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Characterization of a stearoyl-acyl carrier protein desaturase gene from potential biofuel plant, *Pongamia pinnata* L.

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ABSTRACT

A new full length cDNA clone encoding stearoyl-ACP desaturase (SAD) was isolated from seeds of *Pongamia pinnata*, an oil yielding legume plant. The cDNA clone (Pp*SAD*) contained a single open reading frame of 1182-bp coding for 393 amino acids with a predicted molecular mass of 45.04 kDa, and shares similarity with SAD from other plants. Characteristics of the deduced protein were predicted and analyzed using molecular homology modeling; its three dimensional structure strongly resembled the crystal structure of *Ricinus communis* (RcSAD). Southern blot analysis indicated that '*sad*' is a multiple copy gene and was a member of a small gene family. Expression analysis using quantitative real-time PCR revealed that the gene showed marked distinct expression during different stages of seed developments. The results of the expression analysis in this study, combined with existing research, suggest that '*sad*' gene may be involved in the regulation of plant seed growth and development.

1. Introduction

Acyl-ACP desaturase enzymes catalyze the conversion of saturated fatty acids to unsaturated fatty acids by introducing the first double bond into saturated fatty acids. Different groups of enzymes involved in desaturation reaction have been identified in all eukaryotes, cyanobacteria and in some *Bacillus* bacteria as well (Bloomfield and Bloch, 1960; Fulco, 1974). Three types of desaturases are noticeable depending on the kind of compounds esterified to fatty acids (Murata and Wada, 1995). Desaturases identified in plants, animals and yeast are membrane-bound proteins with acyl chain attached to CoA or lipids. There are three types of fatty acid desaturase: acyl-CoA, acyl-ACP, and acyl-lipid desaturases. In plants and cyanobacteria, most desaturation reactions are catalyzed by acyl-lipid desaturases, which introduce unsaturated bonds into fatty acids that are in a lipid-bound form. Acyl-ACP desaturases are present in the plastids of plant cells and introduce the first double bond into fatty acids that are bound to acyl carrier protein (ACP) (Holloway, 1983; Maruta and Wada, 1995; Maruta et al., 1992). Acyl-CoA desaturases are present in animal, yeast and fungal cells, and they introduce unsaturated bonds into fatty acids that are bound to coenzyme A (CoA) (Macartney et al., 1994). The only known soluble desaturase is the plant stearoyl-ACP desaturase (SAD) specific to stearic acid localized in plastids. SAD catalyzes the desaturation of stearoyl-ACP to oleoyl-ACP and plays a key role in determining the ratio of saturated fatty acids to unsaturated fatty acids in plants (Lindqvist et al., 1996) and this ratio is closely related to many functions of plants, especially with regard to acclimatization to low-temperature (Kodama et al., 1995). According to Edgar et al. (1994) the homologs of SAD are also present which involve in the formation of Δ^6-hexadecenoic acid (16:1Δ^6) and oleic acid (18:1 Δ^9) in the seed oil of *Thunbergia alata*.

Although there are other desaturases (SAD homologs) in plants, the SAD catalyzed introduction of the first double bond at position nine in the FA carbon chain is the most important desaturation for the gel state to liquid crystal state transformation of membranes (Los and Murata, 1998). SAD also plays a key role in environmental changes like cold resistance in plants (Kodama et al., 1995; Tasseva et al., 2004), senescence regulation, resistance to fungal infection and mechanical damage (Kachroo et al., 2003; Lea, 2003). Many genes coding for SAD have been cloned from different plants (*Carthamus tinctorius*, *T. alata*, *Ricinus communis*, *Solanum tuberosum*) and the structure and functions of several SAD have been studied (Davydov et al., 2005; Lindqvist et al., 1996). Antisense expression of *Brassica rapa SAD* gene in *Brassica napus* led to dramatically increased stearate levels (up to 40%) in the seeds of transgenic *B. napus* (Knutzon et al., 1992). In the reverse, when the *SAD* gene from *Lupinus luteus* was overexpressed in tobacco, the transgenic tobacco contained very high level of oleic acid (up to 60%) in comparison with control plants (Zaborowska et al., 2002). These literatures imply that *SAD* gene promises to modify the composition of plant fatty acids.

Pongamia pinnata (L.) Pierre, an arboreal legume, is a member of the subfamily Papilionoideae and family Leguminosae, native to tropics and

Abbreviations: ACP, acyl carrier protein; DAF, days after flowering; PpSAD, *Pongamia pinnata* stearoyl-ACP desaturase.

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temperate Asia including part of India, China, Japan, Malaysia, and Australia. The plant has been synonymously known as *Millettia pinnata*, *Pongamia glabra*, and *Derris indica*; commonly it is referred as karanj, pongam, dalkaramch etc. *Pongamia* is drought resistant, semi-deciduous, nitrogen fixing leguminous tree (Nigel and Renee, 2008; Scott et al., 2008). The tree is well suited to intense heat and sunlight and its dense network of lateral roots and thick long tap roots make it drought tolerant (Bobade and Khyade, 2012). Historically, this plant has been used in India and neighboring regions as a source of traditional medicines, animal fodder, green manure, timber, fish poison and fuel. More importantly, *P. pinnata* has recently been recognized as a viable source of oil for the burgeoning biofuel industry (Kesari et al., 2008, 2009; Scott et al., 2008). *Pongamia* has received much attention because of its high content of seed oil (~28–39%, Kesari et al., 2012), which contains high amounts of C18 fatty acids, of which oleic acid and linoleic acid represent about 46 and 27.1%, respectively.

Most research on *P. pinnata* has focused on techniques for extracting oil from seeds (Bobade and Khyade, 2012; Shweta et al., 2004). There were no reports till date on cloning of fatty acid genes in *P. pinnata*. As a first step towards understanding the reaction mechanism and the regulation of expression of fatty acid biosynthetic genes in *Pongamia*, we isolated and characterized a cDNA containing the complete coding region of the fatty acid gene (Pp*SAD*), and analyzed its expression in different tissue types.

2. Materials and methods

2.1. Plant material and growth

Seed sampled at different time intervals was collected from Sila forest range, North Guwahati, Assam, India. Collected samples were rinsed twice with 70% ethanol and immediately stored at −80 °C for further experimental purposes. Seedlings were grown under green house conditions [16 h photoperiod at 28 ± 2 °C with a relative humidity of 75%; light intensity of 125 μmol m^{-2} S^{-1} was provided by fluorescent light (Philips India Ltd., Thane, India)] and the different tissues were collected from grown saplings for expression studies.

2.2. Genomic DNA and RNA isolation

Genomic DNA was extracted from *P. pinnata* leaves according to the protocol described by Kesari et al. (2009). Extracted DNA was purified by ethanol precipitation, and the quality & quantity of the DNA were confirmed both spectrophotometrically and by resolving 0.8% agarose gel in 1× Tris-acetated–EDTA buffer, followed by staining with ethidium bromide (0.5 mg/mL).

Total RNA from different tissue types [root, leaf, stem, flower, cotyledon and different developmental stages of seeds (90-, 180-, 270-, 350-DAF/Day after flowering)] was isolated by the guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987) and recovered by ethanol and sodium acetate precipitation. The RNA quality and relative quantities were estimated by resolving 1 μg RNA on 1.2% agarose/formaldehyde gels with a final concentration of 0.22 M HCHO, followed by staining with ethidium bromide (0.5 mg/mL). Equal intensity of rRNA bands was taken as the criteria for equal quantity and uniform quality of the samples.

2.3. Complementary DNA cloning

Double stranded, blunt-ended cDNA was prepared with a SMART cDNA library construction kit (BD Biosciences Clontech, Palo Alto, CA, USA) using 0.5 μg of poly(A)$^+$ RNA which is separated according to the manufacturer's protocol (Oligotex mRNA mini kit, Qiagen; Cat. No. 70022) from total RNA; isolated from the early immature seeds of *P. pinnata*. The primers for *Pongamia SAD* were designed based on sequence information of the SAD cDNA clone (Accession no. NM_001251324.1 in GenBank) of *Glycine max*. The forward primer SAD1-F (5′-GAAGCCATTCACTCCTCC-3′) was used together with the downstream primer SAD1-R (5′-TCAACTCGACCACTCAAG-3′) in conjunction with the SMART reverse and forward primers to amplify the SAD cDNA from the ds-cDNA population synthesized from the mRNA of the early immature *Pongamia* seeds. The cDNA insert (~1.6 kb) sub-cloned into a pGEM-T Easy vector (Promega, Madison, WI, USA) was sequenced by the dideoxy chain termination method using an automatic sequencer (ABI 777, Macrogen sequencing service, Korea). The nucleotide sequence presented in the current study has been assigned a GenBank accession number of KF192317.

2.4. Sequence analysis

The DNA sequence obtained was compared with sequences deposited in the GenBank database using the Blastn program (Altschul et al., 1997). Sequence data of the corresponding cDNA from different species was analyzed using the ClustalX2.1 package (Thompson et al., 1997). The physicochemical properties of the deduced protein were predicted by Protparam (http://web.expasy.org/protparam/) (Gasteiger et al., 2005). Active sites of the protein sequence were analyzed with the PROSITE database. Protein domains were analyzed by SMART (Schultz et al., 1998). The sub-cellular location of the protein was predicted by the TargetP 1.1 Server (Olof et al., 2000). The secondary and 3D structures of the deduced protein were predicted by PredictProtein (Laszlo et al., 2013) and Swiss-Model respectively. Hydrophobicity analysis was performed on ProtScale (Kyte and Doolittle, 1982), and transmembrane topology prediction was performed using TMHMM Server version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The phylogenetic tree was constructed based on the method suggested by Dereeper et al. (2008).

2.5. Real-time RT-PCR

For real-time RT-PCR, first-strand cDNA was synthesized from 3 μg of total RNA (from various tissues) using Superscript II, according to the manufacturer's protocol (Invitrogen, USA). The RNA quality and quantity were considered to measure the amounts of cDNA and were diluted 1:10 times (~300 ng). The reverse transcribed cDNA samples were used for real time-PCR, which was performed on an ABI Step-One sequence detection system (Applied Biosystems, USA). A PpSAD cDNA fragment (136 bp) was amplified with gene-specific primers SAD2-F and SAD2-R. Initially *GAPDH* gene expression was normalized with various tissues of *Pongamia* to confirm the constitutive expression pattern of the gene. *P. pinnata GAPDH* gene, amplified with the primers *GAPDH*-F and *GAPDH*-R, giving a product of 120 bp, was used as a reference for normalizing the PpSAD cDNA amounts (Table 1). Each PCR was performed in a 25 μL reaction mix containing 1 μL of template cDNA (~300 ng) or the standard, 1× SYBR Premix Ex Taq (TaKaRa, Japan) and 0.3 μM of each primer. Thermal cycling conditions were: 95 °C for 10 s; 40 cycles of 95 °C for 5 s, 60 °C for 31 s; then 95 °C for 15 s, 60 °C for 20 s and 95 °C for 15 s for the dissociation stage. After the real-time PCR, the absence of unwanted by-products was confirmed

Table 1
Primer sequences used in the experiment. Details of the primer sequences used for amplifying the full length PpSAD gene from immature seeds and to verify the differential expression level of *SAD* gene in tissues of *P. pinnata*.

Primer	Sequence (5′–3′)
SAD1-F	GAAGCCATTCACTCCTCC
SAD1-R	TCAACTCGACCACTCAAG
SAD2-F	TGGACAAGGGCATGGACTGC
SAD2-R	TGTTCTTTGGCAAGTCTGGC
GAPDH-F	GCAGGAACCCTGAGGAGATC
GAPDH-R	TTCCCCCTCCAGTCCTTGCT