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CLONING AND EXPRESSION OF *PfSAD* GENE FROM PERILLA FRUTESCENS (L.) BRITT.

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Abstract

A stearoyl-acyl carrier protein desaturase (SAD) gene was cloned from developing seeds of *Perilla frutescens* by RT-PCR, and its bioinformatics analysis and prokaryotic expression were studied. The result indicated that *PfSAD* encoded a polypeptide of 396 amino acids with Mw 45.3 kDa and isoelectric point 6.31. The homological analysis demonstrated that *PfSAD* had high level of homology in amino acid sequence to other plant SAD. The peptide identity of *PfSAD* to SAD of *Salvia miltiorrhiza* was up to 94%. Conservative analysis showed that *PfSAD* contained the classical conserved functional domains of Acyl-ACP desaturase_2 and ferritin-like superfamily. Phylogenetic analysis showed that *PfSAD* had close evolutionary relationship to plants that contained unsaturated fatty acids in seed oil and was most closely related to *S. miltiorrhiza*. Additionally, the *PfSAD* was expressed in *Escherichia coli* BL21 (DE3). The results may provide the basis for engineering the *PfSAD* gene to modify the composition of unsaturated fatty acids in *P. frutescens*.

Introduction

Perilla frutescens (L.) Britt, an annual herb belonging to Lamiaceae, is widely distributed throughout Korea, China and Japan (Kim *et al.* 2016). Its leaves are used as a fresh vegetable and for making pickles, while seeds are widely used as a flavouring agent for traditional diets in Korea. Furthermore *P. frutescens* is a Chinese medicine and a fresh vegetable in the Far East (Nitta *et al*. 2003). Modern research has approved that *P. frutescens* has the function of anti-oxidation (Sikeret *et al.* 2015), aging prevention, blood sugar control, cholesterol control, anti-allergy, anti-microbe, vision and memory improving (Pu *et al.* 2009). The study on *P. frutescens* **is** getting importance in health care, medical research, food and the chemical engineering because the seeds of *P. frutescens* contain more than 90% unsaturated fatty acids (FAs) and about 61% α-linolenic acid (ALA). Its polyunsaturated fatty acids consist of 54~64% ω-3 FAs and 14% ω-6 FAs (Asif 2011). ω-3 FAs include ALA, docosehexaenoie acid (DHA) and eicosapentaenoic acid (EPA), etc. ALA is not only the precursor of DHA and EPA, but also necessary for human health and evolution. It is the basic object in life evolution and has physiological function in improving intelligence and memory, as well as in protecting eyesight (Yuan 2014). So *P. frutescens* has a good application prospect in genetic engineering research. Recently, *P. frutescens* receives much attention for its high content of ALA. The major period for ALA and oleic acid accumulation in *P. frutescens* seed was between 5 and 15 days after flowering, key genes involved in fatty acid and triacylglycerol biosynthesis and metabolism were identified in RNA-seq analysis (Liao *et al*. 2018). Gene coexpression networks were identified from lipid biosynthesis genes of perilla, and two subnetworks that mainly representing the TAG biosynthesis and *de novo* FA synthesis genes were obtained (Zhang *et al*. 2017).

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Change of the ratio of saturated to unsaturated FAs in the membrane lipids has effects on membrane fluidity and function. Saturated FAs are synthesized firstly in plants in two-carbon increments as acyl thioesters of acyl carrier protein (ACP). The stearic acid is a common 18-carbon saturated FA and can directly be changed into unsaturated 18-carbon oleic acid by fatty acid desaturase (FAD) (Fan *et al.* 2007). There are at least 3 FADs such as Δ9-FAD, Δ12-FAD and Δ15-FAD in plants (Fig. 1). Δ9-FAD is encoded by gene *SAD* while Δ12-FAD is regulated by genes FAD2 and FAD6 in *Arabidopsis*. Δ15-FAD is encoded by three genes such as *FAD3*, *FAD7* and *FAD8* in *Arabidopsis*. According to the position of double bond introduced, they are categorized into two groups (i.e., ω-3 including FAD3, FAD7 and FAD8, and ω-6 including FAD2 and FAD6). In *Arabidopsis* and many other plant species, SAD, three ω-3 FAD and two ω-6 FAD have been characterized (Dai *et al.* 2007). FAD3 and FAD7 have also been found in *P. frutescens* (Chung *et al*.1999, Kim *et al.*2008). Recently, a standardized cDNA library was constructed from whole young perilla plants, expressed sequence tags were analyzed, and one new candidate ω-3 fatty acid desaturase gene was found (Lee *et al.* 2014). Additionally, a systematic and comparative study of ω-3 FAD gene family from chia and perilla were reported, the ER-type and FAD7/8 chloroplast-type genes were isolated from these two species (Xue *et al*. 2018).

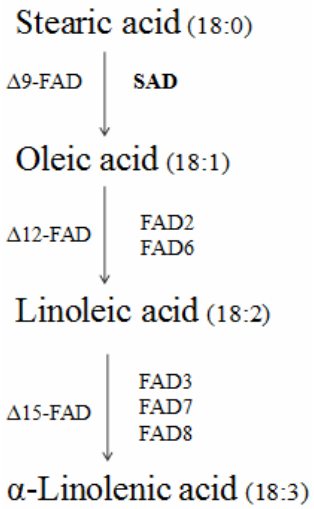

Fig. 1. Biosynthesis of unsaturated fatty acids.

Stearoyl-acyl carrier protein desaturase (SAD, EC1.14.99.6), locating in the plastid stroma, is a pivotal enzyme of FA biosynthesis in higher plants. The insertion of the first double bond is catalyzed by the soluble SAD in stearic acid (18 : 0) turn into the monounsaturated oleic acid (18 : 1) (Lindqvist *et al.* 1996). Since SAD is the sole enzyme in plants, which catalyzes transformation of 18 : 0 to 18 : 1, their activity mainly regulates the homeostasis between saturated FA and monounsaturated FA (Kachroo *et al.* 2007). In plants, many functions are affected by homeostasis, especially to acclimation to low temperature (Kodama *et al.* 1995). Numerous SAD genes have been obtained from different plants, such as *Salvia miltiorrhiza*, *Jatropha curcas* (Luo *et al.* 2006), *Ricinus communis* (Knutzon *et al.*1991), *Cinnamomum longepaniculatum* (Luo *et al.* 2009), safflower (Thompson *et al.* 1991), *Brassica rapa* (Knutzon *et al*. 1992), *B. napus* (Slocombe *et al.* 1992) and *Arachis hypogaea* (Chi *et al.* 2011). Previously many works on the structures and functions of several SAD have been carried out (Thompson *et al.* 1991, Lindqvist *et al.* 1996, Luo

et al. 2006, Luo *et al.* 2009, Chi *et al*. 2011). *SAD* expression of *B. napus* that planted at low temperature was up-regulated, leading to the increase of SAD protein content (Tasseva *et al.* 2004). The over-expression of the *SAD* gene could increase the cold tolerance in plants because of the increased desaturation of FA (De Palma *et al.* 2008). Antisense expression of *B. rapa* SAD gene in *B. napus* resulted remarkably increased stearate levels in seeds of transgenic *B. napus* (Knutzon *et al.* 1992). On the other hand, when the SAD gene from *Lupinus luteus* was over-expressed in tobacco, transgenic tobacco contained very high level of oleic acid (up to 60%) in comparison with control plants (Zaborowska *et al.* 2002). These studies revealed that it is feasible to alter the constituent of plant FA by navigating the SAD gene. But there is no report about the characterization of the SAD gene from *P. frutescens* so far.

In this paper, report on the isolation of SAD from *P. frutescens* based on the homology with formerly characterized SAD genes from *S. miltiorrhiza* by reverse transcription polymerase chain reaction (RT-PCR) is made. Sequence analysis revealed that the segment comprised a full open reading frame (ORF) and had high similarity to other reported SAD in nucleotide sequence besides amino acid sequence. Additionally, the construction of expression vectors and inducible expression of *PfSAD* in *Escherichia coli* BL21 (DE3) was studied. The results will lay the foundation for engineering the *PfSAD* gene to alter the composition of FA in *P. frutescens*.

Materials and Methods

Young seeds of *Perilla frutescens* were collected in the autumn of 2015 from experimental farm of Yunnan Agricultural University and rapidly frozen in liquid nitrogen, then stored in –80ºC refrigerator until use. It was identified by Professor Feng-gen Guo in Yunnan Agricultural University. The voucher specimen (Zhu 20150901) is deposited in the Plant Herbarium of Yunnan Agricultural University in Kunming City, China.

Total RNA was extracted from the developing seeds of *P. frutescens* with the plant tissue RNA extraction kit (TransGen Biotech, Beijing, China) by carefully following the manufacturer's instructions. cDNA synthesis reactions were performed with TransScript One-Step gDNA Removal and cDNA synthesis SuperMix (TransGen Biotech, Beijing, China).

Reverse transcription polymerase chain reaction (RT-PCR) was performed as TransStart® FastPfu Fly DNA polymerase (TransGen Biotech, Beijing, China) with specific primer pair. These primers were designed by Primer 5.0 software (Premier Biosoft Interpairs, Palo Alto, CA) based on conserved sequences of SAD of *S. miltiorrhiza* in GenBank (KF887940) and by using following parameters: Search type was PCR primer and pairs; search ranges was 1~1191; primer length was 20 ± 3 base pair; search mode was automatic. The primer pair with highest score [F: 5'-ATG GCGATGAAGCTGAAT-3'; R: 5'-TCAGA GTTGCACTTCTC-3'] were synthesized by Sangon Biotechnology Co. Ltd., China. The PfSAD gene was PCR-amplified with the conditions as follows: 95°C for 2 min for initial denaturation; 35 cycles of 94°C for 20 s, 55°C for 20 s and 72°C for 30 s; and 72°C for 5 min for the final extension. The PCR products were separated on 1.2% agarose gels, and then the targeted DNA fragments were recovered and cloned into the pLB vector (TIANGEN Biotech, Beijing, China). The ligated products were transformed into *E. coli* (DH5α) cells and the resulting plasmids were obtained as a sequencing template. The sequence data of PfSAD gene was submitted to GenBank and its accession number was KX343938.

The amino acid sequence of PfSAD and other 23 amino acid sequences of SAD downloaded from GenBank were input to the MEGA 4.0 software (Tamura *et al.* 2007). These sequences were aligned first by ClustalW software embedded in MEGA with following parameters: Gap opening penalty=10; gap extension penalties were 0.1 and 0.2 for pair wise alignment and multiple alignment, respectively; Protein weight matrix was gonnet; Residue-specific penalties were ON;

hydrophilic penalties were ON; Gap separation distance=4; Use negative matrix was OFF; End gap separation was OFF; Delay divergent cutoff was 30. The Neighbor Joining phylogenetic tree was constructed based on the poisson correlation of amino acid sequences by MEGA 4.0 with following parameters: gaps/missing data were complete deletion; substitutions to include was All; pattern among lineages was same (Homogeneous); Rates among sites were uniform rates; Phylogeny test and option were bootstrap 1000 replications. The sum of branch length (2.00909992) and 0.05 scale length were shown on the tree and the GenBank accession numbers of 24 species were shown in the parentheses after scientific names. The secondary structure of the deduced protein was predicted by SOPMA (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page = /NPSA/npsa-sopma.html. The tertiary structure of the deduced protein was predicted by SWISS-MODEL http://www.expasy.ch/swissmod/SWISS-MODEL.html and verified by Procheck, Errat and Veryfy_3d softwares (http://services.mbi.ucla.edu/SAVES/).

The PCR products were purified with the TIANgel Midi Purification Kit (TIANGEN Biotech, Beijing, China). According to the instruction of the pEASY-Blunt E1 Expression Kit (TransGen Biotech, Beijing, China), the targeted DNA fragment was ligated with the expression vector and then the ligated product was introduced into Trans-T1 competent cells. The plasmid that harboring the PfSAD gene was named as pEASY-Blunt E1-SAD. The plasmid DNA was isolated from positive transformers that screened through colony PCRs and further verified by DNA sequencing (TSINGKE, Kunming, China). The recombinant plasmids, pEASY-Blunt E1–SAD, were transformed into *E. coli* strain BL21 (DE3). *E. coli* cells harboring pEASY-Blunt E1–SAD were grown at 37°C to an OD_{600} of about 0.7 in LB liquid medium containing 100 mg/ml ampicilli, and then induced for 4 hrs with 0.4 mM IPTG. Cells slang from 1 ml culture, dissolved in 50 µl SDS sample buffer, and heated to 100°C for 3 min. Samples were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which was carried out with a 5% stacking gel and a 12% separating gel and stained by coomassie brilliant blue.

Results and Discussion

A 1191 bp segment of PfSAD was amplified by RT-PCR firstly. Sequence analysis indicated that the cDNA fragment included a complete ORF (GenBank accession KX343938, Fig. 2). Analyzed by DNA tools 5.1, the gene encoded a polypeptide of 396 amino acids with M_w45.3 kDa and isoelectric point 6.31.

A high level of sequence identity was found, when the putative amino acid sequence of PfSAD was compared with amino acid sequences of other SAD, which were available in the NCBI database using the basic local alignment search tool. Among the SADs in GenBank, PfSAD had the highest identity with SAD of *S. miltiorrhiza* (88%), followed by SAD of *Sesamum indicum* (85%), and had more than 70% identity with the great majority of SAD. The polypeptide had two conserved domains, one belonging to acyl-ACP desaturase family with considerable homology in a number of highly conserved blocks (Fig. 3A) and the other belonging to ferritin-like family (Fig. 3B).

The NJ phylogenetic tree of 24 plant SADs (Fig. 4) based on the poisson correlation of amino acid sequences by MEGA 4.0 confirmed that the gene PfSAD cloned by the present belonged to the SAD gene family. PfSAD combined with SAD of *Salvia miltiorrhiza* first study obtained 95% bootstrap support.

Secondary structure prediction by SOPMA revealed that PfSAD protein comprised 45.71% α-helix, 34.09% random coil, 12.88% extended strand and 7.32% β-bridge. The tertiary structure prediction showed that PfSAD protein was a compact globular protein (Fig. 5). The result from 3D model pictures corresponded with the results of secondary structure prediction.

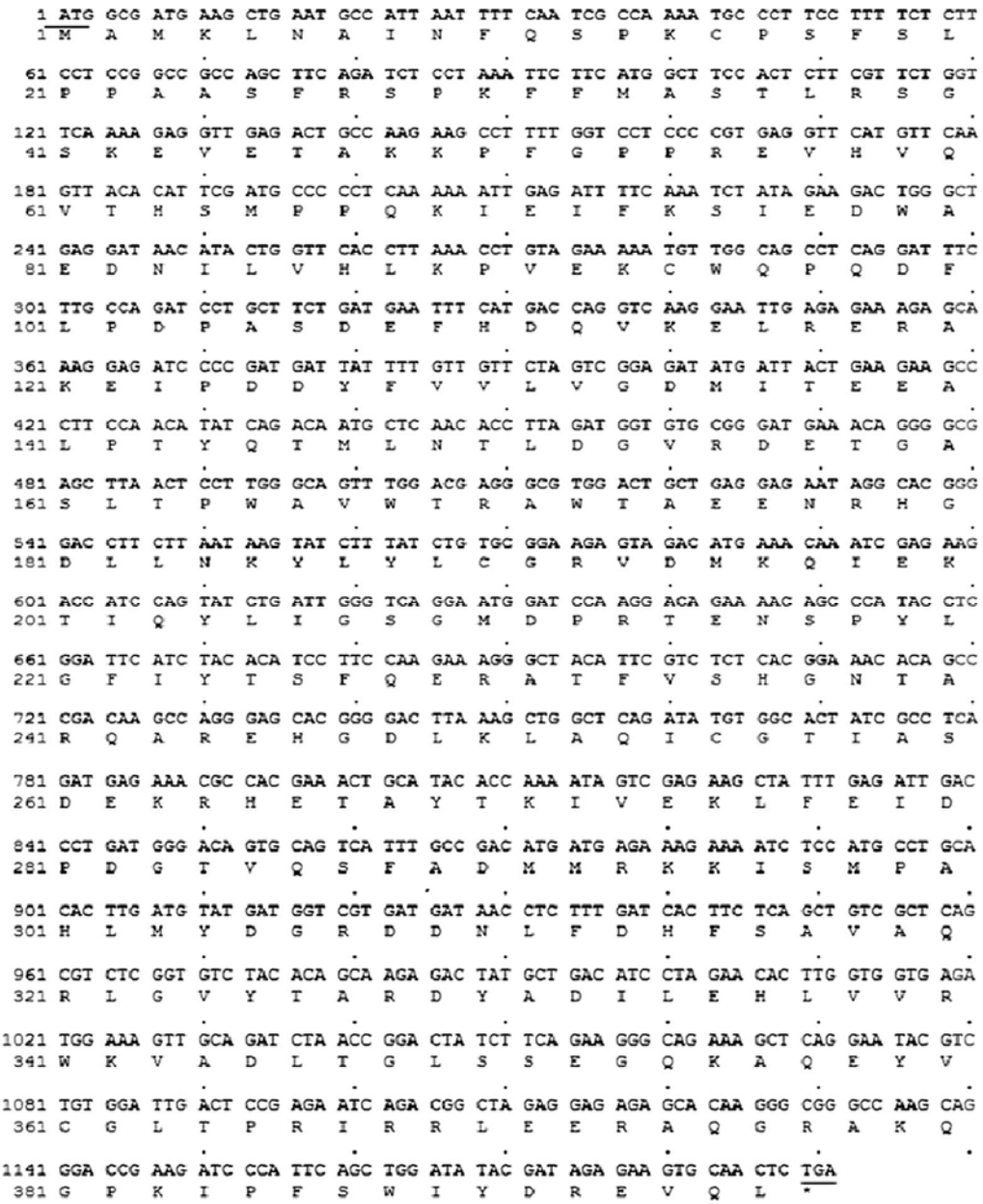

Fig. 2. cDNA sequence and putative amino acid sequence of PfSAD (GenBank accession No. KX343938). The start codon and stop codon are underlined.

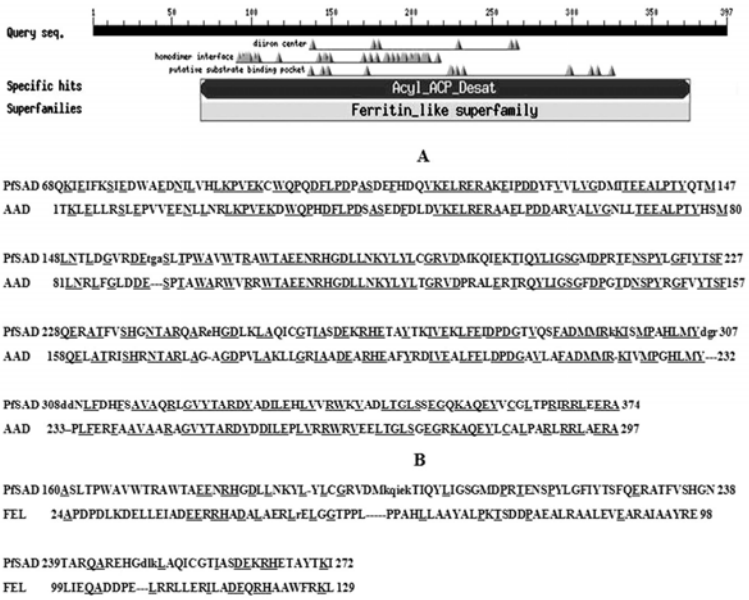

Fig. 3. Comparisons of putative amino acid sequence of PfSAD with NCBI searched domain of acyl-ACP-desaturase family (A) and ferritin-like family (B).

Identical amino acids are underlined. AAD means conservative peptide sequence of acyl-ACP desaturase family (cd01015); FEL stands for conservative peptide sequence of ferritin-like family (cd00657).

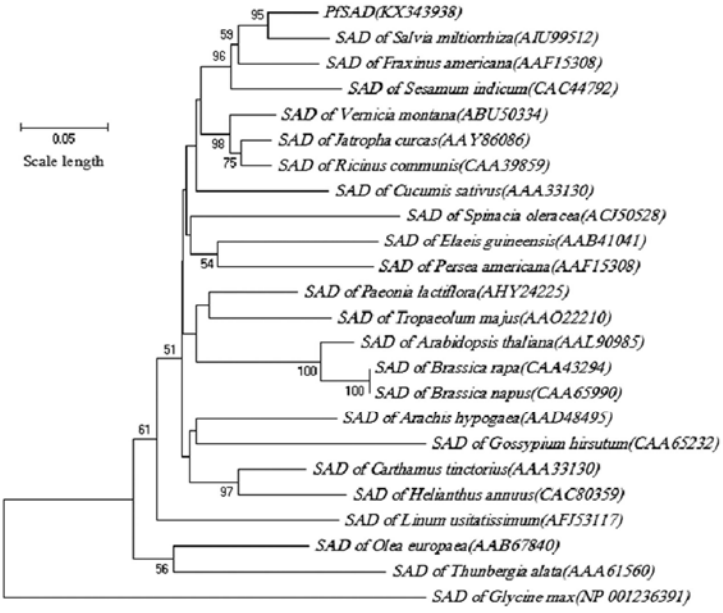

Fig. 4. Neighbor Joining phylogenetic tree of PfSAD and other 23 plant SADs based on the poisson correlation of amino acid sequences by MEGA 4.0. The sum of branch length (2.00909992) and 0.05 scale length were shown on the tree and the GenBank accession numbers of 24 species were shown in the parentheses after scientific names. Bootstrap values of 1000 replicates (≥50%) were displayed near the clades.

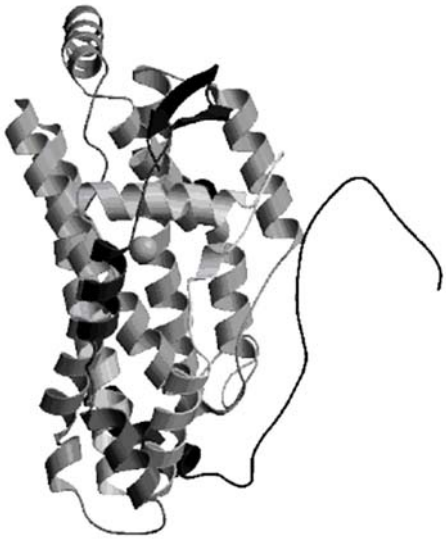

Fig. 5. Tertiary structure prediction of PfSAD protein analyzed by Swiss-Model and verified by Procheck, Errat and Verify_3d softwares.

The gene was transferred to an *E. coli* strain BL21(DE3) vector expression in the pEASY-Blunt E1 Expression System. The extracts of expressing *E. coli* were subjected to SDS-PAGE. The recombinant gave rise to an about 45 kDa protein in response to the IPTG induction (Fig. 6). Stearoyl-ACP desaturase is indicated by the arrow (about 45 kDa).

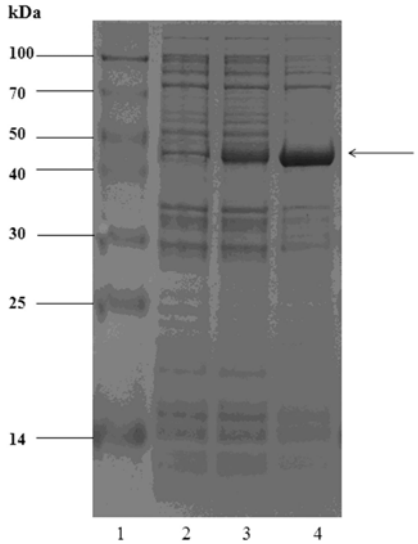

Fig. 6. SDS-PAGE analysis of *P. frutescens* stearoyl-ACP desaturase expressed in *E. coli*.
1 protein marker; 2 lysate from *E. coli* BL21□DE3□harboring pEASY-Blunt E1 with IPTG; 3 lysate from *E. coli* BL21 DE3 harboring pEASY-Blunt E1-SAD without IPTG induction; 4 lysate from *E. coli* BL21 DE3 harboring pEASY-Blunt E1 –SAD with IPTG induction.

P. frutescens receives much attention for its high content of ALA. The biosynthesis of ALA needs three kinds of fatty acid desaturases such as delta-9 FAD (SAD), delta-12 FAD (ω-6 fatty acid desaturases, FAD2 and FAD6) and delta-15 FAD (ω-3 fatty acid desaturases, FAD3, FAD7, FAD8)(Fig. 1). A number of genes encoding ω-3 and ω-6 FAD in *P. frutescens* have been found and submitted to GenBank by gene cloning (Chung *et al*.1999, Xue *et al*. 2017), EST analysis (Lee *et al*. 2014) and transcriptome analysis (Kim *et al*. 2016, Lee *et al*. 2016, Zhang *et al*. 2017, Liao *et al*. 2018, Xue *et al*. 2018) since 1996. Up till now the sequences of 21 genes encoding FAD2 and 26 genes encoding FAD3/7/8 in *P. frutescens* can be downloaded from GenBank but there is no sequence of SAD gene of *P. frutescens* on GenBank. Because SAD catalyzes the first reaction of changing stearic acid (18:0 saturated FA) to oleic acid (18 : 1 unsaturated FA) in the fatty acid biosynthesis pathway in plants and decides the ratio of saturated to unsaturated FA, the research work on SAD gene in *P. frutescens* becomes important and urgent.

In the present experiment a new *SAD* gene was obtained from developing seeds of *P. frutescens* by the technique of RT-PCR using specific primers designed according to *SAD* of *S. miltiorrhiza*. Bioinformatics analysis revealed that both of the nucleotide and deduced peptide sequence of *PfSAD* were highly homological to *SAD* cloned from other plants. Luo *et al.* (2006) indicate that the SAD polypeptide from *J. curcas* has two conserved domains, one belongs to acyl-ACP desaturase family and the other belongs to ferritin-like family. In the present study, these conserved domains were also found in PfSAD polypeptides. The result suggests that PfSAD proteins belong to the acyl-ACP desaturase family and to the ferritin-like family. Additionally, the high amino acid sequence identity of PfSAD to other SAD showed that SAD proteins have been highly conserved during evolution and further proving their key enzymatic roles in FA biosynthesis in plants. Nowadays, the research of SAD has made great progress. For instance, the conformational alterations of SAD diiron centre induced by substrate has been researched with the technique of ENDOR and EPR (Davydov *et al.* 2005). It has been demonstrated that SAD may modulate salicylic acid- and jasmonic acid-mediated defense pathways by regulating the levels of different FA in plastids (Zaborowska *et al.* 2002). The cloning of *PfSAD* gene may enable the research workers to further proof its potential capabilities and modify the ratio of saturated to unsaturated FA in *P. frutescens* by genetic engineering technology.

Acknowledgments

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