

Full Length Research Paper

CsACO4, an ACC oxidase gene regulating male differentiation in cucumber

Yin-Hua Chen^{1,2*}, Xiu-Mei Fu^{1,2}, Hui Wu^{1,2} and Jian zang^{1,2}

¹Hainan Key Laboratory for Sustainable Utilization of Tropical Bioresource, Hainan University, Haikou 570228, P.R China.

²College of Agriculture, Hainan University, Haikou, 570228, P.R China.

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Ethylene has been implicated as a sex-determining hormone in cucumber: Its exogenous application increases femaleness and gynoecious genotypes were reported to produce more ethylene. 1-aminocyclopropane-1-carboxylate (ACC) oxidase (ACO) is the key enzyme in ethylene biosynthesis. In this study, a 1,188 base pair (bp) fragment was amplified from cucumber (Jinyan-4) genome with degenerated primers derived from the ACO amino acid consensus sequence among different plant species. The putative new gene was analyzed by bioinformatic tools. Phylogenetic analysis result of the new gene (CsACO4, GenBank accession number AY450356) was in accordance with the evolution relationship of genetics among various plant species. The result of BLAST showed the sequence presented a very high match with the ACO genes from other plants; the homologue was from 80 to 99%. Using the sequence, a RNA interference (RNAi) transformation vector was constructed through the way of BP cloning. Eleven transgenic plants were obtained. The transgene integrated into cucumber genome was proved with PCR and southern blotting. The morphological exploration showed that the inserted RNAi target fragments could inhibit the endogenous CsACO4 gene expression and that it could regulate male differentiation in the lower nodes in cucumber.

Key words: Cucumber, ACC oxidase gene, RNAi, male differentiation.

INTRODUCTION

Sex expression in cucumber is influenced by genotype and plant hormones. The F and M loci are the genetic controls of sex expression. These loci interact to produce four different sex phenotypes: gynoecious (F-M-), monoecious (ffM-), andromonoecious (ffmm) and hermaphrodite (Fmm). Gynoecious lines produce only female flowers. Monoecious lines produce female and male flowers. Andromonoecious lines produce male and bisexual flowers whereas hermaphrodite lines produce bisexual flowers. Sex expression in cucumber is also influenced by the plant hormone, ethylene. Application of 2-chloroethylphosphonic acid (ethephon), an ethylene-releasing

agent, induces the formation of female flowers. In contrast, aminoethoxyvinyl glycine (AVG), an inhibitor of ethylene biosynthesis, or AgNO₃, an inhibitor of ethylene action, suppresses the development of female flowers and induces male ones (Beyer, 1976; Atsmon and Tabbak, 1979; Takahashi and Suge, 1980; Takahashi and Jaffe, 1984). Accordingly, ethylene appears to act as a sex hormone in cucumber, but the expression level on which ethylene acts to affect sex differentiation is not known.

Ethylene synthesis in plant is basically controlled by ACC synthase (ACS) and ACC oxidase (ACO) (Zarembinski and Theologis, 1994; Balague et al., 1993). Gynoecious cucumber appeared to have an additional copy of ACS gene, which was closely linked to F gene (Trebitch et al., 1997; Sekimoto et al., 1997). In Arabidopsis, five members of the ethylene receptor gene family, ETR1, ERS1, ETR2, EIN4 and ERS2, have been cloned (Chang et al., 1993; Hua et al., 1995). In situ hybridization analysis revealed that the spatial expression patterns of these genes

*Corresponding author. E-mail: yhchen@hainu.edu.cn.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylate; ACO, 1-aminocyclopropane-1-carboxylate oxidase; ACS, ACC synthase; RNAi, RNA interference.

are overlapping but not identical (Hua et al., 1998). To investigate the role of ethylene in the sex expression of cucumber, three ethylenereceptor-related genes, CS-ETR1, CS-ETR2 and CS-ERS were isolated from cucumber (Yamasaki et al., 2000). mRNA accumulation of CS-ACS2 encoding 1-aminocyclopropane-1-carboxylate (ACC) synthase is closely correlated with female flower expression in the cucumber (Kamachi et al., 1997; Yamasaki et al., 2001). The expressions of CS-ERS, CSETR2 and CS-ACS2 in gynoeocious and monoecious cucumbers are upregulated by ethylene, although those in andromonoecious cucumbers were hardly increased by ethylene treatment. In addition, short-day conditions increase female flower production, ethylene synthesis and the expressions of CS-ERS and CS-ACS2 in monoecious cucumbers, but not in andromonoecious cucumbers (Yamasaki et al., 2003).

ACO is also an important gene in ethylene biosynthesis, while the research is few. Kahana showed that ACO gene family members were differentially expressed in pollens of different sexual phenotypes (Kahana et al., 1999). Expression patterns of individual ACO genes in cucumber were highly specialized. The complexity of ethylene response has also been investigated by Deikman (Deikman et al., 1998). In a previous study, the CsACO4 transcription of different sex genotype was shown. In this research, the aim was to isolate homologous gene of the ACO family from different crop species by degenerated primers and to try investigate the function of the fragment of a candidate gene in sex differentiation.

MATERIALS AND METHODS

Plant materials

One genotype of cucumber (*Cucumis sativus*) (monoecious cv Jinyan-4) was used in this experiment. Seedlings of Jinyan-4 were grown in growth chamber for DNA extraction. Genomic DNA was extracted using CTAB method with some modifications (Murray and Thompson, 1980). 0.5 - 1.0 g young leaves were grinded well in 1.5 mL Eppendorf tube, followed by addition of 700 μ L extraction buffer (100 mmol/L Tris-HCl, pH7.5, 500 mmol/L NaCl, 50 mmol/L EDTA, 3% SDS) and incubated for 30 min at 65°C. The supernatant was extracted by one volume of chloroform/isopentanol (24/1) after 5 min centrifugation at 12,000r/min. DNA was precipitated by equal volume of cold isopropyl alcohol and 20 μ L 5 mol/L Na₂Ac solutions, followed by 5 min centrifugation at 13,000r/min. The pellet was rinsed with 75% ethanol and then dried in air, dissolved in TE buffer, digested with RNAase and stored at -20°C.

PCR amplification

Consensus analysis results of 10 ACO sequences with Clustal W1.8.1 revealed that two amino acid sequences were highly conserved. Two degenerated primers (DP1: 5'-A (AG) AA (CT) TGGGG (ATC) TTCTTTGAG-3' and DP2: 5' -TTCAT (AC) GCT TCAAA (AG) CT (ATG) GGCTC-3') were designed from the regions. The 25 μ L PCR reaction mixture contained 1 \times PCR buffer, 1.5 mmol/L MgCl₂, 0.2 mmol/L dNTPs, 0.5 μ mol/L primers, 1.5 unit *Taq* polymerase (Fermentas) and 100 ng templates DNA. PCR was

carried out under the following program: 94°C 4 min; 35 cycles of 94°C 1 min, 53°C 1 min and 72°C 1.5 min; followed by 72°C for 10 min and held on 4°C. The amplified products were separated on a 0.8% agarose gel. The target PCR product was cleaned up by gel extraction kit (Promega), ligated into pGEM-T easy vector (Promega) and transformed into DH 5 α competent cell through heat-shock method (Murray et al., 1980). Positive clones were confirmed by both *Eco*RI digestion and sequencing.

Sequence analysis

Sequence was blasted in GenBank + EMBL + DDBJ + PDB database. Its structure was analyzed with GenScan. Phylogenetic analyses were carried out with Clustal W1.8.1

Construction of plant expression vectors RNAi

The construction method of CsACO4 RNAi vector was described in reference (Chen et al., 2005). Two pair of primers were used. One was designed according to the sequence of *attB* loci and CsACO4 gene; the other was the degenerated primers according to the loci of *attB*. The first round of PCR amplification was conducted with the primers of *attB*1+ CsACO4 and *attB*2+ CsACO4. Product of the primary amplification was used as template for the secondary amplification, with the degenerated primers of *attB*. The target band was inserted into the attP loci through the way of BP cloning. A recombinant plasmid pD511 was obtained and transferred into *Agrobacterium tumefaciens* according to the freeze-thaw methods (Sambrook et al., 1989).

Generating transgenic plant

Seeds of the cucumber were surface-disinfected in 70% ethanol for 30 s and 0.1% HgCl₂ for 20 min and then rinsed three times with sterilized water. The sterilized seeds were placed in one-half strength MS medium and allowed to germinate in the dark at 25°C. Cotyledons from 3-day-old seedlings were excised to three segments and used as explants for regeneration and transformation. Explants were transferred to a pre-culture medium consisting of MS medium supplemented with BA (2.0 mg/L) +ABA (1.0 mg/L) + AgNO₃ (2.0 mg/L) and then placed in the dark at 25°C for 24 h. For transformation, *Agrobacterium* grown to log phase in LB liquid medium (OD₆₀₀: 0.7-0.9) was centrifuged at 3,500 r/min for 10 min and the pellets were resuspended in MS liquid medium. Explants were infected by immersing them in the cell suspension of *Agrobacterium* inoculum for 15 min and then co-cultured in MS medium containing BA (2.0 mg/L) +ABA (1.0 mg/L)+ AgNO₃ (2.0 mg/L) for 3 days. They were then briefly washed with 400 mg/L Cefotaxime (Cef) and placed on agar (0.8%)-solidified shoot regeneration medium supplemented with 2.0 mg/L BA, 1.0 mg/L ABA, 2.0 mg/L AgNO₃, 100 mg/L kanamycin (Kam), and 400 mg/L Cef incubated for 4-5 weeks at 25°C. Green shoots were then transferred to rooting medium (basic medium, 100 mg/L kam and 400 mg/L Cef) for 5-6 weeks and young plants were transferred to zippy pots in a growth chamber at 25°C under a 16 h light/8 h dark cycle. After 1 month in the chamber, plants were grown in a plastic house.

To detect the CsAO4 gene in transformed plant by PCR, total DNA was isolated using the method above. The PCR primer sequences used were designed according to the *NPTII* gene. Forward: 5'-AGACAATCGGCTGCTCTGAT-3'; Reverse: 5'-TCAT TTCGAACCCC AGAGTC-3'. PCR analysis was carried out using the method above.

For Southern blot analysis, about 5 - 10 μ g DNA was digested with *Bam*HI, which was separated on 0.8% agarose gel in 1 \times TAE buffer, followed by blotting onto Amersham Hybond -N+ nylon

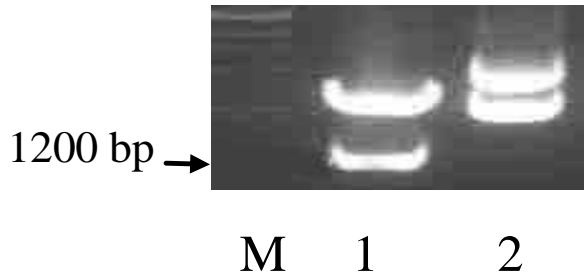


Figure 1. Identification of pGCSACO. M = *N*HindIII marker; 1= Plasmid with insert digested with *Eco*RI; 2 = Plasmid with insert.

membrane with 0.4 M NaOH solution. The membranes were hybridized with a [32 P] dCTP-labeled P35s probe and washed twice with 0.2 \times SSC, 0.1% SDS at 65°C.

Morphological exploration of the transgenic plants

To characterize the function of *CsACO4* gene in sex differentiation, the number of female flowers from transgenic plants with single copy insert was explored as well as the control plants.

RESULTS

Cloning *ACO* gene from cucumber genome

The 1,200 bp band was obtained from Jinyan-4 with degenerated primers DP1 and DP2 and cloned. Recombinant plasmid denominated as pGCSACO was confirmed by *Eco*RI digestion (Figure 1). The recombinants were sequenced.

Sequence analysis

The sequence was analyzed with GenScan. Three exons and two introns were found in the sequence, commencing at the first Met, the length of the deduced ORF (open reading frame) was 717 bp, encoding 239 amino acids. Blast result showed that the predicted ORF was highly homologue to the *ACO* gene of *Cucumis sativus* (99%) (Accession number: AB006806), *Cucumis melo* (97%) (Accession number: X69935), *Prunus persica* (82%) (Accession number: AF129074), *Petunia hybrida* (82%) (Accession number: L21976). The differences between them were at intron and upstream non-coding sequences (Figure 2). The new gene was named *CsACO4* (GenBank accession number AY450356).

Phylogenetic analyses were conducted from the amino acid sequence of *CsACO4* and 10 other *ACO* protein sequences which were top hits of *CsACO4* (Figure 3), including the climacteric and non-climacteric crops. *CsACO4* was clustered first with *ACO* and *ACO3* of cucumber and then with melon, suggesting their relation-

ships during evolution. Climacteric tomato, orange and peach were clustered together which suggested that climacteric and non-climacteric evolution distance, quite in accordance with the analysis from genetics.

Construction of RNAi vectors

The recombinant plasmid pD511 was confirmed by PCR. The T-DNA region of *CsACO4* RNAi vector in pD511 is shown in Figure 4. It was transferred into *A. tumefaciens* strain LBA4404 and the transformed *A. tumefaciens* LBA4404 were confirmed by PCR.

Transgenic plant generation

A total of 27 transgenic plantlets were obtained mediated with *Agrobacterium* transformation. To confirm the presence of the T-DNA in the putative transgenic plants, the T₀ plants were subjected to PCR analysis with the primers specific for both *NPTII* and *ACO4* genes. The expected PCR products of 740 bp representing the *NPTII* gene were detected on the gel from 18 transplants. Plasmid as positive control showed the expected bands while no signal was detected in the negative controls. These results showed that the transgenic plants contained foreign genes. For *ACO4* gene verifying, a P35S-forward primer and *ACO*-reverse primer were used to carry out amplification.

Southern blot analysis was performed with *Bam*HI-digested plant genomic DNA from 18 randomly selected T₀ plants which showed that 11 plants (61%) had hybridization signals when hybridized with the radiolabelled *gus* and P35s gene probe. The plasmid showed a strong signal and the non-transformed control did not show any hybridization signal (Figure 5). This indicated that the foreign genes were inserted into the cucumber genome.

Sex determination in the transgenic plants

The results of the morphological exploration showed that all the transgenic plants (1, 3, 5) had no female flowers in the lower nodes (< 13) (Table 1), while the non-transformed control had 1 - 4 female flowers in the lower nodes (Figure 6). This implied that the inserted target fragment inhibited the endogenous gene expression and it contributed to the male differentiation in the lower nodes. This showed the function of *ACO* gene in the sex differentiation of cucumber.

DISCUSSION

Homologous sequence method is a rapid and convenient way to isolate target gene. It is accomplished using PCR

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AAAATTGGGGTTTCTTTGAGGTGATTCATAGATTAATTATCCTCTCTCTCTCTTTCTCTCTCTCTCTCTCTCTCTGTGAGGTGAT +92
GTCCTGATTTGATTTTAAATATGCAGTTGGTGAACCATGGGATCCCACATGAGTTTTTGGACATGGTTGAGAAGATGACAAGATCATGACA +184
      M V E K M T R D H D
AGAAATGTATGGAAGAGAGGTTTAAGGAGACTGTTCTTAGCAAAGGCTTAGAGGCTGCACAAGCTGAAGTTAATGATATGGATTGGGAAAGC +276
K K C M E E R F K E T V L S K G L E A A Q A E V N D M D W E S
ACCTTCTTCTACGCCATCTCCCGAATCAAACATCTCCAGATGTCTGATCTCGACGAGGAGTATAAGTATGATATTATCTAATTTAAATT +368
T F F L R H L P E S N I S Q M S D L D E E Y K
GTATATAAATGTGTGATAATATTAATCTTTTAGGACATTACTATTCGGATTCTCAACAATTTTAAATCATATGTGAATGATTGGCAGGAAA +460
      K
ATTATGAAGGAATTTGCGAAGAAATGGAGAATCTTGCTGAGGAGTTGTTGGACCTGCTATGTGAGAATCTTGGGTTGGAAAAGGGTTATCT +552
I M K E F A K K L E N L A E E L L D L L C E N L G L E K G Y L
CAAAAATGCTTTCTATGGTTGCGAAAGGCTACATTTGGAACAAAGTTAGCAAATATCCACCGTGTCCCAAGCCCGACCTCATCAAGGGTC +644
K N A F Y G S K G P T F G T K V S K Y P P C P K P D L I K G
TTCGAGCCACACCGACGCCGGTGGCATCATCCTCCTCTCCAAGATGACAAGGTAGGTGGCCTGCAACTCCTGAAAGATGGCGACTGGATC +736
L R A H T D A G G I I L L F Q D D K V G G L Q L L K D G D W I
GATGTGCCCCAATGCGCCATGCCATTGTCGTCAACCTCGGAGACCAACTTGAGGTAATATTATATACTAATACTTATAAAGCAAAGTAGAA +828
D V P P M R H A I V V N L G D Q L E
AAGAAGGACAACGATTAATGATCTATTATCTGCGTTGCATAAAAGATTAGATGGAACATAAGACAATATCCACTTAAGTTTATTCTTAT +920
TAACGTA AAACTGGCAGGTGATC A AAATGGAAGATACAAAAGTGTGATGCCAGAGTGTAAATTACTCAAACAAGTGGAACTGGACGAATG +1012
      I Q K C D A R V L I T Q T S G T G R M
TCGATAGCTTCACTTACAATCCGGGGAGCGACGCGGTGATCTACCCTGCGCCGGCCTAGTAGAGAAAGATGAGGAGAAGAAGGAAGTGA +1104
S I A S F Y N P G S D A V I Y P A P A L V E K D E E K K E V Y
CCCCAAGTTGTGTTTGAAGATTACATGAAGCTGTACCTAGGAGTAAAGTTTCAGGCTAAGGAGCCAAGATTTGAAGCCATGAA +1188
P K F V F E D Y M K L Y L G

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Figure 2. Nucleotide and deduced amino sequences of *CsACO4*. Three exons and two introns were found in the sequence, commencing at the first Met, the length of the deduced ORF (open reading frame) is 717 bp, encoding 239 amino acids. The up shows the nucleotide sequence and the under shows the deduced amino sequence.

or RT-PCR with specific primers, which is designed through the comparison of known gene sequence that is a homolog to the gene to be cloned. The amplified fragment is then confirmed by its sequence. The amino acid sequences of *ACO* from different plants show high conservation, and the homology increased with a closer relationship. Degenerate primers can be designed according to conservative sequence and subsequent PCR amplification of genomic DNA and cDNA generate a fragment of the gene. Full-length gene can be achieved with reverse PCR and adaptor PCR. Comparing several *ACO* amino acid sequences, degenerated primers was designed. Using the degenerated primers, PCR amplification of cucumber genomic DNA generated an 1188 bp

gene fragment. It was confirmed with bioinformatics. No adaptor and its ligation with the restriction-digested genomic DNA was needed in this method and PCR amplification was repeatable and specific. The result of further phylogenetic analysis was in coincidence with the genetic distance.

A number of studies reported a correlation between the degree of femaleness in cucumber and endogenous ethylene production. Gynoecious plants evolved 2 - 3 times more ethylene than monoecious and androecious genotypes (Sekimoto et al., 1997). The detached female floral buds evolved more ethylene than male flower buds. Moreover, the sex-determination locus, *F*, was found to be linked to *CS-ACO2* at a distance of 8.7 cm (Kahana et

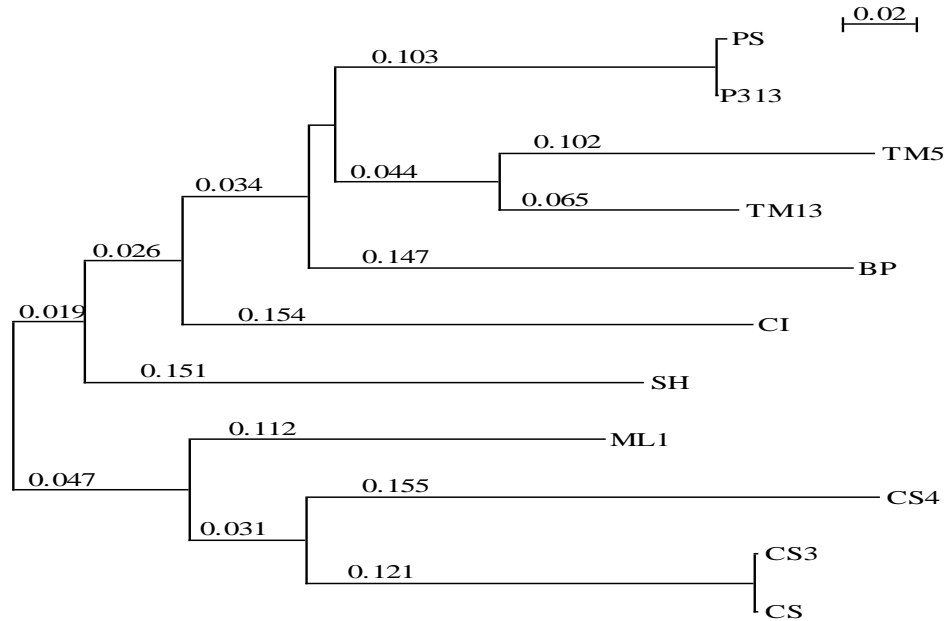


Figure 3. Phylogenetic tree of ACC oxidase sequences by Clustal W1.8.1. The accession number, PS: S41880 (peach), P313: 1909340A (peach), TM5: P24157 (tomato), TM13: P05116 (tomato), BP: AY154649.1 (brich), CI: AF321533 (citrus), SH: AB072040.1 (*Striga hermonthica*), ML1: Q04644 (melon), CS4: AY450356 (cucumber), CS3: AF033583 (melon), CS: AB006806 (cucumber). The number means the Phylogenetic distance conficiency.



Figure 4. T-DNA regions of the expression vector pD511.

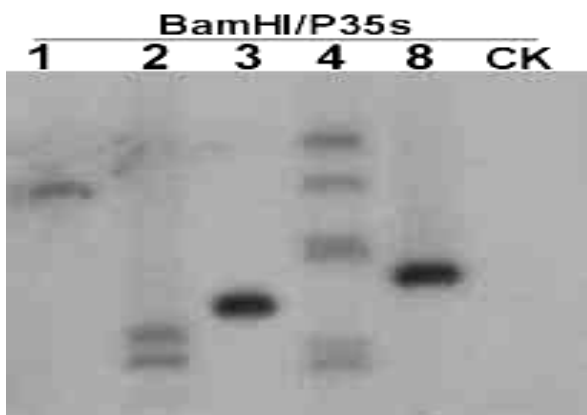


Figure 5. Southern analysis from parts of pD511 transgenic plants (*Bam*HI/ P35s). Lane1 - 4, 8 = transgenic plants; CK = Jinyan-4. The codes indicate number of transgenic plants.

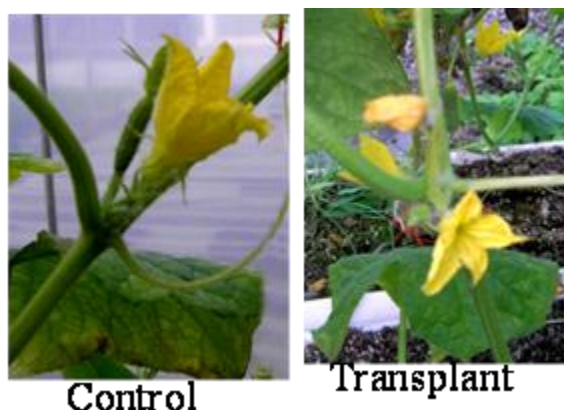
There was a band in the female flower of gynoecious and monoecious genotype. It seemed that the gene expression might be correlated with the femaleness level. To elucidate the relationship between the *ACO* transcript level and sex expression patterns in cucumber exactly, a construct of the RNAi vector of this gene was made and transformed. The morphologic exploration of transgenic plants showed that the *ACO* contributed to male differentiation in the lower nodes. These results were in coincidence with the expression analysis. All these experiments showed the importance of *ACO* gene in sex determination.

In this regeneration system, 3-day-old cotyledons as explants and a novel shoot induction medium (MS medium supplemented with 2.0 mg/L BA, 1.0 mg/L ABA and 2.0 mg/L AgNO_3) were used. It was found that the addition of ABA into shoot induction medium increased the efficiency of shoot organogenesis and induced multiple shoots. Shoot regeneration of cucumber cotyledon was also promoted by appropriate concentration of AgNO_3 (2.0 mg/L), optimally and it was harmful in excess of it. In this research, the regeneration frequency of Jinyan4 was up

al., 1999). The *CsACO4* was isolated and the mRNA of different organs hybridized with the *CsACO4* probe.

Table 1. Sex differentiation of the transgenic plants of cucumber.

Flowers		Numbers of female flowers					Numbers of male flowers				
Position of node		1	3	5	7	9	1	3	5	7	9
Transgenic plants	1#	0	0	0	0	0	1	1	3	1	1
	3#	0	0	0	0	0	0	2	1	1	1
	5#	0	0	0	0	0	1	1	1	2	1
Non-transformed plants		1	1	2	4	3	1	1	2	3	3

**Figure 6.** Sex differentiation of the transgenic plants of cucumber.

to 92.97%. It is believed that this efficient shoot regeneration system will contribute to the production of many transgenic cucumber plants (Tabei et al., 1994, 1998; Trebitsh et al., 1997).

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