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Isolation and Characterization of a Novel 2-Pyrone-Producing Type Ⅲ Polyketide Synthase From *Polygonum cuspidatum**

CHEN Mo[1)], WU Zhi-Jun[1)], WANG Xiao-Wei[1)], HU Hong-Yan[1)],
CAO Jie[1)], CHAI Tuan-Yao[1,2)**], WANG Hong[1)**]

([1)]*College of Life Sciences, University of Chinese Academy of Sciences, Beijing* 101408, China;
[2)]*Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing* 100101, China)

Abstract Derivatives of 4-hydroxy-6-methyl-2-pyrone(2-pyrone) are actively involved in plant resistance to insects and pathogens, and are considered as potential renewable chemical platforms for phloroglucinol and 1,3,5-triamino-2,4,6-trinitrobenzene. 2-Pyrone synthase (2PS), one of the type Ⅲ polyketide synthases (PKSs), is the key enzyme to produce 2-pyrone. In this study, a novel Pc2PS was isolated from *Polygonum cuspidatum* Sieb. et Zucc., a traditional Chinese herb used for the treatment of cough, suppurative conditions, and hypertension. The Pc2PS shared 54% - 56% amino acid sequence identity with 2PSs of other species. Enzymatic activity analysis showed that Pc2PS could catalyze the condensation of one molecule of acetyl-CoA with two molecules of malonyl-CoA at almost the same efficiency as that of three molecules of malonyl-CoA alone for production of 4-hydroxy-6-methyl-2 -pyrone. Thus, the presence or absence of acetyl-CoA in the reaction mixture did not affect catalytic efficiency. This catalytic property of Pc2PS differs from that of previously reported Gh2PSs, which catalyzed malonyl-CoA condensation to form the same products as Pc2PS but at a lower rate in the absence of acetyl-CoA. In addition, we determined the kinetics of Pc2PS with malonyl - CoA alone as the substrate for the first time. *Pc2PS* showed tissue-specific expression in *P. cuspidatum*; *Pc2PS* was predominantly expressed in the root, whereas few transcripts were detected in the leaf. This study enriches the known diversity of 2PS proteins, and provides a novel genetic resource for 2-pyrone biosynthesis.

Key words Type Ⅲ PKS, 2-Pyrone synthase, *Polygonum cuspidatum*
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Type Ⅲ polyketide synthases (PKSs), also termed the chalcone synthase (CHS) superfamily, are relatively conserved enzymes crucial for polyketides biosynthesis that share 30% - 95% sequence identity and a common Cys-His-Asn catalytic triad within a buried active site cavity[1]. It is remarkable that the conserved PKSs produce a diverse group of biologically significant secondary metabolites, including pyrones, chalcones, chromones, stilbenes, and anthraquinones[1]. The type Ⅲ PKS superfamily enzymes are homodimers of which each subunit can perform a complete series of catalytic reactions, including decarboxylation, condensation, and cyclization in a single active site cavity[2]. The diverse functions of type Ⅲ PKSs are mainly reflective of the variety of starter-CoA substrates (acetyl, propionyl, or other aliphatic and aromatic moieties), the number of condensations performed, as well as the type of cyclization and aromatization reactions[1]. The first type Ⅲ PKS to be studied in detail was CHS, which catalyzes the iterative condensation of 4-coumaroyl-CoA with three C2 units from malonyl-CoA to produce a linear tetraketide intermediate, followed by C_6-C_1 Claisen cyclization to form the naringenin

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** Corresponding author.
WANG Hong. Tel:86-10-69672628,E-mail:hwang@ucas.ac.cn
CHAI Tuan-Yao. Tel:86-10-88256343,E-mail:tychai@ucas.ac.cn

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chalcone[3] (Figure 1a). Stilbene synthase (STS) is an additional member of the type Ⅲ PKS superfamily and shares a high amino acid sequence identity with CHS. The reaction of STS is identical to that of CHS, catalyzing one 4-coumaroyl-CoA and three malonyl-CoA molecules to produce the tetraketide intermediate[1]. However, STS synthesizes resveratrol through C_2-C_7 aldol condensation accompanied by a decarboxylative loss of the C_1 carbon (Figure 1b).

In 1995, Helariutta *et al*.[4] isolated a CHS-like gene from *Gerbera hybrida*, a member of the Asteraceae, which showed 74% sequence identity with *Medicago sativa* CHS (MsCHS), but the authors did not identify the products of the novel type Ⅲ PKS. Three years later, the same laboratory reported that the CHS-like protein catalyzed the condensation reaction between acetyl-CoA and two malonyl-CoA molecules, followed by lactonization to synthesize 4-hydroxy-6-methyl-2-pyrone (Figure 1c), and the CHS-like protein was named 2-pyrone synthase (2PS) [5]. Jez *et al*. [6] reported the X-ray crystal structure of Gh2PS1 in 2000, and showed that Gh2PS1 and CHS shared a common three-dimensional fold, similar catalytic residues, and conserved CoA binding sites. Compared with the 28 residues lining the initiation/elongation cavity of CHS, Gh2PS has four amino acid residues changed, which reduced the volume of the active site cavity to one-third that of CHS. A triple mutant combining three point mutations in MsCHS (T197L/G256L/S338I) yields triacetic acid lactone (TAL), which is identical to Gh2PS. These results demonstrate that the volume and shape of the active site cavity influence the function of type Ⅲ PKSs. In 2016, Pietiainen *et al*. [7] further showed that Gh2PS2 and Gh2PS3 not only produced TAL consistent with Gh2PS1, but also formed the strongly antifungal compound 4-hydroxy-5-methylcoumarin.

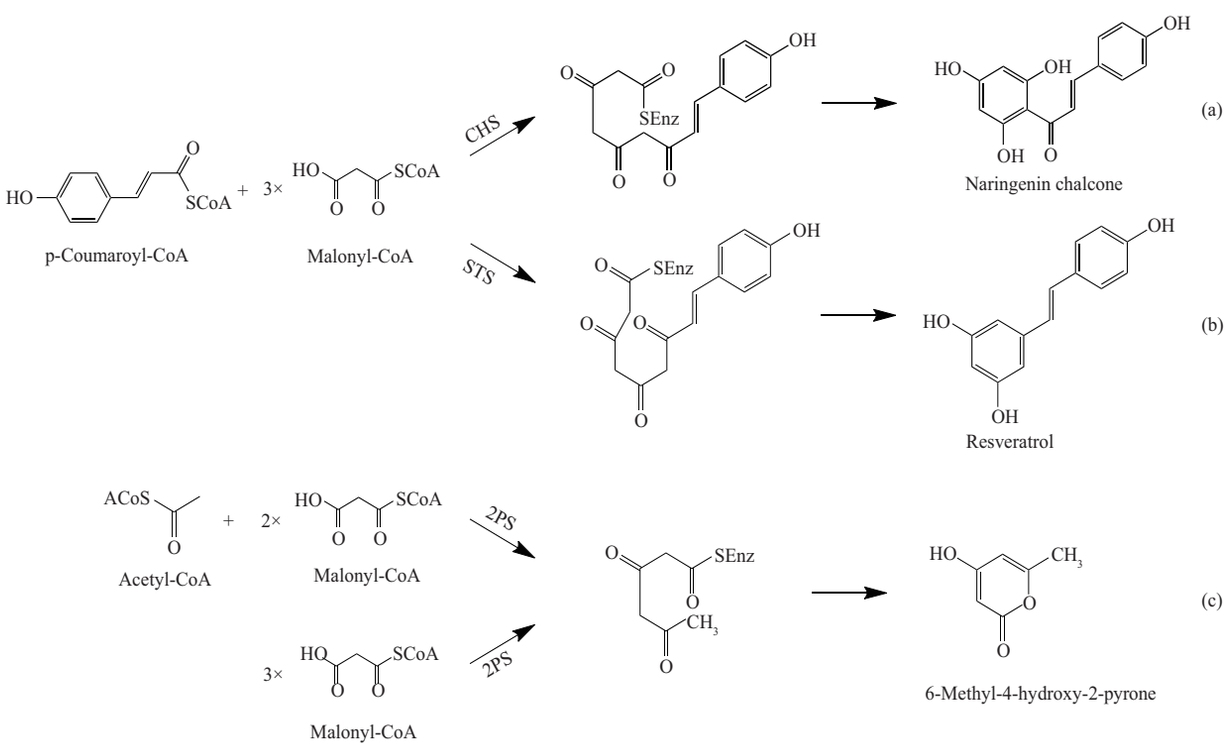

Fig. 1 Reactions catalyzed by CHS, STS and 2PS

(a) CHS catalyzed an iterative condensation of 4-coumaroyl-CoA with three malonyl-CoA and a Claisen cyclization to produce naringenin chalcone; (b) STS catalyzed one 4-coumaroyl-CoA and three malonyl-CoA to form resveratrol by aldol cyclization; (c) 2PS catalyzed acetyl-CoA with two malonyl-CoA or three malonyl-CoA to form 6-methyl-4-hydroxy-2-pyrone by lactonization. CHS, chalcone synthase; STS, stilbene synthase; 2PS, 2-pyrone synthase.

Polygonum cuspidatum Sieb. et Zucc. (Japanese knotweed) belongs to the Polygonaceae and is a medicinal herb widely used in traditional medicine in South-East Asia for treatment of arthralgia, cough, chronic bronchitis, and atherosclerosis[8]. Pharmacological studies have revealed a variety of valuable constituents of *P. cuspidatum*, such as emodin, physcion, resveratrol, piceid, and polydatin, which demonstrate the bioactivities of anti-oxidation, anti-inflammatory, anti-cancer, and liver protection[8]. Such results suggest that *P. cuspidatum* harbors a series of unidentified type Ⅲ PKSs, which construct the basic skeleton of these useful constituents.

Our laboratory has isolated a number of type Ⅲ PKSs from *P. cuspidatum*, such as PcBAS, PcSTS and PcCHS, to elucidate their possible roles in the biosynthesis of the secondary metabolites[9-10]. In the present study, Pc2PS was cloned from *P. cuspidatum* and characterized by means of *in vitro* enzymatic reactions. The enzyme produced a 4-hydroxy-6-methyl-2-pyrone by two decarboxylations condensation reaction between three molecules of malonyl-CoA, or between one molecule of acetyl-CoA and two molecules of malonyl-CoA. In addition, we analyzed the enzyme kinetics of Pc2PS at the optimum temperature and pH, and the expression profile of *Pc2PS* in different tissues. The results enrich the diversity of isolated 2PS proteins and provide a novel gene resource for metabolic engineering of *P. cuspidatum*.

1 Materials and methods

1.1 Plant material and reagents

Acetyl-CoA and malonyl-CoA were purchased from Sigma (St Louis, MO, USA). 4-Coumaroyl-CoA was chemically synthesized in accordance with the method of Beuerle and Pichersky[11]. 4-Hydroxy-6-methyl-2-pyrone was purchased from Aladdin (Shanghai, China). *Polygonum cuspidatum* seeds were obtained from the Institute of Botany, Chinese Academy of Sciences, Beijing, China, and plants were maintained in a greenhouse.

1.2 Cloning of *Pc2PS* cDNA

We performed BLASTn and BLASTx searches against *P. cuspidatum* transcriptome data (unpublished), using some reported PKS genes as the query, and identified several novel PKSs. Using the candidates PKS sequences to design PCR primers, the open reading frame (ORF) of *Pc2PS* was reverse transcribed and amplified from *P. cuspidatum* total mRNA. The sense primer was 5′-ATGGAG-AAATCTTCCGCCAACGC-3′ and the antisense primer was 5′-TTATATAGTGATCGGCACGCTA-CGTAA-3′. The *Pc2PS* gene was subcloned into the expression vector pET-30a using the ClonExpress® Entry One Step Cloning Kit (Vazyme Biotech Co., Ltd, Nanjing, China), which was transformed into *Escherichia coli* expression strain BL21 (DE3).

1.3 Heterologous expression and purification of recombinant protein

The *E. coli* BL21 (DE3) cells containing the ORF of *Pc2PS* were cultured at 200 r/min and 37℃ in 300 ml Luria-Bertani (LB) medium supplemented with kanamycin (50 mg/L). After attaining A_{600} of 0.4–0.6, 0.1 mmol/L isopropyl-D-1-thiogalactopyranoside (IPTG) was added to the culture and the incubation temperature was decreased to 28℃. After 12 h, the bacterial cells were collected by centrifugation at 6000 *g* for 15 min. The purification of recombinant *Pc2PS* was performed according to the method as described[12]. The purified protein was stored at −80℃ for long-term storage.

1.4 Enzyme reaction and product analysis

The standard enzyme assay contained 10 nmol malonyl-CoA and 3.6 μg purified recombinant enzyme in a final 100 μl volume of 100 mmol/L PPB (pH 7.0). For enzyme assays to determine the influence of acetyl-CoA, 10 nmol acetyl-CoA was included in the standard assay solution. As a control, the recombinant protein was inactivated by boiling at 100℃ for 10 min. Incubations were carried out at 30℃ for 30 min and quenched with 5% glacial acetic acid, then extracted with 100 μl ethyl acetate. The ethyl acetate layer was evaporated to dryness under nitrogen, and the residues were dissolved in 50 μl of 50% (*v*/*v*) methanol for HPLC/MS. Assays to determine the optimal pH were performed in triplicate covering the pH range 5.5–8.5. The optimal temperature was identified in triplicate within the range from 15℃ to 50℃.

The products were analyzed by HPLC and LC-MS. Products analysis was performed using an Agilent 1260 HPLC (Agilent, Santa Clara, CA, USA) on a Zorbax SB-C18 column, 5 μm, 4.6 mm × 250 mm (Agilent). The gradient elution was performed with H_2O and CH_3CN, both containing 1%

acetic acid. The HPLC conditions were as follows: 0 – 4 min, 5% – 12% CH_3CN; 4 – 20 min, 12% – 77% CH_3CN; the flow rate was 0.6 ml/min. The detection wavelength was 280 nm for 4-hydroxy-6-methyl-2-pyrone. The retention time of methylpyrone was 12.89 min.

For on-line HPLC/MS analysis, HPLC was carried out using the Agilent 1290 HPLC system coupled to an Agilent 6530 high-resolution Q-TOF mass spectrometer (Agilent) fitted with an ESI source. The mass spectrometer operating conditions were as follows: all spectra were obtained in negative ion mode over a mass range: 50−1 000 *m*/*z*; drying gas flow rate: 5 L/min; dry gas temperature: 300℃; nebulizer 35 psi; and collision energy: 25 V. The LC/MS/MS data were as follows: 4-hydroxy-6-methyl-2-pyrone, R_t = 12.89 min; $[M-H-CO_2]^-$ 81 (100), $[M-H-CH_2-CO]^-$ 83 (9.5), $[M-H-CH_2O_2-CH_2]^-$ 65 (17), $[M-H-CH_2O_2-H_2O]^-$ 63 (4.5). The numbers are *m*/*z* values in atomic mass units with relative intensities in parentheses[6].

1.5 Determination of kinetic parameters

A standard assay, consisting of five concentrations of malonyl-CoA (from 60 to 960 mmol/L) and 7.2 μg purified recombinant Pc2PS protein in a final 100 μl volume of 100 mmol/L PPB (pH 7.0), was used for a kinetic analysis of malonyl-CoA. The experiments were carried out in triplicate. After preincubation of the assay mixture at 30℃ for 5 min, reactions were performed by adding the protein and the mixture was incubated for 30 min. The reactions were quenched with 5 μl glacial acetic acid, and the products were quantified by HPLC. The kinetic parameters were then calculated. Eadie-Hofstee plots were used to derive the K_m and k_{cat} values.

1.6 Phylogenetic tree construction

Forty amino acid sequences for type Ⅲ PKS proteins were aligned using ClustalX (2.1) [13]. A phylogenetic tree was constructed with MEGA version 7.0 using the neighbor-joining method[14]. Statistical support for the tree topology was assessed by means of a bootstrap analysis with 1000 replicates.

1.7 Quantitative real−time PCR

Total RNA of *P. cuspidatum* was extracted from leaves, stems, and roots using the Plant Total RNA Purification Kit (GeneMark, Taiwan, China). The first-strand cDNA was synthesized from 3 μg total RNA using the Hifair™ Ⅱ 1st Strand cDNA Synthesis SuperMix (Yeasen, Shanghai, China) with oligo (dT) primers in accordance with the manufacturer's protocol. Three biological replicates and three technical replicates were used to analyze *Pc2PS* expression.

To explore the expression pattern of Pc2PS transcripts, we performed quantitative real-time PCR in 96-well plates using QuantStudio™ Real-Time PCR Software (Applied Biosystems, USA) with the Hieff™ qPCR SYBR® Green Master Mix (Yeasen, Shanghai, China). The standard reaction mixture contained 5.0 μl of 2× Hieff™ qPCR SYBR® Green Master Mix, 0.2 μl of each primer (10 μmol/L), 1.0 μl diluted (1∶20) cDNA template, and 3.6 μl RNase-free water. The amplification cycle program was 95℃ for 5 min followed by 40 cycles of 95℃ for 10 s, 58℃ for 20 s, and 72℃ for 20 s. The melting curve was produced after 40 cycles at a heating rate of 0.05℃/s, to test the specificity of each primer pair over the temperature range of 60℃−95℃. Three technical replicates were used for each sample. The *NDUFA13* gene was used as a control to normalize the expression level of *2PS* as previously described (unpublished).

2 Results and discussion

2.1 Isolation and characterization of *2PS* from *P. cuspidatum*

In previous works, a number of PcPKSs have been isolated and characterized[9-10,15]. Based on *Polygonum cuspidatum* transcriptome data (unpublished), the full-length sequence of *Pc2PS* was detected by heterologous screening, and then amplified from root-derived cDNA. The ORF of *Pc2PS* was 1 155 bp long and encoded 384 amino acid residences, which had a predicted molecular mass of 41.9 ku and calculated isoelectric point of 5.96 (https://web. expasy. org/protparam/). Multiple alignment analysis demonstrated that the amino acid sequence of *P. cuspidatum* 2PS (the GenBank accession no. MK455782) shared 52% – 83% identity with type Ⅲ PKSs from other plant species. Pc2PS and PcPKS4 were highly similar with 83% identity[15].

Analysis of the multiple alignment revealed that amino acid sequences of type Ⅲ PKSs were highly conserved among the plant species (Figure 2). *Polygonum cuspidatum* 2PS maintained a conserved catalytic triad Cys164, His303, and Asn336

(numbering represents the position in MsCHS)[16]. The majority of the active site residues were conserved in Pc2PS, including Ser133, Ile254, Gly256, Ser338, Pro375, and the "gatekeeper" Phe215 (numbering represents the position in MsCHS). Interestingly, Thr132, Thr197, and Phe265 were substituted by Asn, Gly and Leu, respectively. The active-site residue 197 is thought to control the number of malonyl-CoA molecules condensed. As reported previously, the T197G substitution opens a new tunnel at the base of the cavity, which results in larger polyketide products[17]. In the present study, the T197G substitution in Pc2PS did not result in a larger polyketide. The *R. dauricum* ORS, which contains the T197G substitution, also yield small reaction products, such as orcinol and triacetic acid lactone[18]. The reason for this might be that the large side chain of Met194 blocked the tunnel created by the T197G substitution. Pc2PS could only synthesize the small polyketide 4-hydroxy-6-methyl-2-pyrone, which also might be due to the large side chain of another residue, but mutation experiments are needed to test this hypothesis.

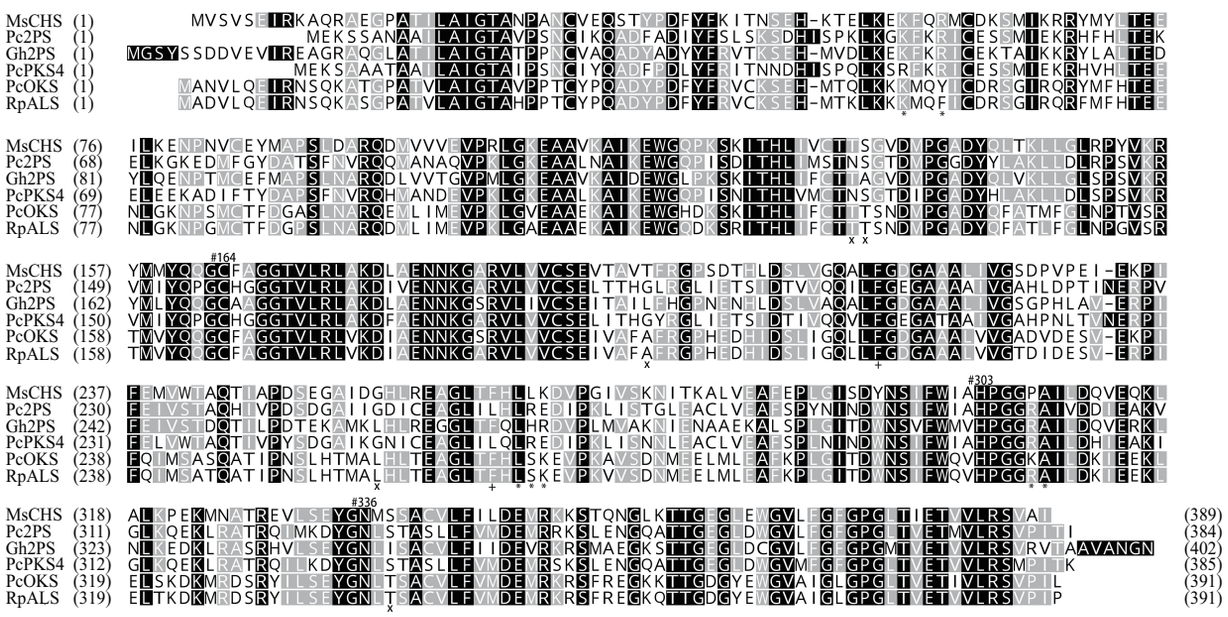

Fig. 2　Comparison of primary sequences of *P. cuspidatum* 2PS and other CHS−superfamily type Ⅲ PKSs

MsCHS，*M. sativa* CHS；Pc2PS，*P. cuspidatum* 2-pyrone synthase；Gh2PS，*G. hybrida* 2-pyrone synthase；PcPKS4，*P. cuspidatum* polyketide synthase 4；PcOKS，*P. cuspidatum* octaketide synthase；RpALS，*R. palmatum* aloesone synthase. The catalytic triad（Cys164，His303 and Asn336） are marked with a hash symbol #；the active-site amino acid residues 132，133，197，256 and 338 are marked with x；the gatekeepers 215 and 265 are marked with +，and the residues for the CoA binding are marked with *.

2.2　Phylogenetic analysis

To reconstruct a phylogenetic tree, we chose all types of PKS, one CHS each from different families of gymnosperms, monocots, and dicots, all members of 2PS, and other types of PKS isolated from *P. cuspidatum* (Figure 3). In the phylogenetic tree, the *P. cuspidatum* 2PS formed a separate cluster with PcPKS4[15], and was grouped with other non-chalcone-forming type Ⅲ PKSs, including STS and benzalacetone synthase (BAS) from *P. cuspidatum*[9,19] and BAS from *Rheum palmatum* (Polygonaceae)[20]. Except for the grouping of PcCHS with the chalcone-forming enzymes, other PcPKSs were clustered together with *Rheum palmatum* BAS and formed an independent branch. They were not only grouped into clusters consistent with their enzymatic function, but also formed a species-specific cluster. Generally, plant STSs form a clade with CHSs, as observed for *Arachis hypogaea* STS and *Pinus strobus* STS[21], but PcSTS was grouped with the non-chalcone-producing type Ⅲ PKSs. The protein sequences of the three non-CHS genes identified from *P. cuspidatum*, namely

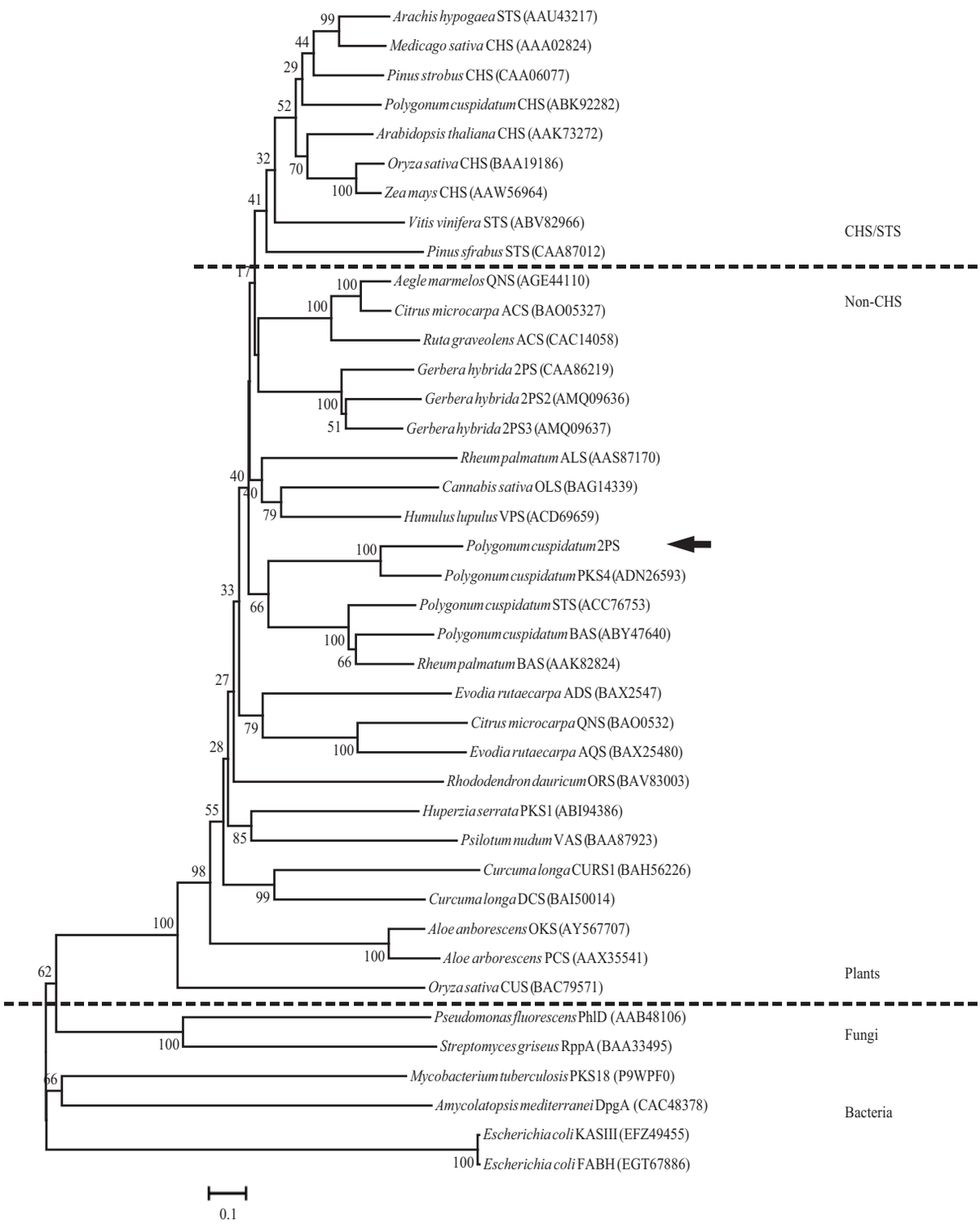

Fig. 3　Phylogenetic tree analysis of CHS superfamily enzymes

Multiple sequence alignment was performed by CLUSTAL W(1.8). The β-ketoacyl carrier protein synthase Ⅲs(FABH and KAS Ⅲ) of *E. coli* was served as an outgroup. The indicated scale represents 0.1 amino acid substitutions per site. 2PS from *P. cuspidatum* is highlighted with arrow. Abbreviations: ACS, acridone synthase; ADS, alkyldiketide CoA synthase; ALS, aloesone synthase; AQS, alkylquinolone synthase; BAS, benzalacetone synthase; BBS, bibenzyl synthase; CHS, chalcone synthase; CURS1, curcumin synthase 1; CUS, curcuminoid synthase; DCS, diketide CoA synthase; DpgA, dihydroxyphenylacetic acid synthase; OKS, octaketide synthase; OLS, olivetol synthase; ORS, orcinol synthase; PCS, pentaketide chromone synthase; QNS, quinolone synthase; STS, stilbene synthase; VAS, phloroisovalerophenone synthase; VPS, valerophenone synthase; RppA, red brown pigment producing enzyme. The Genbank accession numbers are shown in parentheses.

PcBAS, PcSTS, and PcPKS4, were used as queries in a BLAST search of the National Center of Biotechnology Information protein databases. All PKSs with shared identity of more than 75% with PcPKSs were from members of the Polygonaceae, such as *R. palmatum*, *Fagopyrum esculentum*, *Persicaria minor*, *Rheum australe*, and *Fallopia multiflora*. Typically, secondary metabolites are associated with plant resistance. For example, *Aquilaria sinensis* PKS1 abundance is remarkably enhanced by $CdCl_2$ treatment[22]. It has been reported previously that PpORS knockout mutants developed abnormal leaves that showed increased dye permeability and sensitivity to drought[23]. Based on the above-mentioned results, we speculate that the Polygonaceae has undergone rigorous environmental screening prior to the extant species diversification. This screening process resulted in notable divergence between the PKS genes of the Polygonaceae and those of other plant families, hence the PKSs isolated from species of Polygonaceae show within-family identities comparatively higher than those with members of other families.

2.3　Enzymatic activity of Pc2PS

To evaluate its catalytic function, *P. cuspidatum* 2PS was heterologously expressed in *E. coli* with an additional hexahistidine tag at the C terminus. Analysis by SDS-PAGE revealed that the molecular mass of the purified recombinant protein was 42 ku (Figure 4a). The enzymatic activity of the recombinant protein Pc2PS was assayed by the reaction with acetyl-CoA and 4-coumaroyl-CoA together with malonyl-CoA. The HPLC analysis determined that the recombinant Pc2PS produced a new peak at 12.92 min from acetyl-CoA and malonyl-CoA compared with the negative control (Figure 5). The product of the recombinant protein was determined as 4-hydroxy-6-methyl-2-pyrone (TAL) by comparing the HPLC retention time and the UV-spectrum with the reference compound. Analysis of the reaction mixture showed that Pc2PS produced TAL as a single product, consistent with previous

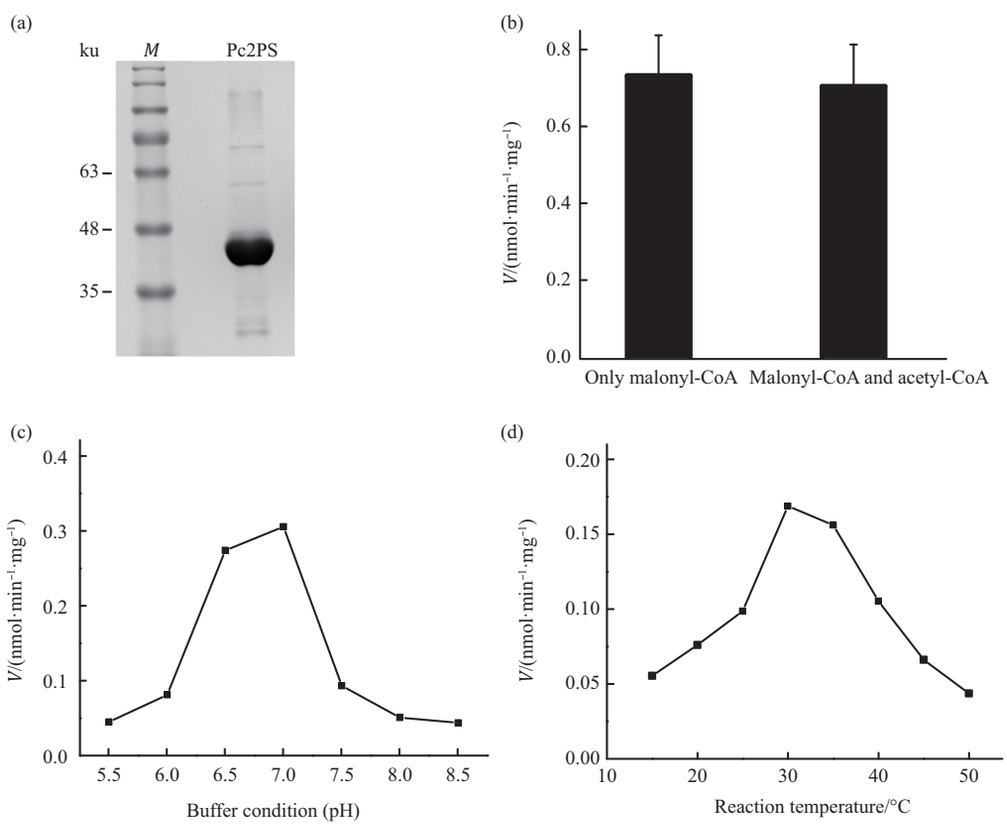

Fig. 4　The properties of recombinant Pc2PS

（a）SDS-PAGE analysis of the purified recombinant 2PS. *M*，molecular mass standards （left）. Pc2PS （right）；（b）The effect of acetyl-CoA on activity of Pc2PS enzymatic reaction；（c）The pH dependency of TAL-forming activity of *P. cuspidatum* 2PS；（d）The temperature dependency of TAL-forming activity of *P. cuspidatum* 2PS.

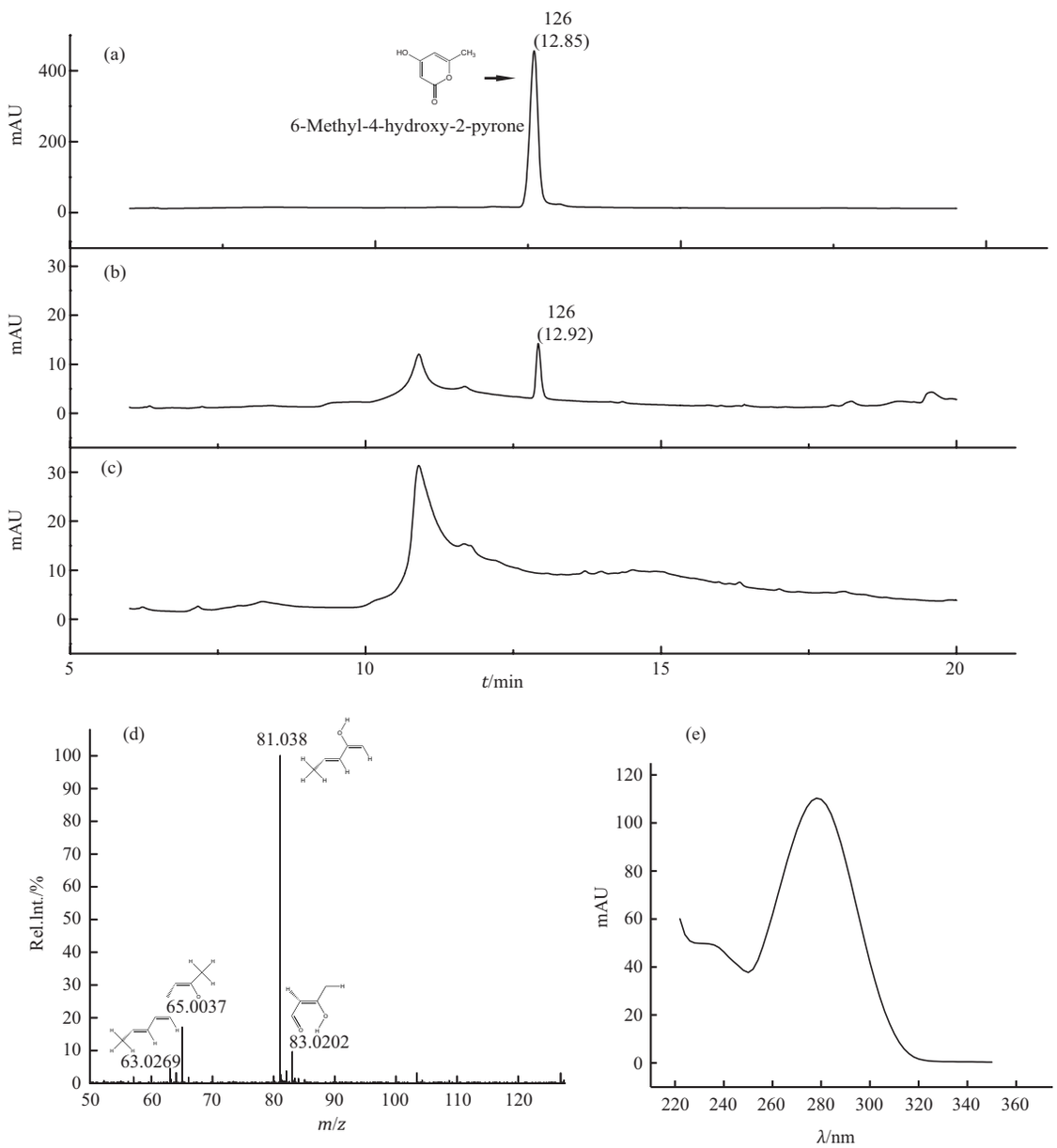

Fig. 5　Product identification by LC−Q−TOFMS

（a）Authentic compound of 4-Hydroxy-6-methyl-2-pyrone；（b）TAL generated by Pc2PS；（c）the products of negative control；（d）The MS/MS fragmentation of Pc2PS products；（e）UV spectra of Pc2PS enzyme reaction products. The chemical structures of product and fragmentation are shown.

reports for Gh2PS1[5]. The LC / MS analysis of the reaction mixture identified an additional compound with m/z of 125 ($[M-H]^-$) in the negative ion mode. The MS / MS analysis of this compound showed that the compound was proposed to be 4-hydroxy-6-methyl-2-pyrone based on data in the METLIN MS / MS Metabolite Database (https://metlin. scripps. edu) and previous publications[5,7]. The *P. cuspidatum* 2PS accepted acetyl-CoA as a substrate, but did not accept 4-coumaroyl-CoA.

As previously reported, 2PSs are able to produce 2-pyrone from malonyl-CoA alone, owing to the ability of 2PS to decarboxylate malonyl-CoA to form acetyl-CoA. However, its decarboxylation activity limited the measurement of K_m and k_{cat} for acetyl-CoA and malonyl-CoA. In addition, absence of acetyl-CoA

in the reaction mixture decreased the rate of synthesis of the product. Thus, the majority of previous works did not measure the enzyme kinetics of 2PS. The Pc2PS was also able to produce TAL from the assay mixture containing malonyl-CoA alone. Interestingly, the rate of synthesis of TAL did not change significantly in the absence of acetyl-CoA (Figure 4b). The reason for this may be that the rate of decarboxylation of malonyl-CoA was much faster than that of synthesis of 2-pyrone, therefore the duration of decarboxylation could be ignored, or that acetyl-CoA did not increase the incorporation from malonyl-CoA in the Pc2PS reaction, as described previously by Eckermann *et al*. [5] Given that the presence of acetyl-CoA had no effect on the activity of the protein, we could measure the kinetics of Pc2PS with malonyl-CoA alone as the substrate.

2.4 Steady−state kinetics analysis

The optimal pH and optimal temperature for 4-hydroxy-6-methyl-2-pyrone-forming activity of Pc2PS were 7.0 and 30℃ in PPB, respectively (Figure 4c, d). Given that Pc2PS was a pH-sensitive type Ⅲ PKS, its catalytic activity decreased rapidly at pH outside the range of 6.5 to 7.0. The steady-state kinetics analysis (Figure 6) showed that recombinant Pc2PS had a K_m value of 978.5 μmol/L and k_{cat} value of 1.186 min^{-1} for malonyl-CoA under the optimal pH and optimal temperature. The catalytic efficiency (k_{cat}/K_m) for TAL formation was 20.2 $s^{-1}\cdot mol^{-1}\cdot L$, which was slightly lower than that of *Aloe arborescens* PCS[17], but was of the same order of magnitude as that of *A. arborescens* octaketide synthase (OKS) [24]. Similar to Pc2PS, both AaPCS and AaOKS accepted malonyl-CoA as a substrate, and their catalytic efficiencies were almost identical in the presence or absence of acetyl-CoA in the reaction mixture. Pc2PS was the first 2PS whose kinetics were measured with malonyl-CoA alone as the substrate.

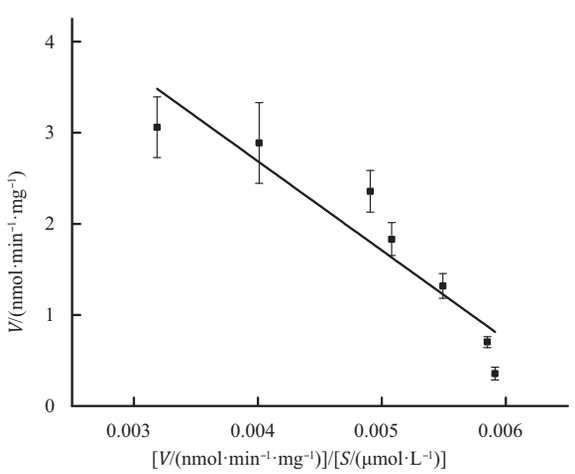

Fig. 6 Eadie–Hofstee plot for *P. cuspidatum* 2PS

V/（nmol · min^{-1} · mg^{-1}） is the reaction rate and *S*/（μmol · L^{-1}） is the substrate （malonyl-CoA） concentration. Each point represents the average of three independent experiments in duplicate.

2.5 Expression pattern

To determine the expression pattern of *Pc2PS* in different tissues, we performed quantitative real-time PCR analysis of cDNA from roots, stems, and leaves. The highest expression level of *Pc2PS* detected was in the roots, followed by the stems, and the lowest expression level was in the leaves (Figure 7). The expression pattern of Pc2PS differed distinctly from those of Gh2PSs. The highest expression level of Gh2PS1 was detected in the leaf blade, scape, bracts, and corolla [5]. The highest expression level of Gh2PS2 was detected in the leaf blade, petals, ovary, and young capitulum. Gh2PS3 was only expressed in the roots[7].

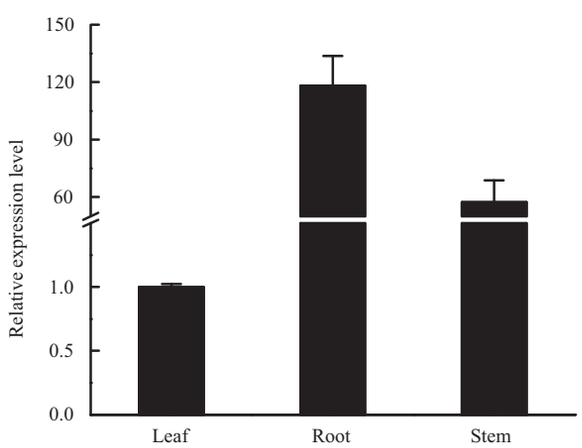

Fig. 7 The expression profiles

Expression profiles of Pc2PS gene in leaf, stem and root of *P. cuspidatum*.

4-Hydroxy-6-methyl-2-pyrone, the product of Pc2PS, is the smallest polyketide produced by type Ⅲ PKSs. In metabolic engineering, TAL has the potential to contribute to renewable chemical platforms through metabolic engineering[25]. In *Gerbera*, TAL was used as a substrate to produce gerberin and parasorboside, the derivative of which inhibit seed germination and fungal growth[5]. Other than several polyketides such as emodin, resveratrol, and piceid, TAL and its

derivatives have not currently been isolated from *P. cuspidatum*. The present expression pattern analysis showed that the expression level of *Pc2PS* in the roots was more than 100 times higher than that in the leaves, which indicated that 2-pyrone-related compounds or other unknown derivatives of TAL might be generated in *P. cuspidatum*. The catalytic efficiency for TAL formation was relatively lower, which might indicate that such compounds were produced, albeit in low concentrations. Therefore, additional studies are needed to isolate 2-pyrone derivative compounds from *P. cuspidatum*.

In conclusion, *P. cuspidatum* 2PS is a novel type Ⅲ PKS that produced 4-hydroxy-6-methyl-2-pyrone as a product from three molecules of malonyl-CoA. Pc2PS is the first 2PS to be isolated from *P. cuspidatum*, and *P. cuspidatum* is the second plant species from which 2PS has been identified in addition to *G. hybrida*. Because the presence or not of acetyl-CoA in the reaction mixture did not affect the efficiency, we determined the kinetics of Pc2PS for the first time. In a future study, site mutations of the active site residues of Pc2PS will be investigated to determine novel key residences which influence the size of products.

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虎杖中一种新的2–吡喃酮合酶基因的克隆和功能研究*

陈　默[1)]　吴志军[1)]　王晓伟[1)]　胡红艳[1)]　曹　杰[1)]　柴团耀[1,2)**]　王　红[1)**]

([1)] 中国科学院大学生命科学学院，北京 101408；
[2)] 中国科学院遗传与发育生物学研究所，北京 100101）

摘要　4-羟基-6-甲基-2-吡喃酮（2-吡喃酮）及其衍生物是一类重要的植物次生代谢产物，具有抗虫、抗真菌等功能，在工业上可用于生产可再生化学平台间苯三酚和1，3，5-三氨基-2，4，6-三硝基苯. 2-吡喃酮合酶（2PS），一种Ⅲ型聚酮合酶（PKSs），是合成2-吡喃酮的关键酶. 本研究以中药材虎杖（*Polygonum cuspidatum Sieb*. et Zucc）为材料，从中分离鉴定了一种新的2-吡喃酮合酶（Pc2PS）. Pc2PS与已知的几种2PSs的氨基酸序列相似性为54%~56%. 通过体外酶促反应鉴定功能发现，Pc2PS可以催化1分子乙酰-CoA与2分子丙二酰-CoA，缩合生成4-羟基-6-甲基-2-吡喃酮；也可以只利用3分子丙二酰-CoA，以相同的效率缩合生成2-吡喃酮. 由此可以看出，乙酰-CoA存在与否并不影响该酶的催化效率. 随后，我们测定了Pc2PS以丙二酰-CoA为单一底物时的酶动力学参数. 虽然之前报道的2PSs也可以只利用丙二酰-CoA生成2-吡喃酮，但与Pc2PS不同的是，乙酰-CoA的缺失会大大降低催化效率. 另外，对*Pc2PS*基因的组织表达特异性检测结果表明，该基因主要在虎杖根中表达，在叶中的表达量很低. 本研究丰富了2PS的种类，并为2-吡喃酮的生物合成提供了基因资源.

关键词　Ⅲ型聚酮合酶，2-吡喃酮合酶，虎杖
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** 通讯联系人.
王红. Tel：010-69672628，E-mail：hwang@ucas.ac.cn
柴团耀. Tel：010-88256343，E-mail：tychai@ucas.ac.cn
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