Cloning and expression analysis of fatty acid desaturase gene *NtSAD1* in *Nicotiana tabacum*

Yangyang Sheng[1], Lijun Yang[2], Xiaojing Xu[2], Junqiu Sun[1], Haixia Li[1], Weihuan Jin[1*], Hongxiang Guo[1*]

[1] College of Life Sciences, Henan Agricultural University, Zhengzhou 450002, Henan, China
[2] Zhumadian Branch of Henan Province Tobacco Company, Zhumadian 463000, Henan, China
[*]Corresponding author: whjin@henau.edu.cn and guohongxiang06@126.com

Abstract: Tobacco K326 (*Nicotiana tabacum L.*) was selected as experimental material, and a △9 Stearoyl-ACP desaturase (*NtSAD*) gene was cloned from the leaf by RT-PCR, which was named as *NtSAD1*. Homology analysis showed that *NtSAD1* gene shared high homology with other plant *SAD* genes and the higher homology among them include tomato 79.71%, Jatropha curcas 79.32%, potato 79.04% and castor bean 79.00%, respectively. It was found that the expressions of *NtSAD1* in leaves, roots and stems were the highest at blossom stage, separately. And the expression of *NtSAD1* in tobacco leaves was the highest in all organs. These results suggest that it is related to oil of tobacco leaves.

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Keywords: *Nicotiana tabacum*; *NtSAD*; cloning; expression analysis

1. Introduction

Tobacco is the most widely commercial leaf crop in the world. The chemical components of tobacco are the internal factors that determine the quality of tobacco. Oil is an important indicator of tobacco quality. The leaves with a lot of oil are bright, and the tissues are soft and elastic. Under the condition of low water content, the leaves can still maintain the oily state and have greater toughness. The fading of leaves does not easily appear after stored. On the contrary, the leaves with less oil are rough and lacklustre, and the tissues elasticity is poor and fragile.

Fatty acids, including saturated fatty acids and unsaturated fatty acids, are important ingredients of tobacco oil. The plant delta 9 Stearoyl-ACP desaturase (SAD) is one of the known soluble desaturase families in plants. There are two sets of desaturase in the plant, which are located in the plasmid and endoplasmic reticulum, and they catalyze desaturation of stearoyl-ACP through introducing a double bond to between C9 and C10 in the chain of fatty acid to form oleoyl-ACPs (Dong et al, 2012). Therefore, SAD is a key enzyme in the plant fatty acid synthesis pathway, and it directly determines the total amount of unsaturated fatty acids and the ratio of saturated and unsaturated fatty acids in vegetable oils.

SAD genes have been cloned from a variety of plants, including *Carthamus tinctorius L*, *Cucumis sativus L*, *Lycopersicon esculentum*, *Brassica rapa*, *Brassica napus* and so on (Thompson et al,1991; Shanklin et al, 1991; Taylor et al, 1992; Knutzon et al, 1992; Slocombe et al, 1992). It has been confirmed that *SAD* expression in developing seeds is higher than that in other tissues in most of the higher plants (Aardra et al, 2007; Zaborowska et al, 2002; Chen et al, 2010; Shah et al, 2000). SAD is encoded by multiple copies of the gene. Although *Arabidopsis* fab2 mutant led to a *SAD* gene inactivation and increased stearic acid content significantly, it could not terminate the synthesis of unsaturated fatty acids (Lighter et al, 1994). Two *SAD* genes were isolated from immature seeds of *Seaamun indicum L*, and they had different expression patterns (Yukawa, et al, 1996). After *SAD* gene was silenced in cotton by RNA interference, the content of stearic acid increased from 20% to 40% in cotton seed oil, and three main fatty acids including palmitic acid, oleic acid and linoleic acid decreased. By aligning the amino acid sequences of *SAD* genes from different species, SAD has been found to have a highly conserved iron redox protein sequence region. However, the other sequence features of *SAD* genes have not been reported yet.

At present, the metabolism of fatty acids in plants is clear, but the relationship between *SAD* gene and tobacco quality has not been reported. In this experiment, the full-length cDNA of *NtSAD* gene was cloned from leaves of tobacco K326, and its sequence analysis was performed. These results provide a theoretical basis for further studying the relationship between *NtSAD* and quality of tobacco.

2. Material and Methods

2.1 Plant material and treatment

Tobacco variety K326 was used as experimental material to clone *SAD1* gene. Tobacco plants were planted in field. At the glomeration stage, blossom stage and mature stage, roots, stems and leaves were respectively sampled for detecting the expression of *SAD1* gene. After these samples were frozen in liquid nitrogen, and then stored at -80 ℃.

2.2 Gene cloning of *NtSAD1*

The Trizol method was used to extract the RNA from tobacco leaves which was used as template for reverse transcription with PrimeScript™RT reagent Kit including gDNA Eraser (Perfect Real Time) kit.

Basing on the *SAD1* gene of *Nicotiana tabacum* in GenBank, we synthesized primers SAD1-F and SAD1-R（Table 1）. The tobacco *SAD1* gene was amplified with cDNA as a template. The PCR conditions were as follows: denaturation at 95 ℃ for 5 min, then 30 cycles (95 ℃ for 30 s, 49 ℃ for 30 s, 72 ℃ for 75 s) followed by 10 min final extension at 72 ℃。

Table 1 Primers used in PCR amplification

Primer name	Primer sequence
SAD1-F	AAAATGGCGTTGAAGTTAAATGC
SAD1-R	AACCTTTGTTTCTTTCATTTGTTGTGC
QSAD1-F	CACCAATGGTCAGCCTTAGATCTC
QSAD1-R	GGAAATCCTGTGGTTGCCAG
actin-F	GGAAACATCGTCCTTAGTGGTG
actin-R	AATCCAGACACTGTACTTGCGT

2.3 Sequence analysis of *NtSAD1* gene

DNAMAN6.0 was used for alignment of nucleotide sequences and BLASTP (http://blast.ncbi.nlm.nih.gov/) on the NCBI website was used for protein prediction. Analysis of physicochemical properties of proteins was performed with ProtParam online software (http://web.pasyasy.org/protparam/). Specialty BLAST (CDD search) and Protein Smart were used for the analysis of conserved area.

2.4 Expression analysis of *NtSAD1* Gene

Total RNA was extracted from the samples of roots, stems and leaves using Trizol solution and treated with RNase free DNase I to remove potential DNA contamination. First-strand cDNA was synthesized using 1 mg of total RNA according to One Step PrimeScript RT-PCR Kit (Perfect Real Time) protocol. TaKaRa SYBR Premix Ex TaqTM II (Perfect Real Time) was used for qRT-PCR on a Bio-Rad IQ5 Real-Time PCR Detection System. The volume of qRT-PCR reaction was 25 ul, and actin was used as the endogenous reference gene. qRT-PCR was performed as described (Li et al, 2016). Three replicates were performed for each sample. The primer pairs used for qRT-PCR are shown in Table 1.

3. Results

3.1Cloning of *NtSAD1* gene

RNA from tobacco leaves was extracted for reverse transcription reaction, and the cDNA was used as template to amplify *NtSAD1* genes. As shown in Figure 1, DNA fragment of 1182bp was obtained. By gene sequencing and Blast analysis, we found that there is 100% homology between the nucleotide sequence and the *NtSAD1* gene.

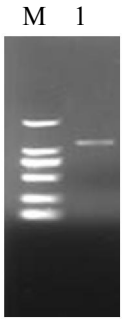

Figure 1. PCR results of *NtSAD1*
M: DL2000, 1: *NtSAD1*

3.2 Homology and molecular phylogenetic tree construction of *NtSAD1* gene

Sequence analysis showed that an open reading frame with a total length of 1182 bp was located 3-191 bp downstream of the 5' end of the amplified 1221 bp cDNA sequence which encoding 394 amino acids. After sequencing, the results show that the fragment is exactly what we need. The 3' untranslated region of 3bp and the 3' non-coding region of 30 bp were flanked on both sides of the coding region (Figure 2).

Using the protein coding region (CDS) of *NtSAD* as reference sequence, homology analysis with Blastn program on NCBI showed that the *NtSAD1* gene shared high homology with other plant *SAD* gene nucleotide sequences. The highest homology among them was tomato 79.71%, Jatropha curcas 79.32%, potato 79.04% and castor bean 79.00%, respectively. The others were as follows: rice 77.21%, flax 78.60%, safflower 78.6%, cucumber 78.48%, groundnut 78.20%, rapeseed 78.04%, sunflower 78.12% and Arabidopsis 76.93% (Figure 3).

3.3 Bioinformatics analysis of NtSAD1 Proteins

The NtSAD1 gene encodes a protein of about 400 amino acids with the molecular mass of about

45.2 kDa and the isoelectric point of 6.29. This protein consists of 18 kinds of amino acids, and Leu is the most abundant amino acid (about 8.9%). There are 55 negatively charged amino acids (Asp+Glu) and 51 positively charged amino acids (Arg+Lys). After searched with NtSAD1 as the query sequence on Protein Smart and NCBI, it was revealed that the plant SAD has a conserved region of 305 amino acids and belongs to the family of acyl-ACP desaturases. The 67-389 amino acid sequence of NtSAD-1 belongs to the conserved region of SAD protein, while there is more than 90% similarity of the amino acids in SAD protein family. SAD protein contains α-helices (accounting for 47.09%), β-sheet (accounting for 14.18%), β-turn (accounting for 9.37%) and free curl (accounting for 29.37%). The 3D structure of NtSAD1 protein was predicted by SWISS MODEL, and it had similar physical and chemical properties with the family of acyl-ACP desaturases (Figure 4).

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1       AAAATGGCGTTGAAGTTAAATGCCTTCAACTTTCAATCTCACAAATACTCTTCTTTTGTT
1          M  A  L  K  L  N  A  F  N  F  Q  S  H  K  Y  S  S  F  V
61      CTTCCACCAATGGTCAGCCTTAGATCTCCAAAGTTTTTCATGGCTGCTAGCCTTCGTTCT
20       L  P  P  M  V  S  L  R  S  P  K  F  F  M  A  A  S  L  R  S
121     GGTACAAAGGAAGTTGAGACGAAGAAGCCCTTTTCTCCTCCTCGTGAGGTTCATGTTCAA
40       G  T  K  E  V  E  T  K  K  P  F  S  P  P  R  E  V  H  V  Q
181     GTAACACACTCTATGCCGCCTCAAAAAATTGAGATTTTTAAATCATTGGAGGATTGGGCG
60       V  T  H  S  M  P  P  Q  K  I  E  I  F  K  S  L  E  D  W  A
241     GATCAAAATTTATTGGTTCACCTGAAATCAGTGGAAAAATGCTGGCAACCACAGGATTTC
80       D  Q  N  L  L  V  H  L  K  S  V  E  K  C  W  Q  P  Q  D  F
301     CTGCCCGATCCTGCTTCAGATGGATTTCATGATCAAGTTAAGGAATTGAGGGAGAGGGCT
100      L  P  D  P  A  S  D  G  F  H  D  Q  V  K  E  L  R  E  R  A
361     AAAGAGATTCCAGATGACTATTTTGTTGTTCTAGTTGGGGATATGATCACAGAAGAAGCC
120      K  E  I  P  D  D  Y  F  V  V  L  V  G  D  M  I  T  E  E  A
421     CTTCCAACATATCAGACTATGCTTAATACATTGGATGGTGTTCGTGATGAAACCGGTGCA
140      L  P  T  Y  Q  T  M  L  N  T  L  D  G  V  R  D  E  T  G  A
481     AGCCCTACTTCTTGGGCCATTTGGACACGGGCCTGGACTGCCGAAGAGAACCGGCATGGT
160      S  P  T  S  W  A  I  W  T  R  A  W  T  A  E  E  N  R  H  G
541     GACCTCCTCAACAAGTATCTTTATCTTTCAGGGAGAGTGGACATGAGAGCAATTGAGAAG
180      D  L  L  N  K  Y  L  Y  L  S  G  R  V  D  M  R  A  I  E  K
601     ACAATCCAATACTTAATTGGATCCGGAATGGATCCTAGGACAGAAAACAATCCATACCTA
200      T  I  Q  Y  L  I  G  S  G  M  D  P  R  T  E  N  N  P  Y  L
661     GGATTTATTTACACATCATTCCAAGAGAGGGCAACTTTTGTCTCCCATGGGAATACAGCT
220      G  F  I  Y  T  S  F  Q  E  R  A  T  F  V  S  H  G  N  T  A
721     AGACTTGCTAAGGAGCATGGAGACATCAAGCTGGCTCAAATATGCGGCATAATTGCTGCA
240      R  L  A  K  E  H  G  D  I  K  L  A  Q  I  C  G  I  I  A  A
781     GACGAGAAGCGCCATGAGACAGCCTACACCAAGATTGTTGAAAAGCTTTTCGAGATCGAC
260      D  E  K  R  H  E  T  A  Y  T  K  I  V  E  K  L  F  E  I  D
841     CCTGATGGCACTGTGCTAGCATTTGCTGATATGATGAGGAAAAAGATATCTATGCCAGCA
280      P  D  G  T  V  L  A  F  A  D  M  M  R  K  K  I  S  M  P  A
901     CACTTGATGTACGATGGGCGCGACGACAACCTCTTTGATCACTTCTCAGCAGTTGCTCAG
300      H  L  M  Y  D  G  R  D  D  N  L  F  D  H  F  S  A  V  A  Q
961     CGTCTCGGTGTTTACACTGCTAAAGACTACGCAGATATATTGGAGTTTCTTGTAGGTAGG
320      R  L  G  V  Y  T  A  K  D  Y  A  D  I  L  E  F  L  V  G  R
1021    TGGAAAGTGGCTGATCTTACTGGATTAACTGGAGAAGGAAGAAAAGCTCAGGAATACGTA
340      W  K  V  A  D  L  T  G  L  T  G  E  G  R  K  A  Q  E  Y  V
1081    TGCGGGTTGCCACCAAGAATCCGAAGATTGGAAGAAAGAGCTCAGGGGAGGGCAAAACAA
360      C  G  L  P  P  R  I  R  R  L  E  E  R  A  Q  G  R  A  K  Q
1141    GGACCAATCATGCCATTTAGCTGGATATATGATAGAGAGGTGAAGCTCTGAGTTGCACAA
380      G  P  I  M  P  F  S  W  I  Y  D  R  E  V  K  L  *
1201    CAAATGAAAGAAACAAAGGTT
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Figure 2. Full-length cDNA sequence and deduced amino acid sequence of NtSAD1

3.4 Expression analysis of *NtSAD1* Gene

Like other *SAD* genes, the expression of *NtSAD1* has tissue specificity. As shown in Figure 5, its expression was the highest in leaves, and the expression was the lowest in roots at the diverse growth stages. The expression of *NtSAD1* in leaves was 10 times more than in roots. In all detected tissues, the expressions of *NtSAD1* were the highest at the blossom stage. Its expression in leaves was the lowest at the mature stage, and the expression in stems and roots were the lowest at the glomeration stage.

4. Discussion

The plant lipoyl-ACP desaturase is localized in plant plastids, and it is the only known soluble.

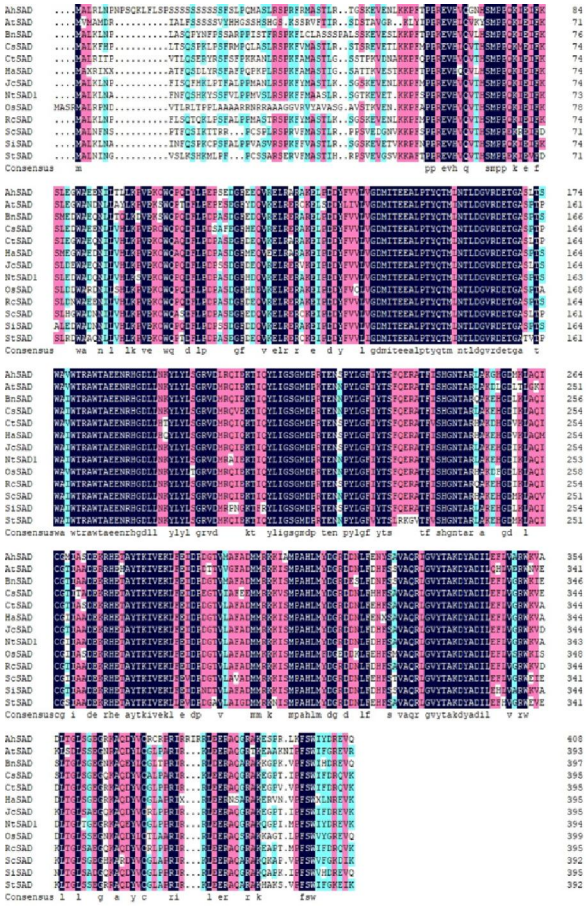
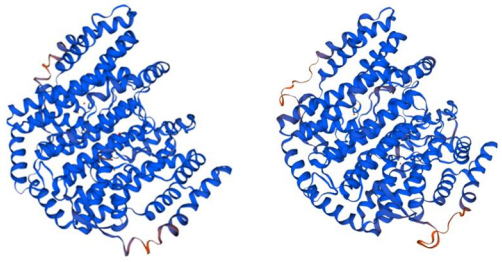


Figure 3. Alignment of amino acid of SAD from *Nicotiana tabacum L* and other plants

AtSAD *(Arabidopsis thaliana)*: AAL90985.1, AhSAD (*Arachis hypogaea)*: AAD48495.1, BnSAD (*Brassica napus)*: CAA65990.1, CtSAD *(Carthamus tinctorius)*: AAA33021.1, CsSAD *(Cucumis sativus)*: AAA33130.1, HaSAD (*Helianthus annuus)*: CAC80359.1, JcSAD *(Jatropha curcas)*: AAY86086.1, NtSAD1 （*Nicotiana tabacum*）: XP_016453362.1, OsSAD (*Oryza sativa*): XP_015622023.1, RcSAD *(Ricinus communis)*: CAA39859.1, SiSAD *(Sesamum indicum)*: CAC44792.1, ScSAD *(Solanum commersonii)*: CAA55535.2, StSAD (*Solanum tuberosum)*: AAA33839.1. Black background indicates 100% sequence homology, pink background indicates amino acid with ≥75%，and sky blue indicates amino acid with ≥50%. desaturase enzyme of the families in

plants, including the Δ9 stearoyl ACP desaturase, the Δ4 palmitoyl ACP desaturase, the Δ6 palmitoyl ACP desaturase and Δ9 myristoyl ACP desaturase and so on. At present, stearoyl-ACP desaturase (SAD) is most widely researched. Here, NtSAD gene was cloned from tobacco leaves. According to the homology analysis of amino acid sequence, we found that NtSAD was highly conserved. The highest homology among them was tomato 79.71%, Jatropha curcas 79.32%, potato 79.04% and castor bean 79.00%, respectively. Plant SAD has a conserved region of 305 amino acid and belongs to the family of acyl-ACP desaturases. At the same time, a ferritin-like conserved region was also found. Therefore, it was suggested that the *NtSAD* gene encodes a stearoyl-ACP desaturase which locates in the chloroplast and catalyzes the dehydrogenation of stearoyl-ACP (18: 0) to produce oleoyl-ACP (16:0).



A B

Figure 4. The 3D structure of NtSAD1 protein and acyl-ACP desaturases.

(A) NtSAD1,（B）acyl-ACP desaturases

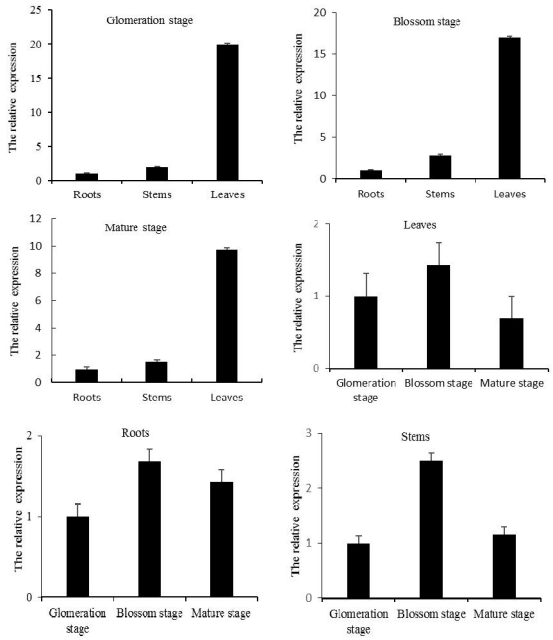


Figure 5. Expression analysis of *NtSAD1* Gene

In 1991, *CmSAD* gene was cloned from safflower for the first time (Thompson et al, 1991). At present, more than 70 *SAD* gene sequences were registered in GenBank, and the structure and function of *SAD* gene have been studied (Zhang et al, 2017). *AtSAD6* gene was found to be related to the synthesis of fatty acid (Klinkenberg et al, 2014). Under drought stress conditions, *AtSAD6* can increase the content of unsaturated fatty acid and reduce the damage degree of *Arabidopsis*. The expression of *SAD* has time and tissue specificity, and it can be highly expressed in plant developing tissues, such as young leaves and the fruit (Slocombe et al, 1994). Here, it was found that *NtSAD1* was highly expressed in tobacco leaves, suggesting that it is related to oil of tobacco leaves.

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Corresponding Author:
Dr. Weihuan Jin and Hongxiang Guo
College of Life Sciences,
Henan Agricultural University
Zhengzhou, Henan, 450002, China
E-mail:whjin@henau.edu.cn,
guohongxiang06@126.com

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