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BRIEF COMMUNICATION

Spatial and developmental expression of key genes of terpene biosynthesis in *Tanacetum parthenium*

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Abstract

Feverfew (*Tanacetum parthenium*) is a medicinal plant belonging to the *Asteraceae* family. To improve understanding terpene metabolism in feverfew, the relative gene expression of four key genes coding 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*HMGR*) and germacrene A synthase (*GAS*) from the mevalonic acid pathway (MVA), as well as 1-deoxy-D-xylulose-5-phosphate reductoisomerase (*DXR*) and hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase (*HDR*) from the methyl erythritol phosphate pathway (MEP), were examined. Target organs and tissues included young leaves (not fully expanded), mature leaves (fully expanded), flowers, stems, roots, and glandular trichomes. *HMGR*, *DXR*, and *HDR* were isolated and sequenced for the first time in feverfew. Real-time quantitative PCR analysis revealed differential expression of these genes in feverfew tissues and developmental stages.

Additional key words: feverfew, mevalonic acid pathway, methylerythritol phosphate pathway, real time PCR.

Feverfew (*Tanacetum parthenium*) belongs to the *Asteraceae* family. It contains diverse terpenes including pharmaceutically active compounds, such as parthenolide or costunolide (Palevitch *et al*. 1997, Liu *et al.* 2011, Majdi *et al.* 2013). The highest amount of parthenolide is in glandular trichomes, flowers, leaves and stems, whereas roots do not contain parthenolide (Majdi *et al.* 2011). In plants, the methylerythritol phosphate (MEP) pathway in plastids and the mevalonic acid (MVA) pathway in cytosol are responsible for terpene biosynthesis (Rohmer *et al.* 1993, Zeng *et al*. 2011). Both pathways produce the universal terpene precursors, isopentenyl diphosphate (IDP) and its allylic isomer, dimethylallyl diphosphate (DMADP). The cytosolic MVA pathway begins by the condensation of three acetyl-CoA to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) (Chappell 1995). The plastidic MEP pathway starts by the condensation of pyruvate and glyceraldehyde-3-phosphate to yield 1-deoxy-D-xylulose-5-phosphate (DXP) (Rohmer *et al.* 1993). Generally, cytosolic IDP/DMAPP serve as precursors for farnesyl diphosphate (FDP, C_{15}) which is converted into sesquiterpenes (C_{15}), triterpenes (C_{30}), and homoterpenes (C_{11}, C_{30}), whereas plastidic IDP/DMAPP serve as precursors for geranyl diphosphate (GDP, C_{10}) and geranylgeranyl diphosphate (GGDP, C_{20}), which yield monoterpenes (C_{10}, from GDP), and diterpenes (C_{20}), tetraterpenes (C_{40}), and prenyl chains (C_{45}) from GGDP (Dudareva *et al.* 2005).

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Abbreviations: DMADP - dimethylallyl diphosphate; DXP - 1-deoxy-D-xylulose-5-phosphate; DXR - 1-deoxy-D-xylulose-5-phosphate reductoisomerase; FDP - farnesyl diphosphate; GAS - germacrene A synthase; HDR - hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase; HMGR - 3-hydroxy-3-methylglutaryl-coenzyme A reductase; IDP - isopentenyl diphosphate; MEP - methylerythritol phosphate; MVA - mevalonic acid; ORF - open reading frame; RACE - rapid amplification of cDNA ends; RGE - relative gene expression.
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In the MVA pathway, HMGR (3-hydroxy-3-methyl-glutaryl-coenzyme A reductase) is the primary rate limiting enzyme. It catalyses the conversion of HMG-CoA to MVA (Goldstein and Brown 1990). The first committed step in the biosynthesis of sesquiterpenes is catalyzed by sesquiterpene synthases which convert linear FDP to various circular sesquiterpenes (Chappell 1995, Irmisch *et al.* 2012).The first committed step in parthenolide biosynthesis is catalysed by germacreneA synthase (GAS) (Majdi *et al.* 2011).

The first committed step in the MEP pathway is catalyzed by 1-deoxy-D-xylulose-5-phosphate reducto-isomerase (DXR) which irreversibly converts DXP to 2-C-methyl-D-erythritol-4-phosphate (MEP) (Takahashi *et al.* 1998).The conversion of 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate (HMBDP) into IDP and DMADP is mediated by 4-hydroxy-3-methylbut-2-enyl-diphosphate reductase (HDR) which is the last step of the MEP pathway (Cordoba *et al*. 2009, Huang *et al.* 2009). A rate-limiting role for HDR has been reported in the biosynthesis of MEP-derived terpenes (Hsieh and Goodman 2005).

Two genes encoding the key enzymes of parthenolide synthesis by the MVA pathway (*HMGR* and *GAS*), and two genes encoding the key enzymes of the MEP pathway (*DXR* and *HDR*) were chosen for our study focused on examining terpenoid production in feverfew. *TpGAS* has been isolated and characterized from feverfew recently (Majdi *et al.* 2011), whereas *HMGR*, *DXR*, and *HDR* genes have not yet been isolated from feverfew. The objective of the present study was to isolate the latter three genes and to examine the expression patterns of all four genes in feverfew tissues, as well as to study their developmental regulation in immature and mature leaves. To this aim, a homology-based PCR cloning strategy was employed for gene isolation followed by quantitative real-time qPCR analysis to survey their expression patterns.

Feverfew (*Tanacetum parthenium* L. Schulz-Bip.) (2*n*=2*x*=18) seeds were obtained from the *Zardband Company*, Tehran, Iran. Plants were grown in a growth chamber with a 16-h photoperiod, a photosynthetic photon flux density of 320 µmol m^{-2} s^{-1} and day/night temperatures of 24/18 °C. A total RNA extraction and cDNA synthesis was carried out as described by Majdi *et al*. (2011). Sequence alignments of published cDNAs from the NCBI database (http://www.ncbi.nlm.nih.gov/) were used to design primers for gene isolation and specific primer pairs were designed based on the conserved regions of cDNAs (Table 1). The amplified fragments were cloned into a *pGEM-T Easy* vector (*Promega*, Madison, USA) and sequenced. 5'-RACE and 3'-RACE were performed using the *SMART-RACE* cDNA amplification kit (*Clontech*, USA). A qPCR was carried out for gene expression analysis with the *iCycler iQ5* system (*BioRad*, Hercules, USA) using the *iQ™ SYBR® Green Supermix* master mix (*BioRad*) in three biological and two technical replicates. *GAPDH* was used as a housekeeping gene for normalization. The ΔCt was calculated according to Livak and Schmittgen (2001). A two step program was used as follows: annealing at 95 °C for 3 min; 40 cycles at 95 °C for 10 s, and a final extension at 55 °C for 30 s. The primer pairs were designed for qPCR analysis using the *Beacon designer* software (*BioRad*, Table 1). Sequences were compared by *BLAST* search in GenBank (http://www.ncbi.nih.gov). Phylogenetic tree analysis of deduced proteins was performed with *PAUP 4.0b10* (Swofford 2003) using the maximum parsimony (MP) method with validation by bootstrapping. Analysis of variance for relative gene expression (RGE) in different tissues was carried out based on a completely randomized design with 3 replications. Mean comparisons were conducted with the Duncan's test using the *SPSS v. 18* software (*SPSS*, Chicago, USA).

The 3' and 5' rapid amplification of cDNA ends (RACE) led to the full length isolation of *TpHMGR* (Gene Bank Accession No. JN005887.1), *TpDXR* (Gene Bank Accession No. AER00470), and *TpHDR* (Gene Bank Accession No. JN005889). A *BLAST* search in GenBank revealed that *TpHMGR, TpDXR*, and *TpHDR* had the highest similarity with their orthologues from *Artemisia annua*, another closely related plant species from the *Asteraceae*, with 91 % (*HMGR*), 98 % (*DXR*), and 93 % (*HDR*) similarity (data not shown). Phylogenetic analysis of each deduced protein with corresponding proteins from other plant species using *PAUP 4.0b10* showed that *Tp*HMGR, *Tp*DXR, and *Tp*HDR also shared the highest similarity with their *A. annua* homologues (Fig. 1*A*-*C*). Deduced proteins including HMGR, DXR, and HDR from *T. parthenium* and *A. annua* were in the same cluster and had the

Table 1. Primer pairs used to clone *HMGR*, *DXR*, and *IDS* in *T. parthenium*, and nucleotide sequences of the primers used in qPCR.

	Gene	Forward 5'- 3'	Reverse 5'- 3'
Cloning	*HMGR*	AATGAACATGGTGTCAAAGGG	CACCAGCCAACACCGAACC
	DXR	GCCACACTTTTCAACAAGGG	ACGAGCAACCTCAACAACAC
	HDR	TCGATACAACATGCCCATGGG	GTCGCTCCTGAGTGGCGTCAC
qPCR	*GAPDH*	GTTGACTTGACTGTGAGACTTGAG	CCTTGAGGTTGCCTTCGGATTC
	HMGR	CTTCCATAGAGGTTGGCACAGTTG	GAGCGTTTGAGCCTGGTGATTC
	GAS	TGCTATCTCGGGTACTTTCAAGG	TTCTCCTCTTATTCTCAACTGTGC
	DXR	CTAATGTCACACTTCTTGCGGAAC	TGTAATCGGAACCAGCCAAAGC
	HDR	CTGAGTGGCGTCACAGATGG	GAAGGGAGAAACAGAGGAGATAGG

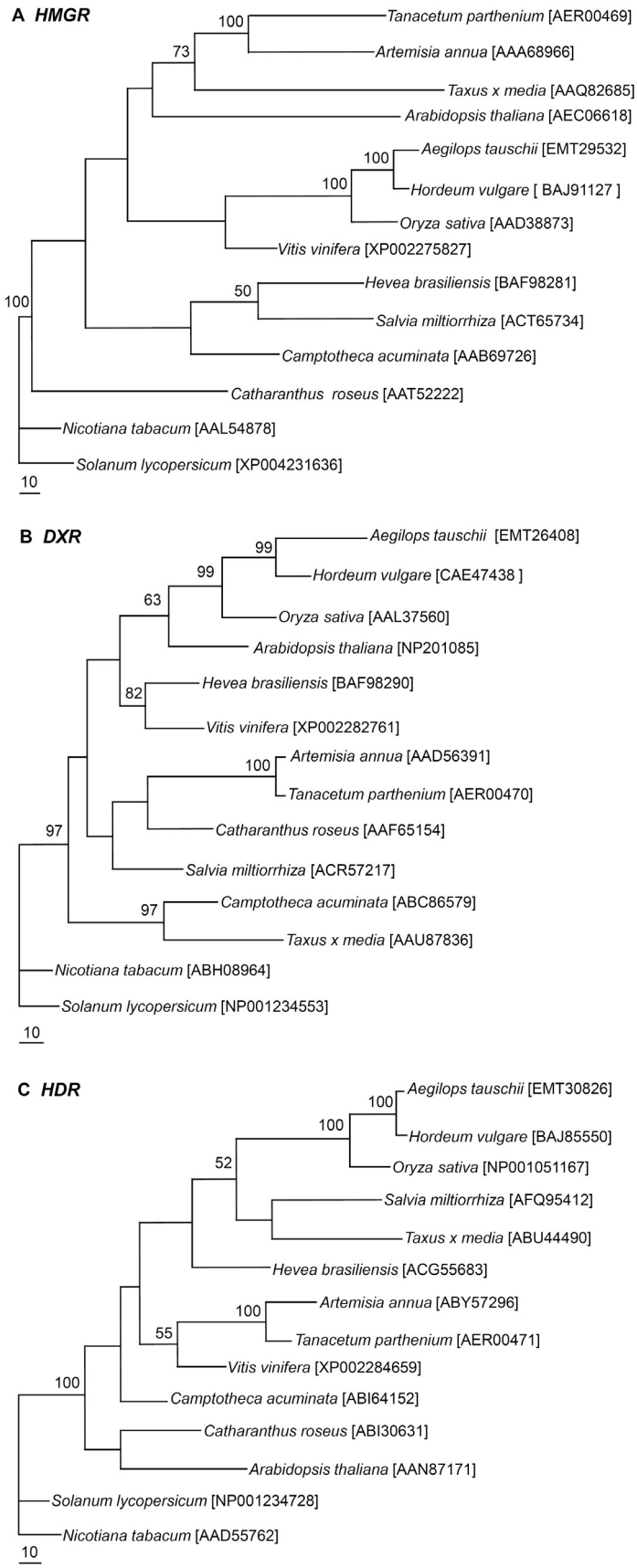

Fig 1. Phylogenic tree analysis of *Tp*HMGR (*A*), *Tp*DXR (*B*), and *Tp*HDR (*C*) with orthologues from other plant species using *PAUP 4.0b10* for maximum parsimony and bootstrap analysis. The numbers on the branches represent bootstrap support for 1 000 replicates. *Bar* = 10 changes.

Table 2. The relative gene expression (RGE) of *TpHMGR*, *TpGAS*, *TpDXR*, and *TpHDR* in different tissues of *Tanacetum parthenium*. Real time qPCR was based on the Ct values. The Ct value for each sample was normalized using the housekeeping gene *GAPDH*. Means ± SE, $n = 3$. Different letters in each row marked significant ($P < 0.05$) differences among tissues according to the Duncan's test. nd - not detected.

Gene	Young leaves	Old leaves	Flowers	Stems	Roots	Trichomes
HMGR	0.33 ± 0.026b	0.18 ± 0.012c	0.61 ± 0.046a	0.20 ± 0.021c	0.07 ± 0.006d	0.64 ± 0.017a
GAS	0.46 ± 0.025c	0.01 ± 0.001d	1.67 ± 0.094b	nd	nd	16.33 ± 1.299a
DXR	0.10 ± 0.009b	0.08 ± 0.006b	0.40 ± 0.023a	nd	0.02 ± 0.001c	0.60 ± 0.049a
HDR	0.49 ± 0.039c	2.70 ± 0.513a	1.12 ± 0.086c	0.13 ± 0.032d	0.06 ± 0.006e	1.55 ± 0.032b

shortest evolutionary distance from each other. A similar trend was observed for *TpGAS*, the previously isolated gene (Majdi *et al*. 2011). In addition, the multiple sequence alignment of *Tp*HMGR, *Tp*DXR, and *Tp*HDR with some homologues from other plant species using *ClustalW* (http://www2.ebi.ac.uk/clustalw) showed a relatively high conservation among plant species (data not shown) which most likely underlies their key roles in biology. The highest sequence similarity between *T. parthenium* and *A. annua* for all the isolated genes suggests that publicly available sequence databases, *e.g.*, glandular trichome ESTs from *A. annua* (Wang *et al*. 2009) might be a useful tool for isolating orthologues from *T. parthenium*.

ANOVA analysis shows that the expression of *TpHMGR, TpGAS, TpDXR*, and *TpHDR* in feverfew significantly differed among tissues ($P < 0.05$; Table 2). The highest expression of *TpHMGR* was detected in flowers and glandular trichomes, followed by young leaves, old leaves, stems, and roots. Nevertheless, the RGE values for *TpHMGR* in all examined tissues were relatively low (0.07 - 0.64). No significant difference was observed for RGE of *TpHMGR* in flowers and glandular trichomes. A low constitutive expression of *HMGR* has been also reported in snapdragon tissues (Dudareva *et al.* 2005). *Arabidopsis* contains two differentially expressed *HMGR* genes, *HMGR1* and *HMGR2*. *HMGR1* is expressed in all tissues with the highest expression in roots and flowers, whereas the expression of *HMGR2* is limited to roots and flowers (Enjuto *et al*. 1994). The *TpHMGR* transcription was lower in old leaves compared to young leaves (Table 2). The higher expression of *TpHMGR* in glandular trichomes and young leaves is consistent with the high activity of parthenolide biosynthesis in these tissues relative to the activity in stems, roots, and old leaves. Consistent with our results, a correlation between the *HMGR* expression and production of artemisinin (an endoperoxide sesquiterpene lactone) has been reported in *A. annua* tissues (Ram *et al.* 2010, Olofsson *et al.* 2011). The highest expression of *TpGAS* was detected in glandular trichomes, followed by young leaves, flowers, and old leaves; it was not expressed in stems and roots (Table 2). The transcription of *TpGAS* was extremely high in glandular trichomes (RGE = 16) compared with other tissues (RGE less than 1.67). The *TpGAS* expression patterns show its spatial and developmental regulation (Table 2). The presence and absence of the *TpGAS* expression is also well coordinated with the presence and absence of parthenolide production in the tissues examined. By far, the highest expression was observed in glandular trichomes, known to be the active site of parthenolide biosynthesis, whereas no expression was detected in roots which do not contain parthenolide (Majdi *et al.* 2011). An apparent decrease in the expression of *TpGAS* in old leaves compared with young leaves might be related to density of glandular trichomes. In line with this idea, in *A. annua*, young leaves show a higher trichome density and produce a higher amount of artemisinin than do old leaves (Arsenault *et al.* 2010). It has been shown that glandular trichome densities in young leaves is much higher than in fully expanded leaves because the original number of trichomes spreads out in over the larger surface area during leaf expansion, *e.g.*, in *A. annua* (Olofsson *et al.* 2011). Changes in the expression of *TpGAS* during feverfew flowering has been reported previously. This indicates that developmental regulation of *TpGAS* exists both in leaves and flowers (Majdi *et al.* 2011). In agreement with our results, the expression analysis of two sesquiterpene synthases in *A. annua*, including amorpha-4,11-diene synthase (*AaADS*) – the first committed step of artemisinin biosynthesis – and germacrene A synthase (*AaGAS*) shows a similar pattern with the *TpGAS* expression in young and old leaves (Olofsson *et al.* 2011). Both of these terpenoids are produced in glandular trichomes. The highest expression of *TpDXR* was detected in glandular trichomes, followed by flowers, young leaves, old leaves, and roots, whereas no *TpDXR* expression was detected in stems (Table 2). In *A. annua* and *A. thaliana*, *DXR* expressions show a similar pattern: it is higher in flowers than in stems or roots (Carretero-Paulet *et al.* 2001, Olofsson *et al.* 2011). The developmental regulation of *DXR* has been reported in *Arabidopsis* (Carretero-Paulet *et al.* 2006), but in the current study, the constitutive gene expression was detected for *TpDXR* in young and old leaves (Table 2). In tomato, a constitutive expression of *DXR* has been reported in ripening fruit (Rodríguez-Concepcion *et al.* 2001). Also in snapdragon flowers, a *DXR* gene expression does not show a diurnal pattern despite that the biosynthesis and emission of MEP-derived isoprenoid volatiles do show a diurnal pattern (Dudareva *et al.*

2005). *TpHDR* was expressed in all tissues. The high expression was found in old leaves, followed by glandular trichomes, flowers, young leaves, stems, and roots (Table 2). The *TpHMGR* and *TpGAS* expressions were 1.8 and 38 fold higher in young leaves than in old leaves, respectively, conversely to the *TpHDR* gene expression which was 5.5 fold higher in old leaves than in young leaves (Table 2). In *Arabidopsis* and *Oncidium*, a *HDR* expression is also detected in all tissues (Huang *et al.* 2009), and transcription is lowest in roots and highest in flowers (Hsieh and Goodman 2005, Huang *et al.* 2009). An interesting observation in our study was the dramatic up-regulation of *TpHDR* in old leaves compared with young leaves. In *A. annua*, an expression of *HDR* is higher in old leaves than other tissues (Olofsson *et al.* 2011), a similar pattern to what we observed in *T. parthenium*. Consistent with this, an up-regulation of *HDR* during tomato ripening has been reported (Lois *et al.* 2000).

In conclusion, *TpHMGR*, *TpDXR*, and *TpHDR* were expressed in all tissues, whereas *TpGAS* was not expressed in stems and roots. *TpHMGR* and *TpGAS*, which both belong to the MVA pathway, were down-regulated in old leaves compared to young leaves, whereas *TpHDR* belonging to the MEP pathway was up-regulated in old leaves. *TpDXR* did not show developmental changes in transcription in feverfew leaves. Except for *TpHDR*, which was highly expressed in old leaves, other genes were highly expressed in glandular trichomes and flowers (the active tissues of parthenolide biosynthesis). Phylogenetic and sequence similarity analyses of the isolated genes from feverfew with homologues from other plant species show that the shortest evolutionary distance exists between them and their homologues from *A. annua*. Beside this, a similar trend was observed for expression patterns of isolated genes in different tissues of *T. parthenium* and *A. annua* indicating that the function of the pathways is conserved between the two species. These results suggest that molecular information from *A. annua* can be used to help pave the way towards terpene biosynthesis elucidation in *T. parthenium*.

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