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ORIGINAL PAPER

Functional expression and characterization of sesquiterpene synthases from *Artemisia annua* L. using transient expression system in *Nicotiana benthamiana*

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Abstract *Artemisia annua* L. produces a number of sesquiterpene synthases, which catalyze the conversion of farnesyl diphosphate to various sesquiterpenes. The cDNAs encoding amorpha-4,11-diene synthase (ADS), a key enzyme in the artemisinin biosynthesis, and *epi*-cedrol synthase (ECS), a complex sesquiterpene cyclization synthase, were cloned into Cowpea mosaic virus-based viral vector (pEAQ-*HT*) with Kozak consensus motif and C-terminal histidine tag. The plasmids were transformed into *Agrobacterium* LBA4404 and, agroinfiltrated into *Nicotiana benthamiana* leaves along with vector (pJL3:p19) containing Tomato bushy stunt virus post-transcriptional gene silencing suppressor. Quantitative PCR was carried out to measure the transcript levels at 0, 3, 6, 9, 12 and 15 days post-infiltration (dpi). The highest relative expression was observed at 9 dpi for both genes. Transiently expressed recombinant proteins of ADS and ECS were confirmed by SDS-PAGE and western blot. Recombinant proteins were extracted from 9 dpi leaves and purified by immobilized metal ion affinity chromatography using histidine tag, which produced yields of 90 and 96 mg kg^{-1} fresh weight of leaves for ADS and ECS, respectively. Activities of the purified enzymes were assayed using gas chromatography–mass spectrometry for product identification and quantification using valencene as internal standard. The recombinant ADS and ECS converted farnesyl diphosphate into amorpha-4,11-diene (97 %) and *epi*-cedrol (96 %) as the major products, respectively. The purified enzymes exhibited the specific activity of 0.002 and 0.01 μmol min^{-1} mg^{-1} protein for ADS and ECS, respectively. The apparent k_{cat} values were 2.1×10^{-3} s^{-1} and 11×10^{-3} s^{-1} for ADS and ECS, respectively.

Key message Agroinfiltration of leaves of *Nicotiana benthamiana* can be used to produce recombinant biosynthetic enzymes as exemplified by two sesquiterpene synthases from *Artemisia annua* in relatively high yields.

Keywords Agroinfiltration · Amorpha-4 · 11-diene synthase · *Epi*-cedrol synthase · *Artemisia annua* · *Nicotiana benthamiana* · Transient expression

Introduction

In recent decades, plant sesquiterpene synthases have attracted considerable research interest because of their importance in plant defense and communication, and for their potential use in various industrial applications. A large number of plant sesquiterpene synthases have been cloned and recombinant proteins produced mostly in bacterial (*Escherichia coli*) expression systems. We have cloned and characterized some sesquiterpene synthases from *Artemisia annua*, such as *epi*-cedrol synthase (ECS) (Mercke et al. 1999), amorpha-4,11-diene synthase (ADS) (Mercke et al. 2000) and farnesene synthase (FS) (Picaud et al. 2005). ADS is a key regulatory enzyme catalyzing the first committed step of artemisinin biosynthesis, i.e. the

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conversion of farnesyl diphosphate to amorpha-4,11-diene (Fig. 1). Artemisinin has during recent years become a very important antimalarial drug, which is widely used in endemic countries (Whitty et al. 2008). The role of ECS in the plant is not established yet and the polycyclic product *epi*-cedrol (Fig. 1), a nonvolatile sesquiterpene alcohol, cannot be isolated from the plant indicating that it may be an intermediate in the biosynthesis of a related metabolite or that the enzyme is not functional in the plant. The cDNAs encoding *ADS* and *ECS* have been isolated, cloned, expressed in *E. coli* and *Saccharomyces cerevisiae* and characterized by several research groups (Mercke et al. 1999, 2000; Hua and Matsuda 1999; Chang et al. 2000; Wallaart et al. 2001; Jackson et al. 2003; Martin et al. 2003; Picaud et al. 2005; Lindahl et al. 2006).

A general problem in producing plant sesquiterpene synthases in bacterial systems is the formation of inclusion bodies. In our own laboratory, we have noticed that highly homologous sesquiterpene synthases behave very differently when expressed in a bacterial host. Some have a much stronger tendency to form insoluble aggregates than the others. ECS is such a protein and the biochemical characterization of this enzyme has been hampered by the fact that it is difficult to produce it in bacterial expression systems. In general, the expression conditions have to be optimized for each enzyme to be produced. We have reported improved yields of recombinant soluble active plant sesquiterpene synthases from *E. coli* by optimization of expression conditions (Picaud et al. 2007).

Plants may be used as a host system for heterologous protein expression of various genes (Kumagai et al. 2000; Escobar et al. 2003; Huang et al. 2010). Such expression systems should be excellent for the production of recombinant plant proteins. Unlike microbial, mammalian and insect cell systems, little or no specialized equipment is required for implementing the plants for the recombinant gene expression and it is easily scalable. Among the many plants studied for recombinant gene expression using plant virus-based vectors, *Nicotiana benthamiana* possesses many favorable attributes. It includes low cost of production, fast growth rate, susceptibility to many plant viruses, high efficiency in genetic transformation, efficient transient expression, ease of purification of recombinant proteins,

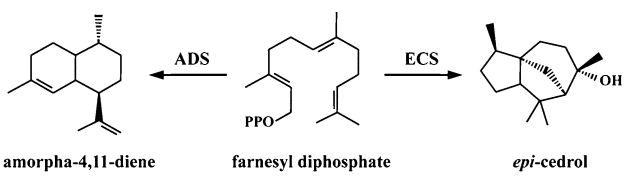

Fig. 1 Reactions catalyzed by amorpha-4,11-diene synthase (*ADS*) and *epi*-cedrol synthase (*ECS*) cloned from *Artemisia annua*. OPP denotes diphosphate moiety

high biomass yield that render it an attractive host for the high level expression and large-scale production of recombinant proteins even though it requires a green house and 6–9 days to obtain the recombinant proteins after agroinfiltration (D'Aoust et al. 2008; Goodin et al. 2008; Sainsbury et al. 2009). *N. tabacum* has been utilized for the molecular cloning of ADS using stable nuclear transformation (Wallaart et al. 2001; Feng et al. 2009). In a recent study, five genes encoding enzymes involved in artemisinin biosynthesis, i.e. farnesyl diphosphate synthase (FDS), ADS, amorphadiene hydroxylase (CYP71AV1), artemisinic aldehyde Δ11(13) double-bond reductase and aldehyde dehydrogenase 1, were transformed into *N. tabacum* for the production of precursors of artemisinin (Zhang et al. 2011). However, it was concluded that the conditions in the transgenic tobacco cells favor reduction of dihydroartemisinic aldehyde to dihydroartemisinic alcohol rather than oxidation to dihydroartemisinic acid (the direct precursor of artemisinin).

During recent years, transient expression by agroinfiltration has been increasingly employed as an alternative to stable transformation to maximize protein expression. Agroinfiltration is flexible and produces the recombinant protein within a few days in the differentiated plant tissues in comparably high yields (Kapila et al. 1997; van der Hoorn et al. 2000; Lindbo 2007b; Sainsbury et al. 2009). Many researchers have used transient expression system based on plant viral vectors and *Agrobacterium tumefaciens* to achieve rapid and high level production of recombinant proteins (Kumagai et al. 2000; Escobar et al. 2003; Lindbo 2007b; Musiychuk et al. 2007; Sainsbury et al. 2009; Huang et al. 2010). Genetically engineered, deconstructed, non-replicating Cowpea mosaic virus (CPMV)-based vectors along with viral suppressor (P19), a gene for post-transcriptional gene silencing from Tomato bushy stunt virus (TBSV) (Lindbo 2007b; Sainsbury et al. 2009) are used in this study as it provides an advantage of high degree of biocontainment without integration of T-DNA into the plant genome. The *N. benthamiana* transient expression using agroinfiltration allows a specific and high level expression of recombinant proteins (Lindbo 2007b; Musiychuk et al. 2007; Sainsbury et al. 2009). These considerations led us to adapt the *N. benthamiana* transient expression system for production of plant sesquiterpene synthases. In our model study, we have heterologously expressed ADS and ECS in *N. benthamiana* and characterized the resulting recombinant enzymes. It may be pointed out that van Herpen et al. (2010) used agroinfiltration of *N. benthamiana* for transient expression of four enzymes of artemisinin biosynthesis, i.e. 3-hydroxy-3-methylglutaryl-CoA reductase, FDS, ADS and CYP71AV1, to produce artemisinic acid. However, artemisinic acid accumulated as a 12-β-diglucoside.

Materials and methods

Plant material

A. annua and *N. benthamiana* were transplanted to individual pots 3 weeks after sowing and grown in a green house maintained at 23 ± 1 °C under 80 % relative humidity under 16 h light/8 h dark photoperiod throughout the study.

Bacterial strains and growth conditions

E. coli (NovaBlue, Novagen) and *A. tumefaciens* strains, LBA4404 (Hoekema et al. 1983) and GV3101 (Holsters et al. 1980), were used in molecular cloning experiments and were routinely cultured at 36 ± 1 and 25 ± 1 °C for *E. coli* and *A. tumefaciens*, respectively, in Luria–Bertani (LB) media using the appropriate antibiotics (Sambrook and Russell 2001).

Construction of plant expression vectors

Standard molecular methods were used to construct plasmids. The *ADS* gene (GenBank accession no. AF138959), previously cloned into the pET28 plasmid, was modified by introduction of a silent mutation in the *Xma*I restriction site using the Megaprimer PCR method (Barik 1995) and *Pfu* DNA polymerase (Fermentas, St Leo-Roth, Germany). The forward primer 1 and reverse primer 2 (Table 1) were used to create a Megaprimer containing a silent mutation of C to T (bold type) at position 846 of *ADS*, thereby eliminating the *Xma*I restriction site in the *ADS* but retaining the serine encoded at this position. The purified Megaprimer (138 bp) as a forward primer and primer 3 (Table 1) as a reverse primer were used to amplify the *ADS*, incorporating the mutation described above, as well as an *Sac*I restriction site at the 5′ end, and an *Xho*I restriction site at the 3′ end. The amplified fragment (939 bp) was digested with *Sac*I and *Xho*I restriction enzymes and ligated into the *Sac*I and *Xho*I restriction sites of *ADS* from the plasmid pET28. The resulting plasmid, pET28 (mut846) was used to amplify the ADS with gene-specific primers 4 and 5 (Table 1) containing Kozak consensus sequence (**GCCACC**) (Kozak 1989) at the 5′ end and the restriction sites of *Nru*I and *Xma*I at the 5′ and 3′ end, respectively.

RNA isolation and first strand cDNA synthesis were performed according to the procedure described elsewhere (Olofsson et al. 2011). The complete open reading frame (ORF) encoding the ECS (GenBank accession no. AJ001539) was amplified from cDNA synthesized from total RNA from *A. annua* using gene-specific primers 6 and 7 (Table 1) containing *Nru*I and *Xma*I restriction sites, respectively, with addition of Kozak sequence at the 5′ end.

The PCR-amplified products of *ADS* and *ECS* genes were purified by the Gel Extraction kit (Fermentas) according to the manufacturer's instructions. The purified products were ligated into pJET1.2 vector and transformed into competent *E. coli* NovaBlue cells by the heat shock method. The sequence integrity of cloned PCR products was verified by nucleotide sequencing. The pJET1.2 vectors containing *ADS* and *ECS* were digested by *Nru*I/*Xma*I and then subcloned into a Cowpea mosaic virus-based plant expression vector, pEAQ-*HT* (Sainsbury et al. 2009) in frame with a C-terminus histidine (His_6) affinity tag resulting in the constructs of pEAQ-*HT*-ADS and pEAQ-*HT*-ECS, respectively (Fig. 2).

Table 1 Nucleotide sequences of primers used

Primer no.	Template	Nucleotide sequence	Application
1	*ADS* fw	5′-CACAAGGAAGAGCTCAGC-3′	Mutation
2	*ADS* rev	5′-CCG**A**GAATACTGTGGCTCATAG-3′	Mutation
3	*ADS* rev	5′-ATACTCGAGTCATATACTCATAGGATA-3′	Mutation
4	*ADS* fw	5′-AGCGTCGCGA**GCCACC**ATGTCACTTACAGAAGAAAAAC-3′	ORF cloning
5	*ADS* rev	5′-AGCGCCCGGGTATACTCATAGGATAAACGAGTAGAG-3′	ORF cloning
6	*ECS* fw	5′-AGCGTCGCGA**GCCACC**ATGTCTCTTATAGTAGAGGATG-3′	ORF cloning
7	*ECS* rev	5′-AATTCCCGGGTGTAAGATGGCATCAACGAAAAGAG-3′	ORF cloning
8	*ADS* fw	5′-GGGAGATCAGTTTCTCATCTATGAA-3′	qPCR
9	*ADS* rev	5′-CTTTTAGTAGTTGCCGCACTTCTT-3′	qPCR
10	*ECS* fw	5′-GCAACAAGCCTACGAATCACTCAA-3′	qPCR
11	*ECS* rev	5′-CGTGAAAAATTAAGGACCCTCATAG-3′	qPCR
12	*Ubi3* fw	5′-GCCGACTACAACATCCAGAAGG-3′	qPCR
13	*Ubi3* rev	5′-TGCAACACAGCGAGCTTAACC-3′	qPCR

Restriction sites are underlined. The mutated base and the Kozak sequences are shown in bold

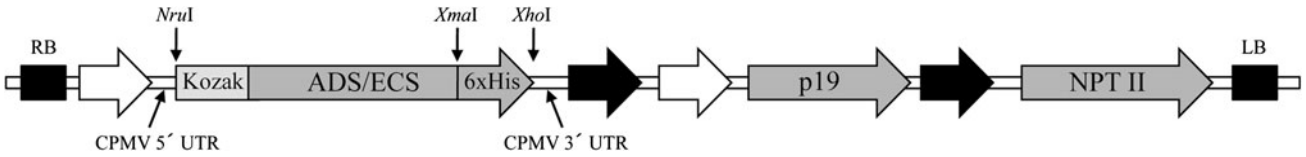

Fig. 2 Schematic representation of T-DNA region of pEAQ-*HT*-ADS and pEAQ-*HT*-ECS for the recombinant transient expression in infiltrated tobacco leaves. *Black boxes* T-DNA borders; *white arrows* CaMV 35S promoters; and *black arrows* Nos terminators

Agrobacterium infiltration into *N. benthamiana* leaves

The binary vectors pEAQ-*HT*-ADS, pEAQ-*HT*-ECS and pJL3:p19 (Lindbo 2007a) were separately transformed into *A. tumefaciens* strain LBA4404 using freeze–thaw method and confirmed by PCR. The pJL3:p19 is a vector expressing post-transcriptional gene silencing (PTGS) suppressor (P19) protein of TBSV. Overnight-grown *Agrobacterium* cultures (50 μg mL^{-1} kanamycin and 25 μg mL^{-1} rifampicin) carrying each vector with an optical density (OD_{600}) of 1.2–1.4 were pelleted by centrifugation at 2000*g* for 20 min. The pellets were resuspended in infiltration solution (10 mM 2-*N*-morpholino-ethanesulfonic acid (MES) pH 5.5, 10 mM $MgCl_2$, and 100 μM acetosyringone). After 2–4 h incubations at room temperature, *Agrobacterium* cultures carrying each expression vector were mixed with an equal volume of *Agrobacterium* culture carrying pJL3:p19 vector and 5–6 mature leaves of 5–8 weeks old *N. benthamiana* plants were co-infiltrated using a 10 mL syringe without a needle from underneath of the leaves. Non-infiltrated leaves [0 days post-infiltration (dpi)], infiltrated leaves at 3, 6, 9, 12 and 15 dpi and leaves infiltrated with pEAQ-*HT* vector (empty vector without any gene construct) with pJL3:p19 at 9 dpi were harvested. Harvested leaf materials were used immediately or frozen in liquid nitrogen and stored at −80 °C until further use.

Relative gene expression using real-time quantitative RT-PCR (qPCR)

RNA extraction, first strand cDNA synthesis and qPCR from the infiltrated leaves at 0, 3, 6, 9, 12, and 15 dpi were carried out as described previously (Olofsson et al. 2011). Briefly, total RNA was isolated from the frozen plant tissue powder (100 mg) using Purelink™ Plant RNA Reagent kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The quality and concentration of RNA were determined by ethidium bromide-stained agarose gel electrophoresis and spectrophotometric analysis (ND-1000, Nanodrop®). The RNA was treated using DNase I (Fermentas) to remove contaminating DNA. RNA (1.0 μg) was reverse transcribed using RevertAid™ H Minus-MuLV reverse transcriptase (Fermentas) primed with 0.5 μg oligo$(dT)_{18}$ primer. The remaining RNA from the first strand cDNA was removed using RNase H (Fermentas) treatment according to the manufacturer's instructions.

The qPCR was performed using specific primers 8 and 9 (Table 1) for ADS and specific primers 10 and 11 (Table 1) for ECS on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA). Real-time PCR reactions were conducted in a final volume of 20 μL using 1 μL of first strand cDNA, 10 μL Power SYBR® Green PCR master mix (Applied Biosystems) and 2 pmol of each forward and reverse primer. Cycling conditions were 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min and dissociation stage at 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s. The specificity of each primer pair was verified by analysing the melting curve of PCR products following the final set of the PCR.

Two biological replicates were used for the measurement, and three technical replicates were analyzed for each biological replicate. Tomato *Ubiquitin* (*ubi3*) gene-specific primers 12 and 13 (Table 1) were employed to amplify the endogenous housekeeping gene *ubi3* in all qPCR samples (Rotenberg et al. 2006). Primer efficiencies were determined according to Pfaffl et al. (2002). Relative gene expressions of *ADS* and *ECS* in relation to housekeeping gene were calculated using the REST 2009 software V. 2.0.13 (Qiagen, Hilden, Germany) (Pfaffl et al. 2002). Cycle threshold (C_q) values were employed to quantify the transcripts in qPCR analysis. Relative gene expression was calculated by comparing the samples with the lowest gene expression.

Total soluble protein extraction and quantification

To extract total soluble protein (TSP), infiltrated leaves and non-infiltrated leaves were grounded in liquid nitrogen to a fine powder and extracted with 1.5 w/v of extraction buffer A [20 mM sodium phosphate, pH 7.0, 0.4 M sodium chloride, 25 mM sodium ascorbate, 25 mM sodium bisulfite, 10 mM magnesium chloride, 5 mM dithiothreitol (DTT), 10 % (v/v) glycerol and 1 % polyvinylpolypyrrolidone (PVPP) and 1.0 % (v/v) plant protease inhibitor cocktail (Sigma, P9599)] at 4 °C. To extract TSP of ECS, the infiltrated and non-infiltrated leaf materials were grounded in liquid nitrogen and thawed in 1.5 w/v of extraction buffer B [100 mM Tris–HCl, pH 7.5; 100 mM potassium fluoride; 5 mM EDTA; 250 mM sucrose;

40 mM sodium ascorbate; 0.2 % BSA), 1 % polyvinylpolypyrrolidone (PVPP) and 1.0 % (v/v) plant protease inhibitor cocktail (Sigma, P9599)] at 4 °C. The protease inhibitors were added to the buffer just prior to use.

Plant soluble proteins containing ADS and ECS were concentrated by ammonium sulfate precipitation, precipitated at 70 % (w/v) and resuspended in phosphate buffered saline (PBS: 10 mM phosphate buffer, 2.7 mM potassium chloride and 137 mM sodium chloride, pH 7.4) before being equilibrated with 15 mL of buffer C [20 mM sodium phosphate, pH 7.4, 0.5 M sodium chloride, and 10 % (v/v) glycerol] with a PD-10 desalting column (GE Healthcare) at 4 °C.

Protein concentrations were determined using the Bio-Rad (Bradford 1976) protein assay (Bio-Rad Laboratories) using bovine serum albumin (BSA) as standard in spectrophotometer (ND-1000, Nanodrop®). Aliquots of each enzyme at different dpi were used for SDS-PAGE and western blot analysis to determine expression efficiency of the recombinant proteins.

Purification of recombinant ADS and ECS

TSP extracts containing the soluble recombinant enzymes expressed with 6×His tag at C-terminus after 9 dpi were purified using immobilized metal ion affinity chromatography (IMAC) and passed through a 5 mL HiTrap Chelating HP column (GE Healthcare) charged with Co^{2+}. After washing with binding buffer (20 mM sodium phosphate, pH 7.4, 0.5 M NaCl, 30 mM imidazole), bound enzyme was eluted using elution buffer (20 mM sodium phosphate, pH 7.4, 0.5 M NaCl and 500 mM imidazole) at 4 °C. The fractions containing the maximum recombinant protein concentration as measured by Bradford protein assay were pooled and desalted with a PD-10 column (GE Healthcare) equilibrated with assay buffer D [20 mM Tris–HCl, pH 7.0, containing 10 mM $MgCl_2$, 10 % (v/v) glycerol, and 10 μM β-mercaptoethanol] for ADS, and assay buffer E [20 mM Tris–HCl, pH 8.5, containing 10 mM $MgCl_2$, 10 % (v/v) glycerol, and 2 mM DTT] for ECS. The eluted fractions were analysed by SDS-PAGE, western blot and stored at −20 °C until use.

SDS-PAGE and western blotting

SDS-PAGE electrophoresis was performed with 30 μg of TSP or 3 μg of purified proteins using NuPAGE™ 1× MES SDS Running Buffer (Invitrogen) on NuPAGE™ 4–12 % Bis/Tris gels (Invitrogen) according to manufacturer's instructions using a Novex™ X-Cell II™ mini horizontal gel electrophoresis unit. After electrophoresis, the gels were either stained with PageBlue™ Protein Staining Solution (Fermentas) following manufacturer's basic protocol or the proteins were electroblotted onto 0.45 μm polyvinylidene fluoride (PVDF) (Millipore, Billerica, MA, USA) membranes using a Novex™ X-Cell II™ Blot module as described by the manufacturer. PageRuler™ Prestained Protein Ladder (Fermentas) was included in SDS-PAGE and western blots as molecular weight markers. Immunoblot detection of transferred proteins in membranes was performed using Anti-His (C-term)-HRP Antibody (Invitrogen) according to the manufacturer's protocol. Bound antibodies were detected using the Amersham ECL Western Blotting detection reagents (GE Healthcare) in accordance with manufacturer's procedures.

Enzyme assays, product identification, and quantification

Purified enzyme of ADS (44 μg) was assayed in assay buffer D and 20 μM farnesyl diphosphate (FDP), and ECS (16 μg) was assayed in assay buffer E and 20 μM FDP in a total volume of 500 μL overlaid with 50 μL of dodecane. The reaction mixtures were incubated in a water bath for 10, 20 and 30 min at 30 °C. Samples were withdrawn from the dodecane phase (1 μL) and subjected to gas chromatography–mass spectrometry (GC–MS) analysis. As a control, the assays were performed without substrate. Samples were analysed by capillary GC on an Agilent 6890 series gas chromatograph equipped with a HP-5MS 5 % phenyl methyl silohexane capillary column (0.2 mm film thickness, 0.25 mm i.d 30 m) and an Agilent 5973 Network Mass Selective Detector. The GC injection port was operated with a carrier gas of helium at a constant flow of 0.7 mL min^{-1} with 1 μL splitless injection mode. The GC was performed with an injector temperature of 40 °C.

To identify and quantify the ADS and ECS produced sesquiterpenes, the oven temperature was programmed from 40 to 50 °C at a rate of 50 °C min^{-1} for 5 min, 50 to 200 °C at rate of 2 °C min^{-1} and from 200 to 300 °C at a rate of 50 °C min^{-1}. The final temperature was maintained for 1 min. Mass spectra were measured in electron impact ionization mode at 70 eV with scanning from m/z 34 to 400 at 3.93 scans s^{-1}. For all experiments, a solvent delay of 4 min was allowed prior to the acquisition of MS data and product peaks were quantified by integration of peak areas using Enhanced Chemstation version E.02.00.493 (Agilent Technologies). Sesquiterpenes were identified by comparing obtained mass spectra and retention indices with those of spectra found in the Mass finder 2.1 database and other libraries. For the quantification of amorpha-4, 11-diene and *epi*-cedrol, a standard curve of (+)-valencene (5–100 ng μL^{-1}) (Fluka) was used. Two replicates were used to quantify the absolute concentration of amorpha-4,11-diene and *epi*-cedrol. Uninfiltrated leaves and leaves infiltrated with empty pEAQ-*HT* vector with pJL3:p19 at 9 dpi were used as negative controls.

The k_{cat} values were calculated from the data obtained from GC–MS. Statistical analysis was carried out using Graphpad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA) and values of $P < 0.01$ were considered to be significant.

Results and discussion

ADS and ECS were transiently expressed in *N. benthamiana*, purified and characterized to confirm the effectiveness of plants as a favorable expression system for the production of plant sesquiterpene synthases.

Cloning and transformation of ADS and ECS

The full length cDNAs of two sesquiterpene synthases from *A. annua* (*ADS* and *ECS*) were amplified by PCR using gene-specific primers and cloned into pEAQ-*HT* vector. The forward primer carried the Kozak sequence upstream of the translation start codon. The reverse primer did not carry a stop codon allowing the addition of a carboxy (C)-terminal histidine tag (six His residues) (Fig. 2). The restriction endonuclease digestion and sequence analysis showed that the genes were inserted in the correct orientation. Recombinant *A. tumefaciens* (LBA4404 and GV3101) cells that harbored plasmids pEAQ-*HT*-ADS and pEAQ-*HT*-ECS (containing *ADS* and *ECS* ORFs) were agroinfiltrated into *N. benthamiana*. Although these plasmids carry a copy of the *p19* gene, we also used a separate plasmid carrying this gene (pJL3:p19) to increase the level of the p19 protein. Both strains were co-infiltrated with an *Agrobacterium* strain carrying the *P19* gene. Between these two strains, LBA4404 exhibited a relatively higher expression of recombinant proteins than GV3101, which confirms the higher efficiency of LBA4404 reported in *N. benthamiana* (Bos et al. 2006) and *Arabidopsis* (Kim et al. 2009) in contrast to previous observations with *N. benthamiana* (Sparkes et al. 2006) and *Solanum tuberosum* (Bhaskar et al. 2009) (data not shown). We performed agroinfiltration in the leaves at various growth stages and found that the infiltration was efficient when using leaves from 5 to 8-week-old *N. benthamiana* plants. Also, we noticed that performing the infiltrations was improved in the afternoon and observed that not providing water 24 h before agroinfiltration, enhanced the efficiency of infiltration.

qPCR

The expression patterns of the *ADS* and *ECS* genes were analyzed using qPCR at 0, 3, 6, 9, 12 and 15 dpi. It has been previously demonstrated that transient gene expression using Tobacco mosaic virus-based vector is significantly up-regulated until 6 dpi and remains at the same expression level up to 15 dpi (Lindbo 2007b; Villani et al. 2009). We thus investigated whether the transient expression of genes with CPMV-based vector were consistent up to 15 dpi or down-regulated. To verify and determine the *ADS* and *ECS* transcripts levels until 15 dpi, we harvested the agroinfiltrated *N. benthamiana* leaves at 0, 3, 6, 9, 12 and 15 dpi and then we performed cDNA synthesis and qPCR. Zero dpi represents leaf samples that were harvested from the plants before infiltration (untreated negative control). To ensure the comparability of transcripts at different time points, all qPCR reactions were performed with equal quantity of total RNA and amplification efficiency was determined for each set of genes by measuring tenfold serial dilutions of 1 µg of cDNA from control and infected leaf samples in duplicate. Amplification efficiencies of *ADS*, *ECS* and the reference gene, *ubi3* were found to be 83, 88 and 86 %, respectively, indicating relevant amplification and accurate quantification of recombinant transcripts by qPCR. Homogeneity and specificity of amplified products were confirmed by melting curve analysis (data not shown). Relative expression was determined by comparing the Cq value of transcripts of different times (3, 6, 9, 12 and 15 dpi) with that obtained at 15 dpi, which exhibited the lowest concentration of transcript (highest Cq value). All Cq values obtained throughout this work are within the linear range of each gene. The results showed significant levels of ADS and ECS transcripts in all of the stages of post-infiltration and the level of transcripts varies greatly at different times after infiltration. We also observed a significant and reproducible increase in expression of both genes (*ADS* and *ECS*) until 9 dpi and a significant decrease at 12 dpi and further, at 15 dpi (Fig. 3). Transcripts of ADS and ECS were not detected in the negative control (0 dpi). The quantitative analysis of ADS and ECS revealed the highest transcript level at 9 dpi with approximately 32- and 16-fold higher, respectively, as compared to that at 15 dpi (Fig. 3). These results are consistent with recent findings showing that upon infection of *N. benthamiana* by potato virus X, a full systemic infection is observed at 9 dpi (Ye et al. 2011). From Fig. 3, it may be concluded that the ECS mRNA is somewhat more stable than the ADS mRNA. This discrepancy may be due to the fact that the higher level expression of recombinant ADS observed triggers a stronger PTGS in *N. benthamiana* leading to a more rapid degradation of ADS mRNA. The high accumulation of CPMV viral proteins, recombinant proteins and P19 proteins in agroinfiltrated leaves of *N. benthamiana* caused premature leaf necrosis resulting in visible cell death in the infiltrated areas ($\sim$0.5 cm^2) at 15 dpi.

Transient expression and purification of ADS and ECS in *N. benthamiana*

The coding regions for *ADS* and *ECS* were expressed in *N. benthamiana* leaves transiently. As both proteins are sesquiterpene synthases, no signal peptide was attached to the N-terminal region of the proteins and it is likely to be expressed in the cytoplasm, which is a site for sesquiterpene biosynthesis (Colby et al. 1998). The recombinant proteins were extracted at 0, 3, 6, 9, 12 and 15 dpi.

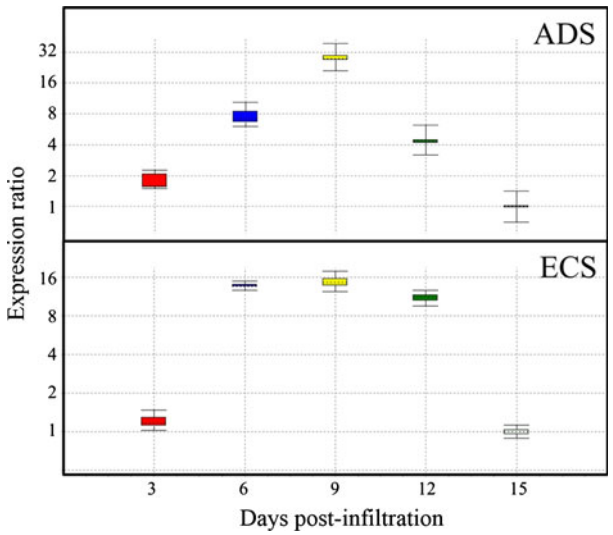

Fig. 3 The relative expression of amorpha-4,11-diene synthase (*ADS*) and *epi*-cedrol synthase (*ECS*) genes was measured by real-time quantitative RT-PCR (qPCR) analysis using cDNA from 3, 6, 9, 12 and 15 dpi (days post-infiltration) with that of 15 dpi The expression levels are normalized to the ubiquitin (ubi3) mRNA. The results are from two separate experiments, each with three technical replicates

To ensure that the recombinant ADS and ECS transcripts correlated with the production of proteins, we performed SDS-PAGE and western blot. SDS-PAGE revealed that the transiently expressed recombinant proteins (TSP and purified) of ADS and ECS from the infiltrated leaves were in accordance with the molecular masses of approximately 65 and 64.7 kDa, respectively (Fig. 4). This is about 1 kDa larger than the predicted native proteins because of the addition of a restriction site of *Xma*I and a 6×His tag at the C-terminal end. In case of ADS, no product was observed in the lane of negative control (0 dpi; Fig. 4a, lane 2), but we are not able to exclude the expression of ECS in the negative control by SDS-PAGE due to the presence of a thick protein band of similar size (Fig. 4b, lane 2). Hence, the expression of recombinant ECS was confirmed by purified protein analysis in SDS-PAGE (Fig. 4b, lane 8) and western blot. Furthermore, we found that the detection of the thick protein band at approximately 65 kDa was due to higher solubility of proteins in extraction buffer B and this band was not seen in the lane of negative control extracted with extraction buffer A. Based on western blot results, it is, therefore, proposed that ADS and ECS were expressed at all the stages of post-infiltration and strongly expressed at 9 dpi (Fig. 4, lane 5). Furthermore, Regnard et al. (2010) also noticed consistent amount of transcripts and proteins at 7 dpi with enhanced green fluorescent (EGFP) gene using autonomously replicating geminivirus shuttle vector. These experiments were repeated three times and the results were reproducible.

The recombinant proteins of the 9 dpi samples were purified by IMAC, which yielded 90 and 96 mg of purified proteins kg^{-1} fresh weight (FW) of leaves for ADS and ECS, respectively. Cane and Kang (2000) and Picaud et al.

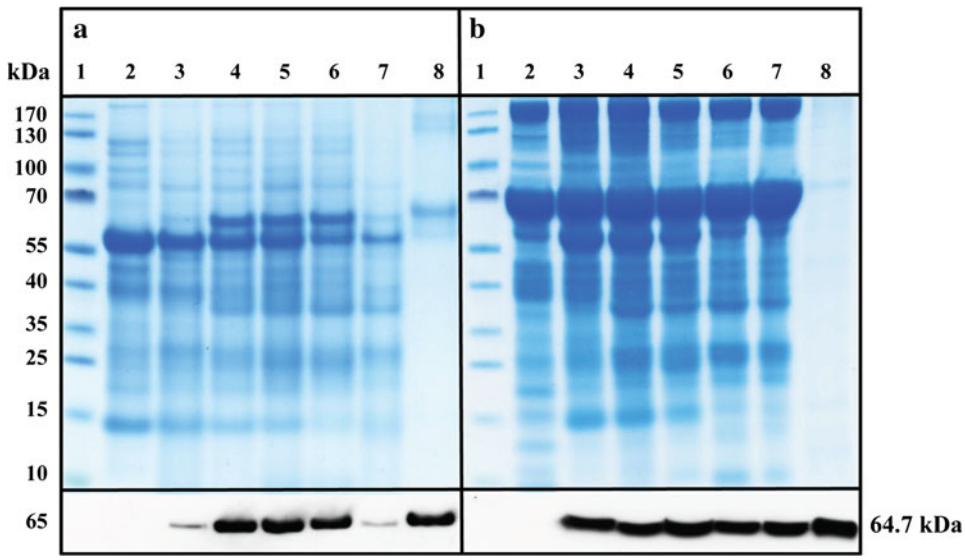

Fig. 4 SDS-PAGE and western blot analysis of recombinant enzymes **a** ADS, **b** ECS. *Lane 1* molecular weight marker; *lane 2* 0 days post-infiltration (dpi) (untreated *Nicotiana benthamiana*: negative control); *lanes 3–7* total soluble proteins (TSP) extracted from the agroinfiltrated leaves of *N. benthamiana* at 3, 6, 9, 12 and 15 dpi, respectively; *lane 8* purified enzyme from TSP at 9 dpi. Molecular mass for each band of the marker is indicated to the left of the panel. Molecular mass of recombinant protein is indicated to the *left* and *right* of the panel in the western blot

(2005) observed recombinant protein (ADS) yields of 10–20 mg L^{-1} using bacterial expression system.

During our purification of ADS and ECS by IMAC, we consistently observed the recombinant proteins in fractions (1 mL) 2–8 as exemplified for ADS in Fig. 5. From this figure, it is evident that the recombinant ADS is not fully purified. An additional purification step such as ion-exchange chromatography is needed to obtain pure enzyme. Furthermore, we found that combination of using the efficient *Agrobacterium* strain LBA4404, co-expressing P19, and maintaining the ambient temperature below 25 °C with relative humidity above 80 % for 24 h after infiltration resulted in an increased expression of both recombinant proteins (data not shown). Generally, environmental factors such as temperature have been recognized to influence the plant–virus interactions. As agroinfiltration with Cowpea mosaic virus-based viral vector has the ability to activate the PTGS in *N. benthamiana*, it might have led to the production of low levels of virus-derived siRNAs which results in a more limited virus-induced gene silencing at 25 °C as compared to the higher temperatures. A recent report revealed that virus-induced silencing was found to be low at 25 °C by quantifying virus-derived siRNAs in *N. benthamiana* (Chellappan et al. 2005). It has long been recognized that humidity influences diverse aspects of plant growth and development. It is, therefore, anticipated that high relative humidity entails higher physiological activities of *N. benthamiana*. It was proposed to be due to high physiological activity, the leaves of *N. benthamiana* would be more susceptible to *Agrobacterial* infections, which might have resulted in higher recombinant gene expression. In *Arabidopsis*, Kim et al. (2009) found that infiltration with LBA4404 and incubation of the plants under 85–90 % of relative humidity for 24 h after infiltration improved the levels of transient expression. Also, it has been reported that co-infiltration of P19 vector expressing RNA silencing suppressor proteins, reduced the PTGS of the co-introduced T-DNA and increased the recombinant protein expression level in *N. benthamiana* (Voinnet et al. 2003).

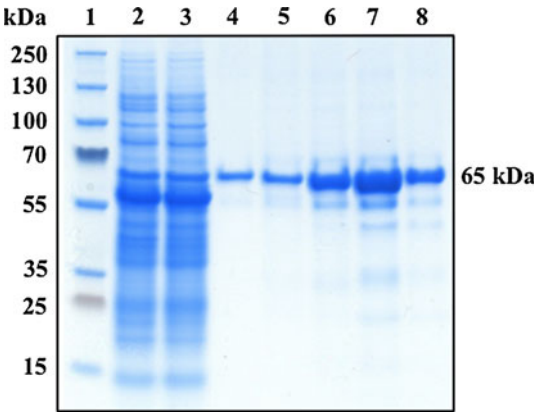

Fig. 5 SDS-PAGE analysis of ADS in cell-free extracts and in different eluted fractions of purified protein after immobilized metal ion affinity chromatography at different concentrations. *Lane 1* molecular weight marker; *lane 2* total soluble protein (30 µg) extracted from agroinfiltrated leaves at 9 days post-infiltration (dpi); *lane 3* total soluble protein (30 µg) extracted from the agroinfiltrated leaves at 9 dpi after ammonium sulfate precipitation; *lane 4* purified protein (4 µg) from eluted fraction 2; *lane 5* purified protein (4 µg) from eluted fraction 4; *lane 6* purified protein (10 µg) from eluted fraction 5; *lane 7* purified protein (47 µg) from eluted fraction 6; *lane 8* purified protein (24 µg) from eluted fraction 7. Molecular mass for each band of the marker is indicated to the *left* of the panel. Molecular mass of recombinant protein is indicated to the *right* of the panel

Functional characterization of transiently expressed ADS and ECS

To demonstrate the enzymatic activity of both proteins, the purified recombinant enzymes were assayed in vitro with FDP as a substrate in a standard sesquiterpene synthase assay.

The products of the sesquiterpene synthase assay were analyzed by GC–MS. This analysis confirmed, as expected that the ADS and ECS produce amorpha-4,11-diene (Fig. 6) and *epi*-cedrol, respectively, as the major products (Fig. 7). The protein purified from negative controls, uninfiltrated leaves and leaves infiltrated with empty pEAQ-*HT* vector with pJL3:p19 at 9 dpi did not produce amorpha-4,11-diene, *epi*-cedrol or other products when assayed with FDP. The cell-free extract from leaves co-transformed with the pJL3:p19 and pEAQ-*HT* empty vectors produced two peaks at $R_t = 41.95$ min and $R_t = 47.68$ min in the chromatograms shown, which were also obtained with purified enzymes of ADS or ECS. Apart of these two peaks, there were two more non-specific product peaks ($R_t = 53.41$ min and $R_t = 57.44$ min) that appeared in the chromatograms produced by the purified enzymes.

Additionally, one and three minor products were observed for ADS and ECS, respectively. Recombinant ADS produced in *E. coli* produced at least 15 minor by-products (Picaud et al. 2005). The enzyme produced in tobacco only produced one by-product, i.e. amorpha-4,7(11)-diene ($R_f = 46.54$) (Fig. 6), indicating that the plant-produced protein is more accurate in its catalysis than the bacterial-produced protein. We have studied the mechanism and stereochemistry of the enzymatic cyclization of FDS by ADS (Picaud et al. 2006) and we can conclude that the two products obtained with the plant-produced enzyme are derived from the final carbocation of the reaction by different deprotonations as the reaction is quenched. In addition to *epi*-cedrol, the recombinant ECS produced small amounts of α-cedrene ($R_t = 42.73$ min), β-cedrene ($R_t = 43.20$ min) and (E)-β-farnesene ($R_t = 52.16$ min) (Fig. 7). These by-products were also

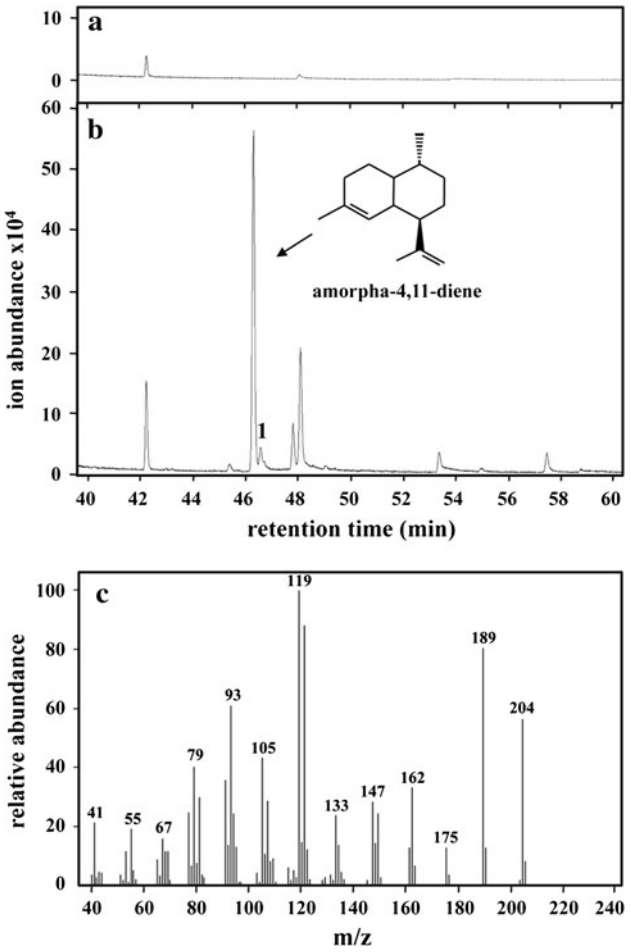

Fig. 6 GC–MS analysis of the products generated from farnesyl diphosphate by purified recombinant amorpha-4,11-diene synthase. **a** Total ion chromatogram of products formed by protein from control plant; **b** Total ion chromatogram of products formed by the purified recombinant amorpha-4,11-diene synthase; *1* amorpha-4,7(11)-diene; **c** Mass spectrum of amorpha-4,11-diene

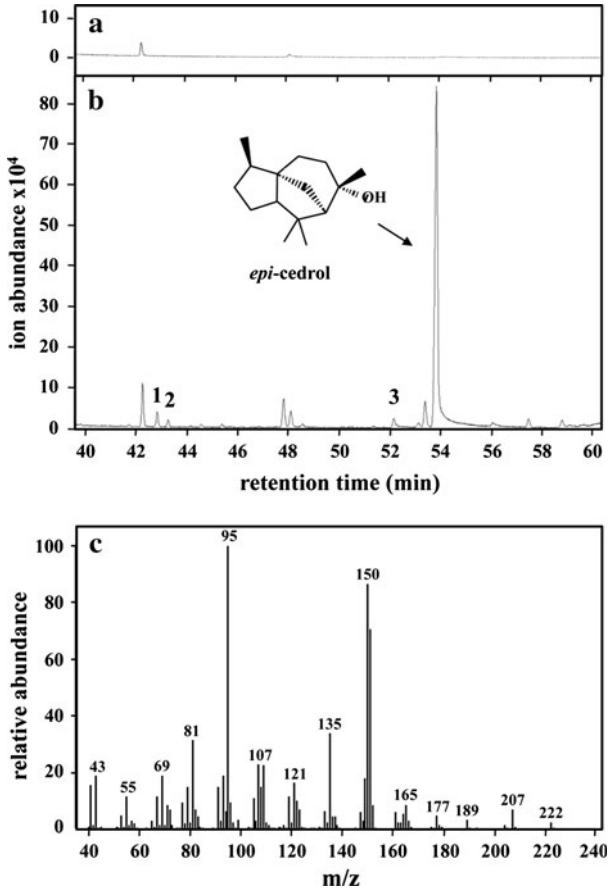

Fig. 7 GC–MS analysis of the products generated from farnesyl diphosphate by purified recombinant *epi*-cedrol synthase. **a** Total ion chromatogram of products formed by protein from control plant; **b** Total ion chromatogram of products formed by the purified recombinant epicedrol synthase; *1* α-cedrene; *2* β-cedrene; *3* (*E*)-β-farnesene; **c** Mass spectrum of *epi*-cedrol

obtained with recombinant ECS produced in *E. coli* (Mercke et al. 1999). The identity of the products was confirmed by comparing its retention time and mass spectra to those found in the Mass finder 2.1. These results confirmed that both *A. annua ADS* and *ECS* genes are functionally expressed in *N. benthamiana*.

Based on the transcript analysis of *ADS* and *ECS* genes, we determined the enzyme activities of both purified ADS and ECS made from leaves at 9 dpi. The products of purified enzymes, amorpha-4,11-diene ($R_t = 46.28$ min) and *epi*-cedrol ($R_t = 53.83$ min) were detected by GC–MS and quantified as 1.9 and 1.7 mg h^{-1} kg^{-1} FW, respectively, using valencene as internal standard. Recently, van Herpen et al. (2010) reported on the production of artemisinin precursors in *N. benthamiana* by transient expression of biosynthetic genes including ADS. After 10 days, the concentration of amorpha-4,11-diene in leaves infiltrated with an ADS construct was reported to be around 0.9 mg kg^{-1} FW. However, the amount of amorphadiene may be considerably higher as it was shown that around 0.5 mg kg^{-1} FW 24 h^{-1} could be collected in the head space. In conclusion, the production of amorphadiene by the purified ADS is considerably higher than that observed in planta, indicating that the in vivo system is operating under non-optimal conditions.

The specific activity and turn-over numbers (k_{cat} values) were determined for ADS and ECS under optimum assay conditions with FDP (20 μM) as substrate at 30 °C. Under our assay conditions, both ADS and ECS were active over 240 min with a linear activity for more than 30 min. The purified enzymes exhibited the specific activity of 0.002 and 0.01 μmol min^{-1} mg^{-1} protein for ADS and ECS, respectively. From these measurements, we calculated the k_{cat} for ADS and ECS to be 2.12×10^{-3} s^{-1} and 11×10^{-3} s^{-1}, respectively, which is in agreement with the previously published values for ADS

($k_{cat} = 4.3 \times 10^{-3}$ s^{-1} at pH 7.5) (Picaud et al. 2005). No value for ECS has been determined earlier due to difficulties to produce the recombinant protein in *E. coli*. As reflected by the low k_{cat} values of ADS and ECS, these enzymes are relatively slow enzyme. This is a general feature of terpene synthases, which exhibit turnover numbers in the range of 0.01–0.3 s^{-1} (Cane 1990). Pre-steady-state kinetic analysis on sesquiterpene synthases implies that plant sesquiterpene synthases operate by a rapid equilibration with FDP to form a precatalytic enzyme–substrate complex, followed by a slightly slower conversion of FDP to hydrocarbons (Mathis et al. 1997; Cane et al. 1997). The reaction is ended by a rate-limiting release of products. It is interesting to note that the k_{cat} value of ECS is considerably higher than that of ADS and other sesquiterpene synthases producing aliphatic products. We suggest that this higher turn-over number is due to a more rapid release of the product as a consequence of the presence of a hydroxyl group, which makes the product more hydrophilic.

To conclude, ADS and ECS were produced in relatively good amounts in the agroinfiltrated intact leaves, and the functional tests showed that the recombinant enzymes showed expected product specificity. Further, the yields of functional recombinant proteins from *N. benthamiana* are generally higher than those from other expression systems, thus providing an avenue to subsequent structural determinations. These considerations led us to adapt the *N. benthamiana* transient expression system when the goal was highly specific and large-scale production of sesquiterpene synthases. Also, our results suggest that the *N. benthamiana* transient expression system using viral vectors described here can be employed for large-scale production of recombinant proteins to study biochemical, biophysical, functions of proteins and for elucidation of their roles in metabolic reactions as well as for industrial applications.

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