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Characterization of raspberry ketone/zingerone synthase, catalyzing the alpha, beta-hydrogenation of phenylbutenones in raspberry fruits

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ABSTRACT

Phenylbutanone raspberry ketone, accumulating in the mature fruits of raspberry (*Rubus idaeus*), imparts the characteristic aroma to the fruits. Here we describe the isolation and characterization of raspberry ketone/zingerone synthase 1 (RZS1), which catalyzed the NADPH-dependent reduction of 4-hydroxybenzalacetone and 3-methoxy-4-hydroxybenzalacetone to raspberry ketone and zingerone (the latter not found in raspberry), respectively. Its apparent K_m values for 4-hydroxybenzalacetone and NADPH were 88 μM and 202 μM, respectively. RZS1 preferred 4-hydroxybenzalacetone to 3-methoxy-4-hydroxybenzalacetone as a substrate by a factor of 1.7, and showed a 6-fold preference for 4-hydroxybenzalacetone over *p*-coumaraldehyde, and no activity for coniferaldehyde. Expression analysis of the *RZS1* gene throughout the plant revealed that its transcript level was highest in mature fruits. We conclude that RZS1 is responsible for hydrogenation of the α,β-unsaturated double bond of phenylbutenones, the final step of the raspberry ketone biosynthesis, in the raspberry fruits.

1. Introduction

Phenylbutanones are a class of compounds comprising the 4-phenyl-2-butanone (C_6–C_4) structure. They are produced in higher plants, and are characteristic constituents in many foods and spices [1]. Furthermore, floral scent bouquet from some plant species contains phenylbutanones. For example, the orchid *Bulbophyllum* flowers emit a mixture of volatiles, including raspberry ketone and zingerone, which act as attractants for males of several fruit fly species belonging to the genus *Bactrocera* [2]. During the interaction between orchid flowers and fruit flies via floral scent, the male flies acquire the fragrance, store, and metabolize it in the pheromone gland to attract conspecific females [3].

4-(4-Hydroxyphenyl)butan-2-one is designated as raspberry ketone because this compound imparts the characteristic aroma to fruits of raspberries (*Rubus idaeus*). The synthesis of raspberry ketone in the raspberry fruits increases during their maturation [4]. Previously, it was thought that raspberry ketone occurred only in raspberry fruits [4]. Recently, either raspberry ketone or its glucoside (lindleyin), or both, have also been found in rhubarb roots (*Rheum palmatum*), raspberry jam orchid (*Dendrobium superbum*), and in pine needles (*Pinus contorta*) [5–7]; however, the natural abundance of raspberry ketone derivatives in these species is very low.

In the biosynthetic pathway leading to raspberry ketone, the C_6–C_4 skeleton of phenylbutanoids is synthesized via a sequential two-step reaction analogous to that catalyzed by chalcone synthase and stilbene synthase [7]. In the first step, benzalacetone synthase (BAS) forms the polyketide intermediate 4-hydroxybenzalacetone through the decarboxylative (Claisen-type) condensation of *p*-coumaroyl-CoA with malonyl-CoA, followed by hydrolysis and decarboxylation (Fig. 1). In the second step, the intermediate product, 4-hydroxybenzalacetone, is reduced to raspberry ketone by a NADPH-dependent reductase that reduces the α,β-unsaturated double bond of the enoyl moiety in the molecules. Reductase activity for 4-hydroxybenzalacetone has been found in the crude protein extracts from raspberry fruits and their tissue cultures [4]; however, there is no report about the reductase gene, which is responsible for the production of phenylbutanoids in higher plants.

In plants, several enone reductases that recognize the α,β-unsaturated double bond of secondary metabolites including 2-(*E*)-nonenal, *p*-coumaraldehyde, (+)-pulegone, and furaneol derivatives [8–15] have been isolated, but none of them have been shown to participate in the phenylbutanone biosynthesis. In *Arabidopsis*

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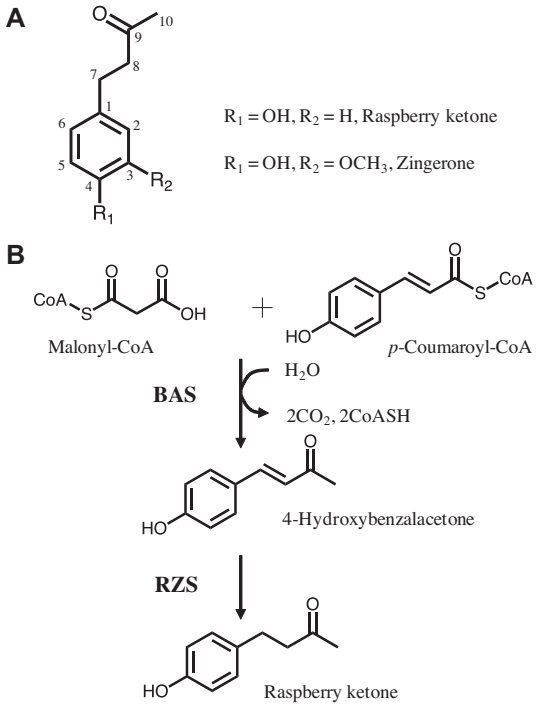


Fig. 1. Structures and biosynthesis of representative phenylbutanones. (A) Structures of raspberry ketone and zingerone. For raspberry ketone, the phenyl-ring substituents are R_1 = OH and R_2 = H. For zingerone, the phenyl-ring substituents are R_1 = OH and R_2 = OCH_3. The carbon numbering system used in this study is shown. (B) Biochemical reactions leading to raspberry ketone in plants. The reactions catalyzed by known enzymes are indicated. The enzyme reported in this study is shown with bold.

thaliana [8] and barley (*Hordeum vulgare*; [9]), it has been reported that the NADPH-dependent 2-alkenal reductase (AER; EC 1.3.1.74) catalyzes the reduction of the α,β-unsaturated bond of short chain 2-alkenals including 2-(*E*)-nonenal and 2-(*E*)-hexenal, which are major toxic products derived from lipid peroxides, into the corresponding saturated aldehydes for detoxification. In recent works, it was found that the cucumber leaves contain alkenal/one oxidoreductase activities capable of reducing both α,β-unsaturated ketones and α,β-unsaturated aldehydes [10]. A Pinaceae (*Pinus taeda*) gene homologous to the *Arabidopsis* AER gene was found to be responsible for the C–C double bond reduction of phenylpropenal [11]. The encoded phenylpropenal alkenal reductase (PPAER) converted various monomeric and dimeric phenylpropenals, such as *p*-coumaraldehyde and coniferaldehyde, into the corresponding phenylpropanals. Whereas the relevant monoterpene double bond reductase PulR, which involves in the conversion of pulegone to (+)-isomenthone and (−)-menthone, was characterized from peppermint (*Mentha* × *piperita*) [12]. These findings led to the suggestion that the homologous genes might be responsible for the final key step to produce raspberry ketone.

In this study, we identified a reductase responsible for the formation of raspberry ketone from 4-hydroxybenzalacetone, and named it raspberry ketone/zingerone synthase (RZS). We cloned two homologous genes, *RZS1* and *RZS2*. The *RZS1* transcript level was high in the mature stage of raspberry fruits, which was the predominant tissue to produce raspberry ketone, whereas *RZS2* was highly expressed in leaves and stems. Furthermore, we found that RZS1, unlike previously characterized phenylpropenal reductases, was highly specific for substrates possessing the α,β-unsaturated double bond in the butenyl side chain. Thus the RZS1 protein is responsible for the last step of raspberry ketone biosynthesis. Our finding identified the missing link in the biosynthetic pathways leading to the phenylbutanone.

2. Materials and Methods

2.1. Chemicals and plant materials

Chemicals were purchased from Tokyo Chemical Industry or Wako Pure Chemicals otherwise stated. 4-Hydroxybenzalacetone was synthesized according to the literature method [16]. mp 111–112 °C (lit [16] 105–107 °C). ^{1}H NMR (300 MHz, $CDCl_3$) δ_H: 2.39 (3H, s), 6.61 (1H, d, J = 15.9 Hz), 6.62 (1H, br.s), 6.90 (2H, d, J = 8.7 Hz), 7.46 (2H, d, J = 8.7 Hz), 7.50 (1H, d, J = 15.9 Hz). Anal. Found: C, 74.12; H, 6.31; N, 0.00. Calcd. for $C_{10}H_{10}O_2$: C, 74.06; H, 6.21; N, 0.00. *p*-Coumaraldehyde was chemically synthesized as described previously [17]. 1-[^{2}H]Dihydro-*p*-coumaraldehyde was chemically synthesized from ethyl *p*-coumarate. Briefly, ethyl *p*-coumarate [17] was reduced with hydrogen in the presence of Pd/C. The resulting ethyl dihydro-*p*-coumarate was protected by ethoxyethyl ether with ethylvinyl ether. Ethyl dihydro-*p*-coumarate ethoxyethyl ether was reduced by lithium alminium deuteride (Aldrich). Ethoxylethyl 1,1-[$^2H_2$]dihydro-*p*-coumaryl alcohol was oxidized with a combination of dimethylsulfoxide and sulfur trioxide-pyridine complex to give 1-[^{2}H]dihydro-*p*-coumaraldehyde. ^{1}H NMR (400 MHz, acetone-d_6) δ_H: 2.58 (2H, t, J = 7.4 Hz), 2.70 (2H, t, J = 7.4 Hz), 6.59–6.64 (2H, m), 6.90–6.98 (2H, m). Raspberry (*R. idaeus* cv. Indian summer) was purchased from the nursery and grown in a garden pot under natural conditions.

2.2. Isolation of full-length cDNA

Total RNA was extracted by using an RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan) and reverse-transcribed using Superscript III reverse transcriptase (Invitrogen, Tokyo, Japan) after the RNA was treated with DNase. For the partial amplification of *RZS* cDNAs, the degenerated primers; DBRDGP2-1 = 5′-GTNATHYTNAARRAYTAYGT-3′, DBRDGPF = 5′-GGNATGCCNGGNYTNACNGC-3′, and DBRDGPR = 5′-CCNCCNACRTTNTCRAARTA-3′ (N = A/T/C/G, H = A/C/T, Y = C/Y, R = A/G), were designed based on the highly conserved sequences within enone reductases including PtPPAER, HvAER, and AtAER. First RT-PCR was carried out with the primer sets of DBRDGP2-1 and DBRDGPR under the conditions described previously [18]. Amplified PCR products for *RZS1* and *RZS2* were used as the template for the second PCR under the same PCR conditions employed in the first PCR with the primer sets of DBRDGPF and DBRDGPR. The resulting PCR products at about 300 bp were cloned into pGEM-T easy vector (Promega) and sequenced. 5′- and 3′-RACE transcripts were performed to obtain the complete coding sequence of *RZS1* and *RZS2* using a SMART RACE cDNA Amplification Kit (Clontech) following the manufacturer's instructions with internal gene-specific primers. Two specific primers; 1DBR3RF = 5′-CTGGGAATCCAAAATTTAATCCAGGA-3′ (a forward primer for 3′-end amplification), were used for *RZS1* amplification. For *RZS2* amplification, two specific primers; DBR33RF = 5′-TTTGCTCACCTAAGAAAGGAGAGAC-3′ (a forward primer for 3′-end amplification) and DBR35RR = 5′-GGCACTTCCAACAACATAGCAACCAG-3′ (a reverse primer for 5′-end amplification), were used. The amplified sequences covered the desired missing sequences.

2.3. RZS expression analysis by semi-quantitative RT-PCR

For spatial and developmental transcript analysis, total RNA was isolated from stems, leaves, and three different developmental stages (green, light-red, deep-red) of fruits. First-strand cDNA was synthesized as described above. The specific primers used in this experiment are as follows; 1DBR3RF (sense primer) and 1DBRRv (antisense primer) for *RZS1*, DBR33RF (sense primer) and 3DBRRv

(antisense primer) for *RZS2*, Ora1ACTF = 5′-GCCAGTGGT CGTACAACTGGTATTG-3′ (sense primer) and Ora1ACTR = 5′-CCTTGATCTTCATGCTGCTTGGAGC-3′ (antisense primer) for *Actin*. For the PCR, different cycles of reactions (94 °C for 15 s, 56 °C for 30 s, 72 °C for 45 s) were performed for each gene.

2.4. Expression and purification of recombinant RZS proteins

Full-length cDNAs were obtained using the following primers; 1DBRFw = 5′-AGCTAGCACCATGGCGAGTGGTGGAGAAATGCAA-3′ (sense for *RZS1*, the *Nhe*I site is underlined), 1DBRRv = 5′-TGCGGCCGCTCACTCTCTGGAAACAACCACCACC-3′ (antisense for *RZS1*, the *Not*I site is underlined), 3DBRFw = 5′-AGCTAGC ACCATG GCGAATGGTGAGCAAGTGATG-3′ (sense for *RZS2*, the *Nhe*I site is underlined), and 3DBRRv = 5′-TGCGGCCGCTCATTCTCGAGAAACAA-CAACAACCT-3′ (antisense for *RZS2*, the *Not*I site is underlined). The amplified DNAs were cloned into pGEM-T easy vector and confirmed by sequencing. The cDNAs were digested with *Nhe*I/*Not*I, and cloned into the *Nhe*I/*Not*I site of pET-28a(+) (Novagen) to encode *N*-terminally His-tagged proteins. Resulting constructs were transformed into *E. coli* BL21 (DE3) pLysS. The cells harboring the plasmid were cultured in Luria–Bertani medium with 50 mg/L kanamycin and 37 mg/L chloramphenicol, and then the recombinant RZS1 and RZS2 proteins were induced with IPTG (final concentration; 0.5 mM) at 20 °C for 16 h. Harvesting and protein purification by affinity chromatography on nickel-nitriloacetic acid agarose (NTA) (Qiagen) were performed using previously described methods [19]. Eluted proteins were desalted on PD-10 desalting columns (GE Healthcare Japan, Tokyo, Japan) into 20 mM Tris–HCl buffer (pH 7.0) with 20% glycerol, and examined by SDS–PAGE gel electrophoresis followed by staining of the gel with Coomassie Brilliant Blue.

2.5. Enzyme assays and HPLC analysis of reaction products

Protein concentration was determined by using the Bradford method [20] with BSA as a standard. The standard reaction mixture contained 0.1 mL of 0.5 M MES-KOH (pH 6.5), 4 μl of 50 mM NADPH, 4 μl of 50 mM substrate dissolved in methanol (final concentration of substrates was 0.5 mM), and 15 μl of purified proteins with water to adjust the final assay volume to 0.4 mL. Incubations were carried out at 25 °C for 20 min, and were stopped with the addition of 15 μl of glacial acetic acid. The products were then extracted with 1 mL of ethyl acetate, and the extract was concentrated by air flow. The reaction products were separated by a reverse-phase HPLC (Shimadzu LC-10A system) on an Ascentis RP-Amide column (4.6 × 250 mm, Supelco). Chromatography was performed with isocratic elution system: 80% methanol and 20% water containing 0.3% glacial acetic acid with a flow rate of 1.0 mL min^{-1}. The absorbance of each reaction product was monitored at 278 nm for raspberry ketone and *p*-dihydrocoumaraldehyde and at 282 nm for zingerone and dihydroconiferaldehyde. The amount of reaction product was calculated based on the standard calibration curve using the authentic compounds.

3. Results and Discussion

3.1. Isolation and sequence analysis of raspberry RZS1 and RZS2

To isolate raspberry cDNAs encoding a double bond reductase capable of reducing 4-hydroxybenzalacetone to produce raspberry ketone, we designed the degenerate oligonucleotide primers based on the highly conserved amino acid sequences of known 2-alkenal reductases (AERs) and used them in a RT-PCR amplification with raspberry fruits RNA. After nested PCR, amplified fragments were obtained and sequenced. Two AER-like sequences were identified and 3′ and 5′-RACE were conducted to obtain full-length sequences of two distinct cDNAs. One cDNA encodes a protein, denominated RiRZS1 (GenBank ID: JN166691), with 348 amino acid residues and the other full-length cDNA contained a 1059-bp ORF encoding protein named RiRZS2 (GenBank ID: JN166692). The deduced amino acid sequences of RiRZS1 and RiRZS2 are 78% identical to each other and most closely related to NtAER with 73–77% identity, followed by AtAER with 69% identity (Table 1). On the other hand, there is no extensive similarity to PtPPAER (less than 50% identity) as well as alkenal/one oxidoreductases (less than 30% identity), which were found recently in cucumber and *Arabidopsis* [10], catalyzing the reduction of both α,β-unsaturated ketone and aldehyde.

3.2. Expression patterns of raspberry RZS1 and RZS2

We determined the transcript levels of *RiRZS1* and *RiRZS2* in different organs of raspberry plants and at the different developmental stages of raspberry fruits by semi-quantitative RT-PCR analysis (Fig. 2). The results indicated the different expression patterns between these two *RZS* genes. Transcript levels of *RiRZS1* increased gradually with the progress of the fruits maturation to reach its maximum at the deep-red stage of fruit ripening, whereas it was very low in leaves and stem tissues. In contrast, *RiRZS2* transcripts accumulated weakly in fruits, but were strongly expressed in leaves and stems (which do not produce any raspberry ketone). Taken together with the accumulation pattern of raspberry ketone during the ripening of raspberry fruits [4], the *RiRZS1* gene most probably participates in the biosynthetic pathway of raspberry ketone in the fruits as compared to *RiRZS2*.

3.3. Enzymatic properties of raspberry RZS1 and RZS2

Heterologous expression systems using *E. coli* cells were used for the characterization of recombinant RiRZS1 and RiRZS2. We purified RiRZS1 and RiRZS2 from *E. coli* overexpression lines by

Table 1
Comparison of amino acid identity for various alkenal/alkenone reductases (%).

	RZS1	RZS2	12-HD/PGR	AtAER	HvAER	NtAER	PulR	PtPPAER
RZS1	–	78.4	43.4	69.3	61.0	76.8	65.2	48.4
RZS2		–	41.4	69.4	61.4	72.8	65.8	45.6
12-HD/PGR			–	41.6	41.1	41.7	37.4	37.5
AtAER				–	63.9	71.1	63.0	44.6
HvAER					–	67.3	56.1	47.5
NtAER						–	67.2	48.3
PulR							–	45.9
PtPPAER								–

12-HD/PGR, 12-hydroxydehydrogenase/15-oxo-prostaglandin 13-reductase from *Cavia porcellus*; AtAER, 2-alkenal dehydrogenase from *Arabidopsis thaliana*; HvAER, 2-alkenal dehydrogenase from *Hordeum vulgare*; NtAER, 2-alkenal dehydrogenase from *Nicotiana tabacum*; PulR, (+)-pulegone reductase from *Mentha x piperita*; PtPPAER, phenylpropenal 2-alkenal dehydrogenase from *Pinus taeda*.

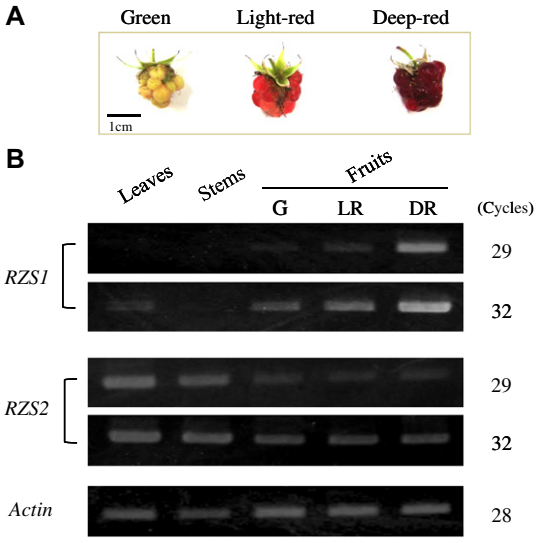


Fig. 2. Different developmental stages of raspberry fruits and tissue-specific expression of *RZS1* and *RZS2* genes. (A) Three different developmental stages of raspberry fruits used in this experiment. (B) Relative expression levels of *RZS1* and *RZS2* genes in the different raspberry tissues. The different developmental stages, green, light red, and deep red are shown with G, LR, and DR, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

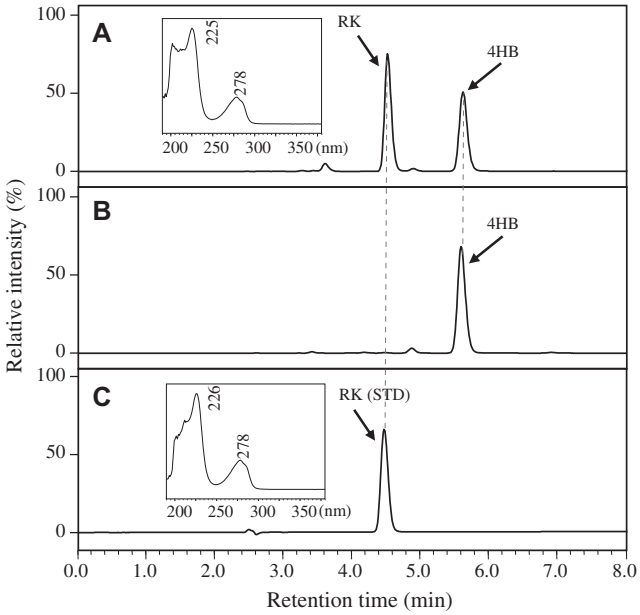


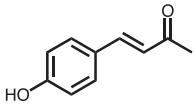
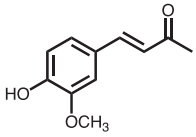
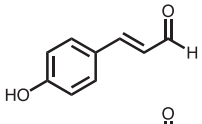
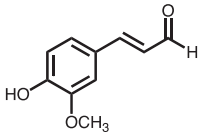
Fig. 3. Product analysis by HPLC of the reaction catalyzed by RZS1. The purified enzyme was incubated with NADPH and 4-hydroxybenzalacetone (4HB) as the substrate, and the extracted product was detected by HPLC using the absorption at 278 nm and compared to the authentic standard raspberry ketone (RK). (A) Reaction catalyzed by RZS1. (B) Reaction catalyzed by RZS1 without NADPH (negative control). (C) Authentic standard of raspberry ketone.

Ni–NTA affinity chromatography, and the resulting proteins with the 37 kDa were confirmed by SDS–PAGE (data not shown). RiRZS1 was tested for the reductase activities by *in vitro* assays with 4-hydroxybenzalacetone. In this experiment, production of raspberry ketone was observed in the presence of NADPH as a cofactor, whereas neither control vector nor absence of NADPH in the reaction mixture generated raspberry ketone (Fig. 3). Detailed characterization of RiRZS1 showed that the enzyme was active in the pH range of 5.5–7.5 with optimal activity at pH 6.5 with MES buffer. The apparent K_m values for 4-hydroxybenzalacetone were 87.8 ± 16.8 μM ($n = 3$) with an apparent k_{cat} value of 0.39 ± 0.04 s^{-1} ($n = 3$). The apparent K_m values for NADPH were 202.1 ± 38.8 μM ($n = 3$).

On the other hand, little raspberry ketone was formed when RiRZS1 was replaced by RiRZS2 for the reductase assay (Table 2). We next tested some of the 4-hydroxybenzalacetone derivatives as potential substrates, including 3-methoxy-4-hydroxybenzalacetone, *p*-coumaraldehyde, and coniferaldehyde, to examine the substrate specificities of RiRZS1. The results of these assays indicated that 4-hydroxybenzalacetone is the most preferable substrate for RiRZS1, followed by 3-methoxy-4-hydroxybenzalacetone and *p*-coumaraldehyde (58.1% and 16.6% relative activities, respectively) (Table 2). Coniferaldehyde, on the other hand, was not a substrate for RiRZS1.

Although PtPPAER activity for 4-hydroxybenzalacetone was not tested, RiRZS1's substrate preference is likely to be different from PtPPAER, having a greater preference for substrates with phenylbutenones including 4-hydroxybenzalacetone and 3-methoxy-4-hydroxybenzalacetone. Its decreased or abolished activities with *p*-coumaraldehyde and with coniferaldehyde, which lack the

Table 2
Substrate specificity of RZS1 and RZS2 from raspberry.

Substrate	Structure	Specific activity (nkat/mg)	
		RZS1	RZS2
4-Hydroxybenzalacetone		7.61 ± 1.03	trace
3-Methoxy-4-hydroxy-benzalacetone		4.42 ± 0.70	trace
p-Coumaraldehyde		1.26 ± 0.17	N.D.
Coniferaldehyde		N.D.	N.D.

methyl group at the end of the side chain, also suggests that the methyl carbonyl group is quite important for either enzyme binding or double bond reduction by RiRZS1.

In conclusion, although many genes encoding various types of NADPH-dependent reductases that recognize the α,β-unsaturated double bond of secondary metabolites have been reported, this work represents the cloning and characterization of RiRZS1, a NADPH-dependent reductase that discriminates the methyl carbonyl group in the butenyl side chain of substrates and plays a crucial role in the biosynthesis of phenylbutanones including raspberry ketone.

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