cDNA Cloning of ACC Synthase and ACC Oxidase Genes in Cucumber Fruit and Their Differential Expression by Wounding and Auxin

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Summary

We cloned and sequenced three cDNA homologs of 1-aminocyclopropane-1-carboxylate (ACC) synthase (pCS-ACS1, pCS-ACS2 and pCS-ACS3) and two of ACC oxidase (pCS-ACO1 and pCS-ACO2) from cucumber (*Cucumis sativus* L.) fruit using RT-PCR (reverse transcription and polymerase chain reaction) and RACE (rapid amplification of cDNA ends) PCR. Conserved amino acid sequences reported for other plant tissues were found in all the cloned ACC synthase and ACC oxidase cDNAs. Among the ACC synthase and ACC oxidase cDNA clones, pCS-ACS1, pCS-ACS3, and pCS-ACO1 were highly homologous to ACC synthase and ACC oxidase genes cloned from other *Cucurbitaceae* family at both the nucleotide and the amino acid levels.

Northern blot analysis showed that wounding induced the accumulation of *CS-ACS1*, *CS-ACS2*, and *CS-ACO1* mRNAs, whereas IAA treatment induced expression of all the three ACC synthase genes and *CS-ACO1*. These expressions paralleled ethylene production in both treatments. Transcripts for *CS-ACO2* were constitutively expressed and were not affected by wounding and IAA treatments. From the expression pattern and the sequence similarities of these genes, we conclude that *CS-ACS1* could be a wound-inducible gene and that *CS-ACS3* is an auxin-inducible one. These results strongly suggest that in cucumber fruit these genes are differentially expressed by wounding and auxin and are involved in the regulation of ethylene biosynthesis at the transcriptional level.

Key Words: ACC oxidase (EC 1.4.3), ACC synthase (EC 4.4.1.14), *Cucumis sativus*, IAA, wounding.

Introduction

In higher plants, ethylene is synthesized from methionine via S-adenosyl-L-methionine and 1-aminocyclopropane-1-carboxylate (ACC) (Yang and Hoffman, 1984; Abeles et al., 1992). Recent studies have revealed that ethylene biosynthesis is mainly regulated by expression of both ACC synthase and ACC oxidase genes at the transcriptional level (Kende, 1993). cDNAs encoding ACC synthase and ACC oxidase have

been cloned from various plant organs. Furthermore, these enzymes are encoded by multigene families, and each of their expressions has been reported to be regulated by developmental, environmental, and hormonal factors (Theologis, 1992; Kende, 1993; Barry et al., 1996).

Fruits can be classified as climacteric or non-climacteric, depending on the presence or absence of an increase in respiration accompanied by ethylene production at the onset of ripening, and their response to exogenous ethylene treatment (Biale and Young, 1981; Alonso et al., 1995). Ripening in climacteric fruits is ethylene-dependent, whereas in non-climacteric fruits, it is ethylene-independent. In the *Cucurbitaceae* family, both climacteric and non-climacteric fruits are found; melons are mainly climacteric; cucumber, winter squash, and zucchini are non-climacteric. Generally, the postharvest handling of climacteric fruits is different from that of non-climacteric ones. Although cucumber fruit produce very little ethylene during maturation and senescence (Biale and Young, 1981), ethylene production is induced by external stimuli such as wounding (Shiomi et al., unpublished data), IAA (Nakagawa et al., 1991; Mathooko et al., 1993), high CO₂ concentra-

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tion (Kubo et al., 1990; Mathooko et al., 1995), and electric currents (Inaba et al., 1991), through activation of both ACC synthase and ACC oxidase, the two key enzymes in the ethylene biosynthetic pathway.

In *Cucurbitaceae* fruits other than cucumber, several ACC synthase genes have been cloned. In winter squash, the isolated cDNA for ACC synthase induced by wounding (*CM-ACSI*, Nakajima et al., 1990) is different from the one induced by IAA (*CM-ACS2*, Nakagawa et al., 1991). Huang et al. (1991) identified a gene encoding ACC synthase (*CP-ACSI*) which is expressed in wounded zucchini fruit tissue. In melon, *ME-ACSI* is the main ACC synthase gene expressed during fruit ripening (Yamamoto et al., 1995). Three putative ACC oxidase genes from melon fruit have been cloned and their differential expression during development and in response to ethylene treatment and wounding has been studied (Lasserre et al., 1996). Although *CM-ACSI*, *CP-ACSI*, and *ME-ACSI* are similar to each other in their nucleotide and amino acid sequences, the first two genes are only expressed in response to external stimuli such as wounding, whereas the latter is expressed during natural fruit ripening. We are interested in elucidating the fundamental differences in the ripening process between climacteric and non-climacteric fruits at the molecular level, using fruits belonging to the same *Cucurbitaceae* family, so that the information might contribute to the improvement of postharvest handling of fruits.

As a first step towards attaining the above goal, we describe the isolation of cDNAs encoding three ACC synthase (*CS-ACSI*, *CS-ACS2*, and *CS-ACS3*) and two ACC oxidase (*CS-ACO1* and *CS-ACO2*) genes from cucumber fruit, and analyzed the expression characteristics of these genes in response to wounding and IAA treatments.

Materials and Methods

Plant material and treatments

Cucumber (*Cucumis sativus* L. cv. Hokushin) fruit were obtained from an experimental farm of Okayama University. Fruit were harvested at commercial maturity (10-14 days after anthesis) and immediately brought to the laboratory.

For wounding, the fruit were cut into slices of 3 mm in thickness, which were further cut into four pieces of similar size, and incubated at 25°C for 12 hr under a humidified condition. For auxin treatment, the slices, cut in a similar manner, were treated with 1 mM IAA and incubated at 25°C for 12 hr as previously described (Mathooko et al., 1993).

Ethylene production

Ethylene production was measured by enclosing the wounded or IAA-treated fruit slices in 40 ml gas tight containers which were incubated at 25°C for 1 hr. Head

space gas was assayed for ethylene concentration by withdrawing a gas sample using a gas-tight hypodermic syringe and injecting it into a gas chromatography equipped with a flame ionization detector and an activated alumina column.

RNA isolation, cloning and sequencing

For total RNA extraction, the fruit slices were frozen in liquid nitrogen and stored at -80°C until used. The frozen tissue was pulverized to a fine powder with a mortar and pestle. Total RNA was extracted by the SDS-phenol method (Sambrook et al., 1989). Poly(A)⁺ RNA was isolated, using Oligotex dT-30 (Takara, Japan) according to manufacturer's instructions. The first strand cDNAs, synthesized from 2 µg of poly(A)⁺ RNA isolated from wound- or IAA-treated fruit, were amplified by RT-PCR (Sambrook et al., 1989) with degenerated oligonucleotide primers (5'-TG GGT YTN GCW GAR AAT CAG CT-3' as the upstream primer and 5'-A RCA AAC WCG RAA CCA MCC TGG-3' as the downstream primer for ACC synthase, and 5'-C SAR AAY TGG GGY TTS TWY GAG-3' as the upstream primer and 5'-TC RAA BCK YGG YTC YTT NG-3' as the downstream primer for ACC oxidase). These primers were synthesized with reference to the conserved amino acid sequences reported for other plant organs (Kende, 1993). The *Sph*I and the *Sal*I restriction site sequences were also included at the 5'-end of both sense and antisense primers, to facilitate subcloning of PCR products. Thirty thermal cycles were performed, each consisting of 1 min at 94°C, 2 min at 37°C, and 3 min at 72°C, using a thermal cycler. The amplified cDNAs were then digested with *Sph*I and *Sal*I and subcloned into pUC118 plasmid vector. The nucleotide sequences of the cDNA inserts were determined by the dideoxy method by overlapping sequences of the smaller fragments digested with various restriction enzymes, using a DNA sequencer (373A, Applied Biosystems). The sequence obtained was searched with GenomeNet database to determine whether or not the cloned cDNA was a fragment of ACC synthase or ACC oxidase.

From the nucleotide sequence of the cDNA fragments determined, gene specific primers were newly synthesized (Table 1) and RACE PCR (Frohman, 1993) was carried out to determine the 5'- and 3'-ends of each fragment with Marathon cDNA Kit (Clontech, USA) according to the manufacturer's protocol. The RACE PCR products were cloned into pCR2.1 vector (Invitrogen, USA) and then screened by overlapping sequences.

Northern blot analysis

Poly(A)⁺ RNA (8 µg), isolated as described from wound- or IAA-treated fruit slices, was separated electrophoretically on 1.0% agarose gels containing 0.66 M formaldehyde and 1 x MOPS (20 mM MOPS, 5 mM

Table 1. Gene specific primers for RACE PCR.

Specific primers	DNA sequence (from 5' to 3')	Position on the sequence ²
CS-ACS 1-5' RACE	CTCAGACATCTTACGTGCAATGCTCACGA	1047 - 1020
CS-ACS 1-3' RACE	GTCCAAAGATATGGAATCCCTGG	958 - 981
CS-ACS 2-5' RACE	CACGAAATCCAGGGAATCCCATG	933 - 911
CS-ACS 2-3' RACE	GCCACTGTCTTTGACAGGCCCGG	801 - 823
CS-ACS 3-5' RACE	CTCCTCTCCTTAAGCACCTCCATGGCGC	958 - 931
CS-ACS 3-3' RACE	GTGTTTGGAGAGTAGTGCTGGG	1247 - 1268
CS-ACO 1-5' RACE	GGTTGACGACAATGGCATGGCGCATT	730 - 705
CS-ACO 1-3' RACE	GGAGAATCTTGCTGAGGAGTTGT	444 - 466
CS-ACO 2-5' RACE	TATTCACACGATCGAGTGCCGGACC	745 - 720
CS-ACO 2-3' RACE	ACAAGTACTCGCCGAATCTCTGC	459 - 481

² CS-ACS 1, AB 006803; CS-ACS 2, AB 006804; CS-ACS 3, AB 006805; CS-ACO 1, AB 006806; CS-ACO 2, AB 006807

sodium acetate, and 1 mM EDTA, pH 7.0) then transferred to nylon membranes (Hybond N, Amersham). Equal loading of RNA samples was ensured by measuring RNA concentrations, using an UV spectrophotometer (UV-1600, Shimadzu, Japan) and hybridizing a control gene, ubiquitin (Callis et al., 1995). Blotting and hybridization were performed according to Sambrook et al. (1989) and Nakatsuka et al. (1997). cDNA probes, of which templates were cloned from RT-PCR products as cDNA fragments, were labeled by random primed DNA labeling kit (Boehringer Mannheim) with ³²P-dCTP at > 3000 Ci mmol⁻¹ (Amersham, Japan). Following hybridization, membranes were washed with 2 x SSPE and 1 x SSPE for 30 min at 60°C and were exposed to imaging plate (Fuji Photo Film, Tokyo) at room temperature prior to analysis for hybridization intensity, using Bio Imaging Analyzer (model BAS 1000, Fujix, Tokyo).

Results

Isolation and identification of cDNA clones

Using degenerated oligonucleotide primers, we cloned five fragments including three different cDNAs for ACC synthase (pCS-ACS1, pCS-ACS2 and pCS-ACS3) and two for ACC oxidase (pCS-ACO1 and pCS-ACO2) based on their deduced amino acid sequences. Using RACE PCR, full length cDNA sequences for each fragment was determined. CS-ACS1 contained an open reading frame of 1479 base pairs encoding 493 amino acids. The open reading frame for CS-ACS2 was 1467 base pairs encoding 489 amino acids, and for CS-ACS3, it was 1443 base pairs encoding 481 amino acids. Highly conserved regions for ACC synthase and the 11 residues which are invariant amino acids conserved in various aminotransferases (Rottmann et al., 1991; Kende, 1993) were also present in the three ACC synthase genes from cucumber fruit (Fig. 1).

The alignment of deduced amino acid sequences showed that each ACC synthase or ACC oxidase gene was highly homologous to the others isolated from

fruits in the Cucurbitaceae (Fig. 1, 2). CS-ACS1 had the highest percentage of sequence homology with ME-ACS1 (Yamamoto et al., 1995), followed by CM-ACS1 (Nakajima et al., 1990) and CP-ACS1 (Huang et al., 1991); 91%, 83%, and 82% at nucleotide, and 96%, 83%, and 83% at amino acid, respectively (Table 2); all of which have been reported to be wound-inducible genes. Although CS-ACS2 did not have a high sequence identity with other ACC synthase genes reported so far, CS-ACS3 had a high percentage of homology with only CM-ACS2 (Nakagawa et al., 1991), an auxin-inducible gene, at both the nucleotide and the amino acid levels, 81% and 90%, respectively (Table 2).

The deduced polypeptides encoded by CS-ACO1 and CS-ACO2 were similar in size, 317 and 314 amino acids, respectively (Fig. 2) and were 73% identical to each other. The identity at the nucleotide level was 66% (Table 3). Amino acids conserved in all members of the Fe(II) ascorbate family of dioxygenases (Lasserre et al., 1996) were also present in CS-ACO1 and CS-ACO2 (Fig. 2). CS-ACO1 was highly homologous to CM-ACO1 (Lasserre et al., 1996) with an identity of 96% at the nucleotide and 97% at the amino acid levels, whereas CS-ACO2 was at most 74% identical to other ACC oxidases at both levels (Table 3).

Expression of ACC synthase and ACC oxidase genes

The expression of CS-ACS1 and CS-ACS2 was induced by both wounding and IAA and the pattern was slightly different (Fig. 3). Maximum CS-ACS1 expression appeared 3 hr after wounding and coincided with the peak for ethylene production, whereas the mRNA level of CS-ACS2 was low and seemed to reach a maximum 6 hr after the treatment (Fig. 3). The level of CS-ACS2 mRNA was highest 6 hr after IAA treatment when IAA-induced ethylene production was also at maximum. The expression of CS-ACS1 was induced 3 hr after IAA treatment and did not change much thereafter. The accumulation of CS-ACS3 mRNA was detectable 3 and 6 hr after IAA treatment but was not detected in the wounded tissue (Fig. 3).

The synthesis of CS-ACO1 mRNA, induced by wounding and IAA, clearly paralleled the changes in ethylene production (Fig. 3). On the other hand, the CS-ACO2 mRNA level was almost constant throughout the treatment period. Transcripts for both CS-ACO1 and CS-ACO2 were at a detectable level even in the control fruit (0 hr).

Discussion

We cloned ACC synthase and ACC oxidase homologs from cDNAs of cucumber fruit, using RT-PCR and both 5'- and 3'-RACE PCR. Sequence analysis and homology search revealed that highly conserved regions and amino acid residues in ACC synthase and ACC oxidase genes which have been reported for other plant tissues were also present in the three ACC synthase and

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CS-ACS1      MG FQ Q T H E T N -- Q V L L S K I A I S D G H G E D S P Y F D G W K A Y D N D P F H P E N N P S G V I Q M G L A E N
CS-ACS2      M V L V S S N D N G N A K K V L S K L A K G N G H G E D S S Y F D G W K A Y D S D P F H P I I N P R G V I Q M G L A E N
CS-ACS3      ----- M K M L S T K A T C N S H G Q D S S Y F L G W E A Y E K N P F D E T S N P N G I I Q M G L A E N
ME-ACS1      M G F H Q T H E T N -- Q V L L S K I A I S D G H G E D S P Y F D G W K A Y D N D P F H P E N N P L G V I Q M G L A E N
CM-ACS1      M E F H Q I D E R N -- Q A L L S K I A V D D G H G E N S P Y F D G W K A Y D N D P F H P E D N P L G V I Q M G L A E N
CM-ACS2      ----- M K M L S T K A T C N S H G Q D S S Y F L G W E A Y E N N P F H T S N P N G I I Q M G L A E N
CP-ACS1A     M G F H Q I D E R N -- Q A L L S K I A L D D G H G E N S P Y F D G W K A Y D N D P F H P E N N P L G V I Q M G L A E N
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CS-ACS1      Q L S P D L L V D W I K K H P K A S I C T D E G L L N F K D I A N F Q D Y H G L P E F R N A I A S F M G K A R G G R V K
CS-ACS2      Q L S F E F V E K W M K N N P R A S I C S V E G I D E F K D I A I F Q D Y H G L P E F R N A V A N F M G K V R G N R V K
CS-ACS3      Q L S F D L L E S W L T K N P D A A S F K R D G K S I F R E L A L F Q D Y H G L P A F K K A L V E F M A E I R G N K V T
ME-ACS1      Q L S P D L I V D W I R K H P K A S I Y T D E G L L N F K D I A N F Q D Y H G L P E F R N A I A S F M G K A R G G R V K
CM-ACS1      Q L S F D M I V D W I R K H P E A S I C T P K G L E R F K S I A N F Q D Y H G L P E F R N G I A S F M G K V R G G R V Q
CM-ACS2      Q L S F D L L E S W L S K N P D A A S F K R D G K S I F R E L A L F Q D Y H G L P A F K K A L V E F M A E I R G N K V S
CP-ACS1A     Q L S F D M I V D W I R K H P E A S I C T P E G L E R F K S I A N F Q D Y H G L P E F R N A I A N F M G K V R G G R V K
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CS-ACS1      F D P S R I V M G G G A T G A S E A V I F C L A D P G D A F L I P S P Y Y A A F D R D L Q W R T R A Q I I P V H C N S S
CS-ACS2      F D P D R V M S G G A T G A H E T M A F C L A D P G E A F L V P V P Y Y P G F D R D L R W R T G V E I V P I C E S S
CS-ACS3      F E A N N I V L T A G A T S A N E T L M F C L A E A G D A F L L P T P Y Y P G F D R D L K W R T G V E I V P I H C T S S
ME-ACS1      F D P S R I V M G G G A T G A S E T V I F C L A D P G D A F L I P S P Y Y A A F D R D L Q W R T R A Q I I P V H C N S S
CM-ACS1      F D P S R I V M G G G A T G A S E T V I F C L A D P G D A F L V P S P Y Y A A F D R D L K W R T R A Q I I R V H C N S S
CM-ACS2      F E A N N I V L T A G A T S A N E T L M F C L A E A G D A F L L P T P Y Y P G F D R D L K W R T G V E I V P I H C T S S
CP-ACS1A     F D P S R I V M G G G A T G A S E T V I F C L A D P G D A F L V P S P Y Y A G F D R D L K W R T R A Q I I R V H C N R S
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CS-ACS1      N N F Q I T R E A L E V A Y K K A E S N I K V K G L I I T N P S N P L G T I Y D R D T L K S L V N F V N D N N I H L V
CS-ACS2      N N F K L T R E A L E T A Y E E A Q K S N I K I K G L I I T N P S N P L G T I Y D R Q T L E T A V S F I N E K N I H L V
CS-ACS3      N G F Q V T Q P A L E Q A Y Q E A Q A R N L R V K G V L V T N P S N P L G T I T M R N E L D L V F D F I T S K G I H L I
ME-ACS1      N N F Q I T R E A L E V A Y K K A E S N I K V K G L I I T N P S N P L G T I Y D R D T L K S L V N F V N D N N I H L I
CM-ACS1      N N F Q V T K A A L E I A Y K K A Q E A N I K V K G V I I T N P S N P L G T I Y D R D T L K L V T F V N Q H D I H L I
CM-ACS1      N G F Q I T S A L E Q A Y K D A Q T R N L R V K G V L V T N P S N P L G T I T M R N E L N L V F D F I T S K G I H L I
CP-ACS1A     N N F Q V T K A A L E I A Y K K A Q E A N M K V K G V I I T N P S N P L G T I Y D R D T L K L V T F V N Q H D I H L I
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CS-ACS1      C D E I Y S A T V F K S P S F I S I A E I I E M D -- H C K K E L I H I L S S L S K D M L P G F R V G I I Y S Y N
CS-ACS2      C D E I Y A A T V F A E P G F I S I S E I D N S D I -- E C D R N L V H V V Y S L S K D M G F P G F R V G I I Y S Y N
CS-ACS3      S D E I Y S G T V F G S P G F V S A M E V L K E R S N E D E E V W K R V H I V Y S L S K D L G L P G F R V G I A I Y S N D
ME-ACS1      C D E I Y S A T V F K S P S F T S I A E I I E M D -- H C K K E L I H I L S S L S K D M L P G F R V G I I Y S Y N
CM-ACS1      C D E I Y S A T V F K A P T F I S I A Q I V E E M E -- H C K K E L I H I L Y S L S K D M L P G F R V G I I Y S Y N
CM-ACS2      S D E I Y S G T V F G S P G F V S A M E V L K E R S S E D E E V W K R V H I V Y S L S K D L G L P G F R V G I A I Y S N D
CP-ACS1A     C D E I Y S A T V F K A P T F T S I A E I V E Q M E -- H C K K E L I H I L Y S L S K D M L P G F R V G I I Y S Y N
               * * * * *
CS-ACS1      D T V V S I A R K M S S F G L V S S Q T Q H L L A A M L S D E F V D N F L V E N S K R L A K R H A R F T K E L E K M G
CS-ACS2      D A V V A C A R K M S S F G L V S S Q T Q Y L I A S M L L D D V F V D N F L A G S A E K L A A R H R N F T K G L A Q V G
CS-ACS3      E M V V A A T K M S S F G L V S S Q T H Y L L S A M L S D K K F T R T Y I S E N Q R L K Q R Q K M L V S G L E K A G
ME-ACS1      D T V V S I A R K M S S F G L V S S Q T Q H L L A A M L S D E F V D N F L V E N S K R L A K R H A R F T E E L E K M G
CM-ACS1      D V V V R R A R Q M S S F G L V S S Q T Q H L L A A M L S D E F V D K F L A E N S K R L A E R H A R F T K E L D K M G
CM-ACS2      D M V V A A T K M S S F G L V S S Q T Q Y L L S A M L S D K K F T I S Y I S E N Q R L K Q R Q K M L V S G L Q K A G
CP-ACS1A     D V V V R R A R Q M S S F G L V S S Q T Q H L L A A M L S D E F V D K F L A E N S K R V G E R H A R F T K E L D K M G
               * * * * *
CS-ACS1      I T C L N G N A G L F V W M D L R K L L K D Q S F E S E M E L W R V I I N E V K L N V S P G S S F R V T E P G W F R V C
CS-ACS2      I G Y L K G S G L F L W M D L R H L L K E K T L E A E M A L W K V I I N E V K L N V S P G S S F H C S E P G W F R V C
CS-ACS3      I K C L E S S A G L F C W V D M R H L L E S D T F E C E L K L W K K I V Y E V G L N I S P G S S C H C T E P G W F R V C
ME-ACS1      I T C L N S N A G L F V W M D L R K L L K D Q S F E S E M E L W R V I I N E V K L N V S P G S S F H V T E P G W F R V C
CM-ACS1      I T C L N S N A G V F V W M D L R R L L K D Q T F K A E M E L W R V I I N E V K L N V S P G S S F H V T E P G W F R V C
CM-ACS2      I N C L D S N A G L F C W V D M R H L L E S D K F E S E L E L W K K I V Y E V G L N I S P G S S C H C T E P G W F R V C
CP-ACS1A     I T C L N S N A G V F V W M D L R R L L K D Q T F K A E M E L W R V I I N E V K L N V S P G S S F H V T E P G W F R V C
               * * * * *
CS-ACS1      F A N M D D N T V D V A L G R I H S F V G I D K K E N N T I P M P P K K K P R E N K L R L S F S F S G R R Y E E G N V
CS-ACS2      F A N M D D N T M D I S I T I R N F V L Q N -- K E V T T K V K K Q F C W R Q S S L E R L S S -- R R L E D --
CS-ACS3      F A N M S Q S T L K L A I R L K S F V Q E L R ----- S V S T P N V S T T T T N I H D S K F S K N I K K N I F T K W
ME-ACS1      F A N M D D N T V G V A L D R I H S F V G I D K K E N T I P M P P K K K H R E N K L R L S F S F S G R R Y E E G N V
CM-ACS1      F A N M D D N T V D V A L N R I H S F V E N I D K K E D N T V A M P S K T R R R E N K L R L S F S F S G R R Y D E G N V
CM-ACS2      F A N M S E S T L K L A V R R L K S F V T E L R ----- S T T T S N H R N ----- H D N K I C K N I K K N I F T K W
CP-ACS1A     F A N M D D N T V D V A L N R I H S F V E N I D K K E D N T V A M P S K T R H R D N K L R L S F S F S G R R Y D E G N V
               * * * * *
CS-ACS1      L K S P H M M S P H S P L V R A K T
CS-ACS2      I M S P H S P L P Q S P H L R A T T
CS-ACS3      V F R Q S V Q D Q A N R K L H Y E R
ME-ACS1      L K S P H M M S P H S P L V R A K T
CM-ACS1      L N S P H T M S P H S P L V I A K N
CM-ACS2      V F R Q S A Q -- E A N R K M Q A E R
CP-ACS1A     L N S P H T M S P H S P L V I A K N

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Fig. 2. Alignment of amino acid sequences deduced from ACC oxidase genes of cucumber (CS-ACO) and melon (CM-ACO1, X95551; CM-ACO2, X95552; CM-ACO3, X95553). Amino acids which are shaded are conserved in all members of Fe(II) ascorbate family of dioxygenases (Lasserre et al. 1996). The arrows indicate the positions of degenerated primers used for RT-PCR.

Table 2. Percentage sequence identity between ACC synthases encoded by multigene families.

	NT→							
		CS-ACS 1	CS-ACS 2	CS-ACS 3	ME-ACS 1	CM-ACS 2	CM-ACS 2	CP-ACS 1A
AA CS-ACS 1		—	67	59	91	83	58	82
↓ CS-ACS 2		64	—	58	67	67	58	67
CS-ACS 3		49	54	—	56	57	81	57
ME-ACS 1		96	67	52	—	79	56	79
CM-ACS 1		83	65	48	85	—	56	96
CM-ACS 2		55	56	90	54	55	—	56
CP-ACS 1A		83	66	52	85	97	55	—

CS-ACS, cucumber (CS-ACS 1, AB 006803; CS-ACS 2, AB 006804; CS-ACS 3, AB 006805); ME-ACS, melon (ME-ACS 1, D 30805); CM-ACS, winter squash (CM-ACS 1, D 01032; CM-ACS 2, D 01033); CP-ACC, zucchini (CP-ACC 1, M 61195); NT, nucleotide; AA, amino acid.

Fig. 1. Alignment of amino acid sequences deduced from ACC synthase genes from cucumber (CS-ACS), melon (ME-ACS1, D30805), winter squash (CM-ACS1, D01032; CM-ACS2, D01033) and zucchini (CP-ACS1A, M61195). Highly conserved regions for ACC synthase are boxed and eleven amino acid residues which are shaded are invariant amino acids conserved in various aminotransferases (Rottmann et al., 1991). The arrows indicate the positions of degenerated primers used for RT-PCR.

Table 3. Percentage sequence identity between ACC oxidases encoded by multigene families.

	NT→				
	CS-ACO 1	CS-ACO 2	CM-ACO 1	CM-ACO 2	CM-ACO 3
AA CS-ACO 1	—	66	96	58	73
↓ CS-ACO 2	73	—	68	57	68
CM-ACO 1	97	73	—	58	73
CM-ACO 2	45	46	45	—	58
CM-ACO 3	76	71	77	45	—

CS-ACO, cucumber (CS-ACO 1, AB 006806; CS-ACO 2, AB 006807);
CM-ACO, melon (CM-ACO 1, X 95551; CM-ACO 2, X 95552; CM-ACO 3, X 95553); NT, nucleotide; AA, amino acid.

Fig. 3. Differential expression of ACC synthase and ACC oxidase genes induced by wounding and IAA. Total RNA was extracted from frozen fruit slices 0, 1, 3, 6 and 12 hr after each treatment as described in Materials and Methods. Each lane contains 8 µg of poly(A)⁺RNA.

the two ACC oxidase homologs of cucumber fruit. Furthermore, the homology search indicated that these genes encode different ACC synthase and ACC oxidase polypeptides (Table 2, 3). This agrees with the finding that both enzymes are encoded by multigene families (Rottmann et al., 1991; Kende, 1993; Barry et al.,

1996).

Northern blot analysis revealed that *CS-ACS1* and *CS-ACS2* were induced by wounding and enhanced by IAA treatment, whereas *CS-ACS3* was induced only by IAA treatment. Because IAA was applied to the fruit slices, the effect of wounding cannot be ignored. Indeed, *CS-ACS1* was expressed in both wound- and IAA-treated slices 3 hr after the treatments. Since *CS-ACS1* is highly homologous to *ME-ACS1* (Yamamoto et al., 1995), *CM-ACS1* (Nakajima et al., 1990) and *CP-ACS1* (Huang et al., 1991), it could also be a wound-inducible gene.

Nakagawa et al. (1991) purified an ACC synthase induced by IAA and determined the partial amino acid sequence in cucumber fruit slices. They cloned a cDNA for IAA-induced ACC synthase from winter squash by using synthesized oligonucleotides based on the determined amino acid sequence, therefore, the IAA-induced cDNA sequences from cucumber and winter squash should be highly homologous. The *CS-ACS3* sequence is highly homologous to that of *CM-ACS2*, and its expression was detected only in the fruit slices treated with IAA, suggesting that *CS-ACS3* is an auxin-inducible gene. However, the reason for the expression of *CS-ACS2* apparently induced by IAA is unknown. Recently, Trebitsh et al. (1997) cloned a fragment of genomic DNA encoding ACC synthase from cucumber, which was designated *CS-ACS1*. Its expression in the shoot apex was induced by auxin. Since the sequence of the cDNA region is more than 99% identical to our *CS-ACS3*, these two genes may most likely be encoding the same ACC synthase polypeptide.

Lasserre et al. (1996) isolated three ACC oxidase genes from melon, of which only *CM-ACO1* is reported to be expressed in the fruit. With respect to the two ACC oxidase genes cloned in this study, *CS-ACO1* is 97% identical to *CM-ACO1* at polypeptide level (Table 3) and the change in its transcript level corresponded with ethylene production while that of *CS-ACO2* was constitutive. This suggests that *CS-ACO1* could be the main ACC oxidase gene expressed when the cucumber fruit is subjected to wounding or IAA treatments.

Our results indicate that the genes we cloned are differentially expressed by wounding and IAA and are involved in the regulation of ethylene biosynthesis in cucumber fruit at the transcriptional level, and that immature cucumber fruit, subjected to wounding or IAA treatments, express very similar ACC synthase and ACC oxidase genes as other fruits in Cucurbitaceae do, especially the ripening melon fruit (Yamamoto et al., 1995). To compare the differences in the ripening process between cucumber and melon fruits, we should use mature cucumber fruit as the experimental subject. Nevertheless, we now have materials to compare differences in the ripening process between climacteric and non-climacteric fruits in the family at the molecular level.

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キュウリ果実のACC合成酵素およびACC酸化酵素cDNAのクローニングと傷害と
オーキシン処理による遺伝子発現塩見慎次郎¹・山本幹博¹・小野貞治¹・垣内勝哉¹・中本純一¹・中務明¹・
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摘 要

キュウリ (*Cucumis sativus* L.) 果実から RT-PCR (reverse transcription and polymerase chain reaction) と RACE (rapid amplification of cDNA ends) PCRにより3種の1-アミノシクロプロパン-1-カルボン酸 (ACC) 合成酵素の cDNA (pCS-ACS1, pCS-ACS2, pCS-ACS3) と2種のACC酸化酵素の cDNA (pCS-ACO1, pCS-ACO2) をクローニングし、塩基配列を決定した。クローニングしたすべてのACC合成酵素およびACC酸化酵素のcDNAには他の植物で既に報告されている保存領域が存在した。ACC合成酵素とACC酸化酵素のcDNAクローンのうち、pCS-ACS1, pCS-ACS3, およびpCS-ACO1は既報の他のウリ科植物の遺伝子の塩基配列ならびにアミノ酸配列と高い相同性を示し

た。

ノーザンブロット分析の結果、傷害処理では主にCS-ACS1とCS-ACO1 mRNAの蓄積が、オーキシン処理ではすべてのACC合成酵素遺伝子とCS-ACO1の発現が誘導され、それらの発現パターンはエチレン生成とほぼ一致した。CS-ACO2は傷害およびオーキシン処理で一定の発現を示した。これらの遺伝子の発現パターンと配列の相同性からCS-ACS1は傷害誘導型、CS-ACS3はオーキシン誘導型であると考えられた。以上の結果より、キュウリ果実では傷害またはオーキシン処理によってこれらの遺伝子が特異的に発現し、転写レベルでエチレン生合成の調節に関与していることが強く示唆された。