Expression profile of phenylpropanoid pathway genes in *Decalepis hamiltonii* tuberous roots during flavour development

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Abstract

To explore the transcriptional control of phenylpropanoid pathway (PPP) involved in vanillin flavour metabolites production in tuberous roots of *Decalepis hamiltonii*, four PPP key genes expressed during the tuber development were identified and their mRNA expression profiles were evaluated using quantitative real-time PCR. Flavour metabolite quantification by HPLC analysis confirmed 10, 170 and 500 µg/g 2-hydroxy-4-methoxy benzaldehyde and 4, 20 and 40 µg/g vanillin in first- (3-month-old plant), second- (18-month-old plant) and third-stage tubers (60-month-old matured plant), respectively. The expression of all four genes phenylalanine ammonia lyase (DhPAL), cinnamate-4-hydroxylase (DhC4H), caffeic acid-*O*-methyltransferase (DhCOMT) and vanillin synthase (DhVAN) increased with flavour development from first stage to second stage. A decrease in expression from 1.9-folds to 0.1-folds and 19.2-folds to 5.2-folds for *DhCOMT* and *DhVAN* was recorded for second stage to third stage, respectively. However, a gradual increase in expression of *DhPAL* (up to 26.4-folds) and a constant expression pattern for *DhC4H* (up to 7.1-folds) was evident from second stage to third stage of flavour development. The decrease in the expression levels of *DhCOMT* and *DhVAN* in third stage shows that the second-stage tubers are more transcriptionally active towards flavour biosynthesis.

Keywords 2-Hydroxy-4-methoxy benzaldehyde · Phenylalanine ammonia lyase · Cinnamate-4-hydroxylase · Caffeic acid-*O*-methyl transferase · Vanillin synthase · Relative expression

Introduction

Elucidation of biosynthetic pathway gene expression of flavour molecules from plant sources is having great significance as this paves the way for better understanding and scale up of these metabolites production (Schwab et al. 2008). The aromatic compound 2-hydroxy-4-methoxy benzaldehyde (2H4MB) is one of the structural isomers of vanillin (Thornell et al. 2000). 2H4MB is widely known for its flavour and is used widely as a flavouring agent (Giridhar et al. 2005). Natural occurrence of 2H4MB in different plants was reported such as in the bark of *Sclerocarya*

caffra Sond and in the roots of *Mondia whitei*, *Rhus vulgaris Meikle* (Kubo and Kinst-Hori 1999), *Periploca sepium* (Wang et al. 2010) *Hemidesmus indicus* (Sreekumar et al. 2000) and *Decalepis hamiltonii* (Giridhar et al. 2005). But the major source for this flavour molecule is from swallow roots of *D. hamiltonii* and *H. indicus* belonging to the family of Asclepiadaceae. In general, aromatic secondary metabolites derived from plant parts are often considered as specialised metabolites, that originate mainly from phenylalanine, tyrosine, and tryptophan (Peled-Zehavi et al. 2015). Phenylalanine-based phenylpropanoid pathway is considered vital for both volatile and non-volatile metabolites (Guterman et al. 2006). Recently, there is a growing interest on metabolic engineering which is emerged as a practical tool to boost up or regulate economically important secondary metabolites in the whole plant or in specific organs (Hirata et al. 2016). However, as a prelude to this, understanding key steps of biosynthetic pathway of targeted metabolite is required. There are many reports on the extracts of this swallow root showing health beneficial activities such as antioxidant, hepatoprotective, neuroprotective, insecticidal

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and microbicidal activities (Chidambaramurthy et al. 2006, reviewed by; Pradeep et al. 2016), where 2H4MB is the major compound. However, the complete biosynthetic pathway of this flavour molecule (2H4MB) is not elucidated in any of the plant system. According to earlier reports, 2H4MB biosynthesis flows through the primary step of phenylpropanoid pathway (PPP) via shikimic acid pathway using aminoxycetic acid and glyphosate which inhibit the starting steps of PPP and shikimic acid pathway (Chakraborty et al. 2008; Kundu et al. 2012). Our recent investigations in *D. hamiltonii* showed addition of ferulic acid to the callus suspension cultures increases vanillin flavour metabolites (Pradeep et al. 2017a, b). Subsequently, we demonstrated the major route of 2H4MB production using phenylpropanoid inhibitors, piperonylic acid, methylene dioxy cinnamic acid (MDCA) and propanil, which inhibit the feruloyl CoA ligase enzyme using in vitro cultured roots (Kamireddy et al. 2017) confirming the biosynthesis of 2H4MB through PPP.

Under this context, four key PPP genes, viz., phenylalanine ammonia lyase (PAL), caffeic acid-*O*-methyl transferase (COMT), cinnamate-4-hydroxylase (C4H), vanillin synthase (VAN) upstream to vanillin biosynthesis, were amplified using degenerate primers. The amplified fragments were sequenced and confirmed by comparing with the available nucleotide databases. The expression profiles of these genes were studied in three flavour developing stages. This study had showed the importance and involvement of these genes in 2H4MB biosynthesis in tuberous roots of *D. hamiltonii* during flavour development.

Materials and methods

Materials

High-performance liquid chromatography (HPLC) grade solvents, viz., ethanol, methanol, acetonitrile and glacial acetic acid were purchased from Merck Millipore (India). Analytical-grade dichloromethane, sodium carbonate (Na_2CO_3), Folin–Ciocalteu's reagent, aluminium chloride (AlCl_3), hydrochloric acid (HCl) and sodium hydroxide (NaOH) were purchased from Sisco Research Laboratory (Mumbai, India). For HPLC analysis, degassed and 0.22- μm membrane-filtered triple distilled water was used.

Plant material

Decalepis hamiltonii Wight & Arn., seeds were collected from the 7-year-old plant in the departmental garden and were kept for germination in garden soil at different intervals. The tuberous roots were collected from 3-month-old plant (Stage-1), 18-month-old plant (Stage-2) and from the

60-month-old mature plant (Stage-3). These tuberous roots were used for RNA extraction and HPLC analysis.

Extraction of 2H4MB and other phenolic compounds

Three different stages of roots weighing 1 g (FW) were extracted with 10 mL of dichloromethane for 2H4MB and ethyl acetate for vanillin (Chidambaramurthy et al. 2006). The extract was filtered through the Whatman filter paper grade 1 to remove the particulate matter and the filtrate was concentrated in a Rotavapor R3000 (Buchi, Switzerland). The concentrate was further dissolved in 1 mL methanol and filtered through 0.22- μm syringe filter before HPLC analysis.

HPLC for 2H4MB

HPLC analysis was performed using a HPLC system equipped with a dual pump and an LD 20 AD UV–Vis detector, Shimadzu (Kyoto, Japan). Separation was achieved on Waters SunFire C_{18} reversed-phase column (4.6×250 mm) (Waters, Milford, MA, USA). Analysis was carried out for sample volume of 20 μL . The gradient elution was opted using mobile phase containing water pH 2.6 adjusted with glacial acetic acid (A) and (B) containing 20% A + 80% Acetonitrile and the flow rate of 1.2 mL/min. (Kamireddy et al. 2017) (Fig. 1).

Mass spectrometric confirmation

HPLC peaks were subjected to electron spray ionisation mass spectrometry (ESI-MS) for the molecular confirmation and identity according to a published procedure with a Waters QTOF-Ultima (Waters Corporation, Micromass Ltd., Manchester, UK) operating at ESI negative mode (Sachan et al. 2005). The following ion optics was used: source temperature: 120 °C, capillary voltage: 3.00 kV; desolvation temperature: 300 °C; desolvation gas: 500 L/h; and cone gas flow, 50 L/h. The mass range was from 100 to 500 m/z , scan speed 1000 amu/s (Fig. 3).

Isolation of total RNA and cDNA synthesis

Total RNA was extracted from 100 mg of plant material (tuberous roots of three stages) using Spectrum Plant Total RNA kit (Sigma, USA) after the tuberous roots were grounded to a fine powder in liquid nitrogen. Genomic DNA contamination was eliminated by treating RNA with recombinant DNase I (RNase-Free DNase from Thermo Scientific). RNA integrity was evaluated on agarose gel electrophoresis. RNA concentration and purity was estimated using NanoDrop 1000 Spectrophotometer (Thermo Scientific). RNA samples with 260/280 ratios ~ 2 was used for cDNA synthesis. cDNA was synthesised for each sample

Fig. 1 HPLC chromatograms showing the separation of 2H4MB and vanillin peaks in different stages of tuber development

with 1 μ g of total RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Lithuania) in a final volume of 20 μ L, following the manufacturer's protocol. The final cDNA samples were diluted tenfold with nuclease free water prior to use in qRT-PCR.

Degenerate primer synthesis, PCR amplification and purification

Till date, there is no gene sequence reported for PPP genes in *D. hamiltonii*. Degenerate primers were designed to find the key regulatory gene sequences of PPP (PAL, CoMT, C4H, and VAN) and housekeeping genes (Actin and GAPDH) in *D. hamiltonii*. Degenerate primers were designed by aligning gene sequences from diverse plant species using Clustal Omega Multiple Sequence Alignment, from the aligned sequences conserved sequences with a stretch more than 20 nucleotides were collected, at the non conserved regions degenerative nucleotides were inserted and these primers were checked for quality using IDT Oligo Analyzer 3.1. Primers with the melting temperature in the range of 58–62 $^{\circ}$ C were selected for PCR reaction. The PCR amplified fragments were eluted from gel using GenElute Gel Extraction Kit (Sigma, USA).

Cloning, gene sequencing and conformation

The eluted gene fragments were cloned into pTZ57R/T vector using InsTAclone PCR Cloning Kit (Thermo Scientific).

Cloned vector was transformed into competent *E. coli* cells of DH5 α strain. The transformed *E. coli* cells were selected on X-Gal- and IPTG-containing medium. Positive colonies for the individual gene were selected and grown for plasmid isolation. Plasmid isolation was carried out using GenElute plasmid miniprep kit (Sigma, USA). Plasmid with insert was confirmed by PCR using extracted plasmid as a template with M13 primers. Plasmids with desired inserts were sequenced using (Applied Biosystem ABI PRISM 310 Genetic Analyzer). The obtained sequence was checked for the homology against the nucleotide repository using nucleotide BLAST; the nucleotide identity (> 70%) and their accession numbers are shown in Table 1.

Primer design for quantitative real-time PCR

Primer pairs for qRT-PCR amplification were designed using previously obtained partial sequences from degenerate primer amplification using OligoExplorer software. Retained parameters were T_m between 60 and 62 $^{\circ}$ C, primer size between 18 and 22 bp, about 50% GC content and amplicon size of 90–150 bp. Amplicon size of PCR products was further checked on 2.5% agarose gels. List of gene-specific primers used for the qRT-PCR is given Table 2.

Quantitative real-time PCR analysis

The obtained three stages of cDNA samples were analysed in triplicates using real-time PCR. qRT-PCR was

Table 1 Specific degenerate primers used for amplification, amplicon size and their BLAST nucleotide identity with accession numbers

Gene name	Primer sequence (5'–3') forward and reverse	cDNA fragment size (bp)	Nucleotide identity (%)	Blast hit (GenBank ID)	Accession number
Actin	TGGACTCTGGTGATGGTGT TCAGCAATACCAGGRAACAT	500	92	<i>Calotropis procera</i> (KU833210.1)	JZ971601
GAPDH	GAGTCCACCGGTGTYTTCAC ACDGTGGTCATSAGACCCTC	260	93	<i>Periploca sepium</i> (AB164360.1)	JZ971600
PAL	GGAGCTHATHAGRTTCTTGAA ACRTCCTGGTTGTGYTGCTC	1080	80	<i>Coffea canephora</i> (JF805760.1)	KY799106
C4H	ATGATGTAYAAAYATGT TCGTTKCCATTRGCCTCAAC	850	85	<i>Catharanthus roseus</i> (Z32563.1)	KY799104
COMT	GGCYTATGGAATGACTGCAT CTCTCTTTCCACCAGGGTT	700	87	<i>Rauvolfia serpentina</i> (KX687825.1)	KY799105
VAN	GGAGGAGATMMAGAAGAGGT CCWCCATTGCAKCCAAAGTT	590	68	<i>Vanilla planifolia</i> (KP278240.1)	MF045895
			79	<i>Glechoma hederacea</i> (KJ775791.1)	

Table 2 Specific primers used for qRT-PCR

Gene name	Primer sequence (5'–3') forward and reverse	Amplicon size (bp)
Actin	TGCAATTCTTCGTCTTGAC CATAGGCTAGCTTTTCCT	138
GAPDH	CTAGCAAGGACGCACCTATG GGGAGCAAGGCAGTTTGTG	105
PAL	GCTTCAAGGATATTCTGGGA GTGATT GTACCACGAAGGGG	102
C4H	GGTGAACCACCCTGAGATC CATACGGAGGCGAAGAGTC	142
COMT	CACCAGGGTTGTGTGCTAAC GATCCTTGCTGAATGTGTTT	101
VAN	AACCACGAAGGGCAATCTC TGAATGTCCAGCAAGATCC	122

performed with a CFX96 Touch Real-Time PCR Detection System (BIO-Rad) using SYBR Green detection chemistry. The reactions were prepared in a total volume of 10 µL, containing 1 µL of diluted cDNA, 0.5 µL (5 µM) of gene-specific primers, 5 µL of 2× SYBR Green Master mix reagents (Faststart Essential DNA Green Master, ROCHE). The standard thermal cycler protocol was used for all PCR reactions, quantification cycles repeated for 40 times.

Data were analysed Using CFX manager, which performed the baseline correction and automatically calculated the threshold for quantification cycles (Cq) values for each reaction. Relative expression of these genes was calculated using the formula proposed by (Pfaffl et al. 2002; Liu et al. 2017) using Actin as a reference gene.

Statistical analysis

The data were expressed as mean ± SD of triplicates and the experiments were repeated twice. Data were subjected to post hoc analysis using Tukey's test (SPSS 17) at significance level of $p < 0.05$.

Results

HPLC quantification of 2H4MB and vanillin

In this study, three different stages of *D. hamiltonii* roots were collected from different ages of plants. These three stages were selected based on maturity, where the first-stage roots were feeble and fibrous in nature, the second-stage roots were stout and tuberous in nature and the third stage completely matured hard swallow roots. Quantification of major flavour compounds 2H4MB and vanillin from these three different stage roots though HPLC analysis (Fig. 1) indicates variation in their content and it varies with the development stage of roots. The amount of 2H4MB quantified in three stages was 10, 170 and 500 µg/g in first, second and third stages of roots, respectively (Fig. 2). Similarly, the amount of vanillin observed in three different stages was 4, 20 and 40 µg/g in first, second and third stages, respectively (Fig. 2). Further, 2H4MB peak mass of 151.3 was confirmed by mass spectrometer (Fig. 3).

Newly identified genes

Using cDNA of *D. hamiltonii* as a template, specific genes were amplified with the synthesised degenerate primers. The amplicons were sequenced and the obtained partial sequences were confirmed by BLASTN analysis against NCBI nucleotide

Fig. 2 Vanillin and 2H4MB content in different stages of *D. hamiltonii* tuber development. Vanillin and 2H4MB were quantified by HPLC during developmental stages of tuber. All the values represented are mean $\pm$ SD of three replicates ($n=3$). Columns with-

out a common lower case letter indicate significantly different values among treatments based on one-way ANOVA and Tukey's test ($p \leq 0.05$)

Fig. 3 Mass spectrometer confirmation of 2H4MB purified from HPLC, with mass peak of 151.3

database. Based on the homology and percent identity, the sequences were identified. Consequently, the sequences for *PAL*, *C4H*, *VAN* and *COMT* are now available for *D. hamiltonii* and the accession numbers of the same are given in Table 1. The proposed biosynthetic pathway of 2H4MB is shown in Fig. 4.

For understanding the phylogenetic relationship of *DhPAL*, *DhC4H*, *DhCOMT* and *DhVAN* genes of *D. hamiltonii* to other plant species, these sequences were aligned with sequences of high similarity sequences and generated a maximum likelihood phylogenetic tree with MEGA 6 programme.

Phylogenetic analysis of PPP genes

DhPAL

DhPAL phylogenetic tree was constructed using five *PAL* sequences from other plants belonging to the eudicots. *Saccharum PAL* sequence was used to out-group the tree, as it belongs to monocot. As predicted, *DhPAL* showed a close relation with asterids showing 100% identity for *Catharanthus roseus* (Fig. 5).

Fig. 4 Hypothesised biosynthetic pathway of 2H4MB in *D. hamiltonii*. PAL phenylalanine ammonia lyase, C4H coumarate 3-hydroxylase, COMT caffeic acid 3-*O*-methyltransferase, VAN vanillin synthase, 4-CL cinnamate-4-hydroxylase

DhC4H

To construct *DhC4H* phylogenetic tree, five *C4H* sequences were taken from different plants belonging to the class eudicot. As expected, *DhC4H* clustered with *Catharanthus roseus C4H* both belonging to Apocynaceae

family. Sequence identity was 82% for *Catharanthus roseus*. *Saccharum* was used to out-group the tree (Fig. 6).

DhCOMT

The phylogenetic tree of *COMT* was constructed by comparing five *COMT* sequences from other plants. *Saccharum* was used to out-group the tree. *DhCOMT* showed 60% sequence identity for *Catharanthus roseus* (Fig. 7). Bootstrap test of 1000 replicates was used to cluster the taxa.

DhVAN

Till date, vanillin synthase gene is reported in only two plant systems (*Vanilla planifolia* and *Glechoma hederacea*). The vanillin synthase we found in *D. hamiltonii* in the present study showed 68% identity with *V. planifolia* and 79% with *G. hederacea* (Table 1).

Expression analysis of the identified genes

The expression analysis of the identified PPP genes was calculated using Actin as the reference gene. The fold change was relative to the expression levels of first stage (Fig. 8). The expression level of *DhPAL*, *DhC4H*, *DhCOMT*, *DhVAN* in the second stage in comparison with control (first stage) are 22.6-, 7.1-, 1.9- and 19.2-folds, respectively, whereas the expression levels of *DhPAL*, *DhC4H*, *DhCOMT*, *DhVAN* in third stage was 26.4-, 7.1-, 0.1- and 5.2-folds, respectively, compared to control. Semi-quantitative PCR was also performed with all the four identified genes, viz., *DhPAL*, *DhC4H*, *DhCOMT*, and *DhVAN* in three stages of flavour development (Supplemental Fig. 1).

Fig. 5 Phylogenetic relationship between various *PAL* sequences was depicted. The analysis involved 13 nucleotide sequences aligned by ClustalW and the phylogenetic tree of *PAL* sequences was constructed using the neighbour-joining method with MEGA 6 (Tamura et al. 2013). Bootstrap test of 1000 replicates was used to cluster the taxa. Bootstrap percentages of > 50 are given. The *Saccharum PAL* sequence was used to root the tree. GenBank ID was put in brackets

Fig. 6 Phylogenetic relationship between various *C4H* sequences was depicted. The analysis involved six nucleotide sequences aligned by ClustalW and the phylogenetic tree of *C4H* sequences was constructed using the neighbour-joining method with MEGA 6 (Tamura et al. 2013). Bootstrap test of 1000 replicates was used to cluster the taxa. Bootstrap percentages of > 50 are given. The *Saccharum C4H* sequence was used to root the tree. GenBank ID was put in brackets

Fig. 7 Phylogenetic relationship between various *COMT* sequences was depicted. The analysis involved 16 nucleotide sequences aligned by ClustalW and the phylogenetic tree of *COMT* sequences was constructed using the neighbour-joining method with MEGA 6 (Tamura

et al. 2013). Bootstrap test of 1000 replicates was used to cluster the taxa. Bootstrap percentages of > 50 are given. The *Saccharum COMT* sequence was used to root the tree. GenBank ID was put in brackets

Discussion

2H4MB and vanillin levels with maturation of tuberous roots

In our study, we observed the increase in flavour compound levels with maturation of tubers as confirmed through HPLC quantification (Fig. 2). Till date, there are no reports on 2H4MB and vanillin levels during maturation of *D. hamiltonii* tuberous roots. However, in *V. planifolia* increase in the levels of vanillin and other flavour components with pod maturation was reported (Palama et al. 2009; Brillouet et al. 2010). The phenolic compounds such as epicatechin and procyanidin B concentration in apple flesh decreased sharply during the early stages of development and the levels remained relatively constant during maturation and storage (Burda et al. 1990). Contrarily, in *Tacinga inamoena* fruit, the phenolic compounds, yellow flavonoids and betalains increase with maturation

(Dantas et al. 2016). Interestingly, in berries of *Vaccinium corymbosum* (Castrejón et al. 2008) and peduncles of *Hovenia dulcis* Thunb (Maieyes et al. 2015), the levels of phenolics decrease with maturation. The possible reason for this decrease could be shift in the pool of total phenolics towards anthocyanin synthesis as these plants start producing anthocyanins in their respective parts during maturation leading to decrease in overall phenolic content (Castrejón et al. 2008). In strawberry, the phenolic compounds changed during maturation; however, no difference in content of flavonoids was detected (Kosar et al. 2004). In *D. hamiltonii*, we observed more than tenfold difference between vanillin and 2H4MB (Fig. 2); this shows 2H4MB-catalysing enzyme is more active in *D. hamiltonii* than vanillin synthase. We also observed that not much difference in flavour content after 3 years of plant development by comparing with 7-year-old plant (data not shown) suggesting that the 2H4MB is stable molecule and the biosynthesis may not be active once the saturation levels are achieved in the plant system.

Fig. 8 Relative expression of key PPP genes during maturation of tuber and flavour development. Relative expression of PAL, C4H, COMT and VAN genes in second and third stages of tuber development. The fold change values are with respect to the first stage. All

the values represent are mean $\pm$ SD of three replicates ($n=3$). Columns without a common lower case letter indicate significantly different values among treatments based on one-way ANOVA and Tukey's test ($p \leq 0.05$)

Expression pattern of four PPP genes in different developmental stages

For the first time, the partial sequences *PAL* (*DhPAL*), *C4H* (*DhC4H*), *COMT* (*DhCOMT*) and Vanillin synthase (*DhVAN*) were obtained by degenerate primers from *D. hamiltonii* tuberous roots. The relationship of *DhPAL* with *PAL* sequences of other Apocynaceae members was checked by phylogenetic analysis. In many of the plant species, *PAL* enzyme coexists in multiple isoforms with various implications in different developmental stages and stress responses (Podstolski et al. 2002; Wanner et al. 1995). Major *PAL* gene expressing in the tubers of *D. hamiltonii* has been presently characterised; the other *PAL* isoforms are to be characterised in different developmental stages and in different parts of the plant. *PAL* is encoded by a family of three or more genes of which *PAL1* and *PAL2* genes are structurally similar and its expression is similar in different plant organs and under different inductive conditions (Wanner et al. 1995). The sub-cellular localisation of *PAL* occurs mainly in cytoplasm, and it may also be associated with some membranous organelles (Jones 1984). In our study, we found a steep increase in *PAL* from first stage to second stage with 22-fold increase and

later from second stage to third stage the increase in *PAL* activity was gradual (Fig. 8). The significant fold increase in *PAL* expression relates to the active role of PPP in *D. hamiltonii* towards 2H4MB biosynthesis. A similar kind of observation was also seen in *Vanilla planifolia* pod development (Fock-Bastide et al. 2014). However, in strawberry, the *PAL* activity decreased with fruit maturation (Cheng and Breen 1991) and this could be due to the shift in pathways towards anthocyanin synthesis during ripening (Castrejón et al. 2008). All three genes (*PAL1*, *PAL2* and *PAL3*) were highly expressed in roots, two genes (*PAL1* and *PAL2*) were expressed in shoots and only *PAL1* was expressed in leaves (Liang et al. 1989). The tissue-specific expression of *PAL* in *Pisum sativum* showed that *PAL1* and *PAL2* accumulated abundantly in roots, moderately in stem and in very low levels in leaves and flowers (Yamada et al. 1992). Abnormal expression of *PAL* leads to unusual phenotypes in tobacco (Elkind et al. 1990).

C4H catalyses the conversion of cinnamate into coumarate; it is one of the key enzyme in PPP which is involved in the biosynthesis of several secondary metabolites (Lu et al. 2006). The cDNA sequences of *C4H* gene reported for different plants have shown to be more conserved (Ehltng

et al. 2006). C4H gene members exist as a multigene family in many plant species; it expresses in various tissues; however, the expression patterns will be different in individual members from each other (Lu et al. 2006). Among C4H1 and C4H2 genes, C4H1 is wound inducible and C4H2 is expressed constitutively (Betz et al. 2001). In *Arabidopsis*, a mutation in C4H caused pleiotropic phenotypes including dwarfism, male sterility and swelling at branch junctions. It also changes the metabolic profile exhibiting reduced lignin monomer production and production of novel hydroxycinnamic ester, cinnamoylmalate which were not found in normal plants (Schilmiller et al. 2009). C4H mostly expressed in roots and cells undergoing lignifications (Bell-Lelong et al. 1997). In Korean black raspberry, during fruit development, C4H gene had a differential expression pattern, where gene expression was first detected in green fruit and then remarkably reduced in yellow fruit, followed by an increase in red and black fruit (Baek et al. 2008). In pepper, the expression level of C4H was constant till 22 days after flowering and sharp increase at 30 days was observed (Cheng and Breen 1991). However, in *D. hamiltonii*, we observed an increase in C4H expression by sevenfolds during tuber maturation and flavour development from first stage to second stage and the expression remained constant during the third stage (Fig. 8). The constant expression of C4H shows its key role in tuber development and flavour synthesis.

COMT is another key enzyme of PPP, which catalyses the formation of ferulic acid from caffeic acid (Pichersky and Gang 2000). COMT is mainly involved in the biosynthesis of monolignols via caffeoyl CoA methyl transferase-mediated pathway (Inoue et al. 1998). COMT also involves in flavour biosynthesis through ferulic acid, where ferulic acid acts as a precursor molecule for vanillin (Gallage et al. 2014) and 2H4MB biosynthesis (Kamireddy et al. 2017). So the expression profile of *DhCOMT* was analysed during the flavour development stages and we found around twofold increases in *DhCOMT* levels in second stage of tubers and in the third stage of tuber maturation the expression levels falls to the basal levels of first stage in comparison with the first stage (Fig. 8). The increase in *DhCOMT* levels at second stage of tuber maturation is directly correlated to the flavour development; this shows active role of *DhCOMT* gene towards flavour biosynthesis in second stage. The downfall of expression levels during the third stage could be due to the feedback inhibition of *DhCOMT* by ferulic acid. Ferulic acid also mediates tuber maturation by lignification, where the soft roots convert into tubers requiring lignification of cells (Sirju-Charran and Wickham 1988). Expression levels of four different COMT genes in different tissues of *Brachypodium* and wheat indicated differential pattern (Wu et al. 2013); whereas in maize, COMT gene expressed highly in roots (Collazo et al. 1992), and in ryegrass, it is expressed abundantly in stem (McAlister et al. 1998).

VAN is a vanillin flavour-synthesising enzyme using ferulic acid as a substrate molecule; this was recently characterised in *Vanilla planifolia* and *Glechoma hederacea* plants (Gallage et al. 2014). Till date, VAN is reported in these two plant systems and now for the first time we are reporting VAN partial sequence from *D. hamiltonii*. VAN belongs to the cysteine proteinases family, as the amino acid sequence contains the typical processing motif from cysteine proteinases. The VAN sequence similarity of *D. hamiltonii* with that of *G. hederacea* was found to be 66% and with *V. planifolia*, the similarity was ~70% (Table 1). In the present study, observations for relative expression of genes indicate that a sharp increase in *DhVAN* expression from first stage to second stage by 19-folds and the expression levels decreased to 5-folds in third stage in comparison with first stage. The sharp increase in *DhVAN* expression caused higher synthesis of vanillin during the second stage. The decrease in *DhVAN* expression during the third stage could be due to the saturation levels attained by the vanillin and, therefore, the feedback inhibition of vanillin on VAN. The continuous accumulation of vanillin throughout second stage to the third stage resulted in higher levels of vanillin in third stage. This also shows that vanillin is a stable compound, and its conversion into other compounds is minimal. Even with 2H4MB the same pattern was observed, where the maximum flavour accumulation happens by third stage (5 years old) and even the matured stage tubers (7-year-old plant tubers) does not show much difference in flavour quantity. Thus the third-stage tubers can be considered as a threshold stage for flavour accumulation as the flavour accumulation would not be much significant post this stage.

Conclusion

In *D. hamiltonii*, the flavour compounds vanillin and 2H4MB increase with maturation of tuber. But this increase was limited to 3 years of maturation and later, much increase in flavour content was not observed. The expression of key PPP genes such as *DhPAL*, *DhC4H*, *DhCOMT* and *DhVAN* increases with flavour content and maturation until the tuber reaches the second stage, and *DhC4H* maintained constant expression even in third stage of maturation. Only *DhPAL* showed parallel increase in gene expression with flavour content throughout the three stages. *DhVAN* and *DhCOMT* genes showed a decrease in their expression from second to third stage of tuber maturation. All the key phenylpropanoid genes are directly or indirectly involved in flavour biosynthesis along with maturation of tubers in *D. hamiltonii*. Second stage of tubers is transcriptionally more active compared with first and third stage of tubers. Based on the metabolic and expression profile of *D. hamiltonii* tubers, we

found harvesting three year old plant tubers will be ideal for flavour extraction where the flavour production is maximum.

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Author contributions PG participated in designing of the study drafting of the manuscript and analysis of results. LV participated in designing of degenerate primers, availing accession numbers to gene sequences and helped in performing the experiments and their analysis. PS performed the HPLC and MS analysis, statistical analysis and participated in drafting the manuscript. NPS involved in discussions and helped in improvement of manuscript. KK designed the study, identified the gene sequences, performed qRT-PCR analysis, analysed the results and drafted the manuscript.

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Compliance with ethical standards

Conflict of interest Authors of this article do not have any conflict of interest in any of the aspects.

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