Characterization of capsaicin synthase and identification of its gene (*csy1*) for pungency factor capsaicin in pepper (*Capsicum* sp.)

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Capsaicin is a unique alkaloid of the plant kingdom restricted to the genus *Capsicum.* Capsaicin is the pungency factor, a bioactive molecule of food and of medicinal importance. Capsaicin is useful as a counterirritant, antiarthritic, analgesic, antioxidant, and anticancer agent. Capsaicin biosynthesis involves condensation of vanillylamine and 8-methyl nonenoic acid, brought about by capsaicin synthase (CS). We found that CS activity correlated with genotype-specific capsaicin levels. We purified and characterized CS (≈35 kDa). Immunolocalization studies confirmed that CS is specifically localized to the placental tissues of *Capsicum* fruits. Western blot analysis revealed concomitant enhancement of CS levels and capsaicin accumulation during fruit development. We determined the N-terminal amino acid sequence of purified CS, cloned the CS gene (*csy1*) and sequenced full-length cDNA (981 bp). The deduced amino acid sequence of CS from full-length cDNA was 38 kDa. Functionality of *csy1* through heterologous expression in recombinant *Escherichia coli* was also demonstrated. Here we report the gene responsible for capsaicin biosynthesis, which is unique to *Capsicum* spp. With this information on the CS gene, speculation on the gene for pungency is unequivocally resolved. Our findings have implications in the regulation of capsaicin levels in *Capsicum* genotypes.

chili | gene cloning | localization

Chili has been domesticated for at least 7,000 years (1). Chili peppers are mainly consumed as food additives in many regions of the globe, including America, because of their unique pungency, aroma, and color (2). Indeed, a quarter of the world's population consumes hot pepper in some form daily (3). Capsaicin, a major alkaloid among capsaicinoids produced only in *Capsicum* fruits (4, 5), has wide applications in the food, medicine, and pharmaceutical industries (4, 6). As a medicine, capsaicin is known to kill some types of cancer cells (7, 8) and provide relief in arthritis and respiratory ailments (9). The pharmaceutical application of capsaicinoids is attributed to its antioxidant, anticancer, antiarthritic, and analgesic properties (6). It is a counterirritant and an analgesic agent (6). A functional cDNA that encodes the capsaicin receptor from sensory neurons has been isolated and characterized (10). The ecological significance of capsaicin in dispersal of chilies has also been reported (11).

Nonpungent peppers are used as vegetable (6), whereas paprika-type peppers are mainly used in food industries for its color, where zero pungency is preferred. The *Capsicum* oleoresins are usually obtained from pungent peppers, which should have 100,000–500,000 Scoville heat units and maximum of 4,000 American Spice Trade Association (ASTA) color units. On the other hand, paprika *Capsicum* oleoresin, used as a colorant in food and cosmetics, should have >40,000 ASTA color units and zero pungency (12). Hence, there is scope for regulating capsaicin biosynthesis in *Capsicum* genotypes to meet the demands of the food, pharmaceutical, and cosmetics industries. Development of transgenic *Capsicum* from zero to high pungency may thus be a practical proposition, which can be achieved by regulating the gene for capsaicin biosynthesis.

Capsaicin is biosynthesized by capsaicin synthase (CS) through the condensation of vanillylamine, a phenyl propanoid pathway intermediate, and fatty acid moieties in placental tissues of *Capsicum* fruits (13, 14) (Fig. 1). We have recently elucidated the regulatory role of 8-methyl nonenoic acid in capsaicin biosynthesis (15). We have reported the biotransformation of phenyl propanoid pathway intermediates to capsaicinoids (16–19) and elicitation (20, 21) of capsaicin in immobilized cell cultures leading to biochemical regulation. We have also elucidated the involvement of calcium calmodulin in regulation of capsaicin production mediated by CS (22).

Identification of the gene for CS with a demonstration of functionality has not been reported so far. However, there are reports of cDNA clones differentially expressed in placental tissues of *Capsicum*. The cDNA clones SB2–66 in Kim *et al.* (23) and *pun1* in Stewart *et al.* (24) are speculated to be involved in capsaicin biosynthesis, which encodes acyl transferases, but the functions of these genes are not known. Moreover, to our knowledge, there are no reports of CS purification and cloning of the CS gene. The gene responsible for capsaicin biosynthesis is required to study pungency regulation in *Capsicum* fruits. Therefore, to identify the gene responsible for capsaicin biosynthesis, we followed the enzyme-to-gene approach. Here we report the purification and characterization of CS, developmental expression of CS during ontogeny of fruit, and the gene encoding same with proven functionality.

Results

Purification of CS. In the study conducted to assay CS activity in placenta of low-, medium-, and high-pungency genotypes, it was noticed that CS activity was highest in high-pungency genotypes M-4 (35.26 units/mg per h) (Table 1). The CS extracted from high-pungency genotype, M-4, was used for further purification and characterization.

Purification of CS. Initially, the entire fraction was subjected to ammonium sulfate fractionation followed by gel filtration. Of the 110 fractions collected, CS activity was performed for every alternate fraction, and active fractions were bulked (Fig. 2*a*). Further purification was achieved on an affinity column of AH Sepharose bound with vanillylamine (Fig. 2*b*) with a specific activity of CS enhancing to 200-fold from that of crude extract

Conflict of interest statement: No conflicts declared.

Abbreviation: CS, capsaicin synthase.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. DQ349223 (*C. annuum*, full-length cDNA), DQ349224 (*C. frutescens*, full-length cDNA), DQ349225 (*C. annuum* genomic DNA), and DQ349226 (*C. frutescens* genomic DNA)].

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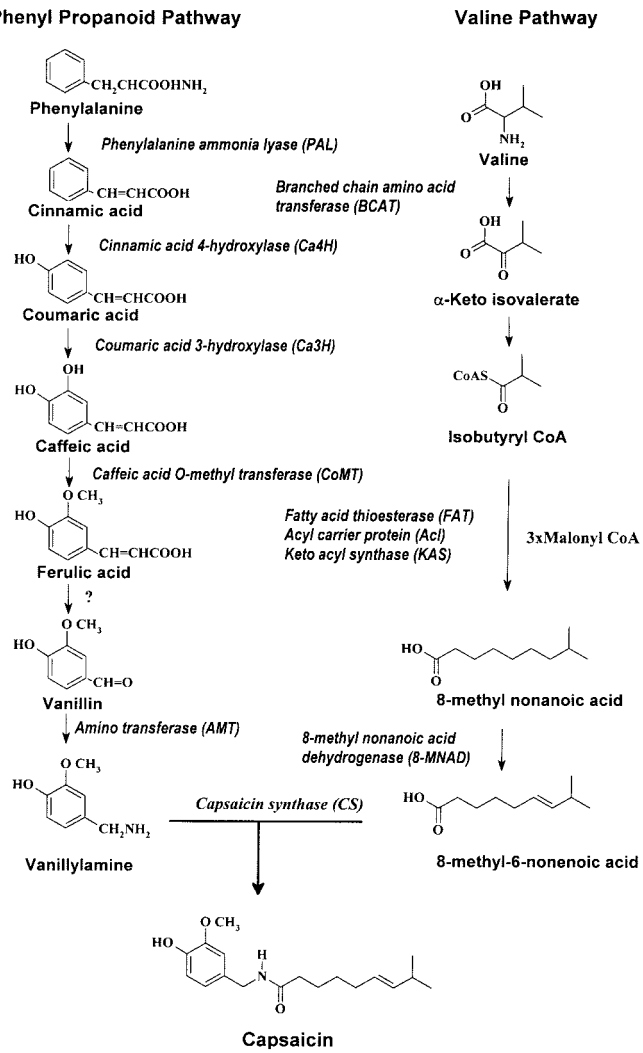

Fig. 1. Capsaicin biosynthetic pathway. PAL, phenylalanine ammonia lyase; Ca4H, cinnamic acid 4 hydroxylase; Ca3H, coumaric acid 3 hydroxylase; CoMT, caffeic acid *O* methyltransferase; pAMT, putative amino transferase; CS, capsaicin synthase; KAS, keto acyl synthase.

(Table 2 and Fig. 2*b*). Thus, CS was purified to electrophoretic homogeneity whose molecular mass was ≈35 kDa (Fig. 2*c*).

Biochemical Characterization of CS. CS was characterized for optimum pH, temperature, K_m and V_{max} values. Maximum CS activity was found at pH 8 (Fig. 3*a*), and 37°C (Fig. 3*b*) was found to be the optimum temperature. The K_m value for vanillylamine was found to be 6.6 ± 0.5 μM, and the V_{max} value was 181 units/mg (Fig. 3*c*). The K_m value for 8-methyl nonenoic acid was found to be 8.2 ± 0.6 μM, and the V_{max} value was 217 units/mg (Fig. 3*d*).

Western Blot to Study Expression of CS. Polyclonal antibodies raised against CS were used as primary antibodies and were highly specific to CS (Fig. 4*A*). Western blot analysis revealed that, during *Capsicum* fruit development, CS and capsaicin levels enhanced concomitantly till the 28 days after anthesis (Fig. 4*B*). Later, as the fruit reached ripening stage, there was reduction in levels of CS, which corresponded with decreased capsaicin content, in accordance with earlier reports (23, 25). There was a distinct positive correlation of CS accumulation with high-, medium-, and low-pungency genotypes. Immunolocalization showed that CS is confined to peripheral cells of placental tissues (Fig. 4*C*), evidently CS is biosynthesized in placental tissues of *Capsicum* fruits.

Identification of Gene Encoding CS. To isolate the CS gene (*csy1*), the N-terminal amino acid sequence of purified CS (35 kDa) was determined (Indian Institute of Technology, Mumbai, India). Degenerate primers were designed for the N-terminal amino acid sequence MIFILTVN, and PCR cloning was performed by using CSF (5′-ATGATHTTYATHYTX-3′) as forward primer and a number of reverse primers (NCBI database) that can pick different classes of acyltransferases (25, 26). Among the combinations tried, use of the reverse primer CSR1 (5′-TTGACCGTAAACTTCCGTTG-3′; based on putative acyl transferase SB2-66 clone from *Capsicum*; ref. 23) provided consistent amplification by PCR using placental-specific cDNA as template (Fig. 7, which is published as supporting information on the PNAS web site). The genomic clones revealed the absence of any introns because there was no size difference between the cDNA and the genomic clone as confirmed by sequencing. The full-length gene was 981 bp (Fig. 8, which is published as supporting information on the PNAS web site). The amplicon was cloned in a T-tailed vector, sequenced and designated as *csy1*. The deduced amino acid sequence of the full-length cDNA of CS is 308 residues long and does not share significant homology with any of the reported amino acid sequences including acyltransferases (Fig. 5 *a* and *b*). The predicted molecular mass of 38 kDa is in accordance with the molecular mass of native CS reported here. The deduced pI value of CS was found to be 8.6. The CS protein is rich in phenylalanine, as evident from deduced amino acid sequence.

RT-PCR Studies. RT-PCR revealed that expression of *csy1* was found only in placental tissues. Significant differences were seen in *csy1* gene transcript levels in high-, medium-, and low-pungency genotypes (Fig. 6*a*). In the high-pungency genotype, *csy1* expression was found to be maximum at 24–35 days after anthesis and restricted to placental tissues (Fig. 6*b*). Moreover, there was no difference in the sequence of genomic clones of

Table 1. CS-specific activity in low-, medium-, and high-pungency genotypes

Genotypes	Pungency, SHU	Capsaicin levels, μM/g	CS specific activity in placental tissues, units*/mg of protein per h
C. frutescens, high pungency (M-4)	5,83,650 ± 2453	72 ± 3.4	35.26 ± 2.21
C. frutescens var AW2, medium pungency	3,19,605 ± 1265	31 ± 2.5	25.44 ± 1.24
C. annuum var. Arka Abhir, low pungency	602 ± 21	0.1 ± 0.01	2.24 ± 0.25

Data recorded on 28th day of anthesis. SHU, Scoville heat units.
*One unit of CS activity refers to 1 nM capsaicin produced per milligram of protein per hour.

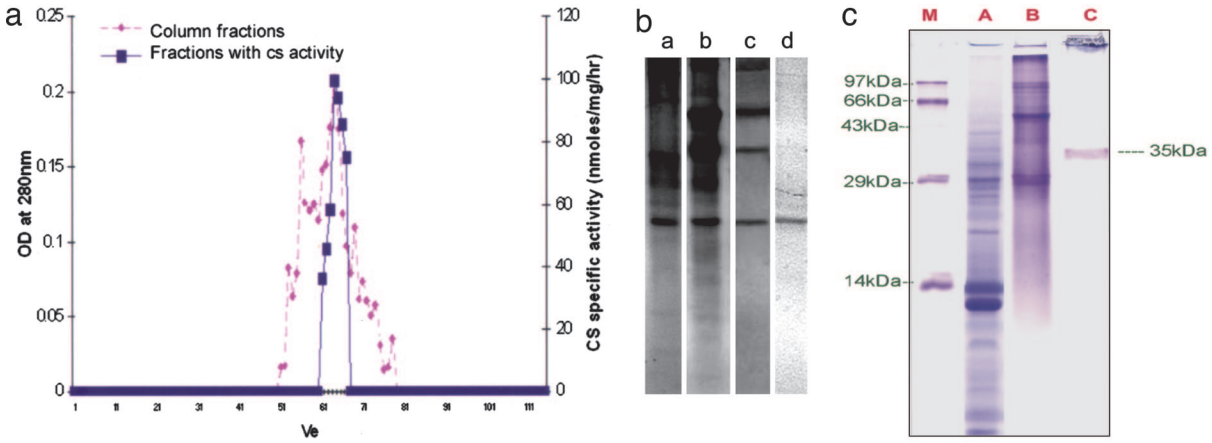

Fig. 2. Purification of CS. (*a*) Elution and specific activity profile of CS through Sephadex G-100. The ammonium sulfate fraction (60%) was loaded in to a gel filtration chromatography column (G-100) that was equilibrated with 0.1 M potassium phosphate buffer at 12 ml/h. Of 110 fractions collected, CS activity was performed for every alternate fraction, and active fractions were bulked and lyophilized. Subsequently lyophilized active CS fractions were loaded to amino H Sepharose column bound with vanillylamine (5 mg/ml). The unbound fractions were collected in 0.1 M potassium phosphate buffer, and the bound fractions were eluted in 1 M NaOH and subjected to CS assay after dialysis against 0.1 M potassium phosphate buffer. (*b*) Native gel (10%) depicting purified CS. Lanes: a, crude placental protein extract; b, 60% ammonium sulfate fractionated placental protein; c, Sephadex G-100 column eluted fractions; d, AH-Sepharose column eluted fraction. (*c*) SDS/PAGE (12.5%) showing purified CS. Lanes: M, protein molecular weight marker; A, ammonium sulphate fractionated placental protein; B, Sephadix G-100 column eluted fractions; C, AH-Sepharose column eluted fraction.

Capsicum annuum (GenBank accession no. DQ349225) and *Capsicum frutescens* (GenBank accession no. DQ349226).

Heterologous Expression of *csy1*. To understand the functionality, *csy1* was expressed in *Escherichia coli* DH5α by using pRESTA expression vector (Fig. 9, which is published as supporting information on the PNAS web site). Western blots confirmed the synthesis of recombinant CS in *E. coli*. The recombinant CS protein showed higher specific activity (62 μM capsaicin per mg of protein per h) than native CS (35 μM capsaicin per mg of protein per h) and was found to be highly specific to substrates of CS, thereby confirming the functionality of *csy1* to be specific to capsaicin biosynthesis.

Discussion

Although there are several reports on genetic regulation of pungency in pepper (23, 24), the gene responsible for the pungency factor capsaicin had been an enigma. The approaches followed by other groups (23, 24) have not resulted in unequivocal evidence for identification of CS gene, which is a committed step in the production of capsaicin, the pungent principle in pepper. In our approach, we started with a high pungent genotype with high level of CS activity for proceeding with purification of CS. Interestingly, the high pungency level correlated with high levels of capsaicin content and CS activity (Table 1). We purified CS to its homogeneity and characterized the same. Although capsaicin is reported to be synthesized in placenta (13, 14), it became evident from immunolocalization studies that it was mainly confined to the peripheral cells of placenta (Fig. 4*C*). Developmental expression studies of CS and capsaicin levels were commensurate (Fig. 4*B*). These results showed close correspondence in levels of capsaicin with CS activity in genotype specific manner (Table 1). Determination of N-terminal sequence was a requirement for a high degree of specificity in identification of the gene. Degenerate primers based on N-terminal sequence for forward and acyltransferase-specific primers for reverse were adopted for PCR, resulting in amplification of a 981-bp fragment. This clone was expressed in *Escherichia coli*, and the recombinant protein showed high level of CS activity. We designated this clone as *csy1*. The transcript analysis of *csy1* correlated with the immunolocalization studies of CS (Figs. 4*b* and 6*b*). The *csy1* sequence does not share significant homology with any of the acyltransferase genes including the genes which were thought to regulate pungency such as *pun1* and *catf2* (24). This observation indicates that *csy1* may be unique to *Capsicum* because capsaicin biosynthesis is confined only to this genus, carrying out condensation reaction between vanillylamine and 8-methyl nonenoic acid and probably not known in any life forms so far. The *csy1* gene reported here has biotechnological applications in regulation of pungency in *Capsicum* because it may help in developing zero- to high-pungency *Capsicum* lines through genetic transformation. Although chili belongs to the Solanaceae family, whose members are otherwise easily amenable to tissue culture and transformation practices, it is highly recalcitrant. Few reports of chili plant regeneration through organogenesis (27, 28) or embryogenesis (29, 30) are available. However, these reports are genotype-specific and, consequently, the regeneration protocol, as well as viable transformation, has to be established for each commercial cultivar for exploiting the potential of genetic engineering. There are isolated

Table 2. Purification of capsaicin synthase from placental tissues of *C. frutescens*

Fractions	Activity, nmol/h	Recovery, %	Protein, mg/ml	Specific activity, units/mg of protein per h	Fold increase
Crude	167	–	4.82	34.58	–
60% Ammonium sulphate fraction	147.47	88.31	1.74	84.28	2.43
Gel-filtration	129.87	77.77	0.94	1,137.95	39.8
AH Sepharose	112.36	67.28	0.01	7,146.00	206.65

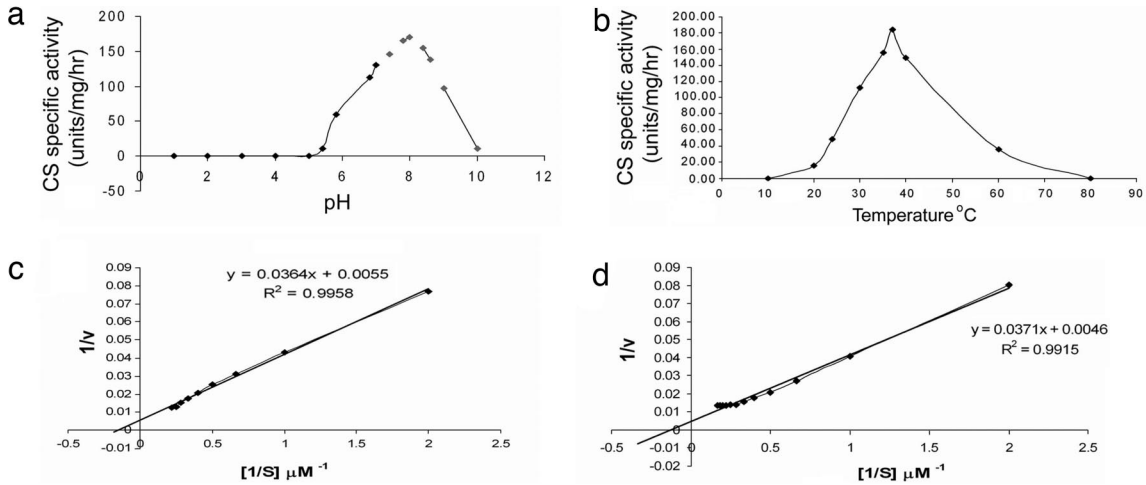

Fig. 3. Biochemical characterization of CS. (*a*) Characterization of CS for optimum pH. The activity of CS was found to be maximum at pH 8. Glycine·HCl (pH 2.2–3.6), acetate buffer (pH 3.6–5.6), potassium phosphate buffer (pH 5.6–7.6), and Tris·HCl buffer (pH 7.6–10) were used. (*b*) Characterization of CS for optimum temperature. Maximum CS activity was found at 37°C. (*c*) Double reciprocal plot of CS using vanillylamine as substrate in high pungency genotype. The K_m value for vanillylamine was found to be 6.6 μM, and the V_{max} value was 181. (*d*) Double reciprocal plot of CS using 8-methyl nonenoic acid as substrate in high pungency genotype. The K_m value for 8-methyl nonenoic acid was found to be 8.2 μM, and the V_{max} value was 217.

reports on genetic transformation in sweet pepper (31, 32) and also hot chili (33). As an extension of the present study, regulating the pungency levels in *Capsicum* cultivars by using *csy1* gene would have wide applications in food, pharmaceutical, and allied industries.

Materials and Methods

Plant Material. *Capsicum frutescens* Mill and *Capsicum annuum* L. genotypes were obtained from the Indian Institute of Horticultural Research (Bangalore, India).

Extraction of Placental Enzymes of *Capsicum* Fruits. One gram of placental tissue separated from 35-day-old *C. annuum*/*C. frutescens* fruits were homogenized in 10 ml of 0.1 M potassium phosphate buffer (pH 6.8) with 100 mg of ascorbic acid and 5 μM 2-mercaptoethanol. The homogenate was centrifuged at 4,000 $\times$ *g* for 30 min at 4°C. The supernatant was used as enzyme extract. The placental extracts were prepared from high-, medium-, and low-pungency genotypes.

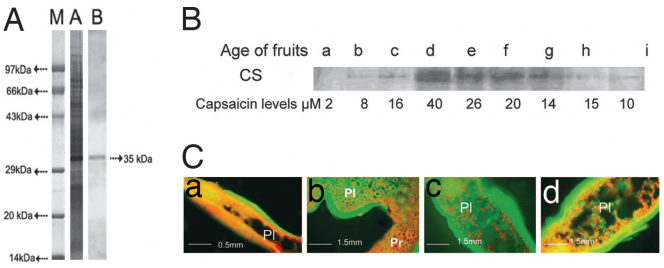

Fig. 4. Expression analysis and localization of CS. (*A*) Specificity of antibody raised against CS as evident from Western blot. Lanes: M, marker; A, SDS/PAGE of crude placental protein extracts; B, Western blot with anti-CS showing the specificity. (*B*) Developmental expression of CS. Lanes: a, 7-day-old placental protein; b, 14-day-old placental protein; c, 21-day-old placental protein; d, 28-day-old placental protein; e, 35-day-old placental protein; f, 42-day-old placental protein; g, 50-day-old placental protein (all high pungent *C. frutescens*); h, 28-day-old placental protein (low pungency genotype Arka Abhir *C. annuum*); i, 28-day-old placental protein (medium pungency genotype Arka Lohit *C. annuum*). (*C*) Immunolocalization of CS in placental tissues of *C. frutescens*. The immunolocalization studies indicated that CS is localized in peripheral cells of placenta of *Capsicum* fruits. (*Ca*) 15-day-old fruit. (*Cb*) 22-day-old fruit. (*Cc*) 30-day-old fruit. (*Cd*) 45-day-old fruit. pl, Placenta; pr, pericarp.

Assay of CS. The activity of CS was assayed (34). The reaction mixture contained 0.5 M potassium phosphate buffer (pH 6.8), 1 μM $MgCl_2$, 1 μM ATP, 5 μM each of vanillylamine, 8-methyl-nonenoic acid, CoA, and 1 ml of enzyme extract in 1 ml of reaction mixture. The reaction mixture was incubated for 2 h at 37°C and terminated by 0.5 M HCl. The reaction mixture was taken into chloroform and later evaporated and resuspended in 100 μl of methanol. Methanol fraction was used for HPLC for determining capsaicin levels. The specific activity of CS was expressed in terms of unit of capsaicin produced per mg of protein per hour. HPLC was used to quantify capsaicin produced by CS (35).

Purification of CS. For purification of CS, crude placental protein extract was brought to 60% ammonium sulfate fraction. The ammonium sulfate fraction was loaded to a gel filtration chromatography column (G-100), which was equilibrated with 0.1 M potassium phosphate buffer at 12 ml/h. The fractions were collected, and CS activity was performed for every alternate fractions and active fractions were bulked. The active fractions were loaded to an affinity column of amino H Sepharose (AH Sepharose) bound with vanillylamine (5 mg/ml). The bound protein fraction was eluted and fractionated by SDS/PAGE. The purity and molecular mass of CS were determined by SDS/PAGE (36). The molecular mass markers were obtained from Bangalore GeNei (Bangalore, India) (97 kDa, phosphorylase B; 66 kDa, BSA; 43 kDa, ovalbumin; 29 kDa, carbonic anhydrase; 14 kDa, lysozyme). Protein was estimated according to Bradford (37).

Developmental Expression and Immunolocalization of CS. Pure CS was used to generate polyclonal antibodies in rabbits (Department of Biochemistry and Nutrition, CFTRI, Mysore, India). Western blots were performed according to Towbin *et al.* (38). To study immunolocalization of CS, fruits were fixed in 2.5% (vol/vol) glutaraldehyde in 0.07 M sodium phosphate (pH 7.4). Samples were dehydrated through 70% alcohol and embedded in paraffin. Sections of 20 μm were cut by using a microtome (Leica, Vienna, Austria) and treated with anti CS antibodies (1:1,000), followed by washing and FITC conjugate (Bangalore GeNei). Samples were observed in a phase-contrast microscope equipped with fluorescence filters (Olympus BX40). Preimmune serum was used as control.

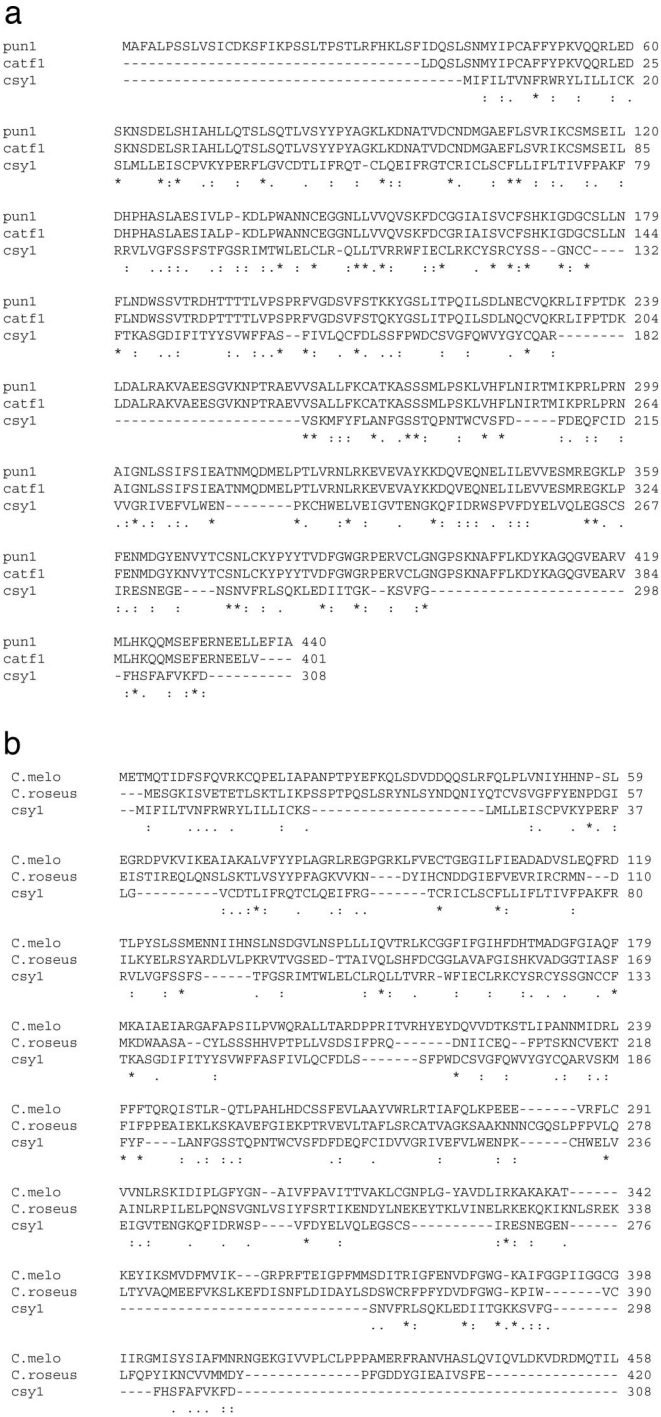

Fig. 5. Amino acid sequence alignment of *csy1* and acyl transferases. (*a*) Multiple sequence alignment of deduced amino acid sequence of *csy1* gene with reported acyl transferases of *Capsicum*. *pun1* and *catf1*, the reported acyltransferases speculated to be involved in regulation of pungency of *Capsicum* fruits, do not share significant homology with *csy1*. (*b*) Multiple sequence alignment of deduced amino acid sequence of *csy1* gene with reported acyl transferases from *Cucumis melo* and *Catharanthus roseus*. The deduced amino acid sequence of *csy1* is unique to *Capsicum* genus and is involved in capsaicin biosynthesis in *Capsicum spp.*

Cloning of the Gene Responsible for Capsaicin Biosynthesis. Purified CS was blotted on PVDF membrane, and the N-terminal amino acid sequence was determined (Indian Institute of Technology, Mumbai, India). Degenerate primers were designed for the N-terminal amino acid sequence (http://arbl.cvmbs.colostate.edu/molkit/rtranslate), and PCR cloning was performed by using forward primer CSF (5′-ATGATHTTYATHYTX-3′) and reverse primers (25 sets) designed to acyltransferases (NCBI database). Placental-specific cDNA was used as a template for cloning. PCR cloning was performed by following 40 cycles at 94°C for 60 s, 52°C for 60 s, and 72°C for 60 s with XT-DNA polymerase (Bangalore GeNei). An aliquot of 10 μl from each PCR was fractionated on a 1.5% (wt/vol) agarose gel in Tris-acetate EDTA buffer. Gels were stained with ethidium bromide solution (0.5 μg/liter) and photographed with a Digital Imaging System (HeroLab, Wiesloch, Germany). The amplicon was cloned in pTZ57R (Qiagen, Valencia, CA) and sequenced at Bangalore GeNei.

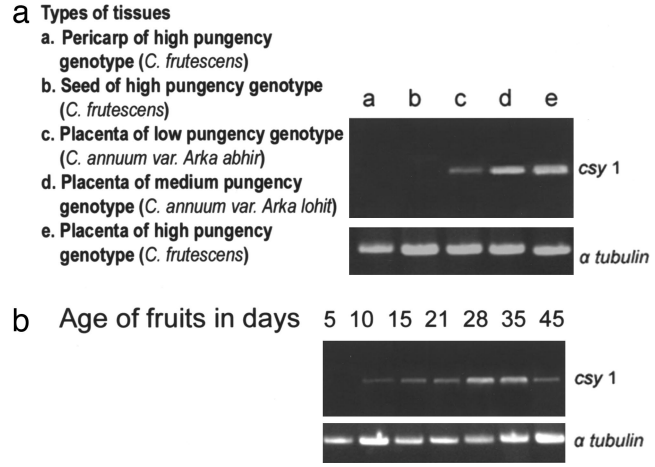

Fig. 6. Expression analysis of *csy1* gene. (*a*) Tissue and genotypes specific expression of *csy1* through RT-PCR. Lanes: a, pericarp of high pungency genotype (*C. frutescens*); b, seed of high pungency genotype (*C. frutescens*); c, placenta of low pungency genotype (*C. annuum* var. Arka Abhir); d, placenta of medium pungency genotype (*C. annuum* var. Arka Lohit); e, placenta of high pungency genotype (*C. frutescens*). (*b*) Developmental expression of expression of *csy1* in high pungency genotypes through RT-PCR.

Sequencing of Clones. PCR was performed to check the presence of inserts by using vector-specific primers M13 for pTZ57 R and T-7 for pRSET A and are sequenced at Bangalore GeNei. The clones obtained through genomic DNA of *C. frutescens* and *C. annuum* were also sequenced. Placental-specific cDNA and also genomic DNA of *C. frutescens* and *C. annuum* encoding for CS were also sequenced. The sequence obtained was checked for homology (39).

Expression Analysis of *csy1* by RT-PCR. Total RNA was extracted by using total RNA extraction kit (Ambion, Austin, TX). To avoid possible RNase contamination, all plastic-wares were treated with 0.1% DEPC (Sigma-Aldrich, St. Louis, MO), and the working area, electrophoresis, tank, and other required materials were treated with RNase Zap (Ambion). *Capsicum* fruits were harvested, and placenta, pericarp, and seeds were separated and frozen in liquid nitrogen; this was immediately followed by RNA extraction. Quality and concentration of RNA were checked on denaturing agarose gel and by absorbance measurements at 230, 260, and 280 nm in a UV spectrophotometer. All of the RNA samples were subjected to DNase (Ambion) treatment to avoid possible artifact amplifications from contaminant genomic DNA.

The *csy1* gene specific primers were designed by using Primer3 software (40). A control PCR was run on extracted RNA samples to check for absence of genomic DNA. First-strand cDNAs were synthesized from 2 μg of total RNA in a 20-μl final volume, by

using Moloney murine leukemia virus reverse transcriptase (Ambion) and oligo(dT) (18 mer) primer (Sigma) following the manufacturer's instructions. The RT-PCR was stopped in the early exponential phase (20 cycles) to maintain initial differences in target transcript quantities (exponential phase of amplification). PCRs were subjected to 22 cycles at 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s with TaqDNA polymerase (MBI Fermentas, Vilnius, Lithuania). The primers used were 5′-ATGTTGCTGGAAATCAGTTGTCCG-3′ (forward) and 5′-TTGACCGTAAACTTCCGTTG-3′ (reverse). An aliquot of 10 μl from each PCR was fractionated on a 1.5% (wt/vol) agarose gel in Tris-acetate EDTA buffer. Gels were stained with ethidium bromide solution (0.5 μg/liter) and photographed with a Digital Imaging System (HeroLab). Intensity of the DNA bands was estimated by intensity histogram. α-Tubulin, used as internal constitutive control, was amplified by using primers 5′-CTGTCAACGACCCCTTCATC-3′ and 5′-CCTGTTGTC-GCCAACGAAGTC-3′. The transcript abundance of CS was quantified by using the intensity histogram.

Heterologus Expression of *csy1*. CS was found negative for glycosylation as studied by the method of McGuckin and McKenzie (41). To generate an expression construct, the *csy1* cDNA clone encoding CS was cloned in frame in expression vector pRSET A (Invitrogen, Carlsbad, CA). Two oligonucleotides were designed for use in PCR. The forward primer ATGTTGCTGGAAAT-CAGTTGTCCG3 encoding MIFILTVN was used in combination with GCTAGTTATTGCTCAGCGG at HindIII site. CS cDNA was amplified by PCR and the product was ligated to pRSET A vector (Invitrogen) followed by sequencing. The plasmids were transformed to DH 5α. Recombinant protein was induced with 1 mM IPTG for 4 h at 37°C. The cells were collected and resuspended in 50 mM Tris·HCl (pH 6.8) containing 0.25% dodecylamaltoside and sonicated. The resulting lysate was centrifuged at 10,000 $\times$ *g* for 15 min, and supernatant was used for recombinant CS assay. Protein extraction, CS assay, and Western blots were performed as explained above for placental-specific native protein.

Statistical Analysis. The mean and standard deviation was calculated according to Tukey's method (42).

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