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Expression of Two 1-Aminocyclopropane-1-Carboxylate Synthase Genes, *CS-ACS1* and *CS-ACS2*, Correlated with Sex Phenotypes in Cucumber plants (*Cucumis sativus* L.)

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Abstract

Ethylene regulates the sex expression in cucumber plants (*Cucumis sativus* L.). We examined expression of two 1-aminocyclopropane-1-carboxylate (ACC) synthase genes, *CS-ACS1* and *CS-ACS2*, at the apices of isogenic gynoecious (*FF*) and monoecious (*ff*) cucumber lines. The transcripts of *CS-ACS1* and *CS-ACS2* were detected at the apices of gynoecious line. On the other hand, only the *CS-ACS2* transcript was detected at the apices of monoecious line. The expression of *CS-ACS2* at the apices was localized to the floral buds that would develop into female flowers. These results suggest that the differentiation of female flowers at the apices of isogenic gynoecious and monoecious cucumber plants is regulated by the levels of both *CS-ACS1* and *CS-ACS2* mRNA at the apices.

Abbreviations

ACC, 1-aminocyclopropane-1-carboxylic acid; RT-PCR, reverse transcription-polymerase chain reaction; STS, silver thiosulphate

Introduction

Cucumber plants (*Cucumis sativus* L.) produce unisexual flowers. The distribution of male and female flowers along the plant axis varies from one plant to the next, giving rise to various sex types (Malepszy and Niemirowicz-Szczytt 1991). Monoecious plants produce male flowers at the base of the main stem, then they produce male and female flowers on the middle part, and finally female flowers are produced at the top. The gynoecious type of cucumber plant produces only female flowers. The female sex phenotype is regulated by the partially dominant allele at the *F* locus (Pierce and Wehner 1990).

It has been suggested that sex expression in flowers of cucumber plants is also regulated, at least in part, by levels of ethylene at the apex. Treatment of cucumber plants with ethephon or with ethylene promoted the development of female flowers (McMurray and Miller 1968, Rudich *et al.* 1969, Iwahori *et al.* 1970). Inhibitors of both ethylene action and biosynthesis suppress female flower development (Beyer 1976, Atsmon and Tabbak 1979). Rudich *et al.* (1972, 1976) reported that a high correlation existed between the evolution of ethylene from apices and the formation of female flowers. The biosynthesis of ethylene in plants has been proposed to be primarily regulated at the level of the 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (Yang and Hoffman 1984). It was reported that the activity of ACC synthase at the apex was higher in gynoecious plants than in monoecious plants (Trebitsh *et al.* 1987).

Two ACC synthase genes have been cloned in cucumber plants (Trebitsh *et al.* 1997, Kamachi *et al.* 1997). Trebitsh *et al.* (1997) reported the identification of an ACC synthase gene (*CS-ACS1*). Monoecious cucumber plants possess a single copy of this gene, whereas gynoecious lines possess at least one additional copy (*CS-ACS1G*), and the *CS-ACS1G* gene is closely linked to the *F* locus (Trebitsh *et al.* 1997). However, they did not show the expression of *CS-ACS1G* at intact shoot apices of gynoecious cucumber and the difference of the expression of *CS-ACS1* between the monoecious and the gynoecious. On the other hand, we have cloned a cDNA (*CS-ACS2*) from cucumber plants (Kamachi *et al.* 1997). Both the timing and the levels of expression of the *CS-ACS2* transcripts at the apices of cucumber plants were correlated with the development of female flowers on the nodes. Although these results indicate the possibility that these genes (*CS-ACS1* and *CS-ACS2*) are involved

in the regulation of sex expression in cucumber plants, the relationship between the expression of the two ACC synthase genes and the development of female flower at the apex remains to be clarified. Therefore, we have examined the expression of *CS-ACS1* and *CS-ACS2* transcripts at intact apices of cucumber plants using isogenic monoecious and isogenic gynoecious lines.

In this paper, we report that both *CS-ACS1* and *CS-ACS2* transcripts were expressed at the apices of gynoecious line and only *CS-ACS2* transcript was expressed at the apices of monoecious line, and discussed a possibility that the transcript detected with the use of *CS-ACS1* probe in this experiment may be the transcript of the *CS-ACS1G* gene.

Materials and Methods

Plant Materials

The seeds of isogenic monoecious (*Cucumis sativus* L.; *ff*) and gynoecious (*C. sativus* L.; *FF*) cucumber lines were derived from cucumber plants (*C. sativus* L., cv. Rensei; *Ff*) at Tohoku Seed Co. (Utsunomiya, Japan). The seeds of F_1 (*Ff*) progeny resulting from these two isogenic lines were also prepared at the Tohoku Seed Company. The F_1 line had a higher degree of female sex expression. The seeds of monoecious cucumber cultivar (*C. sativus L.*, cv. Shimoshirazu) and gynoecious cucumber cultivar (*C. sativus* L., cv. Rensei) were obtained from a local market. All seeds were germinated and grown in soil-filled pots in a greenhouse at 25 ℃ with 12 h of light per day. After growth of the seedlings, the sex of each flower on the first 25 nodes was examined and classified as male or female. A node was designated male if it had at least one male flower, and it was designated female if only female flowers were present on it.

To analyze the gene expression at intact apices of the isogenic monoecious, gynoecious and F_1 lines, the apices were cut off from the seedlings just below the youngest leaf at indicated stages of growth, frozen immediately in liquid nitrogen, and stored at - 80 ℃ prior to extraction of nucleic acids. For studying the localization of expression of *CS-ACS2* transcript in the apex of a cucumber plant, the apices of 25-day-old Rensei and Shimoshirazu plants were prepared as described above, and floral buds and unexpanded leaves from the eighth node to the sixteenth node were excised from the apices under a light-microscope, frozen immediately in liquid nitrogen, and stored at -80 ℃ prior to extraction of nucleic acids.

Isolation of RNA

Total RNA was extracted as described by Prescott and Martin (1987), and then RNA was purified by precipitation in lithium chloride. Poly $(A)^+$RNA was isolated from the total RNA with PolyATtract® mRNA isolation system III (Promega, Inc., Madison, WI, USA).

Preparation of a cDNA Probe

Because the sequence of *CS-ACS3* (Kamachi *et al.* 1997) is identical to that of *CS-ACS1* (Trebitsh *et al.* 1997), the insert of *CS-ACS3* was used as the CS-ACS1 probe. The inserts of *CS-ACS2* and *CS-ACS3* were isolated from the plasmid by electrophoresis and recovered with the Geneclean kit (Bio 101, Inc., Vista, CA, USA) as previously described (Kamachi *et al.* 1997). The cDNA was labeled with [α-^{32}P]dCTP by the random-priming method with a Multiprime™ DNA labeling system (Amersham Pharmacia Biotech, Tokyo, Japan) and used as the probe.

RNA Gel Blot Analysis

Poly $(A)^+$RNA (2 μg per lane) was subjected to electrophoresis on a formaldehyde gel and transferred to a GeneScreen Plus membrane (Du Pont, Boston, MA, USA) as previously described (Kamachi *et al.* 1997). The membrane was hybridized with ^{32}P-labeled *CS-ACS1* probe, and conditions for the hybridization and wash were as described previously (Kamachi *et al.* 1997). Next, the membranes were washed with boiling 0.01x SSC (1x SSC is 0.15 M NaCl, 15 mM sodium citrate) and 0.01% SDS to dehybridize the probe. The membrane was rehybridized with ^{32}P-labeled *CS-ACS2* probe. Autoradiographs were obtained using a Bio-Imaging Analyzer (BAS 5000; Fuji Photo Film Co., Tokyo, Japan). Equal amounts of mRNA in each lane were verified by hybridization with ^{32}P-labeled ubiquitin.

Expression Analysis by RT-PCR

The following pairs of oligonucleotides were used: *CS-ACS1*-specific primers CS1-S1 (5'-GGGTCTTGCCGAGAATCAACTAACA-3') spanning positions 15 to 33 and 146 to 151 of the *CS-ACS1* genomic sequence, and CS1-A1 (5'-GTTGGGTGACTTGGAAGCCGTTGGA-3') spanning position 619 to 643 of the *CS-ACS1* genomic sequence (Trebitsh *et al.* 1997); CS-ACS2-specific primers CS2-S334 (5'-GGAGGAAAA-CTGTGAGGGAGAAGGG-3') and CS2-A776 (5'-GATTGTGGACCGTTGGATCGTTGCT-3'), spanning positions 334 to 358 and 776 to 790 of the *CS-ACS2* sequence, respectively (Kamachi *et al.*

1997). Total RNA was treated with RNase-free DNase I (FPLC*pure* Deoxyribonuclease I; Amersham Pharmacia Biotech, Tokyo, Japan) to eliminate residual genomic DNA. RT-PCR was performed with a GeneAmp RNA PCR Kit (Perkin-Elmer Japan Co. Ltd., Urayasu, Japan) using 1 μg of total RNA. A detailed description of the RT-PCR condition was given in the previous paper (Kamachi *et al.* 1997). The parameters for PCR were 18 cycles of heating at 95 ℃ for 30 sec, at 65 ℃ for 30 sec and at 72 ℃ for 1 min. The number of cycles in the PCR reactions was adjusted so that the amplification of products remained in the linear phase. The PCR products were analyzed by 2% agarose gel electrophoresis and blotted to GeneScreen Plus membranes (Du Pont, Boston, MA, USA). The blots were hybridized with a *CS-ACS1* probe and a *CS-ACS2* probe, respectively, as previously described (Kamachi *et al.* 1997).

Results

Time Course of the Expression of ACC synthase Genes at the Apices of Isogenic Gynoecious and Monoecious Cucumber Lines

To elucidate the relationship between the expression of two ACC synthase genes (*CS-ACS1* and *CS-ACS2*) and sex expression in flowers of cucumber plants, we used a pair of isogenic gynoecious (*FF*) and monoecious (*ff*) cucumber lines. **Fig. 1** shows the patterns of sex expression in flowers of these lines. The gynoecious line produced only female flowers, whereas the monoecious line produced male flowers on several lower nodes, followed by a mixed phase of male and female flowers (**Fig. 1**).

Fig. 2 shows the time course of the expression of *CS-ACS1* and *CS-ACS2* transcripts at the apices of these isogenic gynoecious and monoecious lines. The floral primordia were not observed at the apices of 12-day-old seedlings of gynoecious and monoecious lines and floral buds which sexes were not determined yet were first formed at the apices of 15-day-old seedlings of both lines (data not shown) as reported in a previous paper (Kamachi *et al.* 1997). The transcript of *CS-ACS1* was not detected at the apices of monoecious line; however, the transcript was detected at the apices of gynoecious line at all stages examined by RNA blot analysis with poly (A)$^+$ RNA (**Fig. 2**). The same results were also obtained by DNA blot analysis of a RT-PCR reaction on total RNA isolated from the apices of isogenic gynoecious and monoecious lines (**Fig. 2**). To elucidate the physiological function of the *CS-ACS1* gene, the transcript at the apices of F_1 seedlings between the isogenic gynoecious and monoecious lines were examined. As shown in **Fig. 2**, the *CS-ACS1* transcript was also detected at the apices of F_1 seedlings at all stages examined. The levels of *CS-ACS1* transcript were one half those of the isogenic gynoecious line (**Fig. 2**). As shown in **Fig. 2**, the *CS-ACS2* transcript was not detectable at the apices of 12-day-old seedlings of the gynoecious, monoecious and F_1 lines. The *CS-ACS2* transcript was detected at the apices of 15-day-old seedlings of gynoecious line and the levels increased up to 21 days after planting. Although the *CS-ACS2* transcript was detected at the apices of 18-day-old and 21-day-old seedlings of monoecious line, the levels of the transcript were low compared to those of the gynoecious line. The timing and the levels of expression of the *CS-ACS2* transcript at the apices of F_1 seedlings were nearly the same as those of the gynoecious line (**Fig. 2**).

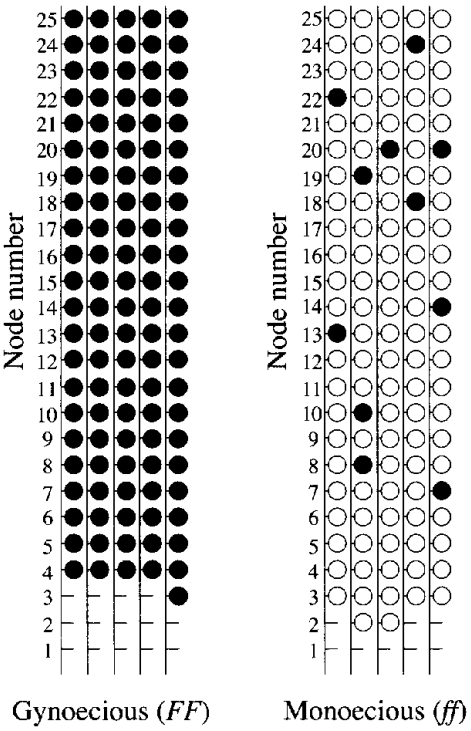

Fig. 1. The patterns of sex expression in flowers of the isogenic gynoecious (*FF*) and monoecious (*ff*) cucumber lines. Plants were grown in soil-filled pots in a growth chamber with 12 h of light per day at 25 ℃. The node number indicates the position of individual nodes along the main shoot. Closed circles, nodes with female flowers; open circles, nodes with male flowers; no circles, vegetative nodes. Data from five plants are presented in each case.

Localization of the Expression of CS-ACS2 Transcript at the Apices of Cucumber Plants

As shown in **Table 1**, a gynoecious cultivar, Rensei, produced only female flowers and a mono-

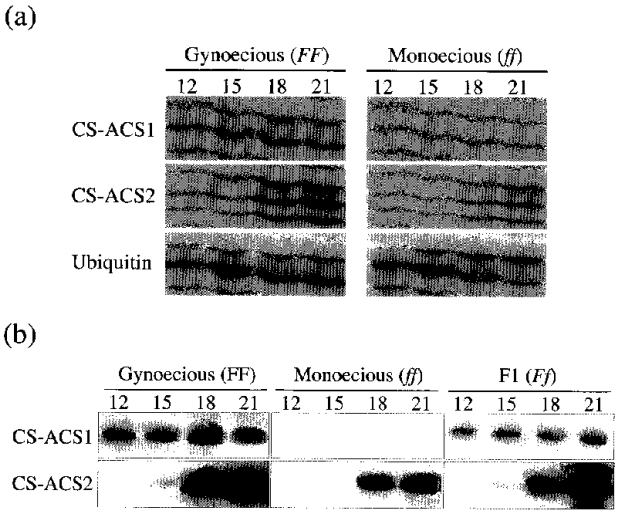

Fig. 2. Time course of the expression of *CS-ACS1* and *CS-ACS2* transcripts at the apices of cucumber plants. (a) The isogenic gynoecious (*FF*) and monoecious (*ff*) cucumber lines were grown under the conditions described in Fig. 1. Poly $(A)^+$ RNA was extracted from the apices of gynoecious and monoecious lines when the plants were 12-day-old (i.e., at the first leaf stage), 15-day-old (i.e., at the two-leaf stage), 18-day-old (i.e., still at the two-leaf stage) and 21-day-old (i.e., at the three-leaf stage). Extracted poly (A)+ RNA (2 _g) was fractionated on a formaldehyde-containing agarose gel, transferred to a nylon membrane and allowed to hybridize with the *CS-ACS1*, *CS-ACS2* and ubiquitin probe. (b) The isogenic gynoecious (*FF*), monoecious (*ff*) and F_1 cucumber lines were grown under the conditions described in Fig. 1. Total RNA was extracted from the apices of these lines when the plants were 12-day-old (i.e., at the first leaf stage), 15-day-old (i.e., at the two-leaf stage), 18-day-old (i.e., still at the two-leaf stage) and 21-day-old (i.e., at the three-leaf stage). *CS-ACS1* and *CS-ACS2* cDNAs were amplified from the total RNA by RT-PCR using a pair of *CS-ACS1*-specific primers (CS1-S1 and CS1-A1) and a pair of *CS-ACS2*-specific primers (CS2-S334 and CS2-A776), respectively, under the conditions described in "Materials and Methods". The PCR products were separated on agarose gels, transferred to nylon membranes and allowed to hybridize with the *CS-ACS1* and *CS-ACS2* probe, respectively.

ecious cultivar, Shimoshirazu, produced only male flowers on the lower nodes as reported previously (Kamachi *et al.* 1997). Therefore, we used these two cultivars and examined the localization of the expression of *CS-ACS2* transcript in the apex. As the apices included floral buds and unexpanded leaves, they were separated under a light-microscope. The expression of *CS-ACS2* transcript was barely detected at the unexpanded leaves included in the apices of both Rensei and Shimoshirazu plants. Although the *CS-ACS2* mRNA was not detected at the floral buds included in the apices of Shimoshirazu plants, the transcript was detected at those of Rensei plants (**Fig. 3**).

Discussion

Sex expression in cucumber plants is mainly determined by two loci, namely, *F* and *m*. The *F* gene is a partially dominant gene that controls femaleness (Pierce and Wehner 1990). The dominant allele at the *m* locus specifies unisexual flowers. A line dominant for *F* (gynoecious) has a higher degree of female sex expression than an isogenic line recessive for this gene (monoecious), as in *MM* or *Mm* plants. As shown in **Fig. 1**, the isogenic gynoecious line (*FF*) used in this experiment had a high degree of female expression than the isogenic monoecious line (*ff*).

Trebitsh *et al.* (1997) have reported the identification of an ACC synthase genomic sequence in cucumber (*CS-ACS1*) that is auxin-inducible in both monoecious and gynoecious cucumber species. Monoecious cucumber possesses a single copy of this gene, whereas gynoecious lines possess at least one additional copy (*CS-ACS1G*) and the *CS-ACS1G* gene is closely linked to the *F* locus (Trebitsh *et al.* 1997). However, they did not show the expression of *CS-ACS1G* transcript at intact apices of gynoecious cucumber. Although they could not detect the expression of *CS-ACS1* transcript at intact apices of gynoecious cucumber plants by RNA blot analysis with total RNA (Trebitsh *et al.* 1997), we could detect the gynoecious-specific expression of the *CS-ACS1* transcript by both RNA blot analysis with poly $(A)^+$ RNA (**Fig. 2**) and DNA blot analysis of a RT-PCR reaction on total RNA prepared from the apices of the isogenic gynoecious cucumber line (**Fig. 2**). The transcript of *CS-ACS1* was detected at the apices of isogenic gynoecious line and not detected at those of isogenic monoecious line at all stages examined. The reason for this discrepancy may be due to the difference in detectable level of the RNA by blot analysis used in the experiments.

As shown in **Fig. 2**, the *CS-ACS1* transcript was detected only at the apices of the isogenic gynoecious cucumber line. In these experiments, we used *CS-ACS1* as the probe for the detection of the

Table 1. Sex expression in the gynoecious (cv. Rensei) and monoecious (cv. shimoshirazu) cucumber plants.

Cultivar	Number of Nodes per plant		
	Female	Male	Aborted
Rensei	17.4 ± 0.2	0.0 ± 0.0	2.6 ± 0.2
Shimoshirazu	0.0 ± 0.0	18.8 ± 0.2	1.2 ± 0.2

The sex of each flower on the first 20 nodes of the main stem was examined and classified as male or female. Results for 5 plants (± SE) are given.

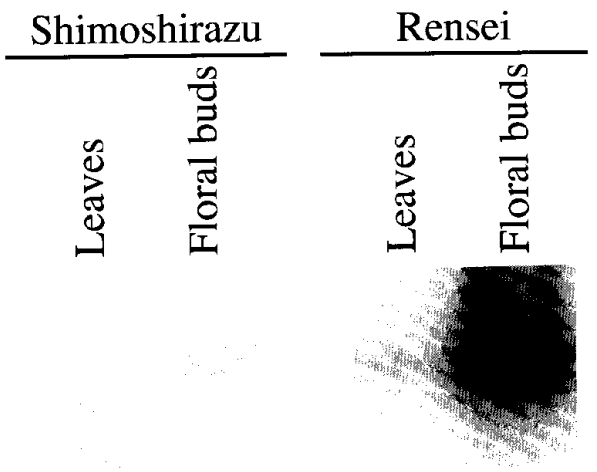

Fig. 3. Expression of *CS-ACS2* at floral buds and unexpanded leaves included in the apices of Rensei and Shimoshirazu cucumber plants. Total RNA was extracted from floral buds and unexpanded leaves from the eighth node to the sixteenth node of 25-day-old Rensei and Shimoshirazu plants. *CS-ACS2* cDNA was amplified from the total RNA by RT-PCR using primers CS2-S334 and CS2-A776 under the conditions described in "Materials and Methods". The PCR products were separated on an agarose gel, transferred to a nylon membrane and allowed to hybridize with the *CS-ACS2* probe.

transcript by RNA gel blot analysis and DNA blot analysis of a RT-PCR reaction. However, Trebitsh *et al.* (1997) reported that the *CS-ACS1* probe hybridized to both the *CS-ACS1* and *CS-ACS1G* genes. Therefore, it is not clear whether the detected transcript by the *CS-ACS1* probe is the *CS-ACS1* transcript or the *CS-ACS1G* transcript. Trebitsh *et al.* (1997) reported that the monoecious cucumber line dose not possess the *CS-ACS1G* gene, though both isogenic gynoecious and monoecious lines commonly possess the *CS-ACS1* gene. They also reported that the *CS-ACS1G* gene is closely linked to the *F* locus. Furthermore, *CS-ACS1* transcript was detected at the apices only when the gynoecious and monoecious cucumber plants were treated with auxin (Trebitsh *et al.* 1997). We could not detect the *CS-ACS1* transcript at intact apices of the isogenic monoecious line but could detect it at those of the isogenic gynoecious line **(Fig. 2)**. Furthermore, we showed that the quantity of the transcript detected at the apices of F_1 (*Ff*) seedlings is one half that of the isogenic gynoecious (*FF*) line **(Fig. 2)**. Therefore, it is likely that the transcript detected with the use of the *CS-ACS1* probe at the apices of both isogenic gynoecious and the F_1 seedlings may be the transcript of the *CS-ACS1G* gene **(Fig. 2)**.

The transcript of *CS-ACS2* was detected at the apices of the isogenic monoecious cucumber line and it was also detected at those of the isogenic gynoecious and the F_1 seedlings **(Fig. 2)**. In a previous paper (Kamachi *et al.* 1997), we showed that the timing and the levels of expression of the *CS-ACS2* transcript at the apices of gynoecious and monoecious cucumber cultivars were correlated with the development of female flowers on the nodes, and that the levels of *CS-ACS2* mRNA at the apices of those cultivars were developmentally regulated. Therefore, from the results shown in **Fig. 2**, it is considered that the expression of *CS-ACS1* (*CS-ACS1G*) transcript at apices of the gynoecious line may hasten the timing of expression of the *CS-ACS2* transcript and increase the levels of expression of the transcript *via* ethylene production at the apices compared to the isogenic monoecious line. The expression of *CS-ACS2* transcript was localized in the apices of cucumber plants. The *CS-ACS2* mRNA was remarkably detected at the floral buds of Rensei plants that would develop into female flowers **(Table 1 and Fig. 3)**. These results suggest that the *CS-ACS2* transcript is expressed only in limited floral buds that will develop into female flowers. In fact, ethylene was thought to act on a floral bud and to induced the development of a female flower. Potentially male buds detached from plants at the bisexual stage reverted to female buds upon addition of auxin *in vitro* (Galun *et al.* 1962, 1963) and it was clarified that the feminizing effect of auxin is mediated by ethylene (Shannon and De La Guardia 1969 and Trebitsh *et al.* 1987). These results and our data reported in this paper suggest

that the expression of *CS-ACS2* transcript at floral buds induces the development of female flowers *via* ethylene formation and the expression of *CS-ACS1* (*CS-ACS1G*) transcript at the apices of the gynoecious cucumber line may hasten the timing of the expression of the *CS-ACS2* transcript and increase the levels of expression of it.

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