A Conserved Mutation in an Ethylene Biosynthesis Enzyme Leads to Andromonoecy in Melons

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Supporting Online Material

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A Conserved Mutation in an Ethylene Biosynthesis Enzyme Leads to Andromonoecy in Melons

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Andromonoecy is a widespread sexual system in angiosperms characterized by plants carrying both male and bisexual flowers. In melon, this sexual form is controlled by the identity of the alleles at the *andromonoecious* (*a*) locus. Cloning of the *a* gene reveals that andromonoecy results from a mutation in the active site of 1-aminocyclopropane-1-carboxylic acid synthase. Expression of the active enzyme inhibits the development of the male organs and is not required for carpel development. A causal single-nucleotide polymorphism associated with andromonoecy was identified, which suggests that the *a* allele has been under recent positive selection and may be linked to the evolution of this sexual system.

Most angiosperms have hermaphroditic flowers containing both male and female organs; nevertheless, sex determination can result in the formation of separate male and female flowers on either the same (monoecy) or different individuals (dioecy). Andromonoecy, where plants carry both male and perfect bisexual flowers, has evolved independently numerous times (*1*) and is found in ~4000 species in 33 angiosperm families (*2*). Several species in the Cucurbitaceae, including cucumber (*Cucumis sativus*) and melon (*Cucumis melo*), have bisexual floral primordia, but often have flowers limited to a single sex. Sex determination occurs by the selective arrest of either the male stamen or female carpel during development (*3*, *4*). In melon, sex determination is governed by the genes *andromonoecious* (*a*) and *gynoecious* (*g*), and the interplay of these two genes results in a range of sexual types (*5*, *6*). Monoecious (*A-G-*) and andromonoecious (*aaG-*) individuals bear male flowers on the main stem and, respectively, female or hermaphrodite flowers on axillary branches, whereas gynoecious (*AAgg*) and hermaphrodite (*aagg*) individuals only bear female and hermaphrodite flowers, respectively (*6*). In addition, cucurbit sex expression patterns can be modified by hormones, such as ethylene, and by environmental factors (*7*, *8*).

In *C. melo*, most plants are monoecious or andromonoecious because of the *a* locus, which has been mapped to a genetic interval of 25.2-centimorgans (*9*). We cloned the *a* locus by constructing high-resolution genetic and physical maps (fig. S1A), used chromosome walking to construct a bacterial artificial chromosome (BAC) contig anchored to the genetic map (fig. S1A), delimited the *a* locus to a single BAC clone, and revealed seven candidate genes within 107 kilobase pairs (kbp) (fig. S1B) (*10*). The *a* locus flanking marker sequences L41 and R5 were used to identify a 14-kbp region containing a gene encoding for a 1-aminocyclopropane-1-carboxylic acid synthase (ACS), designated *CmACS-7* on the basis of homology to the *Arabidopsis ACS-7* gene (At4g26200). ACS is a pyridoxal 5′-phosphate (PLP)-dependent enzyme that catalyzes the first committed, and generally rate-limiting, step in the production of 1-aminocyclopropane-1-carboxylic acid (ACC) from *S*-adenosylmethionine (SAM) in ethylene biosynthesis in higher plants (*11*). Ethylene is then made from ACC by the enzyme ACC oxidase.

A TILLING (targeting induced local lesions in genomes) approach confirmed the role of *CmACS-7* in sex determination (*12*). We identified six mutations in the full *CmACS-7* gene; four silent or intronic and two that led to missense mutations at G19E and D376N (*13*). The G19E change occurs in a highly conserved amino acid position and may affect the function of the protein (*14*), whereas the D376N modification affects a non-conserved amino acid position (Fig. 1A). Backcrosses to the wild type showed that, for more than 100 F_2 plants for each cross, that the D376N mutation, as well as the silent and intronic mutations, had no sexual phenotype (Fig. 1B and fig. S2). In contrast, plants homozygous for the G19E mutation were andromonoecious (Fig. 1B and fig. S2). On the basis of these data, we concluded that *CmACS-7* is the *andromonoecious* gene.

A 14-kbp genomic sequence of melon accessions PI124112 and Védrantais was used to map single-nucleotide polymorphisms (SNPs) linked to *CmACS-7*. The minimal interval between allelic variants was localized to a 2438-bp DNA fragment containing 560 bp of the proximal promoter, as well as exons 1 and 2 and a part of exon 3 (fig. S1C). We identified no differences linked to sex phenotype in the proximal promoter sequence and quantitative reverse transcription polymerase chain reaction (RT-PCR) detected no significant

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difference in the expression level between *aa* and *AA* individuals (Fig. 2A). However, we identified a single missense mutation, A57V, in the CmACS-7 protein (Fig. 1A and fig. S1, C to E). A^{57} is conserved across the ACS protein in seed plants (*15*) (Fig. 1A), and crystallographic studies have determined that the backbone nitrogen of A^{57} forms hydrogen bonds with the α-carboxylate group of the enzyme substrate SAM (*16*), which suggests that it may affect protein function (fig. S3).

To determine whether the andromonoecious phenotype is due to a loss of CmACS-7 enzymatic activity, we assayed ACS activity in vitro by monitoring 5′-methylthioadenosine (MTA) formation at different PLP concentrations. In the presence of high concentrations of PLP, both enzymes showed similar activity (table S1, fig. S4) but at a physiological PLP concentration [~1 μM in the flowers (fig. S5)], the V^{57} ACS isoform activity is undetectable, whereas the A^{57} form retains 50% of its maximum activity (fig. S4). We found that, to reach maximum activity, the V^{57} form needs PLP in amounts greater than 100 times that found naturally (Fig. 1C). These data are consistent with the binding constant (K_d) observed upon PLP binding to the enzyme (table S1). Because the interaction between A^{57} and PLP has not previously been demonstrated, the observed decrease in binding affinity may be due to an improper orientation of SAM in the active site—which may be required for the formation of external aldimine between PLP and SAM (*16*) (fig. S3). We also saw that ACS activity was undetectable in the G19E mutant (Fig. 1C). Thus, on the basis of the loss of the enzymatic activity of the forms containing the natural A57V and the induced G19E mutation, we concluded that an active CmACS-7 enzyme is required for the development of female flowers in monoecious lines, whereas a reduction of enzymatic activity results in hermaphrodite flowers in andromonoecious lines.

In melon female flowers, stamen arrest occurs at stage 6 just after the elaboration of carpel primordia (*17*). Quantitative RT-PCR (Fig. 2A) and in situ hybridization demonstrate that *CmACS-7* mRNA is mainly expressed in female and hermaphrodite flowers at stage 4, when flowers are not morphologically distinguishable (Fig. 2, C and D), and at later stages (Fig. 2, F and G). The early accumulation of *CmACS-7* mRNA was strongly localized in the carpel primordia (Fig. 2, C, D, F, and G). In the male flowers of the PI124112 and Védrantais lines, *CmACS-7* mRNA was not detected at any developmental stages (Fig. 2, B and E). Because *CmACS-7* expression level and pattern were not different between female and hermaphrodite flowers (Fig. 2) and because the loss of CmACS-7 activity accounts for the functional variation, we concluded that CmACS-7–mediated ethylene production in the carpel primordia affects the development of the stamina in female flowers but is not required for carpel development.

To assess the level of polymorphism at *CmACS-7*, we sequenced the entire coding region in a panel of 106 *C. melo* accessions, including 37 monoecious (*AAGG*), 3 gynoecious (*AAgg*), 65 andromonoecious (*aaGG*), and 1 hermaphrodite (*aagg*) individuals (table S2). We observed 21 polymorphisms in the noncoding regions and 9 in the coding sequence. Of the nine coding polymorphisms, two produced amino acid changes, the A57V substitution we identified as responsible for the andromonoecious phenotype and a T230S modification that was observed in only one monoecious accession in a region that is variable among seed plants. The A57V substitution in *CmACS-7* produces a diagnostic loss of an Alu 1 restriction site (fig. S6). We analyzed a germplasm collection of 497 accessions of *C. melo* from different parts of the world with an Alu 1 cleaved amplified polymorphic sequence (CAPS) marker (table S2). All monoecious ($n = 146$) and gynoecious ($n = 3$) accessions identified as harboring the dominant allele *A* contained the A^{57}

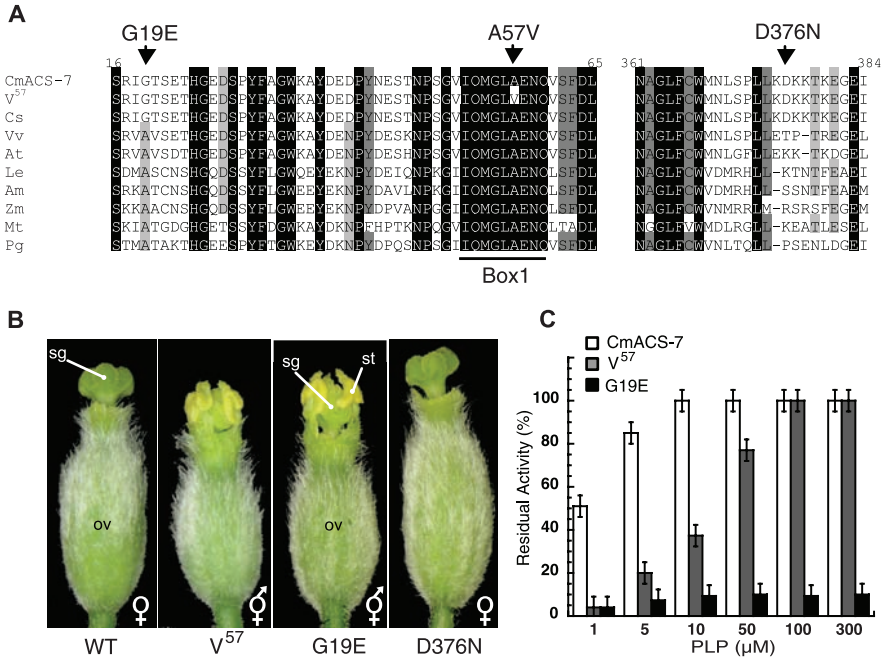

Fig. 1. (**A**) Amino acid alignments of the CmACS-7 and V^{57} isoform with homologous proteins from *Cs* (*Cucumis sativus*, BAF79596), *Vv* (*Vitis vinifera*, CAN66901), *At* (*Arabidopsis thaliana*, AF322390), *Le* (*Lycopersicon esculentum*, AF179247), *Am* (*Antirrhinum majus*, AAC70353), *Zm* (*Zea mays*, AAR25560), *Mt* (*Medicago truncatula*, AAL35745), and *Pg* (*Picea glauca*, ABM60747). Numbers above the alignment indicate the amino acid positions along the CmACS-7 protein. Box 1 indicates a conserved domain in ACS. A57V sequence variation and G19E and D376N EMS-induced mutations are shown above the alignment. (**B**) Flower types from monoecious (WT), andromonoecious (V^{57}), and the G19E and D376N mutants. Male flowers are identical in all the melon types (see SOM). Labels: ov, ovary; sg, stigma; and st, stamen. (**C**) Effect of PLP concentration in the presence of 60 μM SAM on the ACS enzymatic activity of CmACS-7, V^{57}, and G19E protein forms. Residual activities (%) corresponds to the specific activity measured divided by the specific activity measured for CmACS-7 enzyme at 300 μM PLP.

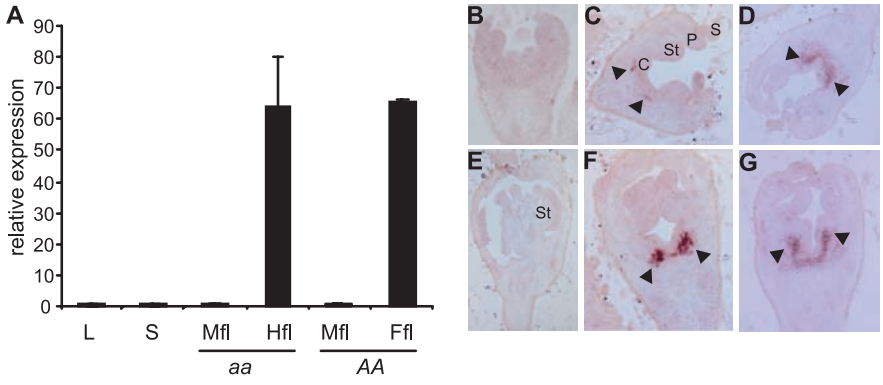

Fig. 2. (**A**) Quantitative RT-PCR of *CmACS-7* in different organs, means ± SD of three biological replicates. L, leaf; S, stem; Mfl, male flower; Hfl, hermaphrodite flower; and Ffl, female flower. Genotypes are indicated in italics below the graph. (**B** to **G**) *CmACS-7* in situ expression at stages 4 (B, C, D) and 7 (E, F, G) of flower development. Male flowers (B and E), hermaphroditic flowers (C and F), and female flowers (D and G). C, carpel; St, stamen; P, petal; and S, sepal.

diagnostic Alu 1 site; all andromonoecious ($n = 347$) and hermaphrodite individuals containing the recessive allele *a* showed results consistent with andromonoecious sex determination via the V^{57} sequence.

To define haplotypes across the entire *CmACS-7* genomic sequence, we used all identified SNPs between the PI124112 and Védrantais lines. Out of the 106 accessions sequenced for *CmACS-7*, a single haplotype, H12, occurred in all the hermaphrodite and andromonoecious accessions, whereas the monoecious and gynoecious accessions contained 11 unique haplotypes (H1 to H11) (Fig. 3). We also compared 1.6 kbp of the promoter region in a subset of 35 melon accessions, but found no differences between hermaphrodite and andromonoecious accessions. In fact, the promoters of the monoecious lines were highly variable. Interestingly, in one monoecious accession, Cantaloup d'Alger, the 1.6-kbp promoter was identical to that of the andromonoecious accessions (fig. S7), which supports the finding that the andromonoecious *CmACS-7* allele is in the coding region.

A maximum parsimony phylogenetic tree created on the basis of the *CmACS-7* gene sequence among accessions indicates that the *a* allele is monophyletic with a single origin derived from the *A* allele (fig. S8). The nucleotide diversity level (π) for this gene in monoecious accessions was 0.0031 (Table 1 and fig. S9A). In contrast, we found no variation in the andromonoecious accessions, consistent with a selective sweep at the *CmACS-7* gene. A haplotype test (*18*) performed under the conservative assumption of no recombination found that the single haplotype within 24 andromonoecious accessions rejected the hypothesis that this site is evolving under neutral evolution ($P < 0.021$). The observed lack of variation at *CmACS-7* was not shared by seven other genes in the melon genome, which suggests that this pattern is not the result of demographic forces. We also conducted a maximum likelihood multilocus HKA test (*19*, *20*) across all six reference genes for which we could obtain the sequence of *C. sativus* ortholog and *CmACS-7*. In this test, a model with selection at *CmACS-7* is significant for andromonoecious accessions compared to a model of neutral evolution across all loci [$P < 0.001$, likelihood ratio test (LRT) = 11.91, df = 1). Finally, the fixation index F_{ST}, a standardized measure of the genetic variance among populations, between andromonoecious and monoecious accessions was highest at *CmACS-7* (mean $F_{ST} = 0.49$) compared with the other genes in the genome (mean $F_{ST} = 0.15$) (Table 1 and fig. S9B). These results are consistent with recent positive selection at the *andromonoecious* allele of the *CmACS-7* gene in *C. melo*.

Linkage between plant hormone synthesis and sex determination also occur in *Mercurialis annua*, where a sex determination gene correlates with the level of zeatin (*21*) and maize, where several mutations affecting sex expression have been identified (*22*). The levels and patterns of polymorphism identified at *CmACS-7* gene are consistent with a recent origin of the andromonoecious allele in this species. These results also suggest that there has been selection for andromonoecy in *C. melo*, which may be due to either selection for resource allocation (*1*) (as males are hypothesized to be less resource intensive than females) or due to the pollen donation hypotheses (*23*) (which suggests that there is a positive correlation between the number of male flowers and male fitness). Both possibilities may be of particular relevance given that *C. melo* is a cultivated species, although greater fruit production in either natural or cultivated species would provide clear selective advantages. Nevertheless, evolutionary ecology theory and experimentation are required to model and assess the selective advantages of andromonoecy in *Cucumis* species, as well as in other plants.

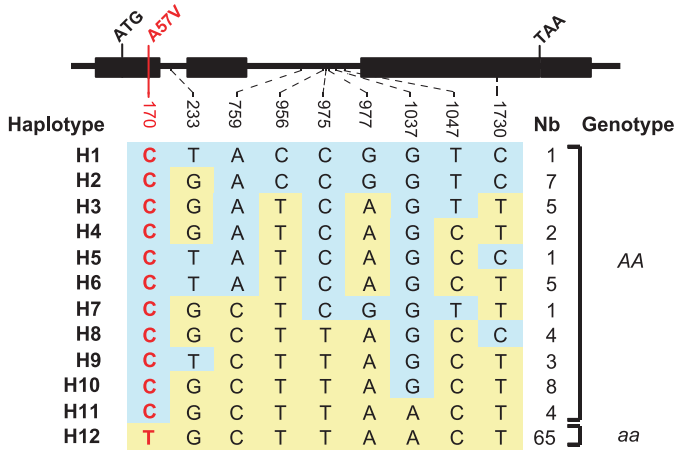

Fig. 3. Haplotypes identified at *CmACS-7*. Each SNP was positioned relative to the first nucleotide of the start codon in PI124112. Nb indicates the number of accessions in each haplotype. Genotype indicates whether the accessions harbor the dominant *A* or the recessive *a* alleles. The SNP at A57V linked with andromonoecy is colored red.

Table 1. Molecular diversity in *CmACS-7* and other melon genes. Nucleotide diversity level (π), Tajima's D, and population differentiation (F_{ST}) levels are indicated. Andro, andromonoecious; mono, monoecious; *Adh1*, alcohol dehydrogenase; *Per-like*, ascorbate peroxidase–like; *Cat2*, catalase 2; *mitoATPg*, mitochondrial ATP synthase g subunit. N.A., not applicable.

Gene	π			Tajima's D			F_{ST}
	Total	Andro	Mono	Total	Andro	Mono	
CmACS-7	0.0023	0.0000	0.0031	−0.9403	n.a.	−0.5937	0.49
Adh-1	0.0027	0.0018	0.0031	−0.1455	−0.4736	−0.281	0.18
Per-like	0.0010	0.0005	0.0014	−0.4764	1.5961	0.0446	0.04
mitoATPg	0.0022	0.0010	0.0027	0.1929	−1.2375	0.3785	0.27
Calmodulin	0.0061	0.0046	0.0069	1.6144	0.5731	1.5624	0.11
Cat2	0.0026	0.0021	0.0027	0.6451	2.3719	0.2689	0.12
Expressed gene	0.0007	0.0002	0.0010	−0.7633	−0.2484	−0.6787	0.32
Shaggy-like	0.0006	0.0003	0.0009	−1.6159	−0.7683	−1.5488	0.00

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13. G19E describes a mutant in which glycine is replaced by glutamic acid at position 19. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Supporting Online Material

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