Differential expression and functional analysis of two short-chain alcohol dehydrogenases/reductases in *Hedychium coronarium*

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ABSTRACT

In this study, the full cDNA sequences of HcADH2 and HcADH3 were cloned from *Hedychium coronarium*. The amino acid sequences encoded by them contained three most conserved motifs of short-chain alcohol dehydrogenase (ADH), namely NAD⁺ binding domain, TGxxx[AG]xG and active site YxxxK. The highest similarity between two genes and ADH from other plants was 70%. Phylogenetic analysis showed that they belonged to a member of the short-chain dehydrogenases/reductases 110C subfamily, but they were distinctly clustered in different clades. Real-time polymerase chain reaction analyses showed that HcADH2 was specifically expressed in bract, and it was expressed higher in no-scented *Hedychium forrestii* than other *Hedychium* species, but was undetectable in *Hedychium coccineum*. HcADH3 was expressed higher in the lateral petal of the flower than in other vegetative organs, and it was expressed the most in *H. coronarium* that is the most scented among *Hedychium* species, and its expression levels peaked at the half opening stage. HcADH2 and HcADH3 had almost no significant expression in leaves, but HcADH2 was expressed in response to external stimuli. The mechanical injury and methyl jasmonate (MeJA) treatment could induce expression of HcADH2 in leaves, whereas HcADH3 could have an induced expression only by MeJA. The recombinant HcADH3 protein, but not HcADH2, expressed in *Escherichia coli*-catalysed conversion of geraniol into citral. It was speculated that HcADH3 had an induced expression in vegetative organ of *H. coronarium* and took part in monoterpenoid biosynthesis in *H. coronarium* flowers, but the role of HcADH2 is relevant only for defensive reactions.

Keywords: expression analysis, function, *Hedychium coronarium*, short-chain alcohol dehydrogenase/reductase

Abbreviations:

ADH, alcohol dehydrogenase; GC-MS, gas chromatography-mass spectrometry; IPTG, isopropyl-1-thio-β-D-galactoside; MeJA, methyl jasmonate; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcription polymerase chain reaction.

INTRODUCTION

Hedychium coronarium is a perennial herb that belongs to the *Hedychium* genus of the Zingiberaceae family. The genus *Hedychium* approximately contains 60 species (Wu, 1994). According to the olfactory sensory

evaluation, four different species of *Hedychium* genus were selected, namely, *Hedychium coccineum* Buch.-Ham (scentless), *Hedychium forrestii* (scentless), *Hedychium gardnerianum* (slightly fragrant) and

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H. coronarium (fragrant) (Fan et al., 2007). *H. coronarium* commonly grows in woodland shrubs at the altitude of 600–2,100 m, which is often cultivated as a horticultural garden plant because of its rich fragrant flowers. It can also be used in excavated medicine and aromatic oil (Wu and Raven, 2001). The volatile compounds mainly consist of monoterpenes, monoterpene derivatives, sesquiterpene and benzenoids, including linalool, (*E*)/(*Z*)- β -ocimene, 1,8-cineole, sabinene, α -thujene, myrcene, α/β -pinene, allo-ocimene, α -farnesene and β -caryophyllene at the blooming stage of *H. coronarium* flowers (Fan et al., 2007; Baez et al., 2011).

H. coronarium is often called a white ginger lily. Its flowers are white and not bright, but they are very fragrant. Therefore, it is an ideal material to study the secondary metabolism of horticultural plant flowers. However, there have been few research on secondary metabolism that is related to the development physiology of *H. coronarium*, such as fragrance, defence, etc. (Yue et al., 2014; Chen et al., 2019). Chemical constituents of the rhizomes of *H. coronarium* had an inhibitory effect on lipopolysaccharide-stimulated production of pro-inflammatory cytokines in bone marrow-derived dendritic cells (Kiem et al., 2011). The new full-length cDNA that encoded farnesyl diphosphate synthase from *H. coronarium* was possibly involved in the biosynthesis of floral and wounding/herbivory-induced leaf volatile sesquiterpenoids (Lan et al., 2013).

Furthermore, short-chain dehydrogenase/reductases (SDRs) are a gene family involved in various secondary metabolic pathways. At present, the research on cloning of SDR genes has mainly been focused on dicots, such as genus *Solanum* (Sonawane et al., 2018), *Catharanthus roseus* (Stavrinides et al., 2018), *Scopolia lurida* (Zhao et al., 2017), *Hevea brasiliensis* (Loh et al., 2016), *Camellia sinensis* (Zhou et al., 2015), *Medicago truncatula* (Pan et al., 2014), *Papaver somniferum* (Chen and Facchini, 2013; Ziegler et al., 2009), *Lycopersicon esculentum* Mill. (Moummou et al., 2012), *Gossypium* spp. (Pang et al., 2010), *Panax ginseng* (Kim et al., 2009), *Solanum tuberosum* L. (Keiner et al., 2002), *Citrullus lanatus* (Thunb.) Matsum. et Nakai (Kim et al., 2003), *Arabidopsis thaliana* (Cheng et al., 2002), etc., while few reports had been on monocots. The momilactone, a synthase (OsMAS/SDR110C-MS1) of *Oryza sativa* with the other four – all falling in the same SDR110C family, had roles in diterpenoid biosynthesis (Kitaoka et al., 2016). The first step of chlorophyll *b* degradation in rice can be catalysed by a membrane-localised SDR (Sato et al., 2008). *Zingiber zerumbet*, a member of the *Zingiber* genus of Zingiberaceae family, is widely cultivated throughout the tropics and the subtropics for its medicinal properties. The rhizome oil of *Z. zerumbet* contains a high content of sesquiterpenoids with zerumbone, and ZSD1 (BAK09296.1) was cloned from *Z. zerumbet* and belonged to a member of the SDRs 110C subfamily (<http://www.sdr-enzymes.org>) which catalysed 8-hydroxy- α -humulene into zerumbone and

involved in secondary metabolism, stress responses and phytosteroid biosynthesis (Okamoto et al., 2011).

The expression of SDRs in plants can be regulated with development or induced by external stimuli. For the former, SDR is often expressed at certain stages during development and in specific tissues, for example, tuber, root, glandular hair of leaves, flower and fruit (Okamoto et al., 2011; Chen et al., 2011). As for the latter, the studies have shown that the tetramer SAD of pea has at least three similar family members (SAD-A, SAD-B and SAD-C), and mRNA expression indicated that SAD expression can be induced by the environment (Scherbak et al., 2011).

Our team has obtained the whole transcriptome sequence of *H. coronarium* (Yue et al., 2015). Among them, there are 10 sequences of short-chain alcohol dehydrogenase/reductase (SCADH). This research analysed the transcriptome of *H. coronarium* to study on secondary metabolism that is related to the development physiology of *H. coronarium* flowers. In this study, two new short-chain alcohol dehydrogenase/reductase genes, *HcADH2* and *HcADH3*, were cloned, respectively. In-depth analysis of expression regulation of these two genes was also performed to understand similarities and differences in the secondary metabolic function of different SDRs in *H. coronarium*. Meanwhile, prokaryotic expressions of recombinant HcADH2 and HcADH3 were also conducted, then different substances were added and the products were examined by GS-MS to determine the catalytic activity of the recombinant enzymes. Further studies are required to clarify their involvement in related secondary metabolic pathways of *H. coronarium* to lay a theoretical basis to fully understand secondary metabolism of *H. coronarium*.

MATERIALS AND METHODS

Plant materials used to clone the genes

H. coronarium was grown in the horticulture chamber in South China Agricultural University under natural light from April to November. The temperature (20–30°C) and the relative humidity of the atmosphere (70–80%) of the environmental conditions were suitable for *H. coronarium* and the plants grew well, and the full-blooming flowers were selected (Fan et al., 2007).

All plant materials were harvested, immediately frozen in liquid nitrogen and stored at –70°C for RNA isolation and quantitative real-time polymerase chain reaction (PCR). All experiments were conducted three times with independently collected plants.

Plant materials for analysis on the temporal and spatial expression of the genes

According to Yue's methods (Yue et al., 2014), different tissues, i.e. flower, bract, leaf, stem, root and rhizome, and different floral parts, i.e. style and stigma, anther,

Figure 1. Photographs of *H. coronarium* flowers in different developmental stages. -40 = 40 h pre-anthesis; -32 = 32 h pre-anthesis; -24 = 24 h pre-anthesis; -16 = 16 h pre-anthesis; -8 = 8 h pre-anthesis; 0 = 0 h anthesis; 8 = 8 h post-anthesis.

filament, outer labellum, lateral petal, sepal, pedicel were harvested from *H. coronarium* for the analysis of gene expression. The development of *H. coronarium* flowers was divided into seven stages (40, 32, 24, 16 and 8 h pre-anthesis, 0 h anthesis and 8 h post-anthesis) (Figure 1). We selected four different species of the *Hedychium* genus, such as *H. coccineum* Buch.-Ham, *H. forrestii*, *H. gardnerianum* and *H. coronarium*. At present, these species of the *Hedychium* genus are widely cultivated in China.

Mechanical injury treatment

According to Lan's methods (Lan et al., 2013), the wounded leaves and control leaves (not wounded, natural leaves) were collected at 0, 3, 6, 12 and 24 h after treatment and immediately frozen in liquid nitrogen.

MeJA treatment

Methyl jasmonate (MeJA) was dissolved in a small volume of ethanol and diluted with distilled water to 300 μ M (Yu et al., 2008). After spraying MeJA, the leaves were rubbed with a layer of lanolin to prevent the volatilisation of MeJA. MeJA-treated and control leaves were harvested at 0, 3, 6, 12, 18 and 24 h after treatment.

All plant materials were frozen in liquid nitrogen and stored at -70°C immediately after harvesting for future isolation of RNA and quantitative real-time PCR assays. All experiments were conducted three times with independently collected materials.

RNA isolation and reverse transcriptase PCR

Total RNA was extracted with the revised hexadecyl trimethyl ammonium bromide (CTAB) method (Zhang et al., 2010). RNA content was determined spectrophotometrically. All RNA extracts were treated with DNase (Takara Bio. Dalian, China). Of note, 1 μ g of RNA was reverse-transcribed in 20 μ l final volume reaction mixture including reaction buffer,

namely 20 $\mu\text{mol} \cdot \mu\text{l}^{-1}$ Rnase inhibitor, 1 $\text{mmol} \cdot \text{l}^{-1}$ dNTP, 1 μg oligo (dT)₁₈ and reverse transcriptase (RT) (M-MLV; Takara Bio. Dalian, China) according to the manufacturer's instructions (Li and Fan, 2011).

Cloning and sequence analysis of the target gene

Our team has obtained the whole transcriptome sequence of *H. coronarium* (Yue et al., 2015). Among them, there are 10 sequences of short-chain alcohol dehydrogenase (SAD), of which HcADH2 and HcADH3 are highly expressed. From the transcriptome of *H. coronarium*, two putative full-length SDRs sequences (comp45280_c0 and comp41433_c0) (Table S1 in Supplementary Materials) were identified as short-chain alcohol dehydrogenase/reductase using the inquiry sequence in BLAST searches. The two genes were clustered and analysed with the known SDR genes, which showed that they were closely related to the genes involved in the secondary metabolism of *H. coronarium*, so these two genes were chosen to carry out further research related to the secondary metabolism of *H. coronarium*. Biological software Primer Premier 5.0 was used to design specific primers (Table S2 in Supplementary Materials) to clone the full cDNA sequences. The primers were synthesised by Shanghai Sangon Biological Engineering Co., Ltd. The full-length cDNAs were amplified using high-fidelity DNA polymerase KOD-Plus (TOYOBO) following the manufacturer's recommendation. The PCR conditions were as follows: 94°C for 2 min, followed by 35 cycles at 98°C for 15 s, 55°C for 30 s, 68°C for 2 min. The product was ligated into the pMD-19 vector after the A base was added on 3' end by TaqDNA polymerase. The obtained plasmid DNA was sequenced.

The predicted protein sequences alignment was performed using the DNAssist version 2.2 software; the identity value was calculated by DNAMAN version 5.2.2 software and the construction of a phylogenetic tree was conducted by alignment on ClustalX at first, followed by neighbour-joining with MEGA software (version 5.05) (Kumar et al., 2004). The numbers at each branch indicate bootstrap percentages from 1,000 replicates. The evolutionary distances were computed using the model of Poisson correction. Positions containing gaps and missing data were eliminated. The scale bar indicates 20% sequence divergence. GenBank accession numbers are shown in Table S3 in Supplementary Materials.

An identity search for translated amino acids was conducted using the National Center for Biotechnology Information (NCBI) BLAST network server (<http://www.ncbi.nlm.gov/BLAST>).

Quantitative real-time PCR assays

Primers for quantitative real-time PCR were designed using Primer Premier version 5.0 (Table S4 in Supplementary Materials), and the dissolution curve suggested that only a single PCR product was amplified by each pair of primers. To ensure the specificity of the primers for amplification of the target gene, we further

sequenced the PCR product of each pair of primers. *GAPDH* was used as the internal control gene in this study.

Real-time RT-PCR was performed in a volume of 20 μ l containing SYBR Premix Ex Taq™ with an ABI 7500 real-time PCR system. The PCR conditions were as follows: 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, annealing at 55°C for 30 s, elongation at 72°C for 30 s. The melting curve was designed to increase 0.4°C every 10 s from 72 to 94°C. All real-time PCR analyses were performed in triplicate with different cDNAs synthesised from three biological replicates. The amplicon was clarified using electrophoresis and sequenced once for identity confirmation. Quantification was done for the analysis of the threshold cycle (Ct) value. According to Manríquez's methods, the relative expression ratios were quantified (Manríquez et al., 2006).

Heterologous expression of HcADH2 and HcADH3 in Escherichia coli

The entire open reading frame of HcADH2 and HcADH3 were constructed using a pET30a vector to express HcADH2 and HcADH3, respectively, as a His-tagged protein (Table S2 in Supplementary Materials). Expression, solubility analysis and purification of the target protein were performed using Yue's methods (Yue et al., 2014). The recombinant plasmid pET30a-HcADH2 and pET30a-HcADH3 were transformed into *E. coli* Rosetta (DE3) competent cells (Invitrogen). Single positive colonies verified by sequencing were inoculated at 37°C overnight, shaking continuously in 5 ml Luria Bertani (LB) liquid medium supplemented with 0.1 mg $\cdot$ ml⁻¹ kanamycin. Then the grown cultures were diluted 1:50 with LB medium under the same conditions as above until OD600 of 0.4–0.5 at 37°C was achieved. Cultures were then induced with 0.05 mM isopropyl-1-thio- β -D-galactoside (IPTG) and incubated for an additional 14 h at 16°C and 180 rpm with shaking. The cells were separated from the medium by centrifugation (5,000 g, 5 min, 4°C) and resuspended in 5 ml lysis buffer (50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 10 mM imidazole). Cells were then sonicated three times (work 3 s with 3 s intervals at 4°C, repeated 30 times) and the resulting suspension was centrifuged at 12,000 g and 4°C for 10 min. The obtained crude extract was loaded onto a Ni-NTA His-Bind Resins (Novagen) to purify His-tagged recombinant protein according to manufacturer's introduction. Partially purified proteins were dialysed against a buffer containing 30 mM 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) (pH 7.5) and 5 mM dithiothreitol (DTT). The purity of the recombinant protein from each elution was estimated using sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE).

Enzyme assay in vitro and production identification

The enzyme assays were performed in a total volume of 1 ml with 20 mM KH₂PO₄, pH 8.0, 50 μ g of an enzyme,

1 mM NAD(H), 10 mM EDTA and 0.1 mM substrate. The mixture was incubated for 1 h at 37°C with shaking (60 rpm), and the reaction was immediately stopped by the extraction of ethyl acetate (150 μ l).

Headspace extraction and injection were conducted using a solid-phase microextraction (SPME) Fiber Assembly 50/30 μ m divinylbenzene/Carboxen™/polydimethylsiloxane (PDMS) (Stable Flex™ for manual holder grey). After incubation, the PDMS fibre was injected into a gas chromatography-mass spectrometer (GC-MS) system for analysis. The GC-MS analyses were performed using an Agilent 7980A GC/5975C MSD. The instrument was equipped with an Agilent DB-5MS capillary column (30 m $\times$ 0.25 mm) and helium as a carrier gas at a constant flow of 1 ml $\cdot$ min⁻¹. The oven temperature was initially maintained at 40°C for 2 min, followed by an increase to 250°C at a rate of 5°C $\cdot$ min⁻¹, and held at 250°C for 5 min. The products were identified by comparison of mass spectra and retention time with authentic standards with the NIST2008 mass spectral library. Controls were carried out in the absence of NAD(H) and an empty vector.

Headspace analysis of floral volatiles

According to Chen's methods (Chen et al., 2019), *H. coronarium* flowers from each stage were enclosed in a 100 ml glass bottle with the addition of ethyl decanoate as an internal standard. The flower volatiles were absorbed by PDMS (with 50/30 μ m divinylbenzene/Carboxen) fibre (Supelco) for SPME for 30 min. Then, the fibre was injected into a GC-MS system (Agilent) for analysis as is described earlier.

RESULTS

Cloning and sequence analysis of HcADH2 and HcADH3

Among the transcriptome of *H. coronarium*, there are 10 sequences of SAD, of which comp45280_c0 and comp41433_c0 are highly expressed. So these two clones were selected and identified as short-chain alcohol dehydrogenase/reductase. The PCR amplification was carried out to get the two genes designated as HcADH2 (MK388796.1) and HcADH3 (MK388797.1) using the full-length primer. The nucleotide sequences of 849 and 903 bp, respectively, were obtained (Figures S1 and S2 in Supplementary Materials). The full-length cDNAs of *HcADH2* and *HcADH3* contained putative open reading frames (ORFs) of 834 and 888 bp, which encoded 278 and 296 amino acid residues with predicted molecular weights of 29.00 and 31.08 kDa, respectively.

The TGxxx[AG]xG motif is present in the sequences of HcADH2 and HcADH3, which is highly conserved in almost all SDRs and is responsible for Rossmann-fold scaffold due to its ability to bind NAD(P) dinucleotides (Joernvall et al., 1995; Kallberg et al., 2002) (Figure 2). Both sequences have the typical motif of YxxxK at the

RWR85228	1	MIRDCAFLN-----RLEGKVALITGGCAKGIGEV	29
HcADH2	1	MAADGSNGNA-----VPASPSRRLEGKVAFITGGAAGSGEAT	37
HcADH	1	MER---R-----RLEGKVAFITGGAAGSGEAT	24
BAK09296	1	MLEGGKVALITGGASGIGESI	21
RWR94702	1	MALFLQKALLRNTSRRLMLKLDWNSSRERNKGRQLSTEAGSCSKLAGKVAITGAASGIGRAT	64
HcADH3	1	MLGMALRVKRG---LAIRTGIRTQQ---QQFSTHP-TPARLAGKVAITGAASGVGRAT	52
MOTIF I			
RWR85228	30	ARVFHQHGAKVVIADIEDDLGLHLVCKDGG-KNAASYVHCDVSKEVDVKNVAVDATVAKFGKLDIM	92
HcADH2	38	ARLFVLHGAKVIGDVRDEFGRAAASSIGGEDVITYVHCDVSKEADVERAVDLAVAKYGRLDIM	101
HcADH	25	VKLFVRHGAKVIVADLQDDKGKALCADLGSDDVASYVHCDVRCESDVKRAVDATVSLHGKLDIM	88
BAK09296	22	ARLFIEHGAKICIVDVQDELQQVVSQRGGDPHACYVHCDVTVEDDVRRAVDFTAEKYGTIDIM	85
RWR94702	65	ASEFIRNGARVVIADVQRQLGEETAKEIG--PNAAFISCDVTQSEISAAIDFAISEHGRLDIM	126
HcADH3	53	AAEFIIHGAQVVLADIQHELKGSVAABIG--PGATFVPCDVTQEPQVAAVVDLAVAKHGRLDIM	114
MOTIF II			
RWR85228	93	FNNAGIVDPWKSNIITNNE--EADFEKVLGVNVKG/VFLGTKHAARVWVPVK-RGSIINNGSIVSV	153
HcADH2	102	FSNAVLDRCRG-VAVAE--ADDFDRVMVNVRG/VFLGTKHAARVMAAGVRGSIINNGSVATV	162
HcADH	89	FNNAGISDPGSSILGEEDTTAVFERVMGVNVLGALLGTKHAGRAMVAAGRGSIIITASLASV	152
BAK09296	86	VNNAGITGDKVIDIRDAD--FNEFKKVEDINVNG/VFLGMKHAARIMIPKM-KGSIVSLASVSV	146
RWR94702	127	YNNAGISGSVVFSITDLE--LSEFDKIMNINVRG/VAGIKHAARVMIIPRR-TGSIILCTASITGI	187
HcADH3	115	YNNAGICGPMTFAVTDVE--LTEFDRVMVNVRS/VAGIKHAARVMIIPRR-AGSIIILCTASITGI	175
MOTIF III			
RWR85228	154	IGGIASHAYVASKHAVLGLTKNAAAEELGQYGRVNCISSEFVLASSITTNFIS---KDDNEIESW	214
HcADH2	163	VAGVASHAYVASKHAVLGLTRSAAEELGQHGIRVNCVSPFAYATSLACDFIH---MDQKQIEQF	223
HcADH	153	VGALGHPVYSCSKHAVLGLARSAAAEELGKHGVRVNCVSPAIVATPLTVSYYQ---TSEEELEGL	213
BAK09296	147	IAGAGPHGYTGAKHAVVGLTKSVAAEELGRHGIRVNCVSPYAVPTRLSMPYLPESMEQEDALRGF	210
RWR94702	188	MGGLSPHAYSISKFATAGVVKSAASELCKYGRVNCISPFAPTEPSTDPMS-VLYPGLDESGV	250
HcADH3	176	VGGIAPLAYSLSKAAVAAVRLSAAELSKHGIRVNCISPASLPTEFGIKAIR-EILPDLEEQRA	238
MOTIF III			
RWR85228	215	ARRI-----ANLKGVLTLNEDLALAAALYLGSDESGYVSGHNFVLDGGFSIVNPSFGLFKQNP	271
HcADH2	224	IGAV-----SNLKGAVLRDDVARAAVYLASDESCYVSGQNIIDGGFTAVNHAFGLFKNS	279
HcADH	214	GEMI-----STLKGVLRLKEDIAEAALFLASDESFRVSGHNLMDG-ASGVTKIFS	263
BAK09296	211	ITFVRS-NANLKGVDLMENDVAEAVLYLATEESKYVSGNLNVLDGGFSIANHTLQVFE	267
RWR94702	251	VEITIQG-AGVLKGANCEVDIAKAAVYLASDDAKYVSGHNLVVDGGFTVFKSIQFPFPDQ	311
HcADH3	239	VEMIELSSAELAGTKCEDVAKAATFLASDEAKYVSGHNLVVDGGFTTSKRLNFSPE	297

Figure 2. Alignment of the deduced amino acid sequences of HcADH2, HcADH3 and alcohol dehydrogenases/reductases from various plant species. RWR85228 = *Cinnamomum micranthum* SDR; HcADH = *Hedychium coronarium* SDR (KF358245); BAK09296 = *Zingiber zerumbet* SDR; RWR94702 = *Cinnamomum micranthum* secoisolariciresinol dehydrogenase. Grey boxes = identical residues; black boxes = similar residues; black triangle = the aspartate at position 52 of HcADH2 and position 67 of HcADH3. SDR, short-chain dehydrogenase/reductase.

active site (Kavanagh et al., 2008). The NAG motif is present in the sequences of HcADH3, while the NAA domain is present in the sequences of HcADH2. The NAG domain is responsible for substance specificity (Oppermann et al., 1997; Filling et al., 2002), which suggests that HcADH2 may catalyse a reaction of substances, different from HcADH3, thus playing a different role in *H. coronarium*. The aspartic acid at position 52 (Asp52) of HcADH2 motifs and the Asp67 of HcADH3 motifs suggest that these two enzymes may prefer NAD over NADP as a coenzyme (Kallberg et al., 2002) (Figure 2).

Bioinformatics analysis of HcADH2 and HcADH3

Alignment of the amino acid sequence revealed that HcADH2 and HcADH3 share 41% identity. They exhibit high identity with SDRs from other species (Figure 2). For example, HcADH2 shows 49% sequence

identity to our identified HcADH (Chen et al., 2019) of *H. coronarium* (Genbank KF358245), while HcADH3 shows 42% sequence identity. HcADH2 shows 59% sequence identity to SDR (Genbank RWR85228.1) and 42% sequence identity to secoisolariciresinol dehydrogenase (SDH) (Genbank RWR94702.1) of *Cinnamomum micranthum* f. *kanehirae*, while HcADH3 shows 47% and 60% sequence identity, respectively (Chaw et al., 2019). HcADH2 and HcADH3 show 49% and 43% sequence identity to the SDR of *Z. zerumbet* (GenBank BAK09296.1), respectively (Figure 2) (Okamoto et al., 2011).

Phylogenetic analysis

Phylogenetic analysis showed that HcADH2 and HcADH3 from *H. coronarium* belonged to a member of the short-chain dehydrogenases/reductases (SDRs) 110C subfamily (Figure 3). HcADH2 and our identified terpene-modified HcADH (Chen et al., 2019) were distinctly clustered in the same clade, whereas

Figure 3. Phylogenetic tree of HcADH2 and HcADH3 with known alcohol dehydrogenase/reductase from other species. Aa = *Artemisia annua*; Ao = *Asparagus officinalis*; At = *Arabidopsis thaliana*; Cm = *Cinnamomum micranthum*; Chl = *Chasmanthium latifolium*; Cl = *Citrullus lanatus*; Cr = *Catharanthus roseus*; Hc = *Hedychium coronarium*; Le = *Lycopersicon esculentum*; Ma = *Musa acuminata*; Mc = *Macleaya cordata*; Mp = *Mentha x piperita*; Nt = *Nicotiana tomentosiformis*; Ol = *Orthocladia laxa*; Os = *Oryza sativa*; Pa = *Parasponia andersonii*; Pb = *Papaver bracteatum*; Pd = *Phoenix dactylifera*; Pm = *Persicaria minor*; Ps = *Paeonia suffruticosa*; Rc = *Ricinus communis*; St = *Solanum tuberosum*; Td = *Tripsacum dactyloides*; Zz = *Zingiber zerumbet*. Scale bars = 20% sequence differences.

HcADH2 distinctly showed a close relationship with several known SDR, such as *Musa acuminata* subsp. *malaccensis* SDH (68% identity), *C. micranthum* f. *kanehirae* SDH (53% identity) (Chaw et al., 2019), *Macleaya cordata* SDR (56% identity) (Liu et al., 2017), *Artemisia annua* glucose/ribitol dehydrogenase (GRD) (53% identity) (Shen et al., 2018), *Asparagus officinalis* momilactone A synthase (MAS) (60% identity) (Figure 3). However, HcADH3 was distinctly clustered in another clade, which showed close relationship with *C. micranthum* f. *kanehirae* SDH (60% identity)

(Chaw et al., 2019), *O. sativa japonica* group MAS (56% identity), *Ricinus communis* SAD (56% identity) and *S. tuberosum* zerumbone synthase (ZSR) (57% identity).

Expression analysis of HcADH2 and HcADH3 genes in different tissues

The real-time RT-PCR analysis showed that *HcADH2* was most highly expressed in bracts. *HcADH2* was also expressed at lower levels in stem, root and rhizome, but it was almost undetectable in leaves and flowers (Figure 4A).

As shown in Figure 4B, transcripts of *HcADH3* were preferentially expressed in flowers, with lower expression in the stem, roots and rhizome, leaf and bract. *HcADH3* transcripts were highly expressed in the lateral petal of the flower, with successively lower expression in the style and stigma, sepal, filament, outer labellum and scant expression in the pedicel and anther (Figure 4C). This result showed that different SDRs genes had differential expression patterns.

Expression analysis of HcADH2 and HcADH3 in different species

The mRNA expression of *HcADH2* and *HcADH3* was analysed in blooming flowers of different species in the *Hedychium* genus (Figure 5A). *HcADH2* gene was highly expressed in no-scented *H. forrestii* and at a very low level in *H. gardnerianum* and *H. coronarium*. It was barely expressed in *H. coccineum*. The highest expression of *HcADH3* was noted in the very scented *H. coronarium*, and only a little expression was observed in *H. forrestii*, *H. gardnerianum* and *H. coccineum* (Figure 5B). *HcADH3* perhaps has a certain correlation with flower fragrance.

Expression analysis of HcADH3 during flower development

The lifespan of a floret in a *H. coronarium* inflorescence is usually only 3 days. During flower development, there was almost scant expression of *HcADH3* mRNA transcripts in petals at 40, 32, 24 and 16 h pre-anthesis (Figure 6). Then, the *HcADH3* expression levels increased sharply at 8 h pre-anthesis (the half opening stage), peaked at this stage and then decreased with the flower development (Figure 6), while the relative terpenes volatile aroma contents also increased sharply at 8 h pre-anthesis (the half opening stage) but peaked at 0 h (the opening stage) stage and then decreased with the flower development (Figure S3 in Supplementary Materials). This may be because the gene expression comes first and the plant traits are displayed later.

HcADH2- and HcADH3-induced expression by mechanical wounding and MeJA treatment in leaves

Both *HcADH2* and *HcADH3* tissue-specific expressions had almost no expression in leaves. Mechanical

Figure 4. The expression analysis of *HcADH2* and *HcADH3* in different tissues of *H. coronarium*. (A) The *HcADH2* relative expression analysis in different tissues using qRT-PCR. (B) The *HcADH3* relative expression analysis in different tissues using qRT-PCR. (C) The *HcADH3* relative expression analysis in different parts of flower using qRT-PCR. OL = outer abellum; Se = petal; LP = lateral petal; Pe = pedicel; An = anther; Fi = filament; SS = style and stigma. Numbers = $\pm$ SD ($n = 3$). Asterisks = statistically significant differences compared with the ones with the highest expression level (* $p < 0.05$; ** $p < 0.01$).

wounding and MeJA treatment caused a significant increase in *HcADH2* mRNA levels in *H. coronarium* leaves (Figure 7A and 7B). The former exhibited a fivefold increase at 3 h in *HcADH2* mRNA levels and the latter showed a 21-fold increase at 12 h compared with those in natural leaves. As shown in Figure 7C, mechanical wounding caused an irregular fluctuation in *HcADH3* mRNA levels in *H. coronarium* leaves, while MeJA treatment induced a significant increase in *HcADH3* mRNA levels which exhibited a sixfold increase at 12 h compared with those in natural leaves (Figure 7D). The results showed that *HcADH2* was an inducible gene in the leaves which might play a defensive role in vegetative organs. However, *HcADH3* expression can only be induced by MeJA treatment but not by mechanical wounding. This suggested that MeJA

induced *HcADH3* expression via the signal pathway other than injury response-related pathways.

The substrate catalysis characterisation of the purified recombinant *HcADH2* and *HcADH3*

The coding regions of *HcADH2* and *HcADH3* were expressed in *E. coli*, and their activity was analysed *in vitro*. The recombinant proteins of both were purified by Ni-affinity chromatography, and their activity was analysed *in vitro*. This experiment focused only on the selection of representatives of different structures of terpenoids as substrates, such as monoterpenoids (geraniol, camphor, linalool) and sesquiterpenoids (farnesol), for catalytic reactions (Table S5 in Supplementary Materials). The purified recombinant *HcADH3* protein can convert geraniol into citral (Figure 8). However, *HcADH3* could

Figure 5. The expression analysis of *HcADH2* (A) and *HcADH3* (B) in different species of *Hedychium*. 1 = *H. gardnerianum*; 2 = *H. forrestii*; 3 = *H. coronarium*; 4 = *H. coccineum* Buch.–Ham. Numbers = $\pm$ SD ($n = 3$). Asterisks = statistically significant differences compared with the ones with the highest expression level (* $p < 0.05$; ** $p < 0.01$).

Figure 6. The expression analysis of *HcADH3* in different developmental stages of *H. coronarium* flower. -40 = 40 h pre-anthesis; -32 = 32 h pre-anthesis; -24 = 24 h pre-anthesis; -16 = 16 h pre-anthesis; -8 = 8 h pre-anthesis; 0 = anthesis (0 h); 8 = 8 h post-anthesis. Numbers = $\pm$ SD ($n = 3$). Asterisks = statistically significant differences compared with the ones with the highest expression level (* $p < 0.05$; ** $p < 0.01$).

not utilise other terpenoids, including camphor, linalool and farnesol, as substrates, while purified recombinant HcADH2 protein could not catalyse geraniol, camphor, linalool and farnesol as substance. These results suggested that the coding region of *HcADH3* cDNA encoded a functional alcohol dehydrogenase/reductase and might participate in dehydrogenation reactions of monoterpenoids, while recombinant HcADH2 protein could not encode or participate.

DISCUSSION

SDRs are divergent NAD(H)- or NADP(H)-dependent enzymes including five major types, namely classical,

extended, intermediate, divergent and complex. They have a wide range of substrate spectra (Kallberg et al., 2002). HcADH2 and HcADH3 belong to the ‘classical’ subfamily of the SDR superfamily. HcADH2 and HcADH3 are an SDR110C, identified on <http://www.sdr-enzymes.org> (Persson et al., 2009), participating in many important secondary metabolisms (Kitaoka et al., 2016).

The temporal and spatial expression patterns of the *HcADHs* can provide insight into the potential roles of a short-chain alcohol dehydrogenase in *H. coronarium*. In higher plants, the function of the short-chain alcohol dehydrogenase gene should be related to gene expression, especially in different tissues, for example, the seeds (Kim et al., 2003), fibres, roots and stems (Pang et al., 2010). The analysis of fragrance-related gene expressions showed that petal epidermal cells had been proposed as the sites for scent production and emission (Vainstein et al., 2001). *PsDFR1* showed the highest transcript abundance in petals, moderate levels in sepals and stamens, low level in leaves and the lowest level in carpels from petals of tree peony (*Paeonia suffruticosa* ‘Cai Hui’) (Zhou et al., 2011). The expression levels of Cucumber (*Cucumis sativus* L.) 18 CsSDR110C genes were different in roots, stems, leaves, male flowers, fruits and tendrils. Among these genes, the expression levels of some CsSDR110C genes can be increased in the light-induced cucumber seedlings dechlorosis process (Wang, 2014). The *HcADH3* gene was also highly expressed in the lateral petal of flower tissues and very low in stem, roots and rhizome, leaf and bract, which implies that *HcADH3* might be a flower-related expression gene.

The expression of qRT-PCR transcript was examined at different floral developmental stages to further understand the potential role of *HcADH3*. In maize (*Zea mays*), a SDR protein, TASSELSEED2 (TS2) can regulate the unisexual flower development (Malcomber and Kellogg, 2006). There was a report on

Figure 7. Expression analysis of *HcADH2* and *HcADH3* mRNA levels in response to external environmental stimuli. (A) *HcADH2* expression at 0–36 h after wounding. (B) *HcADH2* expression analysis of MeJA treatments and natural leaves (control) at different times. (C) *HcADH3* expression at 0–36 h after wounding. (D) *HcADH3* expression analysis of MeJA treatments and natural leaves (control) at different times. Numbers = $\pm$ SD ($n = 3$). Asterisks = statistically significant differences compared with the ones with the highest expression level (* $p < 0.05$; ** $p < 0.01$).

the molecular cloning of GLUCOSE INSENSITIVE1 (GIN1) and ABSCISIC ACID DEFICIENT2 (ABA2), which encoded a unique *Arabidopsis* SDR1. SDR1 was involved in sex determination in maize (Cheng et al., 2002). The *HcADH3* expression levels peaked at 8 h pre-anthesis (the half opening stage), but the expression levels were almost scant at 40, 32, 24 and 16 h pre-anthesis. The *HcADH3* was perhaps involved in related secondary metabolic pathways of *H. coronarium*.

In *Hedychium*, *H. coronarium* is very scented through olfactory sensory evaluation (Fan et al., 2007), but some species, such as *H. coccineum* and *H. forrestii*, have lot of ornamental traits but not scented through olfactory sensory evaluation. *H. gardnerianum* is with faint scent (Wu and Raven, 2001). The transcript level of *HcADH* in the scent species (*H. coronarium*) was highest, but the gene was rarely expressed in the scentless species, such as *H. coccineum* and *H. forrestii*. The change in their expression levels in different species of the *Hedychium* genus was positively correlated with the emission pattern of allo-ocimene (Chen et al., 2019). *HcADH3* genes are highly expressed in the flower

of *H. coronarium*, which illustrates that *HcADH3* is perhaps a flower fragrance-related expression gene, while *HcADH2* genes showed vastly different expression characteristics. But the reasons for the *HcADHs* expression difference in *Hedychium* remain to be further verified experimentally.

In plant defence mechanisms, mechanical injury and MeJA treatments are general methods (Walter, 1992; Weeden and Provvidenti, 1988). Two SDRs *PmADHa* and *PmADHb* were conducted under the exogenous ABA treatment and drought stress in leaves, stems and roots. Exogenous ABA treatment showed that the high expression of *PmADHs* was tested only in roots, whereas the expression of *PmADHa* and *PmADHb* were upregulated in response to drought stress in all organs (Hamid et al., 2018). The expression of SDR1 (unique SDR) showed the dynamic mobilisation of ABA precursors and/or ABA in *Arabidopsis* (Cheng et al., 2002). SalR was a member of SDRs that were active as monomers and possess an extended amino acid sequence compared with classical SDRs from the opium poppy. Amino acid residues

Figure 8. Analyses of products generated by the recombinant HcADH3 enzyme with geraniol as substrate. (A) Total ion chromatogram of the products obtained from the empty vector pET30a. (B) Total ion chromatogram of products obtained from pET30a-HcADH3 with NAD (peak 1, geraniol; peak 2, citral). (C) Mass spectrum of peak 2 (citral). (D) Mass spectrum of authentic citral in the NIST08 library.

conferring salutaridine binding non-specifically reduce (–)-menthone to (+)-neomenthol, which showed that some of these proteins are involved in plant defence (Ziegler et al., 2009). Our results suggested that treatments with mechanical wounding and MeJA in leaves of *H. coronarium* stimulated the expression of *HcADH2*, but *HcADH3* expression was induced only by MeJA treatment. It is possible, therefore, that *HcADH2* and *HcADH3* also have a defensive function in *H. coronarium* vegetative organs.

Hassan et al. (2012) reported that DH I and II *Polygonum minus* geraniol dehydrogenase can catalyse the oxidation of geraniol to geranial, and they might be involved in monoterpene alcohol metabolism and also have their high specificity for allylic alcohols. The recombinant HcADH3 enzyme can use monoterpenoids geraniol as a catalytic substrate. No enzymatic product

was synthesised from camphor, linalool or farnesol, indicating that both the number of carbon bonds of the aliphatic chain and the position of the hydroxyl group, cyclic and acyclic, are crucial for the formation of enzymatic products. This result was in agreement with the study by Hassan et al. However, differently, ZSD1 from *Z. zerumbet*, a member of the SDR superfamily, is involved in phytosteroid biosynthesis. ZSD1 can convert borneol to camphor (Okamoto et al., 2011). Polichuk et al. (2010) analysed that ADH2 belongs to a member of the short-chain alcohol dehydrogenase/reductase (SDR) superfamily. ADH2 showed a strong preference for monoterpene secondary alcohols including carveol, borneol and artemisia alcohol. The short-chain alcohol dehydrogenase/reductase GcSDR was expressed as a recombinant protein in *E. coli* BL21 (DE3) pLysS. Kinetic analysis of recombinant GcSDR

using pyruvaldehyde dimethyl acetal as a substrate has a K_m value of 116.52 mM and a V_{max} value of 720 nmol product formed per minute per milligram (Naseron et al., 2014). The GLYCOALKALOID METABOLISM25 (GAME25) from tomato is a SDR and it catalyses to reduce the C-5,6 double bond in dehydrotomatidine to form tomatidine. The recombinant GAME25 enzyme displayed 3 β -hydroxysteroid dehydrogenase isomerase activity on diverse steroidal alkaloid aglycone substrates and steroidal saponin aglycones (Sonawane et al., 2018). It will perhaps lay a theoretical basis to understand secondary metabolism of *H. coronarium*.

CONCLUSIONS

We have first identified two short-chain alcohol dehydrogenases/reductases in *H. coronarium*. They play different functions in *H. coronarium*. HcADH2 and HcADH3 have an induced expression in the vegetative organ of *H. coronarium*, but HcADH3 is also a flower-related expression gene and HcADH3 has catalytic activity on monoterpene and converts geraniol into citral. Our results would provide new insights into secondary metabolism of *H. coronarium* flowers.

NOTES

The nucleotide sequence reported in this article has been submitted to GenBank database with accession number, HcADH2 = MK388796.1 and HcADH3 = MK388797.1.

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AUTHOR CONTRIBUTIONS

H.C. contributed to all experiments and manuscript preparation. H.C. and Y.F. designed the overall study. Y.Y. performed collection of headspace volatiles and GC-MS analysis. R.Y. performed quantitative real-time PCR assays. Y.F., Y.Y. and R.Y. helped develop the manuscript text.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPLEMENTARY MATERIALS

Figure S1. The RT-PCR amplification products of *HcADH2* and *HcADH3*. M = DNA DL 2000 Marker; 1 = *HcADH2* PCR product; 2 = *HcADH3* PCR product.

>MK388796.1(*HcADH2*)

CCGGCCTGTTGCCATAAACCAAGTAGCTCGTCGTTGTGTTTGGAGAGAGGAGAGGAGGAAGGTAG
 ATAGCGAATTAAGGATTATTATCGTCTTCCACTCAGATGGCGGCTGACGGCAGCAACGGTAATGCAGT
 CCCTGCTTCTCCTTCTCGGAGGTTGGAAGGCAAGGTAGCTTTCATAACCGGCGGAGCCGCCGGTTC
 CGGCGAGGCCACCGCGCGGCTCTTCGTTCTCCACGGCGCGAAGGTGGTGATCGGCGACGTGCGC
 GACGAGTTCGGCCGCGCAGCGGCGTCCAGCATTGGCGGCGAGGACGTGATCACCTACGTGCACTG
 CGACGTGTCCAAGGAGGCAGACGTGGAGCGCGCCGTCGACCTGGCGGTGGCCAAGTACGGCCGG
 CTCGATATCGTTTTAGCAACGCGGCCGTGCTCGACGAATGCCGCGGCGTCGCCGTGCGGGAGGCC
 GACGACTTCGACCGCGTGATGGCGGTCAACGTTGGGGCGTCTTCCTGGGTACCAAGCACGCCGC
 GCGCGCCATGATGGCGGCGGGCGTGCGGGGAAGCATCATCAACAACGGCAGCGTGGCAACGGTGG
 TGGCCGCGGTGGCGTCGCACGCCTACGTGGCGTCAAGCACGCGGTGCTGGGGCTGACGCGGAG
 CGCGGCGGCGGAGCTCGGCCAGCACGGCATCCGCGTCAACTGCGTGTCGCCTTTCGCGTACGCCA
 CGTCGTTGGCCTGCGACTTTATTCACATGGACCAGAAACAGATCGAGCAGTTCATCGGCGCAGTCAG
 CAACCTCAAGGGAGCGGTGCTGAGGGCGGACGACGTGGCGCGCGCCGCTGTCTACCTGGCCAGC
 GACGAGTCTTGCTACGTGACGCGGACAGAACATCATTATCGACGGCGGCTTTACCGCCGTCAACCACG
 CCTTTGGCCTCTTCAAGAACGAGCGATCCCTCGGATCCAGCTGGATTACCTTATGTCGGTGACGGT
 CCGAAAAATGATTATGTGCTATTCAATTGCTCGTCTAAGTTGCTGGTCTTGCTGTGCTTT

>MK388797.1(*HcADH3*)

TTGACAGAAACCATGCTCGGAATGGCTTTGAGAGTTAAGAGGGGATTGGCGATCAGGACTGGAATTA
 GAACGCAACAGCAGCAGTTCTCCACCCACCCAATCCTGCGAGGTTAGCCGGCAAAGTCGCGATCA
 TCACCGGCGCCGCCAGCGGCGTCGGGAGGGCAACAGCCGCAGAGTTCATCCACCACGGCGCACA
 GGTGCTCCTCGCAGACATCCAGCACGAGCTCGGCAAGTCTGTTGCCGCCGAGCTCGGCCCCGGCG
 CCACCTTCGTCCCTTGCGACGTCACCCAGGAGCCCCAAGTCGCTGCCGTGTCGACCTCGCGGTC
 GCCAAGCACGGACGCCTGGACATCATGTACAACAACGCCGGCATCTGCGGACCCATGACCTTCGCC
 GTCACCGACGTGACCTCACCGAGTTCGACCGCGTCATGGCCGTCAACGTCCGGTCAGTGGTGGCA
 GGGATCAAACACGCGGCGCGGGTGATGATCCCGCGCCGCGCCGGGAGCATCCTCTGCACGGCGAG
 CATCACCGGGTTTGTCGGCGGTCTGGCGCCGCTCGCCTATTCTGTGCAAGGCGGCGGTGGCGG
 CTGCAGTGCGGTTATCCGCGGCGGAGCTGAGCAAGCACGGGATAAGGGTGAATTGCATCTCGCCGG
 CCTCGCTGCCGACGCCATTGGGATCAAAGCAATCAGGGAAATCCTCCCTGATTGGAAGAGCAGCG
 GGCGGTGGAGATGATCGAGCTGTCTCGGCGGAGTTGGCGGGGACGAAGTGCAGAGTCAAGACG
 TGGCGAAGGCAGCGACGTTCTGGCTTCAGACGAAGCTAAGTACATCAGCGGGCATAATCTCATGGT
 GGATGGAGGATCACTACATCTAAACGCTTAAATTTTTCACCAGAGTGA

Figure S2. Full-length sequence of *HcADH2* and *HcADH3* genes. Nucleotide sequences with green colour = the primer sequences for full cDNA synthesis. Nucleotide sequences with red colour = initiator codon (ATG), terminators (TGA).

Figure S3. Relative terpenes volatile aroma contents of *Hedychium coronarium* during flower development. Numbers = $\pm$ SD ($n = 3$). Asterisks = statistically significant differences compared with the ones with the highest expression level (* $p < 0.05$; ** $p < 0.01$).

Table S1. Amino acid residues sequences of comp45280_c0 and comp41433_c0

Name	Amino acid residue sequences	Name	Amino acid residue sequences
comp45280_c0	MAADGSNGNAVSPSRRLLEGKV	comp41433_c0	MLGMALRVKRGLAIRTGIRTQQQFSTH
	AFITGGAAGSGEATARLFVLHGAK		PTPARLAGKVAIITGAASGVGRATAAEFI
	VVIGDVRDEFGRAAASSIGGEDVIT		HHGAQVVLADIQHELGKSVADELPGAT
	YVHCDVSKEADVERAVDLAVAKY		FVPCDVTQEPQVAAVVDLAVAKHGRLDI
	GRLDIVFSNAAVLDECRGVAVAEA		MYNNAGICGPMTFAVTDVDLTFEDRVM
	DDFDRVMAVNVVRGVFLGTKHAAR		AVNVRSVVAGIKHAARVMIPRRAGSILC
	AMMAAGVRGSIINNGSVATVVAG		TASITGFVGGGLAPLAYSLSKAAVAAVRL
	VASHAYVASKHAVLGLTRSAAAEL		SAAELSKHGIRVNCISPASLTPFGIKAIRES
	GQHGIRVNCVSPFAYATSLACDFIH		ILPDLEEQRVEMIELSSAELAGTKCEVED
	MDQKQIEQFIGAVSNLKGAVLRAD		VAKAATFLASDEAKYISGHNLMDVGGFT
	DVARAAVYLASDESCYVSGQNIH		TSKRLNFSPE
	DGGFTAVNHAFLFKN		

Table S2. Primer sequences of *HcADH2* and *HcADH3* used in RT-PCR assays

Methods	Forward primer	Reverse primer	PCR product size (bp)
RT PCR	<i>HcADH2</i> TCTTCCACTCAGATGGCGGCT	TCAGTTCTTGAAGAGGCCAAA	849
	<i>HcADH3</i> TTGACAGAAACCATGCTCGGAAT	TCACTCTGGTGAAAAATTTAAGCG	903
PET-30a:HcADH2 vector	CGCGGATCCATGGCGGCTGACGG	CCCAAGCTTTCAGTTCTTGAAGAGGCCAAAAG	
PET-30a:HcADH3 vector	CGCGGATCCATGCTCGGAATG	CCCAAGCTTTCAGTTCTTGAAGAGGCCAAAAT	

Table S3. Sequences description of accession numbers in GenBank on the phylogenetic tree

Accession numbers	Name	Accession numbers	Name
AAB57737.1	Short-chain alcohol dehydrogenase	KM515811.1	Short-chain dehydrogenase/reductase
AAM10204.1	Tropinone reductase	KM515812.1	Short-chain dehydrogenase/reductase
AAU20370.1	Secoisolaticiresinol dehydrogenase	NP_001233856.1	Short-chain dehydrogenase/reductase
AB018559.1	Short-chain alcohol dehydrogenase	OVA14417.1	Short-chain dehydrogenase/reductase
ACZ34296.1	Short-chain alcohol dehydrogenase/reductase	PON43718.1	Short-chain dehydrogenase/reductase
AJO70763.1	Alcohol dehydrogenase	PWA82834.1	Glucose/ribitol dehydrogenase
AK103462.1	Short-chain alcohol dehydrogenase/reductase	RWR74505.1	Short-chain dehydrogenase/reductase
BAG96093.1	Short-chain alcohol dehydrogenase/reductase	RWR85228.1	Short-chain dehydrogenase/reductase
BAG99023.1	Short-chain alcohol dehydrogenase/reductase	RWR94702.1	Secoisolaticiresinol dehydrogenase
BAK09296.1	Short-chain alcohol dehydrogenase/reductase	XP_006366369.1	Zerumbone synthase
DQ384222.1	Short-chain dehydrogenase/reductase	XP_008811702.1	Momilactone A synthase
DQ384263.1	Short-chain dehydrogenase/reductase	XP_009404353.1	Secoisolariciresinol dehydrogenase
EEF41657.1	Short chain alcohol dehydrogenase	XP_009587781.1	Short-chain dehydrogenase/reductase
EF184229.1	Salutaridine reductase	XP_015645551.1	Momilactone A synthase
HQ283448.1	Dihydroflavonol 4-reductase	XP_018675432.1	Secoisolariciresinol dehydrogenase
KF358245	Short-chain dehydrogenase/reductase	XP_020246099.1	Momilactone A synthase

Table S4. Primer sequences used in quantitative real-time PCR assays and their characteristics

Gene name	Primer (forward/reverse) 5'→3'	Amplicon Tm (°C)	Amplicon length (bp)	Amplification efficiency (%)	R2
<i>GAPDH</i>	TAACATCATTCCTCCAGCAGCACT GGTGGATCTCACTGTCAGGCTC	83.20	136	94.8	0.998
<i>HcADH2</i>	TGCTACGTCAGCGGACAGAAC GGACCGTCACCGACATAAGG	90.71	128	79.4	0.991
<i>HcADH3</i>	GTCAGTGGTGGCAGGGAT CAGAGAATAGGCGAGCGG	85.7	124	99.7	0.988

Table S5. Substrate of enzyme catalysed reaction

Substrate	Number of carbon	Structure
Geraniol	C10
Farnesol	C15
L-Linalool	C10
Camphor	C10