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ORIGINAL ARTICLE

A bifunctional type III polyketide synthase from raspberry (*Rubus idaeus* L.) with both chalcone synthase and benzalacetone synthase activity

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Abstract Raspberry ketone accounts for the characteristic aroma of the raspberry fruit. A bifunctional enzyme with both chalcone synthase (CHS) and benzalacetone synthase (BAS) activity is thought to play a crucial role in the synthesis of *p*-hydroxybenzalacetone, yet the in vitro enzymatic properties and reaction products of the CHS/BAS recombinant enzyme from raspberry have not been characterized. In this work, a type III polyketide synthase (PKS) gene (*RinPKS1*) and its corresponding cDNA were isolated from raspberry. Sequence and phylogenetic analyses demonstrated that RinPKS1 is a CHS. However, functional and enzymatic analyses showed that recombinant RinPKS1 is a bifunctional enzyme with both CHS and BAS activity. RinPKS1 showed some interesting characteristics: (1) no traces of bis-noryangonin and 4-coumaroyltriacetic acid lactone could be detected in the enzyme reaction mixture at different pH values; and (2) recombinant RinPKS1 overexpressed in *Escherichia coli* effectively yielded *p*-hydroxybenzalacetone as a dominant product at high pH; however, it effectively yielded naringenin as a dominant product at low pH. Furthermore, 4-coumaroyl-CoA and feruloyl-CoA were the only cinnamoyl-CoA derivatives accepted as starter substrates. RinPKS1 did not accept isobutyryl-CoA, isovaleryl-CoA or acetyl-CoA as substrates.

Keywords Benzalacetone synthase (BAS) · Chalcone synthase (CHS) · Raspberry · *Rubus idaeus* L. · Type III polyketide synthase

Abbreviations

BAS	Benzalacetone synthase
CHS	Chalcone synthase
CoA	Coenzyme A
IPTG	Isopropyl thio-β-d-galactoside
LC-ESIMS	Liquid chromatography electron spray ionization mass spectrometry
OKS	Octaketide synthase
PKS	Polyketide synthase

Introduction

Raspberry ketone [4-(4-hydroxyphenyl)-butan-2-one] is a key flavor molecule with typical raspberry flavor characteristics, and it is frequently used in products such as soft drinks, sweets, puddings and ice creams (Beekwilder et al. 2007). Raspberry ketone is mainly found in the fruit of the raspberry (*Rubus idaeus* L.), but yields are typically very low; the content is usually 1–4 mg kg^{-1} raspberry (Larsen et al. 1991). Up to \$20,000 kg^{-1} may be paid for the natural compound (Beekwilder et al. 2007).

In the fruit of the raspberry, the biosynthesis of raspberry ketone begins with the phenylpropanoid pathway

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(Borejsza-Wysocki and Hrazdina 1994) (Fig. 1). Benzalacetone synthase (BAS) catalyzes a one-step, decarboxylative condensation of *p*-coumaroyl-CoA with malonyl-CoA to produce *p*-hydroxybenzalacetone (Fig. 1) (Borejsza-Wysocki and Hrazdina 1996). In the second step, the double bond in *p*-hydroxybenzalacetone is reduced, resulting in raspberry ketone (Fig. 1). This step is catalyzed by benzalacetone reductase (BAR), and requires the presence of NADPH (Beekwilder et al. 2007).

BAS is a member of the plant-specific, type III polyketide synthase (PKS) superfamily, and it is thought to play a crucial role in the construction of the C_6–C_4 moiety of a variety of medicinally important phenylbutanoids, such as the anti-inflammatory glucoside lindleyin in rhubarb, gingerol and curcumin in ginger plants (Abe et al. 2001), and raspberry ketone in raspberry (Borejsza-Wysocki and Hrazdina 1994, 1996). The type III PKSs are thought to play a crucial role in the construction of the basic skeleton of a variety of aromatic polyketides (Zheng and Hrazdina 2008). Thus far, 14 plant-specific type III PKS enzymes have been identified. Chalcone synthase (CHS) is by far the most common and widely distributed type III PKS in higher plants, and most or all of the divergent plant CHS-like enzymes characterized to date have arisen via extensive duplications and subsequent genetic variations of the CHS gene copy (Austin and Noel 2003). It provides the first committed step in flavonoid biosynthesis by catalyzing the sequential condensation of three acetate units from malonyl-CoA to a *p*-coumaroyl-CoA starter molecule, followed by a Claisen-type

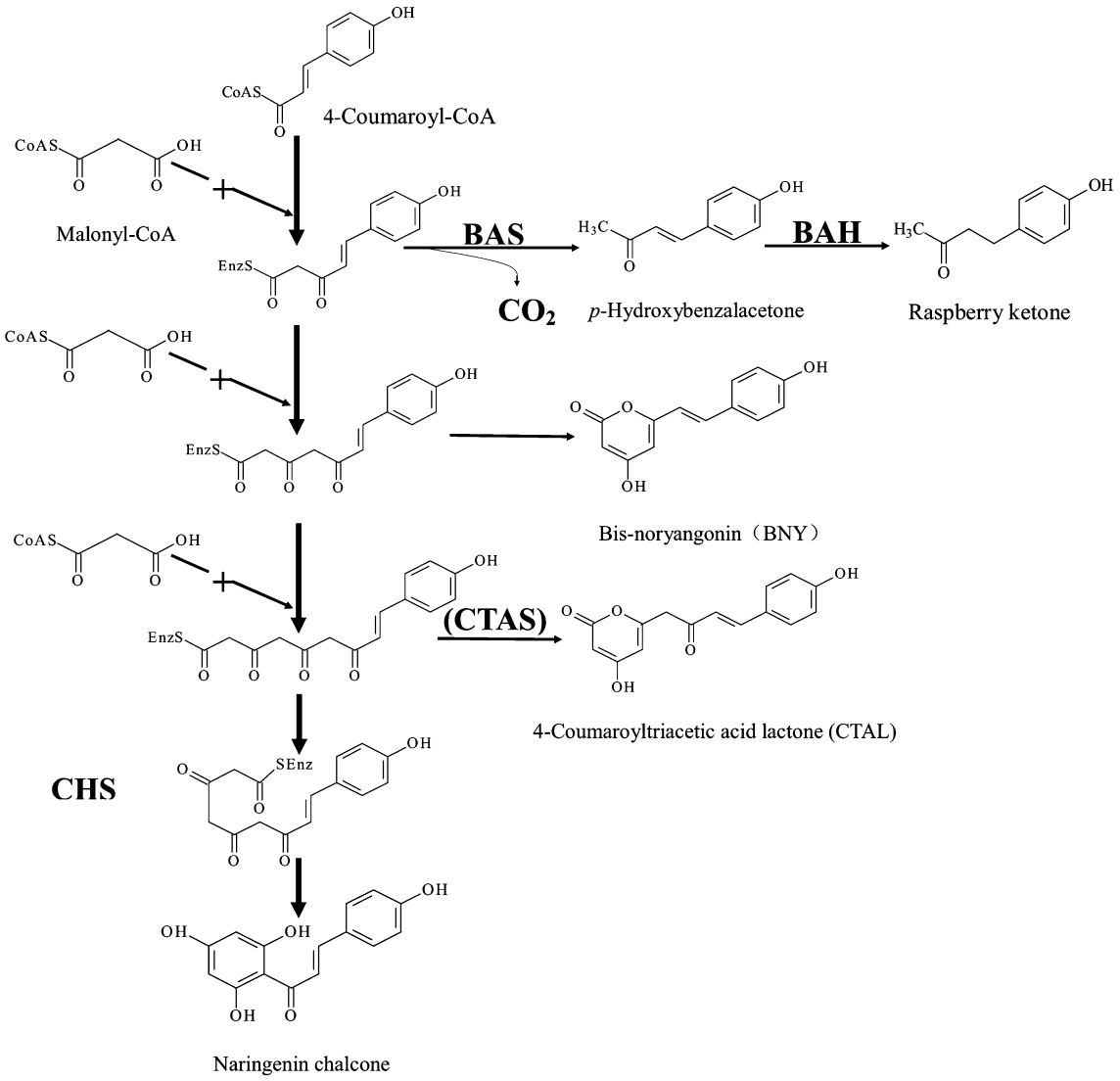


Fig. 1 The reactions for the conversion of 4-coumaroyl-CoA and malonyl-CoA to *p*-hydroxybenzalacetone by the benzalacetone synthase (BAS), and to naringenin by chalcone synthase (CHS). Polyketide pyrones, bis-noryangonin (BNY), and 4-coumaroyltriacetic acid lactone (CTAL) are derailment side products of the type III PKS reactions in vitro when the reaction mixtures are acidified before extraction

cyclization reaction, leading to the formation of an aromatic tetraketide, naringenin chalcone (Fig. 1) (Schröder 1997; Austin and Noel 2003).

The results of a number of studies have shown that *p*-hydroxybenzalacetone is not an early-released side product of CHS, but that this compound is the specific product of BAS (Borejsza-Wysocki and Hrazdina 1994, 1996; Abe et al. 2001; Ma et al. 2009a, b). *Rheum palmatum* BAS is the first reported benzalacetone synthase gene (Abe et al. 2001). In our recent work, two, three-intron type III PKS genes, *PcPKS1* (Ma et al. 2009a) and *PcPKS2* (Ma et al. 2009b), were isolated from *Polygonum cuspidatum*. Phylogenetic and functional analyses revealed that PcPKS1 is a bifunctional enzyme with both CHS and BAS activities, and the recombinant PcPKS2 was found to be a BAS. However, such a true monofunctional BAS has never been identified in raspberry to date. Interestingly, two PKSs, RiCHS (Beekwilder et al. 2007) and RiPKS4 (Zheng and Hrazdina 2008), were described as bifunctional PKSs, exhibiting both CHS and BAS activities in raspberry. However, the BAS activity of RiCHS was found in engineered *Escherichia coli* and yeast in vivo systems, yet no BAS activity could be detected with the recombinant protein, and there was no exact sequence information for RiCHS (Beekwilder et al. 2007). The BAS activity of recombinant RiPKS4 was only detected in crude protein extracts of *E. coli* cells because its poor solubility prevented the isolation of sufficient quantities of protein for enzymatic characterization(Zheng and Hrazdina 2008). In this paper, we describe the cloning of a PKS with both CHS and BAS activities from raspberry, as well as the characterization of its in vitro reaction products.

Materials and methods

Plant material and chemicals

Rubus idaeus plants were maintained in the plant garden and in a greenhouse at the Beijing University of Agriculture, Beijing, China. 4-Coumaroyl-CoA, cinnamoyl-CoA, caffeoyl-CoA and feruloyl-CoA were synthesized as described (Beuerle and Pichersky 2002). Malonyl-CoA, benzoyl-CoA, isobutyryl-CoA, isovaleryl-CoA, acetyl-CoA and naringenin were purchased from Sigma-Aldrich (St. Louis, MO, USA). *p*-Hydroxybenzalacetone (*p*-hydroxyphenylbut-3-ene-2-one) was purchased from Alfa Aesar GmbH & Co. KG (Karlsruhe, Germany).

Cloning of the *R. idaeus PKS* gene

Genomic DNA was isolated from young leaves using a modified cetyltrimethylammonium bromide (CTAB) method (Porebski et al. 1997). Degenerate primers were produced on the basis of the consensus-degenerate hybrid oligonucleotide primers (CODEHOP) strategy using the block maker program (http://blocks.fhcrc.org/blocks/blockmkr/make_blocks.html) as follows: forward, 5′-GGAGGCTGCTGTTAAGGCTATcvmngartgggg-3′ (pRiPKS-c-up) and reverse, 5′-AAAGAACGCAAGCAGAAGAcatrttnccrta-3′ (pRiPKS-c-dw), where n is any nucleotide, v is A or C or G, m is A or C, and r is A or G. The PCR program included a 5-min denaturation at 95 °C, 30 cycles at 94 °C (30 s), 59 °C (40 s) and 72 °C (90 s), and a final 7-min extension at 72 °C. To obtain the full-length gene, thermal asymmetric interlaced (TAIL)-PCR was performed using three gene-specific primers, designed according to the terminal sequences of the core fragment and AD primers (Liu et al. 1995). The PCR conditions were the same as described (Liu et al. 1995).

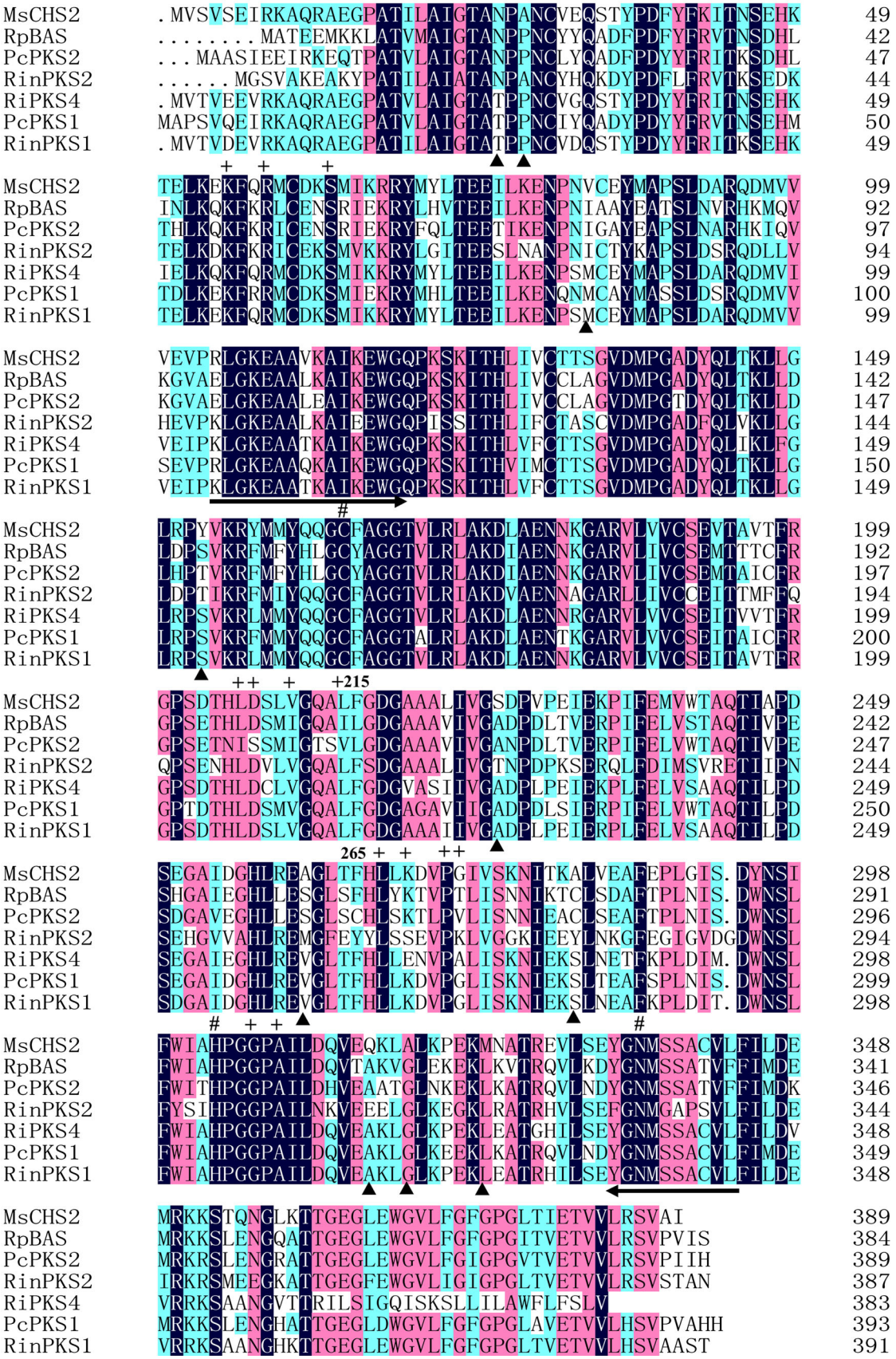
Cloning of *R. idaeus PKS* cDNA

Total RNA was isolated from fruits using the Plant Total RNA Isolation Kit (Autolabtech, Beijing, China). Reverse transcription was conducted at 42 °C using the ImProm-II™ Reverse Transcription System (Promega, WI, USA) and the oligo (dT)-adaptor primer (PdT: CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTTTTTTTTT). A full-length cDNA was amplified using gene-specific primers based on the obtained open reading frame (ORF) sequence: 5′-ATGGTGACCGTCGATGAAGTCCGC-3′ (Pbf-N) and 5′-TCAAGTTGAAGCTGCCACACTGTG-3′ (Pbf-C). The PCR program included a 5-min denaturation at 95 °C, 30 cycles at 94 °C (30 s), 59 °C (40 s) and 72 °C (120 s), and a final 7-min extension at 72 °C. The gel-purified PCR product was ligated into the pMD18-T vector (TaKaRa, Dalian, China) and sequenced.

Heterologous expression in *E. coli* and recombinant enzyme purification

The ORF of the cDNA was amplified using 5′ and 3′ PCR primers: sense, the *Nde*I site is underlined 5′-TATA**GAATTC**ATGGTGACCGTCGATGAAGTCCGC-3′, and antisense, the *Sal*I site is underlined 5′-TATA**CTCGAG**TCAAGTTGAAGCTGCCACACTGTG-3′. The amplified DNA was digested with *Eco*RI/*Xho*I, and cloned into the *Eco*RI/*Xho*I sites of pET-30a (+) (Novagen, Darmstadt, Germany). The recombinant enzyme contained a hexahistidine tag at its C terminus.

After sequencing the ORF on both strands, the recombinant plasmid was introduced into *E. coli* Rosetta-gami™ (DE3) (Novagen) and grown at 37 °C, with shaking at 200 rpm, in 200 ml of Luria–Bertani (LB) medium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RpBAS . . . . . MATEEMKKLATVMAIGTANPPNCYYQADFPDFYFRVTNSDHL 42
PcPKS2 . . . MAASIEEIRKEQTPATVLAIGTANPPNCLYQADFPDYYFRITKSDHL 47
RinPKS2 . . . . . MGSVAKEAKYPATILAIATANPANCYHQKDYPDFLFRVTKSEDK 44
RiPKS4 . MVTVEEVRKAQRAEGPATVLAIGTATPPNCVGQSTYPDYYFRITNSEHK 49
PcPKS1 MAPSVQEIRKAQRAEGPATVLAIGTATPPNCIYQADYPDYYFRVTNSEHM 50
RinPKS1 . MVTVDEVRKAQRAEGPATILAIGTATPPNCVDQSTYPDYYFRITKSEHK 49
MsCHS2 TELKEKFQRMCDKSMIKRRYMYLTEEILKENPNVCEYMAPSLDARQDMVV 99
RpBAS INLKQKFKRLCENSRIEKRYLHVTEEILKENPNIAAYEATSLNVRHKMQV 92
PcPKS2 THLKQKFKRICENSRIEKRYFQLTEETIKENPNIGAYEAPSLNARHKIQV 97
RinPKS2 TELKDKFKRICEKSMVKKRYLGITEESLNANPNICTYKAPSLDSRQDLLV 94
RiPKS4 IELKQKFQRMCDKSMIKKRYMYLTEEILKENPSMCEYMAPSLDARQDMVI 99
PcPKS1 TDLKEKFRRMCDKSMIEKRYMHLTEEILKENQNMCAYMASSLDSRQDMVV 100
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RpBAS KGVAELGKEAALKAIKEWGQPKSKITHLIVCCLAGVDMPGADYQLTKLLD 142
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RpBAS GPSETHLDSMIGQAILGDGAAAVIVGADPDLTVERPIFELVSTAQTIVPE 242
PcPKS2 GPSETNISSMIGTSVLGDGAAAVIVGANPDLTVERPIFELVWTAQTIVPE 247
RinPKS2 QPSENHLDVLVGQALFSDGAAALIVGTNPDPKSERQLFDIMSVRETIIPN 244
RiPKS4 GPSDTHLDCLVGQALFGDGVASIIVGADPLPEIEKPLFELVSAAQTILPD 249
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RinPKS1 GPSDTHLDSLVGQALFGDGAAAIIVGADPLPEIERPLFELVSAAQTILPD 249
MsCHS2 SEGAIDGHLREAGLTFHLLKDVPGIVSKNITKALVEAFEPLGIS. DYNSI 298
RpBAS SHGAIEGHLLESGLSFHLYKTVPTLISNNIKTCLSDAFTPLNIS. DWNSL 291
PcPKS2 SDGAVEGHLLESGLSCHLSKTLPVLISNNIEACLSEAFTPLNIS. DWNSL 296
RinPKS2 SEHGVVAHLREMGFEYYLSSEVPKLVGGKIEEYLNKGFEGIGVDGDWNSL 294
RiPKS4 SEGAIEGHLREVGLTFHLLLENVPALISKNIEKSLNETFKPLDIM. DWNSL 298
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PcPKS2 FWITHPGGPAILDHVEAATGLNKEKLKATRQVLNDYGNMSSATVFFIMDK 346
RinPKS2 FYSIHPGGPAILNKVEEELGLKEGKLRATRHVLSEFGNMGAPSVLFILDE 344
RiPKS4 FWIAHPGGPAILDQVEAKLGLKPEKLEATGHILSEYGNMSSACVLFILDV 348
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RpBAS MRKKSLENGQATTGEGLEWGVLFGFGPGITVETVVLRSVPVIS 384
PcPKS2 MRKRSLENGRATTGEGLEWGVLFGIGPGVTVETVVLRSVPIIH 389
RinPKS2 IRKRSMEEGKATTGEGFEWGVLIGIGPGLTVETVVLRSVSTAN 387
RiPKS4 VRRKSAANGVTTRILSIGQISKSLLILAWFLFSLV 383
PcPKS1 MRKKSLENGHATTGEGLDWGVLFGFGPGLAVETVVLHSVPVAHH 393
RinPKS1 VRRKSAANGHKTTGEGLEWGVLFGFGPGLTVETVVLHSVAAST 391

◀**Fig. 2** Amino acid sequence comparison of seven type III PKSs of plant origin. Multiple sequence alignment was calculated with the DNAMAN package. Black shading shows amino acid identities. The active site residues conserved in the type III PKSs (Cys164, His303, and Asn336, *M. sativa* CHS2 numbering) are marked with #, and residues for CoA binding are marked with +. Amino acid residues are conserved in *P. cuspidatum* PcPKS1 (Borejsza-Wysocki and Hrazdina 1994), *R. idaeus* RiPKS4 (Beekwilder et al. 2005), but are absent from other CHSs (marked with *filled triangle*). The abbreviations for species and accession numbers are: MsCHS2 (*M. sativa* P30074), RpBAS (*R. palmatum* AAK82824), PcPKS2 (*P. cuspidatum* ABY47640), RiPKS4 (*Rubus idaeus* ABV54602), PcPKS1 (*P. cuspidatum* ABK92282)

containing kanamycin (50 μg ml^{-1}) and chloramphenicol (34 μg ml^{-1}). At an absorbance at 600 nm (A_{600}) of 0.6–0.8, 1 mM isopropyl thio-β-d-galactoside (IPTG) was added and the incubation temperature was reduced to 25 °C. After incubation for 4 h, cells were harvested by centrifugation, resuspended in 3 ml of 0.1 M potassium phosphate buffer (pH 7.5), and sonicated on ice for 10 min.

The homogenate was centrifuged at 10,000*g* for 10 min at 4 °C. The supernatant was passed through a column of Ni–NTA His-Bind™ Resin (Novagen) containing Ni^{2+} as an affinity ligand. After washing with 0.1 M potassium phosphate buffer (pH 7.5) containing 0.5 M NaCl and 40 mM imidazole, the recombinant *R. idaeus* PKS was eluted with 0.1 M potassium phosphate buffer (pH 7.5) containing 400 mM imidazole. For long-term storage, the buffer was changed to 0.1 M Tris–HCl (pH 7.5) and 10 % (v/v) glycerol using PD-10 columns (Amersham Pharmacia Biotech, Uppsala, Sweden), and the sample was stored at −80 °C. The efficiency of purification was monitored by SDS-PAGE. The protein concentration was determined by the Bradford method using bovine serum albumin as a standard.

Enzyme reaction and product analysis

The standard assay (300 μl) contained 150 μM starter CoA, 280 μM malonyl-CoA, 0.1 M potassium phosphate (pH 7.0) and 2.0 μg of protein, and was incubated at 30 °C for 60 min, then extracted twice with 700 μl of ethyl acetate and centrifuged at 10,000 *g* for 10 min. Acetic acid (5 % final concentration) was added before the extraction step to detect any side product. After drying under vacuum, the residue was dissolved in 50 μl of 50 % (v/v) methanol. The pH optimum of the assay was determined over a pH range of 5.0–10.0.

Analysis of the enzymatic products was performed by high-performance liquid chromatography (HPLC) on a Kromosil C_{18} reverse-phase column (5 μm, 250 mm × 4.6 mm; Macherey–Nagel, Düren, Germany). A Waters Alliance 2695 HPLC system was connected to a Waters 2487 photodiode array detector, and controlled by a computer running Empower software (Waters, Milford, MA, USA). The eluents were water (A) and methanol (B) at a flow rate of 1.0 ml min^{-1}. The following gradients were used: 20 % B for 2 min, 70 % B for 12 min, 80 % B for 15 min, 95 % B for 16 min, 95 % B for 20 min and 20 % B for 25 min. The detection wavelengths were 289 nm (naringenin chalcone), 323 nm (*p*-hydroxybenzalacetone, 4-CTAL) and 365 nm (bis-noryangonin, BNY). A standard solution of reference compounds was used for quantification.

For on-line, high-performance liquid chromatography/mass spectrometry (HPLC–MS) analysis, liquid chromatography was performed on a Shimadzu LC-10ADvp HPLC system, which was coupled to a Shimadzu LCMS-2010A single quadrupole mass spectrometer with an electrospray ionization (ESI) interface (Shimadazu, Kyoto, Japan). Data acquisition and processing were performed using Shimadzu LC–MS solution software for LC-ESIMS-2010. HPLC separations were conducted under the conditions described above. The optimized MS operating conditions were as follows: all spectra were obtained in positive mode over an *m*/*z* range of 120–350; drying gas flow, 1.5 l min^{-1}; CDL temperature, 250 °C; block temperature, 200 °C; and probe voltage +4.5 kV.

Enzyme kinetics

Kinetic constants were determined using five concentrations of substrate covering the K_m range of 0.2–6.0, and the concentration of the second substrate was the saturation point. The experiment was performed three times with 2.0 μg of purified enzyme in a final volume of 250 μl of 0.1 M potassium phosphate buffer; incubation was at 35 °C for 30 min. The kinetic parameters were calculated for the formation of the major product of the enzyme reactions at the optimum pH. Lineweaver–Burk plots of the data were used to derive the apparent K_m and K_{cat} values (average of triplicates ±SD).

Phylogenetic tree construction

A total of 32 amino acid sequences of plant-specific, type III PKSs were aligned using CLUSTALX, version 1.81 (Thompson et al. 1997). The aligned sequences were first subjected to bootstrapping using the program SEQBOOT in the PHYLIP package. Genetic distances within 100 bootstrap replicates were calculated with the PHYLIP program PROTDIST using a Dayhoff's PAM 001 matrix. Distance matrices were then analyzed with the PHYLIP program NEIGHBOR using the neighbor-joining algorithm. Data from the above calculation were analyzed with the PHYLIP program CONSENSE to obtain bootstrap values reflecting the consistency of the tree branch pattern.

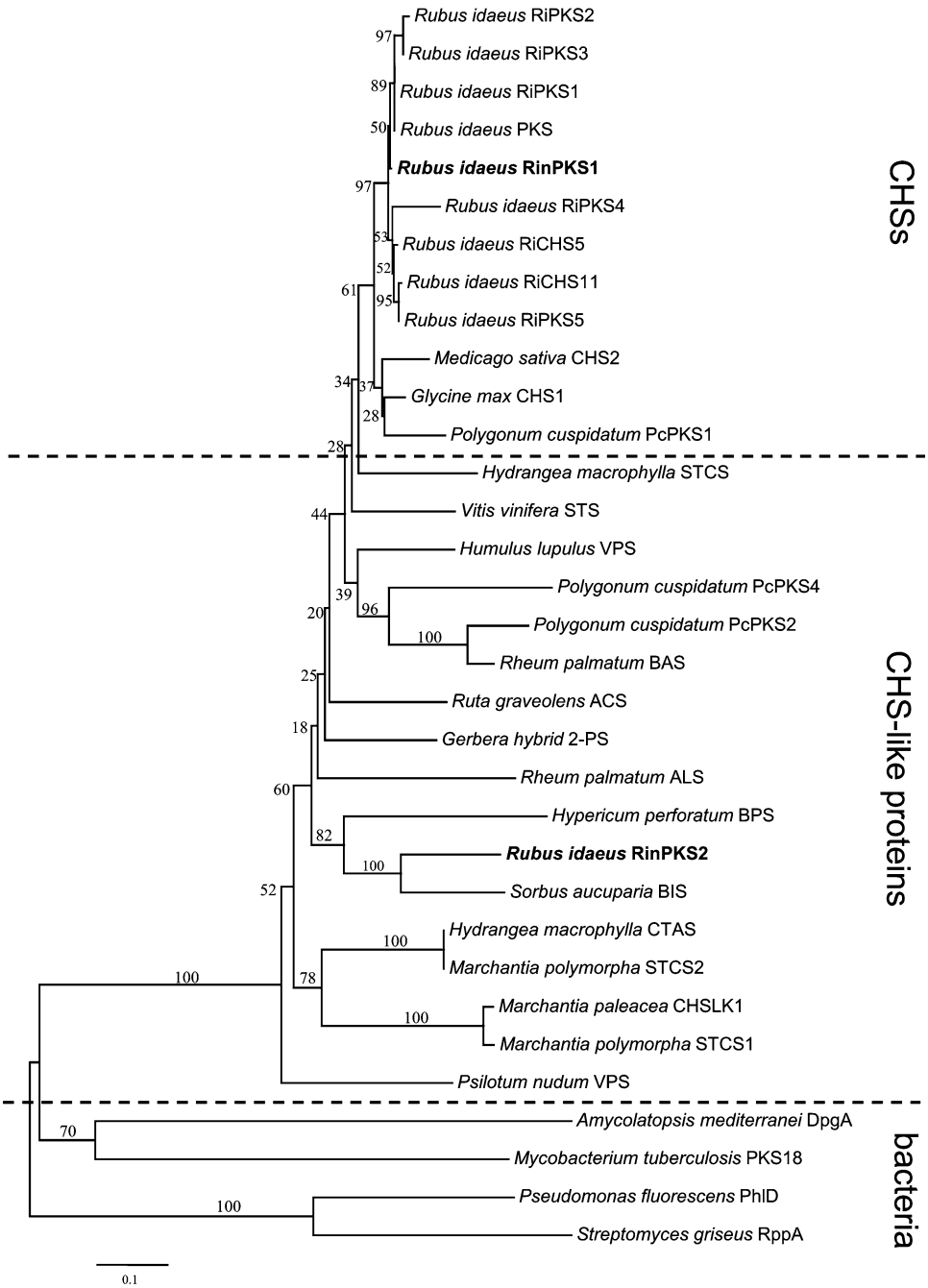

Fig. 3 Neighbor-joining tree of type III PKSs. Numbers at the forks are bootstrap values from 100 replicates. Four bacterial type III PKSs were used to root the tree. The accession numbers used in the analysis are listed in "Materials and methods" section

The final tree was viewed using TREEVIEW WIN32 (Page 1996). The accession numbers used in the analysis are as follows: *R. idaeus* RinPKS1 (RiCHS6, AF400567), RiPKS1 (AAK15174), RiPKS2 (AAK15175), RiPKS3 (AAK15176), RiPKS4 (ABV54602), RiPKS5 (ABV54603), RiCHS5 (AAM90650), RiCHS11 (AAM90651), aromatic PKS (ACF72868), *Medicago sativa* CHS2 (P30074), *R. palmatum* BAS (AAK82824), ALS (AAS87170), *P. cuspidatum* PcPKS1 (ABK92282), PcPKS2 (ABY47640), PcPKS4 (HQ129934), *Vitis vinifera* CHS (CAA53583), *Ruta graveolens* ACS (CAC14058), *Gerbera hybrid* 2-PS (CAA86219), *Sorbus aucuparia* BIS (ABB89212), *Hypericum perforatum* BPS (ABP49616), *Marchantia polymorpha* STCS1 (AAW30009), *M. polymorpha* STCS2 (AAW30010), *Marchantia paleacea* MpCHSLK1 (BAD42328), *Psilotum nudum* VPS (BAA87923), *Humulus lupulus* VPS (BAA29039), *Hydrangea macrophylla* STCS (AAN76182), *H. macrophylla* CTAS (BAA32733) and *Glycine max* CHS1 (ABB30178).

Four functionally characterized, bacterial type III PKSs served as an outgroup: *Pseudomonas fluorescens* PhlD (AAB48106), *Streptomyces griseus* RppA (BAA33495),

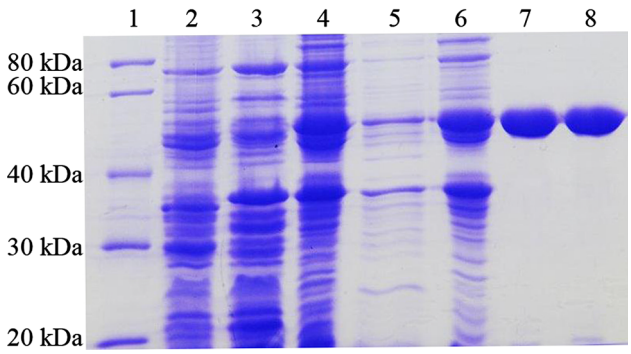

Fig. 4 SDS-PAGE analyses of recombinant RinPKS1 stained with Coomassie brilliant blue. *Lane 1* molecular mass markers with masses indicated in kDa. *Lane 2* total protein from uninduced *E. coli* strain Rosetta-gami^{TM} (DE3) harboring the empty vector pET30a (without insert). *Lane 3* total protein from uninduced *E. coli*. strain BL21-Rosetta (DE3) harboring the His-tagged *RinPKS1* construct. *Lane 4* total protein from BL21-Rosetta (DE3) cells harboring the Histagged *RinPKS1* construct after induction with IPTG for 4 h at 25 °C. *Lane 5* soluble extract from BL21-Rosetta (DE3) cells harboring the Histagged *RinPKS1* construct after induction with IPTG for 4 h at 25 °C. *Lane 6* pellet extract from BL21-Rosetta (DE3) cells harboring the Histagged *RinPKS1* construct after induction with IPTG for 4 h at 25 °C. *Lane 7* recombinant RinPKS1 after Ni^{2+}-chelating chromatography. *Lane 8* recombinant RinPKS1 (5 mg) after passage through a PD-10 columny

Mycobacterium tuberculosis PKS18 (A70958) and *Amycolatopsis mediterranei* DpgA (CAC48378).

Results

Isolation of the *RinPKS1*

Using primers pRiPKS-c-up and pRiPKS-c-dw (Fig. 2), a 700-bp core fragment was cloned, and its deduced amino acid sequence exhibited over 90 % identity with the CHSs of other plants in the raspberry family. Flanking sequences were obtained by TAIL-PCR (Liu et al. 1995), and the resulting gene was designated as *RinPKS1* (Fig. 2). The cDNA of *RinPKS1* was amplified using gene-specific primers for the full-length ORF and the reverse transcription products as template.

Sequence analysis

The full-length *RinPKS1* gene is 1922 bp long and contains an intervening sequence. The intron of the PcPKS3 gene is 385 bp long and is inserted between the first and second nucleotides of Cys60 (numbering of *M. sativa* CHS2), which is conserved in all known PKS genes.

The *RinPKS1* cDNA is 1176 bp in length and encodes a deduced protein of 391 amino acid residues, with a predicted molecular mass of 42.8 kDa and a p*I* of 6.04 (data not shown). The deduced amino acid sequence showed 59–99 % identity with other type III PKSs of plant origin (Fig. 2), 99 % identity with *R. idaeus* RiCHS6 (Kumar and Ellis 2003), 87 % identity with *R. idaeus* RiPKS4 (Zheng and Hrazdina 2008), 85 % identity with *M. sativa* CHS2 (Ferrer et al. 1999), 82 % identity with *P. cuspidatum* PcPKS1 (Ma et al. 2009a), 66 % identity with *R. palmatum* BAS (Abe et al. 2001), 65 % identity with *P. cuspidatum* PcPKS2 (Ma et al. 2009b) and 59 % identity with *Aloe arborescens* octaketide synthase (OKS) (Abe et al. 2005).

R. idaeus RinPKS1 retains the catalytic triad of Cys164, His303 and Asn336, and all identical CoA binding sites (numbering of *M. sativa* CHS2) (Fig. 2). Furthermore, most of the active site residues, including Val98, Thr132, Ser133, Met137, Gly163, Thr194, Gly211, Gly216, Ile254, Ser338 and Pro375, along with the CHS "gatekeepers" Phe215 and Phe265 (Austin and Noel 2003), are well conserved in PcPKS1. However, the CHS active site residues Gln315, Asn325, Thr354 and Gln355 are uniquely replaced with Ala, Glu, Ala and Ala, respectively, in RinPKS1 (Fig. 2). In addition, 10 amino acid residues are conserved in *P. cuspidatum* PcPKS1 (Ma et al. 2009a), *R. idaeus* RiPKS4 (Zheng and Hrazdina 2008) (the bifunctional PKSs exhibiting both CHS and BAS activity), but are different from other CHSs as indicated in Fig. 2.

Phylogenetic analysis

The phylogenetic tree showed that RinPKS1 is located together with all raspberry PKSs in the GenBank database in a cluster containing other typical chalcone-forming enzymes (Fig. 3). The other cluster was formed by all the functionally divergent CHS-like enzymes, including raspberry RinPKS2 (our unpublished results), *R. palmatum* BAS (Abe et al. 2001) and *P. cuspidatum* PcPKS2 (Ma et al. 2009b).

Characterization of RinPKS1

R. idaeus RinPKS1 was heterologously expressed in *E. coli* with an additional hexahistidine tag at its C terminus. The purified enzyme yielded a single band with a molecular mass of 43 kDa on SDS-PAGE (Fig. 4). On a calibrated gel-filtration column, the native molecular mass of the enzyme was about 89 kDa, indicating a homodimeric structure of RinPKS1.

Identification and quantitation of the enzymatic products showed that recombinant RinPKS1 activity was dependent on pH. When incubated with 4-coumaroyl-CoA and malonyl-CoA as substrates at pH 7.5, RinPKS1 catalyzed the formation of naringenin as a major product, along with *p*-hydroxybenzalacetone, which were identified by HPLC and LC–MS via comparison with authentic samples

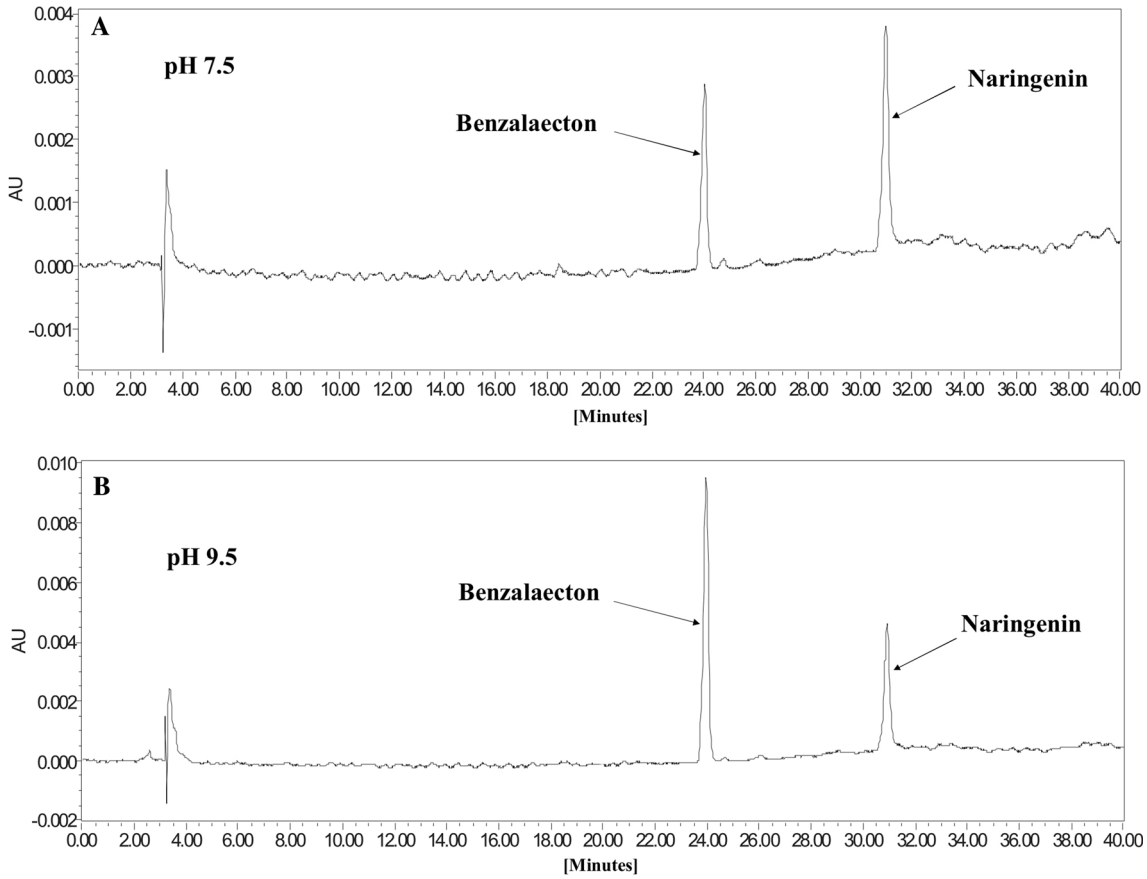

Fig. 5 HPLC elution profiles of enzyme reaction products of the chalcone-forming activity and the *p*-hydroxybenzalacetone-forming activity of the RinPKS1 at pH 7.5 (**a**) and pH 9.5 (**b**). HPLC separation conditions are described in "Materials and methods" section. Note that naringenin chalcone is converted by acid treatment to racemic naringenin through a nonstereospecific ring C closure

Table 1 Substrate specificity of recombinant RinPKS1 from *R. idaeus*

Substrate	Enzyme activity (% of max.)	
	chalcone-forming activity[a]	*p*-hydroxybenzalacetone-forming activity[a]
4-Coumaroyl-CoA	100	100
cinnamoyl-CoA	81	0
caffeoyl-CoA	43	0
feruloyl-CoA	52	77
benzoyl-CoA	22	0
acetyl-CoA	0	0
isobutyryl-CoA	0	0
isovaleryl-CoA	0	0

[a] Substrate specificity was measured for formation of major product of the enzyme reaction at optimum pH

(Fig. 5a). However, at pH 9.5, RinPKS1 effectively yielded *p*-hydroxybenzalacetone as a dominant product, along with naringenin chalcone (Fig. 5b). Moreover, no traces of bis-noryangonin (BNY) and 4-coumaroyltriacetic acid lactone (CTAL) (Fig. 1) could be detected in the enzyme reaction mixture at the different pH values (Fig. 5).

For the chalcone-forming activity, the pH and temperature optima of RinPKS1 were 7.5 and 40 °C, respectively. In addition to 4-coumaroyl-CoA, its derivatives were accepted as starter substrates; however, the relative activities were lower (Table 1). In contrast to CHS, which showed broad substrate specificity towards aliphatic CoA esters, RinPKS1 did not accept isobutyryl-CoA, isovaleryl-CoA or acetyl-CoA as substrates (Table 1). For the chalcone-forming reaction, we determined the kinetic parameters for 4-coumaroyl-CoA ($K_m = 16.42$ μM, $K_{cat} = 2.70$ min^{-1},

K_{cat}/K_m = 2742 M^{-1} s^{-1}) and malonyl-CoA (K_m = 22.83 μM, K_{cat} = 4.28 min^{-1}, K_{cat}/K_m = 3122 M^{-1} s^{-1}).

The pH and temperature optima of the *p*-hydroxybenzalacetone-forming activity of RinPKS1 were 9.5 and 50 °C, respectively. At this pH value, 4-coumaroyl-CoA and feruloyl-CoA were the only cinnamoyl-CoA derivatives accepted as starter substrates (Table 1). RinPKS1 did not accept isobutyryl-CoA, isovaleryl-CoA or acetyl-CoA as substrates (Table 1). For the *p*-hydroxybenzalacetone-forming reaction, we determined the kinetic parameters for 4-coumaroyl-CoA (K_m = 12.89 μM, K_{cat} = 0.65 min^{-1}, K_{cat}/K_m = 840 M^{-1} s^{-1}) and malonyl-CoA (K_m = 41.17 μM, K_{cat} = 0.46 min^{-1}, K_{cat}/K_m = 186 M^{-1} s^{-1}).

Discussion

Raspberry ketone accounts for the characteristic aroma of the raspberry fruit. At present, there is an insufficient amount of raspberry ketone on the commercial market because of the relatively low content of this compound in raspberry (Beekwilder et al. 2007). There is considerable interest in the regulation of raspberry ketone biosynthesis, both to explain and clarify the biosynthetic pathway of raspberry ketone and also for the identification of targets for the biotechnological manipulation of product accumulation. However, the biosynthetic pathway of raspberry ketone and its regulation are less well understood. Hrazdina et al. (Hrazdina et al. 1976) postulated that benzalacetone was the "early release product" of the CHS reaction resulting from the interference of mercaptoethanol with the active center of the enzyme. However, subsequent studies have proven that the production of benzalacetone is not an artefact; instead, this compound is the product of a specific enzyme (Borejsza-Wysocki and Hrazdina 1994, 1996; Abe et al. 2003; Ma et al. 2009a, b). In addition to raspberry, BAS activity has also been detected in pea, apple, grape, tobacco, rhubarb and Japanese knotweed, and the corresponding products, including the glucosylated form, have been found in several plants, such as *R. idaeus*, *Curcuma longa*, *Zingiber officinale* and *R. palmatum* (Borejsza-Wysocki and Hrazdina 1994, 1996; Abe et al. 2003; Ma et al. 2009a, b).

In this study, we described the enzymatic properties of a bifunctional PKS that possesses both CHS and BAS activities. The RinPKS1 sequence of the encoded protein was 99 % identical to *R. idaeus* RiCHS6 (AF400567) (Kumar and Ellis 2003), with two residues out of 391 differing; Glu100 and Ile123 are uniquely replaced with Asp and Val, respectively, in RinPKS1 (numbering of *M. sativa* CHS2) (Fig. 2). Since Asp for Glu and Val for Ile changes can occur by single nucleotide changes, it is possible that RinPKS1 and RiCHS6 are the same, with the minor differences either due to sequencing errors or minor changes in different *R. idaeus* strains. The results of a number of studies have shown that BAS or a bifunctional enzyme with both CHS and BAS activities is thought to play a crucial role in *p*-hydroxybenzalacetone biosynthesis (Beekwilder et al. 2007; Zheng and Hrazdina 2008; Ma et al. 2009a), yet thus far, both the in vitro enzymatic properties (including kinetic parameters) and reaction products of the aforementioned recombinant enzymes have not been characterized in raspberry. Like PcPKS1, a bifunctional CHS with both CHS and BAS activities in *P. cuspidatum* (Ma et al. 2009a), the formation of *p*-hydroxybenzalacetone is an intrinsic property of recombinant RinPKS1: (1) for the enzymatic property, the pH optimum of the *p*-hydroxybenzalacetone-forming activity of RinPKS1 is 9.5, which is similar to that of *R. palmatum* BAS (8.8) (Abe et al. 2003) and *P. cuspidatum* PcPKS1 and PcPKS2 (9.0) (Ma et al. 2009a, b), and RinPKS1 effectively yielded *p*-hydroxybenzalacetone as a dominant product at this pH optimum (Fig. 5b); (2) for catalytic capability, RinPKS1 exhibited a high level of activity for *p*-hydroxybenzalacetone production, and the catalytic efficiency (K_{cat}/K_m) of the BAS activity of RinPKS1 is 840 M^{-1} s^{-1}, which is 12-fold higher than that of PcPKS2 (Ma et al. 2009b) at the pH optimum; and (3) for substrate specificity, at pH 9.5, like PcPKS1 (Ma et al. 2009a), 4-coumaroyl-CoA and feruloyl-CoA were the only cinnamoyl-CoA derivatives accepted as starter substrates (Table 1). In contrast to typical CHSs (Morita et al. 2000; Abe et al. 2002), such as *R. palmatum* BAS (Abe et al. 2001), PcPKS1 and PcPKS2 (Ma et al. 2009a, b), RinPKS1 did not accept isobutyryl-CoA, isovaleryl-CoA or acetyl-CoA as substrates at different pH values (Table 1). In *R. palmatum* BAS, one of the characteristic features is that the active-site Phe215 is uniquely replaced by Leu (Fig. 2). Austin and Noel (Austin and Noel 2003) proposed that the interface of the active site cavity and the CoA-binding tunnel is mediated by the CHS gatekeeper phenylalanines, Phe215 and Phe265. Interestingly, sequence analysis showed that Phe215 and Phe265 are uniquely replaced by Leu and Cys in PcPKS2 (Fig. 2) (Ma et al. 2009b). Like PcPKS1 (Ma et al. 2009a), this mechanism does not apply to RinPKS1 because Phe215 and Phe265 in RinPKS1 are conserved (Fig. 2). Zheng and Hrazdina (Zheng and Hrazdina 2008) postulate that BAS activity of RiPKS4 was created by the sequence variation in the C terminus due to DNA recombination at the 3′ region of its coding sequence (Fig. 2). However, like PcPKS1(Ma et al. 2009a), this speculation could not be proven by the RinPKS1 sequence, no notable similarity was found in the 3′ region between RinPKS1 and RiPKS4 (Fig. 2).

There is evidence that a relationship exists between BAS and CHS in raspberry. The color of the ripe raspberry fruit

is predominantly determined by the formation of red anthocyanins, which are derived from the naringenin chalcone (Beekwilder et al. 2005). A recent study has demonstrated that *RiCHS6* expression did not show a temporal relationship to anthocyanin accumulation, but varied significantly in ripening raspberry fruits, indicating that this gene is regulated in a ripening-dependent manner (Kumar and Ellis 2003). The data provide in vitro and in vivo evidence that RinPKS1 (RiCHS6) can regulate *p*-hydroxybenzalacetone biosynthesis. Apart from our research, summarizing all the CHS/BAS bifunctional enzyme research results in raspberry (Beekwilder et al. 2007; Zheng and Hrazdina 2008), it is tempting to speculate that a bifunctional enzyme with both CHS and BAS activities is most likely to have an important function in the initial reaction of the raspberry ketone biosynthesis pathway.

The fact that *PcPKS1* (a CHS/BAS gene) (Ma et al. 2009a) and *PcPKS2* (a true monofunctional BAS) (Ma et al. 2009b) were both isolated from *P. cuspidatum* may imply that such a case also exists in raspberry. Indeed, when purifying the BAS activity from raspberry, CHS and BAS activities in the same enzyme preparation showed a different response to treatments with 2-mercaptoethanol and ethylene glycol, suggesting that these enzymes are not one and the same molecule (Borejsza-Wysocki and Hrazdina 1996). However, biochemical or molecular strategies to isolate a BAS gene from raspberry were unsuccessful. Interestingly, no functionally divergent, CHS-like gene has been isolated in raspberry to date. The phylogenetic tree showed that all raspberry PKSs in the GenBank database are located in a cluster containing other typical chalcone-forming enzymes (Fig. 3). Recently, a CHS-like gene (*RinPKS2*) was successfully isolated using TAIL-PCR and over-hang extension PCR methods (our unpublished results). For RinPKS2, the phylogenetic analysis showed that it grouped together with *R. palmatum* BAS (Abe et al. 2001) and *P. cuspidatum* PcPKS2 (Ma et al. 2009b) in a cluster containing all functionally divergent, plant-specific, type III PKSs (Fig. 3). To our knowledge, *RinPKS2* is the first isolated CHS-like gene in raspberry. Interestingly, sequence analysis showed that Phe265 is uniquely replaced by Tyr in RinPKS2 (Fig. 2). This result may imply that other functionally divergent, CHS-like genes exist in raspberry. Therefore, further studies are needed to address the functional role of *RinPKS2* and to elucidate whether there are more CHS-like genes in raspberry.

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