Mol Biol Rep (2012) 39:5433–5447
DOI 10.1007/s11033-011-1343-8

Molecular cloning and functional characterization of *Catharanthus roseus* hydroxymethylbutenyl 4-diphosphate synthase gene promoter from the methyl erythritol phosphate pathway

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Received: 6 July 2011 / Accepted: 3 December 2011 / Published online: 13 December 2011

Abstract The Madagascar periwinkle produces monoterpenoid indole alkaloids (MIA) of high interest due to their therapeutical values. The terpenoid moiety of MIA is derived from the methyl erythritol phosphate (MEP) and seco-iridoid pathways. These pathways are regarded as the limiting branch for MIA biosynthesis in *C. roseus* cell and tissue cultures. In previous studies, we demonstrated a coordinated regulation at the transcriptional and spatial levels of genes from both pathways. We report here on the isolation of the 5′-flanking region (1,049 bp) of the hydroxymethylbutenyl 4-diphosphate synthase (HDS) gene from the MEP pathway. To investigate promoter transcriptional activities, the HDS promoter was fused to GUS reporter gene. Agrobacterium-mediated transformation of young tobacco leaves revealed that the cloned HDS promoter displays a tissue-specific GUS staining restricted to the vascular region of the leaves and limited to a part of the vein that encompasses the phloem in agreement with the previous localization of HDS transcripts in *C. roseus* aerial organs. Further functional characterizations in stably or transiently transformed *C. roseus* cells allowed us to identify the region that can be consider as the minimal promoter and to demonstrate the induction of HDS promoter by several hormonal signals (auxin, cytokinin, methyljasmonate and ethylene) leading to MIA production. These results, and the bioinformatic analysis of the HDS 5′-region, suggest that the HDS promoter harbours a number of *cis*-elements binding specific transcription factors that would regulate the flux of terpenoid precursors involved in MIA biosynthesis.

Keywords Promoter · Hydroxymethylbutenyl 4-diphosphate synthase gene · Methyl erythritol phosphate pathway · Monoterpenoid indole alkaloids · *Catharanthus roseus* · GUS activity

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Introduction

Among the plant isoprenoid derivatives, the alkaloids are low-molecular weight, nitrogen-containing compounds found in about 20% of plant species. Their function as secondary metabolites is mainly dedicated to plant defence against herbivores and pathogens [1, 2]. Many of the 12,000 circa known alkaloids have been exploited as pharmaceuticals, stimulants, narcotics or poisons. *Catharanthus roseus* (the Madagascar periwinkle) is one of the main source of monoterpenoid indole alkaloids (MIA) of therapeutic interest, including monomers such as ajmalicine and serpentine, used in the treatment of antiarrythmic heart disorder and anxiety, and heterodimers such as vinblastine and vincristine, used especially for their anti-tumor activities [3–5]. All MIA are synthesized from a common precursor, strictosidine, which results from the condensation of an indole moiety derived from tryptamine and a monoterpenoid component derived from the iridoid glucoside secologanin (Fig. 1). The biosynthesis of secologanin requires both the methyl erythritol phosphate (MEP) pathway and the monoterpene seco-iridoid pathway. The plastidial MEP pathway provides isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) that are condensed to form geraniol through two enzymatic steps. The hydroxylation of geraniol into

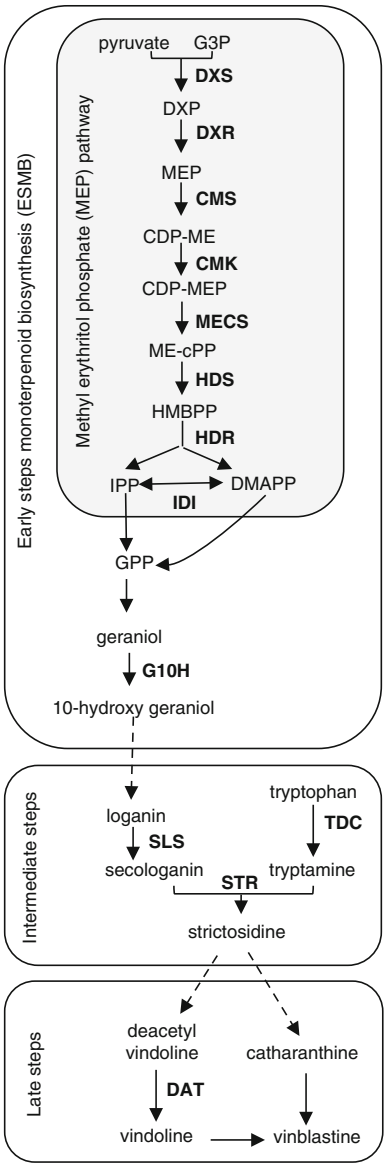

Fig. 1 The pathway for monoterpenoid indole alkaloid biosynthesis in *C. roseus*. *G3P* glyceraldehyde 3-phosphate, *DXP* deoxyxylulose 5-phosphate, *MEP* methylerythritol 4-phosphate, *CDP-ME* 4-diphosphocytidyl-methylerythritol, *CDP-MEP* 4-diphosphocytidyl-methylerythritol 2-phosphate, *ME-cPP* methylerythritol 2,4-cyclodiphosphate, *HMBPP* hydroxymethylbutenyl 4-diphosphate, *IPP* isopentenyl diphosphate, *DMAPP* dimethylallyl diphosphate, *GPP* geranyl disphosphate. Enzymes are indicated in *bold*: *DXS*, DXP synthase; *DXR* DXP reductoisomerase, *CMS* CDP-ME synthase, *CMK* CDP-ME kinase, *MECS* ME-cPP synthase, *HDS* HMBPP synthase, *HDR* HMBPP reductase, *IDI* IPP isomerase, *G10H* geraniol 10-hydroxylase, *SLS* secologanin synthase, *STR* strictosidine synthase, *TDC* tryptophan decarboxylase, *DAT* deacetylvindoline-4-*O*-acetyltransferase

10-hydroxy-geraniol catalyzed by geraniol 10-hydroxylase (G10H) constitutes the first committed step in biosynthesis of secologanin [6]. To date, four genes from the MEP pathway encoding deoxyxylulose 5-phosphate synthase (DXS), deoxyxylulose 5-phosphate reductoisomerase (DXR), methyl-D-erythritol 2,4-diphosphate synthase (MECS) and hydroxymethylbutenyl 4-diphosphate synthase (HDS) have been characterized in *C. roseus* [7–9].

The hormonal regulation of MIA biosynthesis has mainly been studied in undifferentiated *C. roseus* cell suspensions [10]. For instance, in our model system, the C20D cell line, exogenous treatments including phytohormones can modify MIA production. Such cells require the presence of auxin (provided as 2,4-dichlorophenoxy acetic acid, 2,4-D) in the culture medium for their normal growth. Under these culture conditions, C20D cells do not accumulate MIA but their transfer in a 2,4-D free-medium (named inducing-medium, IM) induces MIA biosynthesis [11, 12]. Furthermore, addition of cytokinin, methyljasmonate (MeJa) or ethylene greatly enhances MIA production [13–15]. At the molecular level, a direct correlation between MIA biosynthesis and gene expression of the early steps of monoterpenoid biosynthesis (ESMB) including those catalyzed by DXS, DXR, MECS, HDS and G10H, has been established. Indeed, an increase in the mRNA levels of the ESMB genes can be monitored during MIA biosynthesis whilst no modification of expression occurs for genes encoding enzymes catalyzing intermediate steps of MIA biosynthesis including secologanin synthase (SLS), tryptophan decarboxylase (TDC) and strictosidine synthase (STR) [9]. Such regulation of gene expression appears as a reminiscence of the tissue-dependent pattern of gene expression since the pool of hormone-induced genes (ESMB genes) are specifically expressed in the so-called internal-phloem parenchyma (IPAP) cells while the transcripts of hormone-unregulated genes are restricted to epidermis [9, 16]. In a similar way, protein prenylation events catalyzed by CaaX-prenyltransferases have been showed to be required for ESMB gene expression but not for SLS, TDC and STR [17]. All these results emphasize the importance of a coordinated regulation of the EMSB genes leading to MIA biosynthesis.

Transcription factors are known to play a key role in the regulation of secondary metabolism by controlling biosynthetic pathway gene expression [18]. In *C. roseus*, jasmonate signaling has been extensively studied and several transcription factors have been identified [19, 20]. These include transcriptional activators ORCA2 and ORCA3, two AP2/ERF domain transcription factors [21, 22] as well as repressors, crGBF1 and crGBF2, two G-Box binding factors [23] and ZCT1, ZCT2, and ZCT3, which are members of the transcription factor IIIA (TFIIIA-type; Cys_2/His_2-type) zinc finger protein family [24]. Interestingly, the overexpression of ORCA3 in *C. roseus* cells induced several MIA biosynthetic genes including TDC and STR but not G10H [21]. ORCA3 transactivates the STR promoter via sequence-specific binding to a jasmonate- and elicitor-responsive element (JERE) [21, 25]. The observation that G10H is not regulated by ORCA3 was further confirmed by the characterization of

its promoter, which contains a number of regulatory *cis*-elements involved in regulation of gene expression but no JERE motif [26]. It is likely that the G10H regulation implicates a different transcriptional cascade than those of TDC and STR. As a consequence, the availability and characterization of other gene promoters, such the MEP pathway genes, whose regulation is coordinated with G10H, will help to identify new regulators and to further understand the mechanisms underlying MIA biosynthesis in *C. roseus* cells.

Here, we report on the isolation and characterization of the *C. roseus* HDS promoter. Our analyses suggest that the HDS promoter contains several *cis*-elements and transcriptional enhancers. The isolated promoter can control gene expression in *C. roseus* cells and tobacco plants and is responsive to induction by various hormonal signals in correlation with MIA production. Our results suggest that new families of transcription factors could regulate both the MEP and monoterpene seco-iridoid pathways in *C. roseus* cells.

Materials and methods

Promoter isolation and analysis

The *Catharanthus roseus* HDS promoter sequence was isolated by Thermal Asymmetric Interlaced-PCR (TAIL-PCR) [27, 28]. Sets of nested specific primers were synthesized by Operon Technologies and are listed in Table 1. Specific primers were paired with arbitrary degenerated (AD) random primers AD1, AD2, AD3, AD4, AD5 and AD6 (Table 1) to generate TAIL-PCR amplicons. In this study, three rounds of TAIL-PCR were carried out using the previous PCR as template for the next and employing a common AD primer and nested-gene specific primers in a consecutive manner. All PCR reactions were performed according to Liu and Huang [27] and Li and Gray [28] with the following modifications. Primary TAIL-PCR reaction mixtures contained in a final volume of 20 µl of 1× PCR buffer (Promega), 2 mM $MgCl_2$ (Promega), 0.1 mM each dNTP (Promega), 0.15 µM specific primer, 20 µM AD primer, 1 *U* of GoTaq DNA polymerase (Promega) and 500 ng of genomic DNA, extracted from young leaves of *C. roseus* with the NucleoSpin® Plant kit and according to the manufacturer's instructions (Macherey–Nagel). For secondary reactions, primary PCR products were diluted 40 times in distillated water and 1 µl was added to a 24 µl secondary PCR buffer mixture containing 2 mM $MgCl_2$, 0.1 mM each dNTP, 0.8 U of GoTaq DNA polymerase, 0.2 µM nested specific primer and the same AD primer (2 µM) used in the first reaction. After amplification, secondary PCR products were diluted 1:10 and 1 µl of dilution was transferred to a 1× PCR buffer mixture of 49 µl with concentrations of $MgCl_2$, dNTP, and primers similar to those used for secondary PCR with the only difference that the innermost specific primer is used in this reaction. The cycling conditions were performed according to Li and Gray [28] for the primary PCR and to Liu and Huang [27] for the two additional amplification rounds. An initial denaturation step at 94°C for 2 min was added for each PCR phase. PCR products were analyzed by electrophoresis on a 1.2% agarose gel, extracted from gel and purified with Nucleospin® Extract II kit according to manufacturer's instructions (Macherey–Nagel). The cDNA were cloned into the pGEMT-easy vector (Promega) and further sequenced (Operon technologies). Putative *cis*-elements within the amplified fragment were analyzed using PLACE [29, 30] and PlantCare databases [31, 32].

Construction of the GUS expression cassettes

The transcriptional fusion of the HDS promoter and the GUS gene was achieved using pCambia 1305 or pSCA-cassette GUS vectors. The pSCA-cassette GUS vector was constructed following amplification of the GUS expression cassette of pCAMBIA 1305 (pCamb-35S::GUS; GenBank AF354045; nt 11,263 to nt 2,371) using primers pC1302-35S and pC1302-GFP, and cloning in the pSCA vector (StrataClone PCR Cloning Kit, Stratagene). The resulting pSCA-cassette GUS plasmid (pSCA-35S::GUS) allows the expression of the GUS reporter gene under the control of CaMV35S promoter. To construct HDS promoter-GUS expression vector, a fragment (1,075 bp) containing the 5′ region and the beginning of the ORF (+1 nt to +26 nt) of HDS gene was amplified by PCR using primers pCaHD-SpromPst and pCaHDSpromBgl2 (Table 1) designed to introduce *Pst*I and *Bgl*II restriction sites to the 5′ and 3′ extremity of the PCR products, respectively. The CaMV35S promoter driving the GUS gene expression was removed by a *Pst*I/*Bgl*II digestion of plasmids followed by the cloning of the HDS promoter fragment. The resulting vectors were named pCamb-HDS::GUS and pSCA-HDS::GUS when cloning into the pCambia 1305 and pSCA-cassette GUS vectors respectively.

Construction of plasmids for 5′ deletion experiments

A series of HDS promoter fragments of various lengths were amplified by PCR from pSCA-HDS::GUS plasmid with specific appropriate primers (Table 1). PCR amplification was performed on a final volume of 25 µl of 1× PCR buffer, 3.5 mM $MgSO_4$, 0.4 mM each dNTP, 0.5 µM of each specific primer (Table 1), 0.7 U of *Pfu* DNA polymerase (Promega). The program was initiated by a denaturation step at 94°C for 2 min and was as followed: 94°C for 30 s, 57°C for 30 s and 74°C for 1 min 45 s. After gel purification, each PCR product was cloned into the *Pst*I

Table 1 List of primers used in this study

Primer	Sequence (5′–3′)[a]
AD1	NTCGASTWTSGWGTT
AD2	NGTCGASWGANAWGAA
AD3	WGTGNAGWANCANAGA
AD4	AGWGNAGWANCAWAGG
AD5	NWGSTTMGAACNCGCT
AD6	TGWGNAGWANCASAGA
HDSTAIL1	GGCTACATTCCCAACCATTACTGTGCGTGT
HDSTAIL2	AACAGTTTCTGGACCAGGGTTGGAGTTCTTTA
HDSTAIL3	AGATGCTGGAACTGTTCCGGTCGCCAT
HDSTAIL4	ACCGTTAATCTGCTTCAGCCCACAACCTTC
HDSTAIL5	ACCAGGAATTAATCAAACCTTAACAACGAATTTCGAG
HDSTAIL6	AGCCCAACTACGAAAGAACGACAATTACGATTC
pCaHDSpromPst	AGCTGCAGATTAGTAGAAATAATAATTAAAAAAACAGTTTA AAAAAAATTTAAACTTACGT
pCaHDSpromBgl2	ATAGATCTGATGCTGGAACTGTTCCGGTCGCCATTTCA
hdspromominPst0	AGCTGCAGGTTTAATTTCCCCCTCATACCCTTTC
hdspromominPst1	AGCTGCAGCCCCTAATTTTTTCTGAGACCCATTTC
hdspromominPst2	AGCTGCAGCTGCTCATATATAAGTGCATTCCATTTCCCC
hdspromominPst3	AGCTGCAGCTGTAGTTCGGGGCTTTACTTTCACTCT
hdspromominPst4	AGCTGCAGTGCAAGAACAGCCAACGTTAACAAC
hdspromominPst5	AGCTGCAGGGACAAGAATACAAGTTCTTGTGTTATACCAC
pCaHDSavintronBgl	ATAGATCTGTTCCGGTCGCCATTTCACTAGAAATGGGTCTCAGAA AAAATTAGGGGAAATGGAATGCA
pc1302-35S	GCTTGGATCAGATTGTCGTTTCC
pc1302-GFP	GCCAATATATCCTGTCAAACACTG
RPS9RTfor	AGGCACATAAGGGTTGGAAAG
RPS9RTrev	AGGTCTGATTGATATCCTTCAGT
GEC1	ATTATGGCGACCGGAACAGTTCCA
Hygfor	CGGAAGTGCTTGACATTGGG
Hygrev	TGTTGGCGACCTCGTATTGG
5′-GUSrev	CACGACCAGCTCACCATTGA
3′-HDSfor	CTACTTCAGGAGTCTGAAGATTTTGAG
3′-35Sfor	ACGCACAATCCCACTATCCTT

[a] N indicates A, C, G or T; W indicates A or T; S indicates G or C; M indicates A or C; Incorporated restriction site sequences are underlined

and *Bgl*II sites of pSCA-cassette GUS expression vector. A total of 7 plasmids were constructed corresponding to coordinates −1,049 to +26 (construct 1), −902 to +26 (construct 2), −796 to +26 (construct 3), −694 to +26 (construct 4), −656 to +26 (construct 5), −629 to +26 (construct 6), −600 to +26 (construct 7). Based on pSCA-HDS::GUS1 vector, a supplementary plasmid was constructed lacking the 5′-UTR region located between −600 nt and −6 nt (construct 1Δ).

Transformation of *C. roseus* C20D suspension cells

C. roseus cell suspension cultures (C20D dark-grown strain) were maintained at 24°C under continuous shaking (100 rpm), on a 7-day-growth cycle in the B5 medium of Gamborg et al. [33] supplemented with 58 mM sucrose and 4.5 μM 2,4-D (maintenance medium, MM) as previously described [11]. Prior to particle bombardment, 4 ml of a 3-day old *C. roseus* C20D cell suspension culture was vacuum filtered and plated onto solid MM or a 2,4-D free-medium (induction medium, IM) (8 g l^{-1} agar) supplemented with or without zeatin (5 μM). Plated cells were then cultivated during 48 h in the dark at 24°C before bombardment. Transient transformation of *C. roseus* plated cells was carried out with the Bio-Rad PDS1000/He biolistic particle delivery system, as previously described [34]. The HDS::GUS plasmids used for particle bombardment were isolated and purified from recombinant *E. coli* cultures using the Nucleospin® Plasmid kit following the manufacturer's instructions and approximately 1 μg DNA was used per shot. Following DNA delivery, cells were cultivated 24 h before GUS assays. Two independent

assays were performed with at least five replicates for each construct. GUS reporter gene expression obtained by bombardment with pCamb-HDS::GUS or pSCA-HDS:: GUS vectors was related to GUS gene expression obtained with pCamb-35S::GUS or pSCA-35S::GUS corresponding plasmids respectively, to correct for transformation efficiency.

Establishment, treatments and molecular analysis of stably transformed *C. roseus* cells

Stably transformed *C. roseus* cell lines, containing plasmid DNA pSCA-35S::GUS or pSCA-HDS::GUS and a vector carrying the hygromycin-selection gene [35], were obtained by particle bombardment, as described above. Plated cells were then transferred onto solid selection Gamborg B5 medium (8 g l^{-1} agar) supplemented with 10 μM naphthalene acetic acid (NAA), 1 μM kinetin, 50 mg l^{-1} hygromycin and 250 mg l^{-1} cefotaxime. Plated cells were transferred onto fresh selection medium every week for 1 month, until calli appeared. Then, resistant calli were transferred onto fresh selection medium every month during 3 cell culture cycles. Transformed cells were maintained. For experimental purposes, transgenic cell lines, maintained on MM medium (at 24°C in the dark), were sub-cultured in one of the following media: an IM medium to which zeatin (5 μM) or ethephon (0.5 mM) were added at the third day of culture or MeJa (100 μM) diluted in ethanol added at the fourth day of culture. For MeJa experiments, control cultures were treated with ethanol only.

For molecular analysis of transgenic cell lines, 300 ng genomic DNA extracted with the NucleoSpin® Plant kit according to the manufacturer's instructions (Macherey–Nagel) was used as template to amplify fragments of the transgenes corresponding to transcriptional fusions of the HDS (in HDS-transgenic cells) or 35S (in 35S- (control) transgenic cells) promoter and the GUS gene and a fragment of the hygromycin gene used as selection marker in both HDS- and 35S-transgenic lines. Specific primers of either HDS promoter (3′-HDSfor primer) or 35S promoter (3′-35Sfor primer) were paired with a primer (5′-GUSrev) designed in the coding region of the GUS gene to amplify 592 and 627 bp of transcriptional fusions of HDS or 35S promoters and GUS gene, respectively. The presence of the hygromycin-selection gene was checked by using a specific primer pair (Hygfor and Hygrev) designed in the corresponding coding region. The amplification program was initiated by a denaturation step at 94°C for 2 min and was as followed: 94°C for 30 s, 55°C for 30 s and 72°C for 50 s (35 cycles). PCR products were analyzed by electrophoresis on a 1.2% agarose gel.

Agrobacterium transformation of tobacco leaf discs

Tobacco (*Nicotiana tabacum* L. variety Xanthi) leaf discs were transformed with the *A. tumefaciens* LBA4404 strain containing pCamb-HDS:GUS or pCamb-35S:GUS. Bacteria strains were grown, to an optical density of 0.6 at 600 nm, in Luria-Bertoni medium supplemented with 50 μg l^{-1} kanamycin and 100 μg l^{-1} streptomycin. Leaf discs were co-cultivated with bacteria strains on solid shooting medium (½ Murashige and Skoog (MS), 58 mM sucrose, 1 mg l^{-1} 6-benzyl amino purine (BAP)) at 25°C for 48 h, and then transferred onto solid shooting medium containing 250 mg l^{-1} cefotaxime, to eliminate bacterial carry over, and 50 μg l^{-1} hygromycin, to select for transformed tissues. Transgenic plants were further maintained on solid ½ MS medium supplemented with 58 mM sucrose and transferred to fresh medium every month.

GUS activity assays

Quantification of β-glucuronidase (GUS) activity in *C. roseus* transformed cells and histochemical staining to localize the distribution of GUS activity in tobacco transgenic plants were performed according to the method of Jefferson et al. [36]. For fluorometric assays, transformed cells were first homogenized in protein extraction buffer (50 mM $NaPO_4$ pH 7.0, 10 mM EDTA Na_2, 0.2% Triton X-100, 10 mM β-mercaptoethanol) and centrifuged at 10,000 g for 10 min at 4°C. Protein concentration of the supernatant was determined using Bradford protein assay reagent (Bio-Rad) with bovine serum albumin as standard. For GUS enzyme assay, diluted aliquots of supernatant (from 0.01 to 1 mg/ml) were reacted with 1 mM 4-methylumbelliferyl-β-D-glucoside (MUG) and incubating at 37°C for 1–2 h. The reaction was diluted tenfold in the stop buffer (0.2 M Na_2CO_3) and the enzymatic products were measured by fluorometry upon excitation/emission wavelength of 365/455 nm. GUS enzyme activity was expressed in pmoles 4-methylumbelliferone (4-MU) produced per minute per milligram protein. Presented data are GUS enzyme activity mean ± SD from five distinct bombardment experiments for transient expression assays and from five HDS-transgenic cell culture samples and are expressed relative to the reference condition. Histochemical staining was done according to the protocol described by Jefferson et al. [36] with slight modifications. Plant parts to be stained were incubated overnight in GUS reaction solution, containing 50 mM $NaPO_4$ (pH 7.0), 10 mM EDTA, 0.5 mM each of Kferri- and ferrocyanide in 1% (v/v) Triton-X and 2 mM of 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc; (Glycosynth)) dissolved in *NN*-dimethylformamide. Plant fragments were fixed in FAA (50% ethanol, 5% acetic acid, and 5% formaldehyde)

for 4 h at 4°C, and embedded in Paraplast as previously described [37]. Serial sections (10 μm) were spread on silane-coated slides overnight at 40°C, and paraffin was removed using xylene (two times 15 min) before rehydration in an ethanol gradient series.

HDS transcript analysis by semi quantitative RT-PCR

HDS gene expression was estimated by semi-quantitative RT-PCR. 7-days-old C20D cells were subculture on IM medium supplemented with or without ethephon (IM+E; 0.5 mM) at the third day of subculture. Cells were harvested 48 h after ethephon addition, quickly frozen and ground to powder in liquid nitrogen. Total RNA were extracted with Nucleospin RNA plant kit according to the manufacturer's instructions (Macherey–Nagel). Total RNA (2 μg) were treated with RQ1 RNase-free DNase (Promega) and used for first strand cDNA synthesis by priming with oligod(T_{17}) (0.6 μM). Reverse transcriptions were performed in a 20 μl reaction mixture by use of the SuperScript™ III Reverse Transcriptase (Invitrogen). 1 μl of each RT reaction diluted 10 times, were used for subsequent PCR. HDS and RPS9 cDNA were amplified using GEC1/HDSTAIL1 and RPS9for/RPS9rev primer pairs, respectively (Table 1). PCR amplifications were started by an initial denaturation at 94°C for 2 min and then performed under the following conditions: 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, followed by a final extension at 72°C for 5 min. The number of cycles was, respectively 28 and 30, for RPS9 and HDS genes. Samples (25 μl) were analysed by electrophoresis on a 1.2% agarose gel.

Statistical analysis

Data were analysed with Statistica, version 6.0 (StatSoft Inc., Tulsa, USA). Statistical significance from different treatment was revealed after one-way analysis of variance (ANOVA) test followed by Tukey's test. Asterisks indicate that values are significantly different ($P < 0.05$).

Results

Cloning and sequence analysis of the HDS promoter

To isolate the HDS promoter, Thermal-Asymmetric Inter-Laced (TAIL)—PCR was performed on genomic DNA with specific primers and arbitrary degenerated primers as described in "Materials and methods". A 5′-flanking region fragment of the HDS gene was amplified including 1,049 bp upstream of the initiation codon (Fig. 2). This region encompasses the 141 bp-long 5′-untranslated region (UTR) of the HDS transcript cloned previously [9] (GenBank accession number AY184810). The comparison of these two sequences allowed the identification of an intron located 33 bp upstream of the initiation start codon in the 5′-UTR of HDS and flanked by a 5′ splice site (cag/gtt, −497 to −492, from ATG) and a 3′ splice site (cag/gag,−35 to −30). This suggests in turn that the remaining 109 nucleotides of the 5′-UTR of HDS, at least, include the first non coding exon.

In addition, the analysis of the genomic cloned region, using the PLACE [29, 30] and PlantCARE [31, 32] programs allowed cataloguing a number of potential regulatory motifs corresponding to known *cis*-elements of eukaryotic genes including potential TATA-box (−649 to −643 from ATG) and CAAT-box (−666 to −663 from ATG). Therefore, the genomic region located upstream of the consensus TATA-box was assimilated to the HDS promoter. Other *cis*-elements with roles in regulation of gene expression have been also identified within both the promoter and the intron containing 5′-UTR, and are listed in Table 2 [38–50]. These include *cis*-elements associated with hormone regulations and found in other plant gene promoters: ten Arabidopsis Response Regulator 1 type B (ARR1) transcription factor recognition sequences involved in cytokinin signaling and two sequences critical for cytokinin-enhanced binding; two Ethylene Response Element (ERE)-like motifs as ethylene-responsive element and three pyrimidine-boxes known as to be gibberellic acid responsive elements. We also noted the presence of 19 Dof (DNA binding with one finger) transcription factor recognition core sequences, which could be involved in auxin, jasmonate or ethylene responsiveness as previously reported [51, 52]. In addition, the HDS promoter contains *cis*-elements previously associated with light responsiveness, including the GT1-motif, the I-box core, and the GATA-box (Table 2). The bioinformatic analysis also revealed the presence of ten motifs essential for tissue specific expression and other putative *cis*-acting elements involved in different biological processes such as one MYB-binding site and three W-box recognizing WRKY-type transcription factors. In conclusion, based on the predictive identification of putative *cis*-regulating element, it could be hypothesized that the transcriptional activity of the HDS promoter is regulated by different signals.

The cloned HDS promoter is transcriptionally active and displays a tissue-specific expression in transgenic tobacco plants

A current method to study promoter transcriptional activity relies on the creation of transcriptional fusion of the promoter with a reporter gene encoding GUS or green fluorescent protein (GFP) for instance. A set of transformation vectors has been developed including the widely used pCambia 1305 plasmid (Genbank accession number

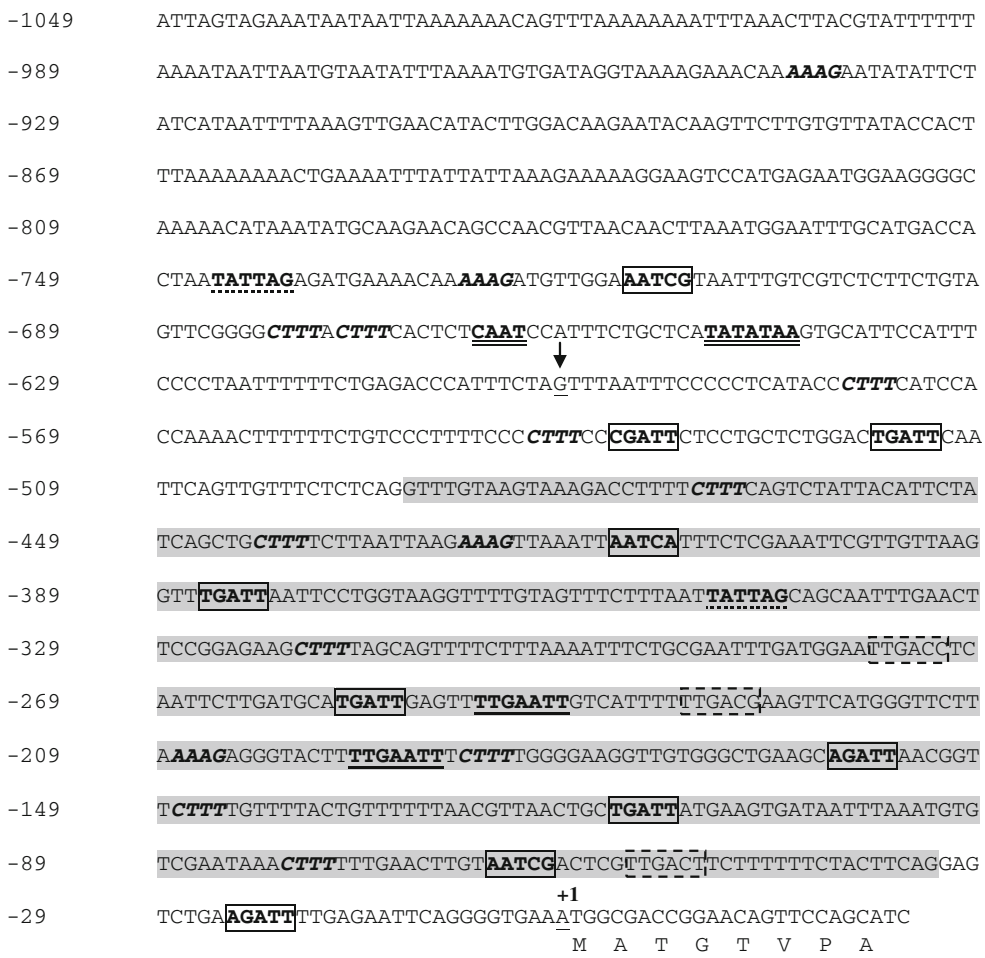

Fig. 2 Nucleotide (nt) sequence of the HDS 5′-flanking region. Numbers indicate the positions relative to the translational start codon ATG with the adenine (*underlined*) assigned at position +1. The first eight amino acids of HDS are shown below the nucleotide sequence. The *arrow* indicates the first nt (*underlined* G) of the 5′-UTR region that included an intron indicated on a *gray background*. The potential TATA and CAAT boxes are shown in *bold* and *double-underlined*. Some regulatory motifs potentially involved in hormonal responsiveness are indicated: the ARR1AT binding sites and CPBCSPOR motifs implicated in cytokinin signaling are respectively framed and *underlined* with *dashed line*; ERELEE4 motifs found in ethylene-inductible promoter genes are in *bold* and *underlined with solid line*; the W-box motifs binding to WRKY transcription factors are framed with *dashed line*; the essential sequence elements (DOFCOREZM) binding Dof proteins are in *bold* and in *italic*

AF354045). This vector displays a hygromycin phosphotransferase gene driven by a double 35S gene from cauliflower mosaic virus (CaMV) adjacent to the reporter GUS cassette including a CaMV35S promoter controlling the GUS reporter gene and a NOS terminator. The transcriptional activity of the cloned HDS promoter was first studied using this vector by replacing the CaMV35S promoter of the reporter GUS cassette by the HDS promoter (including the 5′-UTR region until a region coding the first amino acids) to generate pCamb-HDS::GUS. This vector and the pCambia 1305 empty-plasmid have been subsequently used for agrobacterium-mediated transformation and regeneration of tobacco plants. Histochemical GUS staining performed on transversal section of transgenic tobacco young leaves reveals that both kinds of transformants exhibits GUS activity. Interestingly, while the pCambia 1305 plants exhibited a widely distributed staining, we noted that the pCamb-HDS::GUS displayed a tissue-specific staining (Fig. 3a–b). Such glucuronidase activity is restricted to the vascular region of leaves, excluding the xylem and limited to a part of the vein that encompasses the phloem, in agreement with the previous localization of HDS transcripts in IPAP of *C. roseus* aerial organs [9]. This result shows that the cloned HDS promoter is transcriptionally active and suggests that it contains the *cis*-elements conferring the tissue-specific expression in planta.

Function analysis of the HDS promoter in *C. roseus* suspension cells

To gain insight into the functional role of HDS promoter regions, sequential deletions of the *cis*-elements and fusion of the remaining promoter to the GUS reporter gene were carried out. However, several studies have reported a potential influence of a CaMV35S promoter on the transcriptional activity of a nearby promoter such as in the pCamb-HDS::GUS vector [53, 54]. To determine whether such a construct could influence the transcriptional activity of the HDS promoter and therefore the functional characterization of promoter, we have compared the HDS promoter activity in the pCamb-HDS::GUS plasmid with those obtained in a plasmid devoid of any additional CaMV35S promoter elements. This vector was generated following amplification of the GUS reporter cassette from the pCambia 1305 vector and cloning into the pSCA vector to

Table 2 Regulatory motifs identified in the HDS promoter region

Name of *cis*-element	Sequence[a]	Position from ATG	Function	References
ARR1AT	NGATT	−20, −31, −112, −156, −382, −411, −513, −532, −552, −711	ARR1 binding element involved in cytokinin signaling	[38]
CACTFTPPCA1	YACT	−37, −136, −197, −618, −669, −748, −868, −904	Essential motif for specific expression in mesophyll	[39]
CPBCSPOR	TATTAG	−344, −740	Sequence critical for cytokinin-enhanced binding	[40]
DOFCOREZM	AAAG	−77, −145, −184, −205, −316, −424, −439, −467, −539, −576, −673, −678, −724, −940	Essential sequence element in binding Dof proteins involved in many biological processes	[41]
EBOXBNNAPA	CANNTG	−443, −502	*Cis*-element binding BHLH factor involved in light responsiveness and tissue-specific	[42]
ERELEE4	AWTTCAAA	−189, −240	*Cis*-element found in ethylene-inducible promoter genes	[43]
GATABOX	GATA	−927, −958	*Cis*-element involved in light responsiveness	[44]
GT1CONSENSUS	GRWAAW	−41, −556, −616, −590, −627, −853, −951	Consensus GT-1 binding site in many light-regulated genes	[45]
IBOXCORE	GATAA	−100	Part of light responsive element	[46]
MYB2AT	TAACTG	−124	MYB binding site involved in stress dehydratation	[47]
Pyrimidine box	CCTTTT	−471, −546, −833	GA induction	[48]
TAAAGSTKST1	TAAAG	−301, −352, −478, −840, −915	Target motif for Dof protein	[49]
W-box	TTGACY	−50, −226, −272	WRKY binding site, involved in many physiological processes	[50]

[a] N indicates A, C, G or T; W indicates A or T; Y indicates C or T; R indicates A or G

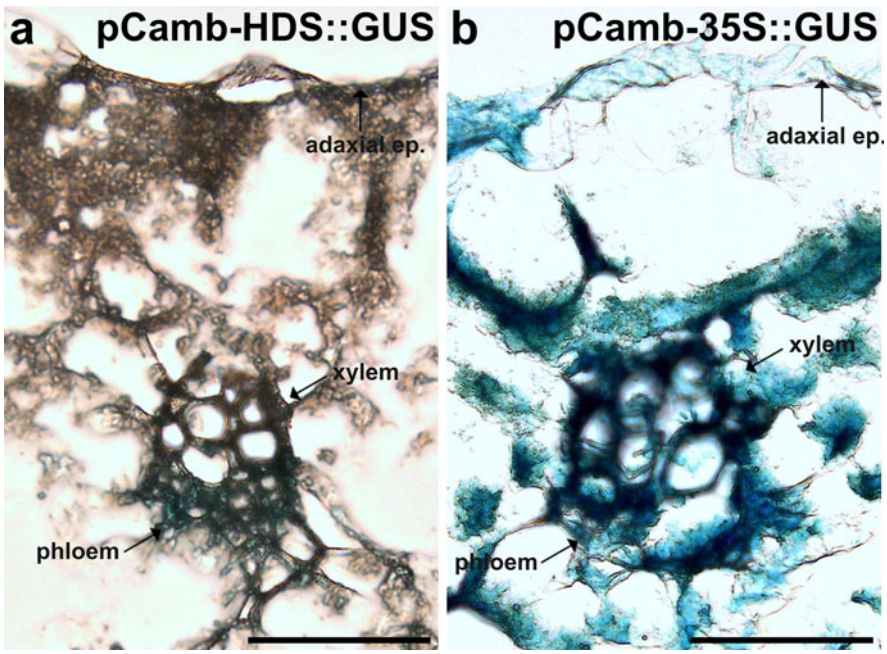

Fig. 3 Histochemical localization of GUS activity in transgenic tobacco young leaves. Tobbaco leaves were stably transformed with pCamb-HDS:GUS (**a**) or pCambia 1305 empty plasmid (**b**). Adaxial ep:adaxial epidermis. *Bars*: 10 μM

generate the pSCA-cassette GUS vector. As previously described for the pCamb-HDS::GUS, the CaMV35S promoter of the reporter GUS cassette was replaced by the HDS promoter including the 5′-UTR region and the region coding the first amino acids, to generate pSCA-HDS::GUS (Fig. 4a–b).

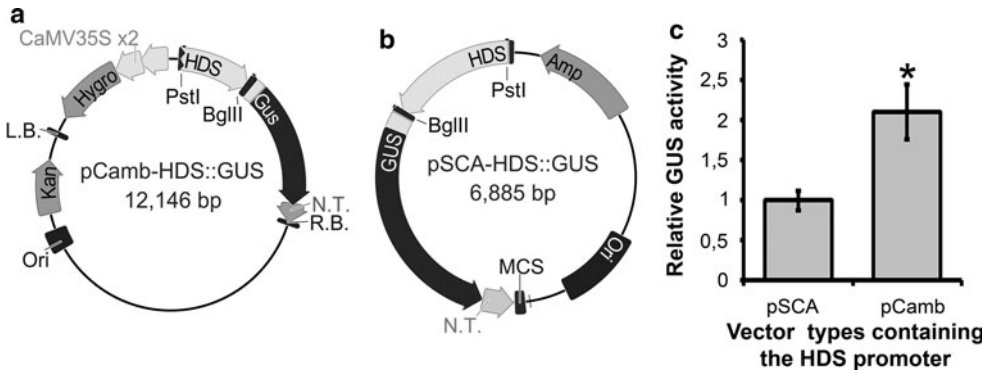

Fig. 4 Comparison of the activity of the HDS promoter cloned into two different reporter vectors. **a** The HDS promoter was cloned into the pCambia 1305.1 plasmid which displays a hygromycin phosphotransferase gene (Hygro) driven by a double CaMV35S gene adjacent to the reporter GUS cassette. The CaMV35S promoter controlling the GUS reporter gene and a NOS terminator (N.T) has been removed by a *Pst*I/*Bgl*II digestion and replaced with the HDS promoter (HDS) including the 5′-UTR region until the initiation codon. **b** The pSCA-HDS::GUS vector has been generated following amplification of the GUS reporter cassette from the pCAMBIA 1305 plasmid and cloning into the pSCA vector. The CaMV35S promoter controlling the reporter gene has been replaced by the HDS promoter following a *Pst*I/*Bgl*II digestion. *Amp* ampicillin resistance ORF, *Gus* GUS plus gene, *HDS* HDS promoter, *Hygro* hygromycin resistance gene, *Kan* kanamycin resistance gene, *L.B.*: T-DNA Left Border, *MCS* multiple cloning site, *N.T.* Nos Terminator, *Ori* pBR322 origin of replication, *R.B.* T-DNA Right Border. **c** HDS promoter activities in transiently transformed *C. roseus* cells. Both constructs were independently introduced in 5-day old *C. roseus* C20D cells plated onto solid MM media by particle bombardment for transient expression assays. Cells were harvested for GUS assays 24 h after transformation. GUS levels obtained with pSCA-HDS::GUS construct was used as reference. Data are mean ± SD from five independent experiments. *Asterisks* indicate that values are significantly different ($P < 0.05$)

Both constructs were independently introduced in *C. roseus* cells by particle bombardment for transient expression assays and the GUS activity was measured and normalized as described in the Materials and methods section. Under our experimental conditions, we noted that cells transiently transformed with the pCamb-HDS::GUS displayed a two-fold higher GUS activity than cells transiently transformed with the pSCA-HDS::GUS (Fig. 4c). Such result demonstrates that the double CaMV35S promoters located upstream of the HDS promoter in the pCamb-HDS::GUS plasmid had influence on the transcriptional activity of the HDS promoter and therefore could interfere with its functional characterization. As a consequence, promoter deletion experiments were exclusively conducted in the pSCA-HDS::GUS vector.

Sequential 5′-deletions of the promoter were performed by PCR and the responsiveness of the deleted versions of the HDS promoter was analyzed by transient assays in *C. roseus* cells grown on IM medium. A schematic map of the eight deleted constructs is shown in Fig. 5, including the GUS activity of each construct expressed relatively to the GUS activity of the full length promoter. The expression level of the full-length promoter including the intron-containing 5′-UTR of HDS (construct 1; Fig. 5) was 468 pmol/MU/min/μg. We observed that the 5′-UTR deletion (construct 1Δ) did not modify the transcriptional activity of the promoter suggesting that this region had no influence on the HDS promoter expression under our experimental conditions.

In contrast, deletion of the HDS promoter from the 5′-end down to nucleotide −902 (construct 2) caused a twofold decrease of the promoter expression. Successive deletions up to −694 (constructs 3 and 4) had no additional effect while further deletion to −656 (construct 5) markedly reduced the promoter strength: less than 25% of the GUS activity remained in comparison to the full-length promoter. Finally, deletions to positions −629 and −600 (constructs 6 and 7) resulted in a quasi-complete disappearance of GUS activity. The promoter region within the construct 5 (−656 to −629) with minimal activity contains the TATA box but no other known regulatory elements and can be therefore considered as the minimal promoter. Moreover, our experiments showed that deletion of the promoter regions between nucleotides −1,049 and −902 or between nucleotides −694 and −656, led to significant reductions in promoter activity suggesting that these regions probably contain potential transcription-enhancing *cis*-regulatory elements. Sequence analysis discussed above, indicates that both regions contain two Dof transcription factor recognition core sequences that may be important for the hormonal responsiveness of the HDS in our experimental conditions.

The HDS promoter activity is induced by cytokinin, methyljasmonate and ethylene in *C. roseus* cells

In *C. roseus* C20 cells, MIA production is induced by the depletion of 2,4-D from the culture medium and accumulation of alkaloids is greatly enhanced when cytokinin, ethylene or methyljasmonate are added [13–15, 55]. To investigate the effects of these hormones on HDS promoter inducibility, we generated stably transformed *C. roseus* cell

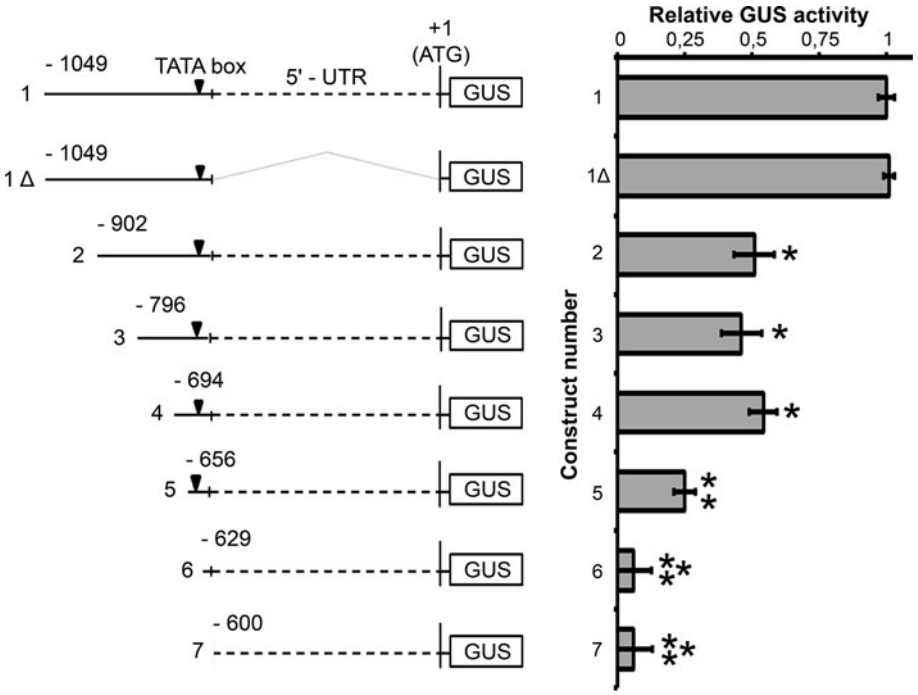

Fig. 5 Deletion analysis of the HDS promoter-GUS constructs. The *left panel* shows a schematic map of the promoter deletion constructs. The position of the translation start codon (ATG) and the putative TATA box are represented by *thin vertical lines* and *triangles*, respectively. *Dashed lines* represent the 5′-UTR region (−600 nt to −6 nt) and *grey lines* pinpoint the deletion of the 5′-UTR region for the construct 1Δ. The *right panel* shows the corresponding GUS activities of each construct. Cells were harvested for GUS assays at 24 h after transient transformation. The data are expressed relative to the GUS activity measured with construct 1, containing the full length HDS promoter and presented with standard deviation. *Asterisks* indicate that values are significantly different ($P < 0.05$). Data are mean ± SD from five independent experiments

lines with pSCA-HDS::GUS vector introduced by particle bombardment. Suspension culture cells were also independently transformed with pSCA-cassette GUS empty-plasmid (pSCA-35S::GUS) to generate a control cell line. To confirm the presence of transgenes corresponding to transcriptional fusions of the HDS or 35S promoter to GUS gene in the genome of C. roseus transformed cells, specific amplifications were performed on genomic DNA extracted from HDS- and control transgenic cell lines. We also checked whether the hygromycin-selection gene was integrated in transformed cells. Molecular analysis of transformed cell lines revealed that HDS- and control transgenic cells possess constructs allowing the transcriptional control of GUS gene by HDS or 35S promoter respectively (Fig. 6). In addition, we also noted that both cell lines have integrated the vector carrying the hygromycin-selection gene. *C. roseus* HDS- and control transgenic cells were grown in media with or without auxin (2,4-D) (MM, IM) or cytokinin (zeatin) (IM+Z) and harvested at day 5 of subculture to determine GUS activity. For data comparison, transient expression assays were also performed in *C. roseus* cells. Indeed, C20D cells were transiently and independently transformed with pSCA-HDS::GUS or pSCA-cassette GUS empty-vectors introduced by particle

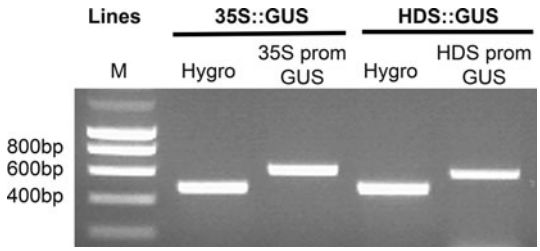

Fig. 6 Molecular analysis of transgenic *C. roseus* stably cell lines. The presence of transgenes corresponding to fusions of either HDS or 35S promoter to GUS gene was detected by specific amplifications on genomic DNA extracted from HDS- and control transgenic stably cells. Amplicons of 592 and 627 bp were respectively obtained from genomic DNA of HDS-transgenic (HDS::GUS line) and control transgenic (35S::GUS line) cells. Amplification of 470 bp of the hygromycin-selection gene was also performed in both transgenic stably cell lines. M: size marker (SmartLadder, Eurogentec)

bombardment and the GUS activity was measured and normalized as described in the "Materials and methods" section. In pSCA-HDS::GUS-transiently transformed cells as well as in HDS-transgenic cells, we clearly observed an induction of HDS promoter activity in IM condition that was enhanced when cells were grown in the presence of cytokinin (IM+Z; Fig. 7a–b). In contrast, in *C. roseus* control transgenic cells, GUS activity under the control of

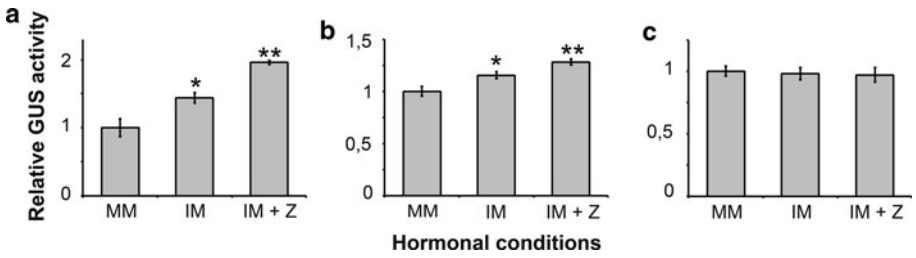

Fig. 7 Effects of auxin depletion and cytokinin addition on HDS promoter expression in transgenic *C. roseus* cells. **a** Analysis of GUS expression in transiently transformed cells. C20D cells plated on solid MM or IM media supplemented with or without zeatin (5 μM), were transiently transformed with pSCA-HDS::GUS and detected for GUS activity 24 h after transformation. **b** and **c** Analysis of GUS activity in HDS- and control-transgenic stably cell lines, respectively. Transgenic cell suspensions were grown on maintenance medium (MM) or a 2,4-D-free medium (IM), supplemented with or without zeatin (IM+Z) at day 3 of subculture. Cells were harvested at day 5 for GUS assays. For all experiments, data are mean ± SD from five independent experiments. *Asterisks* indicate that values are significantly different ($P < 0.05$). GUS activities are expressed relatively to data obtained on MM condition

the 35S promoter, was not affected by the different hormonal treatments (Fig. 7c). These results suggest therefore that the HDS promoter contains regulatory elements implicated in the responsiveness to 2,4-D depletion and zeatin addition. Moreover, measurements of the GUS expression levels showed that the results obtained by transient assays are similar to those obtained in stably transformed cell lines, validating the use of HDS-transgenic cell lines to study HDS promoter activity.

We further investigated the effects of methyljasmonate and ethylene on HDS promoter activity. Indeed, MeJa and ethylene given through ethephon degradation were previously shown to increase MIA accumulation in cell suspension cultures [9, 10, 15]. *C. roseus* HDS-transgenic cells were grown on IM media supplemented either with MeJa (100 μM) or ethephon (0.5 mM). Cells were harvested at day 5 of subculture and GUS activity was measured. Compared to IM condition, an increase of GUS levels was observed when either MeJa or ethephon were added to the culture medium (Fig. 8a–b). These results suggest that the HDS promoter harbours *cis*-elements responsive to both hormones. Using semi-quantitative RT-PCR, we also estimated the expression of the HDS gene in response to ethephon in *C. roseus* C20D suspension cells. We report for the first time an increase of HDS transcript levels 48 h after ethylene treatment (Fig. 8c). This result is in agreement with the observed increase in HDS promoter-controlling GUS activity following treatment with ethephon (Fig. 8b).

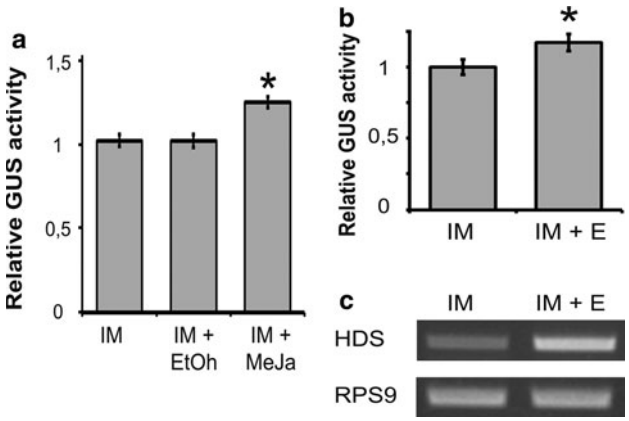

Fig. 8 Effects of methyljasmonate and ethylene on HDS gene. **a** and **b** Analysis of GUS activity in HDS-transgenic stably cell lines. *C. roseus* cells were grown in one on the following media: a 2,4-D-free medium (IM) and IM medium supplemented either with methyljasmonate (100 μM; IM+MeJa), ethanol (IM+EtOH), or ethephon (0.5 mM; IM+E). Cells were harvested 48 h after treatment for GUS assays. Data are mean ± SD from five independent experiments. *Asterisks* indicate that values are significantly different ($P < 0.05$). GUS activities are expressed relatively to data obtained on IM condition. **c** Analysis of HDS transcript accumulation in response to ethylene in C20D cells by semi-quantitative RT-PCR. RPS9 transcript level was used as a loading control. C20D cells were grown in IM or IM supplemented with ethephon (0.5 mM) and harvested 48 h after treatment

Discussion

To investigate the regulatory mechanisms of HDS gene expression, we isolated and characterized its promoter. We cloned a 1,049 nt 5′ non coding region, upstream of the translation initiation codon ATG and containing a 5′-UTR which presents the particularity to contain an intron. Therefore, the HDS gene is composed by a first non-coding exon followed by an intron of 459 bp located closed to the start of the open reading frame of the second exon. Many first introns have been shown to increase gene expression in plants, a regulatory phenomenon named intron-mediated enhancement (IME) [56]. Under our experimental conditions, the HDS promoter activity is not affected by the removal of a region containing the first intron suggesting that the 5′-UTR HDS intron does not act as an IME. Further functional and bioinformatic analyses of the HDS promoter allowed us to determine the minimal promoter but also to pinpoint two promoter regions

containing *cis*-regulatory elements that may play a role in the HDS promoter function.

Measuring transient activity of promoters represents a credible method to analyze promoter activity. Such transformation assays using biolistic were previously described in *C. roseus* cells [57–59], but resulted in a relatively low number of transformed cells in our model system (the C20D cell line). We used an optimized transient transformation procedure recently applied to study the subcellular localization of proteins [34] and tested two different reporter cassettes. In transiently transformed *C. roseus* cells, we demonstrated an increase of the HDS promoter activity when using the pCambia 1305 versus the pSCA-cassette GUS constructs suggesting an influence of the CaMV35S promoter driving the selective marker gene on the expression of nearby cloned genes. We also validate the use of the pSCA-cassette reporter construct for transformation assays by particle bombardment to generate both transiently or stably transformed *C. roseus* cells.

The MIA biosynthetic pathway depends on indole precursor provided by the shikimate pathway and on seco-iridoid precursor derived from the IPP supplied by the MEP pathway. In the past two decades, important progress has been made in regard to the compartmentalization and cellular/tissue specific localizations of the different branches of the pathway. Among them, the early steps of the monoterpenoid biosynthesis (ESMB) occur in internal phloem parenchyma (IPAP) cells in periwinkle aerial organs [9, 60]. Therefore, the pattern of GUS expression under the control of the HDS promoter in transgenic tobacco leaves corroborates with the tissue specific localization of the ESMB genes in *C. roseus*. This is also in agreement with the pattern of GUS expression under the control of the G10H promoter in leaves of transgenic tobacco seedlings [26] and the reports of MEP pathway gene promoter-GUS fusion expressed in the vascular tissues of transgenic Arabidopsis plants [61, 62].

For MIA production, the iridoid branch is usually regarded as limiting in cell and tissue cultures of *C. roseus* [6]. In many plant species, accumulating evidences show that the MEP enzymes contribute to the regulation of the flux of intermediates for the biosynthesis of various isoprenoids such as carotenoids and the phytol part of chlorophylls. In *C. roseus*, we previously demonstrated a transcriptional coordination between the MEP and the seco-iridoid pathways, giving a particular interest to factors regulating the supply of IPP. We particularly focused on phytohormones (auxin, cytokinin, ethylene and methyljasmonate) as their effects have been well described in *C. roseus* cells as having both an influence on MIA production and on MEP pathway gene expression including HDS gene.

In C20D cells, HDS promoter activity is induced by auxin depletion, cytokinin and methyljasmonate as previously reported for the HDS gene expression [9]. We also reported for the first time an increase of HDS promoter activity as well as gene expression by ethylene. Papon et al. [63] previously reported, from 24 to 48 h after cell treatment, the stimulation of G10H gene expression but not of the MEP pathway gene expression (including DXS, DXR and MECS). Our experiments performed by semi-quantitative RT-PCR, clearly showed an increase in the accumulation of HDS transcripts in response to ethylene 48 h after treatment and in accordance with the results of the GUS activity assays in HDS-transgenic cells. Taken together, our results reinforced the role of ESMB genes in controlling metabolic flux leading to MIA accumulation in *C. roseus* cells and suggest that the HDS promoter contains a number of regulatory motifs binding specific transcription factors conferring tissue-specific expression and involved in hormonal responsiveness.

Comparison of the known promoter sequences of the *C. roseus* MIA biosynthetic pathway genes such as TDC and SLS reveals that *cis*-elements binding Dof and WRKY proteins are particularly abundant in the HDS and G10H gene promoters. Dof domain proteins are plant-specific transcription factors with a highly conserved DNA-binding domain, which presumably includes a single C2-C2 zinc finger. Zinc finger proteins like members of the transcription factor IIIA zinc finger protein family (ZCTs) have been previously isolated in *C. roseus* and were shown to act as transcriptional repressors of TDC and STR promoter activity in the regulation of induced secondary metabolism. Dof transcription factors have been suggested to participate in the regulation of vital processes exclusive to plants such as photosynthetic carbon assimilation, light-regulated gene expression, accumulation of seed-storage proteins, germination, dormancy and response to phytohormones [64]. The relative abundance of Dof binding sites in both *C. roseus* HDS and G10H promoters suggest that these specific proteins could play a significant role of ESMB gene expression. The WRKY proteins also contain among their different number of WRKY domains, a zinc-finger like motif and are one the major groups of plant specific transcription factors. They play roles in regulating several different plant processes. These include mediation of immune responses, roles in signaling pathways in response to pathogen attacks and during germination and senescence. Some studies have also reported the WRKY proteins participate in the control of sesquiterpene and benzylisoquinoline alkaloid biosynthesis and their transcriptional induction by methyljasmonate [65–67]. WRKY transcription factors bind to the W-box identified in the HDS and G10H promoters from *C. roseus*. Interestingly, although neither G10H nor HDS promoters contain a JERE element implicated in the jasmonate responsiveness [25], their gene expression is induced by MeJa. Furthermore, G10H is not

regulated by the jasmonate-responsive ORCA3 protein suggesting that the G10H gene regulation utilizes a different transcriptional cascade than TDC and STR [21, 26]. Thus, WRKY proteins could be consider as good candidates to mediate the response to methyljasmonate leading to the MIA biosynthesis induction in *C. roseus*.

In conclusion, we isolated and characterized the MEP pathway HDS gene promoter from *C. roseus*. Functional and bioinformatic analyses reveal that the HDS promoter contains a number of *cis*-motifs that could bind transcription factors involved in the regulation of the isoprenic flux leading to the monoterpene seco-iridoid precursor of MIA. Studies on promoters of the MEP branch constitute a valuable approach to identify new regulatory factors and/or families that could controlled the MIA biosynthesis in *C. roseus* cell suspensions.

Acknowledgments This research was financially supported by the Ministère de l'Enseignement Supérieur et de la Recherche (MENRT) by a grant from the University of Tours. O. Ginis was financed by a fellowship from the Région Centre. We thank Emeline Marais for help in maintaining cell cultures. We also thank Dr Andrew J. Simkin for careful revision of the manuscript.

References

1. Guirimand G, Courdavault V, Lanoue A, Mahroug S, Guihur A, Blanc N, Giglioli-Guivarc'h N, St-Pierre B, Burlat V (2010) Strictosidine activation in Apocynaceae: towards a "nuclear time bomb"? BMC Plant Biol 10:182. doi:10.1186/1471-2229-10-182
2. Roepke J, Salim V, Wu M, Thamm AMK, Murata J, Ploss K, Boland W, De Luca V (2010) Vinca drug components accumulate exclusively in leaf exudates of Madagascar periwinkle. Proc Natl Acad Sci USA 107:15287–15292. doi:10.1073/pnas.0911451107
3. van der Heijden R, Jacobs D, Snoeijer W, Hallard D, Verpoorte R (2004) The catharanthus alkaloids: pharmacognosy and biotechnology. Curr Med Chem 11:607–628
4. Ziegler J, Facchini PJ (2008) Alkaloid biosynthesis: metabolism and trafficking. Annu Rev Plant Biol 59:735–769. doi:10.1146/annurev.arplant.59.032607.092730
5. Guirimand G, Courdavault V, St-Pierre B, Burlat V (2010) Biosynthesis and regulation of alkaloids. In: Pua EC, Davey M (eds) Plant developmental biology—biotechnological perspectives, vol 2. Springer, Berlin, pp 139–160
6. Oudin A, Courtois M, Rideau M, Clastre M (2007) The iridoid-pathway in *Catharanthus roseus* alkaloid biosynthesis. Phytochem Rev 6:259–276. doi:10.1007/s11101-006-9054-9
7. Chahed K, Oudin A, Giglioli-Guivarc'h N, Hamdi S, Chénieux JC, Rideau M, Clastre M (2000) 1-Deoxy-D-xylulose 5-phosphate synthase from periwinkle: cDNA identification and induced gene expression in terpenoid indole alkaloid-producing cells. Plant Physiol Biochem 38:559–566. doi:10.1016/S0981-9428(00)00781-6
8. Veau B, Courtois M, Oudin A, Chénieux JC, Rideau M, Clastre M (2000) Cloning and expression of cDNAs encoding two enzymes of the MEP pathway in *Catharanthus roseus*. Biochim Biophys Acta 1517:159–163. doi:10.1016/S0167-4781(00)00240-2
9. Oudin A, Mahroug S, Courdavault V, Hervouet N, Zelwer C, Rodriguez-Concepcion M, St-Pierre B, Burlat V (2007) Spatial distribution and hormonal regulation of gene products from methyl erythritol phosphate and monoterpene-secoiridoid pathways in *Catharanthus roseus*. Plant Mol Biol 65:13–30. doi:10.1007/s11103-007-9190-7
10. Giglioli-Guivarc'h N, Courdavault V, Oudin A, Crèche J, St-Pierre B (2006) Madagascar periwinkle, an attractive model for studying the control of the biosynthesis of terpenoid derivative compounds. In: Teixeira Da Silva JA (ed) Floriculture, ornamental and plant biology, vol 2. Global Science Books, Isleworth
11. Mérillon JM, Ouelhazi L, Doireau P, Chénieux JC, Rideau M (1989) Metabolic changes and alkaloid production in habituated and non-habituated cells of *Catharanthus roseus* grown in hormone free-medium. Comparing hormone-deprived non-habituated cells with habituated cells. J Plant Physiol 134:54–60
12. Arvy MP, Imbault N, Naudascher F, Thiersault M, Doireau P (1994) 2, 4-D and alkaloid accumulation in periwinkle cell suspensions. Biochimie 76(5):410–416
13. Décendit A, Liu D, Ouelhazi L, Doireau P, Mérillon JM, Rideau M (1992) Cytokinin-enhanced accumulation of indole alkaloids in *Cathranthus roseus* cell-cultures: the factors affecting the cytokinin response. Plant Cell Rep 11(8):400–403. doi:10.1007/BF00234369
14. Décendit A, Petit G, Andreu F, Doireau P, Mérillon JM, Rideau M (1993) Putative sites of cytokinin action during their enhancing effect on indole alkaloid accumulation periwinkle cell suspensions. Plant Cell Rep 12(12):710–712. doi:10.1007/BF00233425
15. Yahia A, Kevers C, Gaspar T, Chénieux JC, Rideau M, Crèche J (1998) Cytokinins and ethylene stimulate indole alkaloids accumulation in cell suspension cultures of *Catharanthus roseus* by two distinct mechanisms. Plant Sci 133:9–15. doi:10.1016/S0168-9452(98)00014-4
16. Guirimand G, Guihur A, Ginis O, Poutrain P, Héricourt F, Oudin A, Lanoue A, St-Pierre B, Burlat V, Courdavault V (2011) The subcellular organisation of the strictosidine biosynthesis in *Catharanthus roseus* epidermis highlights several trans-tonoplast translocations of intermediate metabolites. FEBS J 278(5):749–763. doi:10.1111/j.1742-4658.2010.07994.x
17. Courdavault V, Burlat V, St-Pierre B, Giglioli-Guivarc'h N (2005) Characterisation of CaaX-prenyltransferases in *Catharanthus roseus*: relationships with the expression of genes involved in the early stages of monoterpenoid biosynthetic pathway. Plant Sci 168:1097–1107. doi:10.1016/j.plantsci.2004.12.010
18. Broun P (2004) Transcription factors as tools for metabolic engineering in plants. Curr Opin Plant Biol 7(2):202–209. doi:10.1016/j.pbi.2004.01.013
19. Memelink J, Verpoorte R, Kijne JW (2001) ORCAnisation of jasmonate-responsive gene expression in alkaloid metabolism. Trends Plant Sci 6:212–219. doi:10.1016/S1360-1385(01)01924-0
20. Memelink J (2009) Regulation of gene expression by jasmonate hormones. Phytochemistry 70(13–14):1560–1570. doi:10.1016/j.phytochem.2009.09.004
21. van der Fits L, Memelink J (2000) ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. Science 289:295–297. doi:10.1126/science.289.5477.295
22. van der Fits L, Memelink J (2001) The jasmonate-inducible AP2/ERF domain transcription factor ORCA3 activates gene expression via interaction with a jasmonate-responsive promoter element. Plant J 25:43–53. doi:10.1111/j.1365-313X.2001.00932.x
23. Siberil Y, Benhamron S, Memelink J, Giglioli-Guivarc'h N, Thiersault M, Boisson B, Doireau P, Gantet P (2001) *Catharanthus roseus* G-box binding factors 1 and 2 act as repressors of strictosidine synthase gene expression in cell cultures. Plant Mol Biol 45:477–488. doi:10.1023/A:1010650906695
24. Pauw B, Hilliou FAO, Martin VS, Chatel G, de Wolf CJF, Champion A, Pre M, van Duijn B, Kijne JW, van der Fits L, Memelink J (2004) Zinc finger proteins act as transcriptional repressors of alkaloid biosynthesis genes in *Catharanthus roseus*.

J Biol Chem 279(51):52940–52948. doi:10.1074/jbc.M404391200
25. Menke FLH, Champion A, Kijne JW, Memelink J (1999) A novel jasmonate- and elicitor-responsive element in the periwinkle secondary metabolite biosynthetic gene Str interacts with a jasmonate- and elicitor-inducible AP2-domain transcription factor, ORCA2. EMBO J 18(16):4455–4463. doi:10.1093/emboj/18.16.4455
26. Suttipanta N, Pattanaik S, Gunjan S, Xie CH, Littleton J, Yuan L (2007) Promoter analysis of the *Catharanthus roseus* geraniol 10-hydroxylase gene involved in terpenoid indole alkaloid biosynthesis. Biochim Biophys Acta 1769(2):139–148. doi:10.1016/j.bbaexp.2007.01.006
27. Liu YG, Huang N (1998) Efficient amplification of insert end sequences from bacterial artificial chromosome clones by thermal asymmetric interlaced PCR. Plant Mol Biol Rep 16:175–181. doi:10.1023/A:1007420918645
28. Li ZT, Gray DJ (2005) Isolation by improved thermal asymmetric interlaced PCR and characterization of a seed specific 2S albumin gene and its promoter from grape (*Vitis vinifera* L.). Genome 48:312–320. doi:10.1139/G04-110
29. Prestridge DS (1991) SIGNAL SCAN: a computer program that scans DNA sequences for eukaryotic transcriptional elements. Cabios 7:203–206
30. Higo K, Ugawa Y, Iwamoto M, Korenaga T (1999) Plant cis-acting regulatory DNA elements (PLACE) database: 1999. Nucleic Acids Res 27(1):297–300
31. Rombauts S, Dhais P, Van Montagu M, Rouz P (1999) PlantCARE, a plant cis-acting regulatory element database. Nucleic Acids Res 27(1):295–296. doi:10.1093/nar/27.1.295
32. Lescot M, Déhais P, Moreau Y, De Moor B, Rouzé P, Rombauts S (2002) PlantCARE: a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. Nucleic Acids Res 30(1):325–327. doi:10.1093/nar/30.1.325
33. Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158
34. Guirimand G, Burlat V, Oudin A, Lanoue A, St-Pierre B, Courdavault V (2009) Optimization of the transient transformation of *Catharanthus roseus* cells by particle bombardment and its application to the subcellular localization of hydroxymethylbutenyl 4-diphosphate synthase and geraniol 10-hydroxylase. Plant Cell Rep 28:1215–1234. doi:10.1007/s00299-009-0722-2
35. Courdavault V, Thiersault M, Courtois M, Gantet P, Oudin A, Doireau P, St-Pierre B, Giglioli-Guivarc'h N (2005) CaaXprenyltransferases are essential for expression of genes involved in the early stages of monoterpenoid biosynthetic pathway in *Catharanthus roseus* cells. Plant Mol Biol 57:855–870. doi:10.1007/s11103-005-3095-0
36. Jefferson RA, Kavanagh TA, Bevan MW (1987) Gus fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6(13):3901–3907
37. St-Pierre B, Vazquez-Flota FA, De Luca V (1999) Multicellular compartmentation of *Catharanthus roseus* alkaloid biosynthesis predicts intercellular translocation of a pathway intermediate. Plant Cell 11:887–900. doi:10.1105/tpc.11.5.887
38. Sakai H, Aoyama T, Oka A (2000) Arabidopsis ARR1 and ARR2 response regulators operate as transcriptional activators. Plant J 24:703–711. doi:10.1111/j.1365-313X.2000.00909.x
39. Gowik U, Burscheidt J, Akyildiz M, Schlue U, Koczor M, Streubel M, Westhoff P (2004) cis-Regulatory elements for mesophyll-specific gene expression in the C4 plant *Flaveria trinervia*, the promoter of the C4 phosphoenolpyruvate carboxylase gene. Plant Cell 16(5):1077–1090. doi:10.1105/tpc.019729
40. Fusada N, Masuda T, Kuroda H, Shimada H, Ohta H, Takamiya K (2005) Identification of a novel cis-element exhibiting cytokinin-dependent protein binding in vitro in the 5′-region of NADPH-protochlorophyllide oxidoreductase gene in cucumber. Plant Mol Biol 59(4):631–645. doi:10.1007/s11103-005-0579-x
41. Yanagisawa S, Schmidt RJ (1999) Diversity and similarity among recognition sequences of Dof transcription factors. Plant J 17(2):209–214. doi:10.1046/j.1365-313X.1999.00363.x
42. Hartmann U, Sagasser M, Mehrtens F, Stracke R, Weisshaar B (2005) Differential combinatorial interactions of cis-acting elements recognized by R2R3-MYB, BZIP, and BHLH factors control light-responsive and tissue-specific activation of phenylpropanoid biosynthesis genes. Plant Mol Biol 57(2):155–171. doi:10.1007/s11103-004-6910-0
43. Montgomery J, Goldman S, Deikman J, Margossian L, Fischer RL (1993) Identification of an ethylene-responsive region in the promoter of a fruit ripening gene. Proc Natl Acad Sci USA 90(13):5939–5943
44. Teakle GR, Manfield IW, Graham JF, Gilmartin PM (2002) *Arabidopsis thaliana* GATA factors: organisation, expression and DNA-binding characteristics. Plant Mol Biol 50(1):43–57. doi:10.1023/A:1016062325584
45. Zhou DX (1999) Regulatory mechanism of plant gene transcription by GT-elements and GT-factors. Trends Plant Sci 4(6):210–216. doi:10.1016/S1360-1385(99)01418-1
46. Terzaghi WB, Cashmore AR (1995) Photomorphogenesis. Seeing the light in plant development. Curr Biol 5(5):466–468. doi:10.1016/S0960-9822(95)00092-3
47. Urao T, Yamaguchi-Shinozaki K, Urao S, Shinozaki K (1993) An Arabidopsis myb homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. Plant Cell 5(11):1529–1539. doi:10.1105/tpc.5.11.1529
48. Cercós M, Gómez-Cadenas A, Ho TH (1999) Hormonal regulation of a cysteine proteinase gene, EPB-1, in barley aleurone layers: cis- and trans-acting elements involved in the co-ordinated gene expression regulated by gibberellins and abscisic acid. Plant J 19(2):107–118. doi:10.1046/j.1365-313X.1999.00499.x
49. Plesch G, Ehrhardt T, Mueller-Roeber B (2001) Involvement of TAAAG elements suggests a role for Dof transcription factors in guard cell-specific gene expression. Plant J 28(4):455–464. doi:10.1046/j.1365-313X.2001.01166.x
50. Eulgem T, Rushton PJ, Robatzek S, Somssich IE (2000) The WRKY superfamily of plant transcription factors. Trends Plant Sci 5(5):199–206. doi:10.1016/S1360-1385(00)01600-9
51. Baumann K, De Paolisa A, Costantino P, Gualberti G (1999) The DNA binding site of the Dof protein NtBBF1 is essential for tissue-specific and auxin-regulated expression of the rolB oncogene in plants. Plant Cell 11:323–334. doi:10.1105/tpc.11.3.323
52. Nakano T, Suzuki K, Ohtsuki N, Tsujimoto Y, Fujimura T, Shinshi H (2006) Identification of genes of the plant-specific transcription-factor families cooperatively regulated by ethylene and jasmonate in *Arabidopsis thaliana*. J Plant Res 119(4):407–413. doi:10.1007/s10265-006-0287-x
53. Yoo SY, Bomblies K, Yoo SK, Yang JW, Choi MS, Lee JS, Weigel D, Ahn JH (2005) The 35S promoter used in a selectable marker gene of a plant transformation vector affects the expression of the transgene. Planta 221(4):523–530. doi:10.1007/s00425-004-1466-4
54. Zheng XL, Deng W, Luo KM, Duan H, Chen YQ, McAvoy R, Song SQ, Pei Y, Li Y (2007) The cauliflower mosaic virus (CaMV) 35S promoter sequence alters the level and patterns of activity of adjacent tissue- and organ-specific gene promoters. Plant Cell Rep 26(8):1195–1203. doi:10.1007/s00299-007-0307-x
55. Gantet P, Imbault N, Thiersault M, Doireau P (1998) Necessity of a functional octadecanoic pathway for indole alkaloid synthesis by *Catharanthus roseus* cell suspensions cultured in an auxin-starved medium. Plant Cell Phys 39(2):220–225
56. Rose AB (2008) Intron-mediated regulation of gene expression. Curr Top Microbiol Immunol 326:277–290

57. van der Fits L, Memelink J (1997) Comparison of the activities of CaMV 35S and FMV 34S promoter derivatives in *Catharanthus roseus* cells transiently and stably transformed by particle bombardment. Plant Mol Biol 33(5):943–946. doi:10.1023/A:1005763605355
58. Hilliou F, Christou P, Leech MJ (1999) Development of an efficient transformation system for *Catharanthus roseus* cell cultures using particle bombardment. Plant Sci 140(2):179–188
59. Montiel G, Breton C, Thiersault M, Burlat V, Jay-Allemand C, Gantet P (2007) Transcription factor Agamous-like 12 from Arabidopsis promotes tissue-like organization and alkaloid biosynthesis in *Catharanthus roseus* suspension cells. Metabol Eng 9(2):125–132. doi:10.1016/j.ymben.2006.10.001
60. Burlat V, Oudin A, Courtois M, Rideau M, St-Pierre B (2004) Co-expression of three MEP pathway genes and geraniol 10-hydroxylase in internal phloem parenchyma of *Catharanthus roseus* implicates multicellular translocation of intermediates during the biosynthesis of monoterpene indole alkaloids and isoprenoid-derived primary metabolites. Plant J 38:131–141. doi:10.1111/j.1365-313X.2004.02030.x
61. Estevez JM, Cantero A, Romero C, Kawaide H, Jimenez LF, Kuzuyama T, Seto H, Kamiya Y, Leon P (2000) Analysis of the expression of CLA1, a gene that encodes the 1-deoxyxylulose 5-phosphate synthase of the 2-C-methyl-D-erythritol-4-phosphate pathway in Arabidopsis. Plant Phys 124:95–103. doi:10.1104/pp.124.1.95
62. Carretero-Paulet L, Ahumada I, Cunillera N, Rodriguez-Concepcion M, Ferrer A, Boronat A, Campos N (2002) Expression and molecular analysis of the Arabidopsis DXR gene encoding 1-deoxy-D-xylulose 5-phosphate reductoisomerase, the firstcommitted enzyme of the 2-C-methyl-D-erythritol 4-phosphate pathway. Plant Physiol 129:1581–1591. doi:10.1104/pp.003798
63. Papon N, Bremer J, Vansiri A, Andreu F, Rideau M, Crèche J (2005) Cytokinin and ethylene control indole alkaloid production at the level of the MEP/terpenoid pathway in *Catharanthus roseus* suspension cells. Planta Med 71:572–574. doi:10.1055/s-2005-418663
64. Yanagisawa S (2004) Dof domain proteins: plant-specific transcription factors associated with diverse phenomena unique to plants. Plant Cell Physiol 45(4):386–391. doi:10.1093/pcp/pch055
65. Xu YH, Wang JW, Wang S, Wang JY, Chen XY (2004) Characterization of GaWRKY1, a cotton transcription factor that regulates the sesquiterpene synthase gene (+)-δ-cadinene synthase-A. Plant Phys 135:507–515. doi:10.1104/pp.104.038612
66. Kato N, Dubouzet E, Kokabu Y, Yoshida S, Taniguchi Y, Dubouzet JG, Sato F, Yazaki K (2007) Identification of a WRKY protein as a transcriptional regulator of benzylisoquinoline alkaloid biosynthesis in *Coptis japonica*. Plant Cell Phys 48(1):8. doi:10.1093/pcp/pcl041
67. Ma DM, Pu GB, Lei CY, Ma LQ, Wang HH, Guo YW, Chen JL, Du ZG, Wang H, Li GF, Ye HC, Liu BY (2009) Isolation and characterization of AaWRKY1, an Artemisia annua transcription factor that regulates the Amorpha-4, 11-diene synthase gene, a key gene of artemisinin biosynthesis. Plant Cell Phys 50(12):2146. doi:10.1093/pcp/pcp149