Microsatellite and CAPS Markers for Ethylene-related Genes, 1-Aminocyclopropane-1-Carboxylic Acid (ACC) Synthase and ACC Oxidase Genes, and their Variation in Melon (*Cucumis melo* L.)

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To establish DNA markers for ethylene-related genes in melon (*Cucumis melo* L.), we searched for microsatellite and cleaved amplified polymorphic sequence (CAPS) polymorphisms in two ACC synthase genes (*CMe-ACS1*, *CMe-ACS2*) and two ACC oxidase genes (*CMe-ACO1*, *CMe-ACO3*), and found two CAPSs and six microsatellite markers. Five markers were detected in two genes, *CMe-ACS1* and *CMe-ACO1*, which were expressed during fruit ripening. A highly polymorphic marker was found in *CMe-ACS2*, where the number of simple sequence repeats (TA) was highly variable among cultivars, ranging from seven to thirty-seven. Insertion/deletion of (T)_n and (A)_n were also detected in *CMe-ACS2*. Specific CAPS and microsatellites for vars. *makuwa* and *conomon*, local varieties in East Asia, were detected in four markers, *Acs1-c1*, *Acs2-ms1*, *Aco1-ms2* and *Aco3-ms1*, and could be useful markers for phylogenetic analysis in melon.

Key Words: *Cucumis melo*, ACC oxidase, ACC synthase, CAPS, microsatellite, phylogeny.

Introduction

A microsatellite is characterized by tandem repeats of a simple sequence of a few base pairs, and the number of repeats is variable with the genotype in many cases. Extraordinarily polymorphic microsatellites have been reported in barley, and the number of repeats ranged from 9 to 43 in ribulose-1, 5-bisphosphate carboxylase activase gene and from 9 to 45 in waxy gene (Saghai-Marooft *et al.* 1994). Microsatellites are commonly found in various regions of a gene in many plant species: in the coding region of the LMW-glutenin gene and γ -gliadin gene of wheat (Devos *et al.* 1995), in the 5' untranslated region of waxy gene of rice (Akagi *et al.* 1996), and so on. Although the genetic variability of the microsatellite depends on the locus, highly polymorphic marker can be applicable for fingerprinting or

cultivar identification (Innan *et al.* 1996, Davila *et al.* 1999). On the contrary, species- or variety-specific polymorphism has been used for the analysis of phylogeny and origin of crop plants such as wheat and rice (Lelley *et al.* 2000, Akagi *et al.* 1998). Microsatellite polymorphism has been also analyzed in *Cucurbitaceae* family, and some of the polymorphisms seemed to be genera- or species-specific (Katzir *et al.* 1996). Furthermore, according to their data, polymorphism among oriental melon varieties and other varieties of *C. melo* could be observed at five microsatellite loci, though isozyme analysis failed to detect variety-specific variation in *C. melo* (Kato *et al.* 1998). Such microsatellite markers can be applicable for the study of phylogenetic relationships in *C. melo*.

As another marker, the cleaved amplified polymorphic sequence (CAPS) is characterized by a single nucleotide polymorphism (SNP) at the restriction site and can be easily analyzed by digesting PCR products with restriction enzymes. Nishio *et al.* (1997) succeeded to establish CAPS markers, in the second and the third introns of *S*-locus receptor kinase gene (*SRK*) causing self-incompatibility in *Brassica*, which proved to be useful for the identification of *S* haplotype. These results clearly indicated that microsatellites and CAPSs can be detected in various regions of each gene. Among the genes cloned and sequenced in *C. melo*, the genes related with fruit ripening have been well characterized, and two ACC synthase (*CMe-ACS1*, *CMe-ACS2*) and three ACC oxidase (*CMe-ACO1*, *CMe-ACO2*, *CMe-ACO3*) genes have been reported in *C. melo* (Miki *et al.* 1995, Shiomi *et al.* 1999, Yamamoto *et al.* 1995, Lasserre *et al.* 1996). Microsatellites or CAPS polymorphisms in ACC synthase and ACC oxidase genes and their flanking regions could be applicable as selection markers for ethylene-related characters such as shelf-life and sex expression in melon, as well as molecular markers for phylogenetic analysis in melon.

In this study, microsatellite and CAPS were surveyed around the known sequences of ACC synthase and ACC oxidase genes (Lasserre *et al.* 1996, 1997) of *C. melo*, and polymorphisms detected were characterized by their nucleotide sequences. The application of these molecular markers for phylogenetic analysis within *C. melo* is also discussed.

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Materials and Methods

Plant materials and DNA extraction

Thirteen cultivars from five varieties of *Cucumis melo* were used in this study (Table 1). The seeds of these cultivars were provided by the National Research Institute of Vegetables, Ornamental Plants and Tea (NIVOT), Japan. Seeds were sown on filter paper and grown at 26°C in a 16 h light/8 h dark cycle with a light intensity of 46.5 $\mu\text{Ms}^{-1}\text{m}^{-2}$. Ten-day-old seedlings were separately ground in liquid nitrogen respectively, and total DNA was extracted follow-

ing the procedure of Murray and Thompson (1980) with minor modifications.

Primer design

Specific primer pairs were designed to amplify the respective region containing the simple sequence repeat (SSR), based on the nucleotide sequences of *CMe-ACS1*, *CMe-ACS2* (Miki *et al.* 1995, Shiomi 1998), *CMe-ACO1* and *CMe-ACO3* (Lasserre *et al.* 1997). The details of primer sequences, condition of PCR amplification and target SSR sequence are summarized in Table 2. For CAPS analysis,

Table 1. Melon cultivars used in the present study and their origin

Variety	Cultivar	Origin
var. <i>reticulatus</i>	Earl's Favourite	UK
	Spicy	USA
	Homegarden	USA
	Andes	Japan
	Melon Parental Line Nou 2 gou	Japan
var. <i>cantaloupensis</i>	Charentais	France
var. <i>inodorus</i>	Hamiuri	China
	Honey Dew	USA
	Mirzuchulskaja	Russia
	Spain Noboru 3 gou	Japan (Spanish melon)
	Tendral o Invernale a Buccia Verde	Spain
var. <i>makuwa</i>	Mi-tang-ting	China
var. <i>conomon</i>	Takadashirouri	Japan

Table 2. Characteristics of microsatellite and CAPS markers established in ACC synthase and ACC oxidase genes in *C. melo*

Marker	Primer sequence (5' to 3') Forward/Reverse	Pattern of repeats (SSR)	Amplified region	Annealing temperature	Expected length	No. of alleles detected ¹⁾	Restriction enzymes for digestion	Gene ²⁾
Acs1-ms1	GATCCGGGCGATGCCTTTTGT TTTGTACAGTAATTAATTTTGTC	(TTC) ₆ /(AG) ₆	exon 3 - intron 3	50°C	233 bp	2	HinfI + XhoI	<i>CMe-ACS1</i> AB032935 D30805
Acs1-cl	CACTGCCACCCCATATATC TTTTCTGCTAACCATCTGAAT	—	5' flanking - exon 1	55	1665	2	Hsp92II	<i>CMe-ACS1</i>
Acs1-c2	AATCCTTCCAATCCCTTAGGCAC CAACCAAACCTTAGCTTAT	—	exon 4 - 3' UTR	55	1851	2	HindIII	<i>CMe-ACS1</i>
Acs2-ms1	TCTTTTGTCTTGGTTGTGAGT GATTGCTTTATTTGAATCTTTTG	(TA) ₈	5' flanking	53	200	6	—	<i>CMe-ACS2</i> AB032936 D37937
Acs2-ms2	AAAAATGAAATTGAAATTGGG GTGTGGTTGTGAGTTATGAGG	(AAG) ₄ (A) ₉ /(GAAAA) ₃	5' flanking - 5' UTR	60	304	4	MboI + HinfI	<i>CMe-ACS2</i>
Aco1-ms1	CCACCTCCTCTCTTTCCACATA CTCCTTAAACCTCTCTTCCATAC	(CTT) ₁₈ /(TC) ₉ C(TC) ₄	5' flanking - exon 2	60	605	3	MboI + HaeIII	<i>CMe-ACO1</i> X95551
Aco1-ms2	TGAAGCCATGAAAGCCAATGCTAAT GACCCTTAATCCTTATACTAGC	(AT) ₇	exon 4 - 3' UTR	53	254	2	Tra9I	<i>CMe-ACO1</i>
Aco3-ms1	CAACGACGCCTGCGAAAACT CATCAGCTCATGTGATATCCC	(TTTA) ₂ (TTTC)(TTTG) ₂	exon 1 - exon 2	53	144	2	HindIII + EcoRV	<i>CMe-ACO3</i> X95553

¹⁾ Number of alleles detected in six cultivars sequenced. Only five cultivars were sequenced for Aco1-ms1 and Aco1-ms2.

²⁾ Accession No. of sequence data in GenBank and EMBL sequence databases was also indicated.

primer pairs were designed to amplify 5' and 3' untranslated regions of *CMe-ACS1* (Acs1-c1, Acs1-c2), as also shown in Table 2.

Microsatellite and CAPS analyses

PCR amplification was performed in a volume of 10 µl, containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM or 2.0 mM MgCl₂, 0.5 unit of *Taq* polymerase (Pharmacia), 0.2 mM dNTP, 0.5 µM primer, and 50 ng of template DNA, by using Program Temp Control System PC-700 (ASTEC, Japan). After an initial denaturation step at 95°C for 3 min., 35 PCR cycles at 95°C for 1 min., 50-60°C for 2 min., and 72°C for 2 min., were performed, followed by a final extension at 72°C for 5 min. The optimized annealing temperature depended on primer pair and ranged from 50°C to 60°C. PCR products were digested with restriction enzymes shown in Table 2, by mixing the enzyme and buffer solution with a PCR reaction mixture, and were electrophoresed on 18% nondenatured polyacrylamide gel at 12-14 V/cm constant voltage or on 2% Nusieve 3:1 agarose gel (FMC) at 50 V (Mupid-2, CosmoBio, Japan) constant voltage in the case of Acs2-ms1, Acs1-c1 and Acs1-c2. After electrophoresis, PCR products were visualized with ethidium bromide staining and their polymorphisms were evaluated.

Cloning and sequence

PCR products exhibiting size polymorphisms between several cultivars in the primary screening were sequenced. PCR products were purified with QIA quick PCR Purification Kit (QIAGEN), and ligated into pGEM-T Easy Vector (Promega). The nucleotide sequence was determined by using the Dye-Primer method (Smith *et al.* 1986) with DSQ-1000 DNA sequencer (Shimadzu, Japan).

Results

CMe-ACS1; PCR product of Acs1-ms1 was digested with *HinfI* and *XhoI*. CAPS was detected between melon cultivars among which only 'Spicy' lacked *XhoI* site in intron 3 (Table 3). Microsatellite polymorphism was also detected in intron 3, and 'Spicy' was characterized by (AG)₅ in contrast to (AG)₆ of the others.

CAPS in the 5' flanking region of *CMe-ACS1* was detected in Acs1-c1. According to the original sequence of 'Andes', seven fragments should be expected by digesting the PCR product with *Hsp92II*. All of the expected fragments were detected in most cultivars, while a fragment of 566 bp was missing and two additional fragments of about 400 bp and 166 bp were detected in two cultivars, 'Mi-tang-ting' and 'Takadashirouri' (Fig. 1). Therefore an additional *Hsp92II* site should exist in these cultivars. Moreover, the second largest fragment, corresponding to the most upstream region, was smaller in size (ca. 310 bp) in five cultivars (Fig. 1, Table 3), though the details have not been analyzed.

CAPS in exon 4 of *CMe-ACS1* was detected in Acs1-c2, in which four fragments were expected by digesting with *HindIII*. All of the expected fragments were detected in most cultivars, while two fragments of 945 bp and 247 bp were missing and a fragment of 1192 bp was detected in 'Spicy', 'Homegarden' and 'Mirzuchulskaja' (Table 3). Therefore, a *HindIII* site should be lost by base substitution in exon 4.

CMe-ACS2; Size polymorphism was detected in PCR products of Acs2-ms1 (Fig. 2), which contained SSR of (TA)_n in the 5' flanking region (−2230 bp from transcription starting point). As shown in Fig. 3a, the number of (TA) repeats was highly variable, ranging from seven ('Mi-tang-ting') to thirty-seven ('Mirzuchulskaja'). Furthermore, insertion/deletion of (T)_n and (A)_n were also detected and

Table 3. Microsatellite and CAPS polymorphism in ethylene-related genes detected among thirteen melon cultivars covering five varieties of *C. melo*

Variety	Cultivar	Marker size (bp)							
		Acs1-ms1	Acs1-c1 ¹⁾	Acs1-c2	Acs2-ms1	Acs2-ms2	Aco1-ms1	Aco1-ms2	Aco3-ms1
var. <i>reticulatus</i>	Earl's Favourite	92/+	343/−	+	200	64/149	162	75	53
	Spicy	90/−	310/−	−	214	62/154	165	75	53
	Homegarden	nt	310/−	−	214	63/154	165	75	65
	Andes	92/+	nt	nt	200	64/149	162	75	53
	MPL-No. 2 ²⁾	92/+	343/−	+	200	64/149	155	81	65
var. <i>cantloupensis</i>	Charentais	92/+	343/−	+	218	63/154	162	75	53
var. <i>inodorus</i>	Hamiuri	92/+	343/−	+	200	63/154	165	75	53
	Honey Dew	92/+	343/−	+	200	nt	165	75	53
	Mirzuchulskaja	nt	310/−	−	252	63/154	162	75	53
	Spain ³⁾	92/+	343/−	+	232	63/154	165	75	53
	Tendral ⁴⁾	92/+	343/−	+	232	63/154	165	75	53
var. <i>makuwa</i>	Mi-tang-ting	92/+	310/+	+	196	64/149	155	81	65
var. <i>conomon</i>	Takadashirouri	92/+	310/+	+	196	64/149	nt	81	65

'+', '−': Presence and absence of critical restriction site. nt: Not tested.

¹⁾ Size of smaller fragment was tentatively estimated as ca. 310 bp.

²⁾ Melon Parental Line Nou 2 Gou, ³⁾ Spain Noboru 3 gou, ⁴⁾ Tendral o Invernale a Buccia Verde.

Fig. 1. CAPS polymorphisms detected in Acs1-c1. PCR product was digested with *Hsp92 II*. Lanes 1: 'Earl's Favourite', 2: 'Home-garden', 3: 'Charentais', 4: 'Honey Dew', 5: 'Mi-tang-ting', 6: 'Takadashirouri', M: λ DNA-*HindIII* digest / ϕ X174 RF DNA *HaeIII* digest.

Fig. 2. Microsatellite polymorphisms in Acs2-ms1. Lanes 1: 'Earl's Favourite', 2: 'Charentais', 3: 'Hamiuri', 4: 'Mirzuchulskaja', 5: 'Mi-tang-ting', M: λ DNA-*HindIII* digest / ϕ X174 RF DNA *HaeIII* digest.

six cultivars sequenced were different with each other at the sequence level (Fig. 3a). Therefore, it was clearly indicated that Acs2-ms1 was highly polymorphic and thirteen cultivars were classified into five types simply by the size of PCR product (Table 3).

The PCR product of Acs2-ms2 was digested with *HinfI* and *MboI*, and microsatellite polymorphism was detected in 5' UTR. Size polymorphism was detected in two fragments of 149 bp and 63 bp among three fragments. Repeat numbers of (A) in 149 bp fragment and of (GAAAA) in 63 bp fragment proved to be from six to ten and either three or four, respectively (Fig. 3b). Base substitution from (A) to (G) was also detected in both fragments of 'Tendral o Invernale a Buccia Verde'.

CMe-ACO1; PCR products of Aco1-ms1 were digested with *MboI* and *HaeIII*, and size polymorphism was detected in SSR of (CTT)_n in the 5' flanking region. The number of (CTT) repeats was variable, ranging from fifteen ('Mi-tang-ting') to nineteen ('Tendral o Invernale a Buccia Verde') as shown in Fig. 3c. Deletion of (T) was detected in 'Mi-tang-ting'. Microsatellite polymorphism was also detected in 3' UTR (Aco1-ms2), where the repeat number of (AT) proved to be either seven or ten.

CMe-ACO3; Size polymorphism was detected in Aco3-ms1, in the motif of (TTTA)₂(TTTC)(TTTG)_n in intron 1. Four cultivars, 'Homegarden', 'Melon Parental Line Nou 2 gou', 'Mi-tang-ting' and 'Takadashirouri', were characterized by an insertion of (TTTG)(T)₈ (Table 3).

a) Acs2-ms1		Product size(bp)
Earl's Favourite	FP(15)TAAATGGTAAAGATTAAAGTTGTTT(T) ₁ (TA) ₁ —(AT) ₇ (T) ₁ AGCTTAGATTAT(70)RP	200
Andes	(T) ₁ (TA) ₁ (A) ₁ —(AT) ₇ (T) ₁	200
Charentais	(TA) ₂₀ —(AT) ₇ (T) ₁	218
Hamiuri	(TA) ₁₁ —(AT) ₇ (T) ₁	200
Mirzuchulskaja	(TA) ₁₇ —(AT) ₇ (T) ₁ ...G.....	252
Mi-tang-ting	(T) ₁ (TA) ₁ —AC(AT) ₇ (T) ₁ ...T.....	196
b) Acs2-ms2		Product size(bp)
Earl's Favourite, Andes	FPTAAAGTGGAAAAGA(AAG) ₄ —(A) ₃ GGAT(193)CAAAA—(GAAAA) ₃ ACATCAATCTRP	64, 92, 149
Spicy	(AAG) ₃ G—(A) ₃(GAAAA) ₄	62, 92, 154
Charentais, Spain ¹⁾	(AAG) ₃ G—(A) ₁₁(GAAAA) ₄	63, 92, 154
Tendral ²⁾	(AAG) ₃ G(A) ₃ G(A) ₄GAAGA(GAAAA) ₃	63, 92, 154

Fig. 3. Sequence polymorphisms detected in two microsatellite motifs in CMe-ACS2. PCR product of Acs2-ms2 was digested by *MboI* and *HinfI*, and the fragment size was indicated. FP = Forward primer, RP = Reverse primer. The number in brackets indicates the number of base pairs omitted. Dot denotes identity; dash denotes insertion/deletion. ¹⁾ Spain Noboru 3 gou, ²⁾ Tendral o Invernale a Buccia Verde.

Discussion

The molecular analysis of thirteen melon cultivars of five varieties revealed the existence of significant polymorphism in eight regions of four ethylene-related genes. Polymorphic markers were established in two or three regions within each gene except *CMe-ACO3*. In *CMe-ACS2*, the degree of polymorphism is different between two regions, being hypervariable in *Acs2-ms1* at ca. 2.2 kb upstream from transcription start site and less variable in *Acs2-ms2* at the 5' UTR region (Table 3). In melon, as well as other crop species, various kinds of markers such as isozyme (Staub *et al.* 1987, Okubo *et al.* 1990), RAPD, RFLP (Silberstein *et al.* 1999) and AFLP (Wang *et al.* 1997) have been established. According to Neuhausen (1992), American field type cultivars (var. *reticulatus*) showed the same RFLP type as 'Honey Dew' (var. *inodorus*). Isozyme analysis also failed to detect variety specific variation in peroxidase and acid phosphatase (Kato *et al.* 1998). Therefore, it has so far been difficult to clarify the phylogenetic relationship between melon varieties, by the analysis of isozyme and RFLP markers. On the contrary, microsatellite polymorphism has been analyzed in the *Cucurbitaceae* family (Katzir *et al.* 1996), and five markers seemed polymorphic among oriental melon and other types of melon according to their data. In the present study, CAPS and microsatellite polymorphisms specific to vars. *makuwa* and *conomon*, local varieties in east Asia, were detected in four markers, *Acs1-c1*, *Acs2-ms2*, *Aco1-ms2* and *Aco3-ms1* (Table 3). As to *Aco1-ms2*, Zheng and Wolff (2000) also tried to detect polymorphisms, but all cultivars proved to carry the (AT)₇ allele. On the contrary, ten cultivars of vars. *makuwa* and *conomon* carry (AT)₁₀ allele which is the same as 'Mi-tang-ting' (unpublished data). It was therefore suggested that a polymorphism at *Aco1-ms2* could be regarded as a marker specific to vars. *makuwa* and *conomon*. Although 'Melon Parental Line Nou 2 gou' and 'Homegarden' carry the same allele of *Aco1-ms2* and *Aco3-ms1* as vars. *makuwa* and *conomon*, it can be explained by the use of Chinese melon of var. *makuwa* and Indian melon for the breeding of these cultivars (Takada 1983, Whitaker and Davis 1962).

Another important marker was established in *Acs2-ms1* at ca. 2.2 kb upstream from transcription start site. The nucleotide sequence of *Acs2-ms1* differed among six cultivars sequenced in the present study (Fig. 3a), and the number of (TA) repeats ranged from 7 to 37. Even simply based on the size of PCR product, five alleles were detected among 13 cultivars investigated (Table 3). A highly polymorphic marker should be useful for cultivar identification as well as phylogenetic analysis. The analysis of such markers for east Asian melon should provide important information on the origin and differentiation of vars. *makuwa* and *conomon*.

Plant hormone ethylene is related with many agronomic characters such as sex expression and shelf-life of fruit (Picton *et al.* 1995). Among ethylene-related genes, *CMe-*

ACS1 and *CMe-ACO1* are expressed during fruit ripening (Miki *et al.* 1995, Shiomi *et al.* 1999), *CMe-ACS2* in immature fruit (Yamamoto *et al.* 1995), and *CMe-ACO3* in flower tissue (Lasserre *et al.* 1996), indicating the involvement of these genes in various characters. Sex expression is an important character related to the efficiency of F₁ seed production, and gynoeceious 'Wisconsin-998' has been bred by Peterson *et al.* (1983). Genetic analysis revealed that sex expression is controlled by three genes, *a*, *g* and *m* in melon (Wall *et al.* 1967). As to the molecular marker for sex expression, in cucumber (*Cucumis sativus* L.), close linkage between *F* locus and *CS-ACS1G* (Trebish *et al.* 1997), and the association of allelic variation in *CS-ACS2* with sex determination (Kamachi *et al.* 1997, 2000) have been reported. Although *CMe-ACO3* expressed in flower tissue was analyzed in the present study, genotype for *Aco3-ms1* was the same between 'Earl's Favourite' (normal setting of perfect flower) and 'Honey Dew' (poor setting).

There has been few reports on genetic analysis of shelf-life in melon, probably because of its polygenic nature and large environmental effect. Therefore, a selection marker should be developed to improve the breeding efficiency of shelf-life. DNA polymorphism at eight markers established in ethylene-related genes were compared between melon cultivars with different degree of shelf-life. Although the shelf-life of 'Honey Dew' is longer than that of 'Charentais' (Zheng and Wolff 2000), these two cultivars showed the same type for three markers, *Acs1-ms1*, *Acs1-c1* and *Acs1-c2*. These results suggested that microsatellite and CAPS polymorphisms detected in the present study were not related with shelf-life and sex expression. Therefore, further analysis is necessary to select molecular markers for ethylene-related characters in *C. melo*.

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