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Structural Characterization of ACC Synthase Genes from Melon and Cucumber and their Promoter Activities Determined by GUS Transient Assay

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In order to clarify the differences in the regulatory mechanism(s) of the expression of 1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS) genes during ripening in climacteric melon fruit and non-climacteric cucumber fruit, two sets of their genomic DNA sequences, including ca. 2kb of the promoter regions were determined, using PCR-based methods. ACS genes from melon (*CMe-ACS1, 2*) were structurally similar to their counterpart from cucumber (*CS-ACS1, 2*) in terms of size and position of exons and introns, restriction map, and sequence identity of exons, introns, proximal 5'-flanking promoter regions and splice junction. Southern blot analysis indicated that each ACS gene is present as a single copy. Transient promoter activity was investigated with two constructs of promoter-β-glucuronidase (GUS) fusion, *CMe-ACS1*: GUS and *CS-ACS1*: GUS, in mature mesocarp tissues of the two fruits. In melon disks, GUS activities conferred by the promoters of both *CS-ACS1* (−2098～+42) and *CMe-ACS1* (−2187～+67) were detected, which were decreased by treatment with 1-methylcyclopropene (1-MCP), an ethylene action inhibitor. In cucumber disks, however, only *CS-ACS1*: GUS was expressed; the activity was decreased with 1-MCP, and it was not affected by propylene. These results suggest that the promoter of *CS-ACS1* has a potential to be expressed in the mesocarp tissue of ripening melon fruit, and that the difference in ethylene biosynthesis between melon and cucumber during ripening may be due to the difference in capability of forming trans-acting factor(s), not due to their ACS1 promoter activities.

Key words : *Cucumis sativus* L., *Cucumis melo* L., fruit ripening, GUS transient assay

Introduction

Cucumber and melon belong to the same family, *Cucurbitaceae*. The former is a non-climacteric fruit while the latter is a climacteric fruit[1]. Both fruits have 1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS) and ACC oxidase (ACO) genes, constituting multigene families, and the ACS and ACO genes in each fruit have extremely high nucleotide identity in

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respect of their respective counterpart cDNA[16]. *CMe-ACS1*, a melon ACS gene, is expressed in the placenta and mesocarp tissues of ripening melon[17,22], whereas *CMe-ACS2* is expressed mainly in immature fruit[15]. In cucumber fruit, *CS-ACS1*, a corresponding gene to *CMe-ACS1*, is not expressed even in mature fruits with or without exogenous ethylene. mRNA for *CS-ACS2*, a counterpart gene of *CMe-ACS2*, accumulates in mature mesocarp, which is suppressed by exogenous ethylene[18]. The expression patterns of these genes in immature and mature fruits indicated that the main difference in the ripening process between melon and cucumber may result from the expression patterns of ACS genes, especially the ACS1 gene, during fruit ripening and in response to exogenous ethylene[17,18]. For this reason we decided to determine the promoter activities of ACS1 genes in this study.

It is well known that transcription of a gene is regulated mainly by promoter regions that are located in the 5'-flanking region from the transcription initiation site. Therefore, it is important to identify some specific *cis*-acting element(s) on the ACS genomic sequences, which regulate the expression of these ACS genes by binding with DNA-binding protein(s). AS a first step in this direction, in this study, genomic DNAs encoding ACS from cucumber and melon are structurally characterized, and the results of β-glucuronidase (GUS) transient assay using fusion of ACS1 promoters: GUS reporter gene are discussed.

Materials and Methods

Plant Materials

Cucumber (*Cucumis sativus* L. cv. Honor, a parthenocarpic cultivar) and melon (*Cucumis melo* L. cv. Andes) fruits were grown in greenhouses. Melon fruit were harvested at commercial harvest maturity (mature stage, 45-50 days after pollination) when the fruit had already attained the stage of climacteric rise in ethylene production. Cucumber were also harvested at mature stage (35-40 days after anthesis[18]). A half of the melon and a third of the cucumber fruits were treated with 1-methylcyclopropene (1-MCP) for 16 hrs according to Nakatsuka et al.[11]. Another one third of the cucumber fruit were treated with propylene (5000 ppm) for 24 hrs. Both treatments were performed at the beginning of experiments with intact fruits before the preparation of disks.

DNA isolation and southern blot analysis

Genomic DNA was extracted from the peel tissue of cucumber fruit, and from the young leaves and the mature fruit mesocarp of melon by the method of Murray and Thompson[10]. Genomic DNA (7.5 μg) from melon and cucumber was digested with *Hin*dIII, *Xba*I and *Eco*RI, size-fractionated by electrophoresis in 0.8% agarose gels, and then blotted onto Hybond N Nylon membranes (Amersham). Membranes were hybridized with ^{32}P-labeled cDNA probes prepared from pCS-ACS1 and pCS-ACS2[16] at 37°C overnight in a buffer containing 50% formamide, 5×SSPE, 5×Denhardt's, and 0.1% SDS. After hybridization, membranes were washed twice for 15 min each at 60°C in 0.2×SSPE and 0.1% SDS, and then exposed to imaging plate (Fuji Photo Film, Tokyo) at room temperature.

Cloning and Sequencing of CS-ACS1, CS-ACS2, CMe-ACS1 and CMe-ACS2

From the cDNA sequences of *CS-ACS1*, *CS-ACS2*, *CMe-ACS1* and *CMe-ACS2* reported[16,22], gene specific primers were synthesized and genomic DNA fragments were amplified with genomic DNA as a template by inverse PCR (IPCR) according to Ochman et al.[12] and Triglia et al.[20], and LA PCR *in vitro* cloning kit (Takara) according to the manufacturer's protocol. The conditions for the IPCR were as follows; 1 min 94°C, 2 min 55°C, 3 min+10 sec extension/cycle 72°C for 30 cycles. The amplified genomic DNA fragments *in vitro* were cloned into pUC118 (Takara) or pCR 2.1 (Invitrogen) plasmids and sequenced

using DNA sequencers (373A, ABI and DSQ-1000L, Shimadzu). The sequences of genomic DNAs were determined by overlapping the obtained sequence with the known regions. The putative initiation and termination sites for transcription were determined by comparing the genomic DNA sequence with the full cDNA sequence[16]. Phylogenetic tree was generated using the CLUSTALW program.

Plasmid Construction of ACS Promoter: GUS Reporter Gene

Using the determined sequence of 5'-flanking regions of each ACS1 gene, gene specific primers were synthesized and objective promoter regions of ca. 2 kb were amplified by PCR with genomic DNA from melon and cucumber as templates. Each primer used for PCR contained a site of optimal restriction enzyme which facilitated the subcloning into the pBI221 (Clontech). pBI221 plasmid, which contains CaMV 35S promoter-β-glucuronidase (GUS)-nopaline synthase (NOS) terminator-cassette, was digested with the respective restriction enzyme, and the PCR products were subcloned into the plasmid by replacing CaMV 35S promoter in order to construct the ACS1 promoter: GUS fusion. The constructs with ca. 2 kb promoters were *CMe-ACS1*: GUS (−2181 ~ +67, 2248 bp of promoter) and *CS-ACS1*: GUS (−2136 ~ +69, 2205 bp). A plasmid without CaMV 35S promoter was prepared by removing CaMV 35S promoter from pBI221 (pBI-35S) and the original pBI221 plasmids were also used as control constructs. The fidelity of the constructs was confirmed by the size of the inserts and by sequencing the region around the junction between the pBI221 plasmid and the inserts.

Delivery of Particles by Bombardment

After plasmid DNA constructs were introduced into *Escherichia coli* (JM 109, Takara) and cultured in LB medium, they were purified with QIAprep Spin Miniprep Kit (QIAGEN) and coated onto tungsten particles according to Takeuchi et al.[19] by ethanol precipitation. The DNA-coated particles were delivered with a particle bombardment device (IDERA GIE-III type, TANAKA, Hokkaido) into tissue disks (diameter 1.6 cm, thickness 2 mm) prepared with corkborer and laser blade from the mesocarp tissue of cucumber and melon fruits. The conditions for bombardment were set according to the manufacturer's instruction as follows; accelerating pressure was 6 kg cm^{-2}, stopper-to-target-tissue distance was 5 cm and partial vacuum in the sample chamber was 650-700 mmHg. The bombarded tissues were placed in sterile petri dishes and incubated at 25 °C for 24 h under humidified conditions.

GUS Transient Assay

After being bombarded with particles, the mesocarp disks were incubated for 24 h and then stored at −20 °C until enzyme extraction. The tissues were ground to a powder in liquid nitrogen and mixed well in extraction buffer consisting of 100 mM NaH_2PO_4 (pH 7.0), 20 mM EDTA, 0.2% Triton X-100, 0.2% Sarkosyl and 20 mM 2-mercaptoethanol. The enzyme solution was centrifuged at 15,000 rpm for 10 min at 4 °C. Following the determination of protein content in the supernatant (crude extract) using Bradford reagent[2] (Bio-Rad), an equivalent to 20 μg protein was used for the enzymatic reaction for fluorometric assay. The 200 μl of reaction mixture, including 80 μl of 2.5 mM 4-methyl umbelliferyl glucuronide (MUG) as a substrate and enzyme solution, was incubated at 37 °C for 4 h according to Jefferson et al.[7] and Kosugi et al.[8]. Fluorescence was measured with excitation at 365 nm and emission at 455 nm using a spectrophotofluorometer (F2000, Hitachi). The GUS activity was expressed as pmol 4-MU min^{-1} mg $protein^{-1}$.

Results and Discussion

Structural Characterization of CMe-ACS1, CS-ACS1, CMe-ACS2 and CS-ACS2

Southern blot analysis was performed on

melon and cucumber genomic DNA digested with three restriction enzymes (Fig. 1), using cucumber cDNA probes (Fig. 2), pCS-ACS1 and pCS-ACS2[16]. Apart from cucumber genomic DNA digested with *Xba*I and melon genomic DNA digested with *Hin*dIII and *Xba*I (Fig. 1A), single band was detected. The results corresponded well with the restriction map (Fig. 2), and indicate that each ACS gene in both species is present in one copy per haploid genome.

The genomic sequences of 6241 bp, 6408 bp, 5075 bp and 5640 bp for *CMe-ACS1*, *CS-ACS1*, *CMe-ACS2* and *CS-ACS2*, respectively, were determined in this study. Each sequence included more than 2 kb of 5'-and 80-670 bp of 3'-flanking regions (Table 1). *CMe-ACS1* and *CS-ACS1*, which were highly homologous at cDNA level (91% identical at nucleotide level and 96% at amino acid level, Table 1), were even structurally similar in terms of size and position of exons and introns, and restriction map (Table 1, Fig. 2). Sequence identity in introns and 5'-flanking and 3'-flanking regions was rather low (between 70 and 80%), but 5'-distal promoter region was more divergent with 66% identity (Table 1).

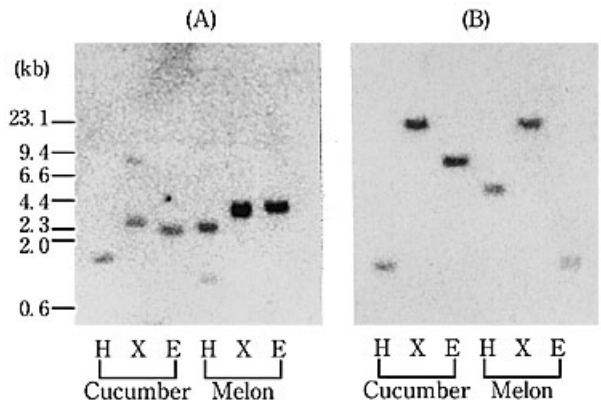

Fig. 1 Southern blot analysis of melon and cucumber ACC synthase genes. Genomic DNA from the two species was digested with three restriction enzymes, *Hin*dIII (H), *Xba*I (X) and *Eco*RI (E), fractionated on an agarose gel, blotted and hybridized with ^{32}P-labeled cucumber ACC synthase cDNAs, pCS-ACS1(A) and pCS-ACS2(B) as probes. Each lane contains 7.5 μg of DNA.

Almost the same structural characteristics were observed between *CMe-ACS2* and *CS-ACS2*, but with higher sequence identity than ACS1 in exons and introns (Table 1, Fig. 2).

All the four genes contained five exons which were interrupted by four introns (Table 1, Fig. 2). ACS genes fall into three classes on the basis of the introns present[4]: four intron genes (zucchini, *CP-ACS1A*, *1B*), three intron genes (*Arabidopsis thaliana*, *AT-ACS1*, *2*, *4*; tomato, *LE-ACS2*; winter squash, *CM-ACS1*; rice, *OS-ACS1*), and two intron genes (tomato, *LE-ACS3*, *4*; potato, *ST-ACS1A*, *1B*). The four genes in the present study contained the same number of intron with *CP-ACS1A* and *CP-ACS1B* from zucchini, belonging to the same family *Cucurbitaceae* with

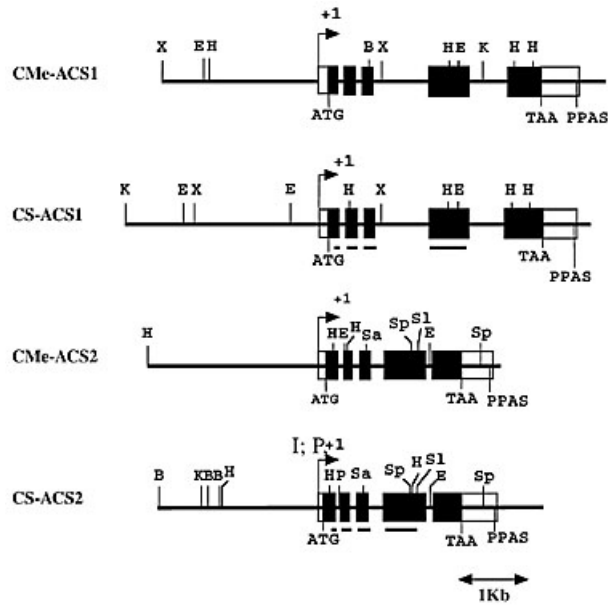

Fig. 2 Partial restriction maps and gene organization of four genomic DNAs encoding ACC synthase genes from melon (*CMe-ACS1*, *2*) and cucumber (*CS-ACS1*, *2*). Coding regions are indicated by filled blocks, and 5'- and 3'-untranslated regions by open blocks. The lines connecting the five exons represent the four introns. The +1 and arrows indicate putative start and direction of transcription. PPAS stands for putative polyadenylation signal. Bars in *CS-ACS1* and *CS-ACS2* indicate the position of templates for preparing probes for southern hybridization. Abbreviations for restriction enzymes are as follows; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Sa, *Sac*I; Sl, *Sal*I; Sp, *Sph*I; X, *Xba*I.

Table 1 Structural characteristics of the *CMe-ACS1, 2* and *CS-ACS1, 2* genes based on the nucleotide sequences determined in this study. *CP-ACS1A* and *CP-ACS1B* from zucchind are also included for comparison

Region		Length (bp)		Identity (%)	Length (bp)		Identity (%)	Length (bp)	
		CMe-ACS1	CS-ACS1		CMe-ACS2	CS-ACS2		CP-ACS1A[f]	CP-ACS1B[f]
Promoter	Distal 5[a]	1681	2196	66	1908	1748	58		
	Proximal 5[b]	500	500	78	500	500	79		
5'UTR[c]		102	127	81	76	62	95	51	64
Exon 1[d]		177	177	95 (97)[e]	183	183	97 (100)[e]	177	177
Intron 1		107	91	69	91	88	88	87	87
Exon 2		132	132	97 (96)	132	132	94 (98)	132	132
Intron 2		103	89	76	111	109	80	83	88
Exon 3		161	161	96 (98)	161	161	94 (98)	161	161
Intron 3		774	796	75	228	224	84	465	1258
Exon 4		567	567	95 (97)	595	592	96 (96)	567	567
Intron 4		562	494	76	96	95	90	86	86
Exon 5[d]		445	445	96 (95)	402	402	97 (98)	445	445
3'UTR[c]		568	549	81	478	489	91		
3'-flanking		362	84	71	114	675	77		
cDNA full		2152	2158	91 (96)	2027	2021	94 (97)		
Total		6241	6408		5075	5460			

a) Promoter region further upstream from −501
b) Promoter region from putative −1 to −500
c) Untranslated region
d) Excluding 5'-and 3- untranslated region, respectively
e) The corresponding protein sequence indentity levels in parentheses
f) Processed from the DDBJ database sequence, accession number M61195

melon and cucumber. Furthermore, the four introns of *CMe-ACS1* and *CS-ACS1* interrupt the coding sequence at the same position as the four introns in the zucchini *CP-ACS1A* and *CP-ACS2B* genes (Table 1)[5].

It is interesting to note that *CP-ACS1A* ,*CS-ACS1* and *CMe-ACS1* share highly homologous cDNA sequences[16] and similar structural characteristics of genomic DNA. Fig. 3 shows a phylogenetic tree generated on the basis of amino acid sequence alignment using the CLUSTALW program. The result indicated that the sequence of the coding region is closely related in *CP-ACS1A*,*CS-ACS1* and *CMe-ACS1*, and between *CS-ACS2* and *CMe-ACS2*. The close relationship between these genes was also observed even when phylogenetic tree was generated using 5'-flanking sequence of 1.5kb (data not shown). These three ACS1 genes have been reported to be wound-inducible[5,16,17,22].

The intron/exon junctions, which are typical of donor and acceptor splice sites, were established by reference to the sequence of each cDNA.

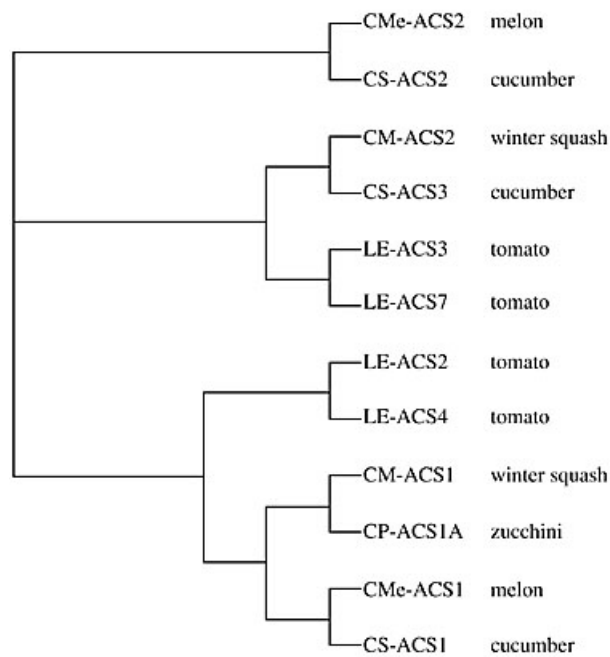

Fig. 3 A phylogenetic tree of ACC synthase genes generated by the CLUSTALW program on the basis of polypeptide amino acid identity. DDBJ accession numbers are as follows: CMe-ACS1, AB032935; CS-ACS1, AB032936; CP-ACS1A, M61195; CM-ACS1, D01032; CMe-ACS2, AB032937; CS-ACS2, AB032938; CM-ACS2, D01033; LE-ACS2, X59145; LE-ACS4, M88487; CS-ACS3, AB006804; LE-ACS3, L34171; LE-ACS7, AF043122.

Comparing the splice junction sequences in ACS genes from melon and cucumber, the sequence was identical between *CMe-ACS1* and *CS-ACS1*, and *CMe-ACS2* and *CS-ACS2*. All the introns started with 'gt' and ended with either 'cag' or 'tag' (data not shown), which concurred with the report by Breathnach and Chambon[3].

In the promoter regions of *CS-ACS1* and *CMe-ACS1*, TATA and CAAT boxes were present. GCC box, which has been reported to be present in ethylene-inducible pathogenesis-related protein genes[14], was not identified in the promoter sequences, but in both *CS-ACS1* and *CMe-ACS1* were identified sequences similar to ethylene-responsive-element in carnation GST1 gene[6], E4 upstream regulatory element[9,12], and some other *cis*-acting elements that are similar to transcription binding sites reported in other plant genes (data not shown).

These results indicate that not only the structural characteristics but also the sequences of melon ACS genes are similar to those of cucumber counterparts, although the sequences in the promoter region, particularly distal 5'-flanking regions are less homologous.

GUS Transient Expression

When fluorometric assay was carried out using mesocarp disks of ripening melon fruits (Fig. 4a), GUS activities conferred by both promoters of *CMe-ACS1* and *CS-ACS1* were detected at rather higher levels than those conferred by CaMV 35S promoter, and were decreased by 1-MCP treatment. This suggests that there may be some ethylene-responsive element present on both promoters. The pattern of change in promoter activity of *CMe-ACS1* by 1-MCP paralleled the results of northern blot analysis[17], in which mRNA abundance for *CMe-ACS1* decreased by the same treatment. These results indicate that the promoters of *CMe-ACS1* and *CS-ACS1* of ca. 2kb are sufficient to direct reporter gene expression in the melon mesocarp tissue, and to respond to ethylene.

When the same constructs were introduced into mature cucumber mesocarp disks, GUS was expressed only by *CS-ACS1*: GUS construct; 1-MCP reduced the activity, but propylene did not affect the activity (Fig. 4b). *CMe-ACS1*: GUS activity, however, was at basal level, and was not affected by these treatments. Since *CS-ACS1* mRNA accumulates only in the mesocarp tissue of immature fruit treated with wounding and auxin, but not in the intact tissues[16,18], the GUS activity conferred by *CS-ACS1* promoter may be due to the effect of wounding (cutting) imposed on the disks during preparation. The fact that *CMe-ACS1*: GUS activity was at basal level, contrary to *CS-ACS1*: GUS activity, suggests that wound-responsive element(s) may be absent on ca. 2kb of *CMe-ACS1* promoter, but may exist on ca. 2kb of *CS-ACS1* promoter. The

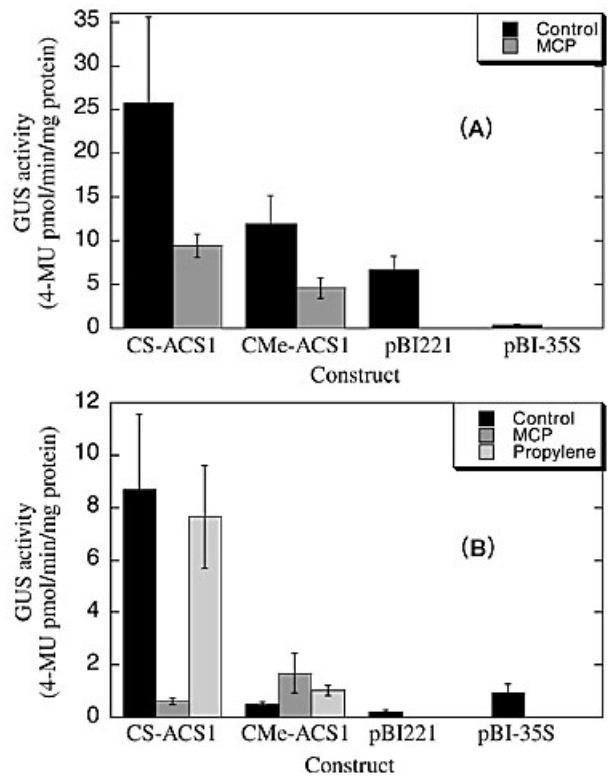

Fig. 4 Promoter expression of CMe-ACS1 and CS-ACS1 in mature melon fruit disks (A), and in mature cucumber fruit disks (B) expressed as GUS transient activity. pBI-35S is a plasmid which was prepared by removing CaMV 35S promoter from pBI-221.

decreased level of *CS-ACS1*: GUS activity by 1-MCP and approximately the same level of activity with the control by propylene (Fig. 4b) coincides well with the results of O'Donnell et al. (13), who reported that ethylene is a requirement for the wound-response transduction pathway.

In previous studies, we clarified that the difference in ethylene biosynthesis between melon and cucumber is due to the expression or lack of expression of ACS1 gene during fruit ripening[17,18]. We had, therefore, expected differences in promoter activity of each ACS1 gene to a large extent. However, the promoter expression of *CMe-ACS1* and *CS-ACS1* was similar at least in mature melon disks. The results of GUS transient assay indicate that the promoter of *CS-ACS1* can potentially be expressed in ripening melon mesocarp, and that the *CMe-ACS1* promoter can not be expressed in mature cucumber mesocarp. This suggests that there may be a difference in the capability of forming *trans*-acting factor(s) for ACS1 promoter between the two fruits. The factor(s) includes a DNA-binding protein, that in turn regulates gene expression. In melon fruit, certain *trans*-acting factor(s) may be synthesized, leading to the transcription of *CMe-ACS1*, but in cucumber fruits such factors may not be synthesized, resulting in failure of *CS-ACS1* expression, thus basal ethylene production, during ripening.

To clarify this issue, further investigations will be required, using more constructs of promoter-reporter gene fusion, so that *cis*-acting elements which are important in regulating the gene expression can be distinctively identified. Moreover, it is important to detect sequence(s) on which DNA binding protein(s) binds, and to characterize these protein(s). Through such investigations, it is possible that the difference(s) in the regulation of *CMe-ACS1* and *CS-ACS1* transcription will be understood more clearly, which may lead to further elucidation of the fundamental differences in ripening characteristics between the two fruits.

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メロンとキュウリの ACC 合成酵素遺伝子の構造的特徴と GUS トランジェントアッセイによるプロモーター活性

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クライマクテリック型果実のメロンとノンクライマクテリック型果実のキュウリの果実追熟に伴う 1-アミノシクロプロパン-1-カルボン酸（ACC）合成酵素（ACS）遺伝子の発現調節機構の相違を明らかにするために，それぞれ 2 種類の ACS のゲムノ DNA 配列(約 2 kb)を PCR 法を基にして決定した．メロンの ACS 遺伝子は，エキソンとイントロンのサイズおよび位置，制限酵素地図，エキソン・イントロン・近位の 5' 上流プロモーター領域・スプライシング部位の塩基配列において，それぞれ対応するキュウリの ACS 遺伝子とよく似た構造をしていた．サザンブロック解析の結果，各 ACS 遺伝子はシングルコピーとして存在すると考えられた．*CMe-ACS1* と *CS-ACS1* の一過的プロモーター活性を β-グルクロニダーゼ（GUS）をレポーター遺伝子として両果実の成熟果肉を用いて調べた．メロン切片では，*CS-ACS1*（−2098〜＋42）ならびに *CMe-ACS1*（−2187〜＋67）のプロモーター発現による GUS 活性が検出され，エチレン作用阻害剤の 1-メチルシクロプロペン(1-MCP)処理によって減少した．しかし，キュウリ切片においては，*CS-ACS1*：GUS のみで GUS が発現し，活性は 1-MCP 処理で減少し，プロピレン処理ではコントロールと同レベルであった．これらの結果より，*CS-ACS1* のプロモーターがメロン成熟果肉組織で発現する潜在能力をもつこと，メロンとキュウリの成熟に伴うエチレン生合成の相違は ACS1 プロモーター活性によるものではなく，両者のトランス因子合成能の相違によることが示唆された．

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