poznań, Poland). Transgenic *S. miltiorrhiza* plants, transformed by CPS-pKGWFS7 plasmid, growing on solid MS medium without 100 mM NaCl were used as controls.

For all treatments, samples were taken at 0, 12, 24, 36, 48, 72, and 96 hours.

RNA isolation and cDNA synthesis

Total RNA isolation was performed in accordance with the protocol given in the Isolate Plant II RNA kit (Bioline, Singapore). Plant leaves were cut off and frozen instantly in liquid nitrogen. Approximately 80–100 mg of plant material was processed. The digestion of putative genomic DNA impurities, by RNase-free DNaseI (4 U/sample) was one of the steps used in the Isolate Plant II RNA kit. To check the efficiency of DNaseI digestion, RNA samples without the subsequent reverse transcription reaction were used as negative controls in quantitative, real-time PCR reaction. The RNA concentration was determined spectrophotometrically using a p300 Nanophotometer (Implen, Germany). The isolated RNA had an $A_{260/A280}$ ratio of 1.6–1.8. The isolated RNA samples were stored at −80°C until analysis.

Each type of RNA samples was obtained in triplicate. The RT-PCR reaction was carried out using an Enhanced Avian HS RT-PCR Kit (Sigma-Aldrich, Poland), according to the manufacturer's protocol. The final concentration of RNA in the reaction mixture was 0.01 μg/μL. The reaction mixture consisted of : 1 μL of dNTPs (1 mM final), 1 μL of anchored oligo $(dT)_{23}$ (3.5 μM final), 2 μL of 10× buffer, 1 μL of RNase inhibitor (20 U), 1 μL of Enhanced Avian Reverse Transcriptase (RT; 20 U), volume of RNA sample (required to achieve final concentration of 0.01 μg/μL), and distilled water up to a final volume 20 μL. The reaction was performed at 42°C for 1 hour. The enzyme was inactivated at 80°C for 5 minutes. Synthesized cDNA was stored at −20°C until analysis. As a reference gene the *ubiquitin* was used. Controls without RT were performed to ensure that the DNase I digestion was complete and samples were not contaminated with genomic DNA.

Real-time PCR

The amount of *GUS* and *ubiquitin* transcripts was analyzed by means of real-time PCR. Experiments were performed in duplicate to ensure reproducibility of the technique. Amplification reactions were performed using Rotor-Gene 6000 (Corbet) and SYBR Green Jump Start Tag ReadyMix™ (Sigma-Aldrich, Poland) according to the manufacturer's instructions.

The primer set 5′-TCAGCGCGAAGTCTTTATAC-3′ (forward) and 5′-ATAACATACGGCGTGACATC-3′ (reverse) produced the *GUS* gene fragment of 162 bp. As a reference, *ubiquitin* gene expression was checked for each sample using the 5′-GTTGATTTTTGCTGGGAAGC-3′ (forward) and 5′-GATCTTGGCCTTCACGTTGT-3′ (reverse) primer set [18,19].

Ethidium-bromide gel electrophoresis and the alignment of primers and *Populus trichocarpa* cDNA sequence (FJ438462.1), confirmed the size of the PCR reaction product to be 192 bp. The same analysis performed for *gus* gene primers and *E. coli gus* gene cDNA sequence (AAA68923.1) validated the size of PCR product to be 162 bp [18,19].

The reaction mixture for both genes consisted of 7.5 μL SYBR-Green ReadyMix, 0.7 μL of each primer, 1 μL of cDNA and distilled water to a final volume of 16 μL. The reactions for *GUS* and *ubiquitin* were carried out in separate tubes. Samples were tested in triplicate, and the means of the obtained C_T values for both *GUS* and *ubiquitin* were calculated. In each experiment, a negative control, also tested in triplicate, was included.

The qPCR reaction parameters were as follows: initial denaturation (95°C, 10 min), denaturation (95°C, 20 s), primer annealing (60°C, 30 s), extension (72°C, 20 s). In total, 40 PCR cycles were performed.

The $2^{-\Delta\Delta C_T}$ method by Livak and Schmittgen [20,21] was used to calculate relative changes in gene expression determined from real-time quantitative PCR experiments. Results are shown as the mean ±*SD* of three determinations in three technical repeats. The Rotor-Gene 6000 Series Software 1.7 (Corbett Life Science, Qiagen) was used to analyze the qPCR results.

Significant differences between plants transformed by CPS-pKGWFS7 or pXK2FS7 as well as material treated by biotic or abiotic factors were assessed by one-way ANOVA. A p value <0.05 was considered to be significant [21].

The normalized *GUS* expression in plants transformed by pXK2FS7 plasmid was 1 (0.52–1.82), while the same parameter in plants transformed by CPS-pKGWFS7 was 0.18 (0.12–0.28; Fig. 1a,b). Therefore, the relative strength of the *CPS* promoter represents 0.18 (0.12–0.28) of that of a strong, constitutively active CAMV35S.

Microarray co-expression studies

Microarray data for *A. thaliana* publicly available through the web interface of the database at http://bbc.botany.utoronto.ca were used for co-expression studies. *Trans*-factors and other protein co-expressed with *A. thaliana CPS* gene (At4g02780) were compared with *trans*-factors revealed by the in silico searches of *S. miltiorrhiza CPS* promoter region. Expression Angler software (University of Toronto, Canada; http://bbc.botany.utoronto.ca) was used to identify *A. thaliana* genes that responds similarly in terms of their gene expression levels relative to the control. All samples in the database are subject to such control. The Pearson correlation coefficient (r) was used to identify the co-regulated genes. The cut-of value for r was set up in the range of 0.65–1.00. Results are formatted after median centering and normalization [22].

Excel was used to calculate the p value, based on the Pearson correlation coefficient (r), using the following formula [23]: $p = TDIST\{ABS[r\ SQRT(\{1 - r \times r\}\ \{n - 2\})], [n - 2], 2\}$, where n is a sample number (224).

Results

The structure of *CPS* promoter and 5′UTR of *CPS* gene

The positions of both, the TIS and translation initiation sites allowed the 5′UTR to be qualified (Fig. 2). The TATA-box was localized −33 nt from the TIS. Furthermore, the sequence around the TIS at adenyl nucleotide 2168 (AGACAA, nt 2164–2169) was closely related to the consensus sequence WnT/aC/t A/cw (where W or w = A or T; n = any nt), found in 217 dicot promoters (Fig. 2) [24].

The in silico analysis of the *CPS* promoter region from *S. miltiorrhiza* indicated a lack of tandem repeats, CpG/CpNpG islands, and microRNA (miRNA) target sequences.

However, the in silico analysis revealed the presence of a pyrimidine-rich segment (PRS) located in the 33-nt fragment 2185–2217 in the 5′UTR, wherein only six nucleotides were not pyrimidines (Fig. 2).

A hypothetical leaf specific *cis*-active motif and corresponding *trans*-factor ASF-2 were identified (Tab. 1, Tab. 2) [25]. The potential influence of *CPS* on leaf development supported the presence of *cis*-active motif for Athb1 *trans*-factor (Tab. 1, Tab. 2) [26,27]. Presence of *cis*-active motif for *trans*-factors DPBF1 and 2 supported the view that *CPS* gene in *S. miltiorrhiza* could participate in embryo development [28].

Our results suggest, that the transcription rate of *S. miltiorrhiza CPS* may potentially depend on the availability of ethylene, auxin, salicylic acid, dark and light (Tab. 1, Tab. 2). The results imply the presence of *cis*-active motifs, which could potentially be recognized by putative *S. miltiorrhiza* homologs of the previously characterized Ethylene Response Factor 1 [29,30]. The *cis*-active motifs associated with response to light and dark were found, together with the respective *trans*-factor GT1 which recognize them (Tab. 1, Tab. 2) [31–33]. However, the other potential *cis*-active elements associated with the response to light and auxin as well as the regulation of the circadian clock were also found, without the corresponding *trans*-factors [34–39].

The outcome of the in silico analysis given in Tab. 1 indicated that *S. miltiorrhiza CPS* promoter activity could be controlled through the numerous stress factors occurring in the internal and external environment of the plant as anaerobic conditions, pathogen infection, dehydration, and protein unfolding [40–43].

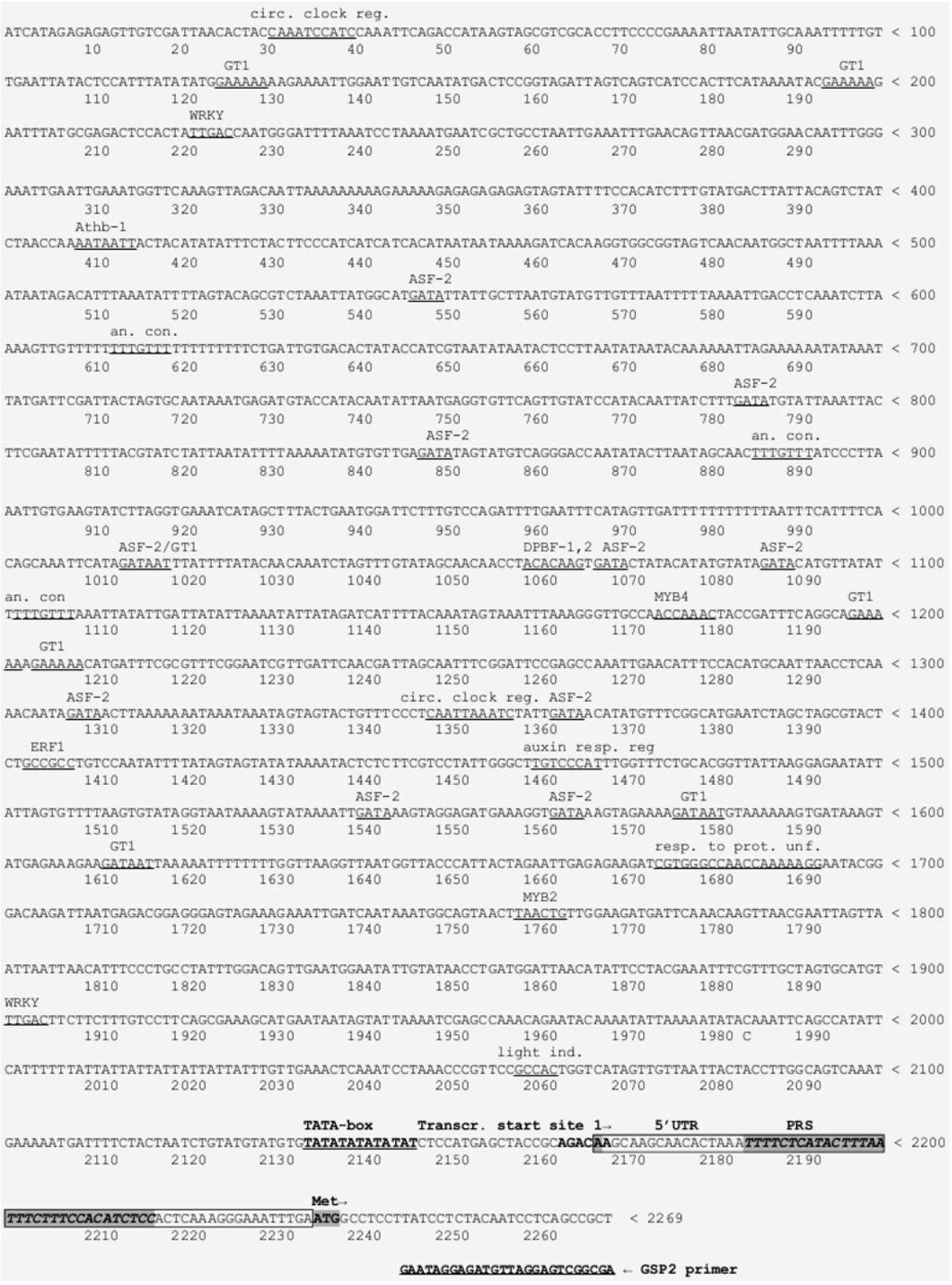

Fig. 2 Sequence of *S. miltiorrhiza CPS* promoter region (nt 1–2269), 5′UTR (nt 2168–2235), and 5′ fragment of *CPS* cDNA (nt 2236–2269). Only strand + is provided. *CPS* gene specific primer GSP2 sequence, TATA-box (nt 2135–2147), and initial Met ATG codon are underlined. Transcription start site at A in the position 2168 and initial Met ATG codon are shaded, pyrimidine rich segment (PRS) in 5′UTR (nt 2168–2235) is shaded and given in italics. Positions of *cis*-active elements were underlined. Following abbreviations were used: an. con. – anaerobic condition; circ. clock reg. – circadian clock regulation; light ind. – light induction; resp. to prot. unf. – response to protein unfolding; auxin resp. reg. – auxin-responsive region.

Tab. 1 The *cis*-active motifs in *S. miltiorrhiza CPS* promoter region and corresponding homologous *trans*-factors from *A. thaliana* and other species, active in hormone, light, and stress-conditions response.

No.	*Cis*-active motif	*Cis*-active motif site	*Trans*-factor and references	Species
Plant embryogenesis				
1	5′-ACACAAG-3′	1060	DPBF-1 and 2 [28]	*A. thaliana*, carrot
Transcription factor expressed in all aboveground plant organs				
Leaf specific transcription factors				
1	5′-GATA-3′	547, 784, 848, 1014, 1068, 1087, 308, 1363, 1541, 1563	ASF-2 [25]	*A. thaliana*, petunia, rice
Leaf development				
1	5′-AATAATT-3′	409	Athb-1 [26,27]	*A. thaliana*
Response to ethylene				
1	5′-GCCGCC-3′	1403	ERF1 [28,29]	*A. thaliana*
Light and hormone response				
2	5′-CAATTAAATC-3′	31, 1349	Circadian clock regulation [34,35]	Tomato
3	5′-GCCAC-3′	2059	Light induction [36,37]	*A. thaliana*
4	5′-GAAAAA-3′	125, 194, 1197, 1204	GT1; light, salt and salicylic acid response [31–33]	*A. thaliana*, pea, oat, rice, tobacco
	5′-GATAAT-3′	1014, 1577, 1612		
5	5′-TGTCCCAT-3′	1459	Auxin-responsive region [38,39]	Pea
Anaerobic conditions				
1	5′-AAACAAA-3′	613, 886, 1102	[40]	*A. thaliana*, maize, pea
Pathogen infection–plant defense				
1	5′-TTGAC-3′	222, 1901	WRKY [41]	*A. thaliana*
Dehydration				
1	5′-TAACTG-3′	1759	MYB2 [42]	*A. thaliana*
Other				
1	5′-CGTGGGCCAAC-CAAAAAGG-3′	1675	Response to protein unfolding [43]	*A. thaliana*

Analysis of *trans*-factors co-expressed with *Arabidopsis thaliana CPS* gene

Large datasets of microarray studies performed on *A. thaliana* allowed to calculate Pearson correlation coefficients (r) among expression level of particular genes. Expression Angler software was used to find out transcription factors and other proteins that are co-expressed with *A. thaliana CPS* gene (At4g02780) and compare them to *trans*-factors putatively interacting with *A. miltiorrhiza CPS* promoter region, predicted by the in silico searches [22,23].

The following microarray dataset compendiums were used: AtGenExpress hormone and chemical, AtGenExpress abiotic stress, AtGenExpress pathogen, and AtGenExpress Plus – extended tissue. The AtGenExpress pathogen compendium contained four genes co-expressed with *A. thaliana CPS* gene. None of them was a *trans*-factor. Many more (224) co-regulated genes, including *trans*-factors were found in the AtGenExpress Plus – extended tissue compendium [22,23]. It was found that $p < 0.00001$ for all obtained r values.

Tab. 2 Molecular properties of *cis*-active elements found in the *S. miltiorrhiza CPS* promoter by in silico searches.

No.	*Cis*-active motif	Function and reference
1	5′-ACACAAG-3′	Sequence found in the proximal region of carrot *Dc3* gene promoter (−117 to −35). Binding of the sequence (consensus ACACNNG) by basic leucine zip *trans*-factors DPBF-1 and 2 regulates plant embryogenesis. The sequence was used to clone cDNA of DPBF-1 and 2 in carrot by yeast one-hybrid system [28].
2	5′-GATA-3′	Element conserved among promoters of light-responsive genes, particularly chlorophyll *a*/*b*-binding proteins (*Cab*). The sequence is recognized by the ASF-2 factor. However, the DNA binding by ASF-2 is not related to light-sensitive but rather leaf-specific expression [25].
3	5′-AATAATT-3′	Sequence AAT(A/T)ATT is a dyad-symmetric fragment recognized by homeodomain and leucine zipper motifs of Athb-1 *trans*-factor. The binding site was verified by a gel mobility shift assays [26,27].
	5′-TGTCCCAT-3′	*PS-IAA4/5* domain A is part of the auxin-responsive region (*AuxRR*) of the pea *PS-IAA4/5* promoter, extending from positions −318 to −135. This region contains two auxin-responsive domains (*AuxRDs A* and *B*) defined by linker scanning mutagenesis that mediate auxin-induced gene expression [38,39].
4	5′-GCCGCC-3′	GCC box is recognized by ERF1 *trans*-factor participating in response to ethylene. The ERF-1 binds GCC box as a homodimer, however, binding to DNA is not necessary to dimerization. The GCC-box was also found in many pathogen-responsive genes such as *PDF1.2*, *Thi2.1*, and *PR4* [29,30].
5	5′-CAATTAAATC-3′	A sequence motif conserved in promoter regions of tomato *Lhc* (Light-harvesting complex) genes. The Lhc complex participates in transmitting circadian rhytmicity. Identical or very similar (consensus CAANNNNATC) motifs were found in promoter regions of circadian controlled *Arabidopsis thaliana Lhc b1*1*, *Lhc b1*2*, *Lhc a3*, and *Lhc a4* genes. The sequence is absent in the promoter of non-circadian expressed *Lhc b* gene of *Pinus contorta* [34,35].
6	5′-GCCAC-3′	The sequence is the core region of sequence over represented in light induced promoters (SORLIP 1) of light-inducible genes regulated by a phytochrome A system. Among other SORLIPs, the SORLIP1 is the most over-represented and most statistically significant element. The localization of SORLIP 1 appears to be strain independent, because the strongest hits are observed when both strands are considered. The core 5′-GCCAC-3′ sequence may be flanked by A at the 5′ and G or A at the 3′ end [36,37].
7	5′-GAAAAA-3′	GT-1 motif found in the promoter of *Glycine max* calmodulin isoform SCaM-4 which plays a role in pathogen- and salt-induced *SCaM-4* gene expression [31].
	5′-GATAAT-3′	Consensus GT-1 binding site (GRWAAW) in many light-regulated genes, e.g., *RBCS* from many species, *PhyYA* from oat and rice, spinach *RCA* and *PETA*, and bean *CHS15*, where R = A/G; W = A/T [32,33].
	5′-AAACAAA-3′	The sequence motif found in majority (9) of 13 analyzed promoters of genes belonging to the ethanol fermentation pathway, that are known to be positively regulated in anaerobic conditions. The group of analyzed genes belongs to the seven different plant species [40].
	5′-TTGAC-3′	The sequence localized in the promoter region of *A. thaliana NPR1* gene. The expression of *NPR1* is induced by pathogen infection or treatment with defense-inducing compounds such as salicylic acid. As a result of *NPR1* overexpression, transgenic plants are more resistant to broad spectrum of microbial pathogens. The activation of *NPR1* promoter depends on recognition of 5′-TTGAC-3′ sequence by *A. thaliana* WRKY18 protein. Mutations of the 5′-TTGAC-3′ sequence abolished their recognition by *A. thaliana* WRKY18, resulting in decreased *NPR1* expression and inhibition of pathogen resistance development [41].
	5′-TAACTG-3′	Gel mobility experiment indicated that the consensus sequence 5′-TAACTG-3′ is recognized specifically by *A. thaliana* MYB2 *trans*-factor. The expression of *MYB2* gene is increased by dehydration and inhibited by rehydration. A beta-glucuronidase reporter gene driven by the *Atmyb2* promoter was induced by dehydration and salt stress in transgenic *Arabidopsis* plants [42].
	5′-CGTGGGCCAAC-CAAAAAGG-3′	Sequence (consensus 5′-CNNNNNNNNNNNNCCACG-3′) is associated with the response to stress induced by protein unfolding (UPR). Analysis of the Affymetrix GeneChip (7372 genes) indicated that the sequence is eight-times enriched in UPR upregulated gene pool of *A. thaliana* [43].

The analysis of obtained data indicated that numerous *trans*-factors participate in such processes as plant morphogenesis, plant defense, response to light, hormones, and osmotic stress as well as regulation of apoptosis (Tab. 3) [44–60]. Comparison of the obtained data with *trans*-factors identified by the in silico searches of *S. miltiorrhiza CPS* promoter showed no common protein (Tab. 1, Tab. 3). Therefore, the potential link between the regulation of *A. thaliana* and *S. miltiorrhiza CPS* gene expression may not be evaluated at the level of particular transcription factor. The common elements in the regulation of both genes are related only to reaction to particular biotic and abiotic factors. According to the above information, the co-expression microarray data indicated positive correlation of *A. thaliana CPS* expression with factors participating in response to red light (AtMYB18/LAF1), circadian clock regulation (CCT motif family protein) and auxin metabolism (AtIDD16; Tab. 3) [45,52,53].

Tab. 3 Transcription factors co-expressed with *CPS* in *A. thaliana*. Pearson correlation coefficient (r) characterizes the co-regulation rate ($p < 0.00001$).

No.	*Trans*-factor	*r*	Function
Plant morphogenesis			
1	AtMYB98	0.654	Pollen development [44]
2	AtIDD16	0.660	Leaf and floral organ morphogenesis [45]
3	NAC domain containing protein 86 (NAC86)	0.660	Sieve element enucleation and differentiation [46]
4	Agamous-like 36	0.672	Seed development [47]
5	Agamous-like 57	0.674	Ovule and seed development [48]
6	AtMYB64	0.699	Female gametogenesis regulation [49]
7	Agamous-like 28	0.715	Flowering regulation [50]
8	RKD5	0.717	Gametophyte development [51]
Light and hormone response			
1	AtMYB18, (LAF1)	0.653	Far-red light signaling pathway [52]
2	CCT motif family protein	0.703	Circadian clock regulation [53]
3	AtIDD16	0.660	Regulation of auxin metabolism [45]
Plant defense			
1	Cysteine/Histidine rich C1 domain family proteins	0.655, 0.663, 0.695, 0.735, 0.736, 0.740	Proteins involved in regulation of higher plant immune system function [54]
Osmotic stress response			
1	AtMYB41	0.678	Osmotic stress response [55]
Regulation of apoptosis			
1	RING/U-box superfamily protein	0.674	Regulation of apoptosis [56]
2	F-box/RNI-like/FBD-like domains-containing protein	0.696	Regulation of apoptosis [57]
Other			
1	AtMYB113	0.668	Anthocyanin biosynthesis [58]
2	NIN-like protein 5, NIN5	0.670	Nitrate signaling [59]
3	RKD5	0.717	Response to nitrogen [51]
4	Gibberellin 2-oxidase	0.689	Gibberellin metabolism [60]

These results resembled data obtained by in silico searches in relation to light and hormone response (Tab. 1). Moreover, microarray data implied that *CPS* gene in *A. thaliana* is co-regulated with cysteine/histidine-rich C1 domain family proteins, playing a role in plant defense processes (Tab. 3) [54]. Previous in silico searches of *CPS* promoter from *S. miltiorrhiza* also implied such a function, played here by WRKY (Tab. 1) [41].

Other examples of closely related functions played by both genes was regulation of apoptosis. The apoptosis was regulated in *A. thaliana* by the putative ubiquitin-ligase and F-box/RNI-like/FBD-like domains-containing protein, both co-expressed with the *CPS* gene (Tab. 3) [56,57]. Also in the *S. miltiorrhiza*, *CPS* promoter was observed a *cis*-active motif participating in the response to protein unfolding (Tab. 1) [43]. The occurrence of a functional link between protein unfolding and apoptosis was supported by the activation of apoptosis pathway in reaction to the presence of unfolded proteins [43,56]. Also, the reaction to plant dehydration could be achieved in the *S. miltiorrhiza CPS* promoter by MYB2 *trans*-factor (Tab. 1) [42]. Microarray data revealed that the *A. thaliana CPS* is co-regulated with AtMYB41, playing a related role in response to osmotic stress (Tab. 3) [55].

Proteins co-expressed with CPS in *A. thaliana* include an enzyme of gibberellin 2-oxidase activity (GA2OX3; Tab. 3) [60]. Although GA2OX3 is not a *trans*-factor, it plays an important role in gibberellin biosynthetic and catabolic processes, implying a regulatory link between gibberellin precursor biosynthesis catalyzed by CPS and next stages of biosynthesis and catabolism of the plant hormone [2,60].

Contrary to previous results, the analysis of obtained microarray data in relation to plant morphogenesis indicated rather different functions as compared to in silico searches. The microarray data implied a role of *A. thaliana CPS* in development of such elements as pollen, sieve, seed, ovule, and gametophyte (Tab. 3) [44,46–51]. According to the in silico searches, the *S. miltiorrhiza CPS* promoter should participate rather in leaf development (Tab. 1) [26,27]. This point of view was supported by identification in *A. thaliana trans*-factor participating in leaf morphogenesis (AtIDD16) [45].

Another example of processes that were revealed only by microarray data and not confirmed by the in silico searches were response to nitrogen and anthocyanin biosynthetic pathway regulation (Tab. 3) [51,58,59].

Calibration of RT-PCR reaction

Calibration curves for *gus* and *ubiquitin* genes were $y = -3.25x - 7.3968$ ($R^2 = 1$) and $y = -3.23x - 6.8942$ ($R^2 = 1$), respectively. RNA samples containing the potential contamination of genomic DNA, that were not treated by reverse transcriptase, indicated no amplification.

Response to biotic and abiotic factors

RT-PCR analysis was used to experimentally confirm the activity of the *CPS* promoter in response to high-salt concentration, salicylic acid, auxin, and gibberellic acid as described in "Material and methods" (Fig. 3a–d). Factors that potentially affect *CPS* promoter activity were selected on the basis of in silico searches (salicylic acid, high-salt, and auxin) and microarray co-expression studies (gibberellic acid). Treatment by auxin did not significantly affect the *gus* gene expression (Fig. 3a).

The response to salicylic acid was observed relatively early, with a 1.75-fold (1.41–2.17) increase in *gus* gene expression observed after 12 hours (Fig. 3c). At later stages, salicylic acid was observed to have an inhibitory effect, with expression being only 0.37-times (0.35–0.39) of control values after 24 hours. Following this, *gus* gene activity increased strongly: 18.57-fold (18.00–19.30) after 72 hours and 13.52-fold (10.41–17.66) after 96 hours.

More time was needed for a positive response to gibberellic acid to be observed than for salicylic acid in our experimental model (Fig. 3b). No response to gibberellin was observed before 24 h, when a 1.49-fold (1.46–1.52) increase in *gus* gene

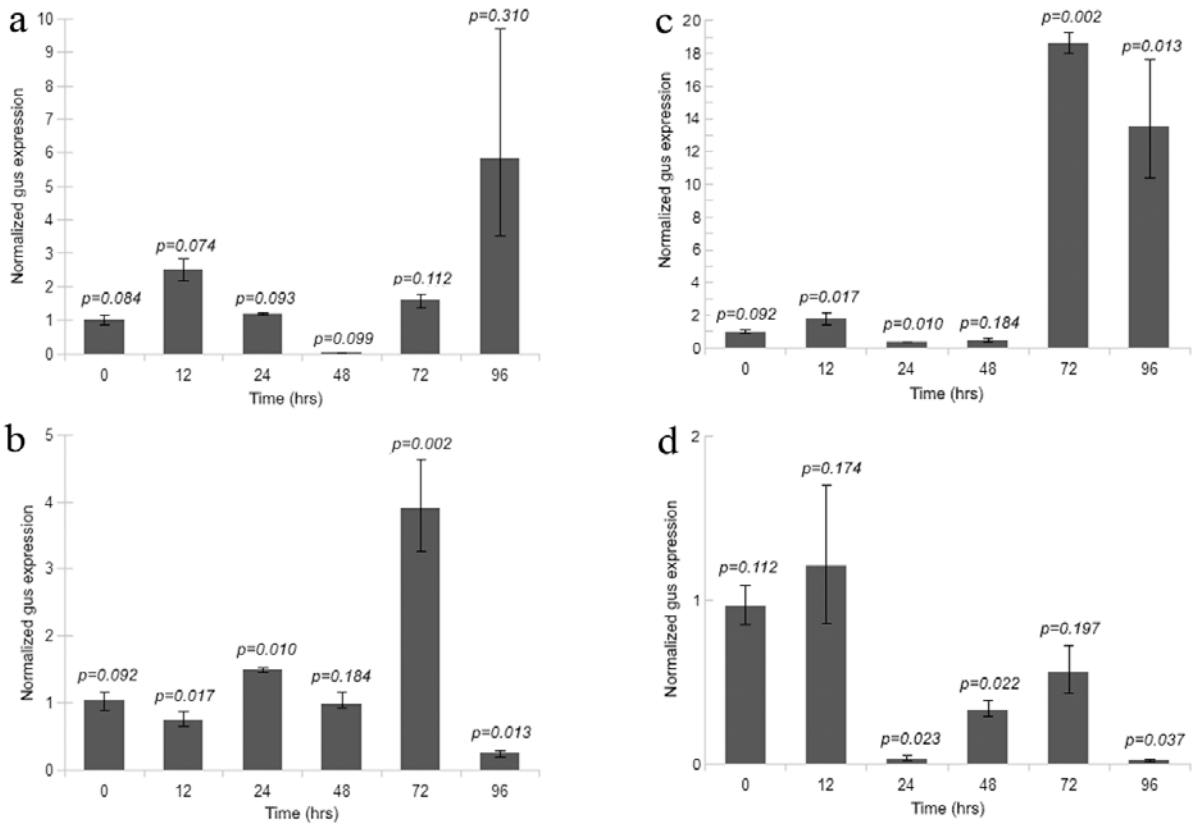

Fig. 3 Temporal changes of *gus* gene expression evaluated at 0, 12, 24, 48, 72, and 96 hours after treatment by auxin (**a**), gibberellin (**b**), salicylic acid (**c**), and 100 mM NaCl (**d**). Results presented as normalized *gus* expression.

expression was seen. Dynamic changes in the rate of *gus* transcription occurred later, since the expression increased strongly after 72 h to 3.90-fold (3.27–4.63), before finally falling to 0.24 (0.20–0.29) after 96 h.

Generally speaking, a putative biphasic or positive feedback loop response was observed for the two analyzed plant hormones [61].

The reaction to 100-mM NaCl treatment, measured as change in *gus* gene expression, was less complex. The decrease was observed after the 24, 48, and 96-h treatment, after which the value fell to below control values 0.03 (0.02–0.05), 0.33 (0.29–0.39), and 0.02 (0.01–0.03), respectively (Fig. 3d).

Discussion

The isolated *CPS* gene fragment indicates moderate activity based on the *GUS* reporter gene expression in transformed *Salvia miltiorrhiza*. The relative strength of isolated fragment represents 0.18 (0.12–0.28) of that of the CAMV35S activity.

The in silico analysis of the *S. miltiorrhiza CPS* promoter revealed a lack of tandem repeats, CpG/CpNpG islands, and miRNA target sites. Therefore, an increased mutation frequency, methylation-dependent control and targeting by miRNAs are unlikely to happen [62–64].

Searching the 5′ UTR for potential transcription regulatory elements, revealed the presence of a 33-nt long PRS, running from nucleotide 2185 to nucleotide 2217 (Fig. 2), that may participate in the proper organization of spliceosomal complexes [65]. The in silico analysis reveals that the *CPS* promoter could play a role in such processes as response to ethylene, dark, light, auxin, and salicylic acid, but these results have not yet been validated experimentally in *S. miltiorrhiza* [29–31,33–39]. Also, the potential influence of such stress factors as anaerobic conditions, dehydration, pathogen infection, and protein unfolding on *CPS* expression should be verified and validated experimentally [40–43].

Comparison of results obtained by the in silico search to the outcome of microarray data, indicated no shared, co-regulated proteins. Only the common biological processes that may be regulated by *CPS* genes in *A. thaliana* and *S. miltiorrhiza* were observed. Therefore, the careful examination of microarray data confirmed and further increased the number of potential processes that may be regulated by *CPS* gene as compared to the in silico search results. Among them are red light response, circadian clock regulation, auxin metabolism, plant defense, regulation of apoptosis, and osmotic stress adaptation [52–54,55–57]. The results of in silico searches and microarray co-expression studies differ with regard to plant morphogenesis. While the in silico studies suggest that *CPS* promoter has a potential role in plant embryogenesis and leaf development, the microarray co-expression tests indicate that the *CPS* promoter has potential participation in a broader range of morphogenesis processes as pollen, sieve, seed, ovule, and gametophyte development [25,28,44–51].

However, all the observed biological processes revealed by the in silico searches and microarray data should be experimentally validated in *S. miltiorrhiza*. Also, novel functions related to nitrogen metabolism and anthocyanin biosynthetic pathway regulation that are revealed by microarray data and are not confirmed by the in silico results require experimental verification by the RT-PCR studies on *S. miltiorrhiza*.

Results of RT-PCR experiments indicated that the *CPS* promoter is not regulated by auxin. Therefore, the *cis*-active element, putatively responding to auxin treatment 5′-TGTCCCAT-3′ (Tab. 1) is rather not active in our experimental model.

Obtained RT-PCR analysis experimentally confirmed that the *CPS* promoter was positively regulated by gibberellin and salicylic acid. The outcome of high-salt treatment suggests the occurrence of negative regulation. The initial increase of *gus* gene expression controlled by *CPS* promoter was seen to fall and then rise again over a longer observation period. This biphasic response found in the case of gibberellin and salicylic acid could suggest that the initial increase of *CPS* activity could produce more gibberellin precursors and finally more endogenous gibberellin, which could stimulate promoter activity at later stages of observation. This hypothesis should be verified by the analysis of endogenous changes in gibberellin occurring in the course of a 96-h period of *CPS* gene stimulation by gibberellin and salicylic acid. Earlier works suggest that increased activity of *CPS* and *ent*-kaurene synthase (KS) are able to increase the concentration of kaurenoic acid. However, the concentration of GA did not significantly change, suggesting that CPS and KS do not affect the efficiency of later stages of gibberellin biosynthesis [66].

The experimentally validated information regarding *trans*-factors recognizing the *cis*-active elements, commonly found in the promoters of crucial, regulatory enzymes, could be used to improve the content of particular secondary metabolites in plant tissues. Similar approaches, based around the concerted regulation of crucial pathway enzymes through overexpression of AtPAP1 *trans*-factor, have facilitated the greater anthocyanin production [67].

An interesting avenue of future research could be the cloning, isolation, and functional characterization of the homologous, or as yet unknown *trans*-factors of *S. miltiorrhiza*. This could be done by using yeast one-hybrid screen technology to search cDNA libraries against known *cis*-active motif sequences from the promoter region [68].

Acknowledgments

Authors would like to thank dr Grażyna Szymańska for technical assistance with figures. Authors are extremely grateful to Dean Elżbieta Mikiciuk-Olasik, the Dean of the Faculty of Pharmacy, Medical University of Łódź, for providing financial support.

Supplementary material

The following supplementary material for this article is available at http://pbsociety.org.pl/journals/index.php/asbp/rt/suppFiles/asbp.3513/0:

Fig. S1 Amplification products generated from genomic DNA isolated from plants transformed by pXK2FS7 (lines 2–7), CPS-pKGWFS7 (lines 8–13) plasmids and untransformed plants (lines 14–19).

Fig. S2 Fluorescence microscope pictures obtained from leaves fragments prepared from plants transformed by CPS-pKGWFS7 and pXK2FS7 plasmids as well as control, untransformed plants.

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