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Molecular Characterization of a Transient Expression Gene Encoding for 1-Aminocyclopropane-1-carboxylate Synthase in Cotton (*Gossypium hirsutum* L.)

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Ethylene performs an important function in plant growth and development. 1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS), the key enzyme involved in ethylene biosynthesis, has been the focus of most ethylene studies. Here, a cotton *ACS* gene referred to as *Gossypium hirsutum ACS1* (*GhACS1*), was isolated. The full-length cDNA of *GhACS1* encodes for a 476-amino acid protein which harbors seven conserved regions, 11 invariant amino acid residues, and the PLP binding active site, all of which characterize ACC synthases. Alignment analysis showed that GhACS1 shared a high degree of identity with other known ACC synthases from different species. Two introns were detected in the genomic DNA sequence, and the results of Southern blot analysis suggested that there might be a multi-gene family encoding for ACC synthase in cotton. From the phylogenetic tree constructed with 24 different kinds of ACC synthases, we determined that GhACS1 falls into group II, and was closely associated with the wound-inducible ACS of citrus. The analysis of the 5' flanking region of *GhACS1* revealed a group of putative *cis*-acting elements. The results of expression analysis showed that *GhACS1* displayed its transient expression nature after wounding, abscisic acid (ABA), and $CuCl_2$ treatments. These results indicate that *GhACS1*, which was transiently expressed in response to certain stimuli, may be involved in the production of ethylene for the transmission of stress signals.

Keywords: ACC synthase, Cotton, Ethylene, RACE, Semi-quantitative RT-PCR

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Introduction

Plants are challenged by a variety of biotic or abiotic stresses throughout their life cycles. In order to survive, they have developed precise mechanisms to accept external signals, and to respond with the appropriate physiological or morphological changes (Murphy *et al*., 2001). In the processes inherent to plant response, stress signals are transmitted by internal signal molecules from the injured position to the entirety of the plant. Thus, the internal signal molecules perform a crucial role in signal transduction. Salicylic acid (SA), jasmonic acid (JA), and ethylene as principal internal signal molecules have been increasingly studied in recent years.

The gaseous plant phytohormone, ethylene, an internal signal molecule, plays a pivotal role in plant growth and development and significantly affects seed germination, root hair development, flower senescence, abscission, and fruit ripening (Johnson and Ecker, 1998). It can also respond to a host of biotic (e.g. pathogenic attack) or abiotic stimuli, including wounding, chilling, ozone, flooding, Li^+, and hypoxia. In higher plants, ethylene biosynthesis proceeds via the Yang cycle (Yang and Hoffman, 1984), as follows: methionine → S-adenosyl-methionine (SAM) → 1-aminocyclopropane-1-carboxylate (ACC) → ethylene. In this pathway, the step in which SAM is converted to ACC via catalysis by ACC synthase is considered to be the rate-limiting step, and ACS is the rate-limiting synthase. ACC synthase, the key enzyme in the synthesis of ethylene, has become the hot spot in the study of ethylene.

Since the first cloning of the *ACS* gene from zucchini (*Cucurbita*) (Sato and Theologis, 1989), a host of *ACS* genes have been isolated and identified from a variety of plant species, including tobacco, *Arabidopsis*, rice, mungbean, wheat, winter squash, apple, potato, tomato, pear, banana, carnation, and rose. (Johnson and Ecker, 1998; Ge *et al*., 2000). Additionally, a multi-gene family has also been detected in these plants. ACC synthase evidences a labile nature and is

present in relatively low abundance in plants (Kende, 1993), but many of them are profoundly regulated as the result of internal or external stimuli. A variety of internal or external stimuli that can induce different expressions of *ACS* genes have been studied until now, including wounding, cytokinin, freezing, Li^{+}, ozone, cycloheximide, Cu^{2+}, IAA, ethylene, and so on. Although a great many *ACS* genes have been isolated and characterized, the majority of them have been derived from model plants including tobacco, *Arabidopsis*, rice, or wheat, or from fruit-specific plants to improve the storage time and ornamentals, prolonging the life of the flower. The transgenic plants obtained using the anti-sense method all evidence an ability to inhibit the production of ethylene. Studies on fruit ripening-related ACC synthase have generated surprising results, but limited data are available regarding the signal transduction of ACC synthase. Cotton (*Gossypium hirsutum*) is an economically important species in China, but no reports regarding its *ACS* genes have yet been filed.

In this study, we selected cotton as an experimental system, isolated a cotton *ACS* gene designated *GhACS1*, and evaluated the expression patterns observed as the result of different treatments. The cloning and characterization of the *ACS* gene from cotton will be a good basic step in the further study of the function of ethylene in stress defenses or signal transduction in the cotton plant.

Materials and Methods

Plant materials and treatments. Cotton (*Gossypium hirsutum* L. cv lumian 22) was used throughout these experiments. The seeds were incubated in Petri dishes between two sheets of moist filter paper at 28°C until germination. Germinated seedlings were grown by aquaculture in tissue culture pots (sterile water) and incubated for an additional 7 days at 28°C with a 16 h light/8 h dark cycle. The 7-day-old plants were employed in different treatments. The edges of the cotton leaves were cut with a pair of clear scissors for wounding treatment. $CuCl_2$ treatment was conducted by placing the cotton roots in 500 μM $CuCl_2$ solution. Abscisic acid (ABA) and methyl jasmonate (MeJA) treatments were applied via the spraying of 100 μM ABA and 100 μM MeJA onto the cotton seedlings. Then, the leaves under each treatment were harvested after appropriate intervals, immediately frozen in liquid nitrogen, and maintained at –80°C until use.

Primers. The primers used in this study are shown in Table 1.

RNA extraction, cDNA synthesis, and genomic DNA preparation. Total RNA was extracted from leaf materials treated via wounding for 30 min using the RNeasy Plant Mini Kit (Qiagen) in accordance with the manufacturer's recommendations. The total RNA obtained was first treated with DNaseI (Promega) in order to remove any potential genomic DNA contamination. The first-strand cDNA was

Table 1. The primers used in this study

Abbreviation	Primer sequence	Description
HP1	5'-CARATGGGHYTNGCNGARAAYC-3'	Degenerate primer, forward
HP2	5'-CCHARNGGRTTDGAWGGRTT-3'	Degenerate primer, reverse
WP5	5'-GCTCATAACAATGCGGTCTGGGTC-3'	5' RACE reverse primer, outer
NP5	5'-CAGCCTCTCTGAACTCAGGCATC-3'	5' RACE reverse primer, nested
AAP	5'-GGCCACGCGTCGACTAGTAC$(G)_{14}$-3'	Abridged Anchor Primer
AUAP	5'-GGCCACGCGTCGACTAGTAC-3'	Abridged universal amplification primer
WP3	5'-GATCGTGACTTAAGCTGGAGAACAG-3'	3' RACE forward primer, outer
NP3	5'-CACTAGAGCTGCCTTGGAAGC-3'	3' RACE forward primer, nested
B25	5'-GACTCTAGACGACATCGA-3'	Universal primer, nested
B26	5'-GACTCTAGACGACATCGA$(T)_{18}$-3'	Universal adaptor primer, outer
P1	5'-AACCATTTCAACACACTCACC-3'	Full length cDNA and genomic sequence primer, forward
P2	5'-GAGCTTCTCACAGTATTGCAC-3'	Full length cDNA and genomic sequence primer, reverse
ProW	5'-CACTCGACCACCCCTCACTTTCCCCAT-3'	LA PCR, primer, outer
ProN	5'-GTCTTCACCATGACCATTACCAGTTGC-3'	LA PCR, primer, nested
C1	5'-GTACATATTGTCGTTAGAACGCGTAATACGACTCA-3'	LA PCR, universal primer, outer
C2	5'-CGTTAGAACGCGTAATACGACTCACTATAGGGAGA-3'	LA PCR, universal primer, nested
PT1	5'-CGATGCTGAAATGGACTTGTGG-3'	Probe primer, forward
RT1	5'-GATGACAATACCATGGAAGTTGC-3'	RT-PCR primer, forward
RT2	5'-TCCACCAATGTTGAGCTTCTC-3'	RT-PCR primer, reverse (Probe primer reverse)
$GhUBI_{-1}$	5'-CTGAATCTTCGCTTTCACGTTATC-3'	Standard control primer, forward
$GhUBI_{-2}$	5'-GGGATGCAAATCTTCGTGAAAAC-3'	Standard control primer, reverse

synthesized using approximately 2 μg of total RNA and the B26 adaptor primer by M-MLV reverse transcriptase (TaKaRa) for 1 h at 42°C.

Genomic DNA was isolated from seedling leaves via the cetyltrimethyl-ammonium bromide (CTAB) technique (Scott and Arnold, 1988). The quality and concentration of RNA and DNA were evaluated via ethidium bromide-stained agarose gel electrophoresis and spectrophotometric analysis.

Cloning of the internal conservative fragment of *GhACS1*. In order to obtain the internal conservative fragment, the HP1 and HP2 primers were designed and synthesized on the basis of the conserved amino acids and nucleotide sequences of the *ACS* genes among *Arabidopsis*, tobacco, citrus, soybean, potato, and mungbean. Reverse-transcription (RT-PCR) was conducted as follows: pre-denaturation at 94°C for 5 min, followed by 35 cycles of amplification (94°C for 50 s, 50°C for 50 s, 72°C for 1 min), and a final extension step at 72°C for 5 min. The PCR products were electrophoresed, purified with an Agarose Gel DNA Purification Kit (TaKaRa), ligated to PMD18-T vector (TaKaRa), transformed into the DH5α *E. coli* strain, then sequenced.

5' RACE. Based on the internal conservative sequence, two specific primers WP5 and NP5 were designed and synthesized. In order to prepare the 5'-ready cDNA, the first-strand cDNA was purified in accordance with the manufacturer's instructions for the Wizard DNA Clean-Up System (Promega), and then polyadenylated at its 5'-end with dGTP using terminal deoxynucleotidyl transferase (TaKaRa); this was followed by ethanol precipitation and resuspension in distilled deionized water. The WP5 primer, coupled with the Abridged Anchor Primer (AAP), were employed in the performance of the first-round PCR. The primary PCR product was diluted 50-fold for secondary PCR using the NP5 nested primer and the Abridged Universal Amplification Primer (AUAP). Both primary and secondary PCR were conducted as follows: pre-denaturation at 94°C for 5 min, followed by 35 cycles of amplification (94°C for 50 s, 60°C for 50 s, 72°C for 1 min). After the final cycle, the amplification was extended for 5 min at 72°C. The PCR products were electrophoresed, purified, and cloned into PMD18-T vector, and sequenced as described above.

3' RACE. Two specific primers, WP3 and NP3, were also designed on the basis of the internal conservative sequence. WP3 and the universal primer, B26, were utilized in the performance of the primary PCR. The PCR products were then diluted 50-fold as the template for secondary PCR with the NP3 primer and the B25 nested universal primer. The general PCR procedures were as follows: pre-denaturation at 94°C for 5 min; 35 cycles of amplification (94°C for 50 s, 56°C for 50 s, 72°C for 1.5 min); and a final extension step at 72°C for 5 min. The PCR products were electrophoresed, purified, and cloned into the PMD18-T vector, and then sequenced.

The full-length cDNA amplification of *GhACS1*. After analysis of the internal, 5' RACE and 3' RACE sequences, the full-length cDNA of *GhACS1* was deduced. The P1 and P2 primers were designed on the basis of the deduced cDNA in order to obtain the full-length sequence via the following PCR procedure: pre-denaturation at 94°C for 5 min, followed by 35 cycles of amplification (94°C for 50 s, 52°C for 50 s, 72°C for 2 min). After the final cycle, the amplification was extended for 5 min at 72°C. The PCR products were electrophoresed, purified, and cloned into the PMD18-T vector, sequenced as described above.

Cloning of the genomic sequence and the 5' flanking region of *GhACS1*. Genomic DNA together with the P1 and P2 primers were employed in the amplification of the genomic sequence. For the amplification of the 5' flanking region, the ProW and ProN primers were designed in accordance with the 5'-end of the genomic sequence. Total genomic DNA (about 5 μg) was completely digested overnight with *Eco*RI at 37°C, then ligated to the cassette, after which the procedures recommended in the instructions of the LA PCR *in vitro* Cloning Kit (TaKaRa) were conducted. The putative *cis*-acting elements in the 5' flanking region were then subjected to a search using the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Southern blot analysis. For Southern blot analysis, total genomic DNA (30 μg/sample) was digested completely with *Eco*RI and *Hin*dIII respectively, separated via electrophoresis on 1% agarose gel, and transferred onto a Hybond-N^+ nylon membrane (Amersham Pharmacia) using the method developed by Sambrook *et al*. (1989). A 500 bp fragment corresponding to the 3'-end of the cDNA (including a portion of the 3' non-coding region) was generated via RT-PCR with the PT1 and RT2 primers, and was labeled with [α-^{32}P] dCTP in accordance with the instructions provided by the manufacturers of the Primer-a-Gene® Labeling System (Promega), then used as the probe of Southern blot analysis. Hybridization was conducted for 24 h at 65°C. After hybridization, the blots were washed four times for 10 min at 42°C in 2 × SSC, 0.2%SDS and 0.2 × SSC, 0.2%SDS respectively, and then exposed to X-ray film (Kodak).

Expression analysis of *GhACS1* via semi-quantitative RT-PCR. Total RNA was extracted from seedling leaves that were differently treated using Trizol reagent (Gibco BRL) in accordance with the manufacturer's instructions. The obtained RNA was initially digested with DNaseI (Promega) to prevent genomic DNA contamination, and the cDNA was synthesized. A cotton polyubiquitin gene (*GhUBI*) generated by the $GhUBI_{-1}$ and $GhUBI_{-2}$ primers was utilized as a standard control in the RT-PCR reactions (Li *et al*., 2005). The RT1 and RT2 primers were designed according to the 3'-end of the cDNA sequence, and were employed to detect *GhACS1* expression under different treatments.

Results

Cloning and characterization of the full-length cDNA of *GhACS1*. With the degenerated primers HP1 and HP2, a fragment of approximately 500 bp was obtained which evidenced a high degree of homology to the *CsACS1* gene (AJ012551). After 5' and 3' RACE with the specific primers WP5, NP5, WP3, and NP3, a 400 bp (about) 5'-end and a 1000 bp (about) 3'-end sequence were generated, respectively. Via the comparison and analysis of the overlapped regions of the three fragments, the full-length cDNA of *GhACS1* was deduced. The entire

sequence of *GhACS1,* approximately 1802 bp, was obtained after RT-PCR with the specific primers P1 and P2, and was identical to the deduced cDNA.

Sequence analysis showed that the entire cDNA sequence of *GhACS1* (GenBank Accession No. DQ355792) was 1802 bp with a 162 bp 5' untranslated region (UTR) and a 212 bp 3' UTR. The cDNA harbored a 1428 bp complete open reading frame (ORF) encoding for a protein of 476 amino acids with a predicted molecular weight of approximately 53.8 kDa, and an isoelectric point of 6.83.

Via comparison of the predicted amino acid sequence of GhACS1 with other known ACC synthases from different plants using DNAman version 5.2.2 (Lynnon BioSoft Company), we determined that GhACS1 evidenced 73, 70, 67, 66.9, 66, and 60% homology with the ACC synthases from citrus, tobacco, potato, soybean, mungbean, and *Arabidopsis,* respectively (Fig. 1). Seven regions that were highly conserved among all ACC synthases (Dong *et al*., 1991) were detected in GhACS1, and each of them harbors at least seven amino acids. The most heavily conserved region included the active-site lysine residue (SLSKDMGFPGFR), which binds to pyridoxal 5'-phosphate (PLP) and Adomet (Yip *et al*., 1990). Eight of eleven invariant residues were conserved in all ACC synthases from various sources, and certain aminotransferases were present in GhACS1 also (Huang *et al*., 1991; Rottmann *et al*., 1991). We also detected four important amino acid residues that were involved in interactions with the PLP cofactor, and the four residues have been studied via site-directed mutagenesis in the apple (White *et al*., 1994) and tomato ACC synthases (Zhou *et al*., 1999; Wong *et al*., 1999). The C-terminal end, which is the least conserved region among all ACC synthases, may correspond to the different protein dimerization and stability of ACC synthase (Rottmann *et al*., 1991; Li and Mattoo, 1994; Zarembinski and Theologis, 1994; Tarun and Theologis, 1998; Vogel *et al*., 1998; Chae *et al*., 2003), thereby suggesting that different ACC synthases may function differently (Gallie *et al*., 2004).

Structure analysis of the genomic sequence of *GhACS1*. After the PCR reaction using cotton genomic DNA as a template and the P1 and P2 specific primers, a fragment of approximately 2360 bp was obtained. On the basis of the comparison with the cDNA sequence of *GhACS1*, the genomic structure was established (Fig. 2A). ACC synthase can be divided into 3 classes, in accordance with the number of the introns. They harbor either 2, 3, or 4 introns, but the position of each intron is conserved far more profoundly among the *ACS* genes (Yoon *et al*., 1999; Hidalgo *et al*., 2005). As has been observed in Fig. 2A, the gene consisted of three exons, which were interrupted by two introns (114 bp and 514 bp respectively) located within the 5'-end near the gene. The two introns all evidenced a high AT content (63% for intron 1, 69% for intron 2) and harbored the conserved GT and AG dinucleotides at the 5' and 3' splice sites, which are all typical structural characteristics of plant introns.

In order to determine whether *GhACS1* belongs to a multi-gene family, Southern blotting was conducted. Genomic DNA isolated from the leaf tissues of cotton plants was completely digested with *Eco*RI and *Hin*dIII, respectively, then hybridized with a labeled 3'-end partial sequence (approximately 500 bp). The restriction map of the *GhACS1* genomic sequence (Fig. 2A) evidences two *Eco*RI sites and one *Hin*dIII site, and no restriction site was detected within the probe sequence. The results of hybridization (Fig. 3) indicated two and four bands in the corresponding lane, thereby indicating that there may exist other *ACS* genes that share some degree of homology with *GhACS1*. These results raise the possibility that a multiple gene family may also encode for ACC synthase in the cotton plant.

Identification of partial putative *cis*-acting elements in the 5' flanking region of *GhACS1*. LA PCR and two specific primers, ProW and ProN, were utilized to isolate the 5' flanking region of *GhACS1*. A 651 bp fragment was generated. The putative transcription starting site was defined on the basis of the cDNA sequence (Fig. 2B).

From the 5' flanking region upstream of the putative transcription site, the TBP binding site (called TATA box) and the CTF binding site (referred to as the CAT box) were identified, and this is consistent with the other published core promoter consensus sequences (Grierson and Covey, 1988; Yamaguchi *et al*., 1998). A 162 bp leader sequence with an AT content of 73.5% separates the transcription and translation start sites. A computer search for the potential putative *cis*-acting elements using the PlantCARE database in the 5' flanking region of the gene revealed 4 different putative *cis*-acting elements that confer gene expression in response to environmental stimuli including light and MeJA. In addition to these elements, elements associated with endosperm expression were also detected (Fig. 2B).

Expression profiles of *GhACS1* under a variety of abiotic stresses (wounding, ABA, and $CuCl_2$). In order to identify the environmental stimuli that induce *GhACS1* expression, we evaluated the effects of abiotic factors including wounding, ABA, and $CuCl_2$ via semi-quantitative RT-PCR. As is shown in Fig. 4A, the wounding of cotton leaves resulted in a dramatic increase in the accumulation of *GhACS1* mRNA within 10 min, followed by a further increase at 20 min, reaching a peak at 30 min. After that point, we noted a decline in expression to undetectable levels at 2 h. The wound-inducible *ACS* genes exist in at least two classes on the basis of induction kinetics. One class of the accumulation kinetics increased rapidly and transiently upon wounding, whereas the other class evidenced relatively slow induction kinetics when stressed by wounding (Ge *et al*., 2000). Thus, the *GhACS1* gene we obtained may be a member of the first class, as it was expressed rapidly and transiently upon wounding.

Studies have indicated that wounding-induced genes may also be affected by wound-related signaling molecules, including

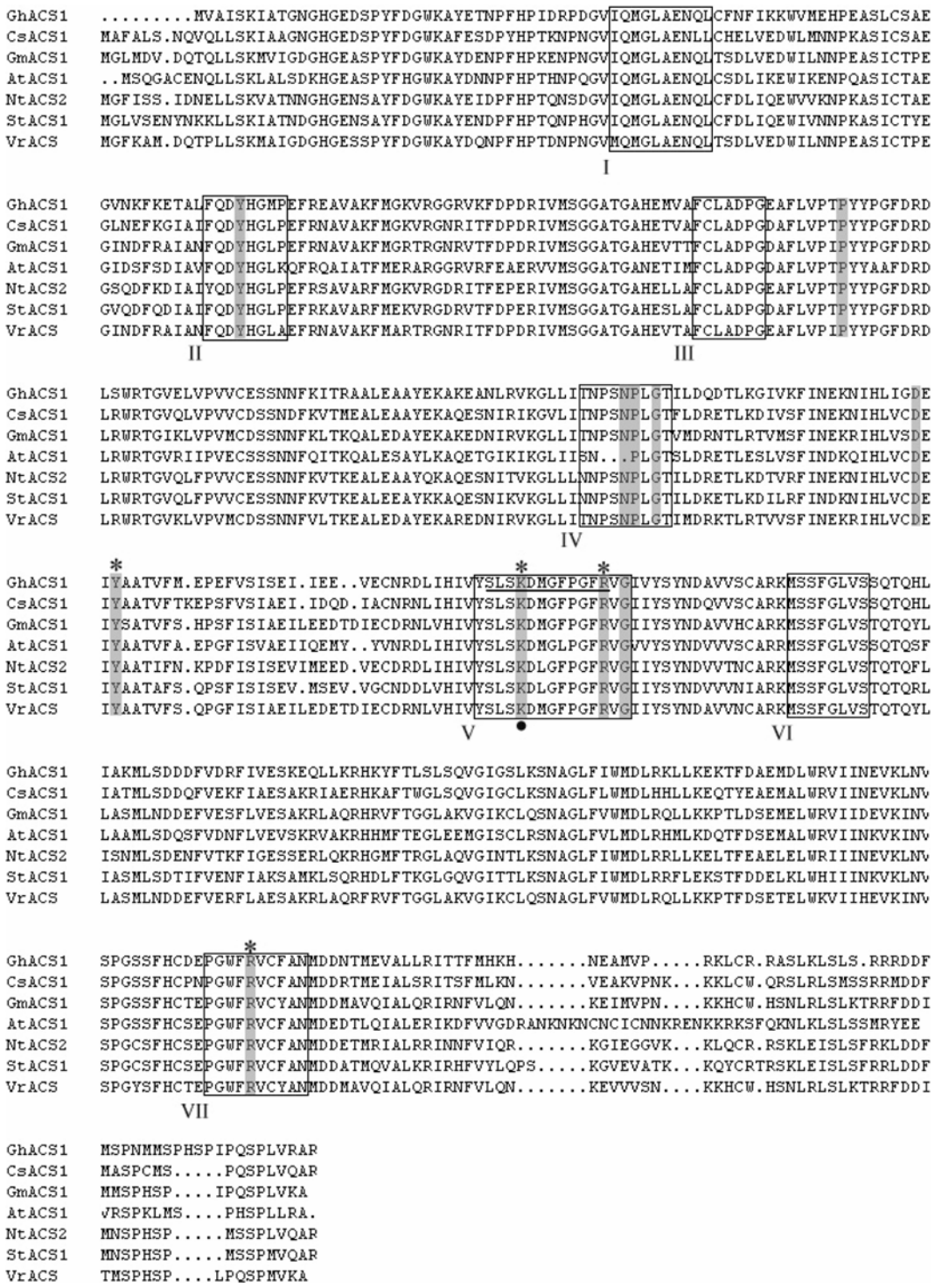

Fig. 1. Alignment of the deduced amino acid sequences of ACC synthases. The amino acid sequences of ACC synthases of GhACS1 (*Gossypium hirsutum*, DQ355792), CsACS1 (*Citrus sinensis*, AJ012551), GmACS1 (*Glycine max*, X67100), AtACS1 (*Arabidopsis thaliana*, NM116016), NtACS2 (*Nicotiana tobacum*, X98492), StACS1 (*Solanum tuberosum*, AB041521) and VrACS (*Vigna radiata*, Z11613) were used for multi-alignment analysis. The seven highly conserved regions among all ACC synthases are boxed. The underlined sequence shows the part of the active site of ACC synthases, and the dot indicates the active site lysine which binds pyridoxal 5'-phosphate (PLP) and Adomet. The 11 invariant amino acid residues conserved in aminotransferases and ACC synthases of various sources are highlighted in grey. The residues which have been studied by site-directed mutagenesis in apple and tomato ACC synthases are marked by asterisk.

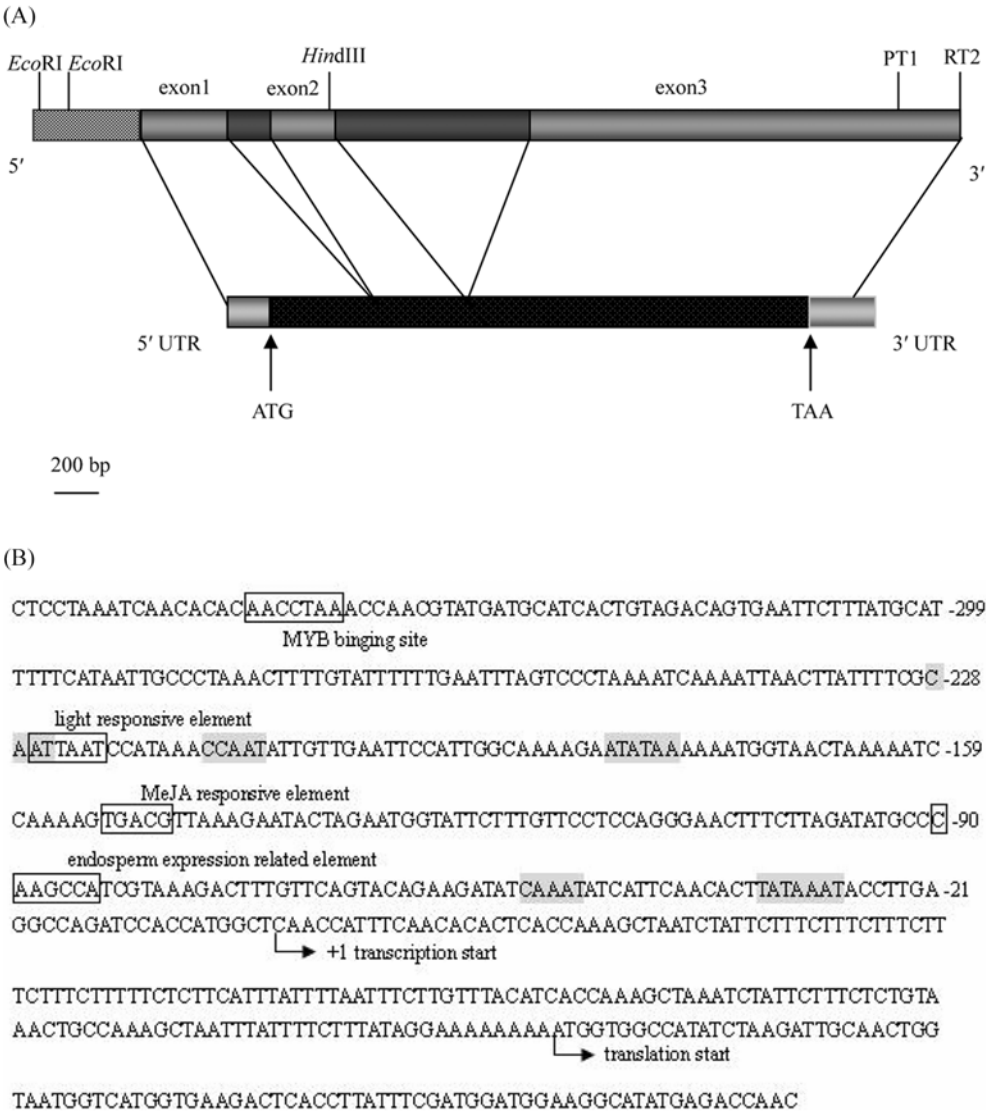

Fig. 2. Schematic representation of the structure and the nucleotide sequence of the 5' flanking region of *GhACS1*. (A) The genomic and mRNA structure of *GhACS1*. Genomic sequence with two introns (114 bp and 514 bp) and mRNA sequence with marked coding region are shown. The 5' flanking region upstream of the exon 1 is shown with lattice. And the selected restriction enzyme sites and the site of the probe in the genomic sequence are marked. (B) Nucleotide sequence of the 5' flanking region. Numbering starts from the predicted transcription initiation site (+1). The transcription and translation sites are indicated with the arrows. The putative core promoter consensus sequences are highlighted in grey, and the putative *cis*-acting elements mentioned are boxed.

oligosaccharin (OGAS) (Creelman *et al*., 1997), systemin (Pearce *et al*., 1991), abscisic acid (Peña-Cortès *et al*., 1989) and jasmonic acid (Farmer *et al*., 1992). Thus, in this study, we also evaluated the expression of *GhACS1* in response to ABA and MeJA (similar to JA) treatments. The results are shown in Fig. 4B. ABA profoundly stimulated the accumulation of the transcript within 30 min after the start of treatment, with a decline occurring at 1 h, after which the levels became undetectable. This result indicated that ABA may be one of the compounds that influences *GhACS1* expression in cotton. However, in response to MeJA treatment, no *GhACS1* expression was detected (data not shown). Our results indicate that the transcript of *GhACS1* can be regulated by ABA, and its expression is quite rapid and transient, occurring just before the 1 h point.

At the same time, we selected $CuCl_2$ as a metal ion inducer of *GhACS1*. The accumulation of mRNA was detected initially at 4 h, with a further increase at 6 h, but after that there were no detectable levels (Fig. 4C). This result suggests that *GhACS1* can be slowly induced but is rapidly expressed in response to Cu^{2+}.

The similar expression patterns of *GhACS1* in response to wounding, ABA, and $CuCl_2$ treatments strongly indicates the transient expression nature of the *GhACS1* gene.

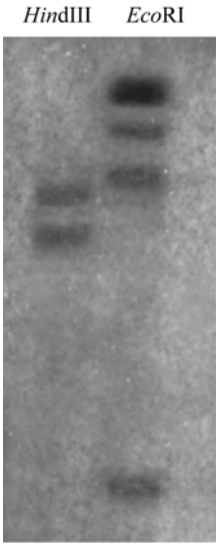

Fig. 3. Southern blot analysis of *GhACS1*. Genomic DNA about 30 μg per lane was digested with *Eco*RI and *Hin*dIII respectively, separated on a 1.0% agarose gel, and then hybridized with a α-^{32}P-labeled 3'-end partial sequence.

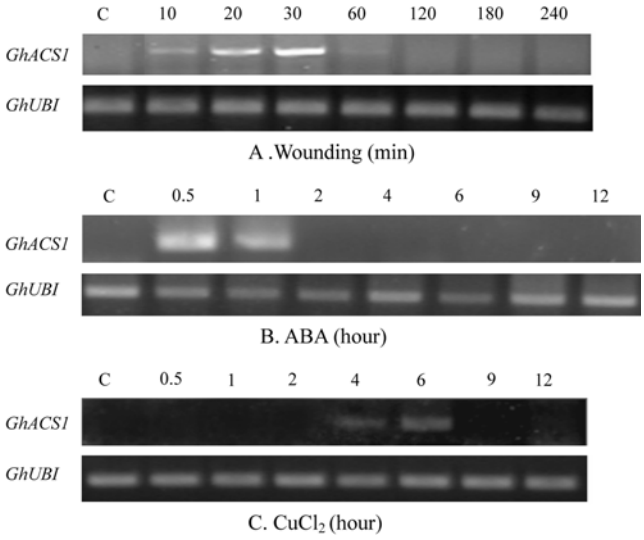

Fig. 4. Expression analysis of *GhACS1* in cotton under different treatments. 7-day-old cotton leaves were used for different treatments, and the no treatment leaves were served as the control. Leaves were harvested at predicted intervals and immediately frozen in liquid nitrogen for RNA extraction. The PCR reaction was repeated at least 3 times. The *GhUBI* gene was used as the standard control to show the normalization of the amount of templates in PCR reactions (lower panel). (A) Expression profiles of *GhACS1* under wounding treatment. (B) Expression pattern of *GhACS1* under 100 μM ABA treatment. (C) Expression of *GhACS1* under 500 μM $CuCl_2$ treatment.

Phylogenetic analysis of GhACS1 with other known ACC synthases. In order to determine the evolutionary relationships existing among different ACC synthases from a variety of plant species, a phylogenetic tree was compiled using DNAman software, and the amino acid sequences used were all deposited in the GenBank database (Fig. 5). The results revealed that these available ACC synthases were classified into three major groups. Studies have demonstrated that ACC synthases from *Arabidopsis* evidence ACS activity, with the exception of those in group III, which includes AtACS10 and

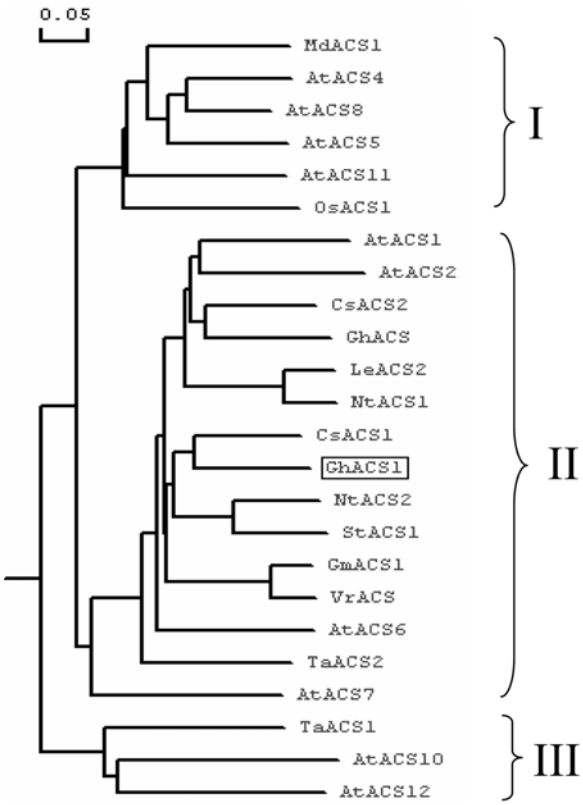

Fig. 5. Phylogenetic relationships of ACC synthases from different species. The tree was generated using the DNAman software. The amino acid sequences of ACC synthases used for construction of the tree are all deposited in the GenBank database under the following accession number: MdACS1 (*Malus x domestica*, DQ137849), AtACS4 (*Arabidopsis thaliana*, NM127846), AtACS8 (*Arabidopsis thaliana*, NM119939), AtACS5 (*Arabidopsis thaliana*, NM125977), AtACS11 (*Arabidopsis thaliana*, NM116873), OsACS1 (*Oryza sativa*, NM001057677), AtACS1 (*Arabidopsis thaliana*, NM116016), AtACS2 (*Arabidopsis thaliana*, NM179241), CsACS2 (*Citrus sinensis*, AJ012696), GhACS (*Gossypium hirsutum*, EF189198), LeACS2 (*Lycopersicon esculentum*, AY326958), NtACS1 (*Nicotiana tobacum*, X65982), CsACS1 (*Citrus sinensis*, AJ012551), GhACS1 (*Gossypium hirsutum*, DQ355792), NtACS2 (*Nicotiana tobacum*, X98492), StACS1 (*Solanum tuberosum*, AB041521), GmACS1 (*Glycine max*, X67100), VrACS (*Vigna radiata*, Z11613), AtACS6 (*Arabidopsis thaliana*, NM117199), TaACS2 (*Triticum aestivum*, U35778), AtACS7 (*Arabidopsis thaliana*, NM118753), TaACS1 (*Triticum aestivum*, U35779), AtACS10 (*Arabidopsis thaliana*, NM104974), AtACS12 (*Arabidopsis thaliana*, NM124548). The GhACS1 studied in this work is boxed.

AtACS12, similar to the Ala or Asp aminotransferases in *Arabidopsis* (Yamagami *et al*., 2003; Iwai *et al*., 2006). As GhACS1 was clustered into group II, we conclude that it may function similarly in cotton, as well.

We also determined that there were many wound-induced ACC synthases in group II, and that GhACS1 is more closely related to that group, which includes CsACS1, CsACS2, NtACS2, GmACS1 and LeACS2 (Ge *et al*., 2000; Rottmann *et al*., 1991; Lincoln *et al*., 1993). More interestingly, another Cu^{2+}-inducible ACC synthase, NtACS1 (Bailey *et al*., 1993) was also discovered to occupy the same evolutionary group as GhACS1. The result also supports previous studies showing that the duplication of ACC synthases occurred prior to the divergence of monocotyledonous and dicotyledonous species (Gallie *et al*., 2004).

Discussion

In plants, wounding can induce the generation of ethylene via the expression of certain *ACS* genes (Sato and Theologis, 1989; Nakajima *et al*., 1990; Lincoln *et al*., 1993). Thus far, many wound-induced *ACS* genes have been isolated from different plants, including winter squash (Nakajima *et al*., 1990), tobacco (Bailey *et al*., 1993; Ge *et al*., 2000), tomato (Lincoln *et al*., 1993), *Arabidopsis* (Arteca *et al*., 1999) and soybean (Liu *et al*., 1993). In this study, we have cloned a novel wound-inducible *ACS* gene from cotton, referred to as *GhACS1*. Comparisons of amino acids indicated that GhACS1 harbors the major characteristics of ACC synthases, including seven conserved regions, 11 invariant amino acid residues, and the active site (shown in Fig. 1). Furthermore, we have determined the expression patterns of *GhACS1* against a variety of stimuli.

The wounding of the cotton leaves induced a rapid and transient expression of *GhACS1* within 10 min, with a peak occurring at 30 min, with a disappearance after 1 h. This is quite similar to the induction kinetics of the soybean *ACS* gene (Liu *et al*., 1993), but sharply different from the expression pattern of the tomato *ACS* gene. After wounding the tomato fruit pericarp tissue, transcript accumulation reached maximum levels at 40 min (Olson *et al*., 1991), but more surprisingly, the high level transcripts can persist for over 16 hours (Li *et al*., 1992). In tobacco, Ge *et al*. identified two classes of *ACS* genes that are responsive to wounding. One class of *ACS* genes can be slowly induced, and achieves a peak at 6 h, in contrast to the observed rapid induction peaking at 2 h (Ge *et al*., 2000). The different expression patterns of the *ACS* genes indicate that there may exist exactly two classes of wounding-responsive *ACS* genes, and they may exploit two different signal transduction pathways.

Since chemical and physical wound response regulators including OGAS, ABA, systemin, and JA may play a positive role in wound signalling, we evaluated the effects of ABA and MeJA on *GhACS1* expression. ABA rapidly induced transcript accumulation within 30 min, but we did not assess accumulation in response to MeJA treatment, which was inconsistent with the reported results of Watanabe *et al*. (Watanabe *et al*., 1998). In the winter squash, that group reported that the wound-induced *WSACS2* was profoundly stimulated by MeJA within 30 min of the commencement of treatment. The observed difference may have been attributable to the different induction kinetics of *GhACS1* and *WSACS2*. When wounding the winter squash mesocarp tissues, *WSACS2* expression was elevated within 40 min and continued to dramatically increase for 120 min. However, *GhACS1* evidences a more rapid and transient expressional nature as compared to *WSACS2*. Although we detected the MeJA responsive element in the 5' flanking region, this was invalid in *GhACS1* gene expression. The different effects of ABA and MeJA upon *ACS* gene expression may provide us with some insight into the relationship existing between wound-stress and wound-induced *ACS* gene expression. This also suggests that different treatments can induce different *ACS* gene expression, and this induction is closely related to the duration of treatment.

Some previous studies have reported that the ethylene and JA signal pathways may interact with one another, co-regulating the expression of some of the genes involved in plant defense (Wang *et al*., 2002). In wound response, ethylene is synthesized via the induction of ACC synthase activity. Two wound response pathways have been proposed. One of these is a JA-dependent pathway, and the other is a JA-independent pathway (Titarenko *et al*., 1997; Leon *et al*., 1998). As *GhACS1* cannot be regulated by MeJA, we suppose that this gene may utilize the JA-independent pathway in order to transmit wound stress signals. This is also very consistent with the theory that two wound-inducible *ACS* genes are present in plants, and they respond to different stimuli and participate in different signal pathways.

The copper ion is considered to be an oxidative stress. Schlagnhaufer *et al*. have demonstrated that two different *ACS* genes can be induced by Cu^{2+} in the potato (Schlagnhaufer *et al*., 1997). *StACS5* was profoundly induced within 30 min and disappear after 2 h, but *StACS4* was initially detected at 2 h, evidencing a decline at 4 h. These two genes evidenced a transient nature in response to Cu^{2+}. In our study, we determined that *GhACS1* can be slowly induced but transiently expressed when treated with Cu^{2+}, a phenomenon similar to that associated with *StACS4*. At the same time, we also assessed the expression of *GhACS1* under NaCl, chilling, and LiCl treatments, but we all failed to detect the transcript of *GhACS1,* as well as in the control. Our results showed that *GhACS1* is an inducible gene but that it is not multi-responsive, it responds only to certain stimuli. Although *GhACS1* can be induced rapidly by wounding and ABA, and slowly induced by copper, it evidences a transient expressional nature under those conditions.

Because *GhACS1* responds to wounding, ABA, and Cu^{2+}, some responsive elements may exist in the 5' flanking region. The 5' flanking region we obtained was a partial sequence, we

detected only light and invalid MeJA responsive elements, thereby suggesting that the *GhACS1* gene may be regulated by light. Thus, there may exist many other elements, especially elements responsive to wounding, ABA, and Cu^{2+}. The further study of the full-length promoter sequence and the transgenic plants will provide more help in evaluating the manner in which this *GhACS1* gene is regulated.

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