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Characterization and functional analysis of the genes encoding 1-deoxy-D-xylulose-5-phosphate reductoisomerase and 1-deoxy-D-xylulose-5-phosphate synthase, the two enzymes in the MEP pathway, from *Amomum villosum* Lour

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Abstract A *DXR* gene, *AvDXR* (GenBank accession no. FJ459894), and a *DXS* gene, *AvDXS* (GenBank accession no. FJ455512), were isolated from the leaves of *Amomum villosum*, one of the most well-known and authentic herbs in South China. The 1,749-bp full-length cDNA of *AvDXR* encoded a peptide of 472 amino acids, and the 2,347-bp full-length cDNA of *AvDXS* encoded a peptide of 715 amino acids. The deduced amino acid sequences of the AvDXR and AvDXS proteins share high homology with DXRs and DXSs from other plant species, and AvDXS belongs to class 1 plant DXS. The characterization based on bioinformatic analysis indicated that the *AvDXR* and *AvDXS* encoded functional proteins as DXR and DXS, respectively. The functional color assay in *Escherichia coli* with pAC-BETA implied that *AvDXR* and *AvDXS* encoded functional proteins that manipulated the biosynthesis of isoprenoid precursors. Both *AvDXR* and *AvDXS* were expressed extensively in the leaves, stems, roots, pericarps and seeds of *A. villosum*. *AvDXS* expression was similar in all tissues investigated, whereas higher levels of *AvDXR* were observed in the fruits, the main part for the accumulation of volatile oil in this plant. *AvDXR* was transformed into tobacco to confirm its function further. Overexpression of *AvDXR* in transgenic T1 generation tobacco increased DXR activity, photosynthetic pigment content and volatile isoprenoid components, and the increase of photosynthetic pigment content was consistent with the *AvDXR* transcription level. This study demonstrated that *AvDXR* plays important role in isoprenoid biosynthesis and it is useful for metabolic engineering.

Keywords 1-deoxy-D-xylulose-5-phosphate reductoisomerase · 1-deoxy-D-xylulose-5-phosphate synthase · Isoprenoid biosynthesis · *Amomum villosum* Lour. · Transgenic tobacco

Introduction

Isoprenoids have diverse biological functions and medicinal activities. *Amomum villosum* Lour. (*Zingiberaceae*), one of the most well-known and authentic herbs in South China, has diverse medicinal functions such as dissipating dampness, warming the spleen, regulating Qi flow and preventing miscarriage, etc. [1]. The medicinal functions of Fructus Amomi (the officinal Latin name of *A. villosum*) depend on its volatile oil, which mainly consist of the monoterpenes, e.g. bornyl acetate, camphor, borneol, limonene, camphene, myrcene, carene-3 and α-terpineol [2]. The isolation and characterization of genes encoding key enzymes involved in isoprenoid biosynthesis could be useful in enhancing the medicinal quality of *A. villosum* and other medicinal plants relying on isoprenoids for their medicinal properties.

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Isoprenoids are universally synthesized through the condensation of the five-carbon compound isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP). In plants, there are at least two distinct pathways providing these isoprenoid precursors: one is the plastid-located 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway and the other is the cytoplast-located mevalonic acid (MVA) pathway [3–7]. Monoterpene biosynthesis is mostly driven by plastidial supply of IPP and DMAPP via the MEP pathway [8]. The transketolase-type condensation of pyruvate with D-glyceraldehyde-3-phosphate to form 1-deoxy-D-xylulose-5-phosphate (DXP) catalyzed by DXP synthase (DXS, EC: 2.2.1.7) is the first step in the MEP pathway [9, 10], and the conversion of DXP to MEP catalyzed by DXP reductoisomerase (DXR, EC:1.1.1.267) is the second regulation step in the MEP pathway [11, 12].

The gene encoding DXS was first cloned and characterized in *Escherichia coli* [13], subsequently followed by the DXS genes cloned from several plants, such as *Mentha* × *piperita* [14], *Arabidopsis thaliana* [9], *Elaeis guineensis* [15] and *Glycine max* [10]. The genes encoding DXR have also been isolated from several plants, such as *A. thaliana* [12], *Croton stellatopilosus*[16], *Zea mays* [17], *Ginkgo biloba* [18], *Salvia miltiorrhiza* [19], and *Camptotheca acuminata* [20]. Both *DXS* and *DXR* were considered potential regulators of flux through the MEP pathway soon after their discovery. Several lines of evidence, including overexpression studies of *DXS* in *Arabidopsis* and tomato [9, 21], support that the step catalyzed by DXS is the limiting step for the biosynthesis of plastidial isoprenoids in plant cells. Furthermore, up-regulation of *DXS* from *Arabidopsis* enhanced production of essential oils in transgenic spike lavender [22]. However, because DXP is an intermediate not only for IPP biosynthesis but also for the biosynthesis of thiamin and pyridoxol, the production of MEP catalyzed by DXR is regarded as the first committed step of the MEP pathway for isoprenoid biosynthesis [8, 12]. Increased isoprenoid biosynthesis has been achieved in transgenic plants with increased *DXR* levels. For example, over-expressing *DXR* from *Synechosystis* PCC6803 in transplastomic tobacco plants increased the content of various isoprenoids [23]. Furthermore, transgenic *Mentha* × *piperita* plants overexpressing *DXR* accumulated substantially more essential oil (~50 % yield increase) without causing a change in monoterpene composition [8]. Both *DXS* and *DXR* can potentially be applied for manipulating biosynthetic pathway by genetic engineering to produce more isoprenoids of commercial significance.

Although there are chemical and pharmacological studies on Fructus Amomi, there has been little molecular research on the genes involved in isoprenoid biosynthesis in *A. villosum*. In this paper, we report the molecular cloning, characterization and function analysis of the *DXR* and *DXS* genes from *A. villosum*. Their biological functions in *E. coli* and expression patterns in different tissues were also investigated and *AvDXR* was transformed into tobacco to confirm its function further.

Materials and methods

Plant materials

Young leaves of *A. villosum* were collected from a 1-year old plant, grown in the medicinal garden of Guangzhou University of Chinese Medicine (China).

For tissue expression analysis, leaves, stems, and roots of 3-month-old seedlings, grown in a breeding lab, were used. Fresh ripe fruits were collected from mature plants growing at Yangchun (China), endemic production area, and stored at −70 °C until used.

Total RNA isolation and first-strand cDNA preparation

The total RNA was isolated using Plant RNA Extraction Kit (Applygen, China), while the total RNA of the fruits, which were divided into pericarps and masses of seeds (including fruit pulp), were isolated and DNA contamination was removed using Column Plant RNAout 2.0 (Tiandz, China). One microgram of the total RNA was used for reverse transcription with 1st Strand cDNA Synthesis Kit (Takara, Dalian) in the presence of oligo (dT) in a 20 μl reaction mixture according to the manufacturer's protocol.

Isolation of *AvDXR*

Degenerate primers AD1, AD2, AD3 and AD4 (Online Supplementary Table 1[1]) were designed based on the high homologous region among the sequences of four monocotyledonous *DXR* genes: AF367205 (from *Oryza sativa*), AJ297566 (from *Z. mays*), AJ583446 (from *Hordeum vulgare*) and AY583784 (from *E. guineensis*). The first-strand cDNA was used as the template for the amplification of the core fragment by nested PCR with primers AD1 and AD3 for the 1st PCR, AD2 and AD4 for the 2nd PCR, using the following PCR program: 94 °C for 3 min, 37 cycles of 94 °C for 0.5 min, 57 °C (1st PCR) or 53 °C (2nd PCR) for 0.5 min, 72 °C for 1 min, and 72 °C for 5 min after the final cycle. The product of the 2nd PCR was purified and sequenced. Subsequently, the core fragment sequence was used to design the gene-specific primers for RACE. AD51 and AD52 were synthesized for 5′-RACE, and AD31 and AD32 were synthesized for 3′-RACE,

[1] All the primers used in this study are shown in Online Supplementary Table 1.

respectively. Then, the 5′-end and 3′-end of the full-length cDNA were obtained by RACE-PCR using SMART RACE cDNA Amplification Kit (Clontech, USA) according to the manufacturer's protocol. After aligning and assembling the sequences of the core fragment, 5′-RACE and 3′-RACE products, the full-length ORF of *AvDXR* was deduced and subsequently obtained by PCR with primers ADO1 and ADO3 using the following PCR program: 94 °C for 3 min, 33 cycles of 94 °C for 0.5 min, 54 °C for 0.5 min, 72 °C for 2.5 min, and 72 °C for 7 min after the final cycle. The PCR product was cloned into pGEM-T Easy vector (Promega, USA) and sequenced.

Isolation of *AvDXS*

Degenerate primers AS1, AS2, AS3 and AS4 were designed based on the high homologous region among the sequences of three monocotyledonous *DXS* genes: NM_001062059 (from *O. sativa*), AY583783 (from *Z. mays*) and EF507248 (from *E. guineensis*). The first-strand cDNA was used as the template for the amplification of the core fragment with AS1 and AS3 for the 1st PCR, AS2 and AS4 for the 2nd PCR, using the following PCR program: 94 °C for 3 min, 37 cycles of amplification (94 °C for 0.5 min, 55 °C for 0.5 min, 72 °C for 1.5 min) and 72 °C for 5 min after the final cycle. The PCR product was purified and sequenced. Subsequently, primers AS51, AS52 and AS31, AS32 designed according to the core fragment sequence were synthesized for 5′-RACE and 3′-RACE, respectively. Then, the 5′-, 3′-end, full-length cDNA and ORF (PCR with primers ASO1 and ASO2) were obtained by the methods mentioned above.

Bioinformatic analysis

Original sequence analysis was performed by using Omiga2.0 and DNAstar software. Comparative analysis of deduced amino acid sequences with other DXRs and DXSs were performed using protein–protein Blast programs (http://www.ncbi.nlm.nih.gov/BLAST/). The multiple alignment analysis was performed by using DNAMAN5.0 software. Prediction of transit peptide of deduced protein was performed by ChloroP server version1.1 (http://www.cbs.dtu.dk/services/ChloroP/). Prediction of the function of the deduced protein was performed with the conserved domains and protein classification programs (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml).

Functional analysis of AvDXR and AvDXS in *Escherichia coli*

The plasmids pAC-BETA and pTrc-AtIPI, provided by Francis X. Cunningham (Department of Cell Biology and Molecular Genetics, University of Maryland, USA) were used to investigate the biological function of AvDXR and AvDXS. The pAC-BETA contains all functional genes of the β-carotene synthesis [24], and also retains a chloramphenicol (Chl) resistance gene. Cells of *E. coli* containing pAC-BETA produce and accumulate β-carotene, resulting in yellow colonies. The pTrc-AtIPI contains a Trc promoter, ampicillin resistance gene and *AtIPI* gene.

The ORF of *AvDXR* was amplified by PCR with primers ADP1 and ADP2 containing *Bgl*II and *Eco*RI restriction sites respectively. After digestion of PCR fragment and plasmid pTrc-AtIPI with *Bgl*II and *EcoR*I, the coding region of *AvDXR* was isolated and cloned into the empty vector pTrc to generate the recombinant plasmid pTrc-AvDXR which was subsequently verified by sequencing.

The ORF of *AvDXS* contains the *Bgl*II restriction site, which is the upstream multiple cloning site of pTrc-AtIPI, therefore the pTrc-AvDXR imported *Sph*I restriction site by primer ADP1 was used as the medial plasmid. The coding region of *AvDXS* was amplified by PCR with primers ASP1 and ASP2 containing *Sph*I and *Eco*RI restriction sites respectively. After digestion of PCR fragment and plasmid pTrc-AvDXR with *Sph*I and *EcoR*I, the coding region of *AvDXS* was isolated and cloned into the empty vector pTrc to generate pTrc-AvDXS.

The pTrc-AtIPI digested by *Pst*I to remove *AtIPI* and then ligated by T4 DNA ligase was used as control. The plasmids pTrc-AvDXR and pAC-BETA as well as the plasmids pTrc-AvDXS and pAC-BETA were co-transformed into *E. coli* TOP10 respectively. The plasmids pTrc and pAC-BETA were also co-transformed and the single plasmid pAC-BETA was transformed into the *E. coli* TOP10 to be used as the negative controls. Transformants were cultured on solid LB medium containing ampicillin (Amp, 150 mg/L) and Chl (50 mg/L) at 37 °C for 1 day and then at 28 °C for 2 day. The color of the transformants accumulating β-carotene was observed as a visible marker to test the function of the target gene.

Expression pattern analysis

Semi-quantitative RT-PCR was used to investigate the expression patterns of *AvDXR* and *AvDXS* in different tissues. The first-strand cDNA of each sample were prepared as mentioned above and then used as templates for PCR. Primers ADR1, ADR2 and ASR1, ASR2 were used to amplify the core fragments of *AvDXR* and *AvDXS*, respectively, while 18SF and 18SR were used to amplify the housekeeping gene, *18S rRNA*, as the internal control to adjust the cDNA dosages to equal amounts. The PCR program was carried out under the following conditions: 94 °C for 2 min, 32 cycles (for *AvDXR*) or 33 cycles (for *AvDXS*) or 28 cycles (for *18S rRNA*) of 94 °C for 0.5 min, 55 °C for 0.5 min and

72 °C for 1 min. The PCR products were separated by agarose gel electrophoresis and band intensity was determined by densitometric analysis. The levels of *AvDXR* and *AvDXS* transcripts in different samples were analyzed and compared by using the intensity ratios of the *AvDXR* and *AvDXS* bands with *18S rRNA*. Semi-quantitative RT-PCR was repeated three times for each sample.

Generation of *AvDXR* transgenic tobacco

The ORF of *AvDXR* was amplified from the plasmid pGEM-AvDXR by PCR with primers ADP3 and ADP4 containing *Xba*I and *Sma*I sites, respectively. Subsequently, the PCR product was digested with *Xba*I and *Sma*I and cloned into the pBI121 binary vector. Thus the *AvDXR* ORF was under the control of CaMV 35S promoter. The recombinant vector, pBI-AvDXR, was transformed into *Agrobacterium tumefaciens* strain EHA105 and then introduced into tobacco (*Nicotiana tabacum* cv. "Zhongyan 90") via *Agrobacterium*-mediated transformation [25]. The transgenic T1 generation tobacco plants were generated as described [25], and only the line(s) showing approximately 3:1 segregation of kanamycin resistance were selected for further study. The molecular screening of *AvDXR* transgenic plants based on DNA was performed by PCR with primers 35S2 and AD53 targeted in CaMV 35S and *AvDXR* 5′-end. Expression analysis of *AvDXR* in transgenic T1 plants was performed as described above but using primers ADR3 and ADR4 instead of ADR1 and ADR2 to evade the endogenous *DXR* in tobacco.

Determination of DXR activity in transgenic tobacco

For extraction of tobacco protein, the first fully expanded leaf samples (150 mg) of 3-month-old tobacco were ground in the ice-bath mortar in 1.5 ml cool buffer (50 mM Tris–HCl [pH7.5], 10 mM β-mercaptoethanol, 10 g/l polyvinylpyrrolidone). After centrifugation (10,000 r/min, 10 min, 4 °C), the supernatant was used to assay the DXR activity using the similar method described previously [23]. The oxidation of NADPH in reactions with or without DXP (Sigma, USA) (final concentration 0.25 mM) was monitored with a spectrophotometer at 340 nm for 6 min in 1 ml reaction buffer (150 mM Tris–HCl [pH7.5], 5 mM $MgCl_2$, 1 mM $MnCl_2$, 4 mM dithiothreitol, 0.075 mM NADPH, 0.25 mL tobacco protein supernatant). One unit of DXR activity was defined as the amount of enzyme catalyzing the oxidation of 1 pmol NADPH (extinction coefficient $= 6.22 \times 10^{-6}$ pM^{-1} cm^{-1}) per min. The protein content in the enzyme extracts was determined using Coomassie Brilliant Blue protein determination kits (NanJing JianCheng Bioengineering Institute, China). Determinations of each plant were performed with two individual samples as replicates.

Determination of chlorophyll and carotenoid contents in transgenic tobacco

Total chlorophylls and carotenoids were extracted from fully expanded leaf samples (50 mg) of 3-month-old tobacco using 5 ml cool 100 % acetone as described by Lichtenthaler and Buschmann [26]. The chlorophyll and carotenoid contents were measured by spectrophotometry and calculated as described [27]. Determinations of each plant were conducted with three individual samples as replicates.

Analysis of volatile isoprenoids

A comparative analysis of volatile isoprenoids in whole flowers was carried out using HS-SPME (headspace solid-phase microextraction) coupled with GC-MS (gas chromatography-mass spectrometry). Four fully developed flowers were harvested from 7-month-old tobacco and placed in a 10 ml headspace vial with a polytertrafluoroethylene septum. Before extraction, the sample was equilibrated for 70 min at 40 °C. Headspace extraction was performed for 30 min at 40 °C with a 100 μm PDMS (polydimethylsiloxane) SPME fiber. The compounds were desorbed by inserting the fiber into GC injector port for 5 min at 250 °C. Before each sampling, the fiber was reconditioned for 30 min at 250 °C. GC-MS analyses were carried out on an Agilent 6890N/5973 GC-MS detector equipped with an HP-5 ms Capillary GC Column (30 m × 0.25 mm × 0.25 μm) (Agilent Technologies, USA). GC oven temperature was programmed at an initial temperature of 40 °C for 1 min with a ramp of 5 °C/min to 280 °C and final time of 10 min. All mass spectra were acquired in electron impact mode at 70 eV with a scan range of 30–500 amu. Compounds were identified by retrieval of authentic standards in mass spectra data base (NIST98).

Statistical analysis

Analysis of variance of all data was performed by the least significant difference (LSD) test at 0.05 or 0.01 probability level when the *F*-test showed a significant ($P \leq 0.05$) or very significant ($P \leq 0.01$) effect using the program SPSS (SPSS Inc.).

Results

Cloning and characterization of *AvDXR* from *A. villosum*

A 1,749-bp full-length cDNA, *AvDXR* (GenBank accession no. FJ459894), was isolated and identified from leaves of *A. villosum.* It has an ORF of 1416 bp, encoding a peptide

of 472 amino acids, a 167-bp 5′-untranslated region and a 166-bp 3′-untranslated region with a polyA tail. The AvDXR protein (ACS26204) has a predicted molecular mass of 51.41 kDa and isoelectric point (pI) of 6.44. Its deduced amino acid sequence is highly homologous to DXR sequences from other plants, e.g. *O. sativa* (BAB78606, 85 % identity), *Z. mays* (ACG33012, 85 % identity), *C. stellatopilosus* (ABO38177, 82 % identity), *H. vulgare* (CAE47438, 80 % identity), *Hevea brasiliensis* (ABD92702, 83 % identity). Multiple alignments of AvDXR and 11 other plant DXRs were performed and a phylogenetic tree was generated (Online Supplementary Fig. 1). Since *AvDXR* was isolated from *Zingiberaceae*, a family of monocotyledons, AvDXR is more homologous to the DXRs from monocotyledons, such as *O. sativa*, *Z. mays* and *H. vulgare*.

Chloroplast (plastid) transit peptides are present in all plant DXRs and direct the enzyme to plastids in which the MEP pathway locates [11, 12, 16–20]. Also, a putative chloroplast transit peptide with a conserved cleavage site of CS-X motif (for AvDXR, X: M) [12, 17] is located at the N-terminus of AvDXR (Online Supplementary Fig. 2). Since DXR catalyses the reduction and rearrangement of DXP in the presence of NADPH [28], the NADPH-binding domains are critical to the catalytic activity of DXR. Two highly conserved NADPH-binding domains, GSTGSIGT and LAAGSNV, were found in the N-terminal region of AvDXR, and two substrate-binding domains, LPADSEHSAI and NKGLEVIEAHY, locate in its mid-region, which is highly homologous to DXRs from other plants and similar to the DXR from *E. coli* [29]. All plant DXRs have a consensus pro-rich region at the N-terminus of the mature protein, which is not present in prokaryotic DXR [12]. The pro-rich domain PPPAWPGRA was also found at the N terminus of AvDXR. According to the result of conserved domains and protein classification, the putative AvDXR protein contains a conserved DXR domain (PRK05447) and belongs to the DXP reductoisomerase superfamily (Online Supplementary Fig. 3).

Cloning and characterization of *AvDXS* from *A. villosum*

A 2,347-bp full-length cDNA, *AvDXS* (GenBank accession no. FJ455512), was isolated from leaves of *A. villosum.* It has a 2,148-bp ORF, encoding a peptide of 715 amino acids, a 35-bp 5′-untranslated region and a 164-bp 3′-untranslated region with a polyA tail. The AvDXS protein (GenBank accession no. ACR02668) has a predicted molecular mass of 77.15 kDa and pI of 7.531. The deduced amino acid sequence of *AvDXS* is highly similar to DXS sequences from other plants, e.g. *E. guineensis* (AAS99588, 86 % identity), *Medicago truncatula* (CAE47438, 86 % identity), *Pueraria montana* var. lobata (AAQ84169, 85 % identity) and *Z. mays* (ABP88134, 84 % identity). It was reported that there are two distantly related classes of DXS in plants (class 1 and class 2) [10, 30]. Multiple alignments of AvDXS with other 18 plant DXSs (ten of them from class 1 and other eight from class 2) were performed, and a phylogenetic tree was constructed (Online Supplementary Fig. 4). The result revealed that AvDXS belongs to class 1 DXS and is more homologous to the DXSs from monocotyledons, such as *Z. mays* and *E. guineensis*.

Plant DXSs also contain a chloroplast transit peptide, which is consistent with the subcellular localization of MEP pathway in plants [10, 14–16]. A predicted chloroplast transit peptide with a conserved cleavage site of VXA motif (for AvDXS, X: C) [15, 31] is located at the N-terminus of AvDXS (Online Supplementary Fig. 5). Thiamin diphosphate (TPP) is the coenzyme of DXS [32]. Similar to other DXSs, AvDXS also contains a TPP-binding domain in the N-terminal, which begins and concludes with the highly conserved sequence -GDG- and -LNDN-, respectively (Online Resource 3). In addition, the histidine residue at position 111 (H111) in the N-terminal of AvDXS shows a good match with the histidine residue (H49) in *E. coli* DXS, which has been reported to be involved in DXS catalysis [33]. The invariant glutamic acid residue, thought to be specific for transketolase activity, was found in the mid-region AvDXS. Moreover, a DRAG domain considered to play important role was found in its C-terminal, which begins and concludes with the highly conserved sequence -DRAG- and -PSD- respectively, similar to other plant DXSs. According to the result of conserved domains and protein classification, the AvDXS protein contains a conserved DXS (PRK05444) domain and belongs to the transketolase superfamily (Online Supplementary Fig. 6).

Function analysis of *AvDXR* and *AvDXS* in *E. coli*

Carotenoids are not ordinarily present in *E. coli*, however *E. coli* introduced with foreign carotenogenic gene clusters is able to produce carotenoids. These genetically engineered *E. coli*, provide a visible color assay that could act as a tool to examine the biological function of molecules involved in isoprenoid biosynthesis [24, 34–38]. To confirm the function of *AvDXR*, the plasmids pTrc-AvDXR and pAC-BETA were co-transformed into *E. coli* strain TOP10. The result showed that *E. coli* containing pTrc-AvDXR and pAC-BETA accumulated significantly higher saffron yellow β-carotene than the control containing the empty vector pTrc and pAC-BETA (Fig. 1A). *Escherichia coli* harboring the single vector pAC-BETA could not grow on LB medium containing Chl and Amp due to the lack of Amp resistance gene.

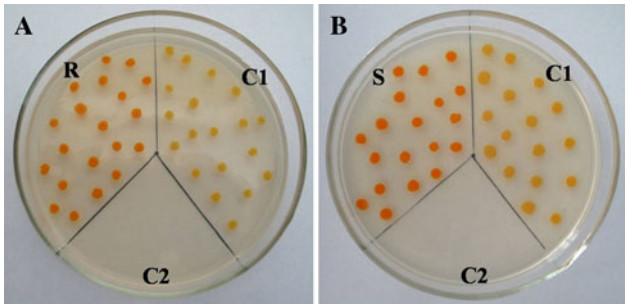

Fig. 1 Functional demonstration of *AvDXR* and *AvDXS* in *E. coli*. The *E. coli* TOP10 was transformed with pAC-BETA and pTrc-AvDXR harboring *AvDXR* (R) or pAC-BETA and pTrc-AvDXS harboring *AvDXS* (S), pAC-BETA and pTrc (C1), and pAC-BETA (C2), respectively

Also, the co-transformations were performed using the method mentioned above to demonstrate that the *AvDXS* encodes the anticipative functional protein. The result showed that *E. coli* containing pTrc-AvDXS and pAC-BETA accumulated significantly higher saffron yellow *β*-carotene than the control with the empty vector pTrc and pAC-BETA, which was similar to that of AvDXR (Fig. 1B).

Expression pattern of *AvDXR* and *AvDXS* in different tissues of *A. villosum*

To investigate the expression of *AvDXR* and *AvDXS* in different tissues, total RNA was isolated from the leaves, stems, roots, pericarps and seeds of *A. villosum*, and subjected to semi-quantitative RT-PCR analysis. The result showed that *AvDXR* was expressed extensively in all tested tissues including non-photosynthetic tissues, e.g. roots, pericarps and seeds, similar to that reported in *G. biloba* [18], *A. thaliana* [12] and *C. acuminata* [20], which implies that *AvDXR* may be a constitutively expressed gene like *DXR*s from other plants. However, *AvDXR* is expressed with the highest transcription levels in fruits, divided into pericarps and seeds, followed by the stems and roots, and lowest in the leaves (Fig. 2). *AvDXS* is also a constitutively expressed gene, expressed strongly in all tested photosynthetic and non-photosynthetic tissues, similar to that reported in *A. thaliana* [39]. Unlike *AvDXR*, the transcript levels of *AvDXS* were indistinctive in all tested tissues (Fig. 2).

Overexpression of *AvDXR* in tobacco increases DXR activity, photosynthetic pigment content and volatile isoprenoid components

To analyze the function of *AvDXR* further, transgenic tobacco plants overexpressing *AvDXR* were generated by *Agrobacterium*-mediated transformation. The transgenic

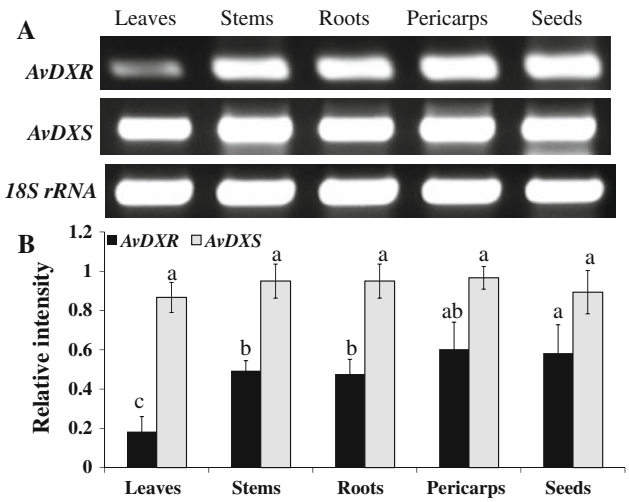

Fig. 2 Expression patterns of *AvDXR* and *AvDXS* in different tissues of *Amomum villosum*. **a** Patterns of *AvDXR* and *AvDXS* transcripts. Total RNA was extracted from leaves, stems, roots, pericarps and seeds (including fruit pulp) of cultivar Yuanguo, respectively. The *18S rRNA* was used as the internal control. **b** Relative intensity of *AvDXR* and *AvDXS* transcripts in comparison to *18S rRNA*. The *same letter* above the columns indicates that the difference is not significant at $P \leq 0.05$

T1 plants of line D2 which showed a kanamycin-resistant segregation ratio of 3:1 were investigated in this study. A total of 12 transgenic T1 plants were identified by PCR (data not shown). Three plants (D2–1, D2–2 and D2–3) showed significant overexpression of *AvDXR* in comparison to the wild-type control by semi-quantitative RT-PCR (Fig. 3A, B), and two of them (D2-1 and D2-2) showed significantly higher DXR activity (Fig. 3C). DXR activity in wild-type control was not detected which may be due to the inadequate sensitivity of determining method used in this work.

Carotenoids and prenyl side-chains of chlorophylls are isoprenoids synthesized via MEP pathway in plastids [40]. Thus, we measured the contents of total carotenoids and chlorophylls of the plants overexpressing *AvDXR* and the wild-type control. Overexpression of *AvDXR* increased the contents of total carotenoids and chlorophylls, and the photosynthetic pigment content showed a positive correlation with the *AvDXR* expression level (Fig. 3d, e). Two plants (D2–1 and D2–3) expressing higher level of *AvDXR* accumulated significantly more carotenoids and chlorophylls than the wild-type control.

Furthermore, volatiles in the flowers were analyzed by HS-SPME combining GC-MS. The transgenic plant D2–1 showing both high levels of *AvDXR* expression and DXR activity were sampled, as well as the wild-type control. The result indicated that overexpression of *AvDXR* increased the volatile isoprenoid components in tobacco flowers. Both the D2–1 and WT flowers released caryophyllene (sesquiterpene), but D2–1 also released santalol (sesquiterpene) and

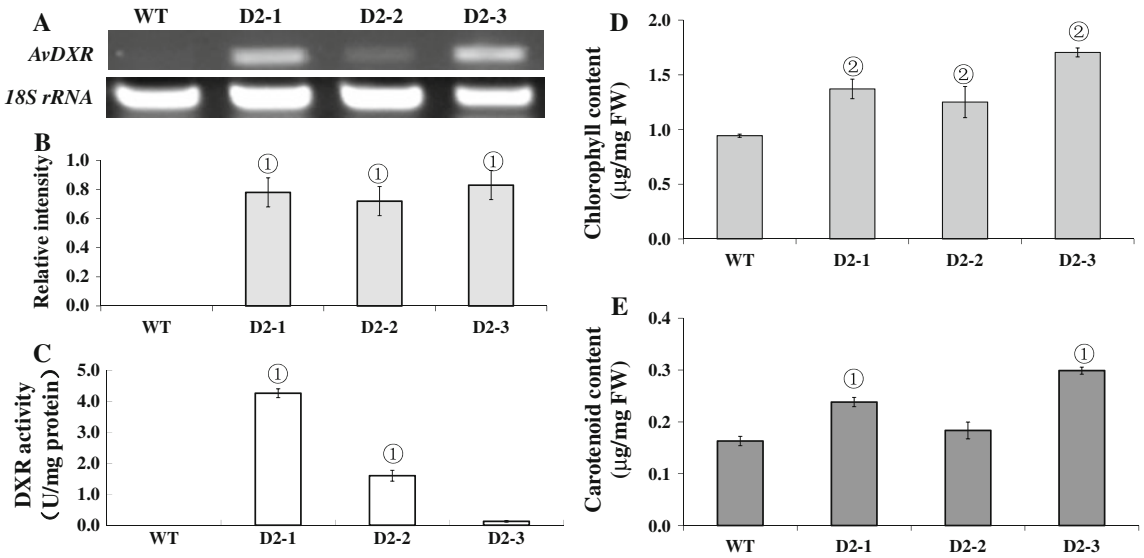

Fig. 3 DXR activity and photosynthetic pigment content of the transgenic tobacco plants in comparison to the wild-type control. **a** The transcript level of of *AvDXR* in wild-type contol (WT) and transgenic plants (D2–1, D2–2, D2–3). The *18S rRNA* was used as the internal control. **b** Relative intensity of *AvDXR* transcripts in comparison to *18S rRNA*. **c** DXR activity. **d** Total chlorophyll (a, b) content. **e** Total carotenoid content. The numbers ① or ② above the columns indicate that the difference in comparison to WT is significant at $P \leq 0.01$ or $P \leq 0.05$, respectively

m-mentha-4,8-diene (monoterpene) which were not detected in WT (Fig. 4). The EI-MS chromatograms of caryophyllene, santalol and m-mentha-4,8-diene were shown in Online Supplementary Fig. 7. However, their contents could not be determined quantitatively because of the limitation of the volatile isoprenoids extracted by HS-SPME.

Discussion

The MEP pathway of isoprenoid biosynthesis plays important roles in many aspects of plant growth and development. Moreover, this plastidial pathway provides isoprenoid precursors for the biosynthesis of many secondary metabolites of medicinal and commercial significance. *Amomum villosum* is a valuable herb plant in South China, and its fruit is used in Chinese medicine, namely Fructus Amomi, which is used to treat digestive disorders effectively. Based on traditional theory in Chinese medicine, the medicinal ingredient of Fructus Amomi is the complex terpenoid compounds in its volatile oil but not separate monomers. Therefore, in order to improve the medicinal quality of *A. villosum* by metabolic engineering, manipulating upstream key-enzymes in isoprenoid biosynthesis is a favorable strategy. Monoterpenes comprise the major components of the volatile oil of *A. villosum* and monoterpenes are mainly synthesized via the MEP pathway [8, 41]. Therefore, we cloned and characterized the encoding genes of DXR and DXS from *A. villosum*, which are the upstream enzymes in the MEP pathway.

The *DXR* gene has been cloned and characterized from several medicinal plants, such as *G. biloba* (*Ginkgoaceae*) [18], *S. miltiorrhiza* (*Labiatae*) [19] and *C. acuminata* (*Nyssaceae*) [20]. Additionally, a conserved *DXR* fragment was also amplified from *Eupatorium adenophorum* (*Asteraceae*) recently [42]. However, to the best of our knowledge, there has been no research on the *DXR* gene as well as *DXS* gene in *A. villosum* or other *Zingiberaceae* plants until now. In this study, the *DXR* gene has been isolated from *A. villosum* successfully and designated as *AvDXR*. Its deduced amino acid sequence shares high homology with DXR superfamily. In addition, *AvDXS* has also been isolated and identified from *A. villosum*. Its deduced amino acid sequence also shares high homology with other plant DXSs and belongs to class 1 DXS. Both the deduced AvDXR and AvDXS contain a chloroplast transit peptide corresponding with the subcellular localization of MEP pathway. According to the result of bioinformatic analysis, *AvDXR* and *AvDXS* are deduced to encode functional proteins which have potential enzymatic functions as DXR and DXS, respectively.

The complementation of an *E. coli* mutant defective in DXR activity provided the proof of the enzymatic function of DXR from *A. thaliana* [12]. Similar complementation assay was performed to identify the function of DXS from *Solanum lycopersicum* [43]. In order to demonstrate the biological function of AvDXR and AvDXS, we used the color complementation method with genetically engineered *E. coli* containing pAC-BETA, which can synthesize *β*-carotene and be applied as model system [34–38]. The

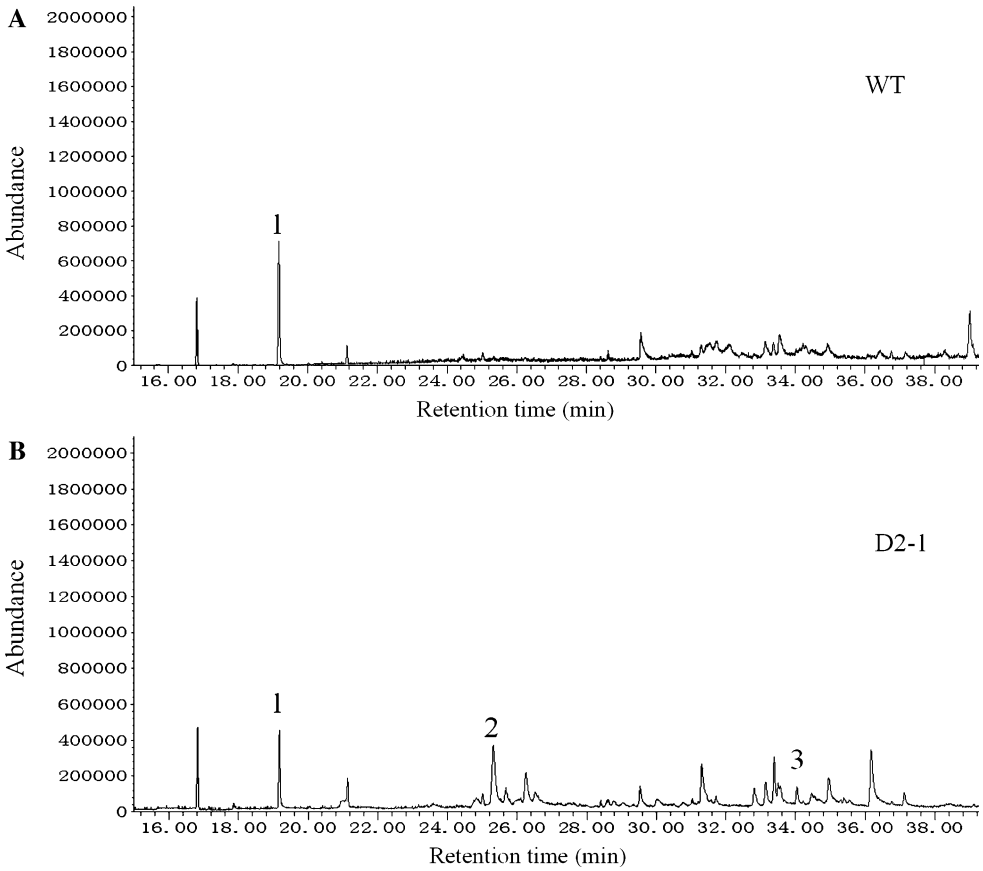

Fig. 4 GC-MS chromatograms of the volatiles in flowers of the transgenic plant D2–1 and wild-type control. **a** The GC-MS chromatogram of wild-type control. **b** The GC-MS chromatogram of transgenic plant D2–1. The numbers *1*, *2* and *3* above the peaks represent caryophyllene, santalol and m-mentha-4,8-diene, respectively

result of the functional color assay provided positive evidence indicating that the *AvDXR* and *AvDXS* encode functional proteins, which increased the accumulation of β-carotene via the MEP pathway in genetically engineered *E. coli*.

The fruit of *A. villosum* is the main part for the accumulation of volatile oil in this plant. The higher expression of *AvDXR* in the fruits indicated that *AvDXR* transcription was consistent with volatile oil accumulation and thus may function in monoterpene precursor biosynthesis, since the volatile oil of *A. villosum* is mainly composed of monoterpenes. However, as for *AvDXS*, its transcript level in pericarps or seeds is similar to other tissues lacking volatile oil. The two distantly related genes, *DXS1* and *DXS2*, encoding DXS in many plant species, are thought to be involved in the biosynthesis of different isoprenoids based on their different expression patterns [10]. For example, *DXS1* is expressed in many developing plant tissues except roots in *M. truncatula*, maize, tomato and tobacco, but *DXS2* is expressed strongly in the mycorrhizal-inducible roots accumulating carotenoids and the monoterpene-synthesizing gland cells of leaf trichomes [30]. Moreover, the *SlDXS1* is ubiquitously expressed in *S. lycopersicum*, whereas *SlDXS2* is expressed in only few tissues, and the *SlDXS2* down-regulation led to a decrease in the monoterpene beta-phellandrene in trichomes [44]. The *AvDXS* isolated in this study belongs to class 1 *DXS* and its expression is not related to the volatile oil accumulation, which suggests that it is not the main regulators for monoterpene biosynthesis in *A. villosum*. The role of *AvDXS* in other isoprenoid biosynthesis, especially the primary isoprenoid biosynthesis remains to be investigated.

In order to analyze the role of *AvDXR* involved in isoprenoid biosynthesis further, transgenic tobacco plants overexpressing *AvDXR* were generated. Overexpression of *AvDXR* in tobacco not only improved the DXR activity, but also increased the photosynthetic pigment content and volatile isoprenoid components. Moreover, the increase of photosynthetic pigment content was consistent with the *AvDXR* transcription level but not the DXR activity. The photosynthetic pigment content of D2–3 was highest among the three transgenic lines but its DXR activity was lowest. It is possible that the overexpression of *AvDXR* may affect the upstream or downstream genes thus change the flux of isoprenoids. It is interesting that overexpression of *AvDXR* increased both monoterpene and sesquiterpene components in the volatile of tobacco flowers, unlike the report about mint in which isoprenoid composition was not changed observably by altering the expression of DXR [8]. Since the DXR is the upstream enzyme in MEP pathway,

overexpression of *AvDXR* provide more IPP precursors in tobacco, consequently stimulate the downstream isoprenoid synthesis. The functional analysis of *AvDXR* in transgenic tobacco indicates that *AvDXR* really plays important role in isoprenoid biosynthesis.

In conclusion, we have successfully isolated and characterized the functional genes encoding DXR and DXS from *A. villosum* for the first time. Compared with *AvDXS*, *AvDXR* may be more valuable for manipulating monoterpene biosynthesis in *A. villosum* by metabolic engineering considering its expressional pattern in *A. villosum* fruits and functional identification in transgenic tobacco.

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