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Original Paper

Biosynthesis of geranial, a potent aroma compound in ginger rhizome (*Zingiber officinale*): Molecular cloning and characterization of geraniol dehydrogenase

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Abstract Fresh ginger (*Zingiber officinale*) rhizome is characterized by a pleasant citrus aroma and pungent flavor. The majority of the aroma-contributing volatiles are monoterpenoids, especially geraniol derivatives such as geranial, geranyl acetate, geraniol and citronellol. In this study, we investigated the interconversion of geraniol derivatives by incorporation experiments using deuterium-labeled geraniol and geranyl acetate. GC-MS analysis revealed that the incorporated geraniol and geranyl acetate were transformed and detected as geranial, geraniol, geranyl acetate and citronellol; however, nerol and neral were hardly detected. Next, we isolated and characterized a cDNA encoding geraniol dehydrogenase (ZoGeDH) by expressed sequence tag database mining. Phylogenic analysis of *ZoGeDH* resulted in its categorization into the cinnamyl alcohol dehydrogenase (CAD) group, along with the previously reported *GeDHs* of sweet basil (*Ocimum basilicum*) and wild perilla (*P. setoyensis, P. citriodora, and P. frutescens*). The recombinant ZoGeDH catalyzed the NADP-dependent oxidation from geraniol to citral. Furthermore, its substrate specificity was the highest for geraniol and nerol, while that for cinnamyl alcohol was 32% of the activity observed for geraniol. The expression levels of *ZoGeDH* in various ginger plant tissues were in accordance with the accumulation of geranial, except in old rhizome.

Key words: Geraniol dehydrogenase (GeDH), cinnamyl alcohol dehydrogenase (CAD), citral, geraniol, geranial, *Zingiber officinale*, rhizome.

Introduction

The rhizome of ginger (*Zingiber officinale*) is one of the most popular spices throughout the world, and possesses a characteristic lemony aroma and pungent flavor. The flavor intensity and balance are commercially important in the quality evaluation of fresh ginger and its products. The volatiles contained in ginger are composed mainly of monoterpenes (e.g., geranial, geranyl acetate and geraniol), sesquiterpenes (e.g., zingiberenes), and phenyl propenes (e.g., eugenol) (Gong et al. 2004; Govindarajan and Connell 1983). It is known that sesquiterpenes such as α-zingiberene and β-sesquiphellandrene are highly abundant in many ginger cultivars. On the other hand, geraniol-related compounds such as geraniol, geranyl acetate and geranial contribute to the fresh lemony aroma of ginger; further, geranial was reported as the most olfactory aroma-active compound in ginger (Nishimura 1995). While these geraniol-related compounds are structurally similar, they differ in aroma properties and threshold level, indicating that their composition reflects the aroma quality of whole ginger rhizome. The contents and composition of these compounds are dependent on the ginger cultivar, and variable according to cultivation area and maturation stage of the rhizome. Previously, we reported that geranyl acetate drastically decreases with increasing geranial levels during the maturation and storage of ginger rhizome (Sekiwa-Iijima et al. 2001). This suggests that the metabolic fluxes from geranyl acetate to geranial occur through geraniol as a key compound, because both of their structures derive from modification of geraniol.

In plants, geraniol is produced from the universal monoterpenoid precursor, geraniol diphosphate (GDP)

Abbreviations: GC-MS, gas chromatography-mass spectrometry; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; EST, expression sequence tags; MTBE, methyl *tert*-butyl ether; GeDH, geraniol dehydrogenase; CAD, cinnamyl alcohol dehydrogenase; ADH, alcohol dehydrogenase.

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by geraniol synthase. Geraniol synthase, a monoterpene synthase, produces geraniol exclusively, removing-OPP from GDP and substituting –OH derived from H_2O (Dong et al. 2013; Iijima et al. 2004; Masumoto et al. 2010; Shishido et al. 2012; Simkin et al. 2013; Vezzaro et al. 2012; Yang et al. 2005). Furthermore, in some plants such as grapes and tea leaves, glycosidically bound forms of geraniol were accumulated and geraniol was produced by enzymatic hydrolysis (Mateo and Jiménez 2000; Mizutani et al. 2002; Wang et al. 2001). As with the biosynthesis of geraniol, a number of enzymatic modifications of geraniol have been characterized in several plant species. Cloning and characterization of geraniol dehydrogenase (GeDH), which is responsible for the reaction between geraniol and citral (a mixture of geranial and neral), was reported in sweet basil (*Ocimum basilicum* cv. Sweet Dani) (Iijima et al. 2006) and native perilla plants (*Perilla setoyensis*, *P. citriodora*, and *Perilla. Frutescens*) (Sato-Masumoto and Ito 2014). As for geranyl acetate, acetyl CoA/geraniol acetyltransferase was characterized for the production of geranyl acetate in rose (*Rosa hybrida*) flower (Shalit et al. 2003) and Palmarosa aroma grass (*Cymbopogon martinii*) leaf (Sharma et al. 2013). 10-Hydroxygeraniol is an important precursor for the biosynthesis of (seco) iridoids, which are pharmaceutically important compounds and include the anticancer agents vincristine and vinblastine (O'Connor and Maresh 2006). Cytochrome P450 enzymes, especially CYP76 family members such as CYP76B6 from *Catharanthus roseus* and CYP76C1 from *Arabidopsis thaliana*, were reported to catalyze the hydroxylation of geraniol (Collu et al. 2001; Hofer et al. 2014; Hofer et al. 2013).

As for the biosynthesis of ginger volatiles, several sesquiterpene synthases such as (*S*)-*β*-bisabolene synthase (Fujisawa et al. 2010) and germacrene D synthase (Picaud et al. 2006) have been characterized. Recently, Koo and Gang comprehensively screened terpene synthases from ginger and turmeric, and functionally characterized 13 monoterpene synthases and 11 sesquiterpene synthases (Koo and Gang 2012). However, the biosynthesis mechanisms of geraniol-related compounds (geranial, geraniol, and geranyl acetate) remain unresolved, although we suggested that geraniol dehydrogenase activity is involved in the accumulation of geranial in ginger rhizome (Sekiwa-Iijima et al. 2001). In this study, we first investigated the biotransformation of geranial from geranyl acetate and geraniol using stable isotope analysis. Next, we conducted molecular cloning of the geraniol dehydrogenase gene, *ZoGeDH*, from ginger rhizome (*Z. officinale* cv. Kintoki) and functionally characterized the recombinant ZoGeDH protein as oxidizing geraniol to citral with $NADP^+$ as a coenzyme. Furthermore, we confirmed that the expression of *ZoGeDH* corresponded to the accumulation of geranial in ginger tissues.

Materials and methods

Plant material

Seed rhizomes of ginger (*Z. officinale* cv. Kintoki) were kindly supplied by Kimura-Noen Co., Ltd. (Aichi, Japan). Seed rhizomes were planted and grown in a greenhouse under natural light and seasonal conditions. After ginger plants were grown to a height of approximately 30 cm, they were harvested and washed with distilled water. The plants were sectioned into 6 tissues: leaf, upper stem, lower stem, young rhizome, old rhizome, and root, frozen with liquid nitrogen, and stored at −80°C until use. For labeled compound incorporation experiments, young seedlings (approximately 20 cm) were harvested and young rhizomes with attached stems were used.

Synthesis of deuterium (D)-labeled geraniol and geranyl acetate

Deuterated geraniol ([1,1-D_2]-geraniol) was synthesized from geranic acid by reduction with deuterated lithium aluminium hydride. Deuterated geranyl acetate was obtained by acetylation of [1,1-D_2]-geraniol with acetic anhydride in pyridine. The synthesized compounds were purified by preparative HPLC and their purity was confirmed as >94% by GC-MS. Each sample was dissolved in DMSO prior to use in experiments.

Incorporation experiment using D-labeled compounds

Ginger plants containing young rhizomes were divided into three groups (eight seedlings per group). A treatment solution (200 ml) containing 1.95 mM of [1,1-D_2]-geraniol or [1,1-D_2]-geranyl acetate with Tween 80 (0.6 mg ml^{-1}) was used to soak the ginger plants. As a control, ginger plants were soaked in a solution containing DMSO without the labeled compounds. After soaking for 18 h, the rhizomes were collected, washed and peeled. The samples were individually frozen in liquid N_2, and powdered with a mortar and pestle before extraction. Samples were prepared in triplicate. Each powdered sample (10 g) was extracted with diethyl ether containing 0.2 mg methyl decanoate (50 ml) with shaking for 15 min. After centrifugation (3000×*g*, 10 min, 4°C), the supernatant was collected. This procedure was repeated twice and the combined supernatants were dehydrated overnight with anhydrous Na_2SO_4. The concentrate was obtained by distillation of diethyl ether at 39.5°C. Since this extract contained a large amount of sesquiterpenes, we fractionated the geraniol-related compounds as previously described (Sekiwa-Iijima et al. 2001).

Isolation of GeDH cDNA and expression in E. coli

Total RNA was extracted from young or old ginger rhizome samples by the cetyltrimethylammonium bromide method in combination with an RNeasy Plant Mini kit (Qiagen GmbH, Hilden, Germany). Construction of cDNA libraries and sequencing of cDNAs to develop an EST database were

carried out at the Kazusa DNA Research Institute (Chiba, Japan) according to a previous report (Ragamustari et al. 2014). In addition, we referred to the Aromatic Rhizome EST database in the University of Arizona (http://www.agcol.arizona.edu/cgi-bin/pave/GT/index.cgi, (Koo et al. 2013)), because this database was opened before we constructed our own database from cv. Kintoki. Comparative analysis using both database accelerated filtering the candidate genes. BLAST searches using full length sequences of *ObGeDH* and *ObCAD* (Iijima et al. 2006) enabled to filter numerous ESTs annotated as encoding alcohol dehydrogenase (ADH) by sequence similarity. Putative ADH cDNAs were assembled into 10 contigs. One contig was more prevalent in rhizome ESTs than other tissues in the Aromatic Rhizome EST database, and was annotated as a CAD. Therefore, this contig was chosen and its full-length cDNA was obtained using reverse transcripts synthesized from young rhizome mRNA as a template. After 5′-RACE was performed using a SMARTer™ RACE cDNA amplification kit (Clontech Laboratories, Inc., Mountain View, CA, USA) according to the manufacturer's protocol, full-length cDNA was amplified with a pair of oligonucleotide primers (5′-CCG AAT TCC ATA TGA TGG CAG AGC TGG GA-3′ and 5′-ACG CGT CGA CCT CTC TTA CGC TTC AGT CAA-3′) using proofreading KOD-plus polymerase (Toyobo, Inc., Osaka, Japan). These primers included the *Nde*I and *Sal*I restriction endonuclease recognition sites, respectively. The PCR products were digested by the corresponding restriction enzymes (Takara Bio Inc., Shiga, Japan) and ligated into the expression vector pET28-(a) to produce a protein with an N-terminal His tag, using a T4 ligase kit (Life Technologies, Inc., Carlsbad, CA, USA) according to the manufacturer's protocol. The constructs were transformed into *E. coli* DH5α competent cells for amplification. After purification with a QIAprep Spin Miniprep Kit (Qiagen), the sequences were confirmed. Next, these plasmids were transformed into Rosetta™ (DE3) pLysS competent cells (Novagen, Madison, WI, USA). Cultures initiated from a single colony were pre-cultured in LB medium containing 50 μg ml^{-1} kanamycin and 20 μg ml^{-1} chloramphenicol overnight with shaking at 37°C. Aliquots were transferred to fresh LB medium containing 50 μg ml^{-1} kanamycin and 20 μg ml^{-1} chloramphenicol, and incubated with shaking (180 rpm) at 37°C until an OD of 0.7 was reached. Expression of the His-tagged ZoGeDH protein was induced by adding isopropyl-β-D-thiogalactopyranoside to the culture media at a final concentration of 0.5 mM. The cultures were incubated for 18 h at 20°C with shaking at 200 rpm and then centrifuged to collect cells. Cells were resuspended in lysis buffer composed of 50 mM Tris-HCl (pH 8.0), 10% (V/W) glycerol, 10 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol and tablets of protease inhibitor cocktail (1 tablet per 50 ml buffer, Complete, Roche Applied Science, Penzberg, Upper Bavaria, Germany). After sonication and centrifugation, the supernatant was used as the crude enzyme. The His-tag fusion of ZoGeDH was affinity-purified using a His GraviTrap kit according to the manufacturer's protocol (GE Healthcare UK Ltd., Buckinghamshire, England).

Preparation of crude enzyme from young ginger rhizome

The crude enzyme extract was prepared from fresh young ginger rhizomes (3 g) with the lysis buffer used for the recombinant protein (15 ml) and polyvinylpolypyrrolidone (0.5 g) by homogenizing on ice. After centrifugation (10,000×g, 15 min, 4°C), the supernatant was used for enzyme assays.

Enzyme assays

Basically, oxidative dehydrogenase assays were performed using a spectrophotometer as described previously (Iijima et al. 2006).

To confirm the products formed in the enzyme-catalyzed reactions, enzymatic assays were performed by incubating 60 μl of the enzyme sample in a final volume of 600 μl buffer containing 50 mM glycine-NaOH (pH 9.5), 1 mM NADP$^+$, and 1 mM geraniol. After incubation for 0.5 h (purified recombinant enzyme) and 4 h (crude enzyme) at 25°C, 300 μl of methyl *tert*-butyl ether (MTBE) was added to the reaction tube, mixed briefly, and centrifuged (10,000×g, 10 min, 4°C). The MTBE layer was directly placed into a GC vial for GC-MS analysis.

GC-MS analysis

Detection of geraniol, citral and geranyl acetate was performed by GC-MS using an Agilent 7890 gas chromatograph equipped with an Agilent 5975 mass spectrometer. Products from the enzymatic assay were detected using a HP-5 (30 m×0.25 mm i.d., 250 μm thickness, Agilent J&W Scientific, Santa Clara, CA, USA) fused silica capillary column. The oven temperature was set from 50°C (2 min hold) to 180°C at a rate of 5°C min^{-1}, and to 250°C at a rate of 15°C min^{-1} with a 1.0-μl injection in split mode (30 : 1). Helium was used as the carrier gas at a flow rate of 1 ml min^{-1}, and the injector temperature was set to 250°C. Mass spectra were obtained under the following conditions: ionization voltage, 70 eV (EI); ion source temperature, 230°C; quadrupole temperature, 150°C. Volatiles from feeding assays were detected with a DB-WAX (60 m×0.25 mm i.d., 250 μm thickness, Agilent J&W Scientific) fused silica capillary column. GC-MS conditions were the same as previously described (Sekiwa-Iijima et al. 2001). Quantification of geraniol-related compounds was performed by selected ion monitoring. Selected m/z of each compound was as follows: m/z 154 (endogenous geraniol), m/z 155 (D_1-geraniol), m/z 156 (D_2-geraniol), m/z 152 (endogenous geranial and neral), m/z 153 (D_1-geranial and D_1-neral), m/z 93 (endogenous geranyl acetate), m/z 95 (D_2-geranyl acetate), m/z 156 (endogenous citronellol), m/z 157 (D_1-citronellol), m/z 158 (D_2-citronellol). A standard curve for each compound was prepared and used for quantification. The concentrations of isotope labeled compounds were calibrated by subtraction of endogenous isotopic intensities.

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA from leaf, upper stem, lower stem, young

rhizome, old rhizome and root of ginger were prepared as described above. RNA isolation was performed in triplicate for each sample. First-strand cDNA from each RNA was synthesized using the Superscript III First-strand synthesis system (Life Technologies) and oligo(dT)$_{20}$ primer according to the manufacturer's instructions. qRT-PCR was performed using SYBR® Premix Ex Taq™ (Takara Bio) with an Applied Biosystems 7500 Fast Real-Time PCR System according to the manufacturer's instructions. The PCR primers used were as follows: forward primer, 5′-TCA CAC AGC CAA GAA CGA ATG-3′, reverse primer, 5′-GGC AGG AGA GAC AGG AGT TGA-3′. As an internal control, actin (accession no. DY357890, Fujisawa et al. 2010) specific primers, 5′-CAG GAG TTA TGG TTG GGA TGG-3′ (forward) and 5′-TTG TAG AAG GTG TGA TGC CAG A-3′ (reverse) were prepared and employed for qRT-PCR. The amplification program was as follows: 95°C for 30 s and 40 cycles at 95°C for 3 s and 63°C for 30 s. Fluorescence was monitored during the cycle to ensure single product amplification.

Sequence analysis

Known protein sequences of GeDH, CAD, and ADH were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/protein/). Alignment of multiple protein sequences was performed using the Clustal W program. Phylogenetic analyses were carried out using the neighbor-joining method using FigTree v1.4.2 program (http://tree.bio.ed.ac.uk/software/figtree/).

Results

Biotransformation of geraniol and geranyl acetate in ginger rhizomes

Previously, we found that geranyl acetate is abundantly contained in young rhizomes of ginger; however, its content levels decrease during maturation in accordance with increasing geranial levels (Sekiwa-Iijima et al. 2001). Therefore, we predicted geranyl acetate and geraniol to be precursors of geranial. Deuterium-labeled geraniol (D_2-geraniol) and geranyl acetate (D_2-geranyl acetate) were synthesized and incorporated into ginger plants. The accumulated labeled compounds in the rhizomes were investigated by GC-MS analysis. Figure 1A shows the contents of endogenous geraniol-related compounds: geraniol, geranial (*trans*-citral), neral (*cis*-citral), geranyl acetate, and citronellol in the control plants. The content of geranial was the highest, 5.2-fold and 4.0-fold higher than that of geraniol and geranyl acetate, respectively. Figure 1B indicates the labeled compounds detected in the plants treated with D_2-geraniol or D_2-geranyl acetate. The extracts contained labeled geraniol-related compounds as well as their endogenous compounds under both treatments, indicating D_2-geraniol and D_2-geranyl acetate were incorporated into the ginger plant

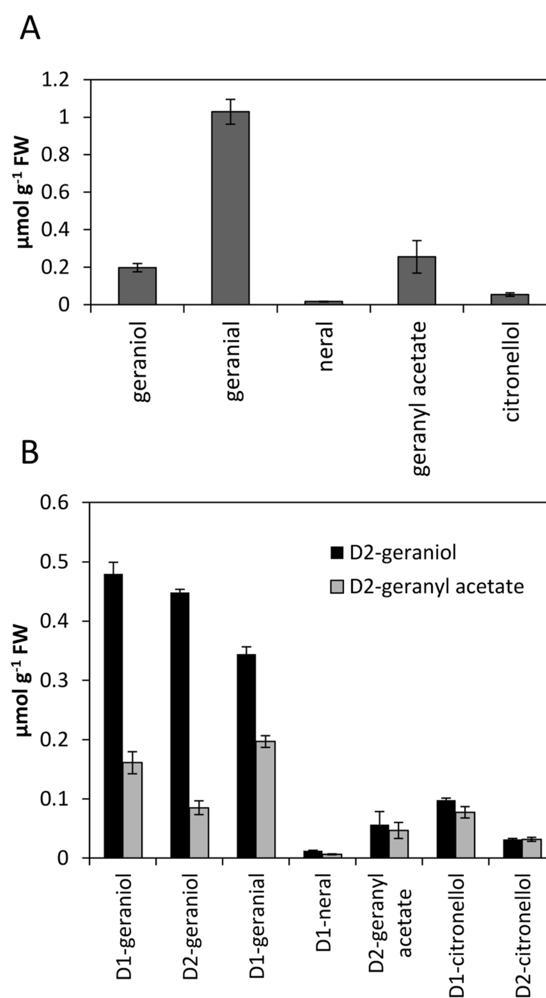

Figure 1. Concentration of geraniol-related compounds in ginger rhizomes. (A) Endogenous concentration and (B) accumulated deuterium-labeled compounds after feeding of D_2-geraniol and D_2-geranyl acetate.

and metabolized to other compounds such as geranial and citronellol. However, the content of labeled geraniol was higher than that of other compounds, even more than that of endogenous geraniol. Especially, treatment with D_2-geraniol accelerated the synthesis of D_1-geraniol and D_1-geranial. D_1-geranial comprised 33.7% of the endogenous geranial content. Other than geraniol, citronellol was also detected as a mixture of D_2- and D_1-labeled compounds. On the other hand, nerol and neral, *cis*-isomers of geraniol and nerol, respectively, were hardly detected in either the control or treated plants. These results indicate that geraniol and geranyl acetate are possible precursors for the formation of geranial and citronellol.

Cloning and phylogenetic analysis of GeDH

There are two possible enzymatic routes to produce geranial from geraniol, catalyzed by geraniol oxidase and geraniol dehydrogenase. Our previous report showed that NADP$^+$-dependent ADH activity for

geraniol was involved in production of citral, whereas geraniol oxidase showed no activity (Sekiwa-Iijima et al. 2001). Therefore, we screened candidate GeDH cDNA using the EST database established by the University of Arizona (Koo et al. 2013) and our domestic sequence collections constructed for *Z. officinale* cv. Kintoki using next-generation sequencing technology. Previously, we isolated two cDNAs encoding GeDH and cinnamyl alcohol dehydrogenase (CAD) from sweet basil, which are responsible for the synthesis of citral from geraniol (Iijima et al. 2006). Therefore, we used these sequences for BLAST searches, and found 3 and 7 contigs annotated as CAD and ADH, respectively. Among them, one contig annotated as CAD was contained in the EST data from rhizome and leaf, and its full-length cDNA (*ZoGeDH*) was obtained after 5′-RACE.

The full-length sequence of *ZoGeDH* was 1,104 bp and encoded a protein 368 amino acids in length. Its deduced molecular weight and theoretical isoelectric point were 39.59 kDa and 6.79, respectively. Figure 2 indicates the alignment of ZoGeDH with other reported plant GeDHs, i.e., *Perilla citriodora* (PcGeDH) and *O. basilicum* (ObGeDH), and a CAD from *O. basilicum* (ObCAD) known to show activity toward geraniol (Iijima et al. 2006; Sato-Masumoto and Ito 2014). The amino acid sequence of ZoGeDH showed 68%, 66%, and 49% identity with that of PcGeDH, ObGeDH, and ObCAD, respectively. ZoGeDH showed conserved NADP- and Zn1-binding domain motifs, and a Zn2 structural motif similar to other plant CADs and GeDHs (McKie et al. 1993; Ragamustari et al. 2013). BLAST search for ZoGeDH using GenBank showed the highest similarity with a predicted mannitol dehydrogenase in *Phoenix dactylifera* (79%, XP_008809345). In functionally identified proteins, CAD3 from *Camellia sinensis* (AEE69008) was highly homologous (72%) to ZoGeDH. Since ZoGeDH exhibited a high degree of homology with CADs from various plants, we performed a phylogenetic

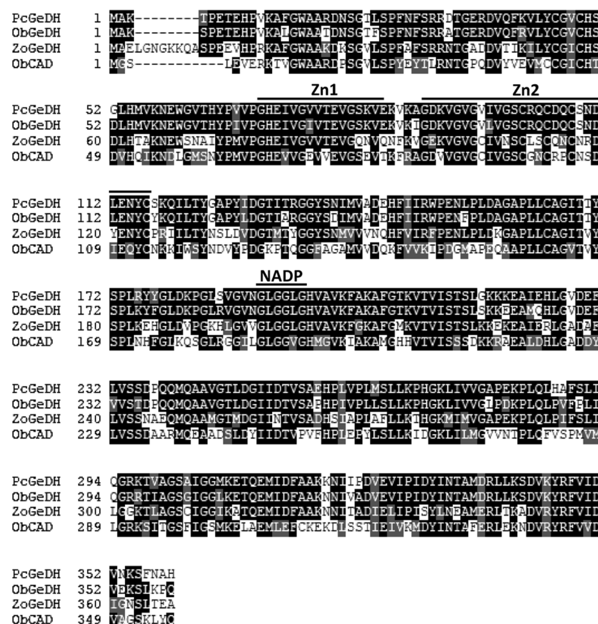

Figure 2. Multiple alignment of amino acid sequences of ZoGeDH (Accession No.: LC002206) in this study with *P. citriodora* GeDH (PcGeDH, Accession No.:AFY63473), *O. basilicum* GeDH (ObGeDH, Accession No.: AAX83107) and CAD (ObCAD, AAX83108). The highlighted letters in black background indicates identical in more than 50% among all sequences, and those highlighted in gray color denote similar ones. The conserved amino acids in the catalytic and binding domains for zinc ion (Zn1 and Zn2), and NADP binding domains are represented as lines above the alignment.

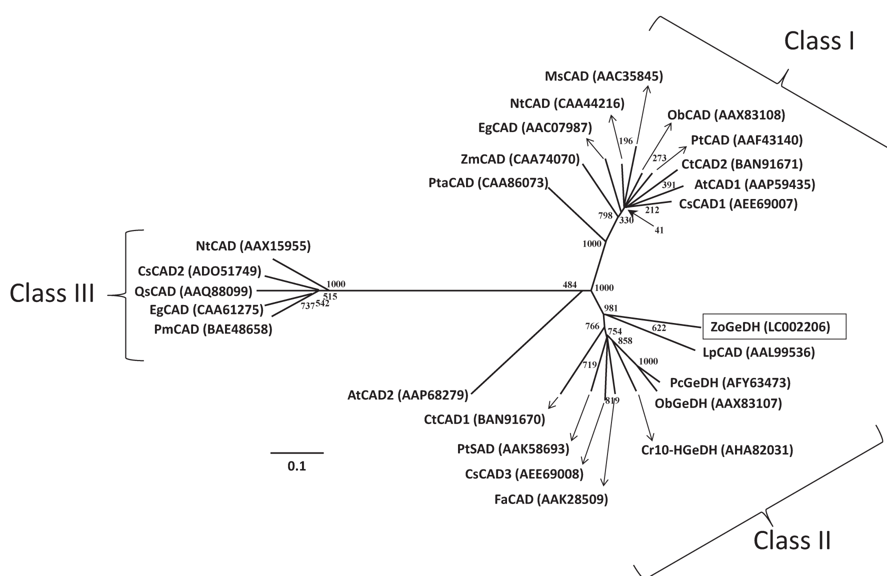

Figure 3. Unrooted phylogenetic tree of GeDHs and CADs created by the neighbor-joining method from amino acid alignment. The ClastalW program (http://clustalw.ddbj.nig.ac.jp) was used for amino acid sequence alignment, and the phylogenetic tree was constructed with FigTree v1.4.2. software. Bootstrap values were obtained from 1000 bootstrap replicates and indicated at the nodes. The scale bar represents 0.1 amino acid changes.

analysis using the amino acid sequences of biochemically characterized plant CADs with annotated CADs from *Arabidopsis thaliana* (Figure 3). It is known that CADs are phylogenetically categorized into three classes (Deng et al. 2013; Ragamustari et al. 2013). ZoGeDH was grouped into the same class as other GeDHs and Cr10-hydroxy GeDH.

Biochemical characterization of purified recombinant ZoGeDH

The open reading frame of GeDH was cloned into an *E. coli* expression vector (pET 28a) and GeDH was heterologously expressed in *E. coli* cells. The resultant His-tagged recombinant protein was purified and subjected to GeDH activity measurements by GC-MS. Purified GeDH produced neral and geranial (citral) by incubation with geraniol and $NADP^+$ (Figure 4C), as did the crude enzyme prepared from fresh ginger rhizome (Figure 4A). Furthermore, nerol was detected, probably as a product of the reverse reaction for neral. On the other hand, geraniol was not converted to citral in the absence of $NADP^+$ either in purified GeDH or in crude enzyme (Figure 4B, 4D). Further, no activity was observed when NAD^+ was substituted for $NADP^+$. The optimum pH for ZoGeDH was 9.5, and 68% of this activity was maintained at a pH range of 8.0–10.0. To characterize the substrate specificity of GeDH, 12 alcohols in addition to geraniol were assessed and their activities were compared (Table 1). GeDH showed equal preference for geraniol and nerol, and the activity with cinnamyl alcohol was 31.6% that for geraniol. The activities for unsaturated alcohols containing a double bond next to the hydroxyl moiety, such as *trans*-2-hexenol and 3-methyl-2-buten-1-ol, were higher than others. Notably, the activity for citronellol, which is characterized by saturation in the C2 and C3 bond of the geraniol skeleton, showed only 16.4% of the activity for geraniol.

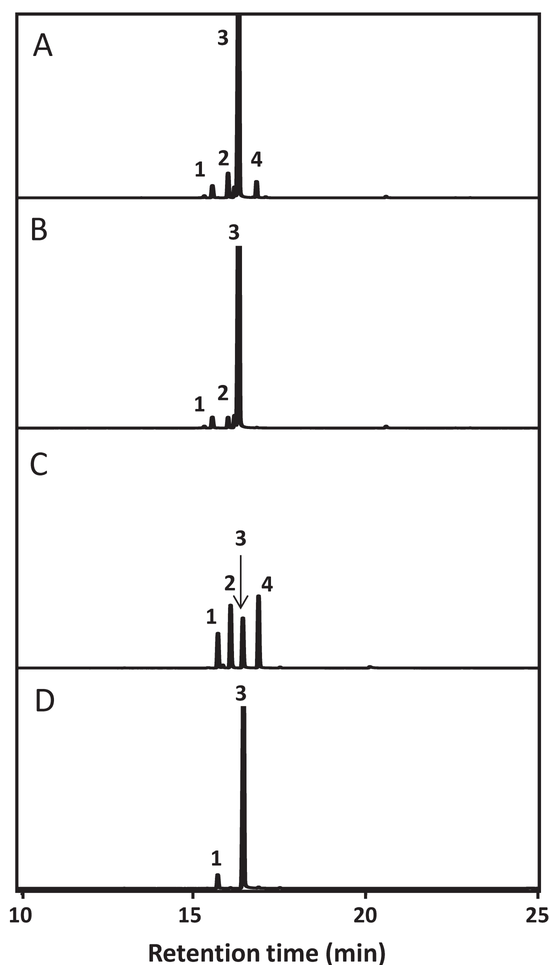

Figure 4. GC-MS profile of the products of GeDH reaction with geraniol. (A) Products after 4 h incubation of geraniol with $NADP^+$ and crude enzyme prepared from young ginger rhizome. (B) Products after 4 h incubation of geraniol with crude enzyme without $NADP^+$. (C) Products after 0.5 h incubation of geraniol with $NADP^+$ and purified ZoGeDH. (D) Products after 0.5 h incubation of geraniol with purified ZoGeDH without $NADP^+$. Peaks 1–4 represent nerol, neral, geraniol, and geranial, respectively.

The K_m, V_{max}, and k_{cat} values for geraniol, nerol and $NADP^+$ were determined under the optimum assay conditions (Table 2). The K_m value for geraniol was 60.9 μM, which was smaller than that of nerol (92.6 μM). The catalytic efficiencies (k_{cat}/K_m, $\mu M^{-1} s^{-1}$) of geraniol, nerol and $NADP^+$ were 0.089, 0.05 and 0.035, respectively.

Expression of ZoGeDH in ginger plants and its relation with geranial content

Total RNAs from the various tissues of ginger cv. Kintoki: leaves, upper stem, lower stem, young and old rhizomes,

Table 1. Substrate specificities of the purified recombinant ZoGeDH.

Substrate	Specificity(% ± SE)
Geraniol	100
Nerol	98.3±2.0
Citronellol	16.4±1.0
3,7-Dimethyl-octanol	11.5±0.5
Cinnamyl alcohol	31.6±0.6
trans-2-Hexenol	26.6±0.8
cis-3-Hexenol	11.9±0.8
Hexanol	24.1±0.6
3-Methyl-2-buten-1-ol	22.9±1.8
3-Methyl-3-buten-1-ol	6.9±0.2
1-Butanol	5.4±0.1
Phenylethylalcohol	9.9±1.7
3-Phenylpropanol	16.2±1.0

Table 2. Kinetic parameters of ZoGeDH.

Substrate	K_m (μM)	K_{cat} (s^{-1})	K_{cat}/K_m($\mu M^{-1} s^{-1}$)
Geraniol	60.9	5.4	0.089
Nerol	92.6	4.6	0.05
NADP	142.4	5.0	0.035

were purified. The expression of *ZoGeDH* mRNA in each tissue was measured by real-time RT-PCR after reverse transcription. The transcription level of *ZoGeDH* for each sample was calculated by normalization with that of constitutive *Actin* gene. Figure 5A indicates the comparative expression levels of *ZoGeDH* in various tissues. In addition, the concentrations of geranial in the corresponding tissues were quantified and shown in Figure 5B. The *ZoGeDH* transcript was expressed only in leaves, stems, and young rhizomes; moreover, its expression was the highest in young rhizomes. The patterns of geranial content were in agreement with the transcript levels of *ZoGeDH* in leaves, stems and roots. However, the content of geranial in old rhizomes was more abundant than in young rhizomes, in contrast to the observed decline in *ZoGeDH* expression. The majority of old rhizomes are derived from seed rhizomes. Therefore, this suggests that the contained geranial in old rhizome is produced prior to maturation, that is, in the previous younger maturation stage.

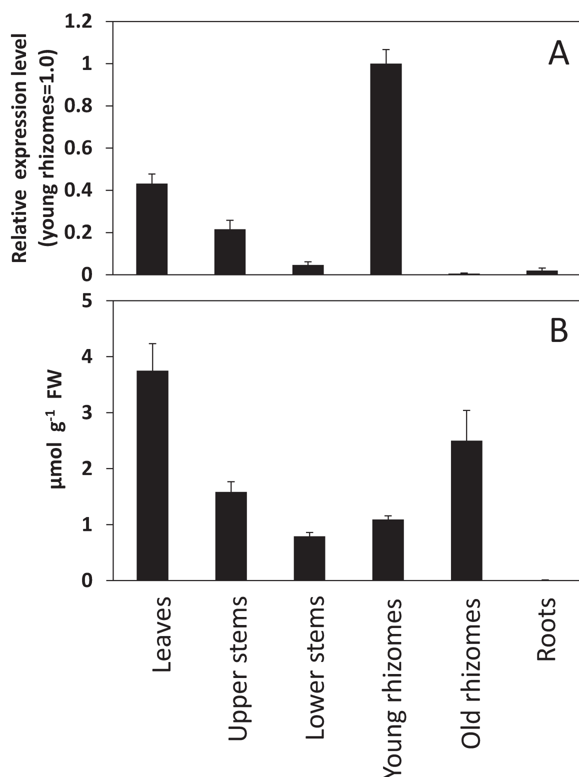

Figure 5. Relative expression levels of the gene encoding ZoGeDH (A) and contents of geranial (B) in various tissues of ginger. qRT-PCR analysis and quantification of geranial were performed in triplicates. The expression level of transcript for each sample was obtained by normalization with transcription level of actin gene. Relative levels of transcripts are shown with young rhizome samples arbitrarily set at 1.0.

Discussion

We investigated the mechanism of biotransformation among geraniol-related compounds in ginger, focusing on geranial synthesis from geraniol and geranyl acetate. The incorporation of deuterium-labeled geraniol and geranyl acetate indicated that they were transferred to geranial with a small amount of neral in ginger rhizomes (Figure 1). The proposed pathway based on GC-MS analysis is shown in Figure 6. In this system, two kinds of labeled geraniol, D_1-geraniol and D_2-geraniol, were detected (Figure 1). Detection of D_2-geraniol can be explained simply as being derived from the incorporated D_2-geraniol or from the hydrolysis of D_2-geranyl acetate by an esterase. On the other hand, D_1-geraniol was suggested to be derived from the reduction of D_1-geranial, initially generated from D_2-geraniol. Thus, these data support that GeDH is involved in the bi-directional reaction between geraniol and geranial.

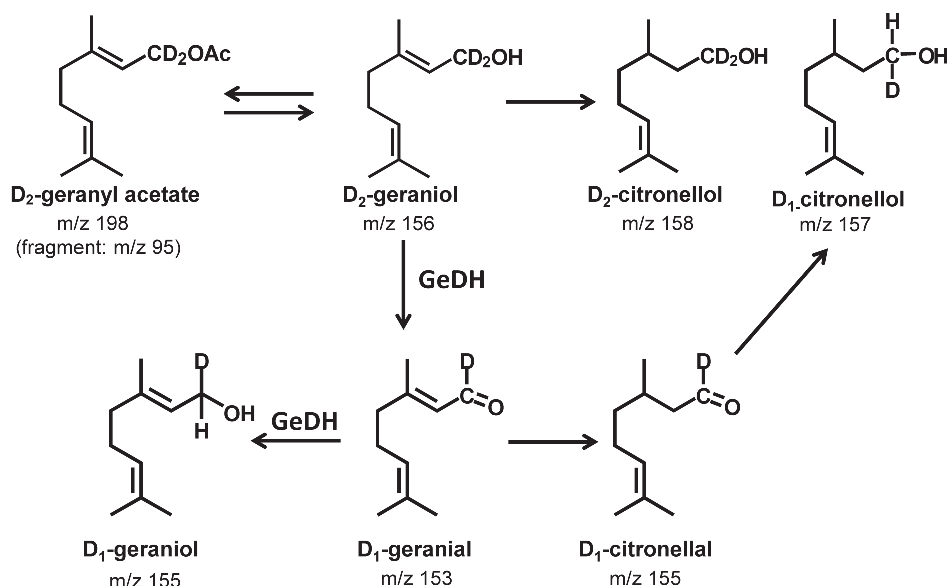

Figure 6. Proposed biotransformation of geraniol-related compounds in ginger rhizomes.

Likewise, citronellol was detected as D_2-citronellol and D_1-citronellol. These compounds are also proposed to be generated directly from D_2-geraniol or via D_1-geranial by reduction. It is known that the abundant amount of geranyl acetate detected in young rhizomes declines to trace levels in old rhizomes (Sakamura 1987; Sekiwa-Iijima et al. 2001), suggesting that geranyl acetate is the key precursor in determining the final amount of geranial. In this study, we observed the biotransformation between geraniol and geranyl acetate as well as between geraniol and geranial. Geranyl acetate was reported to be synthesized from geraniol by acetyl CoA/geraniol acetyltransferase in rose flower (Shalit et al. 2003) and Palmarosa leaf (Sharma et al. 2013). On the other hand, an esterase responsible for the hydrolysis of acetylated alcohol volatiles was recently characterized in wild tomato fruits (Goulet et al. 2012). In a preliminary experiment, we observed these activities using the crude enzyme from ginger rhizome (data not shown). Their enzymatic properties and expression levels should be investigated in detail to clarify the mechanism regulating the composition of geraniol-related compounds.

In this study, a cDNA encoding ZoGeDH from ginger rhizome was identified using the EST database, and the recombinant ZoGeDH was characterized. When we previously isolated *ObGeDH* from *O. basilicum*, it was annotated as a CAD gene prior to being characterized (Iijima et al. 2006). Furthermore, some CADs are known to use geraniol as a substrate (Li et al. 2012). Therefore, we consider it possible that a CAD-like gene in ginger is a candidate for *ZoGeDH*. The finally isolated *ZoGeDH* possessed the conservative domain in CADs and showed sequence similarities with other reported GeDHs and CADs (Figures 2 and 3). Furthermore, the molecular weight of the recombinant ZoGeDH was 39.59 kDa, similar to other CADs (≈40 kDa). While ZoGeDH used cinnamyl alcohol as a substrate, geraniol and nerol were preferred (3-fold greater activity). Furthermore, the kinetic parameters for ZoGeDH for geraniol and nerol are similar to those for ObGeDH (Iijima et al. 2006). Real-time RT-PCR analysis for various tissues of ginger plant indicated that expression levels of *ZoGeDH* are associated with the levels of geranial accumulation. Inconsistent with this trend, however, were the observations in old rhizomes, where the loss of *ZoGeDH* expression was concomitant with the accumulation of abundant geranial. It is likely that geranial in the old rhizomes is synthesized at an early immature stage, and stored during maturation. This indicates that *ZoGeDH* expression decreases or that *ZoGeDH* itself is degraded during the maturation of ginger rhizome.

Geranial and neral are *trans*- and *cis*-isomers, and are transformed into each other by keto-enol tautomerization, which is accelerated at alkaline or acidic condition (Kimura et al. 1982; Wolken et al. 2000). Therefore, citral is usually composed of a mixture of approximately 60% geranial and 40% neral. The optimum pH for GeDH activity in conversion from geraniol to geranial, was pH 9.5. Therefore, in the GeDH assay, while geranial is produced by the oxidation of geraniol, it is suggested to be easily altered to neral by keto-enol tautomerization; thus, both geranial and neral are detected as products. In a number of plants such as lemongrass (*Cymbopogon citratus*) (Singhsangwan et al. 1993), lemon balm (*Melissa officinalis*) (Taherpour et al. 2011), and some varieties of sweet basil (Grayer et al. 1996; Simon et al. 1999) and perilla (Yuba et al. 1995), citral was detected as a mixture of geranial and neral. On the other hand, citral in ginger is composed mainly of geranial (more than 90%) in vivo, although ZoGeDH reacts with geraniol to produce both geranial and neral in vitro. Furthermore, nerol, which is a preferred substrate for ZoGeDH (as is geraniol) is contained at trace levels in the rhizome. This disagreement suggests that the geranial produced by ZoGeDH in vivo is secreted into specialized cells under a structurally stable condition. The ratio of *cis*–*trans* isomerization of citral is reported to be dependent on solvent (Kuwahara et al. 1983). Therefore, the condition where geranial was stored should be the key factor for structural stability of geranial.

Previously, examination of cryo-sections suggested that phenolic compounds such as gingerols in ginger rhizomes are localized in yellow pigmented lipid cells (Zarate and Yeoman 1994). It is not well known where essential oils are synthesized and stored in ginger rhizomes; however, GeDH-produced geranial is assumed to be rapidly secreted to such lipid cells.

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