The Soybean S-Adenosylmethionine Synthetase Gene *GmSAMS1* Confers Resistance to Common Cutworm in Transgenic Tobacco

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Abstract: S-Adenosylmethionine synthetase (SAMS) genes play a role in a variety of plant biological processes involved in plant development and the response to stresses. Here, to evaluate the biological functions of the soybean SAMS genes in plant defense against insects, soybean *GmSAMS1* was cloned from leaves. In the 12 soybean SAMS genes, *GmSAMS1* showed the highest expression level in the leaves. The putative GmSAMS1 protein was highly homologous to other plant SAMS proteins, contained three special conserved SAMS protein domains, and was located in the cytoplasm. Eight motifs in the promoter of *GmSAMS1* were related to abiotic or biotic stress responses. The relative growth rate (RGR) of common cutworm larvae was used to assess the resistance of *GmSAMS1* transgenic tobacco. The RGRs of larvae feeding on both T_0 and T_1 transgenic leaves were significantly lower than that of larvae feeding on control leaves, based on two independent force-feeding experiments. The data indicate that the soybean *GmSAMS1* gene enhances transgenic tobacco resistance to common cutworm.

Keywords: Soybean; S-adenosylmethionine synthetase; Resistance to insects

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大豆 *GmSAMS1* 基因增加烟草对斜纹夜蛾的抗性

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摘 要:腺苷甲硫氨酸合酶基因(S-Adenosylmethionine synthetase,SAMS)在包括发育、胁迫反应等多种植物生物学过程中发挥作用。为评价大豆SAMS基因在植物抗虫中的功能,本研究从大豆叶片中克隆了*GmSAMS1*基因,该基因是12个大豆SAMS基因中在叶片里表达量最高的基因。GmSAMS1蛋白与其它植物SAMS蛋白高度同源,具有3个保守的SAMS结构域,并且定位在细胞膜上。在*GmSAMS1*基因的启动子区包含8个与生物胁迫和非生物胁迫相关的调控元件。以斜纹夜蛾幼虫的相对生长率为指标评价转*GmSAMS1*基因烟草的抗虫性,两个独立的强迫喂养试验结果表明,食用T_0和T_1转基因烟草叶片的斜纹夜蛾幼虫相对生长率显著低于食用对照叶片的斜纹夜蛾幼虫。该结果说明大豆*GmSAMS1*基因提高了转基因烟草对斜纹夜蛾的抗虫性。

关键词:大豆;腺苷甲硫氨酸合酶基因;抗虫性

S-Adenosylmethionine synthetase (SAMS) is a key enzyme that catalyzes the formation of S-adenosylmethionine (SAM) from methionine and ATP[1]. SAM is also known as methionine adenosyltransferase (MAT) and is an important methyl donor used for transmethylation reactions[2]. SAM also serves as the precursor for polyamines and ethylene[3]. Polyamines perform important functions in plant defense against diverse environmental stresses. Ethylene is a plant hormone that plays an important role in inducible defense.

SAMS genes have been isolated and characterized from many plants. SAMS genes are involved in plant development, including root development[4], fruit setting[5], and pollen tube growth[6]. Moreover, SAMS genes are specifically regulated by a variety of environmental factors, including alkali stress[7], cold stress[8], salt stress[9], fungal elicitors[10], flooding stresses and drought stresses[11].

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Soybean (*Glycine max* (L.) Merr) is an important plant nutritional source, providing protein and oil for humans and animals. However, plant damage caused by herbivorous insects reduces the yield and quality of soybean seeds. Some major pests, such as the common cutworm (CCW, *Spodoptera litura* Fabricius), cause soybeans to be unharvestable if they are not controlled[12]. To improve soybean resistance to insects, it is necessary to evaluate and utilize resistance genes, especially endogenous plant genes, which is an eco-friendly strategy for soybean pest management.

In a previous study, we found a soybean SAMS protein and its mRNA were both up-regulated after an attack by common cutworm[13]. Here, we isolated and characterized the soybean SAMS gene, *GmSAMS1*. We analyzed the putative amino acid sequence and the upstream sequence of the *GmSAMS1* gene, overexpressed *GmSAMS1* in transgenic tobacco, and evaluated the insect resistance of the transgenic tobacco. This work will further explore the biological functions of *SAMS* and provide a theoretical basis for the utilization of this gene defensing against insects of soybean and other plant.

1 Materials and Methods

1.1 Plant materials and full-length sequence cloning of soybean SAMS gene

Total genomic DNA was extracted from bulk leaves of 6-8 soybean seedlings (Nannong 99-10) using the DNAquick Plant System (Tiangen, Beijing, China) per the manufacturer's instructions.

The genomic sequence of *GmSAMS1* (*Glyma.15G190500*) is approximately 3,737 bp long and was used as a template to design 3 pairs of specific PCR primers (Table 1). A 50 μL reaction volume of Ex *Taq* polymerase was used according to the manufacturer's specifications (TaKaRa, Dalian, China). The PCR program was as follows: 95℃ for 5 min, 1 cycle, 94℃ for 30 s, 58℃ for 40 s, 72℃ for 90 s, 30 cycles, 72℃ for 10 min, 1 cycle. PCR amplification was performed using a PTC-225 thermal cycler (BIO-RAD, USA). The PCR products were detected with 1% agarose gel electrophoresis, and the products showed clear DNA bands were sequenced by invitrogen (Shanghai, China).

Table 1 Primers used in the isolation of *GmSAMS1*

	Sequence(5′-3′)		Start	Stop	Tm /℃	Product length/bp
The first pair	Forward primer	ACAAAGTCAAATAGCACGAATCACA	1	25	59	2,033
	Reverse primer	AAGTTCAAGTTCAAAGTGGTGAAGG	2,008	2,033	60	
The second pair	Forward primer	AGAAGCCTGATCTACAAATTTTGGC	1,647	1,672	60	1,586
	Reverse primer	CTCTTGAGATCAAGGTTGATGGAGA	3,208	3,233	60	
The third pair	Forward primer	TGTTGAGTACTACAATGACAAGGGT	2,684	2,709	59	1,503
	Reverse primer	GTGTGCATTTTATTGTTTTGCAACG	3,712	3,737	59	

1.2 RNA-seq atlas analysis

To acquire tissue-specific transcript data, a list of 10 *GmSAMS* gene names was entered into the RNA-Seq Atlas of *Glycine max* (http://soybase.org/soyseq/). The gene expression results normalized using the Reads/Kb/Million (RPKM) method were downloaded from this database[14].

1.3 Sequence identification and phylogenetic analysis

The conserved domains of the putative GmSAMS1 protein were predicted by searching the Conserved Domain Database (CDD) of the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/cdd) with default values. Model building and analysis were performed using the SWISS-MODEL server (http://swissmodel.expasy.org/). The crystal structure of *Homo sapiens* SAMS protein (PDB accession number: 2p02.1) was used to build a three-dimensional model of GmSAMS1. The quality of the fit was calculated with SwisspdbViewer 4.0.4.

The promoter position of the *GmSAMS1* gene was predicted by SoftBerry-TSSP (http://linux1.softberry.com/berry.phtml). The cis-regulating elements of the *GmSAMS1* promoter were predicted by PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Thirteen published SAMS protein sequences were downloaded from NCBI. Twelve soybean SAMS protein sequences were downloaded from the soybean database in Phytozome (http://www.Phytozome.net, Wm82.

a2. v1). Multiple alignment of SAMSs were performed with full-length protein sequences, using the ClustalW tool as implemented in MEGA V 6.0 (http://www.megasoftware.net/mega.php). A phylogenetic tree was constructed with MEGA V 6.0 using the neighbor-joining method with the following parameters: Poisson model, pairwise deletion, and bootstrap (1 000 replications, random seed).

1.4 Subcellular localization of the GmSAMS1-GFP fusion protein

The *GmSAMS1* open reading frame (ORF) without the stop codon was inserted in the *attB* sites of the pMDC83 vector using Gateway™ technology according to the Gateway™ manual (Invitrogen, USA), resulting in a translational green fluorescent protein (GFP) fusion at the C-terminus of GmSAMS1 (35S: GmSAMS1-GFP). The recombinant vector pMDC83-GmSAMS1 with the GFP gene and a hygromycin resistance gene was transferred into onion epidermal cells using gene gun. The control cells harbored the empty pMDC83 vector (35S:GFP) with the GFP gene and a hygromycin resistance gene. The location where the gene was expressed was identified by monitoring the fluorescence GFP signals using a confocal laser microscope (Leica CP SP2, Germany).

1.5 Insect bioassay of transformed tobacco

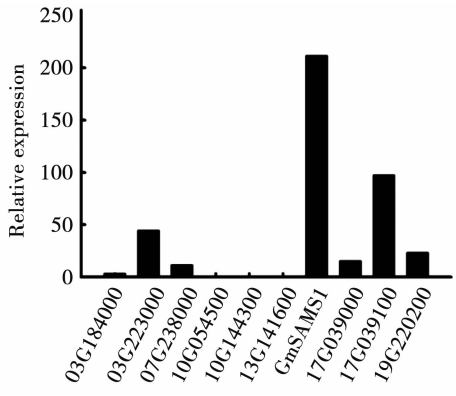
The full-length cDNA of the soybean gene *GmSAMS1* was inserted in the *attB* sites of the pMDC83 vector using Gateway™ technology (Invitrogen, USA). Recombinant vectors under the control of the cauliflower mosaic virus (CaMV) 35S promoter were introduced into *Agrobacterium tumefaciens* strain EHA105 by the freeze-thaw method and transformed into tobacco (*Nicotiana tabacum* cv. SamSun) using the leaf disc transformation method. T_0 and T_1 transformed plants were identified by PCR and RT-PCR.

A force-feeding trial, as previously described by Fan et al.[13], was used to evaluate resistance of the transgenic tobacco. Two independent experiments were conducted under a complete randomized design. One experiment included T_0 and control plants, and the other involved T_1 and control plants. Non-transformed tobacco plants were used as controls. All plants were grown in a greenhouse at 25℃ under a 16 h light/8 h dark photoperiod. Five third-instar CCW larvae in starvation were randomly placed in a culture dish containing one leaf from a transgenic plant or a control plant. Relative growth rate (RGR) of a single larva was used as an index parameter for the insect bioassay. The average weights of five larvae before placement in the dish and after 1, 3 or 4 days were measured and recorded.

2 Results

2.1 Isolation and sequence analysis of the soybean

In our previous study, the soybean SAMS protein showed abundant expression in soybean plants at 6, 12 and 24 h after CCW attack compared with the control[12]. The protein exhibited the greatest similarity to SAMS (AAG17666) from *Brassica juncea* according to mass spectrometry analysis[12]. Using the protein sequence of AAG17666 as a key, 12 putative SAMS proteins were identified in the soybean genome database in Phytozome (Wm82. a2. v1). Among these proteins, the Glyma. 15G190500 protein showed the greatest similarity to AAG17666 and its encoding gene displayed the highest expression level in soybean leaves (Fig. 1). After CCW attack, the mRNA expression of *Glyma. 15G190500* was significantly up-regulated in soybean leaves at 6, 12 and 24 h[12]. Therefore, we isolated the *Glyma. 15G190500* gene from soybean leaf genomic DNA and designated it as *GmSAMS1*. The full length of *GmSAMS1* is 3,737 bp, including a 1,188 bp coding region, a 2,160-bp upstream region, and a 389 bp downstream region. The gene encodes a protein of 395 amino acids.



The raw data were normalized and retrieved from the online database http://soybase.org/soyseq/. The unnamed members were identified by their locus IDs in Phytozome (Wm82. a2. v1). *Glyma. 02G008700* and *Glyma. 03G223000. 2* which lacked expression data in the RNA-Seq Atlas of *Glycine max* in SoyBase are not shown.

Fig. 1 Gene expression profiles of *GmSAMSs* in young leaves

The predicted amino acid sequence, as deduced from the *GmSAMS1* gene, including three conserved S-adenosylmethionine synthetase domains: S-AdoMet _ synt _ N (pfam00438), S-AdoMet _ synt _ M (pfam02772) and S-AdoMet _ synt _ C super family (pfam02773). The S-AdoMet_synt_N is an N terminal domain consisting of 98 amino acids, from the 5th to the 102nd amino acid, the S-AdoMet_synt_M is a central domain consisting of 122 amino acids, from the 119th to the 240th amino acid, and the S-AdoMet_synt _C super family domain is located in the C-terminus and contains 142 amino acids, from the 242nd to the 383rd amino acid. Furthermore, the three-dimensional structure of the GmSAMS1 protein was constructed using the SWISS-MODEL with the human SAMS protein structure (PDB: 2p02.1, Chain A) as a template. The GmSAMS1 protein shares 67% similarity with the human SAMS protein (Fig. 2). Three domains shaped a pseudo 3-fold symmetry of the GmSAMS1 protein. Each one consisted of several α-helices and β-strands. These results confirmed that the GmSAMS1 protein is a typical soybean S-adenosylmethionine synthetase.

The promoter and cis-acting regulatory elements of the *GmSAMS1* gene were predicted with SoftBerry-TSSP and PlantCARE. A proximal promoter was located 836 bp away from the start codon and there were 22 cis-acting regulatory elements in the promoter (Table 2). Eight of these elements were stress-responsive elements or were related to plant defense against disease or insects, including a GT1-motif, GC-motif, ABRE, AT-rich sequence, TCA-element, TC-rich repeats and HSE.

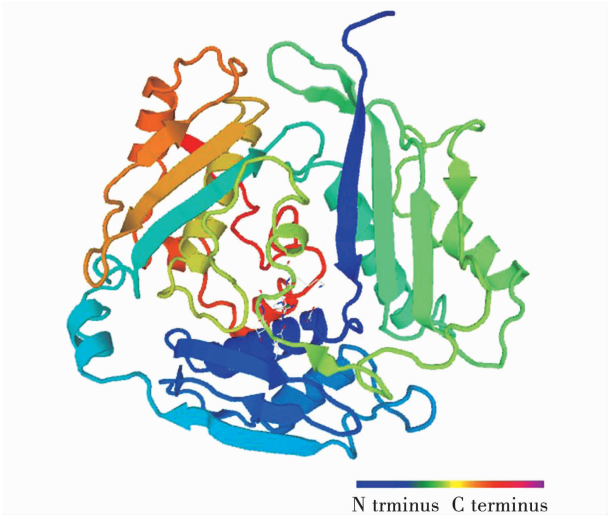


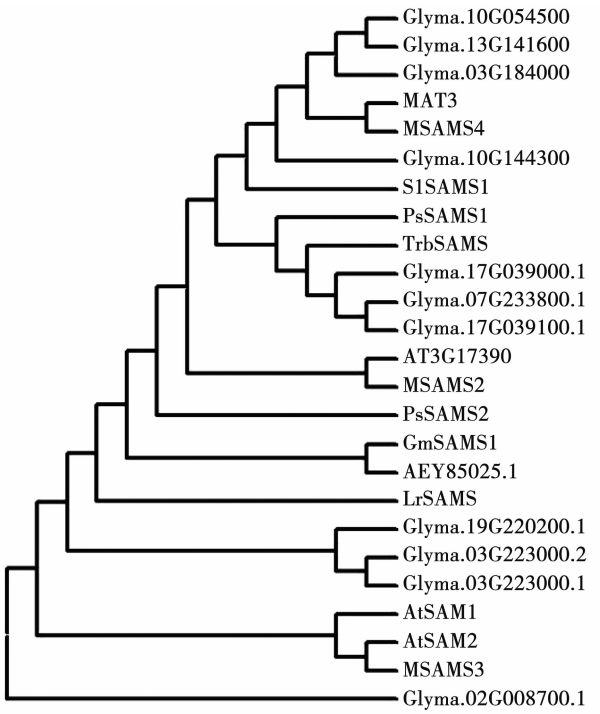
Fig. 2 Predicted tertiary structure of the GmSAMS1 protein established by SWISS-MODE

Table 2 Cis-acting regulatory elements in the promoter of the *GmSAMS1* gene

Number	Motif name	Location	Motif	Function
1	GT1-motif	-27	AATCCACA	SA induction and disease resistance response
2	TATA-box	-31	TATAAAT	Core promoter element around -30 of transcription start
3	GC-motif	-52	CCCCCG	A general stress-responsive element
4	Sp1	-59/-63	CC(G/A)CCC	Stimulates transcription at TATA-containing promoters
5	A-box	-154	CCGTCC	Cis-acting regulatory element
6	CCGTCC-box	-154	CCGTCC	Cis-acting regulatory element related to meristem specific activation
7	CAAT-box	-163	CAAT	Common cis-acting element in promoter and enhancer regions
8	I-box	-170	cCATATCCAAT	Part of a light responsive element
9	ABRE	-193	GCCGCGTGGC	Cis-acting element involved in the abscisic acid responsiveness
10	G-box	-250	CACGAC	A ubiquitous, cis-acting DNA regulatory element
11	Skn-1_motif	-381	GTCAT	Cis-acting regulatory element required for endosperm expression
12	AT-rich sequence	-477	TAAAATACT	Element for maximal elicitor-mediated activation
13	TCA-element	-559	GAGAAGAATA	Cis-acting element involved in salicylic acid responsiveness
14	G-box	-578	TAACACGTAG	Cis-acting regulatory element involved in light responsiveness
15	CCAAT-box	-719	CAACGG	Mybhv1 binding site
16	TC-rich repeats	-770/1,143	ATTTTCTCCA	Cis-acting element involved in defense and stress responsiveness
17	GATA-motif	-809	GATAGGA	Part of a light responsive element
18	ACGT-containing element	-898	AAAACGTTTA	Cis-acting element involved in light responsiveness
19	Box 4	-1,027	ATTAAT	Part of a conserved DNA module involved in light responsiveness
20	HSE	-1,060	AAAAAATTTC	Cis-acting element involved in heat stress responsiveness
21	Box 4	-1,134	ATTAAT	Part of a conserved DNA module involved in light responsiveness
22	HD-Zip 2	-1,217	ATTG	Element involved in the control of leaf morphology development

2.2 Phylogenetic analysis of the putative soybean SAMS protein

The full-length amino acid alignment of the 25 SAMS proteins, including 12 soybean SAMS proteins and 13 other plant SAMS proteins, was used to construct a phylogenetic tree (Fig. 3). Among 12 soybean SAMS proteins, the predicted amino acid sequence of the *GmSAMS1* gene presented the lowest homology to *Glyma.02G008700.1* (80%) and the highly homology to *Glyma.19G220200.1* (97%). Compared with other plant SAMS proteins, as shown in Fig. 3, the GmSAMS1 protein exbitied the highest homology to AEY85025.1 (99%), which is a protein that is down-regulated in pigeon pea under drought[15]. Therefore, *GmSAMS1* might function in the soybean response to stresses similar to its counterpart.

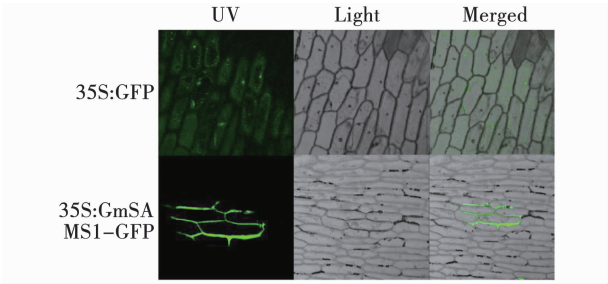


The phylogenetic tree was based on 25 plant SAMS protein sequences and was generated using MEGA6 software (http://www.megasoftware.net/mega.php) with the neighbor-joining algorithm. TrbSAMS (KJ940976) is from *Robinia pseudoacacia*. PsSAMS1 (CAA57580) and PsSAMS2 (CAA57581) are from *Pisum sativum*. MAT3 (AT2G36880), AtSAM1 (AT1G02500), AtSAM2 (AT4G01850) and AT3G17390 are from *Arabidopsis thaliana*. SlSAMS1 (Z24741.1) is from *Solanum lycopersicum*. LrSAMS (JQ219109.1) is from *Lycoris radiata*. MSAMS2 (AAG17666.1), MSAMS3 (AAK71233.1) and MSAMS4 (AAK71234.1) are from *Brassica juncea*. AEY85025.1 is from *Cajanus cajan*. The unnamed members were identified by their locus IDs in Phytozome: *Arabidopsis thaliana* (AT) and *G. max* (Glyma)

Fig. 3 Phylogenetic tree of SAMS proteins

2.3 Subcellular localization of the GmSAMS1 protein

The locations of *GmSAMS1* expression were identified using onion epidermal cells as a transient expression system. The coding region of *GmSAMS1* fused with *GFP* was controlled by the CaMV 35S promoter. As shown in Fig. 4, the GmSAMS1-GFP fusion protein was localized to the cytomembrane of onion epidermal cells, whereas the control (35S: GFP) was distributed throughout the entire cell.



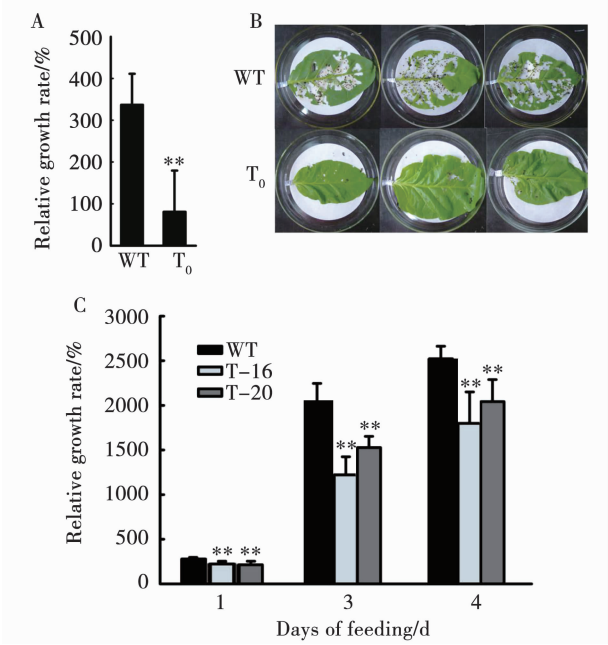
GFP: green fluorescent protein

Fig. 4 Subcellular localization of GmSAMS1 protein

2.4 *GmSAMS1* transgenic tobacco plants acquired resistance to CCW

To determine the role of *GmSAMS1* in plant resistance to CCW, the gene was overexpressed and evaluated in transgenic tobacco. We obtained 12 independent T_0 transgenic plants (35S: GmSAMS1), which were all used in a force-feeding trial to evaluate antibiosis in the transgenic tobacco. Seven non-transgenic plants (wild type) were used as controls. The average RGR value of the CCW larva feeding on the T_0 transgenic tobacco leaves was 81 ± 98% after one day, whereas the average RGR-value of the CCW larva feeding on the control leaves was 337 ± 74 %. The weight gained of the larvae feeding on the transgenic leaves was significantly lower than that of the larvae feeding on the control leaves after one day of feeding (one-tailed t-test, $P = 1.27 \times 10^{-6}$) (Fig. 5A). The transgenic leaves also showed less loss of leaf area than the control leaves (Fig. 5B).

To confirm the results from T_0, we randomly selected two T_1 transgenic tobacco lines, T-16 and T-20 (6 plants per line), from two independent primary transformants and evaluated their antibiosis in a force-feeding trial. Five non-transgenic plants were used as controls. The weights of larvae feeding on transgenic and non-transgenic leaves were recorded before placement in the dish and after 1, 3 and 4 days. After feeding for



A: Relative growth rate(RGR) of the CCW larvae after one day of feeding on control and T_0 transgenic plants; B: The leaves of control and transgenic plants(T_0) after one day of feeding by CCW larvae; C: RGR of the CCW larvae feeding on control and T_1 transgenic lines; WT: Wild-type tobacco used as controls; T_0: T_0 *GmSAMS1* transgenic tobacco plants; **: Significant difference at 0.01; T-16 and T-20: T_1 *GmSAMS1* transgenic tobacco lines.

Fig. 5 Force-feeding trial to evaluate the antibiosis of the transgenic tobacco

one day, the weights of the CCW larvae feeding on the transgenic leaves had increased 224 ± 31% on T-16 and 214 ± 40% on T-20, which was significantly less than the increase observed in larvae feeding on the control leaves (280 ± 17%) (one-tailed t-test, $P = 2.50 \times 10^{-3}$ for T-16 and $P = 3.67 \times 10^{-3}$ for T-20) (Fig. 5C). After feeding for 3 days, the weight of the CCW larvae feeding on the transgenic leaves had increased 1,222 ± 201% on T-16 and 1,526 ± 127% on T-20. Clearly, the weight of CCW larvae feeding on the control leaves (2,036 ± 209%) was significantly higher than that of larvae feeding on the leaves of the two transgenic lines (one-tailed t-test, $P = 5.20 \times 10^{-5}$ for T-16 and $P = 3.69 \times 10^{-4}$ for T-20) (Fig. 5C). After feeding for 4 days, the weight of the CCW larvae feeding on T-16 transgenic leaves had increased 1,798 ± 351% and that of larvae on T-20 transgenic leaves had increased 2,042 ± 246%. The RGRs of the CCW larvae feeding on the control leaves (2,522 ± 140%) were clearly significantly higher than those of the larvae feeding on T-16 and T-20 transgenic leaves (one-tailed t-test, $P = 9.81 \times 10^{-4}$ for T-16 and $P = 1.69 \times 10^{-3}$ for T-20) (Fig. 5C). These results suggest that *GmSAMS1* expression in the transgenic tobacco increases plant resistance to CCW.

3 Conclusion and Discussion

SAMS enzymes are encoded by a multigene family. There are four SAMS genes in *Arabidopsis*[6], four in tomato[7], two in pea[5] and 12 in soybean. We cloned and characterized a soybean SAMS gene, *GmSAMS1*. The polypeptide sequence of SAMS is well conserved among different plant species. The predicted amino acid sequence deduced from the *GmSAMS1* gene was highly homologous to other plant SAMS enzymes reported previously (Fig. 3). The homology between the GmSAMS1 protein and other plant enzyme ranged from 88% (MAT3) to 99% (AEY85025.1).

SAMS genes play a role in a variety of plant biological processes. Most SAMS genes can be induced by different environmental factors. Tomato *SlSAMS1* mRNA and protein respond to alkali stress[7]. *LrSAMS* genes were found to be the most abundantly expressed genes in the cDNA library from NaCl-treated *Lycoris radiata*[9]. *AEY85025.1* was a down-regulated protein in pigeon pea under drought[15]. In the present study, we found that in addition to the abiotic response motifs, there were the biotic stress response motifs located in the upstream sequence of the *GmSAMS1* gene(Table 2). This finding indicated that SAMS genes may also take part in biotic stress responses in plants. In fact, *GmSAMS1* mRNA and protein expression were up-regulated after CCW attack in soybean[13]. The *GmSAMS1* gene was transformed into tobacco, giving the transgenic tobacco more resistance to insects than non-transgenic tobacco(Fig. 5).

SAMS catalyzes the conversion of methionine and ATP into SAM, which serves as the precursor for ethylene and polyamines(PAs)[3]. Ethylene, like jasmonic acid, is an important hormone that works in plant resistance to herbivores and is induced by insect attack. The hormone upregulates the production of defensive proteins and secondary metabolites, including alkaloids, terpenoids, phenolics, and glycosides[16]. These compounds are all toxic to herbivores. In rice and maize, ethylene signaling pathway, as a defensive synergist, has been discovered to improve resistance to chewing herbivores, as in rice resistance to *Chilo sup-*

pressalis and maize resistance to *Spodoptera frugiperda*[16-17]. Although PAs are low molecular weight aliphatic nitrogen compounds different from ethylene, the biogenic amines, as key factors, are involved in plant tolerance to different abiotic and biotic stresses[18]. Therefore, the function of *GmSAMS1* in tobacco resistance to insects might be carried out by the ethylene pathway or the polyamine metabolic pathway. The mechanism of *GmSAMS1* in plant resistance to insects should be the focus of further research.

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