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ORIGINAL ARTICLE

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Isolation of a MADS-box gene (*ERAF17*) and correlation of its expression with the induction of formation of female flowers by ethylene in cucumber plants (*Cucumis sativus* L.)

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Abstract Ethylene regulates sex expression in cucumber (*Cucumis sativus* L.) plants. When the apices of monoecious cucumber seedlings (cv. Shimoshirazu-jibai) were treated with the ethylene-releasing compound, ethephon, female flowers were induced at the nodes. To clarify the action of ethylene in the regulation of sex expression, we attempted to isolate genes whose expression changed during induction of the formation of female flowers at the apices of these cucumber plants upon treatment with ethephon. Using the differential-display method, we identified 20 clones (#1 to #20) that reflected differences in the accumulation of transcripts in apices treated or not treated with ethephon. Sequence analysis of cDNA fragments revealed that the cDNA #17 had the sequence of a MADS-box gene. We isolated the full-length cDNA and showed that it included both a MADS box and a K box, and the corresponding gene was designated *ERAF17*. We examined the expression of *ERAF17* in the apices of cv. Shimoshirazu-jibai and in those of a gynoecious cultivar (Rensei). In these cultivars, the timing and levels of expression of the *ERAF17* transcript were correlated with the development of female flowers. Induction of the synthesis of the *ERAF17* transcript by ethephon occurred within 4 h of the start of treatment and continued for 4 days at least. Expression of *ERAF17* at apices was localized in the floral buds of the gynoecious cultivar, and expression was maintained in female flowers thorough their development. Our results suggest that the induction of the formation of female flowers by ethylene might be regulated by the expression of *ERAF17* in floral buds at the apices of cucumber plants and that expression of this gene might also be involved in the development of female flowers.

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Abbreviations ACC: 1-aminocyclopropane-1-carboxylate · AVG: aminoethoxyvinylglycine · cv.: cultivar · PCR:polymerase chain reaction · RACE: rapid amplification of cDNA ends

Introduction

Cucumber (*Cucumis sativus* L.) plants exhibit three typical patterns of sex expression, being monoecious, gynoecious, or hermaphroditic (Malepszy and Niemirowicz-Szczytt 1991). Monoecious cultivars produce male flowers at the base of the main stem, then they produce male and female flowers on the middle part of the stem, and finally female flowers are produced at the top of the stem. Gynoecious cultivars produce only female flowers. However, immature floral buds of cucumber plants initially have primordia of both stamens and pistils, and unisexuality is established by the stage-specific arrest of pre-formed sex-organ primordia in a particular whorl (Dellaporta and Calderon-Urrea 1993; Grant et al. 1994). Although sex expression in flowers of cucumber plants is determined genetically, ethylene has been shown to induce the formation of female flowers, while gibberellin induces the formation of male flowers (Peterson and Anhder 1960). Treatment of cucumber plants with gaseous ethylene (Iwahori et al. 1970) or with ethephon (2-chloroethylphosphonic acid; McMurray and Miller 1968; Rudich et al. 1969), which releases ethylene in plant tissues, changes sex expression to promote the formation of female flowers. Inhibition of ethylene biosynthesis in gynoecious cucumber plants by aminoethoxyvinylglycine (AVG) and inhibition of the action of ethylene by $AgNO_3$ causes a shift in sex expression from female to male (Beyer 1976; Adams and Yang 1979; Atsmon and Tabbak 1979). In addition, experiments involving combination treatments with plant hormones and inhibitors suggest that ethylene acts

in a more direct manner than gibberellins to influence the sex of cucumber flowers (Yin and Quinn 1995). Furthermore, a high correlation has been observed between the evolution of ethylene and sex expression in cucumber plants (Rudich et al. 1972). The enzyme 1-aminocyclopropane-1-carboxylate (ACC) synthase (EC 4.4.1.14) is a key regulatory enzyme in the ethylene biosynthetic pathway (Yang and Hoffman 1984), and it is encoded by a multigene family. Various cDNAs for genes for ACC synthase that appear likely to be involved in the regulation of sex expression in cucumber plants have been isolated independently in two laboratories (Trebitsh et al. 1997; Kamachi et al. 1997). Kamachi et al. (1997, 2000) reported that both the timing and the levels of expression of the *CS-ACS2* transcript were correlated with the development of female flowers at plant nodes. Furthermore, they reported that the timing of the induction of expression of the *CS-ACS2* gene at the apex corresponded to the timing of the action of ethylene in the induction of the first female flower at the apex of individual gynoecious cucumber plants. Although much information has been accumulated about the role of ethylene in the formation of female flowers on cucumber plants, the mechanisms of action of ethylene in the regulation of sex expression remain to be explored.

Studies of floral homeotic mutants of *Antirrhinum* and *Arabidopsis* led directly to the isolation of the first members of the plant family of MADS-box genes (Sommer et al. 1990; Yanofsky et al. 1990). Further studies of such mutants and of related members of the MADS-box family of genes resulted in the establishment of a universal model for the specification of floral organ identity within a hermaphroditic flower. This model, known as the ABC model, is based on the activities of three classes of genes, most of which have been identified as MADS-box genes, in the determination of four types of floral organ. The ABC model and the genes responsible for functions of A, B and C have been reviewed by Ma (1994), Weigel and Meyerowitz (1994), and Yanofsky (1995). The various MADS-box genes encode transcription factors with a strongly conserved DNA-binding domain, the MADS box, and a moderately well-conserved domain called the K box, which is able to form amphipathic helices that allow dimerization of these transcription factors (Ma et al. 1991; Davies and Schwarz-Sommer 1994). Three MADS-box genes were isolated from cucumber plants as homologs of *AGAMOUS* (Filipecki et al. 1997; Perl-Treves et al. 1998; Kater et al. 1998). Two of them were shown to mediate function C in experiments with transgenic petunia (Kater et al. 1998). However, in analyses of these genes in cucumber plants, no effects of treatments with ethylene or gibberellin were detected (Perl-Treves et al. 1998). Thus, the involvement of these genes in sex determination of cucumber flowers appears unlikely.

We have initiated a study of ethylene-regulated genes that might be related to sex expression in cucumber plants using the differential-display method. In this paper, we report the isolation of a MADS-box gene (*ERAF17*) that appears likely to be related to the induction of the formation of female flowers by ethylene and/or to the development of female flowers at the apices of cucumber seedlings.

Materials and methods

Plant materials

A monoecious cultivar of cucumber (*Cucumis sativus* L., cv. Shimoshirazu-jibai) and a gynoecious cultivar of cucumber (*Cucumis sativus* L., cv. Rensei) were grown in soil-filled pots in a phytotron at 25 °C with 14 h of light and at 20 °C with 10 h of darkness daily. Apices were excised from seedlings just above the youngest leaf at the indicated stages of growth, frozen immediately in liquid nitrogen and stored at –80 °C prior to extraction of nucleic acids.

Treatments

The apices of monoecious cucumber seedlings (cv. Shimoshirazu-jibai) were treated with 500 μM ethephon that contained 0.02% (v/v) Tween 20 at the indicated stages of growth. The apices of gynoecious cucumber seedlings (cv. Rensei) were treated with 100 μM AVG that contained 0.02% (v/v) Tween 20. In control experiments, apices were treated with 0.02% Tween 20. Each apex was treated by placing a piece of absorbent cotton that had been soaked in the appropriate solution on the apex of a growing cucumber plant. Treatments were repeated on three consecutive days at the indicated stages after planting. After further growth of seedlings, the sex of each flower on the first 20 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 it had only female flowers. After treatments, apices were excised from some seedlings and frozen in liquid nitrogen for extraction of nucleic acids.

Isolation of RNA

Total RNA was extracted from the apices of cucumber seedlings as described by Prescott and Martin (1987), and then the RNA was purified by successive precipitations in lithium chloride and in ethanol for use in RNA gel blot analysis. For analysis by the differential-display method, the extracted total RNA was purified by ultracentrifugation at 200,000 *g* for 16 h on a cesium chloride density gradient. Then the purified RNA was treated with DNase (RQ1 RNase-Free DNase; Promega, Madison, Wis., USA) to remove any contaminating DNA. For 5′-RACE (rapid amplification of cDNA ends), poly(A)$^+$ RNA was isolated from the total RNA after ultracentrifugation on a spun column (mRNA Purification kit; Amersham Pharmacia Biotech, Bucks., UK).

Differential-display technique

The differential-display technique was applied as described by Liang et al. (1993). For each condition, four sets of duplicate reactions were performed according to the manufacturer's protocol (RNAmap, mRNA differential-display system; GenHunter Co., Brookline, Mass., USA). Each reaction mixture (total volume, 60 μl) contained 0.6 μg of total RNA, one of the primers $T_{12}MA$, $T_{12}MT$, $T_{12}MG$, or $T_{12}MC$ (where M stands for a mixture of G, A, and C), 20 μM dNTP mixture, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$ and 10 mM (±)-dithiothreitol. Reaction mixtures were heated at 65 °C for 5 min and then at 37 °C for 10 min, after which 150 units of Moloney murine leukemia virus reverse

transcriptase (StrataScript Reverse Transcriptase; StrataGene, La Jolla, Calif., USA) were added. Reaction mixtures were incubated at 37 °C for a further 50 min and then reactions were stopped by heating at 95 °C for 5 min. One-tenth of each mixture after reverse transcription (RT-mixture) was used as the template for polymerase chain reaction (PCR) in a reaction mixture that contained the corresponding T_{12}MN primer in combination with one of 20 arbitrary 10-mer primers for each reaction.

Amplification by PCR was performed using *Taq* polymerase (Perkin-Elmer Japan Co., Urayasu, Japan) in 20 µl of a reaction mixture that contained 2 µl of RT-mixture, 1 µM corresponding T_{12}MN primer, 0.2 µM arbitrary 10-mer, 2 µM dNTP, 0.2 µl α-[^{32}P]dCTP (111 TBq/mmol; ICN Biomedicals Inc., Costa Mesa, Calif., USA), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ and 1 unit of AmpliTaq polymerase (Perkin-Elmer Japan Co.). The parameters for PCR were 40 cycles of heating at 94 °C for 30 s, at 40 °C for 2 min and at 72 °C for 30 s, followed by extension at 72 °C for 5 min in a GeneAmp PCR system (model 9600; Perkin-Elmer Japan Co.). Aliquots of duplicate reaction mixtures after PCR were subjected to electrophoresis on a 6% polyacrylamide/8 M urea sequencing gel to separate amplified cDNAs.

We recognized several bands that differed both in intensity and mobility, and we analyzed bands of cDNAs that were differentially amplified in a consistent manner in further detail. Regions of a gel that contained differentially expressed cDNAs were excised from the dried gel and placed individually in 100 µl of distilled water. The cDNAs that diffused from the gel fragments were re-amplified with the appropriate pair of primers. The primers used in the experiment that led to the isolation of cDNA #17, reported in this paper, were T_{12}MT (5′-TTTTTTTTTTTTMT-3′) and AP-17 (5′-GCAAGGAGTC-3′). The amplified fragment was purified from a 5% polyacrylamide gel. The purified product of PCR was cloned into the pCRII vector (Invitrogen Co., San Diego, Calif., USA) by the method described in the TA Cloning Instruction Manual from Invitrogen.

Preparation of the cDNA probe

cDNA was cleaved by *Eco*RI from pCRII that had been amplified in *Escherichia coli* JM109, purified by gel electrophoresis and recovered. In all of the present experiments, the 3′ sequence of cDNA #17 that had been obtained by the differential-display method was used as the cDNA probe. The cDNA was labeled with α-[^{32}P]dCTP by the random-priming method with a Multiprime DNA Labeling System (Amersham Pharmacia Biotech) for use as the probe.

RNA gel blot analysis

Total RNA was isolated from the apices of the two cultivars (cv. Shimoshirazu-jibai and Rensei) at the indicated stages after planting. Total RNA was subjected to electrophoresis on a 1.17% agarose gel that contained 0.66 M formaldehyde and then transferred to a GeneScreen *Plus* membrane (NEN Life Science Products, Boston, Mass., USA) by capillary action in 10×SSC (1×SSC is 0.15 M NaCl, 15 mM sodium citrate), as recommended by the manufacturer of the membrane. After baking at 80 °C, the membrane was incubated in 1 M NaCl, 1% SDS and 10% dextran sulfate (sodium salt) at 60 °C for 1 h. The denatured ^{32}P-labeled probe and denatured salmon-sperm DNA were then added to the pre-hybridization solution, and the membrane was incubated at 60 °C for 18 h. Post-hybridization washes were performed once for 2 min with 2×SSC at room temperature and twice, successively, for 15 min each with 2×SSC, 1% SDS at 60 °C. The washed membrane was subjected to autoradiography with an intensifying screen or the BioImaging Analyzer (BAS 5000; Fuji Photo Film Co., Tokyo, Japan). Then the membrane was washed with boiling 1×SSC, 0.1% SDS to remove the probe and blots were re-hybridized with ribosomal RNA to ensure that equal amounts of total RNA had been loaded in each lane.

Isolation of full-length cDNA of *ERAF17*

Full-length cDNA corresponding to *ERAF17* was amplified by 5′-RACE. All reactions were performed with a Marathon cDNA Amplification Kit by the method described in the Protocol and Reference Manual from the manufacturer (CLONTECH Laboratories Japan, Tokyo, Japan). The cDNA was reverse-transcribed from poly(A)$^+$RNA that had been isolated from apices of 18-day-old Shimoshirazu-jibai seedlings, treated with ethephon as described above, and it was ligated to the Marathon cDNA adaptor. The 5′-cDNA fragment was amplified by PCR with an *ERAF17*-specific primer (5′-ACGGCTGTGCTCCTCTTGCAAAC-3′), the adaptor primer that was supplied with the kit, and the adaptor-ligated cDNA as the template. PCR was performed with *TaKaRa Ex Taq* (TAKARA SHUZO Co., Tokyo, Japan) in the presence of TaqStart Antibody (CLONTECH). A "hot start" involved incubation at 94 °C for 1 min and was followed by PCR with 30 cycles of heating at 94 °C for 30 s, at 61 °C for 30 s and at 68 °C for 4 min. The products of PCR were cloned into pCRII.

Sequencing of DNA

The cDNA that had been cloned into pCRII was sequenced with an automated DNA sequencer (model 377; Perkin-Elmer) by the dideoxy sequencing method (Sanger et al. 1977) using a Taq Dye Primer Cycle Sequencing kit (Perkin-Elmer). Nucleotide and amino acid sequences were analyzed with GENETYX-MAC software, version 10.1.1 (Software Development Co., Tokyo, Japan). Databases were searched with NCBI BLAST (National Center for Biotechnology Information; Basic Local Alignment Search Tool system 2.0). The predicted amino acid sequence encoded by *ERAF17* and portions of representative MADS-box genes from different plants that included the same part of the sequence (the MADS fragment and the adjacent 110 amino acids) were used for phylogenetic analysis as described by Theissen et al. (1996). A neighbor-joining tree was produced from the results of 1,000 bootstrap replicates using the CLUSTAL W program, version 1.8, of the DNA Data Bank of Japan (Mishima, Japan). The phylogenetic tree was displayed by TreeView software version 1.6 (Page 1996).

Results

Sex conversion in flowers by ethephon

For identification of ethylene-responsive genes that might be related to the induction of the formation of female flowers by ethephon, we examined the conditions under which ethephon effectively induced the conversion of flowers from male to female, using the monoecious cultivar Shimoshirazu-jibai as plant material. As shown in Fig. 1, Shimoshirazu-jibai exhibited stable maleness at the lower nodes of the main stem and no female flowers were formed on the lower nodes. The apices of Shimoshirazu-jibai were treated with 500 µM ethephon, an ethylene-releasing compound, at various stages of growth for 3 days. As shown in Fig. 1, no female flowers were induced on plants that were treated with ethephon 6 days (cotyledon to one-leaf stage) after planting, as was the case on control plants. We could confirm no flower formation at the apices of 6-day-old plants under the light microscope (data not shown). When the apices of 11-day-old plants (one- to two-leaf stage) were treated with ethephon, female flowers were induced on lower nodes, including the first flower. This result indicated

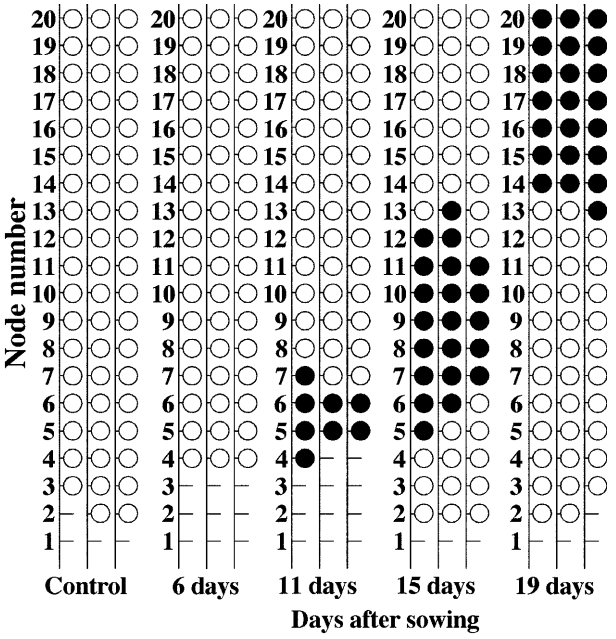


Fig. 1 The timing of the conversion of flowers of cucumber (*Cucumis sativus*) from male to female upon treatment of apices with ethephon. Apices of seedlings (cv. Shimoshirazu-jibai) were treated with or without ethephon (500 μM) for 3 days from the indicated day after sowing. After further growth of seedlings, the sex of each flower on the first 20 nodes was examined and classified as male or female. The node number indicates the position of individual nodes along the main stem. *Open circles* Nodes with male flowers, *closed circles* nodes with female flowers, *no circles* vegetative nodes. Data from three plants are presented in each case

that the flower formation was initiated up to node 7 at the apices of 11-day-old plants and the sex of flower buds was changed by ethephon. The apices of cucumber plants were also treated with ethephon at later growth stages. In the case of 15-day-old plants (two- to three-leaf stage) and 19-day-old plants (three- to four-leaf stage), the plants produced female flowers on higher nodes than did the plants treated with ethephon 11 days after planting. These results indicated that floral buds at nodes 2–13 and nodes 2–20 or more were present at the nodes of 15-day-old and 19-day-old plants, respectively. Furthermore, these results indicated that the sex of flower buds on nodes 2–6 and 2–13 at the apices of 15-day-old and 19-day-old plants, respectively, had already been determined, while that of flower buds on higher nodes differentiating at the apices had not yet been determined and could be changed by treatment with ethephon.

Differential display

Seedlings at the two- to three-leaf stage (15 days old) were chosen for treatment with ethephon because of the effective induction by ethephon of female flowers at this stage. The apices of 15-day-old seedlings were treated or not treated with ethephon for 3 days and apices were cut from seedlings the day after the last treatment. Total RNA was extracted from each sample and 80 sets of differential-display reactions were performed using combinations of 20 arbitrary 10-mers and 4 anchor primers. We detected 92 differentially expressed bands of cDNA (promoted, 62; suppressed, 30) in a series of experiments. We performed RNA gel blot analysis with same samples of RNA as those used for the differential display to remove false-positive clones. As the result, we identified 20 clones (#1 to #20) for which there was a clear difference in the accumulation of transcripts in apices treated with or without ethephon, as determined by RNA gel blot analysis (data not shown).

Isolation and sequence analysis of full-length cDNA #17

We isolated full-length cDNA #17 by 5′-RACE. Figure 2A shows the complete nucleotide sequence of the cloned cDNA and the amino acid sequence deduced from the nucleotide sequence. The cDNA was 819 bp long and contained an open reading frame of 609 bp that encoded a putative polypeptide of 203 amino acids. A homology search revealed that the cDNA corresponded to a gene in the MADS-box gene family, having a MADS box and a K box (Fig. 2A, B). This gene was designated *ERAF17* (ethylene-responsive gene associated with the formation of female flowers 17; GenBank accession number AB046596). The deduced amino acid sequence encoded by *ERAF17* was found to be approximately 50% homologous to that encoded by *TM8*, which is a gene for a MADS-box protein in tomato (*Lycopersicon esculentum*; Pnueli et al. 1991). The genomic southern analysis revealed that the *ERAF17* gene was present as a single copy in both monoecious and gynoecious cucumber plants and that the probe used in this experiment was specific for the *ERAF17* gene (data not shown).

Phylogenetic relationships to other members of the family of MADS-box genes in plants

The family of MADS-box genes is composed of several defined clades of genes whose members have similar respective patterns of expression and strongly related functions, as revealed by phylogenetic analysis (Doyle 1994; Purugganan et al. 1995; Theissen et al. 1996, 2000). Therefore, we performed cluster analysis of *ERAF17* and representative MADS-box genes. Our phylogenetic analysis indicated that *ERAF17* did not belong to any distinguishable clade of any subfamily of MADS-box genes that have been included as participants in the ABC model (Fig. 3). The most similar gene to *ERAF17* was *TM8* from tomato (Pnueli et al. 1991).

The effects of ethephon and AVG on the expression of *ERAF17* and the sex of flowers

Figure 4 shows the expression of *ERAF17* transcripts in the apices of the monoecious cultivar

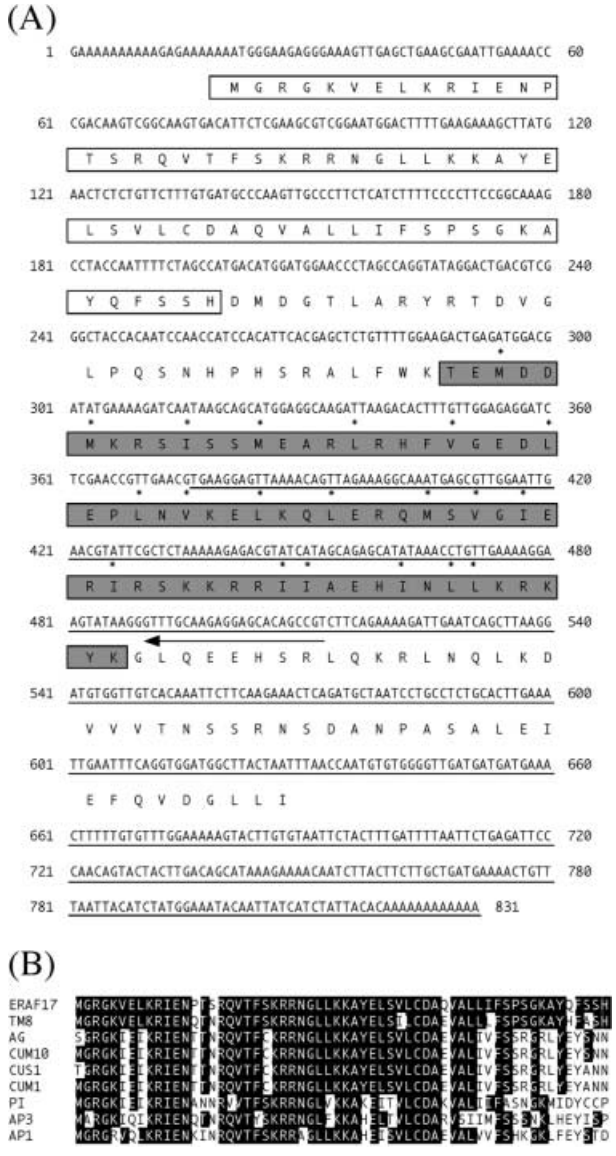


Fig. 2 **A** Nucleotide sequence of *ERAF17* from cucumber and the deduced amino acid sequence. The putative ERAF17 protein is shown in the one-letter amino acid code. The MADS box is represented by an *open box* and the K box is represented by a *gray box*. Within the K box, hydrophobic amino acids (L, I, V, and M) are indicated by an *asterisk*. The specific primer for RACE-PCR is indicated by an *arrow*. The nucleotide sequence that was isolated by differential display is *underlined*. This region was used as the cDNA probe for RNA and DNA gel blot analysis. **B** Alignment of the amino acid sequences of MADS domains encoded by various MADS-box genes. Amino acids identical to those in a consensus sequence based on *ERAF17* are shaded in *black*. The amino acid sequences of the following MADS domains are aligned: ERAF17 (this study); *Arabidopsis* AG (GenBank accession number, S10933), AP1 (Z16421), AP3 (A42095), and PI (D30807); tomato TM8 (X60760); and cucumber CUM10 (AF035439), CUM1 (AF035438), and CUS1 (X97801)

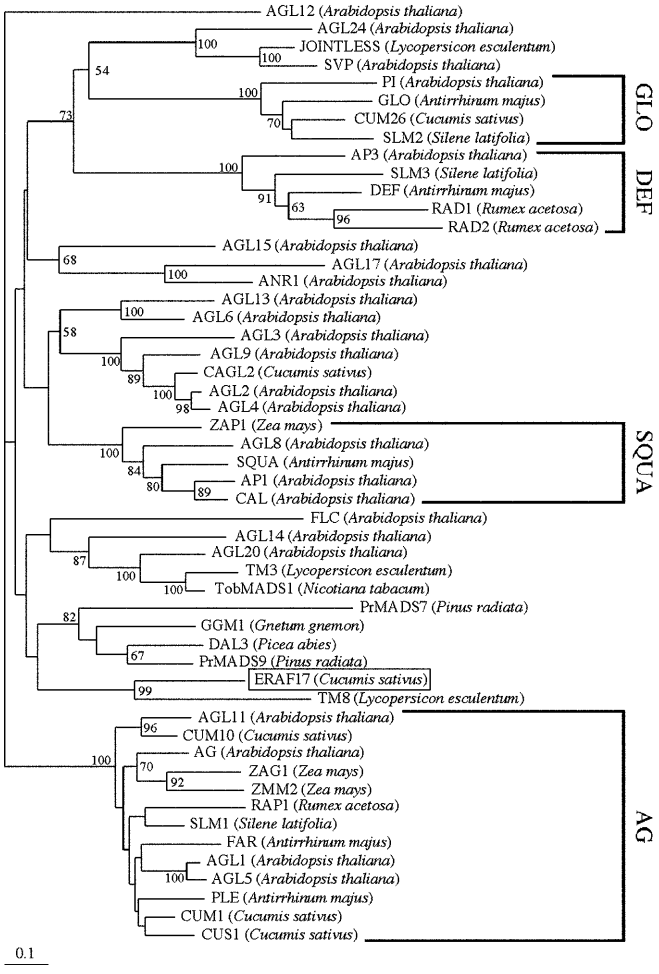


Fig. 3 Dendrogram of MADS-box genes from various plants. The tree is based on the "MADS + 110 domain" (Theissen et al. 1996), which represents the complete MADS-domain and the immediately adjacent I- and K-domains. All known MADS-box genes of *Arabidopsis* and cucumber, representative MADS-box genes of snapdragon, tomato, maize, sorrel and white campion, and five genes of the other species that fell near clades of *ERAF17* are included in the phylogenetic tree. Horizontal branch lengths are proportional to the estimated number of amino acid (a.a.) substitutions per residue (bar = 0.1 a.a. substitution per residue). Names of species from which the respective genes were isolated are given in parenthesis next to the names of proteins. ERAF17 is indicated by an *open box*. The numbers next to some nodes give bootstrap percentages, which are shown only when above 50%. The names of sub-families that include participants in the ABC model are indicated on the right

(Shimoshirazu-jibai) after treatment in the presence or absence ethephon at the indicated stages of growth. The transcript was not detectable in the apices of Shimoshirazu-jibai plants at any stage examined in the absence of ethephon treatment. However, the expression of *ERAF17* was induced in 11-, 15- and 19-day-old plants by ethephon treatment (Fig. 4). The induction of the synthesis of *ERAF17* mRNA by ethephon was correlated with the induction by ethephon of female flowers on the stems of Shimoshirazu-jibai plants (Fig. 1). However, a weak expression of *ERAF17* was detected in 6-day-old plants. It seems that there are a few female flowers formed in some apices of 6-day-old plants.

We treated the apices of gynoecious plants (cv. Rensei), which formed only female flowers, with a

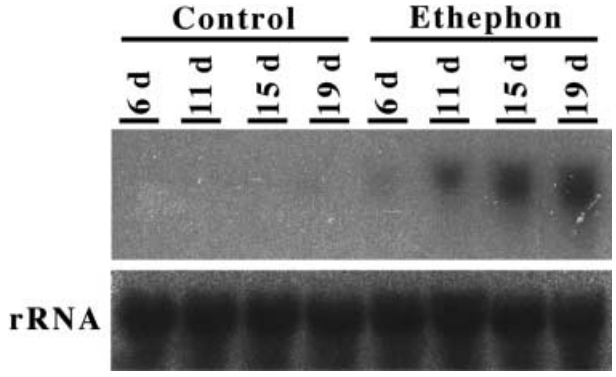


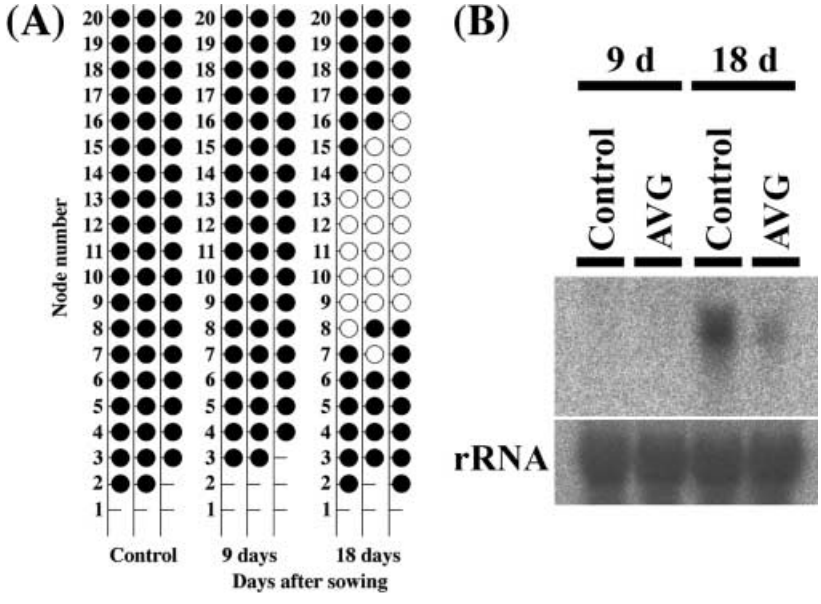
Fig. 4 Stage-dependent accumulation of *ERAF17* mRNA in the apices of monoecious cucumber and the effects of ethephon on such accumulation. The apices of seedlings (cv. Shimoshirazu-jibai) were treated in the presence or absence ethephon (500 μM) for 3 days from the indicated day after sowing. The apices were collected the day after the third treatment in each case. Total RNA (20 μg) was extracted from apices, fractionated on a denaturing agarose gel, transferred to a nylon membrane, and allowed to hybridize with the *ERAF17* probe. The blot was re-hybridized with an rRNA probe to confirm loading of equal amounts of RNA in each lane

Fig. 5 A The effects of AVG on sex expression. Apices of cucumber seedlings (cv. Rensei) were treated or not treated with AVG (100 μM) for 3 days from the indicated day after sowing. After further growth of seedlings, the sex of each flower on the first 20 nodes was examined and classified as male or female. The node number indicates the position of individual nodes along the main stem. *Closed circles* Nodes with female flowers, *open circles* nodes with male flowers, *no circles* vegetative nodes. Data from three plants are presented in each case. **B** Stage-dependent accumulation of *ERAF17* mRNA in gynoecious cucumber plants (cv. Rensei) and the effects of AVG on such accumulation. Apices of seedlings (cv. Rensei) were treated or not treated with AVG (100 μM) for 3 days from the indicated day after sowing. Apices were collected the day after the last treatment. Total RNA (20 μg) was extracted from apices, fractionated on a denaturing agarose gel, transferred to a nylon membrane, and allowed to hybridize with the *ERAF17* probe. The blot was re-hybridized with an rRNA probe as described in the legend to Fig. 4

solution of AVG, an inhibitor of ethylene synthesis, at two stages of growth. As shown in Fig. 5A, no male flowers were induced on plants that had been treated with AVG 9 days (cotyledon to one-leaf stage) after planting, as was the case on control plants. When the apices of 18-day-old plants (two- to three-leaf stage) were treated with AVG, the sex of flowers on lower nodes was unchanged but plants produced male flowers on higher nodes. These results are explained by the facts that the sex of flower buds on the lower nodes had already been determined and that on higher nodes had not yet been determined and could be changed with AVG as the results of ethephon treatment on Shimoshirazu-jibai (Fig. 1). No expression of *ERAF17* was detected in the apices of 9-day-old Rensei plants and AVG had no effect on such expression (Fig. 5B). These results are explained by the observation that no flower formation could be confirmed at this stage under the light microscope. When the apices of 18-day-old plants were treated with AVG, the expression of *ERAF17* was inhibited compared to that in control plants. These results showed that the conversion of flowers from female to male by AVG was correlated with inhibition of the expression of *ERAF17* in apices.

Time course of the induction of expression of *ERAF17* by ethephon

In the above experiment (Fig. 4), we examined the expression of *ERAF17* in the apices of Shimoshirazu-jibai plants using plants that had been treated or not treated with ethephon for three successive days. Therefore, we next examined the time course of the expression of *ERAF17* transcripts in the apices of monoecious cucumber (cv. Shimoshirazu-jibai) after a single treatment with ethephon. As shown in Fig. 6A, B, the *ERAF17* transcript was induced by ethephon 4 h after the start of



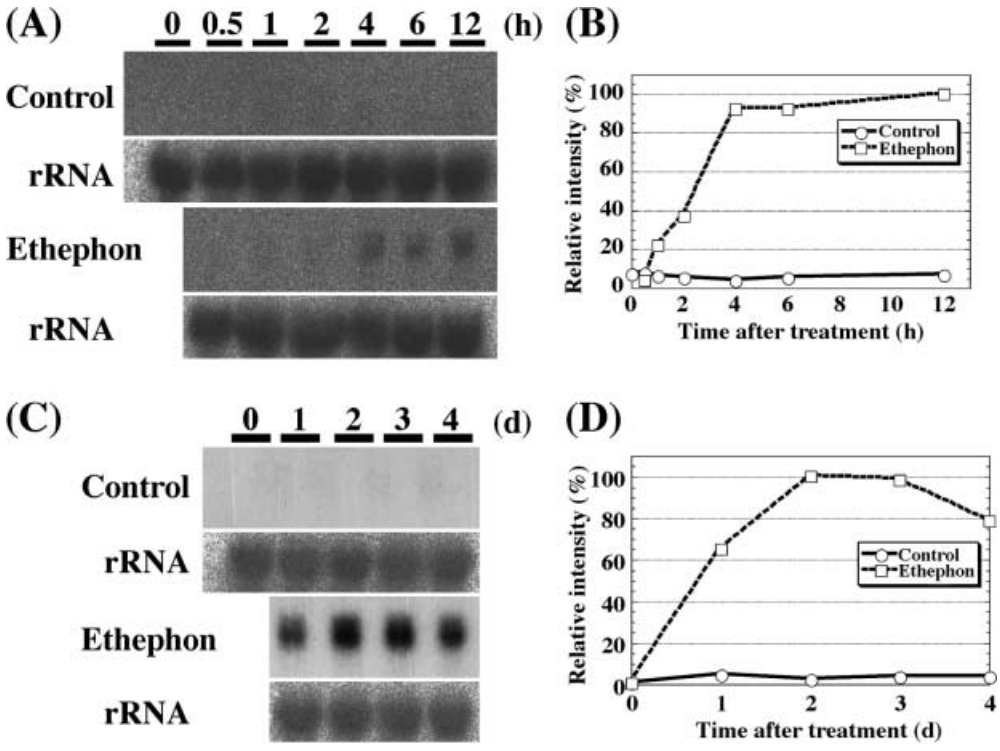


Fig. 6A–D Time course of expression of *ERAF17* transcripts at the apices of cucumber cv. Shimoshirazu-jibai seedlings after treatment with ethephon. A short time-course (**A**) and a long time-course (**C**) are shown. Results of quantification of data using Science Lab 98 Image Gauge (Fuji Photo Film Co., Tokyo, Japan; software version 3.1) are shown in **B** and **D**, respectively. Ethephon (500 μM; containing 0.1% Tween 20) or 0.1% Tween 20 was used to treat Shimoshirazu-jibai apices 18 days after planting. Apices were harvested at the indicated times after treatment. Total RNA (20 μg) was extracted from apices, fractionated on a denaturing agarose gel, transferred to a nylon membrane, and allowed to hybridize with the *ERAF17* probe. The blot was re-hybridized with an rRNA probe as described in the legend to Fig. 4

a single treatment, but it was not detected in controls. Furthermore, the level of the expression remained high for at least 4 days after the treatment (Fig. 6C, D).

Localization of the expression of *ERAF17* mRNA at the apices of cucumber plants

As shown in Figs. 1 and 5A, the monoecious cultivar Shimoshirazu-jibai produced only male flowers and the gynoecious cultivar Rensei produced only female flowers on its lower nodes. Since flower buds were formed at the apices of the cucumber plants, we used these two cultivars to examine the sites of expression of the *ERAF17* transcript in apices. As shown in Fig. 7A, expression was detected in apices only of ethephon-treated Shimoshirazu-jibai plants and in those of Rensei plants treated or not treated with ethephon. The apices included floral buds and unexpanded leaves and we separated those tissues under a light microscope and then extracted the total RNA from each tissue. No expression of the *ERAF17* transcript was detected in unexpanded leaves from the apices of either Shimoshirazu-jibai or Rensei plants. The *ERAF17* transcript was also not detected in the floral buds in the apices of Shimoshirazu-jibai plants, but the transcript was detected in those of Rensei plants (Fig. 7B).

Expression of *ERAF17* in flowers during development

In order to examine the stage-specific expression of the *ERAF17* gene during floral development, we performed RNA gel blot analysis using total RNA that had been extracted from flowers at various stages. As shown in Fig. 8, transcripts of *ERAF17* were barely detectable in male flowers of Shimoshirazu-jibai plants throughout development. By contrast, strong expression of *ERAF17* was detected in female flowers of Rensei plants throughout development as far as anthesis.

Expression of *ERAF17* in floral organs

We next examined the expression of *ERAF17* in the floral organs of mature flowers (harvested at anthesis and the preceding day) by northern blot analysis. As shown in Fig. 9, the expression of *ERAF17* was only detected in sepals, petals and ovaries of female flowers, with the strongest signal being detected in sepals, and an expression in the ovaries was stronger than in the petals.

Discussion

In the present study, we isolated a cDNA clone for a gene designated *ERAF17*, whose expression was induced by treatment with ethephon, an ethylene-releasing compound (Fig. 4), in a monoecious cucumber plant. *ERAF17* was strongly homologous to other MADS-box genes, being most similar to the *TM8* gene from tomato. Phylogenetic analysis showed that *ERAF17* was distinct from floral homeotic MADS-box genes (Fig. 3). According to the phylogenetic tree (Fig. 3), it seems that *ERAF17* and *TM8* belong to the same subfamily. The *TM8* gene has been considered an orphan gene or a new subfamily (Doyle 1994; Purugganan et al. 1995; Theissen

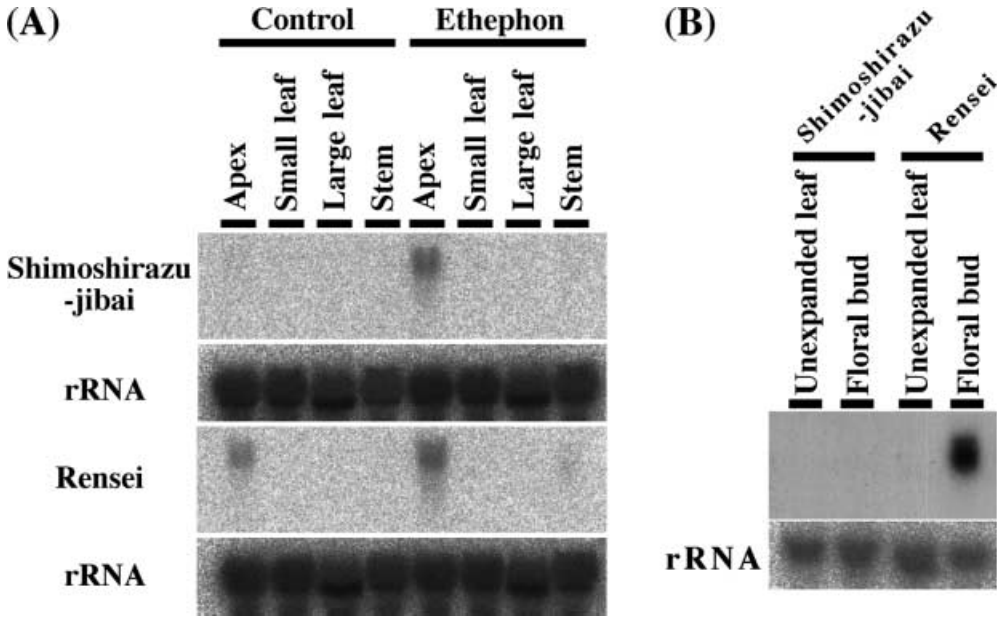


Fig. 7A, B Tissue-specific expression of *ERAF17* and responsiveness to ethylene. **A** Whole cucumber plants were treated or not treated with ethephon (500 μM) by spraying and by application of a piece of absorbent cotton that had been soaked in the appropriate solution on apices 1 day (17 days after sowing) before harvesting. Total RNA was extracted from apices, unexpanded (*small*) leaves, expanded (*large*) leaves and stems of 18-day-old seedlings of Shimoshirazu-jibai and Rensei. **B** Unexpanded leaves and floral buds were separated from each apex of 18-day-old seedlings (three-leaf stage) of Shimoshirazu-jibai and Rensei under a light microscope. Floral buds were excised from the fourth to eighth nodes from the apices. Floral buds were 2–4 mm or 1.5–3 mm long, respectively. Total RNA (20 μg in **A** and 10 μg in **B**) was extracted from each tissue, fractionated on a denaturing agarose gel, transferred to a nylon membrane, and allowed to hybridize with the *ERAF17* probe. The blot was re-hybridized with an rRNA probe (see legend to Fig. 4)

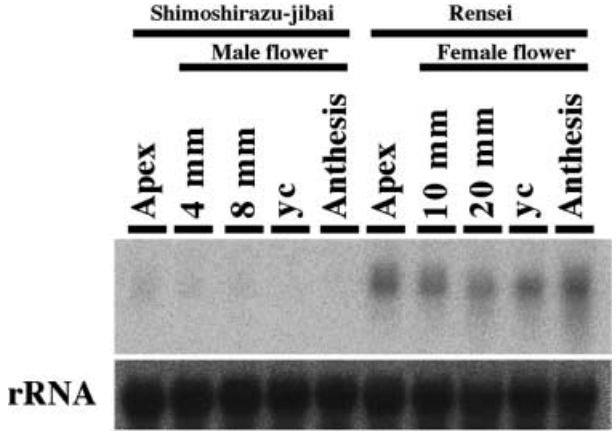


Fig. 8 Expression of *ERAF17* during floral development. Floral buds of cucumber cv. Shimoshirazu-jibai (male) and Rensei (female) were collected and grouped according to respective size and external markers (*yc* corolla becoming yellow) and anthesis. The apices of the two cultivars were also collected 18 days after sowing. Total RNA (12 μg) was extracted from each sample, fractionated on a denaturing agarose gel, transferred to a nylon membrane, and allowed to hybridize with the *ERAF17* probe. The blot was re-hybridized with an rRNA probe (see legend to Fig. 4)

et al. 1996). It was reported that *TM8* is expressed at an early stage of flower development and is repressed in developed flowers but the function of *TM8* is not known (Pnueli et al. 1991). Furthermore, no orthologs to *TM8* from other plants have been characterized. We could not judge whether *ERAF17* and *TM8* have any common features from our phylogenetic tree alone. In this analysis, while we did not obtain any information about the actual role of the product of *ERAF17*, we were able to show that *ERAF17* belongs to a new category of MADS-box genes.

To examine the relationship between *ERAF17* and sex expression, we performed RNA gel blot analysis. As shown in Fig. 4, the expression of *ERAF17* at apices of the monoecious cultivar Shimoshirazu-jibai depended on ethephon treatment and on the growth stage of the plant. The timing of changes in the level of the *ERAF17* transcript appeared to coincide with the timing of the action of ethylene in the formation of female flowers (Fig. 1). When AVG, an inhibitor of ethylene biosynthesis, was used to treat the apices of the gynoecious cultivar Rensei, expression of *ERAF17* was suppressed during the induction of the formation of male flowers (Fig. 5A, B). Moreover, *ERAF17* was expressed only in the floral buds of the gynoecious cultivar (Fig. 7A, B). Recently, Kamachi et al. (2000) reported that the *CS-ACS2* transcript is expressed only in a limited number of floral buds that will develop into female flowers. These results suggest that expression of *ERAF17* is strongly correlated with induction of the formation of female flowers by ethylene. Furthermore, the *ERAF17* transcript was detected 4 h after a single treatment with ethephon (Fig. 6A, B). In cucumber flowers, morphological changes of floral buds to female flowers are observed in ovary development (Atsmon and Galun 1960). However, this morphological change was undetectable in any floral buds at this time under the light microscope (data not shown). Therefore, the

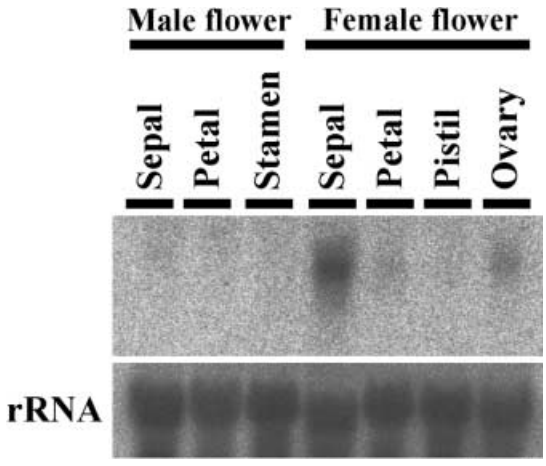


Fig. 9 Expression of *ERAF17* in floral organs. Floral organs of male flowers (sepals, petals and stamens; cv. Shimoshirazu-jibai) and of female flowers (sepals, petals, pistils and ovaries; cv. Rensei) were collected from mature flowers (harvested at anthesis and preceding day). Total RNA (12 μg) was extracted from each sample, fractionated on a denaturing agarose gel, transferred to a nylon membrane, and allowed to hybridize with the *ERAF17* probe. The blot was re-hybridized with an rRNA probe (see legend to Fig. 4)

"early" expression of *ERAF17* appears not to be a result of the development of female flowers but represents a factor that endows femaleness after exposure of primordia to ethylene. The expression of *ERAF17* remained at a high level at least for 4 days after a single treatment with ethephon (Fig. 6C, D), and the transcript was also expressed in female flower buds throughout their development (Fig. 8). It is possible that the role of "late" expression might be different from that of "early" expression. It is well established that the expression of MADS-box genes persists at later stages, when their products perform developmental, or maintenance functions (Bowman et al. 1991; Schwarz-Sommer et al. 1992). Moreover, the patterns of expression of several MADS-box genes suggest that they are involved in embryogenesis and/or in the development of the ovule or fruit (Angenent et al. 1995; Colombo et al. 1995; Heck et al. 1995; Flanagan et al. 1996; Filipecki et al. 1997). Thus, late expression of *ERAF17* might be involved in the development of female flowers and this possibility is supported by the expression of *ERAF17* in ovaries (Fig. 9). Interestingly, the expression of *ERAF17* was detected in sepals and petals of female flowers but not in those of male flowers (Fig. 9); however, we could not explain this difference between male and female flowers.

The possibility that a MADS-box gene might control sex differentiation has been considered in the past. Various studies of class-B and class-C genes (in the ABC model) that are expressed in reproductive organs have been undertaken in dioecious and monoecious plants. Researchers have examined whether MADS-box genes that control the development of reproductive organs are expressed in arrested organ primordia in two dioecious plants, namely, sorrel (*Rumex acetosa*) and white campion (*Silene latifolia*; Ainsworth et al. 1995, and Hardenack et al. 1994, respectively). In sorrel, the transcripts of B-function genes, *RAD1* and *RAD2*, which were expressed in the stamen whorl, were absent from arrested stamen primordia in females. The transcript of the C-function gene *RAP1* was expressed in the inner two whorls in very young flower primordia of both males and females. However, such expression was not detected in arrested fourth-whorl regions of male flowers and in the stamen primordia of female flowers. Although such coincidence might suggest a causal relationship, it is still impossible to determine whether the decrease in level of the transcript caused arrest or vice versa (Ainsworth et al. 1995). In white campion, primordia of opposite sex develop to a more advanced stage, and no differences between male and female flowers were detected in terms of the expression of *SLM1* (a *PLENA* homolog) from the initiation of flowers to meiosis (Hardenack et al. 1994). In the case of monoecious plants, it was reported that *ZMM2*, a putative C-class gene in maize, was expressed in stamen and carpel primordia throughout their development, even though stamen primordia abort in female inflorescences and carpel primordia abort in male ones (Cacharrón et al. 1999). Furthermore, some homologs of *AGAMOUS* genes were also isolated from cucumber plants and analyzed (Perl-Treves et al. 1998). However, they have not been implicated as genes involved in the sex-expression cascade because of their insensitivity to hormonal stimuli. These various observations imply that MADS-box genes that are involved in the ABC model are not involved in sex differentiation in these species.

Cluster analysis of *ERAF17* and other MADS-box genes indicated that *ERAF17* belongs to a clade distinguishable from that of genes in the ABC model (Fig. 3). Moreover the induction of the accumulation of its transcript was detected soon (4 h) after ethephon treatment (Fig. 6A, B). These features are clearly different from those of other MADS-box genes from cucumber. The expression of *ERAF17* was correlated with the formation of female flowers and, thus, it is possible that *ERAF17* might act in a feminizing cascade. In cucumber plants, ethylene might control sex differentiation via the direct or indirect regulation of the expression of *ERAF17*. We recently found that the sequence of a partial cDNA of the *CUS3* gene, isolated from embryogenic cucumber callus (Filipecki 2000; published only in the database, GenBank accession number AJ278013), is identical to the sequence of part of *ERAF17*.

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