Acta Biochim Biophys Sin 2011, 43: 654–661 | © The Author 2011. Published by ABBS Editorial Office in association with Oxford University Press on behalf of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. DOI: 10.1093/abbs/gmr056.
Advance Access Publication 8 July 2011

Short Communication

1-Aminocyclopropane-1-carboxylic acid synthase 2 is phosphorylated by calcium-dependent protein kinase 1 during cotton fiber elongation

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The reaction catalyzed by 1-aminocyclopropane-1-carboxylic acid synthase (ACS) is proposed to be the rate-limiting step in ethylene biosynthesis, which has been found as one of the most up-regulated metabolic pathways during cotton fiber development. However, the transcripts of the identified *ACS* genes did not increase in a similar manner as those of 1-aminocyclopropane-1-carboxylic acid oxidase (*ACO*) genes, implicating a possible post-transcriptional modification or regulatory mechanism. In this work, cotton ACS2 was shown to interact with Ca^{2+}-dependent protein kinase 1 (CPK1). Bacterially expressed and purified recombinant ACS2 was phosphorylated by CPK1 *in vitro* and site-directed mutagenesis studies suggest that ACS2 S460 is a possible phosphorylation site for CPK1. Phosphorylated ACS2 significantly increased ACS activity, leading to elevated ethylene production. We thus speculated that CPK1 is involved in cotton fiber growth regulation by phosphorylating ACS2, which results in enhanced ethylene production *in vitro*.

Keywords 1-aminocyclopropane-1-carboxylic acid synthase (ACS); calcium-dependent protein kinase (CPK); phosphorylation; cotton fiber

Received: January 6, 2011 Accepted: April 15, 2011

Introduction

Ethylene is important due to its role in signaling pathways and critical in the regulation of plant development and serves as a major mediator in response to various biotic and abiotic stresses as well [1–3]. A number of up-regulated genes have been identified by microarray analysis [4–7], laying a foundation for understanding the mechanism of cotton fiber development. Ethylene biosynthesis is one of the most up-regulated metabolic pathways during fiber elongation at 10–15-day postanthesis (dpa), and exogenously applied ethylene stimulates significant fiber growth [5]. Ethylene was found to promote cotton fiber and *Arabidopsis* root hair growth by activating the biosynthesis of UDP-L-Rha and UDP-D-GalA, major components of primary cell walls [8]. Further studies have shown that very long chain fatty acid promotes cotton fiber elongation by stimulating the expression of genes important for ethylene biosynthesis [9]. Indeed, transcripts encoding various lipid biosynthesis genes have been reported to be specifically up-regulated during the period of active fiber cell growth [10,11].

The conversion of S-adenosyl-L-Met (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) and the oxidative cleavage of ACC are two key steps in ethylene production that are catalyzed by ACC synthase (ACS) and ACC oxidase (ACO), respectively [12,13]. The ACS activity is highly regulated in most vegetative tissues in response to various developmental and environmental signals. Therefore, ACS is the rate-limiting enzyme in ethylene production [12,14]. Several studies have revealed that the half-lives of ACS protein range from about 20 min to several hours [15]. The phytohormones, cytokine, and brassinolide are able to prolong the half-life of ACS protein [16,17]. In *Arabidopsis*, ACS2/ACS6 was shown to be phosphorylated by mitogen-activated protein kinases (MPKs), MPK3/MPK6, at the non-catalytic C-terminal domains [18–20]. The over-expression of gain-of-function MPK6 in *Arabidopsis* mutants causes constitutive activation of MPK, resulting in constitutive ethylene production [18]. The ACS stability is mediated by interaction with ETO1 that acts as a substrate adaptor in CUL3-dependent ubiquitin ligase [16,21].

Besides phosphorylation of ACSs by MPKs, plant ACS is proposed to be regulated by calcium-dependent protein kinases (CPKs) phosphorylation by using a synthetic peptide [22]. Tomato LeACS2 is phsophorylated by tomato fruit extracts containing CPK activity [23]. Further studies showed that the stability of LeACS2 is regulated by both CPK and mitogen-activated protein kinase (MAPK) in response to wounding signaling [24]. Over-expression of CPK2 in tobacco plant results in increased ACC, salicylic acid, and jasmonic acid biosynthesis, suggesting a cross-talk between CPK and MPK signaling that controls plant stress

responses [25]. During cotton fiber development, *GhACOs* transcripts were shown to be specifically accumulated in 10 dpa fibers, compared with 10 dpa ovules, whereas, expressions of *GhACS*s are not induced significantly [5]. Therefore, how ACS exerts its role in controlling ethylene synthesis is not understood in fiber cells. In this study, we found that the cotton GhACS2 was phosphorylated by GhCPK1 *in vitro*, and the phosphorylation of GhACS2 increased the ACS activity, leading to elevated ethylene biosynthesis during fiber fast elongation.

Materials and Methods

Materials

Upland cotton (*Gossypium hirsutum* cv Xuzhou 142) was grown in soil mixture in a fully automated greenhouse as reported previously [10]. For *in vitro* ovule culture, 1 dpa (day post-anthesis) fresh ovules were excised from bolls on cotton plant and used directly. For RNA extraction, ovules at different growth stages were frozen and stored in liquid nitrogen immediately after harvest.

RNA extraction and RT-PCR analysis

Cotton ovules were harvested at the indicated time point, frozen in liquid nitrogen immediately until use. The frozen ovules were ground to fine powder with a mortar and pestle using a modified hot borate method [10]. Then, total RNA was extracted from ovules at different stages [5]. First-strand cDNA was synthesized from 5-μg total RNA using the superscript first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, USA). The reverse-transcribed cDNA was used as template for PCR analysis. Gene-specific RT-PCR primers were synthesized commercially (Invitrogen) as listed in **Table 1**. Parallel reactions using cotton UBQ7 primers were performed to normalize the amount of template cDNA added in each reaction. The RT-PCR cycles were as follows: initiation with a 5 min denaturation at 95°C, followed by 35 cycles of amplification with 10 s of denaturation at 95°C, 20 s of annealing,

Table 1 Primers used in this study

Genes	Sequences
GhACO1	5′-CGCCACTTGCCTGAATCTAAC-3′
	5′-CGCCACTTGCCTGAATCTAAC-3′
GhACO2	5′-TGAGGAGAGAGGAGCCACC-3′
	5′-CCCTTAGCCCCTTGATTAGC-3′
GhACO3	5′-CACCAATGGCAAGTACAAAAGT-3′
	5′-GCAAACAACACACATCTACGA-3′
GhACO4	5′-GCTGCTGGACTTGTTCTGTGAG-3′
	5′-GGCAATCACCCTATGCTCCAC-3′
GhACS2	5′-AAAGCCTACGACAGCAGCCCTT-3′
	5′-CATAACTATACGGTTCGGATCA-3′
GhACS6	5′-AAGTCGGTATCGGTTCGTTGAAGAGC-3′
	5′-GGTGATTGAGGTATGGGAGAGTGAGG-3′
GhACS10	5′- GTTATGACAGGGATGTAAAATGGC-3′
	5′- TGTTCTTCTCTCTGGCAAAGTCTA-3′
GhACS12	5′- CGCTTTATTCTACTTCCTCCAACTCT-3′
	5′- TTTCTCAATCAAATCAAAACACAACC-3′
GhACS3	5′-ATGGGGAAAGTGAGGGGAGA-3′
	5′-TGCCAACTCTAAAACCAGGGAAC-3′
GhACS4	5′- GTGCCCGCAAAATGTCCA-3′
	5′-GGAAAGAAGAACCTGGCGAAAC-3′
GhUBQ7	5′-GAAGGCATTCCACCTGACCAAC-3′
	5′-CTTGACCTTCTTCTTCTTGTGCTTG-3′
GhACS2-mut1	5′-GAAAGCAAAATCTTCGCCTCGGCTTCTCTTCGT-3′
	5′-ACGAAGAGAAGCCGAGGCGAAGATTTTGCTTTC-3′
GhACS2-mut3	5′-TGATTGCCCCTCACGCCCCAATTCCTCACGCGCCCCTCGTTCGAG-3′
	5′-CTCGAACGAGGGGCGCGTGAGGAATTGGGGCGTGAGGGGCAATCA-3′

20−30 s of extension at 72°C, and after all cycles another 10 min at 72°C to finish extension.

Ovule culture and inhibitor treatment

For *in vitro* ovule cultures, wild-type (wt) cotton bolls were collected 1 day after flower opening, and sterilized with 10% sodium hypochlorite. The ovules generally floated on the surface of 20 ml liquid media in 50 ml flasks under aseptic conditions and were cultured at 30°C in darkness without agitation. CPK-specific inhibitors, 5 μM trifluoperazine (TFP) and 5 μM *N*-(6-aminohexyl)-5-chloro-1-naphthelenesulfonamid-hydrochloride (W-7), protein kinase A (PKA) inhibitor (H-89, 5 μM), or protein kinase C (PKC) inhibitor (H-9, 5 μM) were added into the media as described previously [5]. The fiber length was measured manually under a dissecting microscope after combing them to upright positions.

Co-immunoprecipitation (Co-IP) assay

Full-length *GhACS2* and *GhACO1* were cloned downstream of the HA-tag in vector pGADT7 with primers ACS2-5P (*Nde*I): 5′-GATCCATATGGAGTTTACGAGAAAGAATCGAC-3′ and ACS2-3P (*Xho*I): 5′-CTAGCTCGAGTTAAGTCCTCGCTCGAACGAGG-3′. Full-length *GhCPK1*, *CPK32*, and *CRK5* were cloned downstream of the c-myc-tag in vector pGBKT7. The primers are as follows: CPK1-5P (*Eco*RI): 5′-CTAGGAATTCATGGGGAATACTTGTGTAGGACCAAG-3′; CPK1-3P (*Sal*I): 5′-ACGCGTCGACCTAAAATTTTAATGCTTCTCTAAACTTG-3′; CPK32-5P (*Eco*RI): 5′-CAATGAATTCATGGGAAATTGCTGTGCTACTCCAT-3′; CPK32-3P (*Sal*I): 5′-ACGCGTCGACTCAACAGCCATTTTTCATTAGTAGA-3′; CRK5-5P (*Nde*I): 5′-ACGCCATATGGGTCTTTGCACCTCCAAACC-3′; CRK5-3P (*Sal*I): 5′-ACGCGTCGACCTATTGAGCTTTTGCGATGGTTC-3′.

In vitro translation of the above proteins was performed using TNT®-coupled wheat germ extract system (Promega, Madison, USA) with [^{35}S]-methionine (PerkinElmer, Massachusetts, USA). The Co-IP assay was carried out using the Matchmaker kit (Becton-Dickinson, San Jose, USA) following the manufacturer's instructions. Gels were dried and scanned using the Typhoon 9200 PhosphorImager (Amersham Pharmacia Biotech, Piscataway, USA).

Preparation of recombinant proteins

cDNAs of *GhACS2* and *GhCPK1* were cloned into pET28a(+) (Novagen, Madison, USA), fused with a gene fragment encoding His-tag at N-termini using the same primers with those used in Co-IP assay. The constructs were verified by sequencing.

GhACS2 mutants, GhACS2-mut1, and GhACS2-mut3, were generated with *GhACS2*-specific primers **(Table 1)** and GhACS2-mut2 was generated by using GhACS2-mut3 as the template and the primers using for GhACS2-mut1 by QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, USA). The constructs were transformed into *Escherichia coli* Rosetta (DE3) cells. For expression of GhACS2, cells were induced with 0.4 mM isopropyl-1-thio-β-galactoside (IPTG) for 6 h at 37°C. For expression of GhCPK1, cells were induced with 0.6 mM IPTG for 18 h at 18°C. His-tagged proteins were purified using nickel columns (Novagen). The recombinant GhACS2 was concentrated using Vivaspin 20 (Sartorius, Hannover, Germany) and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was determined by Bradford method using bovine serum albumin as the standard.

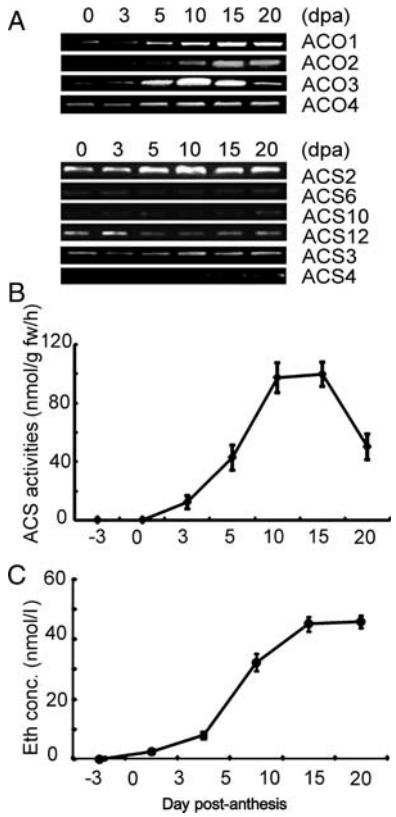

Figure 1 Comparisons of known *ACS* and *ACO* transcript levels, the total ACS activity, and ethylene production during cotton fiber development (A) RT-PCR analyses of the transcripts of the four *ACO* genes (upper panel) and six *ACS* genes (lower panel) identified from the cotton transcriptome. The total RNA samples prepared from wt cotton ovules at defined growth stages (dpa) were used as templates for the analysis. (B) ACS activity analysis throughout the fiber development. The protein extracts prepared from wt cotton ovules at defined growth stages were used for the assay. ACS activity was calculated as nanomoles of ACC produced per gram fresh-weight per hour. (C) Ethylene production from cultivated cotton ovules. The concentration of ethylene produced from cotton ovules harvested at 1 dpa or cultured for a different period of time was measured according to a method described previously [5]. The wt ovules −3, 0, 3, 5, 10, 15, and 20 dpa indicate either RNA or protein extracts prepared from variable developmental stages. All the experiments were repeated three times independently and reported as mean ± SE.

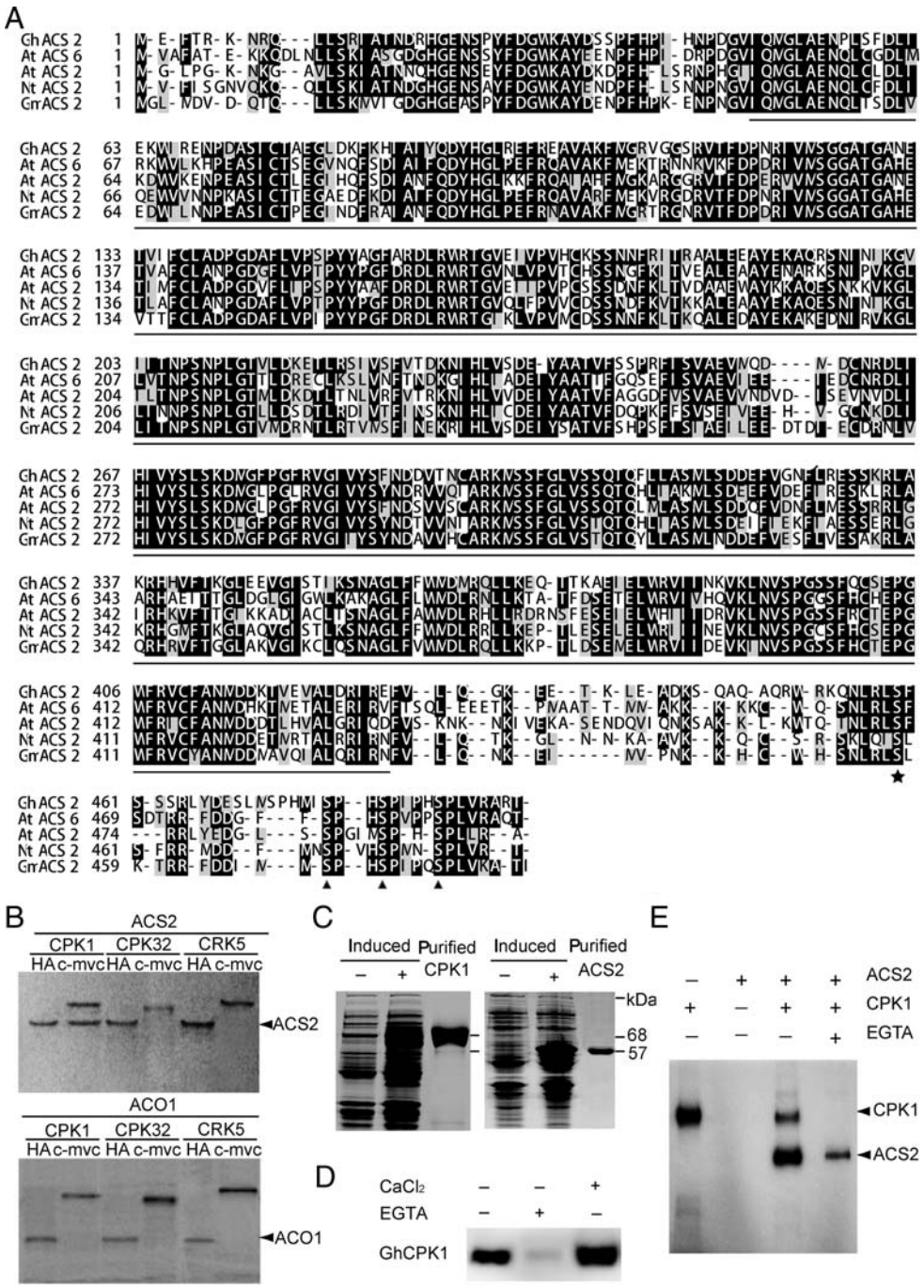

Figure 2 Cotton ACS2 was phosphorylated by recombinant GhCPK1 *in vitro* (A) Multiple sequence alignments of plant ACSs. Gh, *Gossypium hirsutum*; At, *Arabidopsis thaliana*; Nt, *Nicotiana tabacum*; Gm, *Giycine max*. The highly conserved aminotransferase domain is underlined, with the star and the triangles marking putative CPK phosphorylation or MAPK phosphorlyation sites, respectively. (B) Co-immunoprecipitation revealed that cotton CPK1 interacted with ACS2, which was detected by anti-myc antibody and anti-HA antibody. Cotton *ACS2* and *ACO1*, encoding the major transcripts in its class, respectively, were expressed and translated as HA- tagged proteins, and *CPK1*, *32* and *CRK5* were expressed and translated as c-myc- tagged proteins in a wheat germ cell-free system. (C) Purification of recombinant CPK1 and ACS2. The full-length *CPK1* and *ACS2* cDNAs were expressed in *E. coli* as His-tag fusion proteins. The purified proteins were analyzed by SDS-PAGE followed by Coomassie Brilliant Blue staining. (D) CPK1 underwent auto-phosphorylation *in vitro* in a Ca^{2+}-dependent manner. (E) *In vitro* phosphorylation of ACS2 by CPK1 was Ca^{2+}-dependent. '+' and '−', with or without adding the denoted protein or EGTA in the reaction. After electrophoresis, the phosphorylated proteins were visualized by autoradiography.

In vitro phosphorylation

Recombinant GhCPK1 protein (0.2 μg) was incubated with GhACS2 (2 μg) in the buffer containing 20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, and 1 mM DTT. Reactions were started by the addition of 20 μM ATP and 0.1 μCi γ-^{32}P-ATP. After 20 min, reactions were stopped by the addition of loading buffer, and then run on PAGE gel. The phosphorylation levels of ACS were visualized by Typhoon

9200 PhosphorImager. For inhibition of CPK activities, 0.1 mM EGTA was added in the reaction mixture.

ACS activity assay

The ACS activity assay was performed as described previously [26]. Briefly, 500 mg cotton ovules were collected and grounded in liquid nitrogen, subsequently, extracted with two volumes of buffer [100 mM HEPES, pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM DTT, 1 mM Na_3VO_4, 10 mM NaF, 50 mM β-glycerolphosphate, 10 μM pyridoxal 5'-phosphate, 10% glycerol, and complete protease inhibitors (EDTA free; Roche, Mannheim, Germany)]. The mixture was vortexed vigorously and the suspension was used for total ACS activity assay. The assay was performed using 800 μl suspension in 1 ml reaction buffer (100 mM KH_2PO_4, pH 8.0, 1 mM EDTA, 2 mM DTT, 10 μM pyridoxal 5'-phosphate, 500 μM SAM) at 30°C for 2 h. The total ACC contents in the ACS assay were quantified [27]. In the assay, ACC was converted to ethylene immediately via chemical reaction and the produced ethylene was detected by gas chromatography. ACS activity was calculated as nanomoles of ACC production per gram fresh-weight per hour after subtracting the ACC in the suspensions. For detecting the activity of phosphorylated recombinant ACS2, *in vitro* phosphorylation mixture (containing 1 μg purified ACS2) was applied for ACS activity assay.

Statistical analysis

Data were shown as mean ± SE. Statistical analysis was conducted with one-way analysis-of-variance (ANOVA) software combined with Tukey's test. $P < 0.05$ was considered statistical significance.

Results and Discussion

Elevated ethylene biosynthesis during fiber fast elongation correlates to the elevation of ACS activity

The availability of ACC synthesized from SAM by ACS is generally considered to be the rate-limiting step in the ethylene biosynthesis pathway [12]. In this work, transcript levels of four putative full-length cotton *ACOs* and five full-length *ACSs* plus one partial *ACS* from 0 to 20 dpa wt cotton ovule or fibers (10 dpa or older) were analyzed by semi-quantitative RT-PCR. Among all cotton *ACS* genes, full-length *ACS3*, *10*, *12*, and the partial *ACS4* were first reported in the current work. Transcripts for all *ACOs* accumulated significantly from 5 to15 dpa, and declined from 15 to 20 dpa [**Fig. 1(A)**, upper panel]. The levels of most *ACS* transcripts, including *ACS3*, *4*, *6*, *10*, and *12* did not change throughout various fiber developmental stages. The level of *ACS2* showed a slight increase from 3 to 10 dpa [**Fig. 1(A)**, lower panel]. A clear-cut increase in total ACS activity from 3 dpa to 10–15 dpa wt cotton ovules that reached the platform was observed [**Fig. 1(B)**]. The increase in ACS activity coincided with a significant burst in the amount of ethylene released [**Fig. 1(C)**], suggestive of a post-transcriptional, but not transcriptional regulation of ACS.

GhACS2 was phosphorylated by GhCPK1 *in vitro*

As *ACS2* showed most abundant amount of transcripts in any developmental stage [**Fig. 1(A)**], it was used for further studies. Sequence alignment of plant ACSs [**Fig. 2(A)**] revealed that the *GhACS2* we cloned is an ortholog of the previously reported *Arabidopsis* gene, with several potentially conserved phophorylation sites, including a possible CPK phosphorylation site [22] and three putative MAPK phosphorlyation sites [18]. *GhACS2* and *GhCPK1* were expressed and translated as HA- or c-myc-tagged proteins, respectively, in a wheat germ cell-free system, and were subjected to immunoprecipitation analysis. Co-migrated bands were detected in the reaction mixture containing GhCPK1 and GhACS2 using anti-c-myc tag [**Fig. 2(B)**], indicating that GhCPK1 interacted with GhACS2. No interaction was observed between GhCPK1 and GhACO1, between GhACS2 and GhCPK32 or GhACS2 and GhCRK5 [**Fig. 2(B)**], indicating that the interaction between GhACS2 and GhCPK1 is specific and may bear certain physiological importance. GhCPK1 and GhACS2 were overexpressed in *E. coli* cells, and further purified as recombinant proteins having approximately molecular mass of 68 and 57 kDa [**Fig. 2(C)**], which was consistent with their theoretical values, 69.1 and 57.9 kDa,

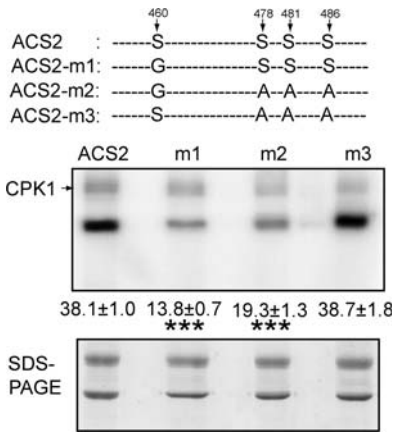

Figure 3 Cotton ACS2 Ser^{460} mutation affected CPK phosphorylation efficiency The potential conserved phosphorylation sites of cotton ACS2 for CPK and MAPK were indicated. ACS2 and its mutated variants were phosphorylated by cotton CPK1 and radioactivity of phosphorylated ACS2 proteins was quantified with a Molecular Dynamics Typhoon 9210 Phosphorimager. Statistical significance of the value was determined using one-way ANOVA software combined with Tukey's test. ***$P < 0.001$. The gel was subsequently stained with Coomassie Brilliant Blue.

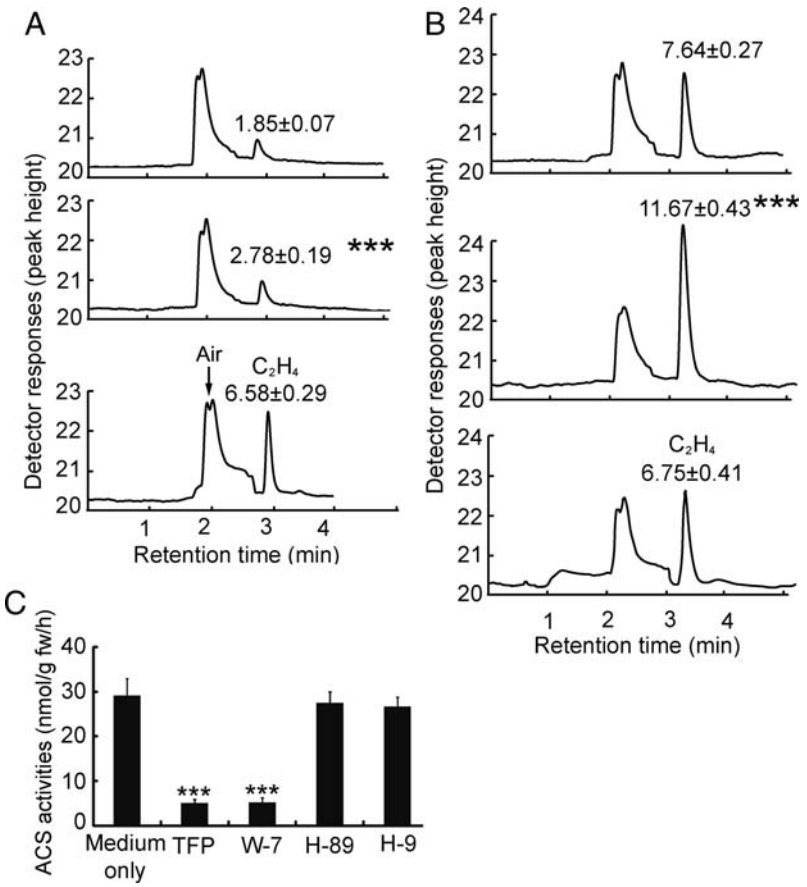

Figure 4 Phosphorylated ACS2 possessed significantly higher ACS activity (A) ACS activity measured in the presence of 10 μM SAM with unphosphorylated ACS2 (upper panel) and phosphorylated ACS2 (middle panel). Bottom panel, 1 p.p.m. ethylene standard. (B) ACS activity measured in the presence of 50 μM SAM with unphosphorylated ACS2 (upper panel) and phosphorylated ACS2 (middle panel). Bottom panel, 1 p.p.m. ethylene standard. Three independent experiments were performed. The figures represented one of the experiments. (C) CPK-specific inhibitors [5 μM TFP and 5 μM *N*-(6-aminohexyl)-5-chloro-1- naphthelenesulfonamid-hydrochloride (W-7)] significantly inhibited ACS activity, but, PKA inhibitor (5 μM H-89) and PKC inhibitor (5 μM H-9) were not effective. Treatment of cotton ovules with different inhibitors was performed as previously described [5]. Statistical significance was determined using one-way ANOVA software combined with Tukey's test. ***$P < 0.001$.

respectively. Ca^{2+} was able to significantly enhance the autophosphorylation of recombinant GhCPK1, whereas the addition of EGTA suppressed the autophosphorylation process substantially [**Fig. 2(D)**], suggesting that GhCPK1 autuphosphorylation is Ca^{2+}-dependent. *In vitro* phosphorylation assay was performed using purified recombinant GhCPK1, GhACS2 in the presence of [γ^{32}P]-ATP. After 20 min incubation of the reaction mix, phosphorylated GhACS2 was observed from the autoradiography that contained SDS-PAGE separated target proteins [**Fig. 2(E)**, the third lane]. In the presence of 1 mM EGTA, a significantly weaker band was detected, showing that GhCPK1 phosphorylated GhACS2 was in a Ca^{2+}-dependent manner [**Fig. 2(E)**, the last lane].

Sequence alignment of GhACS2 revealed that several potential conserved phophorylation sites are present in its amino acid sequence [**Fig. 2(A)**], containing a possible CPK phosphorylation site (S460) [22], and three putative MAPK phosphorlyation sites (S478, S481, S486) [18]. To investigate whether S460 is CPK1 phosphorylating site, recombinant ACS2 proteins with single, triple, quadruple Ser to Gly (G) or Ala (A) mutations were prepared. The ability of these proteins to serve as GhCPK1 substrate was determined by phosphorylation assays. Compared with that of the wt ACS2, the level of CPK phosphorylation was significantly decreased in both the S460G single mutant (ACS2-m1) and S460G/S478A/S481A/S486A quadruple mutant (ACS2-m2) (**Fig. 3**). CPK1 phosphorylation efficiency did not significantly change in the S478A/S481A/S486A triple mutant (ACS2-m3) (**Fig. 3**, mid panel), indicating that GhACS2 S460 is a possible phosphorylation site for the enzyme. This is supported by the recent finding that LeCDPK2 phosphorylated LeACS2 at the same site [24].

Phosphorylation of GhACS2 by GhCPK1 increased ACS activity

To understand the physiological importance of ACS phosphorylation, we assayed the activity of the purified recombinant GhACS2 or the *in vitro* phosphorylated GhACS2 using SAM as the substrate. The enzymatic product, ACC, was

converted to ethylene immediately, in the assay, via a chemical reaction catalyzed by Hg^{2+} and NaOCl [27]. The activities of the native and phosphorylated ACS2 were 1.85 ± 0.07 and 2.78 ± 0.19 mol/mg/h, respectively, using 10 μM SAM as the substrate [**Fig. 4(A)**], and were 7.64 ± 0.27 and 11.67 ± 0.43 mol/mg/h, respectively, using 50 μM SAM as the substrate [**Fig. 4(B)**]. Compared with that of the unphosohorylated form, we saw ~50% increase of the enzyme activity from phosphorylated ACS, under low or high substrate concentration. We cannot exclude the possibility that phosphorylation of GhACS2 by GhCPK1 may also increase the stability of GhACS2, leading to sustained high-level ethylene production. Further, treatment of wt ovules with CPK inhibitors, TFP, and *N*-(6-aminohexyl)-5-chloro-1-naphthelenesulfonamid-hydrochloride (W-7) [28], significantly suppressed total ACS activity [**Fig. 4(C)**], suggesting that CPK activity is important for the function of cotton ACS.

The identification of cotton ACS2 as the substrate of CPK1 uncovers an important regulatory mechanism of ethylene biosynthesis during cotton fiber development. It has been reported that, in *Nicotiana tabacum*, CPK signaling activated ethylene-mediated biological processes following both biotic and abiotic stress stimuli [25]. Interestingly, ethylene is found to increase cytosolic $[Ca^{2+}]$ via activation of Ca^{2+}-permeable channel in tobacco cells [29]. MAPK cascade including MKK9-MPK3/MPK6 can activate ethylene biosynthesis [18,20,30] or ethylene signaling via the transcription factor EIN3 [19]. We propose that the regulation of *ACO* genes at the transcript levels [5] together with post-translational regulation of ACS shown in the present study may synergistically contribute to promote the ethylene burst required for fast fiber cell elongation. Future analysis will focus on how ethylene biosynthesis and signaling is regulated by CPK, which may shed new lights on the molecular mechanisms controlling key aspects of cotton fiber development.

Accession numbers

Sequence data from this work can be found in Genbank/EMBL databases under following accession numbers: GhCPK1 (FJ938290), GhCRK5 (AAZ83348), GhCPK32 (FJ938291), GhACS2 (ABM66813), GhACS6 (DQ122174), GhACS3 (JF508502), GhACS12 (JF508503), GhACS10 (JF508504), GhACS4 (JF508505), GhACO1 (DQ116442), GhACO2 (DQ116443), ACO3 (DQ116444), ACO4 (DQ122175), and GhUBQ7 (DQ116441).

Funding

This work was supported by grants from the National Basic Research Program of China (2010CB126000) and the National Natural Science Foundation of China (90717009).

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