A cucurbit androecy gene reveals how unisexual flowers develop and dioecy emerges

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Understanding the evolution of sex determination in plants requires identifying the mechanisms underlying the transition from monoecious plants, where male and female flowers coexist, to unisexual individuals found in dioecious species. We show that in melon and cucumber, the androecy gene controls female flower development and encodes a limiting enzyme of ethylene biosynthesis, ACS11. ACS11 is expressed in phloem cells connected to flowers programmed to become female, and ACS11 loss-of-function mutants lead to male plants (androecy). CmAACS11 represses the expression of the male promoting gene CmWIP1 to control the development and the coexistence of male and female flowers in monoecious species. Because monoecy can lead to dioecy, we show how a combination of alleles of CmAACS11 and CmWIP1 can create artificial dioecy.

Most angiosperms are hermaphroditic, producing exclusively bisexual flowers. Sex determination is a developmental process that leads to unisexual flowers in ~10% of angiosperm species (1). About half of these are monoecious species, with male and female flowers on the same plant, and half are dioecious species, with separate male and female individuals (2). The evolutionary pathways from hermaphroditism have been modeled, but empirical evidence is scanty (2–4). Dioecy appears to have evolved most frequently via monoeomy through a series of mutations that alter the ratio of male and female flowers. Dioecy may have also evolved through emergence of unisexual individuals that coexist with hermaphrodites, creating gynodioecy or androdioecy populations (3–6).

Cucurbitaceae is a large plant family of species that display mostly unisexual flowers. Out of some 800 species investigated, 460 are monoecious and 340 are dioecious, and there have been numerous shifts between monoecy and dioecy (7). Several Cucurbitaceae species, including cucumber and melon, show different sexual morphs (fig. S1). In monoecious melon (Cucumis melo), most of the flowers are male, and female flowers develop at the youngest lateral branches of the growing vines. Cloning of naturally occurring mutations leading to transition from monoecy to different sexual morphs has brought insight to the sex-determination pathway (8, 9). The andromonoecious gene CmAACS-7 controls stamens development in female flowers (9). The gynoecious gene CmWIP1 controls male flower development, and its loss of function leads to purely female plants (8). However, it is not known how male and female flowers coexist on the same plant, in monoecious species, and how purely male plants can emerge from monoecious or hermaphroditic populations.

Because solely male plants have not been described in melon, we investigated natural polymorphisms in the related species cucumber (Cucumis sativus) (10). Cucumber is also monoecious, and its sex determination system is conserved with melon (11). Cucumber plants homozygous for the androecious allele (a) bear only male flowers (12).

We cloned the androecious gene (a) in cucumber using a positional cloning strategy (fig. S2) that involves a cross between the monoecious line

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**Fig. 1. Molecular characterization of alleles of ACS11. (A)** Alignments of CsACS11 with homologous proteins from Cme (Cucumis melo), Cma (Momordica charantia), At (Arabidopsis thaliana), Ph (Petunia hybrida), Sl (Solanum lycopersicum), Vv (Vitis vinifera), Pt (Populus trichocarpa), and Pg (Picea glauca). Boxes 1 and 6 indicate conserved domains in ACS proteins. TILLING mutations in CsACS11 and CmAACS11 are shown above the alignment in green and orange, respectively. (B) Cucumber flower types from monoecious plant (WT), androecious (A843), and mutants G39R, W58*, and P437L. (C) Melon flower types from monoecious plant (WT) and mutants G72E, L45F, and S295F. OV, ovary; SG, stigma; ST, stamen. (D and E) ACS enzymatic activity of WT and mutants (D) CsACS11 and (E) CmAACS11 isoforms.
Oman and the androecious line Erez. Sequence analysis of the (a) locus revealed a single nonsynonymous nucleotide deletion, ΔA43, within exon 3 of Csa2G3353460. This 1-base pair (bp) deletion leads to a premature stop codon, suggesting that Erez is null for Csa2G3353460 (fig. S2, C and D). Csa2G3353460 encodes a 1-aminocyclopropane-1-carboxylic acid synthase (ACS), hereafter CsACS11 (fig. S3). ACS catalyzes the rate-limiting step in ethylene biosynthesis, the production of 1-aminocyclopropane-1-carboxylic acid (ACC) from S-adenosylmethionine (SAM). Ethylene is then made from ACC by the ACC oxidase (13). To investigate whether this 1-bp deletion is associated with androecy, we resequenced Cs4CSII in a panel of 50 cucumber accessions of different sexual morphs. In contrast to the 1-bp deletion, none of the observed polymorphisms was associated with androecy, supporting that the 1-bp deletion underlies androecy in cucumber (figs. S4 and S5).

Because there is no efficient cucumber transformation protocol, we produced a TILLING (Targeting Induced Local Lesions in Genomes) collection from a monoecious cucumber line and screened for mutations in CsACS11. We identified 10 induced mutations with seven silent, one nonsense (W58*), and two missense mutations (G39R and P437L) (table S1) (14). G39R occurs in a highly conserved amino acid codon position and is predicted to affect the function of the protein, whereas the P437L modification affects a nonconserved amino acid (fig. 1A and fig. S5). Crosses support that the P437L and silent mutations have no impact on the sex of the plant, whereas plants homozygous for the G39R or W58* mutations were androecious, with no female flowers (fig. 1B and table S1). On the basis of these data, we concluded that CsACS11 is the androecious gene (4).

To test whether the genetic determinant controlling female flower development is conserved in Cucumis, we screened for mutations in the melon ortholog of CsACS11, MEO33C010779, hereafter CmACS11 (fig. S3). CmACS11 and CsACS11 share 92% amino acid sequence identity and are syntenic (figs. S5 and S6). We isolated 10 silent or intronic changes and three missense mutations—L45F, G72E, and S295F—in CmACS11 (fig. 1A and table S1). L45F and S295F mutations are in highly conserved amino acid positions and are predicted to affect the function of the protein, unlike the G72E mutation, which is in a nonconserved amino acid position (table S1 and fig. S5). We back-crossed CmACS11 nonsense mutant lines to the wild type and observed no effect on the sex of the plant correlated with the G72E mutation, nor for silent and intronic mutations (fig. 1C and table S1). In contrast, plants homozygous for L45F or S295F mutations were androecious (fig. 1C). We therefore conclude that CmACS11 controls female flower development in melon and that ACS11 function evolved before the divergence of Cucumis melo and Cucumis sativus, ~10 million years ago (10).

In the parental line Erez, the androecious allele encodes a truncated form of ACS11. In the TILLING screens, all induced mutations leading to androecy are predicted to be loss-of-function (fig. 1 and figs. S5 and S7). We expressed the ACS11 mutant proteins and assayed their activity in vitro (11). As expected, the mutations that lead to androecy—W58*, A4843, L45F, G39R, and S295F—all display low to undetectable ACS activities, whereas mutations not affecting the sex of the plant, G72E and P437L, have activities comparable with those of the wild type (Fig. 1, D and E). We conclude that ACS11-mediated ethylene production is necessary for the development of female flowers in monoecious Cucumis species, whereas loss of ACS activity leads to female-to-male transition. Consistent with this, ACS11 loss-of-function mutants treated with the ethylene-releasing agent Ethephon developed female flowers (fig. S8).

ACS11 mRNA can only be detected in female flower buds from monoecious cucumber and melon plants at stage 4 (fig. 2, A to C and G) (15), in which the bud sex is not morphologically distinguishable, with expression at later stages (fig. 2, D and I). No ACS11 expression was detected in vegetative tissues or male flowers (fig. 2, E and H, and fig. S9). ACS11 mRNA was strongly localized in vascular bundles of female flowers in both internal and external fasicular phloems (fig. 2F and fig. S10) but not in the extrafascicular phloem (16). This strong signal corresponds to the companion cell sieve element complex in the phloem connected to flower buds with a developing carpel (fig. S10, C and E). In the andromonoeious plants—developing male and hermaphrodite flowers—ACS11 is highly expressed in the phloem of hermaphrodite flower buds but not in male buds (fig. S11). This supports that ACS11-mediated ethylene production is required for the development of the carpel in monoecious and andromonoeious Cucumis species.

Expression of CmWIP1 inhibits carpel development, and the nonexpression or loss-of-function of CmWIP1 releases this inhibition (8). Hence, CmACS11 function is antagonistic to CmWIP1 function in flowers programmed to develop carpels. To test the hypothesis that CmACS11 may be the repressor of CmWIP1, we analyzed...
the expression of CmWIP1 in flowers that do or do not express CmAACSII and in flowers impaired in CmAACSII function. CmWIP1 and CmAACSII expression was diametrically opposite, with CmWIP1 highly expressed in male buds but not in female buds that express CmAACSII (Fig. 2B). Expression of CmWIP1 is reactivated in CmAACSII loss-of-function mutants (Fig. 3A). We also generated and phenotyped Cmwip1Cmacs11 double mutants, which were gynoecious like the CmACSII single mutant (Fig. 2B), indicating that CmWIP1 is epistatic to CmAACSII. These data also imply that CmAACSII acts upstream of CmWIP1 in the sex-determination pathway. We thus generated and characterized double and triple CmACSII, CmWIP1, and CmAACS-7 mutants (Fig. 3B). The results support a model in which expression of the carpel inhibitor, CmWIP1, is dependent on nonexpression of CmAACSII, and expression of the stamina inhibitor, CmAACS-7, is dependent on nonexpression of CmWIP1 (Figs. 2B and 3C). In monoecious and andromonoecious plants, male flowers result from nonexpression of CmAACSII, which permits CmWIP1 expression (Figs. 2H and 3C and fig. S11). Female flowers develop on the branches because of expression of CmAACSII that represses the expression of CmWIP1. Consequently, the nonexpression of CmWIP1 releases the expression of CmAACS-7 that inhibits the stamina development (Fig. 3C). If nonfunctional CmAACS-7 is expressed, hermaphrodite, instead of female, flowers develop (Fig. 3C). Androecious plants result from a loss of function of CmAACSII, leading to expression of CmWIP1 in all flowers on a plant. Gynoecious plants are obtained by inactivation of CmWIP1 function, and hermaphrodite plants are obtained by inactivation of CmWIP1 and CmAACS-7 (Fig. 3C).

In angiosperms, dioecy is believed to arise most frequently via monoecy and less frequently from other sexual systems, such as gynodioecy (1, 3). Dioecy is theorized to arise from a minimum of two mutations, in which one results in female (1) and the other in male individuals (2). CmWIP1 and CmAACSII fit a single sex-determination model and can explain the development of unisexual flowers (Fig. 3C). We produced a 1:1 sex ratio segregating dioecious population (G test) (17) by crossing female plants homozygous for the recessive alleles Cmwip1 and Cmacs11 and male plants of Cmwip1/CmACSII and Cmacs11/CmACSII genotype (Fig. 4A). Thus, a combination of alleles of genes controlling monoecy theoretically can result in dioecy (Fig. 4B).

The cloning of the androecy gene and its integration into a genetic model of sex determination indicate the molecular underpinnings of how unisexual flowers coexist and how their relative numbers could be modulated on the same plant in monoecious species, and provide a possible route toward dioecy. The expression of CmACSII in the phloem shows that ethylene is a likely signal to be controlling the sex of flowers on the branches. Nevertheless, it still unknown whether ACSII mRNA, protein, ACC, or ethylene is the signal. Ethylene signaling controls inhibition of stamina development, through expression.

**Fig. 3. Expression and function of CmAACSII, CmWIP1, and CmAACS-7 correlates with sex. (A)** Quantitative RT-PCR of ACSII, ACS-7, and WIP1 in stage 4 male flower buds, collected from the main stem (M45F-MS) or the first three nodes of the lateral branches (L45F-LB), of an androecious (male) Cmacs11/L45F plant. Shown are the mean ± SD of three biological replicates. (B) Male flowers result from nonexpression of CmAACSII, which represses the expression of CmWIP1. (C) Model of the sex-determination pathway in melon integrating CmAACSII, CmWIP1, and CmAACS-7 genetic and functional information.

**Fig. 4. Engineering dioecy from monoecious melon. (A)** Fixation of Cmacs11 loss-of-function alleles at the population level and the maintenance of the dominant allele of CmWIP1 at the heterozygous level in male plants lead to a dioecious mating system. (B) Segregation analysis of two consecutive crosses between male plant of WIP1/wip1 acs11/acs11 genotype and female plant of wip1/wip1 acs11/acs11 genotype shows dioecy. Sex ratio was calculated as females/(females + males). G statistical test (G) is indicated with the level of significance [not significant (ns), P > 0.05].
of CmACS7 as well as the development of the carpel through expression of CmACS5II. This is likely due to a tight control of the kinetics of the production of this hormone during sex determination. Because ethylene seems to be a major hormone in sex determination in angiosperms (28), it is likely that our model of sex determination in a monoecious plant can be used as a framework for investigations of sex determination in other plant families. Furthermore, this work may allow easier breeding and optimization of the synchronization of male and female flower development on the same plant so as to improve fruit yields in nonmodel, cultivated Cucurbitaceae species.

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NONHUMAN GENOMICS

The Symbiodinium kawagutii genome illuminates dinoflagellate gene expression and coral symbiosis

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Dinoflagellates are important components of marine ecosystems and essential coral symbionts, yet little is known about their genomes. We report here on the analysis of a high-quality assembly from the 1180-megabase genome of Symbiodinium kawagutii. We annotated protein-coding genes and identified Symbiodinium-specific gene families. No whole-genome duplication was observed, but instead we found active (retro) transposition and gene family expansion, especially in processes important for successful symbiosis with corals. We also documented genes potentially governing sexual reproduction and cyst formation, novel promoter elements, and a microRNA system potentially regulating gene expression in both symbiont and coral. We found biochemical complementarity between genomes of S. kawagutii and the anthozoan Acropora, indicative of host-symbiont coevolution, providing a resource for studying the molecular basis and evolution of coral symbiosis.

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ingoflagellates are alveolates, with the mostly parasitic apicomplexans as their closest relatives (fig. S1A). Members of the genus Symbiodinium are essential photosynthetic endosymbionts in coral reefs (7). Dinoflagellates show enigmatic genetic and cytological characteristics, including permanently condensed chromosomes and a high proportion of diverse methylated nucleotides, and often feature large nuclear genomes (up to 250 Gb) (2). We report a 0.935-Gbp assembly of the 1.18-Gbp genome of Symbiodinium kawagutii (figs. S1B and S2), a Clade F strain originally isolated from a Hawaiian reef ecosystem (3). A high-quality S. kawagutii genome assembly corresponding to ~80% of the genome was achieved from ~151-Gbp Illumina genome shotgun sequence (~130x genome coverage) (tables S1 to S4 and fig. S3). Genome annotation revealed 36,850 nuclear genes, with ~68% occurring in families (1.69 genes per family) (table S5). Only ~9% (3280) of S. kawagutii genes were in tandem arrays (1279 clusters) (table S6), with 2 to 10 repeats (76% being ≤4 repeats) per array. The genome encodes the common metabolic pathways expected for typical photosynthetic eukaryotes (fig. S4 and table S7), and we found genes involved in sexual reproduction, cyst formation and germination, and telomere synthesis (table S8). The telomeric motif (TTTAGGG)n was identified at the ends of scaffolds and was also detected by fluorescence in situ hybridization (fig. S1B).

Globally, our analysis revealed extensive genomic innovation in dinoflagellates. A total of 20,112 gene families were clustered from the genomes of S. kawagutii and eight other species representing higher plants, chlorophytes, rhodophytes, diatoms, phaeophytes, alveolates, and cnidarians. S. kawagutii has 12,516 gene families, of which 7663 were gained in the ancestor of Symbiodinium (Fig. 1A and table S9). These genes were enriched in metabolic gene ontologies (table S10). When the gene families were normalized to z scores to balance the effect of different total gene numbers, 96 gene families had shrunk (table S11) and 265 gene families had expanded in Symbiodinium (table S12). The LINE-1 reverse transcriptase (a retroelement) is the most highly expanded family.
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