Plant Biotechnology 29, 185–189 (2012)
DOI: 10.5511/plantbiotechnology.12.0418a

Note

Characterization of the casbene synthase homolog from Jatropha (*Jatropha curcas* L.)

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Received January 17, 2012; accepted April 18, 2012 (Edited by K. Akashi)

Abstract The high oil content of Jatropha (*Jatropha curcas* L.) seeds makes Jatropha an attractive resource for the production of sustainable bioenergy. However, the Jatropha seed kernels also contain antinutrients and various toxins that persist in the oil and seed cakes and pose a safety risk. Since phorbol esters (PEs) are the major contributor to toxicity, a better understanding of PE biosynthesis is expected to elucidate an effective strategy for the utilization of Jatropha plants. In this study, a *Jatropha curcas* casbene synthase homolog (*Jc*CSH) with high sequence similarity to casbene synthases (CSs) from *Ricinus communis*, *Euphorbia esula*, and *Sapium sebiferum* was cloned from Jatropha leaf tissue. CS has been hypothesized to catalyze the first step of phorbol biosynthesis. *JcCSH* encodes a protein that contains a chloroplast transit peptide and a DDXXD motif that is conserved among known terpene cyclases. *JcCSH* was expressed in seedlings, mature leaves, and the flesh of developing fruits, but not in developing seeds. Our results suggest that *JcCSH* is widely involved in casbene biosynthesis in various tissues other than seeds.

Key words: Casbene synthase, Jatropha (*Jatropha curcas* L.), phorbol ester.

Recently, Jatropha (*Jatropha curcas* L.) has attracted much attention as a potential resource for the production of sustainable bioenergy, following concerns about the depletion of fossil fuels and global warming. Jatropha, a perennial shrub that originated in Mexico and Central America and belongs to the Euphorbiaceae family, is widely distributed between the tropics of Cancer and Capricorn. The seed kernels are rich in oil (44–62%) and proteins (22–35%), making this plant a favorable feedstock not only for biodiesel but also for protein (Makkar et al. 1998). However, it is hard to utilize the Jatropha seed cakes for animal feed after extracting the oil, because several antinutrients and various toxins, such as curcin, trypsin inhibitors, and phorbol esters (PEs), are contained in the seed kernels (Makkar et al. 1998). Among these harmful substances, the major contributor to toxicity is PEs (Makkar et al. 1998). PEs are tetracyclic diterpenoids that occur in the seeds, sap, and latex of plants of the Thymelaeaceae and Euphobiaceae families. Six PE derivatives were identified from Jatropha oil and their structures were solved by nuclear magnetic resonance (NMR) analysis. They were all found to contain 12-hydroxy-16-deoxyphorbol (Adolf et al. 1984; Haas et al. 2002). Since a PE isolated from Jatropha is known to have tumor-promoting activity (Hirota et al. 1988), it is critical to eliminate the risk of PEs toxicity if Jatropha is to be used commercially. One effective means of doing so is to shut down and/or modify the biosynthesis of PEs at the molecular level.

PEs are composed of a tetracyclic diterpene, named tiglian, and two fatty acids, which are esterified to C13 and C16 of the tiglian skeleton (Haas et al. 2002). The biosynthetic pathway of PEs is currently poorly understood. The only presumed step is the first cyclization of tiglian by casbene synthase (CS), during which geranylgeranyl pyrophosphate is converted to a monocyclic diterpene, casbene. Macrocyclic diterpenoids, including tigliane, are thought to be biosynthesized from casbene, through several catalytic conversion or modification steps (Figure 1; Schmidt 1987). Casbene was isolated as the antifungal diterpene from castor bean (*Ricinus communis*), and stress-treated castor bean seedlings were found to exhibit high levels of CS activity (Moesta and West 1985). The cDNA sequence corresponding to *CS* was first determined from castor bean (*RcCS1*; Mau and West 1994). Recently, five *CS*

Abbreviations: CS, casbene synthase; Jatropha, *Jatropha curcas* L.; PE, phorbol ester.
This article can be found at http://www.jspcmb.jp/
Published online June 15, 2012

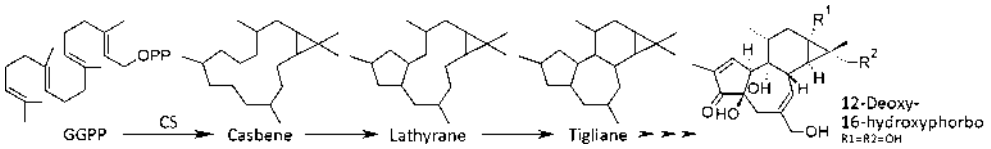

Figure 1. Hydrocarbon skeletons of casbene, lathyrane, and tigliane and structure of phorbol. A geranylgeranyl pyrophosphate (GGPP) is cyclized into casbene (a monocyclic diterpene) by casbene synthase (CS). Phorbol structure is based on a tetracyclic diterpene known as tigliane, which is assumed to be synthesized from casbene through lathyrane (a dicyclic diterpene) as intermediate. All six PEs identified from Jatropha oil contain 12-hydroxy-16-deoxyphorbol.

candidates were isolated from *R. communis*, *Euphorbia esula*, and *Sapium sebiferum* (*RcCS1*, *RcCS2*, *RcCS3*, *EeCS*, and *SsCS*; Kirby et al. 2010). Overexpression of these genes in yeast revealed that *Rc*CS1, *Rc*CS3, *Ee*CS, and *Ss*CS synthesized casbene as the primary product; however, *Rc*CS2 synthesized a different primary product, neocembrene, which has been identified as a trail-following pheromone in termites (Kirby et al. 2010). This finding supports the hypothesis that similar enzymes participate in the production of casbene and neocembrene (Crombie et al. 1980). Prompted by these studies and in an effort to further our understanding of PEs biosynthesis in Jatropha, we sought to isolate the Jatropha *CS* gene.

In order to clone Jatropha *CS*, we designed degenerate primer sets that target a highly conserved region of the eight *Rc*CS proteins registered in the NCBI databank (Supplemental Table 1). An accession of Jatropha, NBF-1, which was collected from the Philippines by Nippon Biodiesel Fuel Co., Ltd. (http://www.nbf-web.com/index.html), was used in this study. Since PEs had been detected in mature leaves of NBF-1 by liquid chromatography-mass spectrometry (LC-MS) analysis (Matsukawa and Kajiyama, personal communication), we decided to use mature leaves for the cloning of the *CS* gene. Total RNA was isolated by using a Plant RNA Isolation Mini Kit (Agilent), and genomic DNA was degraded by treatment with RQ1 RNase-Free DNase (Promega). First-strand cDNAs were synthesized from 2 µg total RNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche) and 50 pmol oligo-$(dT)_{15}$ primer. Amplification of cDNA fragments corresponding to the *CS* gene was performed by using the primers presented in Supplemental Table 1 and KOD-plus Polymerase (TOYOBO), and the amplified fragments were cloned into a pGEM-T Easy vector (Promega). The cloned fragments were sequenced by an ABI PRISM® 3100 Genetic Analyzer (Life Technologies), and we obtained only one species of sequence from the clones. Based on the sequence information of the fragment, we designed *CS*-specific primers (Supplemental Table 1) to isolate full-length cDNA sequence by Rapid Amplification of cDNA Ends (RACE) using a GeneRacer Kit (Life Technologies).

We successfully isolated a 2016-bp full-length cDNA sequence, which we named *J. curcas* casbene synthase homolog (*JcCSH*) (Accession number, AB687998). The whole genomic sequence of Jatropha was revealed in 2011, and nine *CS* genes (*JcCS1-9*) had been predicted by *in silico* analysis (Sato et al. 2011). Among them, the sequence information is publically available only for *JcCS1*, *3*, *4*, *6*, *7*, and *9* through international databases (DDBJ/GenBank/EMBL). *JcCSH* is not identical to any of the predicted *JcCS* genes, but corresponds approximately to the predicted gene model of JcCB0049821.10 in Jatropha Predicted Gene (http://www.kazusa.or.jp/jatropha/), which has the highest identity of nucleotide sequence, 96.6%, with *JcCSH*. We suppose that the sequential difference between *JcCSH* and JcCB0049821.10 might be attributed to genomic polymorphisms between plant materials. Following this idea, phylogenetic tree analysis with known CS amino acid sequences showed a well-separated branch of *Jc*CSH and JcCB0049821.10 with bootstrap value of 100%, from the other *Jc*CSs (Figure 2A). The phylogenetic tree also indicated that the CSs fall into two distinct groups, a group including *Jc*CSH, JcCB0049821.10, *Jc*CS1, *Rc*CS1, *Rc*CS3, *Ee*CS, and *Ss*CS, and a group of the other *Jc*CSs. Of note, the chloroplast transit peptides are found in all CSs of the former group, but in none of the latter group, by using TargetP predictor program (Emanuelsson et al. 2000; http://www.cbs.dtu.dk/services/TargetP/) (Figure 2B).

JcCSH putatively encodes a protein possessing three characteristic features; a chloroplast transit peptide, a DDXXD motif, and conserved cysteine and histidine residues (Figure 2B). The chloroplast transit peptide, which is composed of 52 amino acids, is located in the N-terminus of *Jc*CSH (Figure 2B). We performed a transient expression analysis of *Jc*CSH fused to a fluorescent protein (YFP) in leaf protoplasts derived from *Arabidopsis thaliana* (Columbia) (Wu et al. 2009) using laser scanning confocal microscopy LSM700 (ZEISS). The coding region of *Jc*CSH, except for the stop codon, was amplified by PCR, cloned into the pENTR™/D-TOPO® vector (Life Technologies), and then integrated into the Gateway® destination vector pA35GY by LR Clonase™ (Life Technologies). In pA35GY, the Gateway *att*R cassette (Life Technologies) is located between the 35S promoter and the YFP coding sequence followed by the NOS terminator, and the fragment from pENTR™/D-TOPO® vector is fused to N-terminal of YFP. The

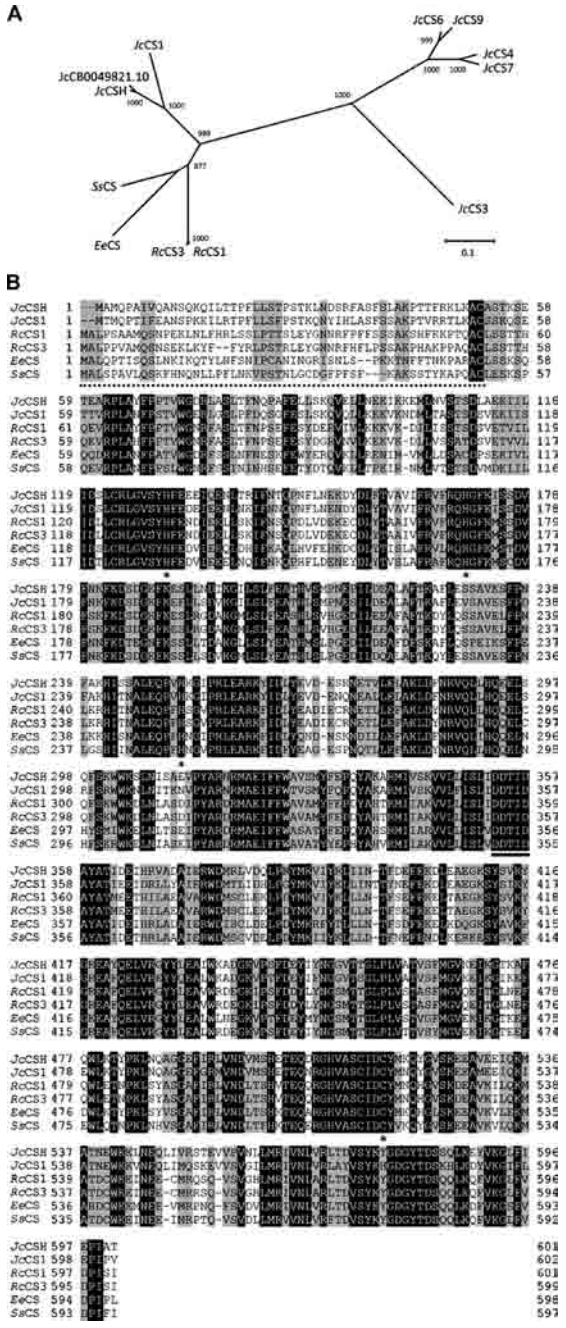

Figure 2. Casbene synthases (CSs) of Jatropha and other Euphorbeaceae species. Unrooted phylogenetic tree of CSs (A). The phylogenetic tree was established with the amino acid sequences of *Jc*CSH and other CSs, namely, *Jc*CS1, 3, 4, 6, 7, and 9 from *J. curcas* (Genebank ID, BAJ53213, BAJ53216, BAJ53218, BAJ53219, BAJ53220, and BAJ53221, respectively); *Rc*CS1 and *Rc*CS3 from *R. communis* (Genebank ID, L32134 and XM_002513297, respectively); *Ee*CS from *E. esula* (Genebank ID, GU332591); and *Ss*CS from *S. sebiferum* (Genebank ID, GU332590) by the neighbor-joining method. Numbers are bootstrap values and given for clades that received support values of over 80% (1000 resamplings). The scale (0.1) represents a 10% change in sequences. Amino acid sequence alignment of the six CS proteins (B). We introduced gaps (marked with dashes) to maximize the similarities. Identical and similar residues are shaded with black and gray, respectively. The predicted chloroplast transit peptide sequence is underlined with a dotted line and the DDXXD motif is underlined. The highly conserved histidine and cysteine residues are indicated with asterisks.

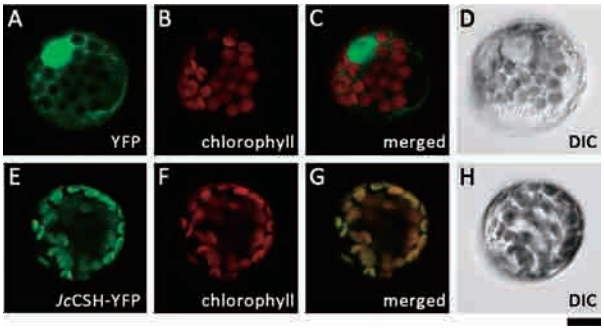

Figure 3. Subcellular localization of *Jc*CSH. A fluorescent protein (YFP) or *Jc*CSH fused to YFP (*Jc*CSH-YFP) was transiently expressed in *A. thaliana* leaf protoplasts and examined by laser scanning confocal microscopy. Confocal (A–C, E–G) and differential interference contrast (DIC; D and H) images of protoplasts expressing YFP alone (A–D) or *Jc*CSH-YFP (E–H). Confocal images of YFP signal (A and E), chlorophyll autofluorescence (B and F), and the merged images of YFP and chlorophyll (C and G). The protoplast expressing YFP alone exhibited fluorescence throughout the cytoplasm and the nucleus (A). In protoplasts expressing *Jc*CSH-YFP, the fluorescent signal colocalized with chlorophyll autofluorescence (E–G), indicating that *Jc*CSH localized to the chloroplast. Bar=10 μm.

backbone of pA35GY plasmid is identical to a general cloning vector, pBI221 (Clontech). *Jc*CSH-YFP was localized to the chloroplast (Figure 3), which is consistent with the hypothesis that CS enzymes, like other enzymes that catalyze the biosynthesis of plant diterpenoids, are present and functional in chloroplasts (Kirby and Keasling 2008). The DDXXD motif is conserved among known terpene cyclases, and is assumed to be a catalytic domain for protonation-initiated cyclization (Prisic et al. 2007). This motif is also involved in coordinating divalent cations, such as Mg^{2+} or Mn^{2+}, during the regulation of catalytic activity of the diterpene cyclases (Mann et al. 2010). The DDTID motif sequence is conserved among *Rc*CS1, *Rc*CS3, *Ee*CS, and *Ss*CS, which were shown to synthesize casbene (Kirby et al. 2010; Mau and West 1994), *Jc*CS1, and *Jc*CSH (Figure 2B). In *Rc*CS2, which is reported to be a neocembrene synthase (Kirby et al. 2010), the corresponding sequence is DDTFD. Thus, sequence differences in the DDXXD motif appear to impart different reaction properties to the terpene cyclases. In addition, the conserved cysteine and histidine residues, which are presumed to be involved in the interaction with metal ions (Mau and West 1994), are also found in *Jc*CSH.

To elucidate the gene expression pattern of *JcCSH*, we performed quantitative RT-PCR analysis. Total RNAs were isolated from seedlings (10 days after germination), mature leaves, the flesh of developing fruits, and developing seeds (Figure 4). RNA isolation, DNase treatment, and first-strand cDNA synthesis were performed as described above. Approximately 200 ng of cDNA was used as template for the quantitative PCR analysis using FastStart DNA Master SYBR Green I

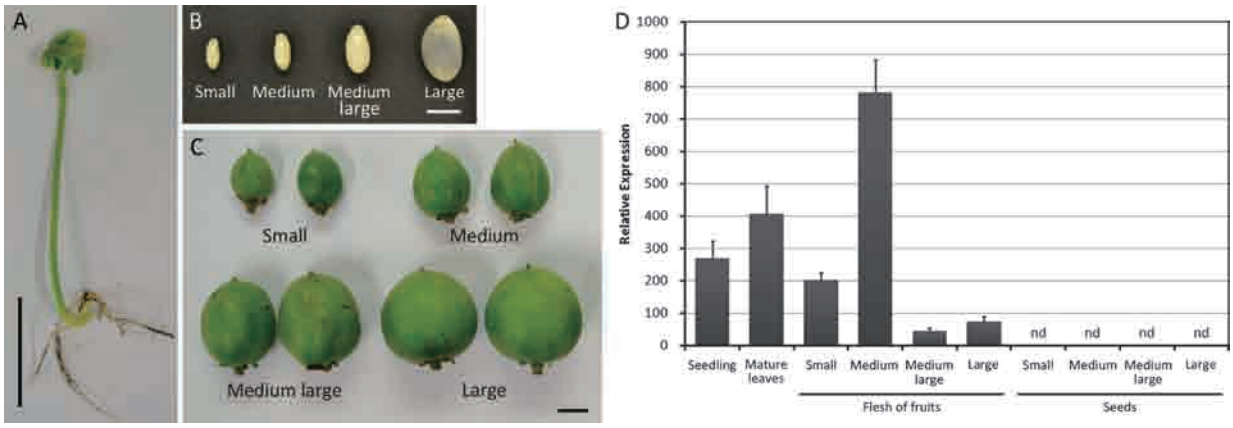

Figure 4. Expression pattern of *JcCSH* in various organs. Ten-day-old Jatropha seedling (A). Developing Jatropha seeds (B). Developing Jatropha fruits (C). Developing fruits were categorized based on their sizes: small, <2 cm in length; medium, 2–2.5 cm; medium large, 2.5-3 cm; large, >3 cm. The relative expression levels of *JcCSH* normalized to the amounts of *18S rRNA*, as determined by quantitative RT-PCR analysis (D). Flesh and seeds were obtained from the fruits. Bars=5 cm in A and 1 cm in B and C. Bars in D indicate SD (n=3). nd, not detected.

(Roche) and a Roche LightCycler® 480. The sequences of the gene-specific primers for *JcCSH* and the *18S rRNA* gene (Zhang et al. 2008), which was used as the internal control, are listed in Supplemental Table 1. By the sequencing analysis, we confirmed that the PCR fragments amplified with *JcCSH* gene-specific primers correspond exactly to the partial sequence of *JcCSH*.

JcCSH was expressed in seedlings, mature leaves, and the flesh of developing fruits, but not in developing seeds (Figure 4). These data imply that casbene is biosynthesized in most organs of Jatropha, regardless of their developmental stages. Lathyrane-type diterpenes and phorbol were found in various tissues of other Euphorbiaceae plants, e.g., jatrowediol from *J. weddelliana* stems (Brum et al. 2001), multifidone from *J. multifida* stems (Das et al. 2009), and sapintoxin A from unripe *S. indicum* fruit (Taylor et al. 1981). Thus, the *JcCSH* gene is expected to be involved in the biosynthesis of a broad range of diterpenes, including phorbol.

Interestingly, *JcCSH* was not expressed in developing seeds (Figure 4). This fact gave rise to two possible hypotheses regarding the biosynthesis of PEs in seeds. The precursors of phorbol and/or PEs may be synthesized in distinct organs, such as fruits and/or leaves, and then be transported to the seeds. In tobacco plants, nicotine is synthesized in the roots and transported to the aerial parts (Katoh et al. 2005). Similar mechanisms could underlie PEs accumulation in Jatropha seeds. Alternatively, *CS* genes other than *JcCSH* may function in PEs biosynthesis in seeds. Information about the genomic DNA sequence of Jatropha allowed us to predict at least nine *CS* genes *in silico* (Sato et al. 2011), and importantly, *JcCSH* is not identical to any of the nine predicted *CS* genes. Besides these nine *CS* genes, a keyword search using Jatropha Predicted Gene of the Kazusa DNA Research Institute (http://www.kazusa.or.jp/jatropha/) identified 43 distinct sequences that have high similarity to *RcCS*, *EeCs*, and *SsCS*. These facts suggest that the genes underlying the biosynthesis of casbene and its derivatives are multifaceted in Jatropha, and that the spatiotemporal regulation of *CS* genes is involved in PE biosynthesis. Moreover, previous work on *Rc*CSs showed that it is difficult to distinguish casbene synthase from neocembrene synthase based on their amino acid sequences, because these sequences are highly similar (Kirby et al. 2010). We have to analyze the enzyme activities of the predicted Jatropha *CS* genes, including *JcCSH*, to elucidate the casbene biosynthetic pathway in Jatropha.

Our results regarding the *JcCSH* gene raise the possibility that PEs can be specifically removed from the seed by well-controlled modification, i.e., by inhibiting PEs transportation into seeds or by suppressing the seed-specific expression of *CS*. As PEs act as an insecticide (Kumar and Sharma 2008), their accumulation in vegetative tissues should benefit Jatropha plants by preventing insect damage. Further studies of PEs biosynthesis will facilitate the development of effective breeding programs that will improve the utility of Jatropha.

Acknowledgements

We thank Dr. Tetsuya Matsukawa and Dr. Shin'ichiro Kajiyama (Kinki University) for LC-MS analysis of PEs. We thank Ms. Nobuko Shizawa and Ms. Shoko Mizobuchi (NAIST) for their technical assistance. This work was supported by the Kanagawa Prefectural Government Program for Human Resources Utilization by Bio-venture Companies.

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