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Aromatic and pyrone polyketides synthesized by a stilbene synthase from *Rheum tataricum*☆

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Dedicated to Meinhart H. Zenk on the occasion of his 70th birthday

Abstract

A cDNA encoding a stilbene synthase, RtSTS, was isolated from the rhizomes of Tatar rhubarb, *Rheum tataricum* L. (Polygonaceae), a medicinal plant containing stilbenes and other polyketides. Recombinant RtSTS was expressed in *E. coli* and assayed with acetyl-coenzyme A (CoA), *n*-butyryl-CoA, isovaleryl-CoA, *n*-hexanoyl-CoA, cinnamoyl-CoA and *p*-coumaroyl-CoA as primers of polyketide synthesis. RtSTS synthesized resveratrol and a trace amount of naringenin chalcone from *p*-coumaroyl-CoA, supporting the enzyme's identification as a resveratrol-type stilbene synthase (EC 2.3.1.95). Bis-noryangonin and *p*-coumaroyl triacetic acid lactone (CTAL)-type pyrones were observed in minor amounts in the reaction with *p*-coumaroyl-CoA and as major products with cinnamoyl CoA. As well, such pyrones, and not aromatic polyketides, were identified as the only products in assays with aliphatic and benzoyl CoA esters. Acetonyl-4-hydroxy-2-pyrone, a pyrone synthesized from acetyl-CoA, was identified as a new product of a stilbene synthase. Using Northern blot analysis, *RtSTS* transcript was found to be highly expressed in *R. tataricum* rhizomes, with low transcript levels also present in young leaves. This expression pattern correlated with the occurrence of resveratrol, which was detected in higher amounts in *R. tataricum* rhizomes compared with leaves and petioles using HPLC. Few stilbene synthases have been found in plants, and the identification of *RtSTS* provides additional sequence and catalytic information with which to study the evolution of plant polyketide synthases.

Keywords: *Rheum tataricum* L.; Polygonaceae; Tatar rhubarb; Polyketide synthase; Stilbene synthase; Chalcone synthase; Resveratrol; Pyrone

1. Introduction

The chalcone synthase superfamily of polyketide synthases (PKSs) are key enzymes in the biosynthesis of many plant natural products including the ubiquitous flavonoids and related anthocyanins, and stilbenes, pyrones, phloroacylphenones and acridone alkaloids of more restricted distribution (Schröder, 2000). The enzymes responsible are homodimeric type III PKSs that, using aromatic or aliphatic CoA esters as reaction primers, carry out sequential decarboxylation and condensation reactions with one, two or three C_2 units derived from malonyl-CoA. The prototype enzyme of the superfamily is chalcone synthase (CHS) (EC 2.3.1.74), which has been extensively investigated at the biochemical (Schröder, 1999; Jez and Noel, 2000) and structural level (Ferrer et al., 1999). CHS uses *p*-coumaroyl-CoA to form naringenin chalcone via a Claisen-type cyclization, with this compound further cyclizing to give naringenin. A closely related plant PKS, stilbene synthase (STS) (EC 2.3.1.-), also uses phenylpropanoid CoA starter esters but cyclizes the tetraketide intermediate in an aldol-type reaction to yield resveratrol, from *p*-coumaroyl-CoA (EC 2.3.1.95), or pinosylvin, from cinnamoyl-CoA (EC 2.3.1.146).

☆ The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL/DDBJ with the accession number AF508150.

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Nomenclature

ACS	Acridone synthase
BAS	Benzalacetone synthase
BBS	Bibenzyl synthase
BNY	Bis-noryangonin
CoA	Coenzyme A
CHS	Chalcone synthase
CID	Collision-induced decomposition
CTAL	*p*-Coumaroyl triacetic acid lactone
CTAS	*p*-Coumaroyl triacetic acid synthase
IPTG	Isopropyl-β-D-thiogalactopyranoside
PKS	Polyketide synthase
2-PS	2-Pyrone synthase
RACE	Rapid amplification of cDNA ends
RtSTS	*Rheum tataricum* stilbene synthase
SDS	Sodium dodecyl sulfate
SSC	Saline sodium citrate
STS	Stilbene synthase
UPM	Universal primer mix
VPS	Valerophenone synthase

Plant PKSs are catalytically flexible, usually forming several products in vitro, and accepting a variety of physiological and non-physiological substrates. Both chalcone and stilbene synthases convert *p*-coumaroyl-CoA to the major products naringenin chalcone or resveratrol, respectively, as well as to the byproduct pyrones bis-noryangonin (BNY) and *p*-coumaroyl triacetic acid lactone (CTAL) (Yamaguchi et al., 1999). The latter are formed by derailment after two or three condensation reactions with malonyl-CoA, respectively. Additionally, cross reactivity between CHS and STS has been demonstrated with CHS forming resveratrol and STS forming naringenin in assays with *p*-coumaroyl-CoA, albeit in small amounts (Yamaguchi et al., 1999). CHSs and STSs accept aliphatic CoA esters as reaction primers. For example, *Scutellaria baicalensis* CHS converts isovaleryl-CoA to phloroacylphenones (via a correct Claisen cyclization) (Morita et al., 2000), while *Arachis hypogaea* STS forms BNY-type pyrones rather than aromatic products with this substrate (Morita et al., 2001). CHS and STS also utilize thiophene, furan and halogenated analogues of *p*-coumaroyl-CoA to form unnatural polyketides (Abe et al., 2000; Morita et al., 2001). The ability of plant PKSs to accept different CoA primers and carry out various condensation and cyclization reactions (Jez et al., 2002) makes this enzyme class, along with terpene cyclases (Greenhagen and Chappell, 2001), one of the major generators of carbon skeleton diversity in natural products.

As part of a study of the biosynthesis of polyketides present in medicinal plants, we investigated the CHS superfamily members present in *Rheum tataricum* L. (Polygonaceae), or Tatar rhubarb. *Rheum* species are a rich source of polyketides including phenylbutanoids, anthraquinones, naphthalenes and stilbenes (Kashiwada et al., 1988). Members of this genus are also used as medicinal plants (Foust, 1992), particularly in Asian traditional medicine, and stilbenes have been reported to mediate the antioxidant activity of *Rheum* extracts (Matsuda et al., 2001). It is interesting to note that the first in vitro evidence for STS activity was shown with protein extracts of *Rheum rhaponticum* rhizomes (Ruprich and Kindl, 1978), although the enzyme responsible was never isolated using biochemical or molecular approaches. Abe et al. (2001) recently reported the isolation of a cDNA encoding benzalacetone synthase (BAS), a PKS involved in phenylbutanoid biosynthesis, from the leaves of *Rheum palmatum*. In this paper, we describe the cloning of *RtSTS*, a resveratrol-type STS from *Rheum tataricum* rhizomes and the characterization of its in vitro reaction products.

2. Results and discussion

A homology-based approach with degenerate oligonucleotide primers that correspond to conserved regions of plant PKSs was used to amplify gene fragments from *Rheum tataricum* rhizome RNA by RT-PCR. Rhizomes were targeted because many of the polyketides of interest, including stilbenes and anthraquinones, are synthesized in this tissue. In this manner, two 584 bp fragments, *RtPKS1* and *RtPKS2*, that showed similarity to CHS superfamily members were isolated. Since these fragments showed 98.5% similarity at the nucleotide level, only *RtPKS1* was selected for further analysis. The cDNA regions 5′ and 3′ from this fragment were obtained using RACE PCR. The full-length *RtSTS* cDNA was 1429 bp encoding a protein of 391 amino acids with a predicted molecular weight of 43.0 kDa. The 59 bp 5′ untranslated region contained a stop codon in-frame with the presumed start codon indicating the cDNA was full length. The deduced amino acid sequence of *RtSTS* is shown in Fig. 1, as a multiple sequence alignment with other plant CHS superfamily members. Southern blot analysis (data not shown) found that three copies of *RtSTS* are present in the *R. tataricum* genome.

RtSTS was expressed in *E. coli* and purified by immobilized metal affinity chromatography on a cobalt resin to give homogeneous recombinant enzyme (Fig. 2). In order to determine the catalytic activity of RtSTS, recombinant enzyme was incubated with ^{14}C-malonyl-CoA and the potential starter CoA esters, acetyl-CoA, *n*-butyryl-CoA, isovaleryl-CoA, *n*-hexanoyl-CoA, cinnamoyl-CoA and *p*-coumaroyl-CoA. The resulting radioactive products were resolved by reversed-phase thin layer chromatography. We acidified enzyme assays

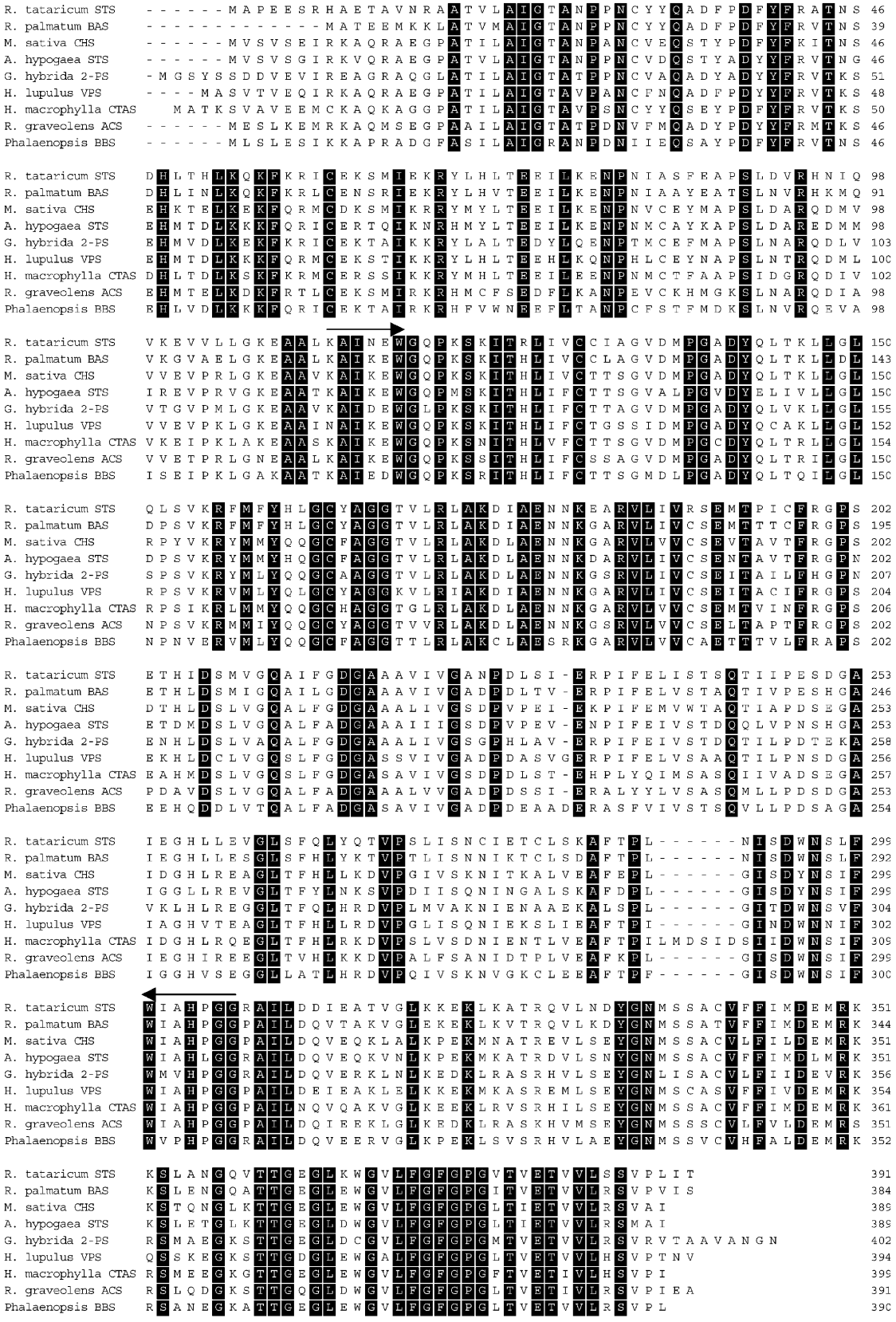


Fig. 1. Alignment of deduced amino acid sequence of *RtSTS* from *Rheum tataricum* with the plant CHS superfamily members *Rheum palmatum* BAS, *Medicago sativa* CHS, *Arachis hypogaea* STS, *Gerbera hybrida* 2-PS, *Humulus lupulus* VPS, *Hydrangea macrophylla* CTAS, *Ruta graveolens* ACS and *Phalaenopsis* BBS. Conserved motifs to which degenerate PCR primers were directed are shown with arrows. Shaded letters indicate conserved amino acid residues. Hyphens denote gaps introduced to maximize similarity. Accession numbers of proteins are found in the Experimental.

before extraction to increase recovery of pyrones (Yamaguchi et al., 1999) and prevent cyclization of chalcones to their corresponding flavanones. As shown in Fig. 3, RtSTS accepted both aliphatic and aromatic CoA esters as primers for polyketide synthesis and produced multiple products from each. The major product formed in the *p*-coumaroyl-CoA assay co-chromatographed with resveratrol, suggesting that the *Rheum* PKS encoded an STS.

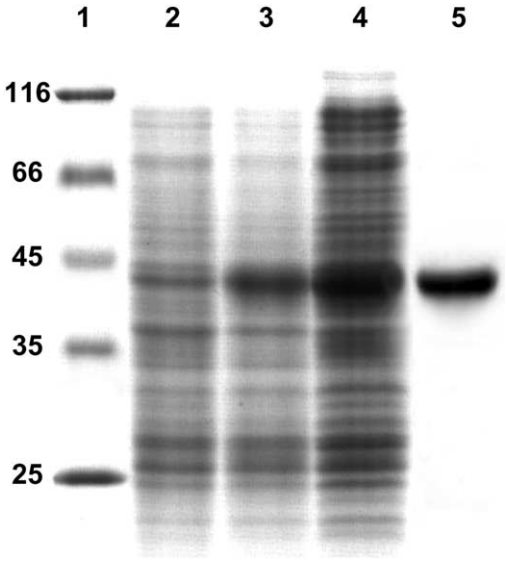


Fig. 2. SDS-PAGE analysis of recombinant RtSTS stained with Coomassie blue. Lane 1, molecular mass markers with masses in kDa indicated; Lane 2, uninduced cells; Lane 3, cells grown for 12 h at 28 °C after induction with IPTG; Lane 4, crude cell lysate and Lane 5, RtSTS (2 μg) purified by affinity chromatography with a Talon cobalt resin.

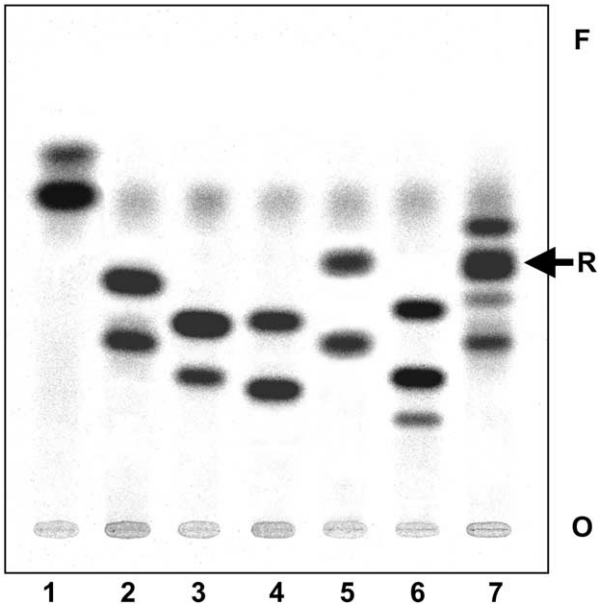
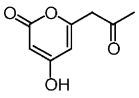
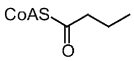
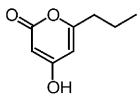
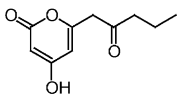
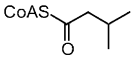
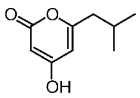
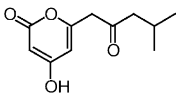
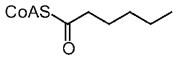
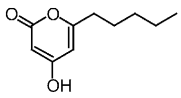
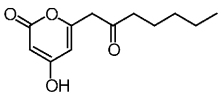
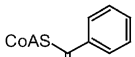
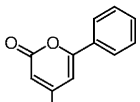
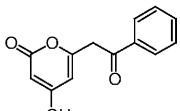
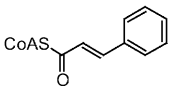
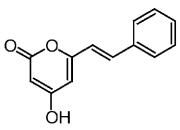
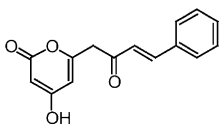
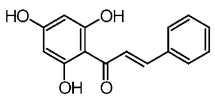
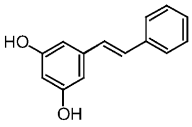
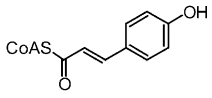
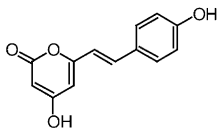
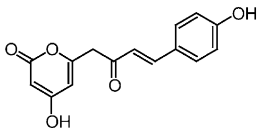
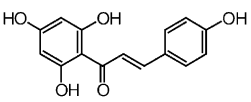
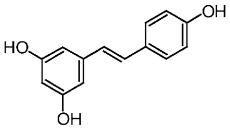


Fig. 3. Reversed-phase TLC analysis of RtSTS reaction products. The reactions were performed with 1.5 μg of purified enzyme, [2-^{14}C]malonyl-CoA and acetyl-CoA (lane 1), *n*-butyryl-CoA (lane 2), isovaleroyl-CoA (lane 3), *n*-hexanoyl-CoA (lane 4), benzoyl-CoA (lane 5), cinnamoyl-CoA (lane 6), *p*-coumaroyl-CoA (lane 7). O = TLC origin, F = solvent front, R = position of resveratrol standard.

To conclusively identify the polyketide products, RtSTS was incubated with unlabelled malonyl-CoA and the starter CoA esters in scaled up in vitro reactions and the resulting product mixtures analyzed by LC–MS and LC–MS/MS. Products were identified by their parent ions and collision-induced decomposition (CID) spectra. We have previously published the results from the LC–MS analysis of a series of polyketides formed enzymatically from CHS (Samappito et al., 2002); therefore, only a graphical summary of the enzymatic products of RtSTS is presented here (Table 1). RtSTS formed aromatic products only with *p*-coumaroyl-CoA and cinnamoyl-CoA. Incubation with *p*-coumaroyl-CoA gave resveratrol (**7e**) (*m*/*z* 229, $[M+H]^+$ and *m*/*z* 227, $[M-H]^-$) as the major product, demonstrating that RtSTS is an STS. Resveratrol was identified by its electrospray CID spectrum of the $[M-H]^-$ ion (*m*/*z* 227) displaying key ions at *m*/*z* 185 ($[M-H-CH_2CO]^-$) and *m*/*z* 143 ($[M-H-2CH_2CO]^-$) in comparison with an authentic sample. Smaller amounts of BNY-type (**7b**) and CTAL-type (**7c**) pyrones, and naringenin chalcone (**7d**), were also detected in the *p*-coumaroyl-CoA assay. This spectrum of four products, including the naringenin chalcone formed as a cross-reaction product, has been previously described for *Arachis hypogaea* STS (Yamaguchi et al., 1999). RtSTS synthesized BNY-type (**6b**) and CTAL-type (**6c**) pyrones as well as a small amount of pinocembrin chalcone (**6d**) and pinosylvin (**6e**) from cinnamoyl-CoA. Pinocembrin chalcone was clearly identified by its parent ion (*m*/*z* 257 $[M+H]^+$) and the typical collision-induced fragments at *m*/*z* 153 (trihydroxybenzoyl ion) and *m*/*z* 131 (cinnamoyl ion), while pinosylvin (**6e**) was detected by the LC–MS analysis showing a peak with masses of *m*/*z* 213 ($[M+H]^+$) and *m*/*z* 211 ($[M-H]^-$). Only three products were visible in the ^{14}C-labeled assay with cinnamoyl CoA (Fig. 3), the least polar (lower R_f value) of which likely corresponds to an unresolved mixture of pinocembrin chalcone and pinosylvin.

With benzoyl-CoA and the aliphatic substrates tested, RtSTS catalyzed only the formation of BNY-type (**1b**, **2b**, **3b**, **4b**, **5b**) and CTAL-type (**1c**, **2c**, **3c**, **4c**, **5c**) pyrones as a result of derailment after two and three condensation reactions. With some notable exceptions, the exclusive formation of pyrones by plant PKSs seems to be an indicator that the starter CoA ester in the reaction is not the physiologically relevant substrate for the enzyme under assay. This phenomenon was observed in the assays of RtSTS with aliphatic and benzoyl CoA substrates. Pyrones also form as byproducts in reactions with correct substrates but the amounts are generally minor compared to the main aromatic product. In a limited number of cases, pyrone formation appears to

Table 1
Enzymatic products of RtSTS with different starter CoA esters

Substrate	Products			
 Acetyl-CoA (**1a**)	 *m/z* 127 $[M+H]^+$ (**1b**)	 *m/z* 169 $[M+H]^+$ (**1c**)		
 *n*-Butyryl-CoA (**2a**)	 *m/z* 155 $[M+H]^+$ (**2b**)	 *m/z* 197 $[M+H]^+$ (**2c**)		
 Isovaleryl-CoA (**3a**)	 *m/z* 169 $[M+H]^+$ (**3b**)	 *m/z* 211 $[M+H]^+$ (**3c**)		
 *n*-Hexanoyl-CoA (**4a**)	 *m/z* 183 $[M+H]^+$ (**4b**)	 *m/z* 225 $[M+H]^+$ (**4c**)		
 Benzoyl-CoA (**5a**)	 *m/z* 189 $[M+H]^+$ (**5b**)	 *m/z* 231 $[M+H]^+$ (**5c**)		
 Cinnamoyl-CoA (**6a**)	 *m/z* 215 $[M+H]^+$ (**6b**)	 *m/z* 257 $[M+H]^+$ (**6c**)	 Pinocembrin chalcone *m/z* 257 $[M+H]^+$ (**6d**)	 Pinosylvin *m/z* 213 $[M+H]^+$ (**6e**)
 *p*-Coumaroyl-CoA (**7a**)	 *m/z* 231 $[M+H]^+$ (**7b**)	 *m/z* 273 $[M+H]^+$ (**7c**)	 Naringenin chalcone *m/z* 273 $[M+H]^+$ (**7d**)	 Resveratrol *m/z* 227 $[M-H]^-$ (**7e**)

be the true catalytic activity of plant PKS enzymes. 2-Pyrone synthase from *Gerbera hybrida* forms 6-methyl-4-hydroxy-2-pyrone as its major product in vitro, with the involvement of this enzyme in pyrone metabolism supported by antisense "knockout" of 2-pyrone synthase in *G. hybrida* (Eckermann et al., 1998). A *p*-coumaroyl triacetic acid synthase from *Hydrangea macrophylla* may also represent an example of a pyrone-forming PKS, although this enzyme has been suggested to yield a linear tetraketide in vivo (Akiyama et al., 1999)

The formation of 6-acetonyl-4-hydroxy-2-pyrone (tetracetic acid lactone) (**1c**) (*m/z* 169 $[M+H]^+$), in addition to 6-methyl-4-hydroxy-2-pyrone (triacetic acid lactone) (**1b**) (*m/z* 127 $[M+H]^+$), when RtSTS was incubated with acetyl-CoA is notable. Compound **1b**

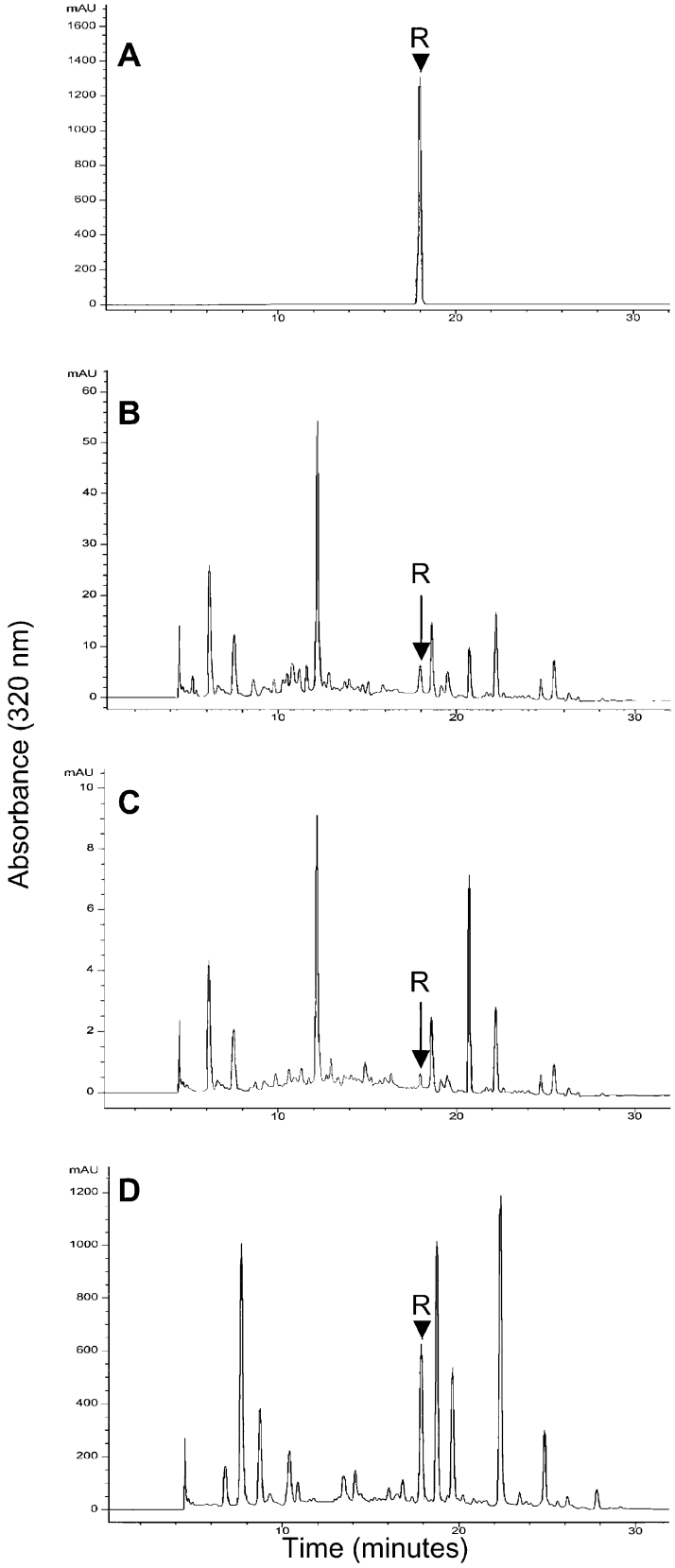


Fig. 4. Reversed-phase HPLC analysis of resveratrol in different tissues of *Rheum tataricum*. Methanolic extracts of each tissue were acid hydrolyzed before analysis to cleave sugar residues. Chromatograms represent (A) resveratrol standard, (B) leaf extract, (C) petiole extract and (D) rhizome extract. Note that the scale of the absorbance (y) axis differs in each of the panels due to the large variation in resveratrol content between the tissues analyzed. Chromatograms were obtained at 320 nm by photodiode array detection.

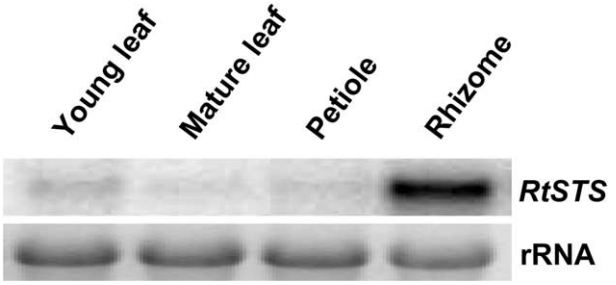


Fig. 5. Northern blot analysis of *RtSTS* expression in different tissues of *Rheum tataricum*. The lower panel shows equal loading of RNA as determined by ethidium bromide-staining of ribosomal RNA.

has been previously described as the main product formed from the use of acetyl-CoA as a starter CoA ester by *Gerbera hybrida* 2-pyrone synthase (Eckermann et al., 1998) and CHS from *Petroselinum crispum* (Schüz et al., 1983). 6-Acetonyl-4-hydroxy-2-pyrone (**1c**) is a new enzymatic product from a plant PKS, although we have also observed it as an enzymatic product from *Senna alata* CHS (Samappito et al., 2002). Its synthesis is analogous to the formation of CTAL and likely involves the condensation of acetyl-CoA with three C_2 units derived from malonyl-CoA followed by a pyrone-type cyclization. This compound has not been reported previously from plants,

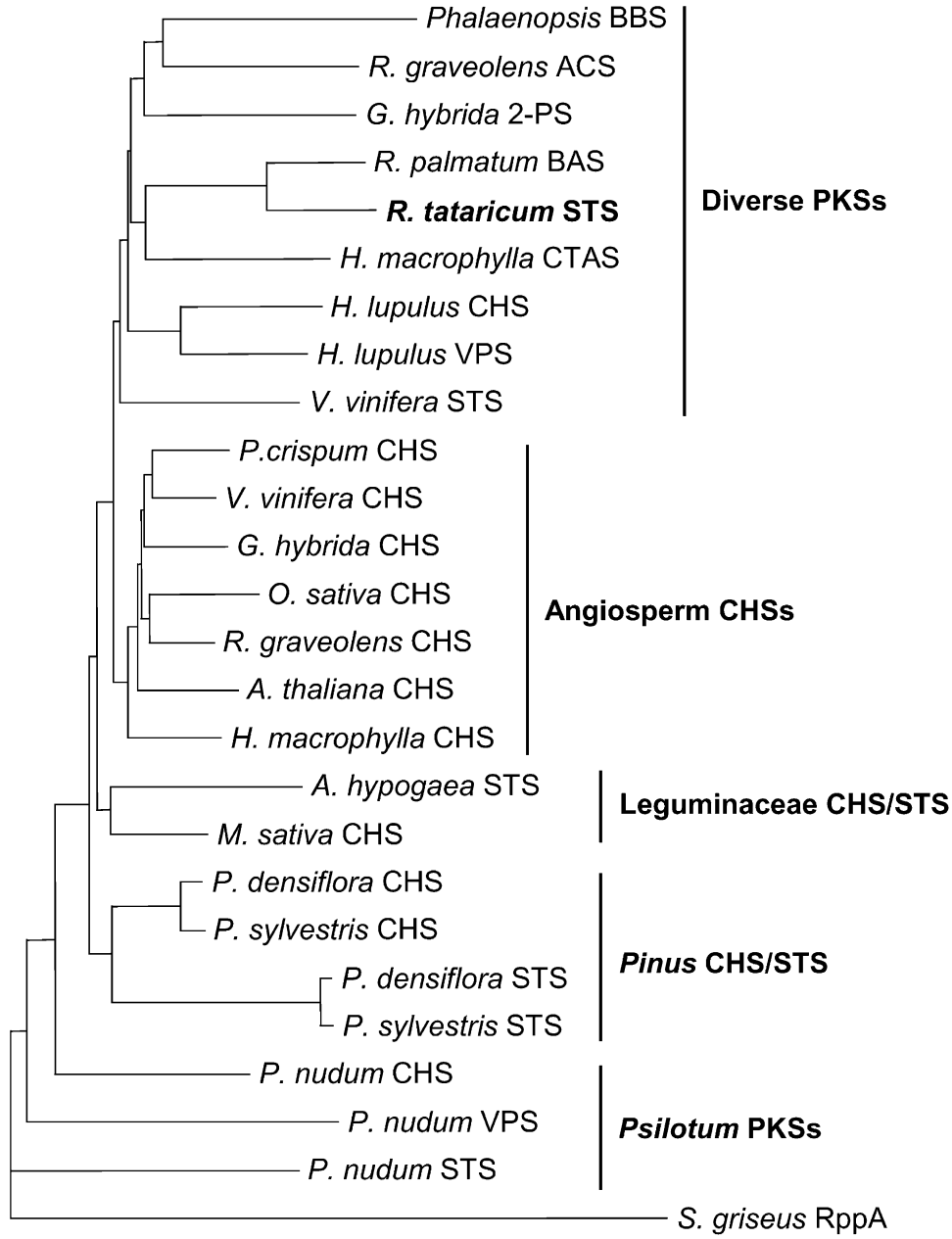


Fig. 6. Comparison of the deduced polypeptide sequence of *RtSTS* with members of the CHS superfamily. The relationship tree was constructed using ClustalX (Thompson et al., 1997). *Streptomyces griseus* RppA was used as an outgroup. Accession numbers of proteins used to construct the phylogeny are found in the Experimental.

although it has been isolated as a polyketide metabolite of *Penicillium stipatatum* (Bentley and Zwitkowits, 1967).

To confirm that stilbenes were present in the tissue from which the *RtSTS* cDNA was isolated, we analyzed leaf, petiole and rhizome extracts of *Rheum tataricum* by HPLC. Acid hydrolysis of extracts was used to cleave sugar residues and release stilbenes as their aglycones. Resveratrol was detected in all tissues, with higher amounts occurring in the rhizomes (Fig. 4). To the best of our knowledge, this is the first report of resveratrol from *R. tataricum*. The presence of this compound provides further evidence that the in vivo function of RtSTS is the biosynthesis of resveratrol. The distribution of resveratrol correlated with the expression pattern of the *RtSTS* transcript as determined by Northern blot analysis of total RNA isolated from young leaves, mature leaves, petioles and rhizomes (Fig. 5). Young leaves showed low expression levels, and the transcript was virtually absent from older leaves and petioles. A high expression level was detected in rhizomes where resveratrol levels were also highest.

A comparison of the relationships between *RtSTS* and other chalcone synthase superfamily members (Fig. 6) shows *RtSTS* to be closely related to *Rheum palmatum* BAS (82% amino acid identity) and forming a group with functionally diverse enzymes such as *Ruta graveolens* acridone synthase (ACS), *Gerbera hybrida* 2-pyrone synthase (2-PS) and *Vitis vinifera* STS. This group appears to be distinct from groups composed of angiosperm CHSs, and from PKSs from the Leguminosae (*Arachis* and *Medicago*), *Pinus* and *Psilotum*. Abe et al. (2001) analyzed the relationships of *Rheum palmatum* BAS and characterized the group to which this enzyme belongs as more primitive than flavonoid forming CHSs. While the sequences and methods used for analysis of sequence relationships may differ from those used in this study, the close relationship of *R. palmatum* BAS with *RtSTS* and *V. vinifera* STS and *Humulus lupulus* CHS suggests that no evolutionary conclusions as to the primitive or advanced nature of PKSs can be drawn on the basis of a simple comparison of sequence and enzyme function. The close relationship of *Rheum palmatum* BAS and *R. tataricum* STS supports a trend, first noted by Tropf et al. (1994), that functionally distinct enzymes that occur in the same plant taxa are more similar to each other than they are to functionally identical proteins that occur in more distantly related taxa.

STSs are not common in the plant kingdom. In comparison to the more than 100 genes encoding CHS that have been isolated from different species of higher plants (as reported in Swiss-Prot, 2002), STSs have been found in only four plant genera: *Arachis* (Schröder et al., 1988), *Vitis* (Melchior and Kindl, 1990), *Pinus* (Fliegmann et al., 1992) and *Psilotum* (Yamazaki et al., 2001). This is due both to the restricted distribution of stilbenes and to the inability to separate stilbene from CHSs based on sequence analysis; some sequences annotated as CHSs in sequence databases may in fact encode STSs. The cloning and characterization of a resveratrol-type STS from the medicinal plant *Rheum tataricum* provides a new example of a plant PKS participating in the biosynthesis of the pharmacologically and agriculturally important stilbene, resveratrol. It will be interesting to obtain the sequence of CHSs from *Rheum* and *Arachis*, so that a pairwise comparison of co-occurring CHS and STSs from *Vitis*, *Pinus*, *Arachis*, *Psilotum* and *Rheum* may be undertaken.

3. Experimental

3.1. Chemicals

Acetyl-CoA, malonyl-CoA, *n*-butyryl-CoA, isovaleryl-CoA, *n*-hexanoyl-CoA, benzoyl-CoA, naringenin and resveratrol were purchased from Sigma. 2-^{14}C-Malonyl-CoA (55 mCi/mmol) was from Biotrend Chemikalien (Cologne, Germany). *p*-Coumaroyl-CoA and cinnamoyl-CoA were kindly provided by D. Knöfel of the Abteilung Sekundärstoffwechsel, Leibniz-Institut für Pflanzenbiochemie (IPB), Halle.

3.2. General methods

Rheum tataricum was grown from seed in the greenhouse at the IPB, Halle. Total RNA was isolated as described by Salzman et al. (1999). Poly(A)$^+$ RNA was isolated with Oligotex beads (Qiagen) according to the manufacturer's instructions. For Northern blot analysis, 10 μg total RNA was resolved by electrophoresis and transferred to a nylon membrane (Hybond N, Amersham Pharmacia Biotech). The full-length cDNA was excised by restriction enzyme digest and labeled with ^{32}P using random primers. Hybridization was performed at 65 °C overnight and the membranes were then washed three times for 15 min with 2×SSC and 0.1% SDS at 65 °C. Radioactivity was visualized by phosphorimaging.

For Southern blot analysis, genomic DNA was isolated from young leaves according to Dellaporta et al. (1983). DNA (10 μg) was digested with *Apo*I, *Bcl*I and *Bst*XI (each cutting once within the reading frame), resolved by electrophoresis on a 0.8% agarose gel, and capillary blotted onto a Hybond N nylon membrane. The cDNA labeled as above was used as a hybridization probe as described for Northern blot analysis. Radioactivity was visualized by phosphorimaging.

3.3. Amplification of partial cDNAs from R. tataricum

First-strand cDNA was synthesized from 5 μg of total RNA isolated from *R. tataricum* rhizomes using Superscript II reverse transcriptase (Life Technologies).

One microlitre of the first-strand cDNA was used as a PCR template with degenerate primers 5′-AA(A/G)GC(C/T)AT(A/C)GAIGA(A/G)TGGGG-3 and 5′-CCACCIGG(A/G)TGI(A/G)CAATCC-3′ based on those described by Helariutta et al. (1995) and *Taq* polymerase (Promega). Cycling conditions consisted of an initial denaturation at 94 °C, 3 min; 30 cycles of 94 °C, 30 s; 48 °C, 30 s and 72 °C, 1 min, followed by 10 min at 72 °C. A PCR product of the expected length (575–600 bp) was purified by gel electrophoresis, ligated into pGEM-T Easy (Promega) and sequenced. Comparison of several fragments showed that two sequences of 98.5% similarity were amplified from *R. tataricum* rhizomes.

3.4. 5′ and 3′ RACE PCR

A RACE PCR kit (SMART technology; Clontech) was used to synthesize cDNA from total RNA isolated from *R. tataricum* rhizomes. 3′ RACE was performed using the gene-specific primer 5′-CATAGACTCCATGGTAGGGCAAGC-3′ and universal primer A mix (UPM) supplied by the manufacturer to amplify a 755 bp DNA fragment. Cycling conditions consisted of an initial denaturation at 94 °C, 5 s; 30 cycles of 94 °C, 5 s; 68 °C, 10 s and 72 °C, 3 min, followed by 7 min at 72 °C using Advantage polymerase (Clontech). Similarly, 5′ RACE used the gene-specific primer 5′-GCCGCACCGTCACCAAATATTGC-3′ and UPM to amplify a 781 bp DNA fragment. Both 3′ and 5′ RACE PCR products were gel purified, ligated into pGEM T-Easy and sequenced.

3.5. Expression in E. coli

To express the cDNA in *E. coli*, the open reading frame was cloned into pET14b (Novagen), which contains a hexahistidine N-terminal fusion tag. Amplification was performed using primers 5′-AATAGTCATATGGCACCGGAGGAGTCG-3′ (the NdeI site is underlined) and 5′-TTTAAAGGATCCTCAGGTAATTAGCGGCAC-3′ (the *Bam*HI site is underlined) with the 5′-RACE cDNA as template and *Pfu* polymerase (Promega). The 1.2 kb PCR product was digested with *Nde*I and *Bam*HI, gel purified and ligated into *Nde*I/*Bam*HI-digested pET-14b. Cloning of the expression construct was confirmed by sequencing. The plasmid was transformed into *E. coli* BL21(DE3) cells, 1 l cultures in LB broth containing 50 μg/ml ampicillin were grown at 37 °C until OD_{600} ~0.6 and protein expression induced with 1mM IPTG. Induced cultures were grown at 28 °C for 12 h, harvested by centrifugation and frozen at −80 °C.

3.6. Purification of recombinant RtSTS

Frozen *E. coli* cells were resuspended in buffer containing 50 mM Tris–HCl (pH 7), 500 mM NaCl, 2.5 mM imidazole, 10% (v/v) glycerol, 10 mM β-mercaptoethanol, 1% Tween-20 and 750 μg/ml lysozyme. Cells were incubated for 30 min on ice followed by a brief sonication. The lysate was clarified by centrifugation and the recombinant protein bound to Talon resin (Clontech). After washing with lysis buffer lacking detergent and lysozyme, recombinant protein was eluted with buffer containing 50 mM Tris–HCl (pH 7), 500 mM NaCl, 250 mM imidazole, 10% (v/v) glycerol, 10 mM β-mercaptoethanol. The protein-containing fractions were pooled and the buffer exchanged to 50 mM HEPES (pH 7), 10 mM β-mercaptoethanol and 10% (v/v) glycerol using a gel filtration column (PD10, Amersham Pharmacia Biotech). Recombinant RtSTS was determined to be pure by electrophoresis on a denaturing polyacrylamide gel (Laemmli, 1970). Purified enzyme was stored at −20 °C before use.

3.7. Enzyme assays

Polyketide synthase activity was measured by the conversion of starter CoA esters and [2-^{14}C]malonyl-CoA into reaction products. The standard enzyme assay contained 100 mM HEPES buffer (pH 7), 20 μM starter CoA and 5 μM [2-^{14}C]-malonyl-CoA (12,000 dpm) and 1.5 μg RtSTS in a 50 μl reaction volume. The assay mixture was incubated for 30 min at 30 °C. The reaction was stopped by addition of 5 μl 10% (v/v) HCl and was extracted twice with 100 μl ethylacetate. The combined organic phase was evaporated to dryness and the products separated by thin layer chromatography (RP18, Merck) developed in MeOH–H_2O–acetic acid (70:30:1). Selected reactions were co-chromatographed with a resveratrol standard. The ^{14}C-labelled products were visualized by phosphorimaging.

3.8. Mass spectrometric analysis of enzymatic products

To identify the enzymatic products, scaled up reactions containing 75 mM Tris–HCl (pH 7), 50 μM starter CoA, 100 μM malonyl-CoA and 5 μg purified enzyme in a 200 μl reaction volume were used. The reaction proceeded for 1 h at 30 °C before acidification and extraction with ethyl acetate. After drying in vacuo, the residue was dissolved in methanol and analyzed by LC–MS. Positive and negative electrospray ionization (ESI) mass spectra were obtained with a Finnigan MAT TSQ 7000 instrument (electrospray voltage 4.5 kV; heated capillary temperature 220 °C; sheath and auxillary gas nitrogen) coupled to a Micro-Tech Ultra-Plus MicroLC system equipped with an RP18-column (5 μm, 100×1 mm, SepServ, Berlin). For all compounds, a gradient system was used that ranged from H_2O:CH_3CN 90:10 [each containing 0.2% (v/v) acetic acid] to 10:90 over 15 min, followed by isocratic elution with a 10:90 mixture of both solvents for 10 min; flow

rate 70 μl/min. The collision-induced dissociation mass spectra during an HPLC run were recorded with a collision energy of −20 or −25 eV for positive ions as well as +20 or +25 eV for negative ions, respectively; collision gas: argon, collision pressure: 1.8×10^{-3} Torr.

3.9. HPLC analysis of R. tataricum rhizomes

To measure resveratrol in the leaves, petioles and rhizomes of *R. tataricum*, 1 g fresh plant material was frozen in liquid nitrogen and ground to a fine powder in a mortar and pestle. Ten milliliters of 80% (v/v) MeOH containing 3% (v/v) HCl were added and the extract was hydrolyzed at 100 °C for 60 min in a sealed tube. After centrifugation (10,000×*g*, 10 min) the clarified extract was evaporated, dissolved in water, and extracted with ethylacetate. The organic layer was dried in vacuo, the residue dissolved in 50 μl 50% (v/v) methanol and 10 μl were analyzed by HPLC. The HPLC system consisted of a Hewlett Packard Series 1100 instrument with a Eurospher-100 RP18 column (5 μm, 250×4 mm, Knauer). Chromatographic separation was performed using a solvent system of (A) H_2O containing 2% CH_3CN and 0.2% phosphoric acid and (B) CH_3CN containing 2% H_2O and 0.2% phosphoric acid (B) with a linear gradient of 20–60% B over 30 min. Flow rate was 0.6 ml/min with detection at 320 nm. All HPLC solvent percentages are expressed v/v.

3.10. Accession numbers

The GenBank accession numbers of the CHS superfamily members used to construct the relationship tree are *Phalaenopsis* BBS (P53416), *Ruta graveolens* ACS (S60241), *R. graveolens* CHS (CAC14059), *Gerbera hybrida* 2-PS (CAA86219), *G. hybrida* CHS (S56699), *Rheum palmatum* BAS (AAK82824), *Rheum tataricum* STS (AF508150), *Hydrangea macrophylla* CTAS (BAA32733), *H. macrophylla* CHS (BAA32732), *Humulus lupulus* VPS (BAA29039), *H. lupulus* CHS (BAB47196), *Vitis vinifera* STS (CAA54221), *V. vinifera* CHS (CAA53583), *Petroselinum crispum* CHS (CAA24779), *Oryza sativa* CHS (CAA61955), *Arabidopsis thaliana* CHS (BAB11121), *Arachis hypogaea* STS (P20178), *Medicago sativa* CHS (P30074), *Pinus densiflora* CHS (BAA94594), *P. densiflora* STS (BAA94593), *Pinus sylvestris* CHS (CAA43166), *P. sylvestris* STS (CAA43165), *Psilotum nudum* CHS (BAA87922), *P. nudum* VPS (BAA87923) and *P. nudum* STS (BAA87925).

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