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Cloning, characterization, and expression of 1-deoxy-D-xylulose-5-phosphate reductoisomerase gene from *Dioscorea zingiberensis*

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Abstract

Diosgenin, mainly extracted from some *Dioscorea* species, is the most important starting material for the production of steroidal drugs. It is believed that diosgenin in *Dioscorea* is synthesized from isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP, the isomer of IPP) produced by the cytosolic mevalonate pathway. So far, the possibility of the plastidic 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway for biosynthesis of diosgenin remains unclear. The key enzyme in the MEP pathway is 1-deoxy-D-xylulose 5-phosphate-reductoisomerase (DXR, EC: 1.1.1.267). In this study, a DXR gene, named *DzDXR* (GenBank accession number KY131955), was isolated from *Dioscorea zingiberensis*. The *DzDXR* has an open reading frame of 1 413 bp encoding a protein of 470 amino acid residues. The function of *DzDXR* was verified by a colour enhancement assay in the *Escherichia coli* cells harbouring the plasmid pAC-BETA. Bioinformatic analyses revealed that *DzDXR* had a putative plastid transit peptide at the N-terminal region and was highly homologous to other plant DXRs, especially to those in monocotyledons. During the growth period of *D. zingiberensis*, the expression of *DzDXR* was found significantly high in leaves and very low in tubers, and the highest expression was observed in mature leaves in summer. In contrast to the *DzDXR* transcription, diosgenin was present predominantly in tubers and in minute quantities in leaves. Because diosgenin is very likely formed mainly in the cytosol of mature leaf cells of *Dioscorea* and the plastidic IPP and DMAPP produced by the MEP pathway can be transported into the cytosol, the consistently high expression of *DzDXR* detected in mature leaf of *D. zingiberensis* implies that the MEP pathway might play a significant role in diosgenin biosynthesis.

Additional key words: β -carotene, diosgenin biosynthesis, *Escherichia coli*, MEP pathway, plasmid pAC-BETA.

Introduction

Diosgenin has multiple biological activities and has been used for production of numerous steroidal drugs for more than half a century (Patel *et al.* 2012, Chen *et al.* 2015). Although diosgenin occurs in many plants, it is mainly obtained from some *Dioscorea* species. To date diosgenin biosynthetic pathway is not fully known. Isotope feeding studies have proved that cholesterol is the precursor of diosgenin in *Dioscorea* (Bennett *et al.* 1965, 1970, Joly

et al. 1969a,b, Stohs *et al.* 1969) and Dewick (2009) proposed a pathway from cholesterol to diosgenin. Cholesterol is one of phytosterols that exist ubiquitously in plants. Phytosterols are synthesized in the cytosol from isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) (Piironen *et al.* 2000, Suzuki and Muranaka 2007).

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Abbreviations: DMAPP - dimethylallyl diphosphate; DXP - 1-deoxy-d-xylulose 5-phosphate; DXR - 1-deoxy-d-xylulose 5-phosphate reductoisomerase; IPP - isopentenyl diphosphate; MEP - 2-C-methyl-D-erythritol 4-phosphate; ORF - open reading frame; qPCR - quantitative polymerase chain reaction; RACE - rapid amplification of cDNA ends.

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Plants have two independent pathways for production of IPP and DMAPP, the mevalonate pathway in cytoplasm and the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway in plastids. Traditionally, it is believed that these two pathways are strictly compartmentalized: isoprenoids such as phytosterols, triterpenes, and sesquiterpenes are from the cytosolic IPP and DMAPP, while other isoprenoids like isoprene, monoterpenes, diterpenes, tetraterpenes, carotenoids, phytols, tocopherols, and plastoquinone are from the plastidic IPP and DMAPP (Lichtenthaler 1999, Bick and Lange 2003, Hemmerlin *et al.* 2012, Vranová *et al.* 2013). Based on these findings, it is concluded that diosgenin is derived from the cytosolic IPP and DMAPP. However, the crossflow between the two pathways has been reported in some plants like *Antirrhinum majus* (Dudareva *et al.* 2005), *Arabidopsis thaliana* (Laule *et al.* 2003), *Catharanthus roseus* (Schuhr *et al.* 2003), *Coluria geoides* (Skorupinska-Tudek *et al.*

2008), *Salvia miltiorrhiza* (Yang *et al.* 2012a), tobacco (Hemmerlin *et al.* 2003), and tomato (Gutensohn *et al.* 2013). Moreover, it has been found that the transport of isoprenoid precursors from the plastids to the cytosol is more effective (Bick and Lange 2003, Schuhr *et al.* 2003). So far, whether the MEP pathway is involved in diosgenin biosynthesis in *Dioscorea* is unclear. Clarifying this issue is not only scientifically interesting but also important for breeding elite cultivars of *Dioscorea*. Cloning the MEP pathway genes of *Dioscorea* will help us to investigate this issue using molecular techniques. 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR; EC 1.1.1.267) catalyzes the formation of MEP from 1-deoxy-D-xylulose 5-phosphate (DXP), a committed step in the MEP pathway (Carretero-Paulet *et al.* 2002, Hasunuma *et al.* 2008). In this paper, we report cloning, characterization, and expression analysis of a *DXR* gene from *D. zingiberensis*, which is cultivated in China for diosgenin production.

Materials and methods

Dioscorea zingiberensis C.H. Wright plants were collected in Shiyan, Hubei Province, China, and grown in the field at Hubei University, Wuhan, China. Total RNA was extracted from the young leaves using *TRIzol* reagents (Invitrogen, Carlsbad, CA, USA) and then reverse transcribed into cDNA using *M-MLV RTase* cDNA synthesis kit (*TaKaRa*, Dalian, China) according to the user's manual.

Cloning of full length cDNA of *D. zingiberensis* DXR gene (*DzDXR*): Degenerate oligonucleotide primers DXR-F and DXR-R (Table 1 Suppl.) were designed according to the conserved regions of known plant DXRs to amplify a partial cDNA fragment of *D. zingiberensis* DXR gene. Degenerate PCR was conducted in a volume of 20 mm³ containing 14 mm³ of ddH₂O, 2 mm³ 10× *TransTaq HiFi II* (*TranGen Biotech Co.*, Beijing, China) buffer, 2 mm³ of 10× *GC* enhancer, 0.4 mm³ of 10 mM dNTP mix, 0.4 mm³ cDNA, 0.4 mm³ of 10 μM DXR-F, 0.4 mm³ of 10 μM DXR-R, and 0.4 mm³ of *TransTaq HiFi* DNA polymerase under the following conditions: 94 °C for 3 min, then 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min, and a final extension at 72 °C for 10 min. Based on the partial cDNA sequence, primers DXR-GSP1 and DXR-GSP2 (Table 1 Suppl.) were synthesized to clone the 5' and 3' ends of the cDNA fragment by rapid amplification of cDNA ends (RACE), respectively. The RACE PCR was performed using *SMART^{TM}* RACE cDNA amplification kit (*Clontech*, Mountain View, CA, USA) following the manufacturer's instructions. The full-length cDNA of *D. zingiberensis* DXR gene, designated *DzDXR*, was obtained by assembling the partial cDNA fragment and the 5' and 3' end sequences *via ContigExpress* software. The intact open reading frame (ORF) of *DzDXR*,

named *DzDXR*-ORF, was amplified with primers DZR(ORF)-F and DXR(ORF)-R (Table 1 Suppl.) using a program of pre-denaturation at 94 °C for 3 min, followed by 35 cycles of at 94 °C for 30 s, at 59 °C for 30 s, and at 72 °C for 90 s, and an extension of at 72 °C for 8 min.

The above amplification products, *e.g.*, the partial cDNA fragment, 5' and 3' end cDNA, and *DzDXR*-ORF, were inserted into pMD18-T vector (*Promega*, Madison, WI, USA) and transformed into competent *Escherichia coli* (strain TOP10). In each transformation, at least five positive recombinant clones were sequenced.

Bioinformative and functional analysis of *DzDXR*: The ORF of *DzDXR* was identified and the amino acid sequence was deduced by *ORF Finder* on NCBI (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). Multiple sequence alignment of DXRs was conducted using the *DNAMAN* program. The presence of signal peptides was predicted by using the *TargetP 1.1* server (<http://www.cbs.dtu.dk/services/TargetP-1.1>) and the *ChloroP 1.1* server (<http://www.cbs.dtu.dk/services/ChloroP/>).

Plasmids pAC-BETA and pTrcAtIPI were used to test the function of *DzDXR*. The pAC-BETA contains a chloramphenicol resistance gene and four functional genes necessary for β-carotene biosynthesis, *crtE*, *crtB*, *crtL*, and *crtY*. The pTrcAtIPI contains an ampicillin resistance gene and an *AtIPI* gene.

After the vector containing *DzDXR*-ORF was digested with *Pst* I and *Not* I *DzDXR*-ORF was recovered and subcloned into the plasmid pTrcAtIPI that was digested by the same enzymes to remove the insert *AtIPI*, creating a recombinant plasmid pTrc-DzDXR. The pTrcAtIPI was also digested by *Pst* I to remove *AtIPI* and then self-ligated by T4 DNA ligase to yield a control vector pTrc. Both pTrc-DzDXR and pAC-BETA were co-transformed into

competent *E. coli* (strain TOP10F'). The *E. coli* TOP10F' cells were also transformed with plasmid pTrc alone or together with plasmid pAC-BETA and used as controls. The transformants containing both pTrc-DzDXR and pAC-BETA or both pTrc and pAC-BETA were cultured on solid Lurie and Broth (LB) medium supplemented with 50 mg dm⁻³ chloramphenicol and 100 mg dm⁻³ ampicillin, and the transformants containing pTrc were cultured on solid LB medium with 100 mg dm⁻³ ampicillin at 37 °C. Single colonies of the above transformants and wild type *E. coli* TOP10F' cells were cultured in 2 cm³ liquid LB medium at 37 °C for 1 d. These cultures were streaked on an antibiotic free LB agar plate and incubated at 37 °C for 1 d and at 28 °C for 2 d. The function of *DzDXR* was evaluated based on the colour of the transformants.

Expression analysis of *DzDXR* in *D. zingiberensis*: *DzDXR* transcriptions in *D. zingiberensis* were analyzed using real-time quantitative (q)PCR. The young leaves, mature leaves, newly formed tubers (young tubers), and old tubers were collected from three field-grown plants in the middle of April, July, and September 2017. Total RNA was extracted and cDNAs were synthesized as mentioned above. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as an internal control. The primer pair *DzDXR*(RT)-F and *DzDXR*(RT)-R and the primer pair *GAPDH*-F and *GAPDH*-R (Table 1 Suppl.) were used to amplify the transcripts of *DzDXR* and *GAPDH*, respectively. The PCR reactions were performed using *SYBR Premix ExTaq*^{TM} (*Tli RNaseH Plus*) (*Takara*) on the *Bio-Rad CFX96*^{TM} real-time PCR system. The conditions were: 95 °C for 3 min, 40 cycles of 95 °C for 15 s and 60 °C for 30 s, and a final dissociation stage at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. Triplets of all the samples were run, and the specificity of each

primer pair was validated by a dissociation curve. The Pfaffl (2001) method was used to calculate the relative expression of *DzDXR*. Because young tubers in April and young leaves in September were not available, data on *DzDXR* expression in these tissues were absent.

Diosgenin determination: Leaves and tubers were harvested from three plants in late October. Old and young tubers were separated and sliced. All the samples were dried at 60 °C and then grounded to powder. Diosgenin extraction was done as described by Mou *et al.* (2015) with some modifications. Briefly, about 100 mg of the powder was added into a 50 cm³ flask containing 20 cm³ of 1 M sulfuric acid and kept at 125 °C for 3 h for hydrolysis. The hydrolysate was filtered and washed with distilled water until it became neutral. The hydrolysate together with the filter paper was extracted three times with petroleum ether and dried under vacuum on a rotary evaporator. The petroleum ether extract was dissolved in 2 cm³ methanol and filtered before subjected to analysis. Reverse-phase high performance liquid chromatography (HPLC) was employed for quantitative determination of diosgenin. The chromatographic analysis was performed on an *Agilent 1100* HPLC system (*Agilent Technologies*, Palo Alto, CA, USA) using water + methanol (10:90, v/v) as the mobile phase with a flow rate of 1 cm³ min⁻¹ and detected at 210 nm (Huang *et al.* 2008). A calibration curve was generated with diosgenin standard (*Sigma-Aldrich*, St. Louis, USA) and diosgenin content was calculated by the method of external standards.

Data analysis: Data on *DzDXR* transcription and diosgenin content were statistically analyzed by *ANOVA* and Tukey's test at 1 % significance using *IBM SPSS* program.

Results

A specific, 773-bp cDNA fragment was amplified by degenerate PCR. Then, a 5' end of 807 bp and a 3' end of 956 bp were obtained by RACE PCR. Finally, a full length cDNA of 1 643 bp was generated by assembling the partial cDNA fragment and its 5' and 3' end sequences, which was designated *DzDXR* (GenBank accession number KY131955). The *DzDXR* has an ORF of 1 413 bp, a 5' untranslated region (UTR) of 42 bp and a 3' UTR of 188 bp. The intact ORF of *DzDXR* was obtained by PCR. Sequencing results of all the PCR products indicated that only one *DXR* gene was identified.

DzDXR encodes a polypeptide of 470 amino acid residues. *BLAST* analysis of *DzDXR* at the *NCBI* server revealed that *DzDXR* shared a high sequence similarity with other plant *DXRs*, especially with those of monocotyledons, for examples, 87 % identity to *Lilium longiflorum* *DXR* (AHJ57307) and 86 % identity to

Narcissus tazetta (ADD82536), *Oryza sativa* (AAL37560), *Sorghum bicolor* (XP_021311303), and *Zea mays* (XP_008655547) *DXRs*. Multiple sequence alignment of *DzDXR*, *E. coli* *DXR*, and other plant *DXRs* is shown in Fig. 1 Suppl.. Like other plant *DXRs*, *DzDXR* has an extension at the N-terminal side that is not present in the *E. coli* *DXR*. The extension is poorly conserved but enriched in Ser residues, which is thought as the typical features of plastid transit peptides (Heijne *et al.* 1989), and contains a Pro rich region defined as P(P/Q)PAWPG(R/T)A motif (Carretero-Paulet *et al.* 2002). Data analysis with both the *TargetP 1.1* program and the *ChloroP* program predicted that *DzDXR* has a plastid localization signal in the first 46 amino acids. Based on the predicted value, the putative cleavage site of *DzDXR* (Fig. 1 Suppl.) was at the Cys-Ser-Met motif, which is consistent with the prediction of Carretero-Paulet

et al. (2002) that the processing site of plant DXRs is at the N terminus of a conserved Cys-Ser-X motif (X is any of the hydrophobic residues Ala, Val, or Met). The two conserved NADPH binding motifs [GSTGS(I/V)GT and LAAGSN(V/I)] and the two conserved substrate (DXP) binding motifs (LPADSEHSAI and NKGLEVIEAHY) found in other DXRs were also present in *DzDXR* (Fig. 1 Suppl.).

IPP and DMAPP are also the precursors for carotenoids. *E. coli* has the MEP pathway producing IPP and DMAPP, but cannot synthesize carotenoids due to lack of other genes necessary for carotenoid biosynthesis. After the plasmid pAC-BETA is introduced into *E. coli*, the transformed cells can produce β -carotene and have a yellow colour (Cunningham *et al.* 1996, Cao *et al.* 2010). If *DzDXR* encodes a functional DXR, the *E. coli* cells harbouring both pAC-BETA and pTrc-*DzDXR* can produce more IPP and DMAPP and then more β -carotene, and therefore should be yellower than those bearing pAC-BETA or both pAC-BETA and pTrc. As expected, the cells co-transformed with pAC-BETA and pTrc-*DzDXR* were obviously yellower than the control cells containing both pAC-BETA and pTrc (Fig. 1A,C), demonstrating that *DzDXR* is a functional gene. Like the wild type cells, the cells transformed with the vector pTrc were white as they were unable to synthesize carotenoids (Fig. 1B,D).

The qPCR analysis of *DzDXR* transcripts showed that during the growth period of *D. zingiberensis*, *DzDXR*

expressions had a similar expression patterns in different organs (Fig. 2A). Regardless of seasons, *DzDXR* was expressed highly in mature leaves, moderately in young leaf, and lowly in tubers. The expression of *DzDXR* in mature leaf was much higher in July than in April and September, indicating that *DzDXR* expression in leaves was related to the seasons. Unlike *DzDXR* transcription, most of diosgenin was present in tubers, especially in old tuber (Fig. 2B). The average content of diosgenin in the old tubers, young tubers, and mature leaves were 67.4, 51.3, and 8.6 mg g⁻¹(d.m.), respectively.

Fig. 1. Functional demonstration of *DzDXR* in *E. coli* cells. A - transformants containing pAC-BETA and pTrc-*DzDXR*; B - wild type cells; C - transformants containing pAC-BETA and pTrc; D - transformants containing only pTrc.

Fig. 2. Expression of *DzDXR* (A) and diosgenin content (B) in different organs of *D. zingiberensis*. Data on *DzDXR* expression in young tuber in April and in young leaf in October were absent due to that the tissues were not available. Means $\pm$ SEs, $n = 3$, different letters indicate significant differences at $P < 0.01$.

Discussion

Some *Dioscorea* species produce significant quantities of diosgenin. For instances, diosgenin content in tubers of

D. composita and *D. zingiberensis* are usually over 3 %, even over 10 % (Ding *et al.* 1981, Datta and Datta 1984,

Hua *et al.* 1989). The existence of large amounts of diosgenin in these plants implies that they can efficiently produce IPP and DAMPP for diosgenin biosynthesis.

A positive correlation between *DXR* gene expression and production of isoprenoids has been observed in many species, such as *Amomum villosum* (Yang *et al.* 2012b), *A. thaliana* (Carretero-Paulet *et al.* 2002), *Catharanthus roseus* (Veau *et al.* 2000), *Medicago truncatula* (Bede *et al.* 2006), *Populus × canescens* (Mayrhofer *et al.* 2005), rose (Wang and Yao 2012), *Salvia miltiorrhiza* (Yang *et al.* 2012a), and transgenic peppermint (Mahmoud and Croteau 2001) and tobacco (Hasunuma *et al.* 2008, Zhang *et al.* 2015). In present study, it was found that the expressions of *DzDXR* did not match the amounts of diosgenin in the leaves and tubers of *D. zingiberensis* at all. But this does not mean that the MEP pathway is not involved in diosgenin biosynthesis in *Dioscorea* because secondary metabolites (intermediates and end products) can be transported from the synthesis sites to the other sites (Nagata *et al.* 2002, Burlat *et al.* 2004, Li and Hu 2009). Bennett *et al.* (1963) found that the shoot of *D. spiculiflora* was the major site of synthesis of phytosterols and sapogenins and believed that they were rapidly translocated to the tuber. Barker *et al.* (1966) also indicated that diosgenin was formed in the shoots of *D. deltoidea* and *D. sylvatica* and transported to the tubers. In fact, almost all of diosgenin in *Dioscorea* occurs in glycoside

form named dioscin, which undoubtedly can facilitate its movement. Recently, it was reported that the expression of the genes encoding squalene synthase (a crucial enzyme in biosynthesis of phytosterols) and 3-*O*-sterol glycosyltransferase (an enzyme catalyzing the first glycosylation step of diosgenin during dioscin biosynthesis) and the content of the first glycosylation product (diosgenin-3-*O*-glucoside) were much higher in the mature leaves than in the tubers of *D. zingiberensis*. These data strongly suggest that diosgenin was synthesized mainly in mature leaves of *D. zingiberensis*. Our results showed that during the growth period, *DzDXR* was expressed consistently and significantly in mature leaves. This observation suggested that the MEP pathway was active in mature leaves of *D. zingiberensis* and significant amounts of IPP and DMAPP were formed there. Since transport of IPP and DMAPP produced by the MEP pathway from the plastids into the cytosol can occur in some plants, it was assumed that the MEP pathway might play an important role in the biosynthesis of diosgenin in *D. zingiberensis*. A further study on this assumption is necessary. One of strategies could be to produce transgenic *D. zingiberensis* plants over- and under-expressing *DzDXR* and to analyze the relationship between *DzDXR* expressions and diosgenin yields in transgenic and non-transgenic plants.

References

- Barker, E.A., Riartin, J.T., Wilson, A.P.: The distribution of diosgenin in *Dioscorea* spp. - *Ann. appl. Biol.* **58**: 203-211, 1966.
- Bede, J.C., Musser, R.O., Felton, G.W., Korth, K.L.: Caterpillar herbivory and salivary enzymes decrease transcript levels of *Medicago truncatula* genes encoding early enzymes in terpenoid biosynthesis. - *Plant mol. Biol.* **60**: 519-531, 2006.
- Bennett, R.D., Heftmann, E.: Biosynthesis of *Dioscorea* sapogenins from cholesterol. - *Phytochemistry* **4**: 577-586, 1965.
- Bennett, R.D., Heftmann, E., Joly, R.A.: Biosynthesis of diosgenin from 26-hydroxycholesterol in *Dioscorea floribunda*. - *Phytochemistry* **9**: 349-353, 1970.
- Bennett, R.D., Heftmann, E., Preston, W.H., Jr., Haun, J.R.: Biosynthesis of sterols and sapogenins in *Dioscorea spiculiflora*. - *Arch. Biochem. Biophys.* **103**: 74-83, 1963.
- Bick, J.A., Lange, B.M.: Metabolic cross talk between cytosolic and plastidial pathways of isoprenoid biosynthesis: unidirectional transport of intermediates across the chloroplast envelope membrane. - *Arch. Biochem. Biophys.* **415**: 146-154, 2003.
- Burlat, V., Oudin, A., Courtois, M., Rideau, M., St-Pierre, B.: 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, 2004.
- Cao, X., Zong, Z., Ju, X., Sun, Y., Dai, C., Liu, Q., Jiang, J.: Molecular cloning, characterization and function analysis of the gene encoding HMG-CoA reductase from *Euphorbia pekinensis* Rupr. - *Mol. Biol. Rep.* **37**: 1559-1567, 2010.
- Carretero-Paulet, L., Ahumada, I., Cunillera, N., Rodríguez-Concepción, M., Ferrer, A., Boronat, A., Campos, N.: Expression and molecular analysis of the *Arabidopsis* *DXR* gene encoding 1-deoxy-D-xylulose-5-phosphate reductoisomerase, the first committed enzyme of the 2-C-methyl-D-erythritol 4-phosphate pathway. - *Plant Physiol.* **129**: 1581-1591, 2002.
- Chen, Y., Tang, Y.M., Yu, S.L., Han, Y.W., Kou, J.P., Liu, B.L., Yu, B.Y.: Advances in the pharmacological activities and mechanisms of diosgenin. - *Chin. J. Nat. Med.* **13**: 578-587, 2015.
- Cunningham, F.X., Pogson, B., Sun, Z., McDonald, K.A., DellaPenna, D., Gantt, E.: Functional analysis of β and ϵ lycopene cyclase enzymes of *Arabidopsis* reveals a mechanism for control of cyclic carotenoid formation. - *Plant Cell* **8**: 1613-1626, 1996.
- Datta, S.K., Datta, K.: Chemodifferentiation of diosgenin in *Dioscorea composite*. - *Phytochemistry* **33**: 2684-2685, 1984.
- Dewick, P.M. (ed): Medicinal Natural Products: A Biosynthetic Approach (3rd Ed.). - John Wiley & Sons, Chichester 2009.
- Ding, Z., Zhou, L., Wang, Y., Tang, S.: Factors influencing diosgenin content of *Dioscorea zingiberensis*. - *Chin. traditional herbal Drugs* **12**: 34-35, 1981.

- Dudareva, N., Andersson, S., Orlova, I., Gatto, N., Reichelt, M., Rhodes, D., Boland, W., Gershenson, J.: The nonmevalonate pathway supports both monoterpene and sesquiterpene formation in snapdragon flowers. - *Proc. nat. Acad. Sci. USA* **102**: 933-938, 2005.
- Gutensohn, M., Orlova, I., Nguyen, T.T., Davidovich-Rikanati, R., Ferruzzi, M.G., Sitrit, Y., Lewinsohn, E., Pichersky, E., Dudareva, N.: Cytosolic monoterpene biosynthesis is supported by plastid-generated geranyl diphosphate substrate in transgenic tomato fruits. - *Plant J.* **75**: 351-363, 2013.
- Hasunuma, T., Takeno, S., Hayashi, S., Sendai, M., Bamba, T., Yoshimura, S., Tomizawa, K., Fukusaki, E., Miyake, C.: Overexpression of 1-deoxy-D-xylulose-5-phosphate reductoisomerase gene in chloroplast contributes to increment of isoprenoid production. - *J. Biosci. Bioeng.* **105**: 518-526, 2008.
- Heijne, V. G., Stepphuhn, J., Herrmann, R.G.: Domain structure of mitochondrial and chloroplast targeting peptides. - *Eur. J. Biochem.* **180**: 535-545, 1989.
- Hemmerlin, A., Harwood, J.L., Bach, T.J.: A raison d'être for two distinct pathways in the early steps of plant isoprenoid biosynthesis? - *Proc. Lipid Res.* **51**: 95-148, 2012.
- Hemmerlin, A., Hoeffler, J.F., Meyer, O., Tritsch, D., Kagan, I.A., Grosdemange-Billiard, C., Rohmer, M., Bach, T.J.: Cross-talk between the cytosolic mevalonate and the plastidial methylerythritol phosphate pathways in tobacco bright yellow-2 cells. - *J. biol. Chem.* **278**: 26666-26676, 2003.
- Hua, Z.P., Ding, Z.Z., He, S.A., Sheng, C.G.: Research on correlations between climatic factors and diosgenin content in *Dioscorea zingiberensis*. - *Acta pharm. sin.* **24**: 702-706, 1989.
- Huang, W., Zhao, H., Ni, J., Zuo, H., Qiu, L., Li, H., Li, H.: The best utilization of *D. zingiberensis* C.H. Wright by an eco-friendly process. - *Bioresour. Tech.* **99**: 7407-7411, 2008.
- Joly, R.A., Bonner, J., Benett, R.D., Heftmann, E.: Conversion of cholesterol to an open-chain saponin by *Dioscorea floribunda*. - *Phytochemistry* **8**: 857-859, 1969a.
- Joly, R.A., Bonner, J., Benett, R.D., Heftmann, E.: The biosynthesis of steroidal saponins in *Dioscorea floribunda* from doubly labelled cholesterol. - *Phytochemistry* **8**: 1709-1711, 1969b.
- Laule, O., Furholz, A., Chang, H.S., Zhu, T., Wang, X., Heifetz, P.B., Gruissem, W., Lange, M.: Crosstalk between cytosolic and plastidial pathways of isoprenoid biosynthesis in *Arabidopsis thaliana*. - *Proc. nat. Acad. Sci. USA* **100**: 6866-6871, 2003.
- Li, J., Hu, Z.: Accumulation and dynamic trends of triterpenoid saponins in vegetative organs of *Achyranthus bidentata*. - *J. integr. Plant. Biol.* **51**: 122-129, 2009.
- Lichtenthaler, H.K.: Pathway of isoprenoid biosynthesis in plants. - *Annu. Rev. Plant Physiol. Plant mol. Biol.* **50**: 47-65, 1999.
- Mahmoud, S.S., Croteau, R.B.: Metabolic engineering of essential oil yield and composition in mint by altering expression of deoxyxylulose phosphate reductoisomerase and menthofuran synthase. - *Proc. nat. Acad. Sci. USA* **98**: 891-892, 2001.
- Mayrhofer, S., Teuber, M., Zimmer, I., Louis, S., Fischbach, R.J., Schnitzler, J.P.: Diurnal and seasonal variation of isoprene biosynthesis-related genes in grey poplar leaves. - *Plant Physiol.* **139**: 474-484, 2005.
- Mou, Y., Zhou, K., Xu, D., Yu, R., Li, J., Yin, C., Zhou, L.: Enhancement of diosgenin production in plantlet and cell cultures of *Dioscorea zingiberensis* by palmarumycin C₁₃ from the endophytic fungus, *Berkleasmium* sp. Dzf12. - *Trop. J. Pharm. Res.* **14**: 241-248, 2015.
- Nagata, N., Suzuki, M., Yoshida, S. and Muranaka, T.: Mevalonic acid partially restores chloroplast and etioplast development in *Arabidopsis* lacking the non-mevalonate pathway. - *Planta* **216**: 345-350, 2002.
- Patel, K., Gadewar, M., Tahilyani, V., Patel, D.K.: A review on pharmacological and analytical aspects of diosgenin: a concise report. - *Nat. Prod. Bioprospect.* **2**: 46-52, 2012.
- Pfaffl, M.W.: A new mathematical model for relative quantification in real-time RT-PCR. - *Nucl. Acids Res.* **29**: 2002-2007, 2001.
- Piironen, V., Lindsay, D.G., Miettinen, T.A., Toivo, J., Lampi, A.M.: Plant sterols: biosynthesis, biological function and their importance to human nutrition. - *J. Sci. Food. Agr.* **80**: 939-966, 2000.
- Schuh, C.A., Radykewicz, T., Sagner, S., Latzel, C., Zenk, M.H., Arigoni, D., Bacher, A., Rohdich, F., Eisenreich, W.: Quantitative assessment of crosstalk between the two isoprenoid biosynthesis pathways in plants by NMR spectroscopy. - *Phytochem. Rev.* **2**: 3-16, 2003.
- Skorupinska-Tudek, K., Poznanski, J., Wojcik, J., Bienkowski, T., Szostkiewicz, I., Zelman-Femiak, M., Bajda, A., Chojnacki, T., Olszowska, O., Grunler, J., Meyer, O., Rohmer, M., Danikiewicz, W., Swiezewska, E.: Contribution of the mevalonate and methylerythritol phosphate pathways to the biosynthesis of dolichols in plants. - *J. biol. Chem.* **283**: 21024-21035, 2008.
- Stohs, S.J., Kaul, B., Staba, E.J.: The metabolism of ¹⁴C-cholesterol by *Dioscorea deltoidea* suspension cultures. - *Phytochemistry* **8**: 1679-1686, 1969.
- Suzuki, M., Muranaka, T.: Molecular genetics of plant sterol backbone synthesis. - *Lipids* **42**: 47-54, 2007.
- Veau, B., Courtois, M., Oudin, A., Chénieux, J.-C., Rideau, M., Clastre, M.: Cloning and expression of cDNAs encoding two enzymes of the MEP pathway in *Catharanthus roseus*. - *Biochim. biophys. Acta* **1517**: 159-163, 2000.
- Vranová, E., Coman, D., Gruissem, W.: Network analysis of the MVA and MEP pathways for isoprenoid synthesis. - *Annu. Rev. Plant Biol.* **64**: 665-700, 2013.
- Wang, H., Yao, L.: Cloning and expression profile of 1-deoxy-D-xylulose 5-phosphate reductoisomerase gene from an oil-bearing rose. - *Russ. J. Plant Physiol.* **61**: 548-455, 2014.
- Yang, D., Du, X., Liang, X., Han, R., Liang, Z., Liu, Y., Liu, F., Zhao, J.: Different roles of the mevalonate and methylerythritol phosphate pathways in cell growth and tanshinone production of *Salvia miltiorrhiza* hairy roots. - *PLoS ONE* **7**: e46797, 2012a.
- Yang, J., Adhikari, M.N., Liu, H., Xu, H., He, G., Zhan, R., Wei, J., Chen, W.: 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. - *Mol. Biol. Rep.* **39**: 8287-8296, 2012b.
- Zhang, H., Niu, D., Wang, J., Zhang, S., Yang, Y., Jia H, Cui, H.: Engineering a platform for photosynthetic pigment, hormone and cembrane-related diterpenoid production in *Nicotiana tabacum*. *Plant Cell Physiol.* **56**: 2125-2138, 2015.