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ORIGINAL ARTICLE

Molecular and functional characterization of *CpACS27A* gene reveals its involvement in monoecy instability and other associated traits in squash (*Cucurbita pepo* L.)

Cecilia Martínez · Susana Manzano · Zoraida Megías · Alejandro Barrera · Adnane Boualem · Dolores Garrido · Abdelhafid Bendahmane · Manuel Jamilena

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Abstract A number of *Cucurbita pepo* genotypes showing instable monoecy or partial andromonoecy, i.e. an incomplete conversion of female into bisexual flowers, have been detected. Given that in melon and cucumber andromonoecy is the result of reduction of ethylene production in female floral buds, caused by mutations in the ethylene biosynthesis genes *CmACS7* and *CsACS2*; we have cloned and characterized two related *C. pepo* genes, *CpACS27A* and *CpACS27B*. The molecular structure of *CpACS27A* and its specific expression in the carpels of female flowers during earlier stages of flower development suggests that this gene is the *Cucurbita* ortholog of *CmACS7* and *CsACS2*. *CpACS27B* is likely to be a paralogous pseudogene since it has not been found to be expressed in any of the analyzed tissues. *CpACS27A* was sequenced in Bolognese (Bog) and Vegetable Spaghetti (Veg), two monoecious inbred lines whose F2 was segregating for partial andromonoecy. The *Bog* allele of *CpACS27A* carried a missense mutation that resulted in a substitution of the conserved serine residue in position 176 by an alanine. Segregation analysis indicated that this mutant variant is necessary but not sufficient to confer the andromonoecious phenotype in squash. In concordance with its involvement in stamen arrest, a reduction in *CpACS27A* expression has been found in bisexual flower buds at earlier stages of development. This reduction in *CpACS27A* expression was concomitant with a downregulation of other ethylene biosynthesis and signaling genes during earlier and later stages of ovary development. The role of *CpACS27A* is discussed regarding the regulation of ethylene biosynthesis and signaling genes in the control of andromonoecy-associated traits, such as the delayed maturation of corolla and stigma as well as the parthenocarpic development of the fruit.

Keywords Andromonoecy · Ethylene · Sex determination · Stamen arrest · Parthenocarpy · Unisexual flower development

Abbreviations

ACC	1-Aminocyclopropane-1-carboxylic acid
ACO	ACC oxidase
ACS	ACC synthase
AI	Andromonoecy index
AVG	Aminoethoxyvinylglycine
CTR	Constitutive triple response
ETR, ERS	Ethylene receptors
EIN3	Ethylene insensitive 3
STS	Silver thiosulphate
PLP	Pyridoxal phosphate

Introduction

The study of sex determination in cucurbit species has facilitated the understanding of the molecular evolutionary

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C. Martínez · S. Manzano · Z. Megías · A. Barrera · M. Jamilena (✉)
Departamento de Biología y Geología, Agrifood Campus of International Excellence (ceiA3), Universidad de Almería, La Cañada de San Urbano s/n, 04120 Almería, Spain
e-mail: mjamille@ual.es

A. Boualem · A. Bendahmane
Unité de Recherche en Génomique Végétale, INRA/CNRS-URGV, 2, Rue Gaston Crémieux, 91057 Evry cedex, France

D. Garrido
Departamento de Fisiología Vegetal, Universidad de Granada, Fuentenueva s/n, 18071 Granada, Spain

mechanisms that lead to the development of unisexual flowers in plants. The most widely cultivated species of the Cucurbitaceae family, *Cucumis melo*, *Cucumis sativus*, *Citrullus lanatus* and *Cucurbita pepo* are all monoecious, although in *Cucumis*, lines have been described that are andromonoecious, gynoecious, hermaphrodite or androecious. The genetic control of sex in *C. melo* and *C. sativus* is under the control of two major genes: *a* (*andromonoecious*) and *G* (*Gynoecious*) in melon (Kenigsbuch and Cohen 1990), and F (Female) and M (Monoecious) in cucumber (Galun 1962; Kubicki 1969). The combinations of these genes produce the main sexual phenotypes of these two species: hermaphrodite, monoecious, andromonoecious and gynoecious. In *C. lanatus* and *C. pepo* most of the genotypes described to date are monoecious, although in squash the lines Bolognese (Bog) and Vegetable Spaghetti (Veg) have been reported to be almost gynoecious or androecious and therefore produce a high percentage of female or male flowers, respectively (Manzano et al. 2010a).

The most important factor regulating sexual expression in cucurbit species is the phytohormone ethylene, controlling the transition from male to female flowering, the ratio of female to male flowers, and sex determination of individual floral buds (Takahashi and Jaffe 1984; Takahashi et al. 1982; Atsmon and Tabbak 1979; Byers et al. 1972; Owens et al. 1980; Kamachi et al. 1997; Trebitsh et al. 1997). The *F* and *M* loci of cucumber have been cloned and shown to encode two different members of the ACC synthase family (CsACS1G and CsACS2, respectively), involved in ethylene biosynthesis (Trebitsh et al. 1997; Mibus and Tatlioglu 2004; Boualem et al. 2009; Li et al. 2009). The *A* locus of melon also encodes for the ethylene biosynthesis enzyme CmACS7, while the gynoecious locus *G* encodes for the transcription factor CmWIP1 (Martin et al. 2009). Andromonoecy in both cucumber and melon is the consequence of a loss of function mutation in either the *CmACS7* or *CsACS2* genes, indicating that their specific expression and consequently the specific production of ethylene in the carpels during earlier stages of female flower development are required to arrest the development of stamens (Boualem et al. 2008, 2009). Expression of *CmWIP1* leads to carpel abortion, resulting in the development of unisexual male flowers. *CmWIP1* also represses *CmACS7* expression, favoring the development of stamens and the production of male flowers (Martin et al. 2009). Similarly, the expression of cucumber *CsACS2* is induced by *CsACS1G*, and maintained by a positive feedback regulation (Li et al. 2012).

To date, no andromonoecious genotype has been described in *C. pepo*, but sex determination in individual floral buds of squash appears to be regulated by ethylene in the same way as in melon and cucumber (Manzano et al. 2010a, b, 2011). In the present paper, we have identified a number of squash cultivars that show instable monoecy or partial andromonoecy when grown under high-temperature conditions. We have cloned and characterized two squash homologous genes to *CmACS7* and *CsACS2*, and studied their implication in this partial andromonoecy of *C. pepo*. We discuss the involvement of this and other biosynthesis and signaling genes not only in the arrest of stamen development, but also in other associated traits during female flower development, including a delay in the maturation of petals and stigma as well as the parthenocarpic development of the fruit.

Materials and methods

Plants and culture conditions

Supplemental Table S1 shows the different *C. pepo* cultivars that have been screened in this work for monoecy instability. They consist of 9 commercial hybrids and 58 traditional cultivars conserved at the Gemplasm Bank at the Polytechnic University of Valencia (COMAV), and the University of Almería (BSUAL). In addition, we have also analyzed two inbred lines of *Cucurbita pepo* subspecies *pepo*, Veg and Bog, which contrast regarding both the ratio of female to male flowers and ethylene production and sensitivity (Manzano et al. 2010a, 2011), as well as the F1 and three independent F2 populations derived from the cross Bog × Veg. These generations, together with the selfing progenies of the hybrids Cavili and Argo, were used to determine the inheritance of monoecy instability in *C. pepo*.

After a period of 10–15 days in nursery, plants were transplanted and grown in a greenhouse in Almería (Spain) following standard local commercial practices for both plant nutrition and pest and disease control. Given that monoecy instability was only noticeable when the daytime temperature reached over 30 °C, the essays were all carried out in spring/summer. Moreover, to demonstrate that daytime temperatures were responsible for the induction of monoecy instability, plants of the cultivars Cavili and Argo, as well as the F2 population of Bog × Veg, were grown under controlled conditions in two chambers with the same photoperiod but with two temperature regimes: 25 or 35 °C. In the first chamber, treatment consisted of 14 h light at 25 °C and 10 h night at 20 °C. In the second, plants were grown in the same conditions but with a central segment of 7 h at 35 °C during the light period. Relative humidity was maintained between 50 and 60 %.

Evaluation of monoecy instability and sex expression in *C. pepo*

C. pepo is a monoecious species that produces male and female flowers in the same plant foot. Under

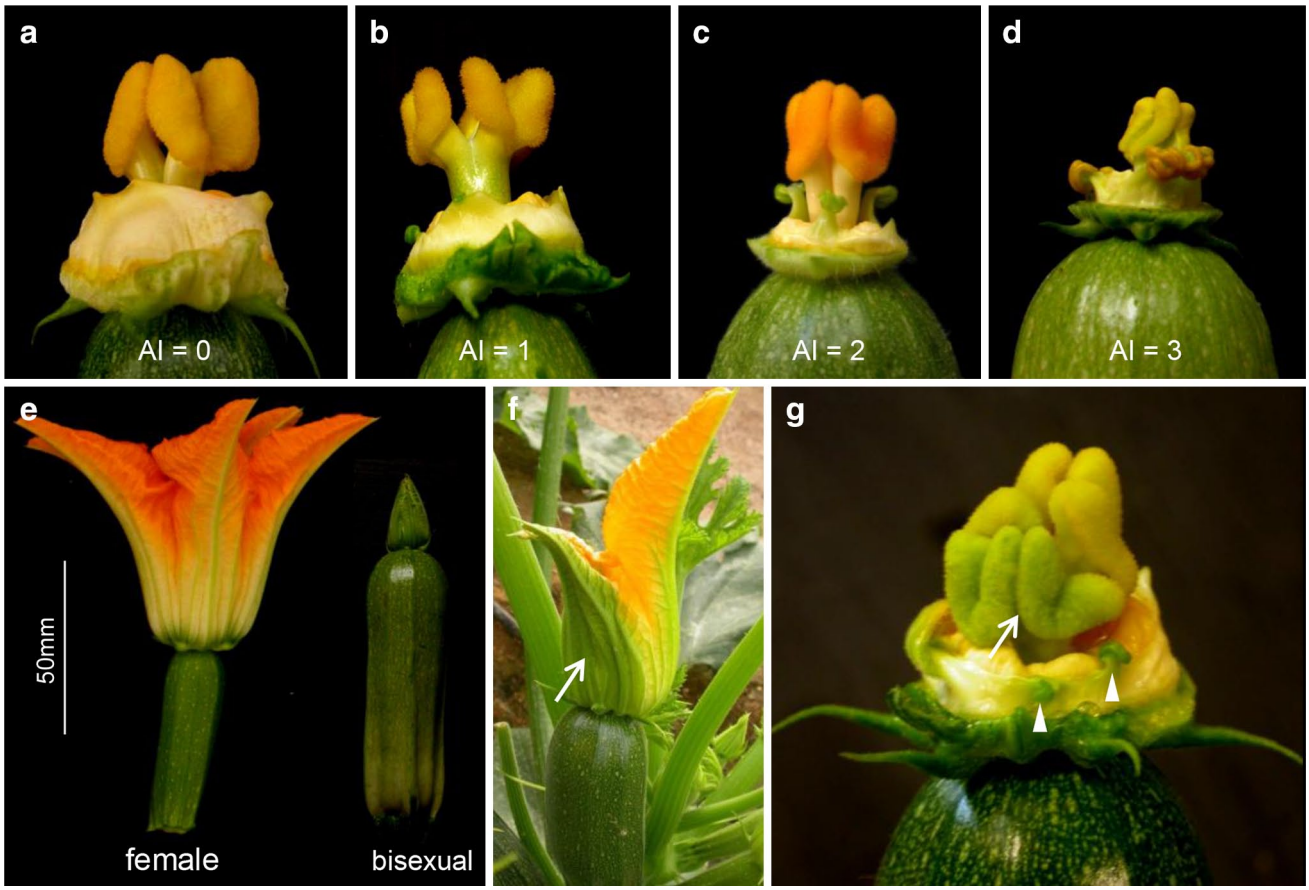

Fig. 1 Female flower phenotypes. **a–d** Classification of pistillate flowers and their andromonoecious index (*AI*), based on the degree of stamen development. Petals were removed for the visualization of stamens and pistils. **e** Comparison of ovary and petal development in female (AI = 0) and bisexual flowers (AI = 3) of the partially andromonoecious cultivar *Argo*. **f–g** Chimeric flowers showing delayed development of petals and stigma (*arrows*) only in the flower section that develops stamens (*arrowheads*). **g** Magnification of the flower in **f** after removing petals

high-temperature conditions (spring and summer), certain cultivars of *C. pepo* are unstable monoecious or partially andromonoecious, i.e. female flowers are converted into bisexual ones with different degrees of stamen development. Given the variation in temperature during the growing season, some of the pistillate flowers developed only primordial stamens, while others develop complete stamens with pollen. To evaluate monoecy instability in the different cultivars and populations, we defined an andromonoecy index (AI) for each flower, plant, population and cultivar. Pistillate flowers were scored from 0 to 3 according to their degree of stamen development. Female flowers with no stamen development were scored as AI = 0, while bisexual flowers with complete stamens and anthers able to produce pollen were scored as AI = 3. A score of 1 was assigned to flowers with primordial stamens, and 2 to flowers with medium-sized stamens and anthers (Fig. 1). Based on the flower scores, the AI of each plant in a population was calculated as the average score for at least five flowers. The AI of a cultivar, inbred line or F1 population was calculated from at least 10 plants with a minimum of 5 pistillate flowers evaluated per plant. Plants and genotypes with an AI of between 0 and 0.9 were considered to be monoecious, while those with scores of between 1 and 3 were considered unstable for monoecy or partially andromonoecious.

The evaluation of sex expression was based on both the number of initial nodes with male flowers before the production of the first female flower and the percentage of female or male flowers per plant in the first 30 nodes of the main stem.

Evolution of ethylene production throughout female and bisexual flower development

Ethylene production was measured in female and bisexual flowers at different stages of development, as were the expression of *CpACS27A* and other ethylene genes. Female flowers were collected from F2 plants of the cross

Bog × Veg that were completely monoecious (AI = 0), while bisexual flowers were obtained from F2 plants showing the most extreme phenotype for partial andromonoecy (AI ≥ 2). According to flower bud length, we separated nine stages of floral development, including anthesis (Manzano et al. 2010b): S0, flower buds less than 2 mm; S1, 2–4 mm; S2, 4–8 mm, sepals are longer than petals; S3, 8–12 mm, sepals are lightly shorter than petals, stigma is green; S4, 13–17 mm; S5, 18–25 mm, stigma starts to become yellow; S6, 26–35 mm; S7, floral 36–50 mm, stigma yellow and petals become yellow; anthesis, petals are open, stigma yellow and receptive.

Ethylene was determined in three replicates per sample, each one containing three female or bisexual flowers at the same stage of development. Floral buds were excised and incubated at room temperature for 6 h in hermetic glass containers in the dark. Ethylene production was determined by analyzing 1 ml of gas from the headspace on a Varian 3900 gas chromatograph apparatus fitted with a flame ionization detector. The instrument was calibrated with standard ethylene gas.

Cloning and molecular characterization of *CpACS27A* and *CpACS27B*

Degenerated primers *CpACS27F2*/*CpACS27R2* and *CpACS27ARACE BF*/*CpACS27ARACEER*, derived from conserved region in *CmACS7* and *CsACS2*, were used to amplify two genomic PCR fragments of 650 and 582 bp (base pair) that corresponded to *CpACS27A*. Specific primers from these two initial fragments were combined with primers in 5′ UTR and 3′ UTR of melon *CmACS7* to complete the whole gene sequence. *CpACS27_2FWD* and *CpACS27RTREV* originated a fragment of 1,350 bp in the 5′ region of the gene, while *CpACS27AFWD RACE* and *CmACS_1Rev* produced an overlapping fragment of 933 bp in the 3′ region.

The initial fragment of 871 bp from *CpACS27B* was obtained by the combination of primers *CpACS271F* and *CpACS273R*. The combination of *CpACS27BRTFWD2*, a specific primer from this *CpACS27B* fragment, and the degenerated primer *CpACS27RACEER* was then used to amplify the final sequence of 1,376 bp, containing the end of the second exon, the second intron and the almost complete third exon. Sequence data from *CpACS27A* and *CpACS27B* can be found in the GenBank/EMBL data libraries under accession numbers KF113530 to KF113533.

Alignments have been performed using the BLAST alignment tools at NCBI (http://www.blast.ncbi.nlm.nih.gov/) and Clustalw at GenomeNet Database Resources (http://www.genome.jp/tools/clustalw/). Phylogenetic relations have been studied using MEGA4 software (Tamura et al. 2007), which allowed the alignment of proteins and the construction of phylogenetic trees using the UPGMA method (Sneath and Sokal 1973), with 2,000 replicates bootstrap (Felsenstein 1985). The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling 1965). There were a total of 516 positions in the final dataset.

Protein 3D models for the CpACS27A enzyme were generated by the Cn3D software at NCBI (http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml). The model files 1IAY.pdb and 1IAX.pdb from *Solanum lycopersicon* were used to study the position of the amino acid changes between *B* and *V* alleles.

Genotyping *B* and *V* alleles of *CpACS27A*

We have detected a single nucleotide polymorphism (SNP) between Bog and Veg inbred lines that produce an amino acid substitution of a serine to an alanine in residue 176 of the CpACS27A protein. The respective alleles of the gene *CpACS27A* in Bog and Veg were called *B* and *V*, respectively. As the change affected a *Taq I* restriction site of the gene, we used a CAP marker to genotype these two alleles in different cultivars and segregating populations. DNA was isolated from frozen young leaves using the commercial DNeasy® Plant Mini Kit (Qiagen). 15–35 ng of purified DNA was used to amplify by PCR a 1,351-bp fragment of *CpACS27A* with primers *CpACS27_2FWD* and *CpACS27RTREV*. 200 ng of each PCR product was then digested with the enzyme *Taq I* (Roche) for 4 h at 65 °C. Results were resolved in agarose gels at 1.3 %. The *B* allele generated four fragments of 493, 423, 352, and 63 bp, while the *V* allele showed five fragments of 438, 423, 352, 63 and 55 bp.

Expression analysis by quantitative RT-PCR

Gene expression analysis was performed in three replications per sample. Each replication was the result of an independent extraction of total RNA from three flowers at the same stage of development, except for smaller flowers, whose RNA isolation was performed individually to avoid mixing female and bisexual flowers. RNA was extracted according to the protocol of the Aurum Total RNA Mini kit (Biorad). The remaining DNA in RNA samples was eliminated by digestion with RQ1 RNAse-free DNAse (Promega). Before cDNA synthesis, the absence of DNA in RNA samples was verified using 2 µl of RNA samples as template in PCRs with primers *CpACS27EXMOFWD* and *CpACS27RTEXREV* from *CpACS27A*, located at both sides of the second intron of the gene, and therefore differentiating the products derived from DNA and cDNA.

Once it had been established that RNA samples contained no DNA, 600 ng of RNA was used for cDNA synthesis using *iScript Reverse Transcription Supermix for RT-qPCR*

(Biorad). The expression of the genes was then evaluated through quantitative RT-PCR using the *Rotorgene* thermocycler (Qiagen) and *Power SYBR Green PCR Master Mix* (Qiagen). The q-PCR primers for each gene are shown in Supplemental Table S2. Those of *CpACS27A* were designed to avoid DNA amplification, one in exon 3 and the other between exons 2 and 3. To avoid any possible cross-amplification with DNA, and before any q-PCR experiment, the size of the PCR products for each pair of primers was checked in agarose gels, and sequenced. Quantitative RT-PCRs consisted of 40 cycles with the following three steps: 20 s at 95 °C, 15 s at 59 °C and 20 s at 60 °C for the genes *CpACS27*, *CpACS1*, *Cp18S* and *CpACTIN*; and 20 s at 95 °C, 15 s at 57 °C and 20 s at 60 °C for genes *Cp1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID OXIDASE* (*CpACO1*), *CpETHYLENE RECEPTOR1* (*CpETR1*), *CpETHYLENE RESPONSE SENSOR1* (*CpERS1*), *CpCONSTITUTIVE TRIPLE RESPONSE1* (*CpCTR1*), *CpCTR2*, *CpETHYLENE INSENSITIVE3.1* (*CpEIN3.1*) and *CpEIN3.2* Genes *CpACS2-CpACS7* were analyzed in a two-step program consisting of 95 °C for 5 s and 61 °C for 30 s.

Relative expression of each gene was determined by the comparative Ct (*Cycle Threshold*) method using *C. pepo 18S* ribosomal RNA and *ACTIN* genes as internal standards. To use this method, we first demonstrated that the efficiency of amplification for each amplicon was roughly equivalent, regardless of the amount of template cDNA. The absolute value of the slope of ΔCt (Ct of the target gene-Ct of the reference gene) versus serial dilutions of cDNA for a given sample must be <0.1. The relative expression of each gene was then calculated relative to a calibrator sample using the formula $2^{-\Delta\Delta Ct}$, where ΔΔCt is the difference between the ΔCt of each sample and the ΔCt of the calibrator sample.

In situ hybridization

For in situ hybridization, probes were labeled with digoxigenin-11-UTP using the nucleic acid labeling kit (Boehringer). The *CpACS27A* cDNA in the plasmid vector pGEM-T was used as a template to synthesize digoxigenin-labeled sense and antisense RNA with T3 and T7 RNA polymerases. Sample preparation and hybridization were performed using a protocol modified from those described in Jackson (1991), Kronenberger et al. (1993) and Nikovics et al. (2006).

Results

Monoecy instability in *C. pepo*: identification of genotypes and inheritance pattern

Under winter conditions, when day temperatures did not exceed 30 °C, all the analyzed cultivars of squash were considered to be monoecious, developing both male and female flowers in the same plant foot. However, under spring/summer conditions, when day temperatures rise above 30 °C, certain squash cultivars showed an instable monoecy or a partial andromonoecy, characterized by the development of both bisexual and male flowers in the same plant foot (Supplemental Table S1). In addition to genetic factors, these differences in sex expression are likely to be caused by high temperature, since plants of the same cultivars that were grown in controlled chambers under the same regime of humidity, light and photoperiod, but with a daytime temperature of 25 or 35 °C, developed bisexual flowers only under the higher temperature (data not shown). Under high-temperature conditions, therefore, female flowers were converted into partial bisexual flowers with different degrees of stamen development, from undersized stamen in some flowers to complete stamens with pollen in others (Fig. 1a–d). These bisexual flowers also showed a delayed development and maturation of petals and a higher growth rate of the ovary, which resulted in marketable parthenocarpic fruits before the aperture of the corolla (Fig. 1e). Some of the bisexual flowers showed a chimeric appearance because delayed growth and maturation only affected those petals and stigmas located in the flower section that developed stamens (Fig. 1f–g).

Cultivars showing this partial conversion of female into bisexual flowers were considered to be instable for monoecy or partially andromonoecious. Since temperature varied over the growing season, the conversion of female into bisexual flowers did not affect all the flowers in each plant equally. The bisexuality or andromonoecy index of each pistillate flower was scored according to stamen development. Complete female flowers with no stamen development were scored as AI = 0 (Fig. 1a); while complete bisexual flowers having complete stamens with anthers and pollen were scored as AI = 3 (Fig. 1d). Pistillate flowers with incipient anthers were scored as AI = 1 (Fig. 1b), and those with medium-sized anthers were scored as AI = 2 (Fig. 1c). The AI of each plant and genotype was then calculated as the average score of a minimum of five pistillate flowers in each plant, and at least 10 plants for each cultivar.

Plants and cultivars with an average AI that ranged between 0 and 0.9 were considered to be stable for monoecy or monoecious, while those with an average AI of between 1 and 3 were considered to be instable for monoecy or partially andromonoecious (Supplemental Table S1). Out of a total of 67 cultivars, we have identified 3 commercial hybrids and 26 traditional cultivars that were partially andromonoecious under high-temperature conditions (Supplemental Table S1). We also evaluated two contrasting inbred lines for ethylene sensitivity and sexual expression, Bog and Veg (Manzano et al. 2010a). The

Table 1 Segregation of monoecious: partial andromonoecious plants in the F2 generation of the cross Bog × Veg, and in the selfing progenies of the F1 hybrids Cavili and Argo

Generation	No. of monoecious plants[a]	No. of partially andromonoecious plants[a]	Expected segregation	χ^2	*P*
Parental line Bog	30	0	–	–	–
Parental line Veg	30	0	–	–	–
F1 (Bog × Veg)	30	0	–	–	–
F2.1 (Bog × Veg)	68	18	13:3	0.17	0.67
F2.2 (Bog × Veg)	76	17	13:3	0.02	0.89
F2.3 (Bog × Veg)	113	27	13:3	0.01	0.91
Pooled F2 (Bog × Veg)	257	62	13:3	0.13	0.71
Cavili F1	0	30	–	–	–
Cavili F2	30	65	1:3	1.69	0.19
Argo F1	0	30	–	–	–
Argo F2	18	61	1:3	0.15	0.69

[a] F2 plants were classified in monoecious or partially andromonoecious on the base of their AI mean values, scored from at least five flowers per plant. Plants with AI = 0–0.9 were phenotyped as monoecious, while those with AI = 1–3 were considered partially andromonoecious

inbred lines, as well as the F1 generation derived from the cross Bog × Veg, showed a stable monoecious phenotype (Supplemental Table S1). Nevertheless, the three analyzed F2 populations derived from the cross Bog × Veg exhibited segregation for AI (Table 1). The F2 segregation ratio was closed to 3:1, but the observed pattern of inheritance cannot be explained by just one major gene. The segregation ratio of monoecious to partially andromonoecious in the three F2 populations fitted the 13:3 ratio (Table 1), the expected ratio if partial andromonoecious phenotype in this population is conferred by the interaction of two independent genes, one dominant and one recessive. The F2 populations derived by self-pollination of the F1 hybrids Cavili and Argo fitted the 3:1 ratio for partially andromonoecious to monoecious (Table 1), which suggests that in these two cultivars there is only one major segregating gene for the andromonoecious phenotype.

Cloning and molecular characterization of *CpACS27A* and *CpACS27B*

Given that in melon and cucumber the andromonoecious phenotype is caused by a mutation in the orthologs genes *CmACS7* and *CsACS2*, we have cloned and characterized two squash *ACS* genes homologous to *CmACS7* and *CsACS2*. Degenerate primers (Supplemental Table S2), which were designed based on the alignment of *CsACS2* and *CmACS7*, were used in PCRs with Argo genomic DNA as a template. Two initial PCR fragments of 650 and 871 bp were cloned and sequenced. The combination of specific primers from these two fragments as well as primers from conserved regions in the 5′ and 3′ regions of *CmACS7* and *CsACS2* allowed us to clone and sequence 1,886 and 1,376 bp genomic fragments from two highly homologous genes called *CpACS27A* and *CpACS27B*. The genomic fragment of *CpACS27A*, and its corresponding cDNA of 1,533 bp, contained the complete coding sequence of the gene. The *CpACS27B* genomic fragment was, however, incomplete, and no transcript of this gene was detected in the different tissues analyzed by RT-PCR, which suggests that *CpACS27B* was likely a paralogous pseudogene.

Figure 2 shows the molecular structure of *CpACS27A* and *CpACS27B*. The structure of *CpACS27B* was deduced from the alignment of the partial sequence of this gene with the complete sequence of *CpACS27A*. *CpACS27A* was found to have the same structure as *CmACS7* and *CsACS2*: three exons and two introns (Fig. 2), but it differed from other described *C. pepo ACS* genes, which, like *CpACC1A* and *CpACC1B*, are more complex and contain five exons (Sato et al. 1991). The homology of *CpACS27A* exons with those of *CpACS27B*, *CmACS7*, *CsACS2* and *CitACS4* was very high (Supplemental Table S3). The highest identity was found with exons 2 and 3 of *Citrulus lanatus CitACS4* (Supplemental Table S3). No homology was found among introns of these genes, however. Moreover, although the first intron has the same length in *CpACS27A*, *CmACS7* and *CsACS2*, the length of the second intron varied considerably not only among genes of different species, but also between genes *CpACS27A* and *CpACS27B* of *C. pepo* (Fig. 2).

The deduced CpACS27A protein contains 445 amino acids. Homology analysis with other ACS enzymes in public databases revealed 89 % identity with CsACS2 and CmACS7 (Fig. 3a). After multiple sequence alignment and a clustalw analysis, these three enzymes were clustered together with *Arabidopsis AtACS7* in the branch corresponding to ACS type III, but separately from type I and II ACS enzymes (Fig. 3b). Type III ACS proteins are truncated at the C-terminal end, and consequently lack the CDPK phosphorylation motif of type I, and the MAPK6 phosphorylation motif of type I and II ACS enzymes (Yoshida et al. 2005; Zhang et al. 2012). The deduced partial amino acid sequence of CpACS27B showed a high number of

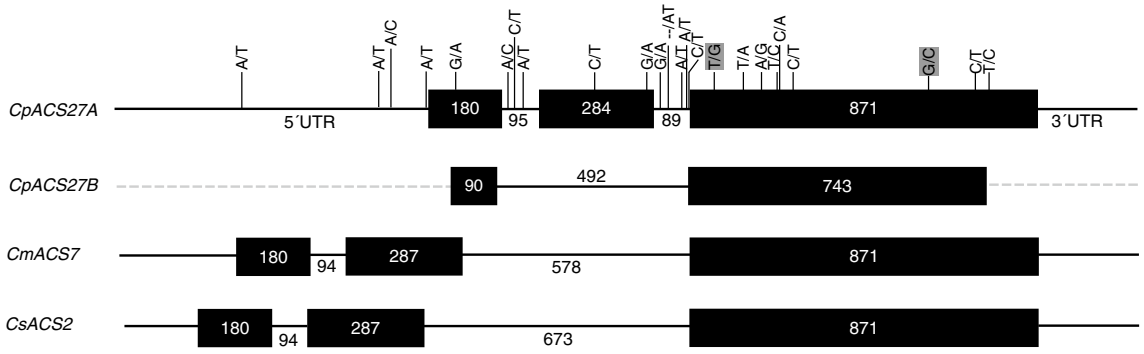

Fig. 2 Comparison of the structure of *CpACS27A* and *CpACS27B* of *C. pepo*, with that of *CmCAS7* and *CsACS2* of *C. melo* and *C. sativus*, respectively. The numbers indicate the size of the three exons (*filled boxes*) and the two introns (*black lines*) in bps. *Grey dashed line* indicates unknown sequence in *CpACS27B*. In *CpACS27A*, the distribution of a single nucleotide polymorphisms between *Bog* and *Veg* inbred lines is shown. Missense changes producing amino acid substitutions in the protein are highlighted in *grey*

substitutions, insertions and deletions in conserved domains of the protein (data not shown), again indicating that *CpACS27B* is a non-functioning pseudogene that has accumulated a number of mutations in its evolution.

Expression of *CpACS27A* was determined by real-time RT-PCR, using *C. pepo* 18S and *ACTINE* as reference genes. As also occurs for *CmACS7* and *CsACS2*, *CpACS27A* was found to be specifically expressed in pistillate flowers, both in the female flowers of monoecious plants and in female and bisexual flowers of partially andromonoecious plants (Fig. 4). The expression in bisexual flowers was, however, about four times lower than in female flowers (Fig. 4). No *CpACS27A* transcript was found to be accumulated in male flowers, stems or leaves (Fig. 4). The slight expression detected in the apical shoot was likely derived from female and bisexual floral buds that this tissue includes along with male flowers and the apical meristem (Fig. 4).

To compare the expression of *CpACS27A* during the development of female and bisexual flowers, pre-anthesis flower buds were separately classified by their size in eight stages of development (Fig. 5a). Throughout flower development, the maximum expression of *CpACS27A* was found in the smallest female floral buds considered (S0, flower bud length less than 2 mm). Subsequently, gene expression decreased gradually to cease completely at stage S5 (Fig. 5b). In bisexual flowers, the gene expression pattern was similar, although the expression level was lower than in female flowers at all stages of development and declined more rapidly than in female flowers, with no transcripts detected after stage S3. No gene expression was detected at anthesis (Fig. 5b) or at post-anthesis stages of flower development (data not shown). Ethylene production increased throughout the development of female and bisexual flowers up to anthesis, and it was lower in bisexual than in female flowers (data not shown).

In medium-sized pistillate buds, where it was possible to separate the different floral organs, *CpACS27A* expression was specifically detected in the pistils of female and bisexual flowers, in which the ovary showed much higher expression than the style and the stigma (Fig. 6). No expression was observed in the petals, nectaries or stamens of bisexual flowers (Fig. 6). To establish the spatial expression pattern of *CpACS27A*, we also performed in situ hybridization in female flowers at different stages of development (Fig. 6). At very early stages of female flower development, before the appearance of the ovary, *CpACS27A* transcripts were found to accumulate specifically in carpel primordia (Fig. 6b). In later stages, when the ovary is already formed, gene transcripts were also found in ovules (Fig. 6c).

Expression of other ethylene biosynthesis and signaling genes in female and bisexual flowers

Since ethylene is able to autoregulate its own production and signaling, and given that the traits associated with partial andromonoecy, i.e. the higher growth rate of the ovary and the delay in the maturation of petals and stigma, could be controlled by ethylene in later stages of female flower development (Martínez et al. 2013), we have compared the expression patterns of other ethylene biosynthesis and signaling genes previously found to be upregulated throughout the development of the female flower (Manzano et al. 2013). The expression was analyzed in the ovaries of female and bisexual flowers at S2, S3 and S5 stages of flower development. We found a correlation between the expression of *CpACS27A* and that of other ethylene genes whose expression is upregulated later in the development of the female flower. In fact, while the expression of all the analyzed ethylene genes was upregulated throughout the development of female flowers, meanwhile the expression in bisexual flowers was maintained at lower level or

a

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CpACS27A        MAIEIELDQNPAVELSQIGMSETHGEDSPYFAGWKAYDENPYNETTNPSGVIQMGLAENQ
CmACS7          *****DIE***T****R**T*******************D****S***********V***
CsACS2          ******IE**SS****R**T************C******D****S***************

CpACS27A        VSFDLVEEYLEQNSD-VLQANSSGFREDALFQDYHGLLSFRTAMAGFMEQIRGGRARFDP
CmACS7          *****L*****E*CEGEGNYLN*****N*********F***S**GS***E******K***
CsACS2          *****L*****E*CEGEGNYLN*****N*********F***S**GS***E******K***

CpACS27A        NRVVLTAGATAANELLTFILANPGDALLVPTPYYPGFDRDLRWRTGVKIVPIHCDSANNF
CmACS7          ************************************************************S***
CsACS2          ************************************************************S***

CpACS27A        QITPKSLEAAYNNAIAMKMKVRGVLITNPSNPLGATIQRSTIEEILDFVTRKNIHLVSDE
CmACS7          *****A**E***S*ME**I************************D****************
CsACS2          *****A**E***S*TE**I*********S**************D****************

CmACS7          IYSGSVFSSAEFTSVAEVLESRSYKNAERVHIVYSLSKDLGLPGFRIGTIYSYNDKVVTT
CsACS2          *********D***********G**********************V*************
CpACS27A        ***********I******A**G**********************V*************

CpACS27A        ARRMSSFTLISSQTQRFLASMLSNRKFTEKYIKMNRDRLKKRYEMIIEGLRTAGIDCLKG
CmACS7          ****************************K*************************E**E*
CsACS2          ****************************N*************************E**E*

CpACS27A        NAGLFCWMNLSPLLKDKKTNTDGEIELWKRILKEVKLNISPGSSCHCSEPGWFRVCFANM
CmACS7          ******************-KE****I*********************************
CsACS2          ******************-KE****I************L*********************

CpACS27A        SEHTLHVALDRIHCFVEKLKKEDEAN
CmACS7          **K*********RR*M*RM***N***
CsACS2          **K*********RR*M*RM***N***
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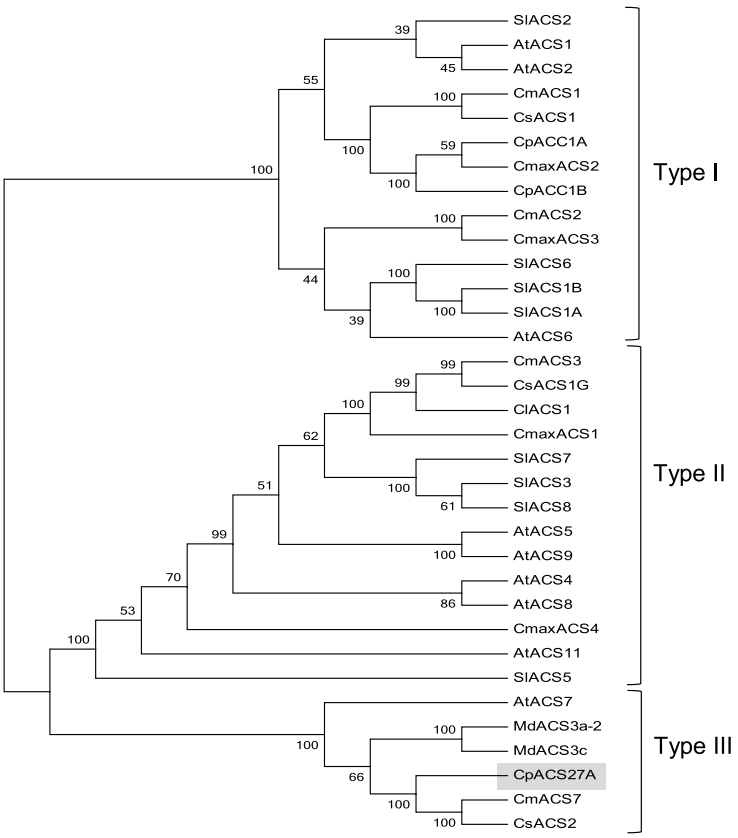
b



◀**Fig. 3** **a** Alignment of *C. pepo* CpACS27A protein with *C. melo* CmACS7 and *C. sativus* CsACS2. Amino acid changes between variants of CmACS7 and CsACS2 in monoecious (CmACS7 allele *A*, ACG70849.1; CsACS2 allele *M*, BAF79596.1) and andromonoecious lines (CmACS7 allele *a*, ACG70850; CsACS2 allele *m*, ACT78959.1), as well as between CpACS27A alleles in *Bog* (allele *B*) and in *Veg* (allele *V*), are highlighted in *grey*. **b** Evolutionary tree performed for 35 ACS proteins from different plants: *Arabidopsis thaliana* (AtACS1, AAM91649.1; AtACS2, AAG50097.1; AtACS4, Q43309.1; AtACS5, Q37001.1; AtACS6, Q9SAR0.2; AtACS7, AEE85169.1; AtACS8, Q9T065.1; AtACS9, Q9M2Y8.1; AtACS11, AEE82593.1), *Cucurbita máxima* (CmaxACS1, P23599.1; CmaxACS2, AAA91152.1; CmaxACS3, BAB47124.1; CmaxACS4, BAB47123.1), *Cucurbita pepo* (CpACC1A, AAA33111.1; CpACC1B, AAA33112.1; CpACS27A, KF113530), *Cucumis melo* (CmACS1, BAA83618.1; CmACS2, BAB18464.1; CmACS3, ACO83163.1; CmACS7, ACG70849.1), *Cucumis sativus* (CsACS1, BAA93714.1; CsACS1G, ABI33818.1; CsACS2, ACG70849.1), *Citrullus lanatus* (ClACS1, AFI49625.1), *Malus x domestica* (MdACS3a-2, AEP82201.1; MdACS3c, BAE94692.1) and *Solanum lycopersicon* (SlACS1A, AAF97614.1; SlACS1B, AAF97615.1; SlACS2, P18485.2; SlACS3, NP_001234026.1; SlACS5, NP_001234156.1; SlACS6, NP_001234164.1; SlACS7, AAK72432.1; SlACS8, AAK72431.1). The tree was inferred using the UPGMA method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2,000 replicates) is shown next to the branches

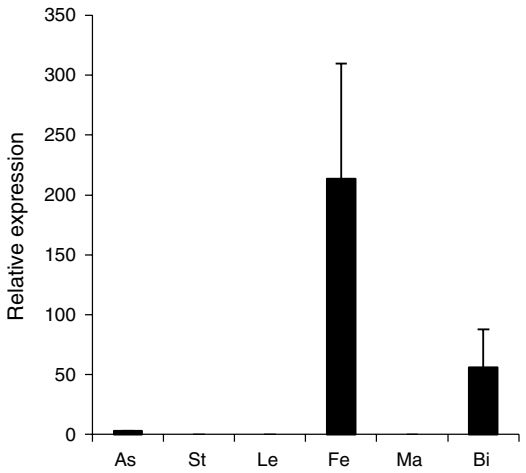


Fig. 4 Relative expression of *CpACS27A* in different tissues of *C. pepo* cv Argo. The values represent the mean and standard deviation of three biological replicates. *As* apical shoot, *St* stem, *Le* leaf, *Fe* female flowers, *Ma* male flowers, *Bi* bisexual flowers. The Q-PCR analysis was performed in the earliest stage of flower development

even downregulated (Fig. 7). This was true not only for the biosynthesis genes *CpACS1*, *CpACS4*, *CpACS5*, *CpACS6* and *CpACO1*, but also for the ethylene receptor genes *CpETR1* and *CpERS1*, as well as for the ethylene signaling genes *CpCTR1*, *CpCTR2*, *CpEIN3.1* and *CpEIN3.2*, indicating a differential regulation of ethylene genes during ovary development in bisexual and female flowers, even at stage S5, when the expression of *CpACS27A* is already lost (Fig. 7).

Cloning and sequencing of *CpACS27A* in contrasting genotypes

Given the segregation for monoecious to partially andromonoecious in the F2 plants derived from the cross Bog × Veg, we have searched for polymorphisms between the *CpACS27A* gene in Bog and Veg, and analyzed their possible co-segregation with this trait in the F2 population. 24 SNPs were detected between Bog and Veg *CpACS27A* sequences (Fig. 2). Exons 1 and 2 were found to be highly conserved, showing only three SNPs between Bog (*B*) and Veg (*V*) alleles; exon 3 showed 10 SNPs in 874 bp; and the two small introns contained 7 SNPs in 95 and 89 bp, respectively. The other four SNPs were located in the 5′ UTR of the gene (Fig. 2). Only 2 of the 24 detected SNPs produce aminoacid substitutions, one at position 176 (S/A), and another at position 355 (E/D). The residue S^{176} in Veg (allele *V*) was found to be highly conserved among ACS proteins. In fact, only 16 out of 100 ACS proteins with similarities to CpACS27A had no S at this position. This residue was also conserved in CmACS7 and CsACS2 of melon and cucumber, respectively (Fig. 3), and was located in one of the 25 conserved motifs detected by Zhang et al. (2012) among 121 ACS and ACS-like proteins of different origins, close to Pyridoxal 5′ phosphate (PLP) and $SO4^{2-}$ binding sites of the enzyme (Supplemental Fig. S1). The residue E^{355} in Veg (allele *V*) was found to be conserved among type III ACS enzymes, including CmACS7 and CsACS2 (Fig. 3), although it is not located close to any known functional motif, and 27 % of the analyzed ACS proteins showed no E at this position. Based on these results we considered that Veg has a WT allele for *CpACS27A* gene (allele *V*), while Bog is likely to have a mutated variant (allele *B*).

To know whether either of these two mutations could be responsible for the partial andromonoecious phenotype, we have genotyped the detected two missense mutations of this gene in 69 different cultivars of *C. pepo*, as well as in the F2 population derived from the cross Bog × Veg. The E^{355} mutation was detected in no *C. pepo* cultivar except in the Bog inbred line, suggesting that it could be a cultivar-specific variation. However, the A^{176} mutation was also detected in 5 of the 67 commercial and traditional cultivars analyzed. These five cultivars (AFR-12, S-4, A-19, V-185, V-142) showed a partial andromonoecious phenotype when grown under high-temperature conditions (Supplemental Table S1). However, other cultivars that were also phenotyped as partially andromonoecious, including the hybrids Cavili, Argo and Parthenon, did not carry the A^{176} variant. The monoecy stable cultivars were all homozygous for the WT allele *V* (Supplemental Table S1).

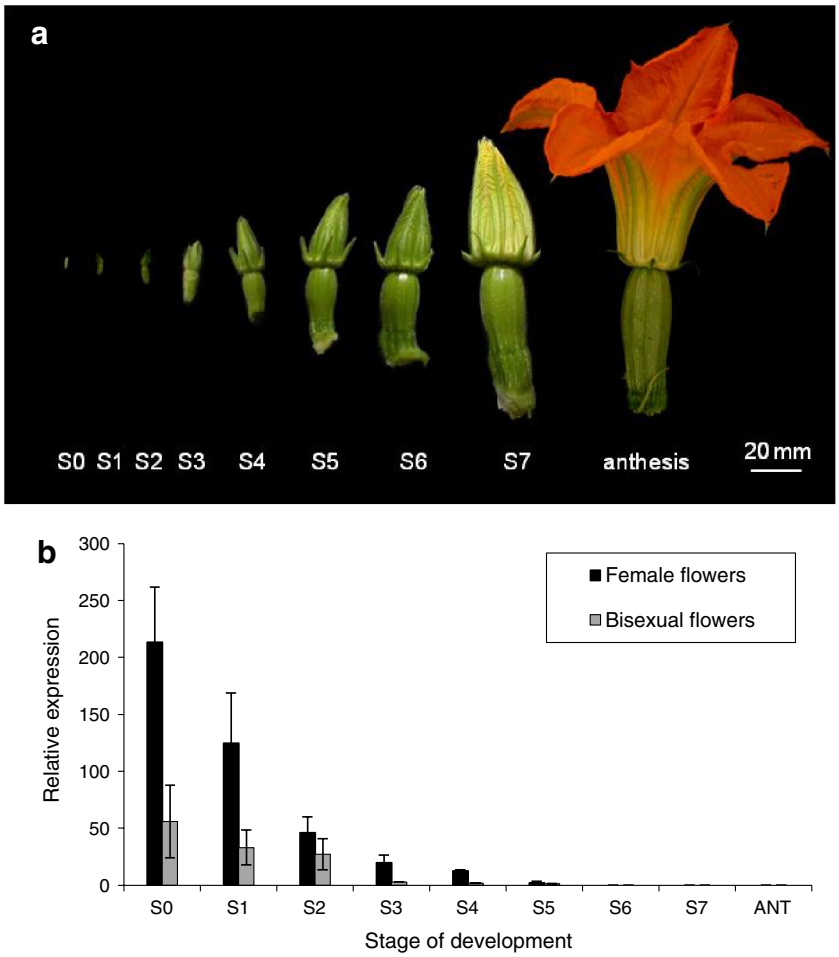


Fig. 5 Expression of *CpACS27A* during the development of female and bisexual flowers. **a** Stages of development of analyzed pistillate flowers. **b** Expression of *CpACS27A* in female and bisexual flowers at different stages of flower development. Flowers were sampled from the F2 population of the cross Bog × Veg, segregating for monoecy to partial andromonoecy. The gene expression in S0 corresponds to complete flowers. For the rest of the stages, it was possible to separate the different floral organs, and gene expression was measured in the ovaries, the tissue in which the gene is most expressed. Each value represents the mean and the standard deviation from at least three replicates. *Error bars* indicate standard deviation

The possible co-segregation of A^{176} mutation with the partial andromonoecious phenotype was studied in 84 F2 plants of the cross Bog × Veg, 54 monoecious and 30 partially andromonoecious (Table 2). All partially andromonoecious plants carried the mutated allele *B* (genotype *BB* o *BV*), but none of them were homozygous for the WT allele (*VV*). However, the monoecious stable plants showed any of the genotypes: *BB*, *BV* or *VV*. It seems, therefore, that the mutated allele derived from Bog (*B*) was necessary but not sufficient to confer the partial andromonoecious phenotype. Accordingly, *BB* and *VV* F2 plants differed significantly as regards their average AI, and the mutated allele *B* increased this index (Table 3). However, no association was found between either of the two alleles of this gene and the number of initial nodes before the transition to female flowering, or with the percentage of female flowers per plant (Table 3). Neither was a significant difference found between monoecious and partially andromonoecious plants for these two sexual expression traits, suggesting that andromonoecy did not co-segregate with a higher number of female or male flowers per plant.

Discussion

Environmental, hormonal and genetic factors controlling partial andromonoecy in *C. pepo*

Sex determination in *C. pepo* is known to be regulated by ethylene. In fact, treatments with the ethylene inhibitors AVG (aminoethoxyvinylglycine) and STS (silver thiosulphate) not only delay the transition to female flowering and reduce the number of female flowers per plant, but also induce the conversion of female into bisexual flowers (Manzano et al. 2011), indicating that the hormone is involved in the arrest of stamen development in female flowers, and therefore in monoecy stability.

In this paper, we have identified a number of *C. pepo* cultivars in which this incomplete conversion from monoecy to andromonoecy occurs when they are grown under greenhouse spring–summer conditions. Temperature is likely to be the main environmental factor, since sex conversion can be reproduced in controlled chamber by increasing only the daytime temperature from 25 to 35 °C. Daytime temperature of growth is likely to reduce the production of ethylene

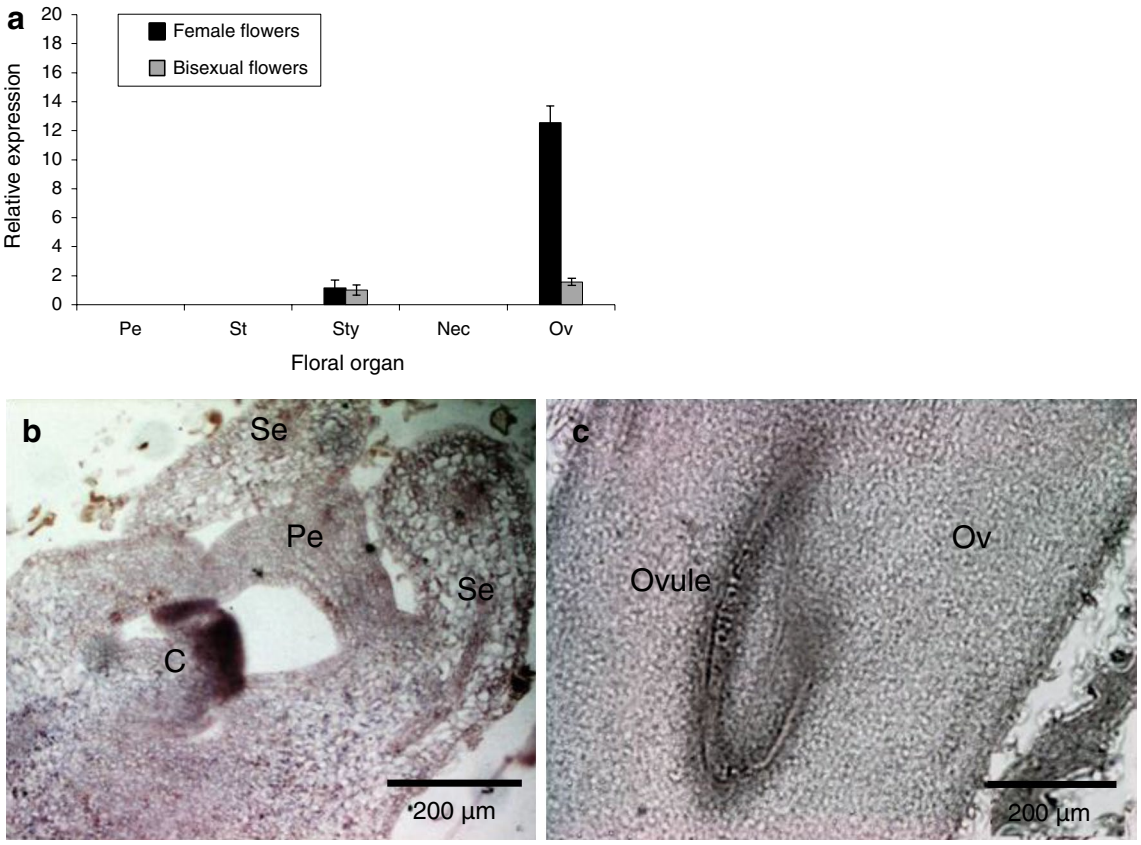


Fig. 6 Expression of *CpACS27A* in floral organs of female and bisexual flowers of *C. pepo* at different stages of development. **a** Relative expression of *CpACS27A* in female and bisexual floral organs at stage S4. Flowers were collected from monoecious and partially andromonoecious F2 plants resulting from the cross *Bog* × *Veg*. Data represent the mean of three independent biological replicates. *Error bars* indicate standard deviation. **b** In situ hybridization of *CpACS27A* on a longitudinal section of a complete female flower at stage S0, before the formation of the ovary. The gene is only expressed in carpel primordia. **c** In situ hybridization of *CpACS27A* on the ovary of a female flower at stage S2. Note the high accumulation of gene transcripts in ovule primordia. *Se* sepal, *Pe* petal, *St* stamen, *C* carpel, *Sty* style and stigma, *Nec* nectary, *Ov* ovary

in the floral buds, which results in monoecy instability. In cucumber, environmental factors such as day length and temperature can alter ethylene production and the formation of male or female flowers (Rudich et al. 1972a, b; Yamasaki et al. 2003), a process that appears to be mediated by sugar signaling (Miao et al. 2011).

The effect of the temperature on monoecy stability is genotype dependent and only affected 29 of the 67 analyzed cultivars (Supplemental Table S1), which indicates that this trait, although modulated by the temperature, is also genetically regulated. The 3:1 segregation ratio of partially andromonoecious to monoecious plants in the F2 generation of the hybrids Cavili and Argo indicated that andromonoecy in these two cultivars is controlled by one temperature-sensitive dominant gene which we named *Andromonoecious* (*A/a*). This situation differs from that found in the two cultivated species of the genus *Cucumis*, cucumber and melon, where andromonoecy is caused by the action of one recessive gene (Kubicki 1969; Kenigsbuch and Cohen 1990; Boualem et al. 2008, 2009; Li et al. 2009). On the other hand, the partially andromonoecious plants in the F2 of the cross Bog × Veg seem to be conferred by two independent genes, one dominant and one recessive. Assuming that the dominant gene is the same as that segregating in Cavili and Argo F2 (gene *A*), the recessive one would correspond to another locus that we have called *Monoecious* (M/*m*). Partially andromonoecious F2 plants would be *A_mm*, while monoecious ones would be *A_M_*, *aaM_*, or *aamm* (Supplemental Fig. S2). These two genes could be involved in ethylene biosynthesis or sensitivity because both Bog and Veg differ in ethylene production and sensitivity (Manzano et al. 2010a, 2013). Manzano et al. (2011) observed that the reduction of ethylene production or sensitivity by AVG or STS treatments was effective in Bog (and other genotypes) but not in Veg to induce partial andromonoecy. It seems therefore that Bog carries the gene that is able to

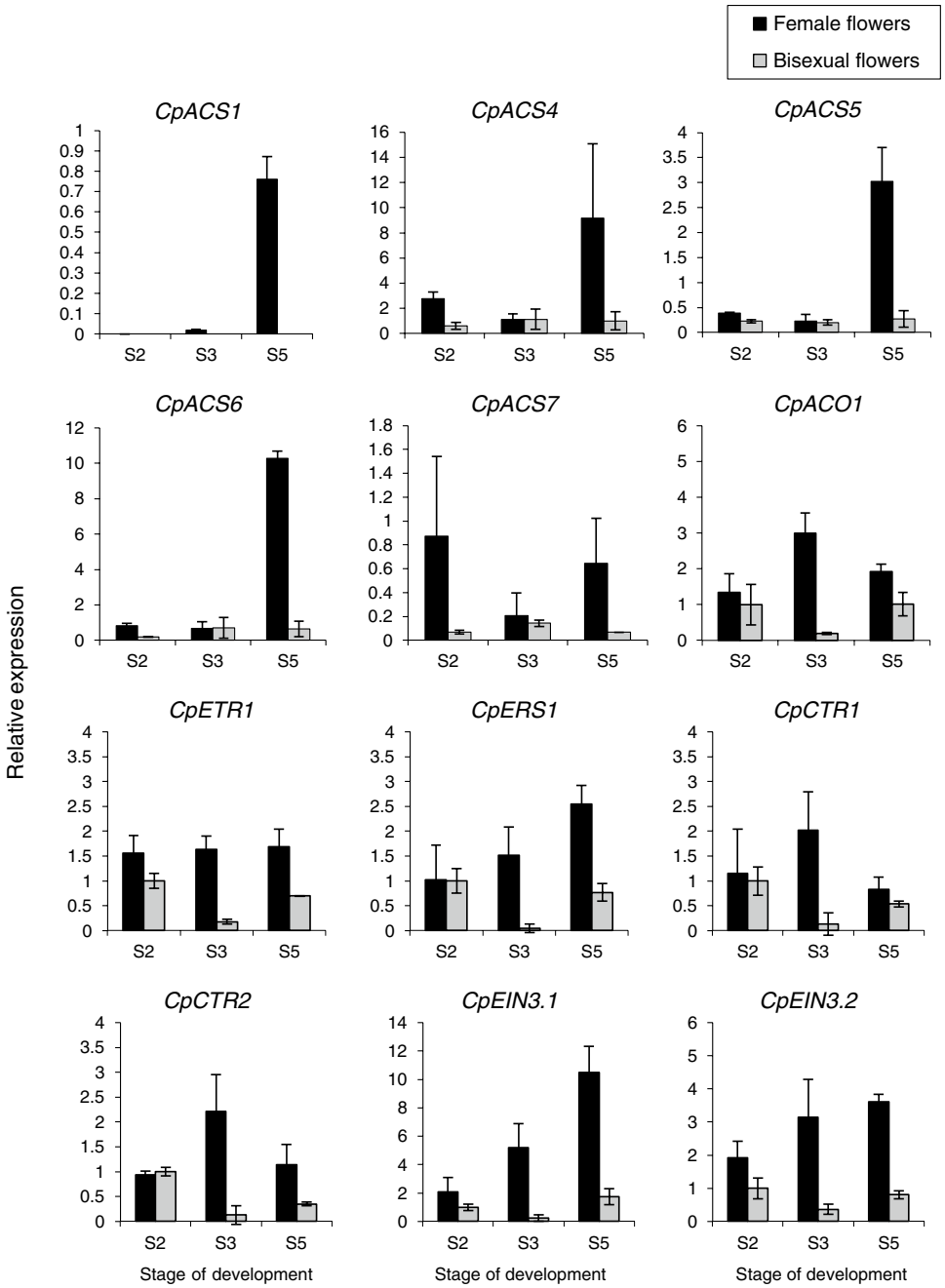


Fig. 7 Relative expression of ethylene biosynthesis, perception and signaling genes in female and bisexual flowers at different stages of floral development. It is presented as data of stages S2, S3 and S5 at which sexual phenotypes are clearly distinguishable. Each data point represents the mean of three replicates with three ovaries/fruits each. *Error bars* indicate standard deviation

Table 2 Segregation of *B* and *V* alleles of *CpACS27A* among monoecious and partial andromonoecious plants of the F2 population derived from the cross Bog × Veg

Genotype F2 plants	Monoecious (No. of plants)	Partial andromonoecious (No. of plants)
BB	15	17
BV	15	13
VV	24	0
Total	54	30

confer partial andromonoecy in squash, but that to act it requires a low level of ethylene in the floral buds.

Implication of *CpACS27A* in the partial andromonoecy of *C. pepo*

Given that in squash the production of female and bisexual flowers is controlled by ethylene in the same way as in melon and cucumber (Manzano et al. 2011), it is likely that the gene responsible for this function in squash is the

Table 3 Mean phenotypic values for andromonoecious index, female flowering transition and percentage of female flowers per plant in parental lines Bog and Veg, and in the F1 and F2 progenies of Bog × Veg cross

Population	Genotype	Andromonoecious index (AI)	Nodes before female flowering	Female flowers per plant (%)
Bog	*BB*	0.2 ± 0.1a	2.9 ± 0.4a	84.9 ± 9.3c
Veg	*VV*	0.0 ± 0.0a	19.4 ± 0.5c	26.0 ± 3.0a
F1	*BV*	0.1 ± 0.1a	6.0 ± 0.4b	64.8 ± 2.0b
F2	*BB*	1.4 ± 0.2b	6.0 ± 0.4a	62.3 ± 4.6a
	BV	1.1 ± 0.1b	5.7 ± 0.6a	60.1 ± 4.9a
	VV	0.1 ± 0.1a	6.9 ± 0.6a	52.2 ± 5.3a
F2	Monoecious		5.2 ± 0.3a	62.4 ± 4.5a
	Partially andromonoecious		4.8 ± 0.3a	70.9 ± 2.8a

Different letters indicate statistical differences among genotypes at $P \leq 0.05$

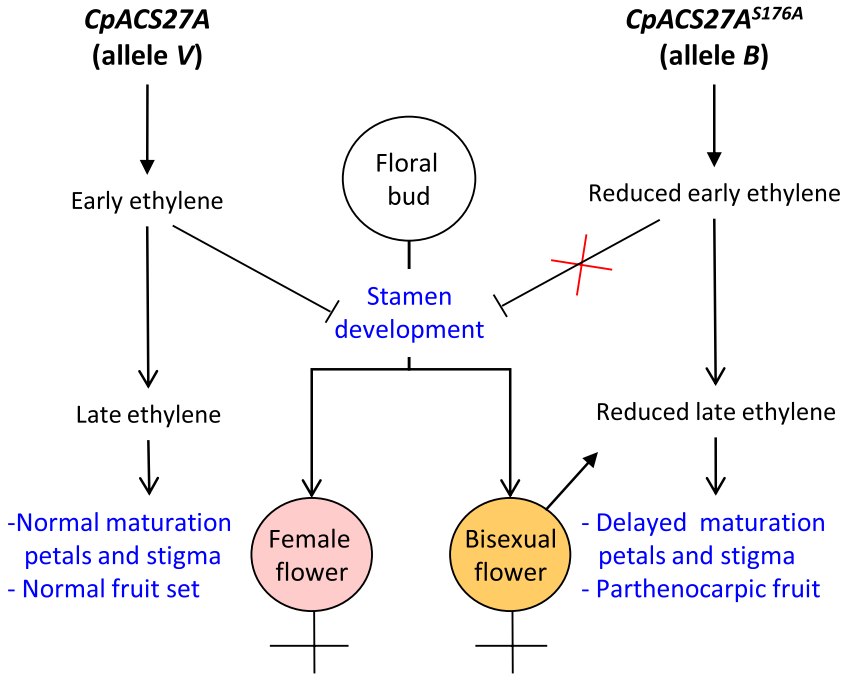


Fig. 8 Diagram indicating the function of *CpACS27A* gene. The ethylene forming enzyme CpACS27A regulates early ethylene production in the pistillate floral bud, which arrests stamen development and leads to the formation of a female flower. This also induces later ethylene production and signaling genes, likely involved in floral organ maturation and fruit set control. The mutant CpACS27A^{S176A} is unable to produce enough early ethylene to arrest stamen development, leading to the formation of bisexual flowers. Later ethylene production and signaling are also reduced, which delay the maturation of floral organs and induce the parthenocarpic development of the fruit

ortholog of *CmACS7* or *CsACS2*. We have isolated two *ACS* genes from *C. pepo*, *CpACS27A* and *CpACS27B*, showing a very high sequence identity and the same molecular structure as *CmACS7* and *CsACS2*. *CpACS27B* seems to be a pseudogene because no transcripts were found in any of the analyzed tissues. However, the expression of *CpACS27A*, as that of *CmACS7* and *CsACS2*, was specific to carpel primordia in early stages of floral development, when the arrest of stamen development must take place. Moreover, the encoded CpACS27A protein shows 89 % identity with melon and cucumber enzymes CmACS7 and CsACS2, and no other protein has been detected with such a high degree of similarity in the proteome of *C. pepo*. Based on these data, we concluded that *CpACS27A* is likely the squash ortholog of *CmACS7* and *CsACS2*, and could be involved in the arrest of stamens during the determination of female flowers (Fig. 8). As also occurs in melon and cucumber, we have found that the partial andromonoecy of squash is associated with a reduction of *CpACS27A* expression in the floral buds which results in the development of bisexual flowers. Although we have detected that other ethylene biosynthesis and signaling genes were also downregulated in the bisexual floral bud, *CpACS27A* was the earliest *ACS* gene to be expressed in the floral meristem, coinciding with the time of stamen arrest and sex determination. The other genes are induced much later in female flowers and are probably involved in the development and maturation of floral organs rather than in sex determination.

We have detected a nucleotide polymorphism between Bog and Veg lines, producing a serine to alanine amino acid substitution at position 176 in Bog *CpACS27A* (*CpACS27A*^{S176A}, or allele *B*). This mutation, which affected a

very conserved motif not only in ACS, but also in ACS-like and ATT proteins (Zhang et al. 2012), has been detected in 5 of the 29 andromonoecious cultivars studied in this paper (Supplemental Table S1), but in none of the stable monoecious cultivars. Moreover, the mutation was present not only in all partially andromonoecious F2 plants derived from the cross Bog × Veg, but also in some of the monoecious ones (Table 2). These data indicate that the Bog haplotype of *CpACS27A* (allele *B*) is necessary to confer andromonoecy in squash (Fig. 8), and could correspond to the temperature-sensitive allele encoded by the andromonoecious dominant *A* locus defined previously. Although the mutation $^{S}176^{A}$ was not detected in all the identified partially andromonoecious cultivars, this variation can be used for the selection of stable monoecious genotypes in squash.

As previously discussed, the other gene responsible for andromonoecy in squash could be a gene that regulates the expression of *CpACS27A*. In cucumber and melon, *CsAC2* and *CmACS7* are regulated by the genes controlling gynoecy: *CsACS1G* in cucumber (Trebitsh et al. 1997) and *CmWIP1* in melon (Martin et al. 2009). Given that we have found no co-segregation between andromonoecy and the delayed flowering and lower proportion of female flowers of *Veg*, it is unlikely that the gene that regulates andromonoecy in squash corresponds to *CpWEAK ETHYLENE INSENSITIVE* (*CpWEI*), the locus that confers maleness to *Veg* while reducing ethylene sensitivity (Manzano et al. 2010a).

Regulation of other ethylene genes and traits associated with partial andromonoecy

Andromonoecy in squash is associated with a delay in the maturation of petals and stigma, as well as with a premature development of the ovary and a parthenocarpic development of the fruit, processes that must be regulated later than the arrest of stamen development in the female flower. In comparison with female flowers, the lack of stamen arrest and the downregulation of *CpACS27* in very early stages of bisexual flower development were found to be associated with the downregulation of other ethylene biosynthesis and signaling genes during later stages of bisexual flower development, and even in the few days after anthesis (Martínez et al. 2013). It is known that ethylene can autoregulate its own production and sensitivity (Nakatsuka et al. 1998; Barry et al. 2000; Liu et al. 2008). A positive feedback regulation mechanism has recently been found for *CsACS2* (Li et al. 2012). It appears therefore that the ethylene produced in the pistil at the earliest stages of flower development by *CpACS27A*, which determines the sexual fate of the bud towards a female flower, may also activate other genes involved in ethylene biosynthesis and signaling during later stages of development (Fig. 8). The reduced expression of *CpACS27A* (or a non-functional allele such as *CpACS27A*^{S176A}) would diminish ethylene production and signaling in the female floral meristem, as we have observed in the ovary of squash bisexual floral buds, inhibiting later developmental processes that depend on ethylene, including maturation and opening of the corolla, the development of the ovary, and the set and early development of the fruit (Martínez et al. 2013). The andromonoecious locus of melon (*a*) also has a pleiotropic effect on the size and shape of melon ovary and fruit (Risser 1984; Périn et al. 2002). The accumulation of *CsACS2* in the developing ovary of cucumber has also suggested that the gene is involved not only in the maintenance of femaleness but also in ovule or ovary development (Saito et al. 2007). In squash, we have found that the ethylene produced by CpACS27A in the pistils can not only promote the development and maturation of the stigma and the corolla, but also inhibit the development of the ovary. During the days immediately after anthesis, reduced ethylene production is also necessary to induce fruit set and early fruit development, while an induction of ethylene production and signaling is observed in the ovary of aborted flowers 3 days after anthesis (Martínez et al. 2013). This effect could be mediated by the regulation of other ethylene biosynthetic and signaling genes which, as we have found in squash, are activated later in the developing ovary (Fig. 8).

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