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A Wound-induced ACC Synthase Gene of Moso Bamboo Shoot

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Abstract: The present study was carried out to clone and sequence 1-aminocyclopropane-1-carboxylate synthase (ACC synthase, pBA-ACS) from a sliced moso bamboo shoot using reverse transcription and polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends-PCR (RACE-PCR). The cDNA clone of pBA-ACS was 1793 bp in length and contained a 5'-untranslated region of 126 bp, an open reading frame of 1446 bp encoding 482 amino acids and 3'-untranslated region of 221bp containing stop codone. The pBA-ACS was highly homologous to ACC synthase genes from rice, followed by apple and arabidopsis. In northern analysis, expression of pBA-ACS mRNA was enhanced in wounding tissue until 24 h and coincided with the peak of ACS synthase activity but disagreed with ethylene production. The ACC synthase activity suggested that the increase might be the response to the wounding associated with harvest.

Key words: ACC synthase, moso bamboo shoot, northern analysis

Introduction

The plant hormone ethylene, plays an important role in regulating of various physiological responses during plant growth and development. These include fruit ripening, flower sense cence and abscission in response to external stresses. Ethylene is synthesized from methionine via s-adenosylmethionine (SAM) and ACC in higher plants (Adams and Yong, 1979). ACC synthase (EC 4.4.1.14) and ACC oxidase (EC 1.4.3) play essential roles in this pathway. ACC synthase catalyzes the conversion of SAM to ACC and the reaction is regarded as the rate limiting-step, whereas ACC oxidase catalyzes the oxidation of ACC to ethylene and is called as the ethylene-forming enzyme. The accumulation of ACC synthase gene correlated with ethylene production, while large accumulation of ACC oxidase gene was observed in preclimactreric raise (Liu *et al*., 1999). Expression and sequence of ACC synthase has been investigated in many fruits and vegetables, such as tomato (Olson *et al*., 1991; Lincoln *et al*., 1993), apple (Yip *et al*., 1991), banana (Liu *et al*., 1999), broccoli (Pogson *et al*., 1991), pumpkin (Sato *et al*., 1991) winter squash (Nakajima *et al*., 1990). Matsui *et al*. (2001) reported the cloning and characteristic of the ACC oxidase gene and the relation between the enzyme activity and gene expression from wounded bamboo shoot. Moso bamboo is usually harvested and it is very perishable after harvest. If ACC synthase genes of the bamboo shoot will be completely elucidated like in tomato, longer shelf life of bamboo shoot may be produced by using antisense RNA technology.

In this study, we report the cloning and characterization of the ACC synthase gene from wounded Moso bamboo shoot the changes in the enzyme activity and gene expression during storage. The gene structures and the deduced peptide sequences will be also analyzed to clarify the phytogenic relationship among several ACC synthase in other plant species.

Materials and Methods

Plant materials: Moso bamboo shoots were harvested a farmer's field in Kagawa, Japan in April 1999. The shoots were immediately brought to the laboratory, cut into 3 mm in thickness after peeling off the bracts and incubated at 25°C for 0, 8, 16 and 24 h under humid and dark conditions. After the treatment, samples were immediately stored at -80°C until RNA needed for extraction.

ACC synthase activity assay and ethylene production: Frozen tissue (2 g) was added in liquid nitrogen and grounded in a cooled mortar and pestle with 4 ml of extraction buffer (400 mM K-phosphate pH 8.5, 10 µM pylidoxal phosphate (PLP), 0.5% mercaptoethanol and 20% glycerol). The resulting homogenate was filtered through 4 layers Kimwipe and the filtrate was charged on a dry Sephadex G-25 column (1.5 i.d. x 7 cm) prepared by centrifuging at 3,000 rpm for 3 min at 4°C just before use. The elute solution recentrifuged at 3,000 rpm for 5 min was used for the enzyme assay, according to the method described by Lizada and Yang (1979). To 0.4 ml of the

elute solution, 0.2 ml of 50 mM Hepes-KOH buffer containing 10 μM PLP and 200 μM SAM was added and afterwards it was incubated at 30 °C for 15min, followed by mixing with 400 μl of stopping reagent [Bleach-NaOH (2:1, v/v) and 10 mM $HgCl_2$ in the ratio of 1:3 (v/v)]. A blank experiment was carried out using ice bath instead of 30°C and maintaining the concentration of reagent and the same procedure. After immediately putting a stopper, the tube was shaken vigorously for 15 sec and kept in the ice bath for 3 min. Ethylene produced in the head space (1 ml) of capped tubes was determined by gas chromatography (GC).

RNA extraction, isolation and amplification of poly $(A)^+$ RNA: Total RNA was extracted by the ISOGEN method (Nippon gene, Osaka, Japan) according to the manufacturer's protocol. Poly $(A)^+$ RNA was prepared from the total RNA by Oligo(dT)-cellulose chromatography (BIO-RAD, Tokyo, Japan) as described by Watanabe (1989). To isolate ACC synthase fragments, degenerate oligonucleotide primers homologous to conserved regions of ACC synthase were synthesized. The degenerate oligonucleotide primers for PCR were synthesized to two amino acid domains conserved in various ACC synthase genes, IQMGLAE for the sense primer (5'-TYCARATGGGTCTHGCDGAA-3') and AGLFCWV for the antisense primer (5'-ACCCARCARAASARDCCNGC-3'),respectively. The first strand cDNAs, synthesized from 2 g of poly$(A)^+$ RNA isolated from wound-treated bamboo shoots, were performed with a SuperScript ™ Preamplification System for first strand cDNA (BRL, Tokyo, Japan) containing RT. In order to produce automatically cohesive end after a digestion of uracil, uracil primers containing the degenerate oligonucleotide primers mentioned above were synthesized. One is 5'-CUACUACUA-IQMGLAE for the sense primer and the other is 5'-CUACUACUA-AGLFCWV for the antisense primer. The uracil primers were amplified by PCR. The parameters for PCR were 50 cycles of heating at 94°C for 30 s, at 57 and 72°C for 30 sec. The PCR products were then digested with uracil DNA glycosilase and cloned into the pAMP1 vector (BRL, Tokyo, Japan) by the methods of the Instruction manual. Full-length cDNA encoding bamboo cDNA was amplified by RACE method. All reactions were performed with Marathon ™ cDNA Amplification Kit by the methods described in the manual (CLONETECH, Tokyo, Japan). The cDNA was synthesized from poly $(A)^+$ RNA isolated from sliced bamboo shoots and it was ligated to the Marathon cDNA adaptor. The 5'cDNA fragment was amplified by PCR using bamboo shoot cDNA specific primer (5'-CGGTCGACGAGGTCCTTGGACAGGCTGTACACG-3') and the adaptor primer that was supplied in the kit as primers and the adaptor-ligated cDNA as a template. The 3' cDNA fragment was amplified by PCR using the bamboo shoot cDNA specific primer (5'-GTCGACAGCCTGTCCAAGGACCTCG-3') and the same adaptor primer in the kit as primers and the adaptor-ligated cDNA as a template. The parameters for PCR were 30 cycles of heating at 94 and 60°C for 30 sec and at 70°C for 3 min. The PCR products were cloned into the pSPORT 1 vector (BRL, Tokyo, Japan).

Sequencing of DNA: The cDNA inserts excised with Not I and Sal I from pAMP 1 vector and cloned into the pSPORT 1TM Not I-Sal I-cut (BRL, Tokyo, Japan). A series of deletion mutants were performed with deletion kit (Nippon gene, Osaka, Japan) by exonuclease III. DNA sequencing was performed by the cycle sequencing methods using GATCR-biocycle sequencing kit and a DNA sequencer GATC 1500 Long-Run system (GATC GmbH, Konstanz, Germany).

Sequence data analysis: Sequence analysis was performed using computer software from the GENETYX-MAC Version 7. Homology searches with the Genebank and the EMBL databases were performed using the Homology programme in the software. The phylogenetic tree was also constructed with the UPGMA method in the software.

Preparation of the digoxigenin (DIG)-UTP-labeled RNA probe: The cloned RT-PCR product including the coding region of ACC synthase gene was cleaved by Not I and Sal I from the pSPORT 1 vector that had been amplified in *Escherichia coli* (DH-5α) and it was purified by gel electrophoresis and recovered. Antisense DIG-labeled RNA probes were prepared using cloned RT-PCR product and the DIG RNA labeling kit (Bohringer Mannhaim) according to the manufacturer's instruction.

Northern blot hybridization: Ten μg of Poly $(A)^+$ RNA was subjected to electrophoresis on a 1.0% agarose gel that contained 0.66 M formaldehyde and transferred to Hybond-N^+ (Amersham) by capillary action with 20 x SSC, according to manufacturer's instruction. After drying the membrane, the RNAs were fixed with UV. The membrane was preincubated in 5 x SSPE, 5 x Denhart's solution and 0.5% SDS at 60°C for 3 h and then probed with the gene-specific antisense DIG-labeled RNA probe at 60°C for 24 h. The membrane was washed once with 2 x SSPE containing 0.1% SDS for 10 min at room temperature, once with 1 x SSPE plus 0.1% SDS for 15 min at 65°C and twice

with 0.2 x SSPE containing 0.1% SDS for 15 min each at 65°C. After allowing sheep anti-DIG Fab fragments conjugated to alkaline phosphatase to bind to the DIG-labeled probe, the excess antibody was removed. Signals were detected by color reaction using 5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium salt as the substrate. RNA samples were ensured by measuring UV spectrometer (UV-1200, Shimazu, Japan) and hybridizing an internal control gene, bamboo actin cDNA.

Results

Isolation and identification of cDNA: The cDNA clone of pBA-ACS was 1793 bp in length and contained a 5'-untranslated region of 126 bp, an open reading frame of 1446 bp encoding 482 amino acids and 3'-untranslated region of 221 bp containing stop codon (Fig. 1). The alignment of deduced amino acid sequence showed that ACC synthase gene was highly homologous to the others isolated from other plant (Fig. 2). pBA-ACS had the highest percentage of sequence homology with rice (M96673) followed by wheate ACS2 (U35778) and mung bean (AB000679); 87.8, 68.2 and 63.9% at nucleotide, respectively and had the highest percentage of sequence homology with rice (M96673) followed by apple (L31347) and mung bean (AB000679); 86.7, 64.1 and 63.9% at amino acid respectively, all of which have been to be wound-inducing genes (Table 1).

Table 1: Percentage of nucleic acid and amino acid identity between ACC synthase from bamboo shoot and the other plants in database

Plants	Nucleic acid (%)	Amino acid (%)
Apple-ACS (L31347)	63.4	64.1
Arabidopsis-ACS (U23481)	62.9	63.6
Banana-ACS (AF080258)	62.9	54.6
Mung bean-ACS (AB000679)	63.9	63.9
Pea-ACS (AF016458)	62.3	63.9
Pear-ACS (X87112)	63.2	63.4
Rice-ACS (M96673)	87.8	86.7
Tomato-ACS (m34289)	53.9	51.6
Wheat-ACS1 (U35779)	55.4	47.3
Wheat-ACS2 (U35778)	68.2	56.1
Citrus-ACS (AJ012551)	56.3	54.4
Papaya-ACS (U68216)	54.9	51.2
Tabaco-ACS (X65982)	54.8	50.6

Bamboo shoot-ACS (AB085172) is calculated as 100%

Wound-inducing ethylene synthesis: Ethylene production was induced in the shoot after slicing [Fig. 3 (A)]. The production rate started to increase after slicing, reaching a peak at 16 h after slicing and then started to decline. The rate of production was about four times higher than that of initial (0 h) production rate.

Expression of ACC Synthase gene: The expression of pBA-ACS was induced by wounding. Maximum pBA-ACS expression appeared 24 h after wounding and coincided with the peak of ACC synthase activity (Figs. 3B and 4) which disagreed with ethylene production. A phylogenetic tree was reconstructed from an alignment of the deduced amino acid sequence from the Moso bamboo shoot ACC synthase with other ACC synthase in the database. The pBA-ACS (accession no. AB085172), ACS from rice (M96673) and arabidopsis (U23481) clustered together strongly, with the closest relationship Moso and rice. Other wound associated ACC synthase are indicated in Fig. 5.

Rice and bamboo shoot belong to monocotyledon plant and other apple and pea belong to dicotyledon plant.

Discussion

To understand ethylene biosynthesis, this study isolated a cDNA for ACC synthase from bamboo shoot which is wounding-related. This clone, pBA-ACS, had highly sequence similarity to rice, mung bean and soybean cDNA that were also associated with wounding. Northern blot analysis revealed that the expression pBA-ACS increased in sliced bamboo shoot until 24 h of storage period at 20°C. The sliced bamboo shoot produced a considerable amount of ethylene in response to the wounding and ACC synthase activity was also induced in the shoot. The increase in ACC synthase activity occurred concurrently with the increase in the abundance of pBA-ACS transcription (Fig. 4) suggesting well that the transcription of pBA-ACS led to the increase in wound-induced ACC synthase activity but disagree ethylene production in the bamboo shoot. The expression of ACC oxidase in the previous paper (Matsui *et al.*, 2001) and ACC synthase in bamboo shoot increased coincidently with the increase in ethylene production. Liu *et al.* (1999) have reported that the expression of the ACC oxidase and synthase genes in flesh banana were observed before and onset of climacteric rise, respectively. Therefore, the highest ethylene in Fig. 3 (A) might be included the ethylene produced strongly by ACC oxidase. The highest ACC oxydase activity (Matsui *et al.*, 2001) occurred 8 h ahead of the highest ACC synthase activity. This phenomenon suggested that the accumulation of pBA-ACS mRNA correlated with the relation of ethylene production, while the accumulation pBA-ACO mRNA was observed at earlier stage of wounding.

The phylogenetic tree was constructed form an optical alignment of proteins using the UPGMA method of GENETIX-MAX software. Accession numbers of sequences (from top to bottom of the tree): L31347, X87112, AB000679, AF016458, U23481, AB085172, M96673, AF0180258, U35778, AJ012551, U68216, X65982, M34289, U35779.

Fig. 1: Nucleic acid sequence and derived polypeptide sequence of the pBA-ACS cDNA. The arrows indicate the positions of degenerated primers used for RT-PCR and RACE-PCR

Fig. 2: Alignment of amino acid sequences deduced from bamboo shoot (AB085172), rice (M96673), apple (L31347), pea (AF016458), banana (AF080258) and mung bean (AB000679)

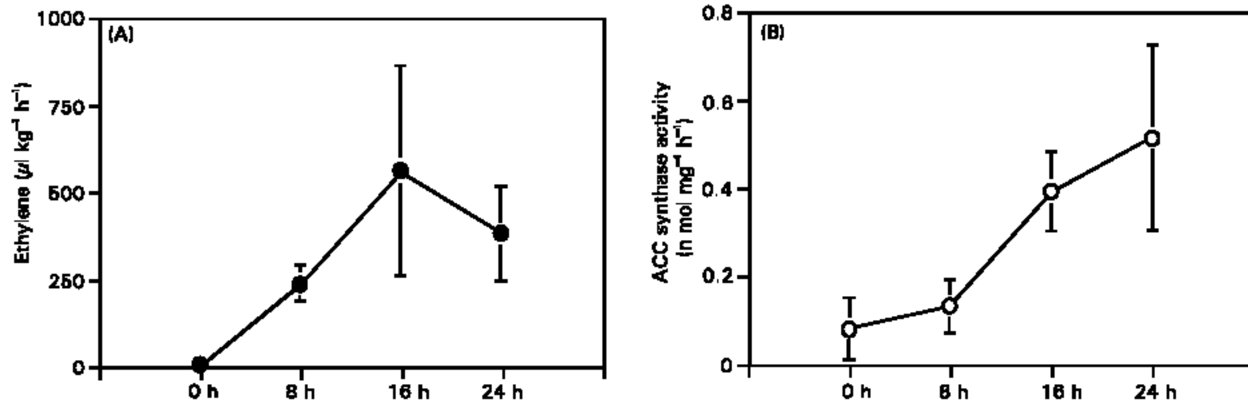

Fig. 3: Change in ethylene production (A) and ACC synthase activity (B) after wounding treatment of bamboo shoot. Each point represents the mean of 3 replication. The bar shows the standard error (SE)

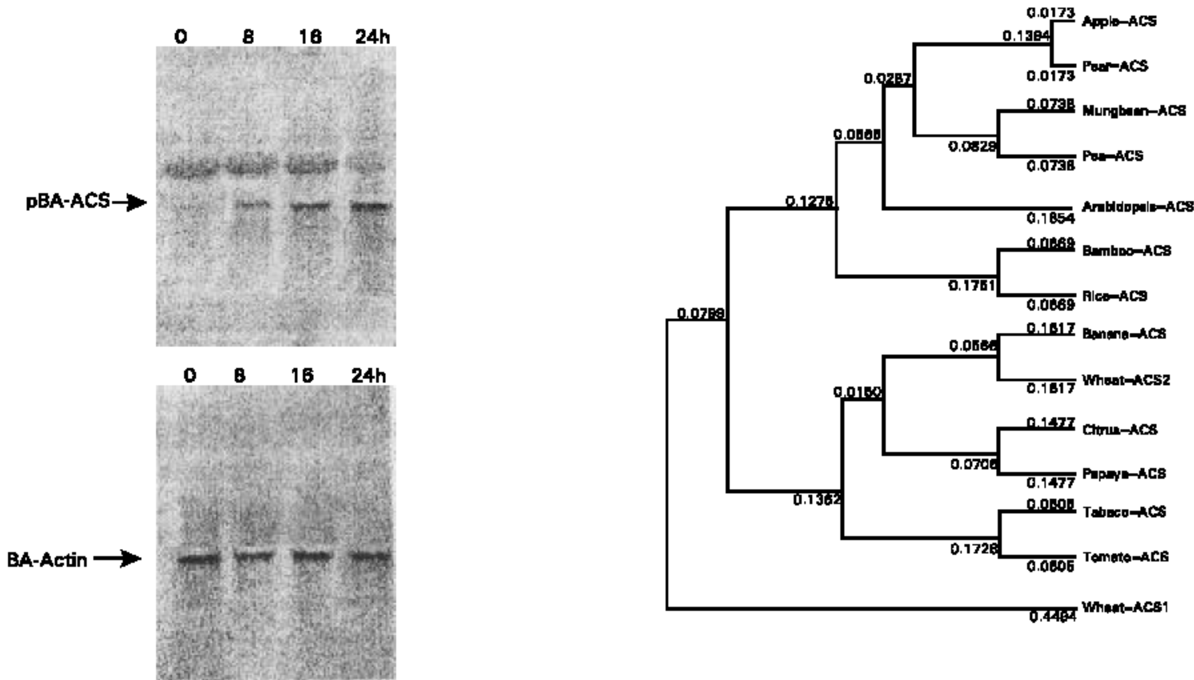

Fig. 4: Northern analysis of total RNA (10 µg per lane) expressed form bamboo shoot following wounding treatments. RNA was extracted from tissue after 0, 8, 16 and 24 h of wounding treatments

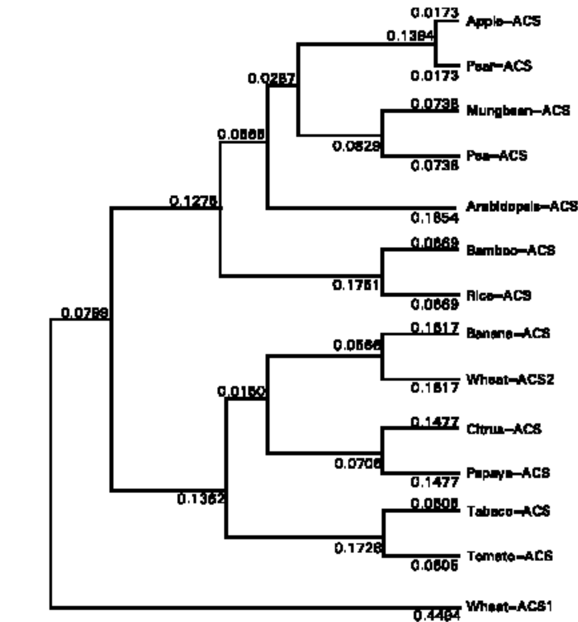

Fig. 5: Phylogetic analysis of ACC synthase amino acid sequences from bamboo shoot and other ACC synthase in the database

ACC synthase can be induced by various factors, such as fruits ripening (Nakatsuka *et al.*, 1997), auxin (Kim *et al.*, 1992; Yi *et al.*, 1999), senescence of flower (Rottmann *et al.*,1991; Have and Woltering, 1997), elicitor (Oetiker *et al.*, 1997), carbobn dioxiside stress (Mathooko *et al.*, 1999) and ozone induce (Schlagnhaufer *et al.*, 1995). The result was consisted with that observed in wound-induced winter squash (Nakajima *et al.*, 1990), zucchini (Huang *et al.*, 1991) tomato (Lincoln *et al.*, 1993) and so on. Phylogenetic analysis of ACS sequences has revealed the at least two major branches that contain characteristic conserved amino acid sequences, monocotyledon and dicotyledon. The pBA-ACS belonged to the subgroup wound-induced monocotyledon as it was highly homologous to rice ACS. Therefore, it can be concluded that induction of ethylene production in harvested bamboo shoot is regulated by transcription of pBA-ACS in response to the wounding associated with harvest.

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