BIOLOGIA PLANTARUM 62 (2): 250-260, 2018 DOI: 10.1007/s10535-017-0766-z

Activation of polyketide synthase gene promoter in *Cannabis sativa* by heterologous transcription factors derived from *Humulus lupulus*

G.S. DURAISAMY, A.K. MISHRA, T. KOCÁBEK, and J. MATOUŠEK*

Institute of Plant Molecular Biology, Biology Centre of the Academy of Sciences of the Czech Republic, Branišovská 31, CZ-37005 České Budějovice, Czech Republic

Abstract

Cannabis sativa, an annual herbaceous plant, produces a wide variety of secondary metabolites among which delta-9-tetrahydrocannabinol (THC) is the most important one. The dissection of biosynthetic pathway(s) of this compound and its regulation by transcription factors (TFs) is an important prerequisite for efficient biotechnological manipulation of its secondary metabolome. A polyketide synthase (PKS) of *C. sativa* catalyzes the first step of cannabinoid biosynthesis, leading to the biosynthesis of olivetolic acid. Cloning and analysis of *PKS* promoter based on online *PLACE*, *Plant CARE*, and *Genomatix Matinspector* professional databases, indicated that *PKS* promoter consisted of *cis*-elements such as TATA-box, CAAT-box, W-box, Myb-box, E-box, and P-box. Plant expression vector PKS::*GUS* was constructed in such a way that the ATG of the *PKS* gene was in the frame with the β-glucuronidase (*GUS*) coding region. Using a combinatorial transient *GUS* expression system in *Nicotiana benthamania* leaves, it was shown that heterologous TFs such as *Hl*WRKY1, *Hl*MYB3, *Hl*WDR1 and *Hl*bZIP1 from *Humulus lupulus* significantly activated *PKS* promoter. Moreover, Tombusvirus p19 core protein, which is known for silencing suppressor functions, acted in our combinatorial transient expression system as an enhancer of *PKS* promoter activity along with hop TFs. Our analyses suggested the involvement of the hop derived TFs (*Hl*WRKY1, *Hl*MYB3, *Hl*WDR1 and *Hl*bZIP1A) and p19 in the activation of *PKS* gene promoter, which could be used for the genetic manipulation of *C. sativa* to enhance the cannabinoid production.

Additional key words: β-glucuronidase, bZIP1, MYB3, RT-qPCR, Tombusvirus p19 core protein, WDR1, WRKY1.

Introduction

Cannabis sativa L. is native to Central Asia and is one of the oldest domesticated annual dioecious plants (Small and Cronquist 1976). Several compounds have been identified in this plant such as flavonoids, stilbenoids, alkaloids, lignanamides, phenolic amides, and the most significant cannabinoids, which are C_{21} terpenophenolic compounds with bioactive properties (Downer and Campbell 2010). Divergent selection based on the application, classified *C. sativa* into two varieties, hemp (*C. sativa* var. *sativa*) and marijuana (*C. sativa* var. *indica*) (Alghanim and Almirall 2003). Hemp variety is used as a source of industrial fiber, seed oil, and topical ointments (Hillig 2005), while marijuana variety contains psychoactive constituent, Δ9-tetrahydrocannabinolic acid (THCA) (Giacoppo *et al*. 2014). In addition, THCA alleviates neuropathic pain (Russo *et al*. 2005), increases the tolerance to chemotherapy (Flores-Sanchez and Verpoorte 2008) as well as to anorexia in people suffering from AIDS (Haney *et al*. 2007). The first enzyme in the cannabinoid pathway is a type III polyketide synthase (PKS), which require the association of olivetolic acid cyclase (OAC) to catalyze the condensation of hexanoyl-CoA with three molecules of malonyl-CoA to yield olivetolic acid (OA) (Gagne *et al*. 2012). OA reacts with geranyl pyrophosphate to form cannabigerolic acid (CBGA), which is converted by oxidocyclase enzymes to major cannabinoids THCA and cannabidiolic acid (CBDA) (Taura *et al*. 2007). The

Submitted 9 May 2017, *last revision* 13 July 2017, *accepted* 2 August 2017.
Abbreviations: bZIP1 - basic-leucine Zipper Domain 1; CHS - chalcone synthase; GUS - β-glucuronidase; MU - 4-methylumbelliferone; MYB3 - myeloblastosis proto-oncogene family of R2R3; PKS - polyketide synthase; 35S - 35S *cauliflower mosaic virus* promoter; TF - transcriptional factor; TBSV - *Tomato bushy stunt virus*; THC - tetrahydrocannabinol; THCA - Δ9-tetrahydrocannabinolic acid; WDR1 - WD repeat containing protein; WRKY1 - transcription factor containing WRKYGQK motif; X-Gluc - 5-bromo-4-chloro-3-indolyl-β-D-glucuronide.
Acknowledgements: The work was supported by the Czech Science Foundation project (GACR 13-03037S) and by the Institutional support RVO: 60077344. The first two authors equally contributed to this work.
* Corresponding author; e-mail: jmat@umbr.cas.cz

non-enzymatic decarboxylation of THCA and CBDA leads to the formation of their neutral form Δ9-tetra-hydrocannabinol (THC) and cannabidiol (CBD), respectively (Gagne *et al*. 2012). The co-existence of cannabinoids, flavonoids, and stilbenoids in *C. sativa* could be correlated to different enzymes of the PKS family, which has been classified into three types (Fischbach and Walsh 2006). Among them chalcone synthase (CHS, EC 2.3.1.74) and stilbene synthase (STS, EC 2.3.1.95) are the most studied enzymes from the group of type III PKSs, which exist exclusively in plants and bacteria (Austin and Noel 2003).

The THCA biosynthesis pathway is similar to the bitter acid humulone biosynthesis pathway in *Humulus lupulus* (hop), where chalcone synthase-like enzyme, belonging to the polyketide synthases (PKSs) group, catalyze the condensation of coumaroyl CoA with malonyl CoA to form first intermediate chalconaringenin (Matoušek *et al*. 2007). In hop several members of *PKS* group have been identified. They can be categorized into chalcone synthase (*CHS)* and valerophenone synthase (*VPS*) gene families. The complexity of the promoter elements of the *CHS*_H1 genes in hop and involvement of ternary complexes of transcription factors (TFs), *Humulus lupulus* Myb2*, Humulus lupulus* bHLH2, and *Humulus lupulus* WDR1 through protein:protein interactions to exert combinatorial activation of expression of genes involved in flavonoid biosynthetic pathways has been shown (Matoušek *et al*. 2012). This ternary complex is highly organized and each subunit fulfills specific functions such as binding to DNA, activation of expression of a target gene, or stabilization of the TF complexes (Hichri *et al*. 2011). Furthermore, computational analysis of *CHS*_H1 promoter motif in hop, predicted the existence of W-, P-, Myb-, G- and H- binding boxes suggesting that promoter regulation and expression of prenylated chalcones depend on the interaction of more than one TF (Duraisamy *et al*. 2016). The regulation of the flavonoid biosynthesis pathway by ternary complexes has also been shown in *Pisum sativum* (Hellens *et al*. 2010), *Arabidopsis thaliana* (Hichri *et al*. 2011), and *Lotus japonicus* (Yoshida *et al*. 2010).

The *Tomato bushy stunt virus* (TBSV) p19 is one of a class of plant and animal virus proteins that suppresses the host defense RNA silencing process (Hearne *et al*. 1990). This protein possesses two independent silencing suppressor functions, viral siRNA binding and the induction of microRNA miR168l and it subsequently controls the argonaute protein1 (AGO1) accumulation, both of which are required to efficiently cope with the RNA-silencing based host defence (Várallyay *et al*. 2014). Since, p19 exhibits host dependent activities, therefore it has been hypothesized that this protein may interact with one or more host TFs (Chu *et al*. 2000).

Although *C. sativa* genome has been sequenced, the involvement of TFs in the production of secondary metabolites has not been widely studied. Hop is the closest relative of *C. sativa* and the involvement of hop TFs in secondary metabolite production has been well characterized (Matoušek *et al*. 2006, 2010, 2012, 2016). The present work entails new insights of the activation of *PKS* promoter of *C. sativa* by transient combinatorial expression of hop TFs with or without p19. The present work entails new insights of the trans-activation of *Cannabis sativa* PKS promoter by transient combinatorial expression of hop TFs. We believe that such information may lead to a better understanding of PKS promoter activation pattern during cannabinoid biosynthesis including potential involvement of RNA silencing processes.

Materials and methods

Plants and cultivation: Seeds of *Cannabis sativa* L. var. *indica* (marijuana) obtained from commercial source (*AutoMaris*, Valencia, Spain), were sown in commercial growing substrate in pots and placed in climatic chamber (*Weiss Gallenkamp*, Loughborough, UK). Seedlings were grown under natural irradiance supplemented with artificial irradiance [170 μmol m^{-2} s^{-1} PAR] to reach a 16-h photoperiod, a temperature of 25 ± 3 °C, and an air humidity of 50 - 60 %. Similar conditions were used to grow *Nicotiana benthamiana* Domin plants, and young leaves over 1 cm long were used for the transient transformation assays. Leaf samples were collected from *C. sativa* plants for DNA isolation and *N. benthamiana* for RNA isolation. All collected samples were immediately immersed in liquid nitrogen and stored at -80 °C until analyses.

The promoter sequence analysis of *PKS* gene and PCR amplification: *PKS* gene sequence along with 2 kb upstream sequence of the transcription start site, which includes the promoter region of *C. sativa*, was retrieved from the *Cannabis Genome Browser* database (http://genome.ccbr.utoronto.ca) based on the reported draft genome sequence of *C. sativa* (*NCBI* accession number AGQN00000000). The promoter prediction software *BPROM* (*SoftBerry*, Mount Kisco, NY, USA) was used to identify possible promoters of the up-stream of *PKS* candidate genes. The *PKS* promoter sequence was analyzed using following publicly available databases: *SOGO* (https://sogo.dna.affrc.go.jp), *Plant CARE* (http:// bioinformatics.psb.ugent.be/webtools/plantcare/html/), and *Genomatix* (https://www.genomatix.de/solutions/ genomatix-software-suite.html). The analyzed promoter motifs were used for primer designing and PCR amplification.

Genomic DNA from leaves of *C. sativa* was extracted using cetyltrimethyl amonium bromide (CTAB) method (Saghai-Maroof *et al*. 1984). The designed pPKS711 forward (5'-GGTCAAGAAAAGTTCCCTACC-3') and reverse (5'-ACTTTTGTCACCTACATATACAT-3') primers were used for PCR amplification of *PKS* promoter region. PCR reaction mixtures in a final volume

of 20 mm^3 consisted of 50 ng genomic DNA, 0.25 μM each specific forward and reverse primers, 0.6 units of *Hot Start Ex Taq* polymerase (*TaKaRa Bio,* Nojihigashi, Japan), 1× Taq buffer and 200 μM dNTPs mixture. The PCR amplification was carried out in the thermal cycler (*Bio-Rad,* Hercules, USA) under following conditions: an initial denaturing step at 94 °C for 2 min, followed by 40 cycles consisting of denaturation at 94 °C for 30 s; annealing at 57 °C for 45 s; extension at 72 °C for 2 min, and final extension at 72 °C for 10 min. The product size (~720 bp) was confirmed by 1.2 % agarose gel electrophoresis. The PCR product was isolated from agarose gel using *QIAquick* gel extraction kit (*Qiagen,* Hilden, Germany), reamplified with pPKS711-Eco (5'-AAGAATTCGGTCAAGAAAAGTTCCCTACC-3'), and pPKS711-Xho (5'-AACTCGAGGGTCAAGAAA AGTTCCCTACC-3') primers using two high fidelity polymerases (*Roche Molecular Biochemicals*, Basel, Switzerland) and subsequently cloned into *pCR-Script SK(+)* vector following manufacturer's instructions (*Stratagene*, La Jolla, CA, USA). Positive transformants were selected randomly and inserts were sequenced using an *ABI377* sequencer (*Applied Biosystems,* Foster City, CA, USA) with T3 and T7 primers.

Vector construction and combinatorial transient expression: The *PKS* promoter region was amplified using pPKS711-F and pPKS711-R primers and promoter fragment was ligated into the plant vector pBGF-0 (Chytilová *et al*. 1999) using *Eco*RI and *Xba*I restriction sites in the sense orientation adjacent to the β-glucuronidase (*GUS*) coding region as described earlier (Matoušek *et al*. 2006). The infiltration with the vector pBGF-0 (promoter-less *GUS* plasmid) was used as negative control, while 35S::GUS/GFP fusion cassette was used as a positive control to compare the promoter activity (Fig. 1).

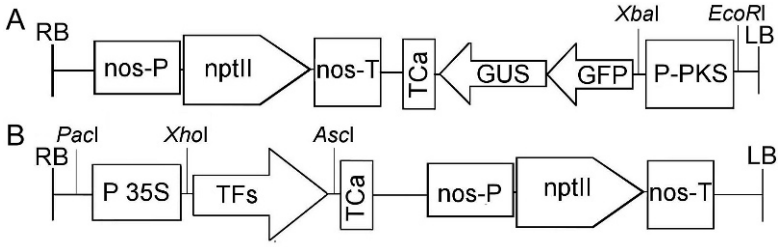

Fig. 1. Schematic representation of expression cassettes within the T-DNA region of plant vectors used in this study. General cassette of the vector pBGF-0 harboring the PKS promoter (P-PKS) (*A*) and pLV-07 bearing the 35S *cauliflower mosaic virus* promoter driven transcription factor (TF) from hop (*B*). RB and LB - right and left T-DNA borders, respectively. *Npt*II designates the neomycin phosphotransferase gene for resistance to kanamycin. This gene is driven by the nopalin synthase promoter (nos-P). Terminators of CaMV (TCa) are shown. Restriction enzyme sites XbaI and EcoRI (*A*) and PacI, XhoI, and AscI (*B*) used for promoter and hop TF integration in expression cassette are also shown.

Previously cloned hop TFs such as *Hl*MYB3 (Matoušek *et al*. 2007), *Hl*bZIP1 (Matoušek *et al*. 2010), *Hl*WDR1 (Matoušek *et al*. 2012) and *Hl*WRKY1 (Matoušek *et al*. 2016) were used for the transient expression experiments. In addition, the vector containing silencing suppressor *Tombusvirus* p19 (Voinnet 2005) was obtained from Dr. Oliver Voinnet (Institut de Biologie Moléculaire des Plantes, Zürich) and used in *A. tumefaciens* transformation for transient expression experiments.

Histochemical staining and fluorometric assay of GUS activity: *Agrobacterium tumefaciens*, strain LBA 4404 carrying appropriate gene constructs was cultured on LK (Langley and Kado 1972) medium supplemented with 50 mg dm^{-3} kanamycin (*Sigma*, St. Louis, USA) and incubated at 28 °C. A 10 mm^3 loop of confluent bacterium was re-suspended in 1×10^4 mm^3 of infiltration medium (10 mM $MgCl_2$, 0.5 μM acetosyringone), diluted to an absorbance A_{600} of 1.0, and incubated at room temperature without shaking for 2 h before infiltration.

For the transient expression analysis, *A. tumefaciens* LBA4404 suspension harboring ProCsPKS::BGF was mixed with individual or multiple combination of *A. tumefaciens* LBA4404 suspension containing hop TFs gene construct with or without p19 in equal volume. An infiltration to *N. benthamiana* leaf was performed as described earlier (Voinnet *et al*. 2003). The treated leaf tissues after 3 d of infiltration were homogenized in extraction buffer (50 mM phosphate buffer, pH 7.0, 10 mM EDTA, 0.1 % *Triton X-100*, 0.1 % sodium lauryl sarcosine, and 10 mM β-mercaptoethanol) by freezing in liquid nitrogen and grinding with a pestle and mortar and β-glucuronidase (GUS) activity was assayed using a fluorometric assay as described earlier (Jefferson *et al*. 1987). Concentrations of the generated fluorescent dye 4-methylumbelliferone (MU) was measured using the *VersaFluor*™ fluorometer (*Bio-Rad*, Hercules, USA) with excitation at 365 nm and emission at 455 nm. The fluorimeter was calibrated with a fresh preparation of MU (100 nM) as standard. At least three independent experiments were performed from three independent lines.

Histochemical staining for GUS activity was performed using 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc) as a chromogenic substrate dissolved in dimethylsulfoxid to the final concentration of 1 mg dm^3 (Solís-Ramos *et al*. 2010). Tissues were vacuum

infiltrated with X-Gluc reaction buffer (100 mM sodium phosphate buffer, pH 7.2, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 0.2 % *Triton X-100*, and 1 mg cm^{-3} X-gluc) for 15 min and incubated at 37 °C overnight. After incubation, pigments and chlorophylls were removed from green tissues by a series of 70 % ethanol treatments and tissue samples were observed for the presence of blue staining (Jefferson *et al*. 1987).

RNA isolation and reverse transcription quantitative PCR (RT-qPCR): RT-qPCR was used to analyze *GUS* gene transient expression in leaves of *N. benthamiana* 3 d after infiltration with *Agrobacterium* bearing vector construct. Total RNA was isolated from 100 mg of leaves using *Concert*™ plant RNA purification reagent (*Invitrogen*, Carlsbad, CA, USA). After RNA purification DNA cleavage was carried out using *RNeasy* plant total RNA kit, *Qiagen*, Hilden,Germany) and further treated with DNase I (*DNA-freeTM* kit, *Ambion*, Austin, TX, USA) to remove the DNA contamination. The synthesis of cDNA was performed using oligo$(dT)_{18}$ primer and *SuperScript™ III* reverse transcriptase as per manufacturer's instruction (*Invitrogen*). A total of 5 mm^3 of 50 times diluted cDNA was used for a 20 mm^3 of PCR reaction mixture consisting of 2 × *SYBR Premix Ex Taq* (*TaKaRa*) and 375 nM *GUS* gene forward (5′-ACAGCCAAAAGCCAGACAGAG-3′) and reverse (5′-GCGTAA GGGTAATGCGAGGTA-3′) primers. The amplification was carried out and a PCR program consisted of an initial denaturing step at 95° C for 5 min, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s, and 72 °C for 30 s. The product size was confirmed by melting analysis and 2 % agarose gel electrophoresis. The specificity of the individual PCR amplification was verified by melting curve analysis following the thermal denaturing cycle at 60 - 95 °C with 1 °C increments for 5 s between each step. Threshold values (C_T) were generated from the *IQ 5* software tool (*Bio-Rad*). The relative mRNA ratio was calculated according to the method of Livak and Schmittgen (2001). To normalize the results, the hop specific *Hl*-GAPDH gene (accession No. ES437736) was used as internal control. Mean values and SDs were obtained from three biological replicates.

Comparative phylogenetic analysis: To elucidate the evolutionary relationship and degree of conservation of hop TFs, the phylogenetic tree was constructed from hop and other plant species including *C. sativa*. It was based on the amino acid sequences of hop TFs *Hl*WRKY1 (accession No. CBY88801), *Hl*MYB3 (accession No. CAM58451), *Hl*bZIP1 (accession No. CAZ15514) and *Hl*WDR (accession No. CBK62755) with *MEGA 5.0* software using the neighbor-joining method, and the reliability was set to 1 000 bootstrap replicates (Tamura *et al*. 2011).

Results

PCR amplification with designed *PKS* promoter primers and sequence analysis indicated that *PKS* promoter region consisted of 711 bp nucleotide. Sequence comparison of hop *Hl*chs_H1 promoter and *PKS* promoter using *Geneious 9.0* software (*Biomatters*, Auckland, New Zealand) showed the significant sequence similarity (~68 %) and specific *cis*-acting elements conservation within these two promoters region (Fig. 2). *SOGO* analysis of *PKS* promoter revealed the position of TATA-box (required for the accurate initiation of gene transcription) at -181 (TATA) from transcription start site. The promoter regulatory consensus elements such as two CAAT-boxes, important for transcription initiation were identified at positions -60 and -395. The well known TFs binding motifs, two W-boxes (GGTCA) and an E-box (CAAATG) were present at -238, -392 and -240 respectively (Fig. 2). The similar type of functional elements such as Myb-P and MYB boxes were positioned at -375 and -354, respectively. MYB and E-box motifs are found in a number of plant promoters and serve as binding site for R2R3 MYB and basic helix loop-helix (bZIP) TFs, respectively (Matus *et al*. 2010, Duraisamy *et al*. 2016).

The predicted PKS promoter, which contains key *cis*-elements required for sufficient expression, was inserted in binary vector pBGF upstream of a *GUS* reporter gene to observe the interaction of hop TFs with *cis*-elements (Fig. 1). The expression analysis of CsPKS::GUS fusion promoter segments in *N. benthamiana* under normal condition was examined. GUS activity was measured by fluorometric assays in extracts from leaves 3 d after agro-infiltration. The maximum transient GUS activity was reached 86 h after infiltration and it declined thereafter, depending on the TF or TFs combination infiltrated. We examined individual hop TFs and different combination of TFs (*Hl*WRKY1 + p19, *Hl*WDR1 + p19, *Hl*bZIP1 + p19, *Hl*MYB3 + p19, *Hl*bZIP1 + *Hl*WRKY1, *Hl*bZIP1 + *Hl*WRKY1 + p19, *Hl*bZIP1 + *Hl*MYB3, *Hl*bZIP1 + *Hl*MYB3 + p19 *Hl*MYB3 + *Hl*WRKY1, *Hl*MYB3 + *Hl*WRKY1 + p19, *Hl*WRKY1 + *Hl*WDR1, and *Hl*WRKY1 + *Hl*WDR1 + p19) (Fig. 3). In individual TF co-infiltration assay, GUS activity [pmol(MU) mg^{-1}(f.m.) min^{-1}] was the highest for *Hl*MYB3 (9.0) followed by *Hl*bZIP1 (5.2), *Hl*WRKY1 (3.8), *Hl*WDR1 (3.5), and comparatively low activity was noticed in p19 (2.3) (data not shown). In different combinations of TFs, the highest GUS activity was noticed for *Hl*WRKY1+*Hl*WDR1+p19 combination followed by *Hl*MYB3+*Hl*WRKY1, p19+*Hl*WDR1, *Hl*WRKY1+*Hl*WDR1, and *Hl*bZIP1 +*Hl*WRKY1 (Fig. 3). Results indicated that the activation of *PKS* promoter was regulated by several heterologous TFs derived from hop. Moreover, MYB, MYB-P, E and W binding boxes have been predicted in the *PKS* promoter region, suggesting their important regulatory

role for interaction with TFs either independently or in combinatorial way. This finding was in accordance with the knowledge about the regulation of the secondary metabolites in hop (Matoušek *et al*. 2012, 2016).

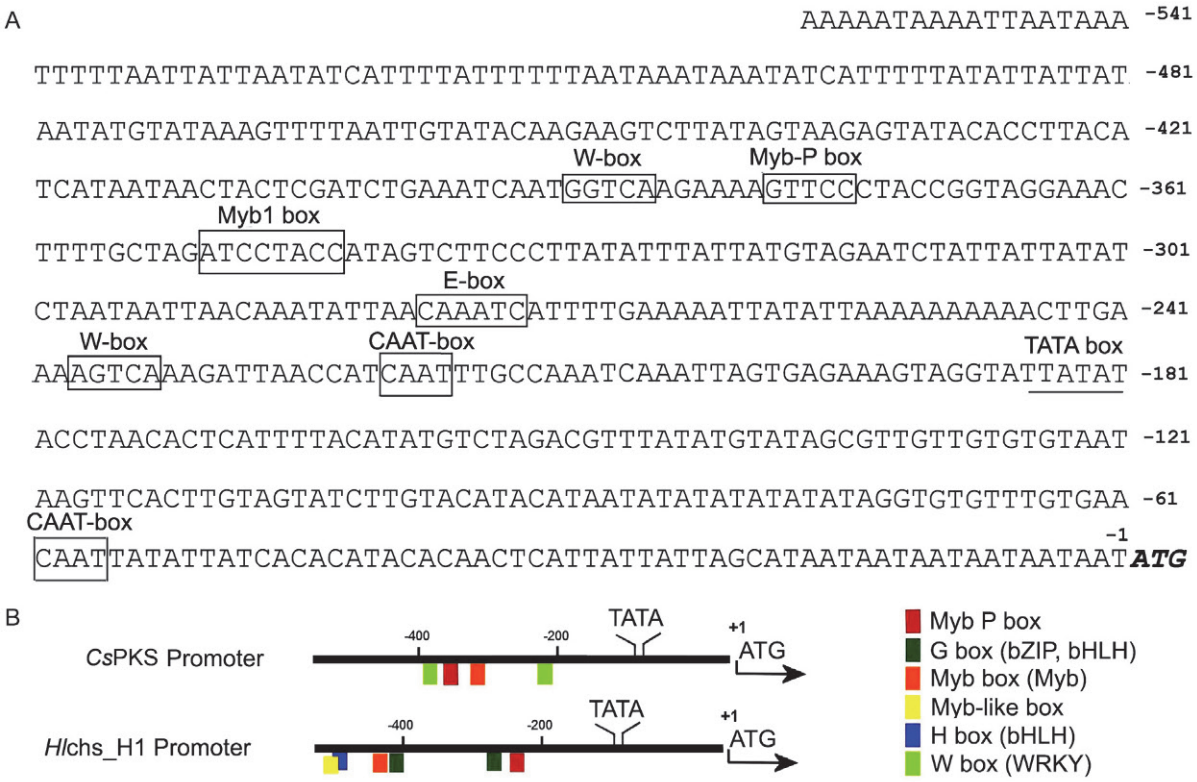

Fig. 2. Schematic representation of *PKS* promoter nucleotide sequences: the TATA-box. and all putative *cis*-elements are shown (*A*). Comparison of hop chalcone synthesis promoter (*Hl*chs_H1) with cannabis *PKS* promoter (CsPKS) in terms of different binding boxes distribution and location.

The histochemical GUS staining experiments were performed to analyze the GUS gene expression pattern driven by PKS promoter in *N. benthamiana* infiltrated with hop TFs and p19. The leaf infiltrated with *Hl*WRKY1+*Hl*WDR1+p19 and *Hl*WRKY1+ *Hl*WDR1 showed intense blue colour among the six combinations (Fig. 4). The detectable low and noticeable background were observed in leaves infiltrated with *Hl*bZIP1+ *Hl*WRKY1 and *Hl*MYB3+*Hl*WRKY1 respectively. This observation was consistent with fluorometric data. As expected, no GUS activity was detected in leaves that were not infiltrated with hop TFs. This was similar to the the activity displayed by the transgenic lines containing the promoter-less vector control.

In addition to measuring GUS activity, we also analyzed the expression of *GUS* gene in leaf tissues of *N. benthamiana* plants infiltrated with hop TFs (with or without p19) by RT-qPCR method. The result showed high *GUS* gene expression in leaf tissues infiltrated with *Hl*WRKY1+*Hl*WDR1+p19 (100 %) followed by *Hl*MYB3+*Hl*WRKY1 (97 %), *Hl*WRKY1+*Hl*WDR1 (66 %) and *Hl*bZIP1+ *Hl*WRKY1(52 %) (Fig. 5). In individual combinations of hop TF with p19, *GUS* gene expression was higher in *Hl*WDR1+p19 (35 %) than in *Hl*WRKY1+p19 (23 %). These results support our fluorometric measurements. In addition, combinatorial expression of *Hl*WRKY1 and *Hl*WDR1 TFs along with p19 resulted in enhanced activation of *PKS* promoter in *N. benthamiana* suggesting its probable role either in stabilization of *Hl*WRKY1 and *Hl*WDR1 TFs or interaction with *PKS* promoter.

To investigate the evolutionary relationship of *H. lupulus* TFs with *C. sativa*, a neighbor-joining tree with 1 000 bootstrap was constructed (Fig. 6). Phylogeny tree based on amino acid alignment revealed that *Hl*Myb3, *Hl*WDR1, *Hl*WRKY1, and *Hl*bZIP1 TF families of hop clustered together with those of *C. sativa* TFs, suggesting significant similarities between them. Furthermore, the presence of some similar groups and sub-groups in comparative phylogeny revealed the conserved nature of these TFs genes during angiosperm evolution.

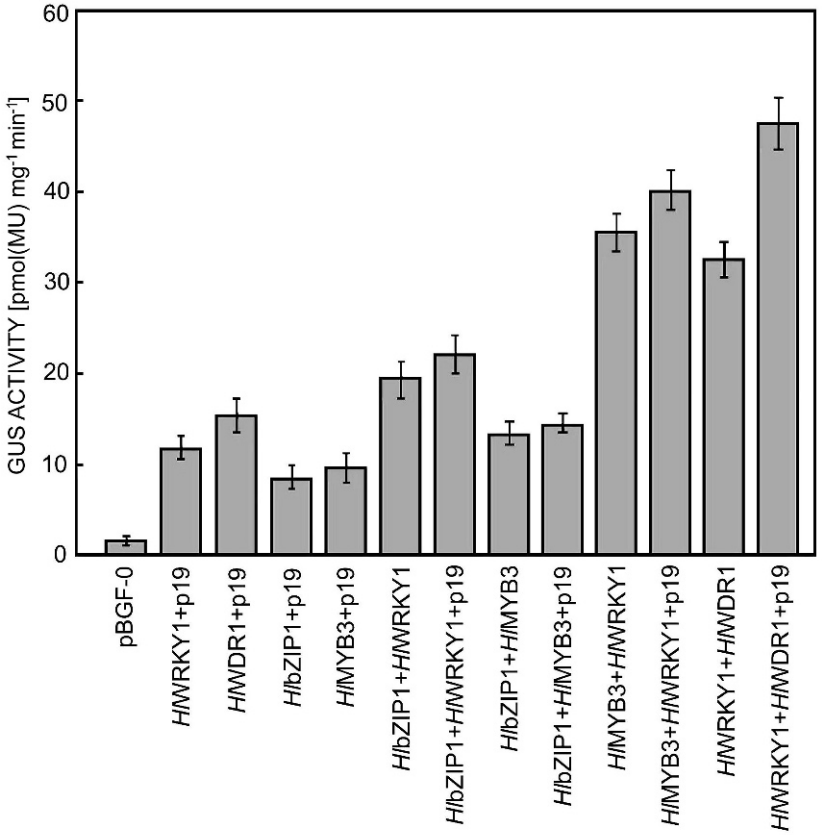

Fig. 3. The GUS activity in *N. benthamiana* leaves co-infiltarated with pPKS:GUS/GFP construct and pLV-07 plant vector harbouring the 35S-driven transcription factors from hop (*Hl*Myb3, *Hl*WDR1, *Hl*WRKY1, and *Hl*bZIP1) individual or in different combinations and with or without *Tombusvirus* p19. Means ± SEs, $n = 3$.

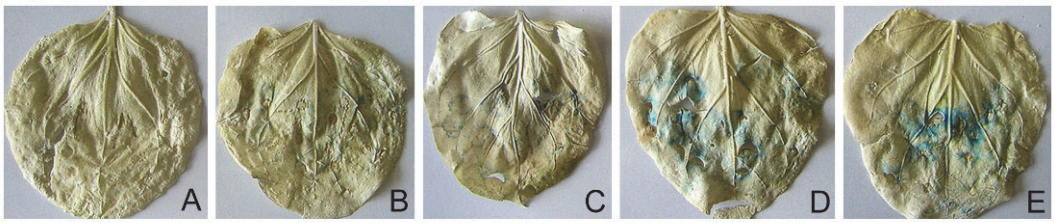

Fig. 4. Histochemical staining of GUS activity of *N. benthamiana* leaves co-infiltrated with PKS:GUS/GFP construct and pLV-07 plant vector harboring the 35S-driven transcription factor from hop with following combination *Hl*WRKY1+*Hl*WDR1(*B*); *Hl*MYB3+ *Hl*WRKY1 (*C*); *Hl*MYB3+*Hl*WRKY1+p19 (*D*); *Hl*WRKY1+ *Hl*WDR1+p19 (*E*); pBGF-0 (*A*) was used as control.

Discussion

Cannabinoids are among the best known group of natural products and more than 80 different cannabinoids have been found so far (Van Bakel *et al*. 2011). Several therapeutic effects of cannabinoids have been described and the discovery of an endocannabinoid system in mammals marked a renewed research interest in these compounds (Flores-Sanchez *et al*. 2010). The transcriptional regulation of cannabinoid biosynthetic pathway is still yet not completely studied. In the present study, we cloned the *PKS* promoter and identified expressions with different hop TFs (*Hl*MYB3, *Hl*bZIP1, *Hl*WRKY1, and *Hl*WDR1) along with p19, which could be potentially used for future genetic manipulation with the aim to increase cannabinoid production.

Several genes encoding transcription factor families, including R2R3-MYB, bHLH, WD40, WRKY, bZIP, and MADS-box factors, are involved in the transcriptional control of flavonoid biosynthesis genes (Bomal *et al*. 2008). Many of these have been identified in *Arabidopsis* (Stracke *et al*. 2007), maize (Bomal *et al*. 2014), petunia

(Shimada *et al*. 2006), *Antirrhinum majus* (Mano *et al*. 2007), hop (Matoušek *et al*. 2012, 2016), and other plant species. As mentioned above, different *cis*-acting elements serve as binding sites for different class of TFs such as MYB, bZIP, WDR, and WRKY to regulate gene expression (Zhang *et al*. 2013). Thus it is reasonable to assume from bioinformatics analysis of the *PKS* promoter region that those TFs are involved in the transcriptional regulation of the *PKS* promoter.

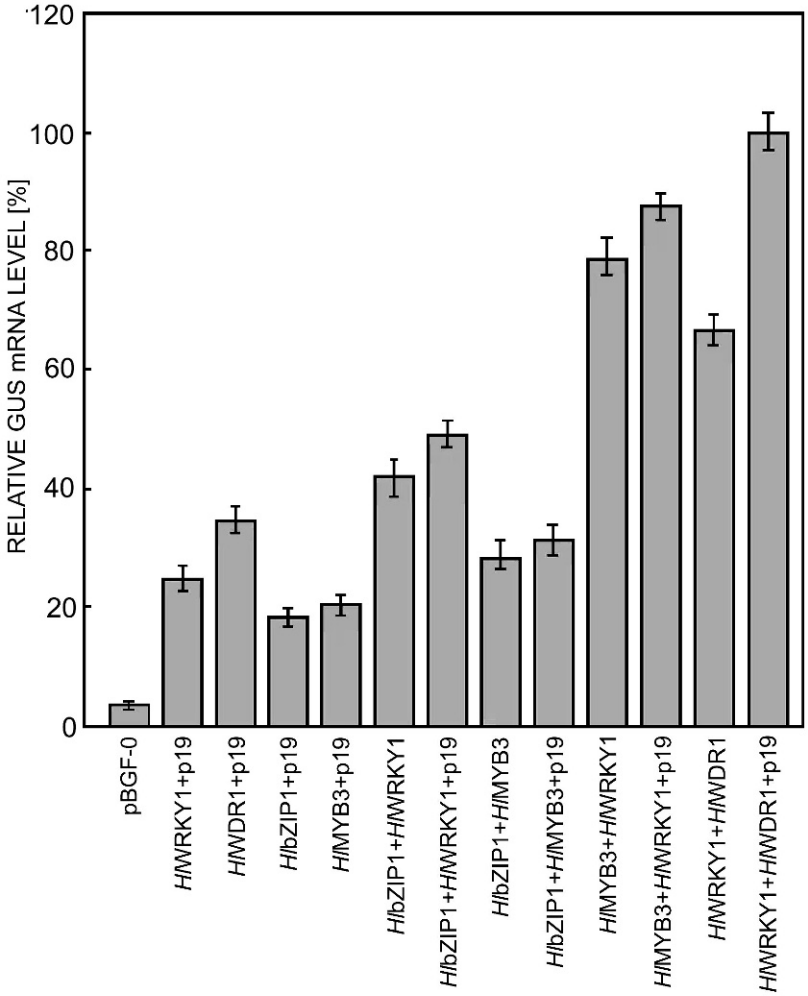

Fig. 5. RT-qPCR analysis of *GUS* gene expression in *N. benthamiana* leaves co-infiltarated with PKS:GUS/GFP construct and pLV-07 plant vector harbouring the 35S-driven transcription factors from hop (*Hl*Myb3, *Hl*WDR1, *Hl*WRKY1, and *Hl*bZIP1) individually or in different combinations and with or without *Tombusvirus* p19. Means ± SEs, $n = 3$.

The *Tombusvirus* derived p19 has two independent silencing suppressor functions: viral siRNA binding and the miR168-mediated *AGO1* control, both of which are required to efficiently cope with the RNA-silencing based host defense (Várallyay *et al*. 2014). The intriguing observation in combinatorial expression was the combination of p19 with *Hl*WRKY1 and *Hl*WDR1, which leads to higher *PKS* promoter activation compared to other combinations. This observation is similar to lupulin gland specific gene expression by combinatorial action of *Hl*WRKY1 and *Hl*WDR1 as described recently (Matoušek *et al*. 2016). In *N. benthamiana*, low expression of *p19* caused altered leaf morphology, delayed the time of the appearance of developed secondary stem, and the constitutive expression of *p19* interferes with the aberrant RNA pathway of gene silencing. These findings indicate that the *p19*-targeted post-transcriptional gene silencing (PTGS) pathway plays a role in plant development (Silhavy *et al*. 2002, Li *et al*. 2014). Evidence for the existence of small interfering (si)RNA that target WRKY1 in hop has been recently documented (Matoušek *et al*. 2016). Thus, it could be possible that expression of WRKY specific siRNA regulates mRNA population of WRKY and p19 further interacts with WRKY specific siRNA to modulate the promoter activation of *PKS*. In addition, phosphorylation appears to be a very important step in the activation of WRKY protein. In *Arabidopsis* WRKY33 was shown to be a direct phosphorylation target of MPK3/MPK6 following the infection by *Botrytis cinerea* (Mao *et al*.

2011). In tobacco, overexpression of the MAP kinase SIPK triggers cell death through the phosphorylation of WRKY1 (Menke *et al*. 2005). In addition, ten miRNA families are involved in signal transduction in tobacco including Nta-miRn58a miRNA, which targets mitogen-activated protein kinase kinase (MAPKK) (Guo *et al*. 2011). Sequential activation of kinases within MAPKK cascades is a common and evolutionary-conserved mechanism of signal transduction (Guo *et al*. 2011). Thus, there is further possibility that *p19* interaction with Nta-miRn58a might cause *PKS* promoter activation. However, these two models certainly warrant further investigation.

The unique advantages of promoters derived from plant genes make them a potentially powerful tool for improving plant secondary metabolite production. Hence, tissue-specific and inducible promoters are preferred as experimental tools to analyze the effects of TFs to regulate biosynthetic pathway (Huda *et al*. 2013). In this respect, the *PKS* promoter possesses interesting and original properties of possible practical value in biotechnological applications, especially for economically valuable medicinal plants. During our attempt to identify the hop TFs expressions in *PKS* promoter, we used an efficient transient expression assay systems of *N. benthamiana via* leaf agro-infiltration (Voinnet *et al*. 2003). The GUS gene reporter system is one of the most effective techniques employed in the study of gene regulation in plant molecular biology (Fior and Gerola 2009). The *GUS* gene expression and its protein activity in leaves of *N. benthamiana* were significantly up-regulated by various hop TFs such as *Hl*WDR1, *Hl*bZIP1, *Hl*MYB3 and *Hl*WRKY1, as evident from bioinformatics of the *PKS* promoter region which revealed their corresponding interactions within the promoter region. In the similar kind of framework, cloning and expression of *Rosea1* (R2R3-MYB) TF from *Antirrhinum majus* directly activate the anthocyanin biosynthesis in *Gossypium hirsutum* (Gao *et al*. 2013).

Histochemical staining of *N. benthamiana* leaf, infiltrated with *PKS* promoter and hop TFs (*Hl*WDR1, *Hl*bZIP1, *Hl*MYB3, and *Hl*WRKY1) showed *PKS*

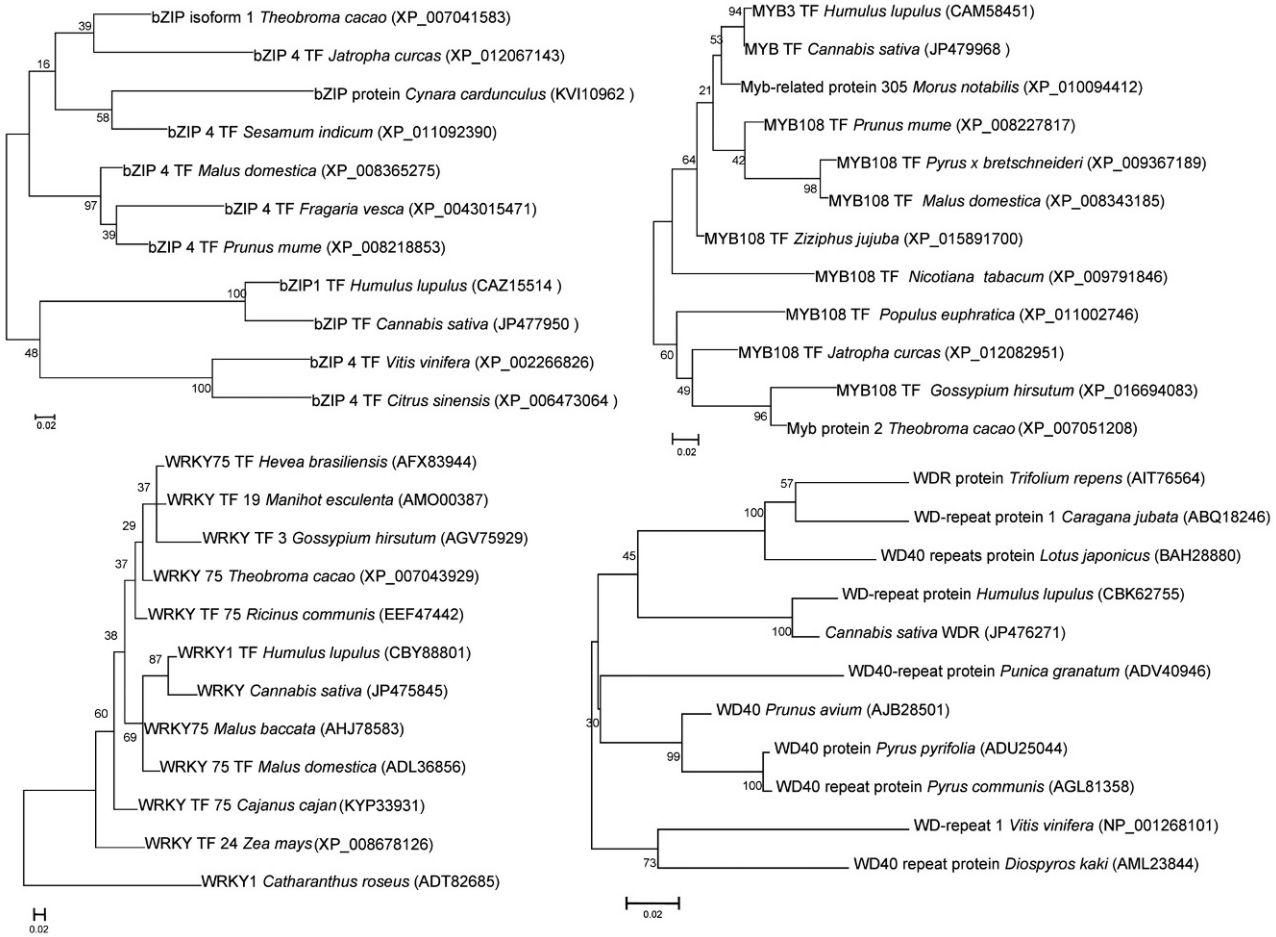

Fig. 6. Phylogenetic analysis of hop transcription factors. The maximum-likelihood trees were constructed based on the amino acid sequences aligned using *CLUSTALW* and phylogenetic inferences were obtained using the neighbor-joining method (*MEGA* software). GenBank accession numbers are indicated in parentheses. Numbers at the nodes are bootstrap values obtained by repeating 1 000 times the analysis to generate a majority consensus tree. Bootstrap values are shown near the tree nodes. The *scale bar* corresponds to 0.02 estimated amino acid substitutions per site.

promoter activation in the form of *GUS* expression. This kind of expression pattern was also reported for *Catharanthus roseus* (*Cr*WRKY1) promoter using heterologous system (Yang *et al*. 2013).

RT-qPCR analysis in *N. benthamiana* showed that the PKS promoter was up-regulated 53-fold 3 d after infiltration, whereas *GUS* gene was only induced 6.1-fold (data not shown). This difference may be due to expression of the endogenous gene driven by the native promoter not being similar to the heterologous *GUS* gene fused with the same promoter, as reported in previous studies (Venter and Botha 2010). We have noticed that the promoter driving *GUS* expression exhibited unwanted background expression, although the level was low. One possible reason is that the agro-infiltration was associated with induced *GUS* gene expression. Our study suggested that *CsPKS* promoter along with characterized TFs of hop and *Tombusvirus* derived p19 could be used as model system to enhance production of cannabinoids. The comparative phylogenetic analysis of hop TFs such as *Hl*WDR1, *Hl*bZIP1, *Hl*MYB3, and *Hl*WRKY1 with respect to their corresponding TFs gene families in *C. sativa* indicated their functional proximity. Therefore, it would be intriguing to clone TF homologous to *Hl*WDR1, *Hl*bZIP1, *Hl*MYB3 and *Hl*WRKY1 TFs from *C. sativa* and observe their role in cannabinoid production, which is the aim of our future work.

Conclusion

Although genes encoding PKSs have been cloned and studied from many other plants, we present the first report of *PKS* promoter activation with hop TFs. Trans-activation of *C. sativa* PKS promoter with TFs isolated from *H. lupuls* was revealed using transient expression system in the *N. benthamiana* leaf sectors. An addition of silencing suppressor p19 with combinatorial expression of *Hl*WRKY1 and *Hl*WDR1 TFs led to enhance activation of *PKS* promoter suggesting its probable role either in stabilization of *Hl*WRKY1 and *Hl*WDR1 TFs or interaction with PKS promoter. From this study, we can conclude that *PKS* promoter along with hop TFs with or without *Tombusvirus p19* could be effectively used as a model system to enhance the production of cannabinoids.

References

Alghanim, H.J., Almirall, J.R.: Development of microsatellite markers in *Cannabis sativa* for DNA typing and genetic relatedness analyses. - Anal. bioanal. Chem. **376**: 1225-1233, 2003.

Austin, M.B., Noel, J.P.: The chalcone synthase superfamily of type III polyketide synthases. - Nat. Prod. Rep. **20**: 79-110, 2003.

Bomal, C., Bedon, F., Caron, S., Mansfield, S.D., Levasseur, C., Cooke, J.E.K., Blais, S., Tremblay, L., Morency, M.J., Pavy, N., Grima-Pettenati, J., Séguin, A., MacKay, J.: Involvement of *Pinus taeda* MYB1 and MYB8 in phenylpropanoid metabolism and secondary cell wall biogenesis: a comparative *in planta* analysis. - J. exp. Bot. **59**: 3925-3939, 2008.

Bomal, C., Duval, I., Giguere, I., Fortin, É., Caron, S., Stewart, D., Boyle, B., Séguin, A., MacKay, J.J.: Opposite action of R2R3-MYBs from different subgroups on key genes of the shikimate and monolignol pathways in spruce. - J. exp. Bot. **65**: 495-408, 2014.

Chu, M,, Desvoyes, B., Turina, M. R., Noad, R., Scholthof, H.B.: Genetic dissection of tomato bushy stunt virus p19-protein-mediated host-dependent symptom induction and systemic invasion. - Virology **266**: 79-87, 2000.

Chytilová, E., Macas, J., Galbraith, D.W.: Green fluorescent protein targeted to the nucleus, a transgenic phenotype useful for studies in plant biology. - Ann. Bot. **83**: 645-654, 1999.

Downer, E.J., Campbell, V.A.: Phytocannabinoids CNS cells and development: a dead issue?. - Drug Alcohol Rev. **29**: 91-98, 2010.

Duraisamy, G.S., Mishra, A.K., Kocabek, T., Matoušek, J.: Identification and characterization of promoters and cis-regulatory elements of genes involved in secondary metabolites production in hop (*Humulus lupulus*. L). - Comput. Biol. Chem. **64**: 346-352, 2016.

Fior, S., Gerola, P.D.: Impact of ubiquitous inhibitor on the GUS gene reporter system: evidence from the model plants *Arabidopsis*, tobacco and rice and correction methods for quantitative assays of transgenic and endogenous GUS. - Plant Methods **5**: 19-29, 2009.

Fischbach, M.A., Walsh, C.T.: Assembly-line enzymology for polyketide and nonribosomal peptide antibiotics: logic, machinery, and mechanisms. - Chem. Rev. **106**: 3468-3496, 2006.

Flores-Sanchez, I.J., Linthorst, H.J.M., Verpoorte, R.: *In silicio* expression analysis of *PKS* genes isolated from *Cannabis sativa* L. - Genet. mol. Biol. **33**: 703-713, 2010.

Flores-Sanchez, I.J., Verpoorte, R.: Secondary metabolism in cannabis. - Phytochem. Rev. **7**: 615-639, 2008.

Gagne, S.J., Stout, J.M., Liu, E., Boubakir, Z., Clark, S.M., Page, J.E.: Identification of olivetolic acid cyclase from *Cannabis sativa* reveals a unique catalytic route to plant polyketides. - Proc. nat. Acad. Sci. USA **109**: 12811-12816, 2012.

Gao, Z., Liu, C., Zhang, Y., Li, Y., Yi, K., Zhao, X., Cui, M.L.: The promoter structure differentiation of a MYB transcription factor *RLC1* causes red leaf coloration in empire red leaf cotton under light. - PLoS ONE **8**: e77891, 2013.

Giacoppo, S., Mandolino, G., Galuppo, M., Bramanti, P., Mazzon, E.: Cannabinoids: new promising agents in the treatment of neurological diseases. - Molecules **19**: 18781-18816, 2014.

Guo, H.X., Kan, Y.C., Liu, W.Q.: Differential expression of miRNAs in response to topping in flue-cured tobacco (*Nicotiana tabacum*) roots. - PLoS ONE **6**: 1-15, 2011.

Haney, M., Gunderson, E.W., Rabkin, J., Hart, C.L., Vosburg, S.K., Comer, S.D., Foltin, R.W.: Dronabinol and marijuana in HIV-positive marijuana smokers. Caloric intake, mood, and sleep. - J. Acquir. Immune Defic. Syndr. **45**: 545-554, 2007.

Hearne, P.Q., Knorr, D.A., Hillman, B.I., Morris, T.J.: The complete genome structure and synthesis of infectious RNA from clones of *Tomato bushy stunt virus*. - Virology **177**: 141- 151, 1990.

Hellens, R.P., Moreau, C., Lin-Wang, K., Schwinn, K.E., Thomson, S.J., Fiers, M., Frew, T.J., Murray, S.R., Hofer, J.M.I., Jacobs, J.M.E.: Identification of Mendel's white flower character. - PLoS ONE **5**: 1- 8, 2010.

Hichri, I., Barrieu, F., Bogs, J., Kappel, C., Delrot, S., Lauvergeat, V.: Recent advances in the transcriptional regulation of the flavonoid biosynthetic pathway. - J. exp. Bot. **62**: 2465-2483, 2011.

Hillig, K.W.: Genetic evidence for speciation in *Cannabis* (*Cannabaceae*). - Genet. Resour. Crop Evol. **52**: 161-182, 2005.

Huda, K.M.K., Banu, M.S.A., Pathi, K.M., Tuteja, N.: Reproductive organ and vascular specific promoter of the rice plasma membrane Ca^{2+}ATPase mediates environmental stress responses in plants. - PLoS ONE **8**: e57803, 2013.

Jefferson, R.A., Kavanagh, T.A., Bevan, M.W.: GUS fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. - EMBO J. **6**: 3901-3907, 1987.

Langley, R.A., Kado, C.I.: Studies on *Agrobacterium tumefaciens*. Conditions for mutagenesis by N-methyl-N′-nitrosoguanidine and relationships of *A. tumefaciens* mutants to crown gall tumor induction. - Mutat. Res. **14**: 277- 286, 1972.

Li, F., Huang, C., Li, Z., Zhou, X.: Suppression of RNA silencing by a plant DNA virus satellite requires a host calmodulin-like protein to repress *RDR6* expression. -PLoS Pathog. **10**: e1003921, 2014.

Livak, K.J., Schmittgen, T.D.: Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}$T method. - Methods **25**: 402- 408, 2001.

Mano, H., Ogasawara, F., Sato, K., Higo, H., Minobe, Y.: Isolation of a regulatory gene of anthocyanin biosynthesis in tuberous roots of purple-fleshed sweet potato. - Plant Physiol. **143**: 1252-1268, 2007.

Mao, K., Wang, K., Zhao, M., Tao, X.T., Klionsky, D.J.: Two MAPK-signaling pathways are required for mitophagy in *Saccharomyces cerevisiae*. - J. cell. Biol. **93**: 755-767, 2011.

Matoušek, J., Kocábek, T., Patzak, J., Bříza, J., Siglová, K., Mishra, A.K., Duraisamy, G.S., Týcová, A., Ono, E., Krofta, K.: The "putative" role of transcription factors from HlWRKY family in the regulation of the final steps of prenylflavonid and bitter acids biosynthesis in hop (*Humulus lupulus* L.). - Plant mol. Biol. **92**: 263-277, 2016.

Matoušek, J., Kocábek, T., Patzak, J., Füssy, Z., Procházková, J., Heyerick, A.: Combinatorial analysis of lupulin gland transcription factors from R2R3MYB, bHLH and WDR families indicates a complex regulation of *chs_H1* genes essential for prenylflavonoid biosynthesis in hop (*Humulus lupulus* L.). - BMC Plant Biol. **12**: 27, 2012.

Matoušek, J., Kocabek, T., Patzak, J., Skopek, J., Maloukh, L., Heyerick, A., Fussy, Z., Roldàn-Ruiz, I., De-Keukeleire, D.: HlMyb3, a putative regulatory factor in hop (*Humulus lupulus* L.), shows diverse biological effects in heterologous transgenotes. - J. Agr. Food Chem. **55**: 7767-7776, 2007.

Matoušek, J., Kocábek, T., Patzak, J., Stehlík, J., Füssy, Z., Krofta, K., Heyerick, A., Roldán-Ruiz, I., Maloukh, L., De-Keukeleire, D.: Cloning and molecular analysis of HlbZip1 and HlbZip2 transcription factors putatively involved in the regulation of the lupulin metabolome in hops (*Humulus lupulus* L.). - J. Agr. Food Chem. **58**: 902-912, 2010.

Matoušek, J., Vrba, L., Škopek, J., Orctová, L., Pešina, K., Heyerick, A., Baulcombe, D., De Keukeleire, D.: Sequence analysis of a "true" chalcone synthase (chs_H1) oligofamily from hop (*Humulus lupulus* L.) and PAP1 activation of chs_H1 in heterologous systems. - J. Agr. Food Chem. **54**: 7606-7615, 2006.

Matus, J.T., Poupin, M.J., Cañón, P., Bordeu, E., Alcalde, J.A., Arce-Johnson, P.: Isolation of *WDR* and *bHLH* genes related to flavonoid synthesis in grapevine (*Vitis vinifera* L.). - Plant mol. Biol. **72**: 607-620, 2010.

Menke, F.L., Kang, H.G., Chen, Z., Park, J.M., Kumar, D., Klessig, D.F.J.: Tobacco transcription factor WRKY1 is phosphorylated by the MAP kinase SIPK and mediates HR-like cell death in tobacco. - Mol. Plant-Microbe Interact. **18**: 1027-1034, 2005.

Russo, E.B., Burnett, A., Hall, B., Parker, K.K.: Agonistic properties of cannabidiol at 5-HT_{1A} receptors. - Neurochem. Res. **30**: 1037-1043, 2005.

Saghai-Maroof, M.A., Soliman, K.M., Jorgensen, R.A., Allard, R.W.: Ribosomal DNA spacer-length polymorphism in barley: Mendelian inheritance, chromosomal location, and population dynamics. - Proc. natl. Acad. Sci. USA **8**: 8014-8018, 1984.

Shimada, S., Otsuki, H., Sakuta, M.: Transcriptional control of anthocyanin biosynthetic genes in the *Caryophyllales.* - J. exp. Bot. **58**: 957-967, 2006.

Silhavy, D., Molnar, A., Lucioloi, A., Szittya, G., Hornyik, C., Tavazza, M., Burgyan, J.A.: Viral protein suppresses RNA silencing and binds silencing generated, 21 to 25 nucleotide double stranded RNAs. - EMBO J. **21**: 3070-3080, 2002.

Small, E., Cronquist, A.: A practical and natural taxonomy for *Cannabis*. - Taxon 2**5**: 405-435, 1976.

Solís-Ramos, L., Nahuath-Dzib, S., Andrade-Torres, A., Barredo-Pool, F., González-Estrada, T., De la Serna, E.: Indirect somatic embryogenesis and morphohistological analysis in *Capsicum chinense*. - Biológia **65**: 504-511, 2010.

Stracke, R., Ishihara, H., Huep, G., Barsch, A., Mehrtens, F., Niehaus, K., Weisshaar, B.: Differential regulation of closely related R2R3-MYP transcription factors controls flavonol accumulation in different parts of the *Arabidopsis thaliana* seedling. - Plant J. **50**: 660-677, 2007.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S.: MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. - Mol. Biol. Evol. **28**: 2731-2739, 2011.

Taura, F., Sirikantaramas, S., Shoyama, Y., Yoshikai, K., Shoyama, Y., Morimoto, S.: Cannabidiolic-acid synthase, the chemotype-determining enzyme in the fiber-type *Cannabis sativa*. - FEBS Lett. **581**: 2929-2934, 2007.

Van Bakel, H., Stout, J.M., Cote, A., Tallon, C.M., Sharpe, A.G., Hughes, T.R., Page, J.E.: The draft genome and transcriptome of *Cannabis sativa*. - Genome Biol. **12**: 1-17, 2011.

Várallyay, E., Oláh, E., Havelda, Z.: Independent parallel functions of p19 plant viral suppressor of RNA silencing required for effective suppressor activity. - Nucl. Acids Res. **42**: 599-608, 2014.

Venter, M., Botha, F.C.: Synthetic promoter engineering. - In:

Pua, E.C., Davey, M.R. (ed.): Plant Development and. Biological and Biotechnological Perspectives. Vol. 1. Pp. 393-414. Springer-Verlag, Berlin - Heidelberg 2010.

Voinnet, O., Rivas, S., Mestre, P., Baulcombe, D.: An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. - Plant J. **33**: 949-956, 2003.

Yang, Z., Patra, B., Li, R., Pattanaik, S., Yuan, L.: Promoter analysis reveals *cis* regulatory motifs associated with the expression of the WRKY transcription factor *CrWRKY1* in *Catharanthus roseus*. - Planta **238**: 1039-1049, 2013.

Yoshida, T., Fujita, Y., Sayama, H., Kidokoro, S., Maruyama, K., Mizoi, J., Shinozaki, K., Yamaguchi-Shinozaki, K.: AREB1, AREB2, and ABF3 are master transcription factors that cooperatively regulate ABRE-dependent ABA signaling involved in drought stress tolerance and require ABA for full activation. - Plant J. **61**: 672-685, 2010.

Zhang, B., Wang, O., Qin, J., Liu, S., Sun, S., Liu, H., Kuang, J., Jiang, G., Zhang, W.: *cis*-acting elements and *trans*-acting factors in the transcriptional regulation of Raf kinase inhibitory protein expression. - PLoS ONE **8**: e83097, 2013.