RESEARCH PAPER

The ethylene receptors \textit{CpETR1A} and \textit{CpETR2B} cooperate in the control of sex determination in \textit{Cucurbita pepo}

Alicia García\textsuperscript{1,\textdagger}, Encarnación Aguado\textsuperscript{1}, Cecilia Martínez\textsuperscript{1}, Damian Loska\textsuperscript{2}, Sergi Beltrán\textsuperscript{2}, Juan Luis Valenzuela\textsuperscript{1}, Dolores Garrido\textsuperscript{3}, and Manuel Jamilena\textsuperscript{1,\ast}

\textsuperscript{1} Department of Biology and Geology, Research Centers CIAIMBITAL and CeAiA3, University of Almería, 04120 Almería, Spain
\textsuperscript{2} Centro Nacional de Análisis Genómico (CNAG), 08028 Barcelona, Spain
\textsuperscript{3} Department of Plant Physiology, University of Granada, 18071 Granada, Spain.

\ast Corresponding author: mjamille@ual.es

Received 23 April 2019; Editorial decision 4 September 2019; Accepted 10 September 2019

Editor: Frank Wellmer, Trinity College Dublin, Ireland

Abstract

High-throughput screening of an ethyl methanesulfonate-generated mutant collection of \textit{Cucurbita pepo} using the ethylene triple-response test resulted in the identification of two semi-dominant ethylene-insensitive mutants: \textit{etr1a} and \textit{etr2b}. Both mutations altered sex determination mechanisms, promoting conversion of female into bisexual or hermaphrodite flowers, and monoecy into andromonoecy, thereby delaying the transition to female flowering and reducing the number of pistillate flowers per plant. The mutations also altered the growth rate and maturity of petals and carpels in pistillate flowers, lengthening the time required for flowers to reach anthesis, as well as stimulating the growth rate of ovaries and the parthenocarpic development of fruits. Whole-genome sequencing allowed identification of the causal mutation of the phenotypes as two missense mutations in the coding region of \textit{CpETR1A} and \textit{CpETR2B}, each one corresponding to one of the duplicates of ethylene receptor genes highly homologous to Arabidopsis \textit{ETR1} and \textit{ETR2}. The phenotypes of homozygous and heterozygous single- and double-mutant plants indicated that the two ethylene receptors cooperate in the control of the ethylene response. The level of ethylene insensitivity, which was determined by the strength of each mutant allele and the dose of wild-type and mutant \textit{etr1a} and \textit{etr2b} alleles, correlated with the degree of phenotypic changes in the mutants.

Keywords: \textit{Cucurbita pepo}, ethylene, ethyl methanesulfonate, mutants, sex determination, fruit set.

Introduction

Monoecious and dioecious plant species produce unisexual flowers (male or female) either in the same plant (monoecy) or in separate plants (dioecy). They are believed to be derived from a hermaphrodite ancestor, by different mechanisms, which result in the suppression of either stamen or carpel primordia development during the formation of female or male flowers, respectively (Dellaporta and Calderon-Urrea, 1993; Pannell, 2017). The genetic control mechanisms underlying sex determination in plants are diverse, ranging from heteromorphic sex chromosomes, as occurs in the dioecious species \textit{Silene latifolia} and \textit{Rumex acetosa}, to a number of non-linked genes, as occurs in the monoecious species of the family Cucurbitaceae (Jamilena \textit{et al.}, 2008; Pannell, 2017)

Cultivars of Cucurbitaceae species, including \textit{Cucumis sativus} (cucumber), \textit{Cucumis melo} (melon), \textit{Citrullus lanatus} (watermelon), and the species of the genus \textit{Cucurbita} (pumpkins and
many squashes), are mainly monoecious, although certain cultivars of *C. sativus*, *C. melo*, and *C. lanatus* are andromonoecious, producing male and hermaphrodite flowers on the same plant (Malepszy and Niemirowicz-Szczytt, 1991; Perl-Treves, 2004; Boualem et al., 2009; Manzano et al., 2016). Both monoecious and andromonoecious plants go through two flowering phases of development: an initial phase in which the plant produces only male flowers, and a second phase in which the plant alternates the production of pistillate and male flowers (Perl-Treves 2004; Manzano et al., 2013, 2014; Zhang et al., 2017). The transition to pistillate flowering, and the number of pistillate flowers per plant, vary within the different cultivars of each species. In *Cucumis*, natural genetic variation includes other sexual phenotypes, such as gynoecious (a plant producing only female flowers) and androecious (a plant producing only male flowers). Neither the andromonoecious phenotype nor the gynoecious and androecious phenotypes have been observed in the genus *Cucurbita*, although some cultivars show a partially andromonoecious phenotype characterized by the occurrence of male and bisexual flowers, that is, pistillate flowers with partially developed stamens and no pollen (Martínez et al., 2014).

Sex determination mechanisms in cucurbit species are controlled by ethylene. Under treatments that reduce ethylene biosynthesis or perception, the monoecious plants are converted into partially or completely andromonoecious ones, demonstrating that ethylene participates in the sex identity of female flowers, and that an ethylene threshold is required to arrest stamen development in the female flower (Manzano et al., 2011; Zhang et al., 2017). Besides the control of individual floral buds, ethylene also participates in the control of sex expression within the plant. A reduction in ethylene biosynthesis or perception delays the transition to pistillate flowering and reduces the number of pistillate flowers per plant in *C. sativus*, *C. melo*, and *C. pepo*, but has the opposite effect in *C. lanatus* (Manzano et al., 2011, 2014). By contrast, treatment with ethylene or ethylene-releasing agents induces the production of male flowers in *C. lanatus* but promotes the production of pistillate flowers in the other species (Rudich et al., 1969; Byers et al., 1972; Den Nijs and Visser, 1980).

The genes and mutations responsible for cucurbit sex phenotypes are currently being sought. The arrest of stamen development in the female flowers of the different species requires the functioning of the ethylene biosynthesis orthologs *CmAACS7*, *CsAACS*, *CpACS27A*, and *CitACS4*, which are specifically expressed in the female flowers of *C. melo*, *C. sativus*, *C. pepo*, and *C. lanatus*, respectively. Loss-of-function mutations in these genes led to andromonoecy in *C. melo*, *C. sativus*, and *C. lanatus* (Boualem et al., 2008, 2009; Ji et al., 2016; Manzano et al., 2016), but to only partial andromonoecy in *C. pepo* (Martínez et al., 2014). The androecious and gynoecious phenotypes also resulted from two independent mutations: in *C. melo*, androecy resulted from a mutation in the *CmAACS11* gene (Boualem et al., 2015), while gynoecy was produced by a mutation in the *CmAACS11* gene (Boualem et al., 2009). *CmAACS11* represses the expression of *CmAACS11* to permit the coexistence of male and female flowers in monoecious species (Boualem et al., 2015).

Having performed extensive screening of an ethyl methanesulfonate (EMS)-generated mutant collection of *C. pepo* in the search for ethylene-insensitive mutants (Garcia et al., 2018) in order to gain further insights into the genetic network regulating sex determination in cucurbits, in this paper we present a molecular and functional characterization of two semi-dominant mutations that affect two ethylene receptors of *C. pepo*: *CpETR1A* and *CpETR2B*. These mutations confer ethylene insensitivity on the plant, resulting in the conversion of female flowers to bisexual or hermaphrodite ones; that is, monoecy to andromonoecy. The mutations also alter the development and maturity of different floral organs in pistillate flowers, including ovaries and fruit.

### Materials and methods

#### Plant material

The ethylene-insensitive mutants analyzed in this study were selected from a high-throughput screening of a *C. pepo* mutant collection by the triple-response assay (Garcia et al., 2018), consisting of shortening and thickening of hypocotyls and roots in seedlings germinated in the dark with an external input of ethylene (Bleecker et al., 1988). The *etr1a* and *etr2b* mutants analyzed in this paper correspond to the *ein2* and *ein3* mutants isolated by García et al. (2018).

Before phenotyping, *etr* mutant plants from each family were crossed for two generations with the background genotype MUC16, and the resulting BC2 generation was selfed to obtain the BC2S1 generation. Given that homozygous *etr1a* and *etr2b* mutants were female sterile, they were always derived from selfed progenies of BC heterozygous plants. Sterility also prevented us from obtaining double homozygous mutants. The heterozygous double mutants (wt/*etr1a* wt/*etr2b*) were obtained by crossing heterozygous wt/*etr1a* as female and homozygous or heterozygous wt/*etr2b* as male, and genotyping the offspring for the causative mutations. The double heterozygous plants were also female sterile, preventing us from obtaining the double homozygous *etr1a*/*etr1a* *etr2b*/*etr2b*.

#### Phenotyping for sex expression and sex determination traits

First, BC2S1 or BC2S2 plants from *etr1a* and *etr2b* mutant families were classified according to their level of triple response to ethylene in wild-type (WT), intermediate (wt/*etr*), and ethylene-insensitive mutants (*etr*/*etr*), and then transplanted to a greenhouse and grown to maturity under local greenhouse conditions without climate control, and under standard crop management, in Almería, Spain.

For the ethylene response assay, seeds were germinated for 2 days in the absence of ethylene and then placed in a growth chamber containing 50 ppm ethylene in darkness for 5 days. The identified mutants showed more elongated hypocotyls and roots than WT, resembling seedlings grown in air. The ethylene sensitivity of each mutant genotype was estimated by using three replicates with at least 20 seedlings of the same genotype. Ethylene sensitivity was assessed as the percentage of reduction in hypocotyl length relative to air-grown seedlings: \( \frac{(H_0-H_1)}{H_0} \times 100 \), where \( H_0 \) corresponds to the hypocotyl length of air-grown seedlings, and \( H_1 \) to the hypocotyl length of ethylene-treated seedlings. The final ethylene sensitivity was relativized to the WT seedling response, considering that the WT has 100% ethylene sensitivity, and ethylene insensitivity was calculated as (100–ethylene sensitivity).

The sex phenotype of each plant was determined according to the sex of the flowers in the first 40 nodes of each plant. A minimum of 30 WT, 30 wt/*etr*, and 30 *etr* plants were phenotyped for each mutant family. The sex expression of each genotype was assessed by determining the node at which plants transitioned to pistillate flowering, and the number of male or pistillate flower nodes. The sex phenotype of each individual pistillate flower was assessed by the so-called andromonoecy index (AI) (Martínez et al., 2014; Manzano et al., 2016). Pistillate flowers were separated into three phenotypic classes that were given a score from 1 to 3 according to the degree of their stamen development: female (AI=1), showing no stamen development; bisexual (AI=2), showing partial development of stamens and...
no pollen; and hermaphrodite (AI=3), showing complete development of stamens and pollen. The average AI of each plant and genotype was then assessed from the resulting AI score of at least five individual pistilate flowers from each plant, using a minimum of 30 plants for each genotype. The growth rates of male and female flowers in each of the WT and mutant plants were assessed by measuring the length of ovaries and petals every 3 days in at least 12 flowers of each genotype, starting with flowers ~2 mm in length. The anthesis time was estimated as the number of days taken for a 2 mm pistilate or male floral bud to reach anthesis. The effect of the *etr1a* and *etr2b* mutations on the vegetative vigor of each plant was assessed by determining the average plant height, the total number of nodes, and the average internode length in the main shoot of WT and mutant plants at 60 days after transplantation.

Identification of *etr1a* and *etr2b* mutations by whole-genome sequencing analysis

To identify the causal mutations of the *etr1a* and *etr2b* phenotypes, WT and mutant plants, which were both derived from BC2S1 segregating populations, were subjected to whole-genome sequencing (WGS). In total, 120 BC2S1 seedlings from each mutant family were assessed by using GATK’s HaplotypeCaller in gVCF mode (McKenna et al., 2010), with a minimum of 30 samples for each genotype. Variants were called from ‘good-quality bases’, selected according to the following filter parameters: base quality ≥17, mapping quality ≥20, read depth ≥8. Variants were marked with Picard v1.110. In each sample we counted all the SNPs with a frequency of ≥0.01, with a fold coverage of 5–10. The average fold-effective coverage of all the samples was between 15.5 and 17.2, and the percentage of genomic bases with a fold coverage of ≥20 was between 78.4 and 83.3% (see Table S1). Gene expression analysis was performed by using quantitative RT–PCR. Gene expression analysis was performed by using quantitative RT–PCR. Gene expression analysis was performed by using quantitative RT–PCR. Gene expression analysis was performed by using quantitative RT–PCR. Gene expression analysis was performed by using quantitative RT–PCR. Gene expression analysis was performed by using quantitative RT–PCR. Gene expression analysis was performed by using quantitative RT–PCR.

Bioinformatics and statistical analyses

Alignments were performed using the BLAST alignment tools at NCBI (http://www.blast.ncbi.nlm.nih.gov/) and Clustal Omega at EMBL–EBI (https://www.ebi.ac.uk/Tools/msa/clustalo/). The phylogenetic relations of ETR1, ETR2, and ERS1-like ethylene receptors were studied using MEGA7 software (Kumar et al., 2016), which allowed the alignment of proteins and the construction of phylogenetic trees using the Maximum Likelihood method based on the Poisson correction model (Zuckerand and Pauling, 1965), with 2000 bootstrap replicates. The protein sequences (Supplementary Table S4) were obtained using The Arabidopsis Information Resource (https://www.arabidopsis.org/) and the Cucurbit Genomics Database (http://cucurbitgenomics.org/).

Multiple data comparisons were obtained by analysis of variance with the significance level P<0.05, and each two averages were compared using Fisher’s least significant difference method.

Results

eetr1a and eetr2b are two semi-dominant ethylene-insensitive mutations affecting sex determination

eetr1a and eetr2b are two independent, ethylene-insensitive mutant families that were found after screening a mutant collection of *C. pepo* for ethylene triple response (García et al., 2018). To ensure accurate phenotyping, mutant plants were backcrossed with the background genotype MUC16 for two generations, and then selfed. The resulting BC2S1 or BC3S1 generations segregated for three ethylene-response phenotypes in etiolated seedlings: ethylene-sensitive plants (WT), intermediate plants (wt/etr), and ethylene-insensitive plants (etr) (Fig. 1). The segregation ratio of the three ethylene triple–response phenotypes in BC2S1 and BC3S1 generations indicated that the two ethylene-insensitive mutations were semi-dominant, and that the intermediate phenotype corresponded to heterozygous plants (wt/etr1a or wt/etr2b) (Supplementary Table S5) (García et al., 2018).
Role of ethylene perception in *Cucurbita* sex determination

WT plants responded to ethylene with reduced length of the roots and the hypocotyl, and also increased hypocotyl thickness, in comparison to those grown in air. In contrast, the length of the hypocotyl and roots of ethylene-treated homozygous *etr1a* and heterozygous *etr2b* seedlings was reduced to a lesser extent in relation to those grown in air (Fig. 1); this indicated that the level of insensitivity of the two mutants was not total, as they possessed a partially ethylene-insensitive phenotype. Assuming that WT plants are completely ethylene sensitive (0% ethylene insensitivity), we estimated the percentage of ethylene insensitivity in homozygous and heterozygous seedlings of the two mutants. The homozygous *etr1a* seedlings showed a more severe ethylene-insensitive phenotype than the homozygous *etr2b* seedlings (85.5% ethylene insensitivity for *etr1a/etr1a* versus 63.7% in *etr2b/etr2b*). Heterozygous mutants displayed an intermediate percentage of ethylene insensitivity (51.1% in *wt/etr1a* and 43.5% in *wt/etr2b*) (Fig. 1).

Given that ethylene is the main regulator of sex determination in cucurbits, we assessed whether the *etr1a* and *etr2b* mutations altered the sexual phenotype of the plants. Staminate and pistillate flowers in the first 40 nodes of WT, heterozygous, and homozygous mutant plants were assessed (Supplementary Fig. S1). All plants showed two sexual phases of development. In the first phase, plants produce only male flowers. The second phase starts after the pistillate flowering transition and is characterized by the production of male and pistillate flowers alternately (Fig. 2A). The duration of these two phases of sexual development was affected by the *etr* mutations. Homozygous *etr1a* and *etr2b*

**Fig. 1.** Ethylene triple-response phenotypes of WT and heterozygous and homozygous single and double mutants for *etr1a* and *etr2b*. When grown in air, both the WT and the mutants showed the same growth. When exposed to ethylene, the WT responded with drastic reductions in the length of the hypocotyl and roots, while the mutants had a minor but differential response. The average level of ethylene sensitivity (ES) was assessed as the percentage of reduction in hypocotyl length relative to air-grown seedlings, and assuming that WT is 100% ethylene sensitive. The percentage ethylene insensitivity was then calculated as (100–ES). Assessments were performed in three replicates with at least 20 seedlings for each genotype.

**Fig. 2.** Effect of *etr1a* and *etr2b* on sex expression. (A) Schematic representation of the distribution of male and female flowers in WT and *etr1a* or *etr2b* (*etr*) mutant plants. The male and female phases of development, and the node at which plants start to produce pistillate flowers (pistillate flowering transition), are indicated. Blue = male flower; red = female flower; yellow = bisexual or hermaphrodite flower. (B) Comparison of pistillate flowering transition in WT plants and single and double mutants. (C) Comparison of the number of pistillate flowers per plant in WT and single and double mutant plants. Error bars represent SE. Different letters in (B) and (C) indicate statistically significant differences (P<0.05) between samples.
mutants showed a significant delay in pistillate flowering transition, as well as a reduction in the number of pistillate flowers per plant (Fig. 2B, C). Heterozygous etr1a and etr2b mutants showed an intermediate phenotype for both traits (Fig. 2B, C), confirming the semi-dominant nature of the two mutations.

The most evident phenotypic alteration in the etr1a and etr2b mutants was the conversion of monoecy into partial or complete andromonoecy. WT plants produced exclusively female flowers after the pistillate flowering transition, indicating a complete arrest of stamen development in pistillate flowers (Fig. 3A). In the homozygous etr1a mutants, almost all female flowers were transformed into hermaphrodite flowers, showing fully developed stamens with fertile pollen (Fig. 3B). In etr2b mutants, a high percentage of female flowers were transformed into bisexual (pistillate flowers with immature stamens) or hermaphrodite flowers; however, a small number of female flowers remained (pistillate flowers with immature stamens) or hermaphrodite flowers in WT plants and etr1a and etr2b mutants (Fig. 3B), indicating that with regard to the sex of the flower, etr1a and etr2b mutations are recessive, and therefore not semi-dominant as shown for ethylene triple response and sex expression. Given that the degree of stamen development in pistillate flowers was variable, plants were classified according to the AI, ranging from AI=1 (monoecious) to AI=3 (andromonoecious) (Fig. 3A). All ethylene-sensitive WT plants showed an average AI of 1. Homozygous plants containing either etr1a or etr2b mutations displayed an average AI of 2.8 and 1.8, respectively, indicating that the etr1a mutation rendered plants almost completely andromonoecious, while the etr2b mutation rendered them partially andromonoecious. The average AI for heterozygous plants was 1.1 for both etr1a and etr2b types, very similar to that of WT plants (Fig. 3C).

etr1a and etr2b alter petal and ovary/fruit development and affect plant vigor

Table 1 and Fig. 4 show the effects of the etr1a and etr2b mutations on petal and ovary/fruit development. In the hermaphrodite flowers of etr1a, the petal growth rate was reduced and resembled petal development in male flowers. Petal maturity and subsequent anthesis of the flower were delayed (Fig. 4B). Anthesis time, which is the period of time taken for a 2 mm floral bud to reach anthesis and to open, was longer in male WT flowers (average 22 days) than in female WT flowers (average 14 days) (Table 1). Hermaphrodite etr1a flowers took an average of 22 days to reach anthesis (range 15–40 days). Under the greenhouse conditions used, several hermaphrodite flowers did not reach anthesis; the petals remained green and closed for more than 40 days (Fig. 4B). The development to maturity of petals in bisexual flowers of etr2b was also delayed in comparison with WT female flowers. However, the delay was less pronounced than in etr1a (Fig. 4B, Table 1). No alterations in petal development or anthesis time were observed in etr1a and etr2b male flowers, and no change was found in petal development or anthesis time of female flowers of etr1a or etr2b heterozygous plants (Fig. 4B; Table 1).

Significant differences in ovary size were detected between WT and etr1a and etr2b pistillate flowers (Fig. 4). While WT ovaries reached ~8 cm at anthesis and then aborted, the flowers of etr1a and etr2b reached 16–40 cm (or even more) at anthesis (Fig. 4C). These can be considered as parthenocarpic fruits because the

---

**Table 1. Anthesis time of pistillate and male flowers in etr1a and etr2b mutant families**

| Family | Flower | Anthesis time (days) |
|--------|--------|----------------------|
|        |        | wt/wt | etr/etr | etr/etr |
| wt/wt  | Pistillate | 14.3±1.2 b | 14.8±1.4 a | 22.7±1.7 a |
| wt/etr | Male    | 22.4±0.7 a | 23.1±1.8 a | 22.7±1.8 a |
| etr2b  | Pistillate | 13.8±1.3 b | 14.3±1.1 b | 20.4±2.0 a |
| etr2b  | Male    | 22.4±0.5 a | 21.6±1.1 a | 22.7±1.7 a |

Different letters within the same row indicate significant differences between means (P<0.05).
Fig. 4. Effect of etr1a and etr2b mutations on the growth rate of petals and ovaries of male and pistillate flowers. (A) Images of pistillate flowers in WT and either etr1a or etr2b mutants (etr) at 7, 14, 20, or 24 days after the flower’s ovary reached 4 mm in length. The WT pistillate flower reached anthesis at ~15 days, and in the absence of pollination, the floral organs abscised and the fruit aborted 2–3 days after anthesis. In the two etr mutants, the anthesis time was markedly delayed, but the fruit grew normally in the absence of pollination (parthenocarpic fruits). (B) Comparison of the growth rate of WT and mutant petals. Flowers were labeled when their ovaries were 4 mm long, and then measured every 3 days for 25 days. P = pistillate flowers, M = male flowers. Yellow circles indicate the time at which more than 80% of the flowers reached anthesis. (C) Comparison of the growth rate of WT and mutant ovaries/fruits over a period of 25 days. Error bars represent SE.
flowers remained closed, rendering them unavailable for pollination. During the first 16 days, the growth rates of WT and mutant ovaries were similar. After 16 days, WT ovaries aborted, while those of homozygous etr1a and etr2b flowers maintained their growth up to anthesis. Since the anthesis time of etr1a and etr2b pistillate flowers was achieved much later than that of WT plants, the size of the mutant ovary at anthesis was much larger (Fig. 4C). The ovary growth rate of heterozygous wt/etr1a flowers was intermediate between that of wt/wt and homozygous etr1a/etr1a (Fig. 4C), while heterozygous wt/etr2b ovaries displayed the same growth rate as WT plants. Pollination was attempted in homozygous mutant flowers that reached anthesis, but none of the fruits in the homozygous etr1a and etr2b mutant plants were able to set seeds under the given conditions. Since the pollen of these mutants is fertile, these results indicate female sterility associated with the etr1a and etr2b mutations. As no pure seed could be obtained from etr1a or etr2b homozygous mutants, they were maintained by selfing heterozygous mutant plants.

Vegetative development was enhanced in mutant adult etr1a/etr1a and etr2b/etr2b plants. Table 2 shows the differences in plant height, number of nodes, and internode length of plants grown under the same conditions. The two mutations were associated with increased plant growth rate and height compared with WT plants. These differences in height and growth rate were mainly due to an increase in the internode length, which was approximately twice as high in mutants as in WT plants. The number of nodes developed by WT and mutant plants was, however, very similar (Table 2).

**Phenotype of etr1a and etr2b double mutants**

The interaction between the etr1a and etr2b mutations was studied in double-heterozygous plants for both mutations. As mutant bisexual and hermaphrodite flowers were sterile, heterozygous wt/etr1a plants were pollinated with pollen from homozygous etr2b/etr2b plants. The progeny segregated as 1:1 for single-heterozygous wt/wt, wt/etr2b and double-heterozygous wt/etr1a wt/etr2b plants. However, double-heterozygous plants were also female sterile, which made it impossible to generate double-homozygous plants bearing the two mutations. The reciprocal cross produced the same results.

Double-heterozygous mutants showed a more severe ethylene-insensitive phenotype (61.3% insensitivity) than single-heterozygous mutants (51.1% insensitivity for wt/etr1a and 43.5% for wt/etr2b), and their phenotype resembled the triple response of etr2b/etr2b (Fig. 1). The maleness effect of the etr1a and etr2b mutations was also enhanced in the double-heterozygous wt/etr1a wt/etr2b plants compared with the phenotype of the single-heterozygous mutants. The female flowering transition was delayed up to an average of 17.9 nodes, and pistillate flower production was also significantly reduced to about 4.3 flowers resulting from the first 40 nodes of the plant, again resembling the phenotype exhibited by the homozygous single mutants (Fig. 2B, C).

In the double-heterozygous wt/etr1a wt/etr2b plants, most female flowers were converted into bisexual or hermaphrodite flowers (average AI=2.3), contrasting with single-heterozygous plants, which produced nearly 100% female flowers (average AI=1.1) (Fig. 3B, C). Thus, the combination of the two etr mutations, albeit in heterozygous conditions, had a similar effect on the flower sexual phenotype as the homozygous single mutations (Fig. 3B, C). These data therefore indicate that the two mutations have an additive effect on the sex phenotype of pistillate flowers, and that the effect is dependent upon the number of mutant alleles for the two loci.

**Identification of the etr1a and etr2b mutations**

The causal mutations of the etr1a and etr2b phenotypes were identified by WGS. One DNA WT bulk and one DNA mutant bulk were made for each mutant family, each bulk consisting of a pool of DNA from 20 plants from the same BC2S1 segregating population. With regard to the mutant bulk, only the ethylene-insensitive plants that showed the most strongly andromonoecious phenotype were selected, in order to assure that they were homozygous for the mutations. The four DNA bulks were subjected to WGS and the results were mapped against the C. pepo reference genome version 3.2. The identified SNPs were filtered by using different criteria. Common variants between samples of the two families were discarded, as they were considered likely to correspond to spontaneous nucleotide polymorphisms in the MUC16 genetic background (García et al., 2018). SNPs corresponding to canonical EMS mutations (G>T and G>A) were selected, and filtered for their quality and depth, as well as for their mutant allele frequency (AF), in WT and mutant DNA samples. We expected the WT bulks to present the genotype 0/0 (AF=0) and the mutant bulks to present the genotype 1/1 (AF=1).

Assuming minor contamination of the bulks with some heterozygous plants, we discarded SNPs with AF<0.8 in the mutant samples and AF>0.2 in the WT samples. After filtering, two EMS candidate mutations were selected for etr1a and one candidate mutation was selected for etr2b (Supplementary Table S1). The sequences surrounding the candidate mutations (±500 bp) were then used in BLAST searches against the DNA and protein databases at NCBI, which detected an EMS canonical C>T transition in both etr1a and etr2b families, located in the coding region of the ethylene receptor genes CpETR1A and CpETR2B, respectively (Table 3). To verify that these were the causal mutations of the etr1a and etr2b phenotypes, more than 200 plants segregating for either etr1a or etr2b were genotyped for the WT and mutant alleles of CpETR1A and CpETR2B. The results demonstrated a perfect co-segregation of the etr1a and etr2b sex phenotypes with

| Plant height (cm) | Node number | Internode length (mm) |
|-------------------|-------------|-----------------------|
| WT                | 93.9^b      | 47.1^a                | 28.8^a                 |
| etr1a/etr1a       | 112.5^a     | 50.7^a                | 24.1^a                 |
| WT                | 85.43^b     | 47.2^b                | 13.5^b                 |
| etr2b/etr2b       | 113.3^a     | 48.5^a                | 28.8^a                 |

Different letters indicate significant differences between wild-type (WT) and homozygous mutants (P<0.05).
mutations in \( \text{CpETR1A} \) and \( \text{CpETR2B} \), respectively (Table 4). The other candidate mutation for \( \text{etr1a} \) segregated independently of the mutant phenotype.

With regard to the ethylene triple response, the plants that were homozygous for the WT alleles were sensitive to ethylene, and those that were homozygous for the mutant alleles of \( \text{CpETR1A} \) or \( \text{CpETR2B} \) were ethylene insensitive; heterozygous plants showed intermediate triple-response phenotypes (Table 4). Moreover, plants that were homozygous for any of the identified mutations were all andromonoecious, while those that were either homozygous for the WT allele or heterozygous were all monoecious (Table 4). This finding demonstrates that mutations identified in the two ethylene receptor genes \( \text{CpETR1A} \) and \( \text{CpETR2B} \) co-segregated with the ethylene-insensitive phenotype and andromonoecy of the \( \text{etr1a} \) and \( \text{etr2b} \) mutants.

### Gene structure of \( \text{CpETR1} \) and \( \text{CpETR2} \)

The \textit{de novo} assembly of the \( \text{C. pepo} \) genome, published recently, revealed a whole-genome duplication, which occurred just before the speciation event that created the genus \( \text{Cucurbita} \) (Sun \textit{et al.}, 2017; Montero-Pau \textit{et al.}, 2018). Accordingly, we found that the genomes of the \( \text{Cucurbita} \) species contain two paralogs for each of the ethylene receptors \( \text{ETR1} \), \( \text{ERS1} \), and \( \text{ETR2} \). No \( \text{ERS2} \)- or \( \text{EIN4} \)-like receptors were found in the genomes of these species. The \( \text{C. pepo} \) genome has two \( \text{ETR1} \) duplicates (\( \text{CpETR1A} \) and \( \text{CpETR1B} \)), which showed more than 90% homology and mapped on chromosomes 7 and 11, and two \( \text{ETR2} \) duplicates (\( \text{CpETR2A} \) and \( \text{CpETR2B} \)), which showed more than 89% homology and mapped on chromosome 8. The duplicates maintained the same molecular structure: six exons and five introns for the two \( \text{CpETR1} \) paralogs, and three exons and two introns for the two \( \text{CpETR2} \) paralogs (Fig. 5A, B).

The \( \text{etr1a} \) mutation was located at nucleotide position 284 of the first exon of the \( \text{CpETR1A} \) gene, and the \( \text{etr2b} \) mutation was located at nucleotide position 1018 of the first exon of the \( \text{CpETR2B} \) gene (Fig. 5A, B). The deduced \( \text{ETR1A} \) and \( \text{ETR2B} \) receptors had the same domains as those of the Arabidopsis homologs: an ethylene-binding domain with three transmembrane segments; one GAF domain; one histidine-kinase domain; and a response regulator receiver domain. The two mutations resulted in an amino acid substitution in each protein: A95V in the ethylene-binding domain of \( \text{CpETR1A} \), and E340K in the coiled-coil domain between the GAF and histidine-kinase domains of \( \text{CpETR2B} \) (Fig. 5A, B). All \( \text{ETR1} \)- and \( \text{ETR2} \)-like proteins in the NCBI database were found to contain the WT amino acid at these two particular positions, indicating that the amino acids affected by the two mutations are highly conserved in very different plant species (Supplementary Figs S2 and S3).

### Table 3. Genotype, depth (DP), and allele frequency (AF) of causal mutations of the \( \text{etr1a} \) and \( \text{etr2b} \) phenotypes

| DNA bulk | Causal mutations |
|----------|------------------|
|          | LG07: 6 891 436 (C>T) | LG03:11 824 350 (C>T) |
| Genotype | DP | AF | Genotype | DP | AF |
|----------|----|----|----------|----|----|
| \( \text{etr1a} \) WT | 0/0 | 20 | 0.15 | 0/0 | 19 | 0 |
| Mutant | 1/1 | 20 | 1 | 0/0 | 9 | 0 |
| \( \text{etr2b} \) WT | 0/0 | 16 | 0 | 0/0 | 10 | 0 |
| Mutant | 0/0 | 8 | 0 | 1/1 | 14 | 1 |

### Table 4. Co-segregation analysis of the \( \text{CpETR1A} \) and \( \text{CpETR2B} \) mutations with the ethylene-insensitive phenotypes in \( \text{BC2S1} \) populations segregating for \( \text{etr1a} \), \( \text{etr2b} \), and double mutants

| Segregating population | \( \text{CpETR1A} \) or \( \text{CpETR2B} \) genotypes | Triple response to ethylene | Sexual phenotype | Total |
|------------------------|---------------------------------|-----------------------------|-----------------|-------|
|                        | Genotype                         | Sensitive                   | Intermediate    | Insensitive | Monoecious | Andromonoecious |       |
| \( \text{etr1a} \) 0/0 | 80                              | –                           | –               | –           | 80         | 61             | 226  |
| 0/1                   | –                               | 61                          | –               | –           | 61         | 234            |       |
| 1/1                   | –                               | –                           | 85              | –           | 85         | 87             |       |
| \( \text{etr2b} \) 0/0 | 85                              | –                           | –               | –           | 85         | 62             | 234  |
| 0/1                   | –                               | 62                          | –               | –           | 62         | 87             |       |
| 1/1                   | –                               | –                           | 87              | –           | 87         | 47             |       |
| Double mutants 0/0; 0/1 | –                 | 22                          | –               | –           | 22         | 25             | 25   |
| 0/1; 0/1             | –                               | –                           | –               | –           | 25         | 25             |       |
Effects of etr1a and etr2b on ethylene receptor gene expression

The expression patterns of the mutated *CpETR1A* and *CpETR2B* and their duplicated paralogs (*CpETR1B* and *CpETR2A*) were studied in leaves, roots, shoots, and shoot apices of WT plants (Fig. 6). The four genes were expressed in all the analyzed tissues, suggesting that both duplicates maintain their expression in these tissues.

We also investigated whether the *etr1a* or *etr2b* mutations alter the patterns of expression of the four *ETR* genes. Gene expression was compared in WT and mutant female floral buds at very early stages of development, that is, when stamen arrest takes place in the WT female flower (stage T0), and at 1 day before anthesis of WT flowers (stage T5); in the latter case, we separated the flower into the ovary and a tissue comprising the petals, style, and stigma (Fig. 7). The *etr1a* mutation inhibited the expression of *CpETR1A* and *CpETR2A* in the tissue that comprised the petals, style, and stigma of T5 flowers, and also reduced the transcription of *CpETR2B* in T0 flowers (Fig. 7). By contrast, the *etr2b* mutation inhibited the expression of *CpETR1B* and *CpETR2A* in T0 floral buds, and the expression of *CpETR1A* in the ovary of T5 flowers.
Discussion

etra and etrb are two missense mutations in ethylene receptors leading to semi-dominant ethylene insensitivity

Ethylene is perceived by a family of two-component histidine kinase receptors that repress the ethylene signaling cascade in the absence of ethylene but become inactivated upon ethylene binding (Hua and Meyerowitz, 1998). Mutations in ethylene receptor genes fall into two main categories: (i) dominant gain-of-function mutations, conferring ethylene insensitivity, and (ii) recessive loss-of-function mutations that have little effect as single mutations but show a constitutive ethylene response in combination, for example, in double, triple, and quadruple mutants of Arabidopsis. Both etra and etrb of C. pepo correspond to the first type of mutation, since they are semi-dominant and result in plant ethylene insensitivity (Fig. 1).

In Arabidopsis dominant mutants, the ethylene-insensitive phenotypes are caused by single amino acid substitutions in the transmembrane ethylene-binding domain of any of the five ethylene receptors described in this species (Bleecker et al., 1988; Chang et al., 1993; Guzmán and Ecker, 1990; Hua et al., 1995, 1998; Wang et al., 2009). The etra mutation described here is also a missense mutation (A95V), situated in the third transmembrane domain of the N-terminal ethylene-binding site of CpETR1A (Fig. 5), which causes a strong reduction in ethylene sensitivity in etiolated seedlings (Fig. 1). The mutation is contained in a conserved segment of the protein, close to the T94M mutation of Arabidopsis ETR1, which is known to disrupt the ability of the receptor to bind ethylene and to strongly affect ethylene sensitivity (Wang et al., 2006; Resnick et al., 2008). The etrb mutation, however, is a missense mutation (E340K) within the coiled-coil domain between the GAF and histidine-kinase domains of CpETR2B (Fig. 5). Given that this domain does not participate in ethylene binding, it is likely that the ethylene insensitivity of etrb is caused by a lack of transduction of the ethylene signal, as has been suggested for other dominant ethylene-insensitive mutations (Hall et al., 1999). Thus, etra may disrupt the ethylene-binding site, and etrb may alter ethylene signal transduction, but both mutations

![Fig. 7](image-url) Relative expression of CpETR1 and CpETR2 ethylene receptor genes in female flowers of C. pepo. The relative level of each transcript was quantified by qRT–PCR in three independent replicates of each tissue. T0 corresponds to complete female flowers 2 mm in length; T5 corresponds to pre-anthesis-stage female flowers, separated into the ovary and a tissue comprising the petals, style, and stigma (PSS). The comparison of gene expression was performed between homozygous WT and homozygous mutant flowers derived from plants in the same segregating population. Different letters indicate statistically significant differences (P<0.05) between samples.

![Fig. 8](image-url) Model of sex determination in cucurbit species, integrating the function of the ethylene receptors ETR1 and ETR2 of C. pepo with other sex-determining genes identified in C. melo and C. sativus (ACS11, ACS2, WIP1, and ACS2/7). The ethylene biosynthesis enzymes ACS11 and ACS2/7 have different spatiotemporal expression patterns, causing the arrest of carpels or stamens required for a flower to develop as male or female, respectively (left panel). The two biosynthetic pathways are connected by the transcription factor WIP1, which represses the transcription of ACS2/7, and is negatively regulated by the ethylene-producing enzymes ACS11 and ACO2 (Boualem et al., 2015; Che and Zhang, 2019). The ethylene receptors ETR1 and ETR2 should perceive and transmit signaling of the ethylene synthesized by both the ACS11 and ACS2/7 pathways. The inhibition of the ACS2/7 ethylene response releases the arrest of stamens in female flowers, resulting in the production of bisexual or hermaphrodite flowers (andromonoecy). On the other hand, the inhibition of the ACS11 ethylene response can induce WIP1, which enhances the arrest of carpels and so leads to the formation of male flowers. Red and dotted lines in the right panel indicate increased or decreased effects, respectively, produced by the etra and etrb mutations.
should convert the CpETR1A or CpETR2B receptors to a constitutive signaling-on state that represses the ethylene response (Fig. 8). The differing levels of ethylene insensitivity shown by single *etr1a* and *etr2b* mutants of *C. pepo* could indicate that CpETR1A (subfamily I) has a more prominent role in ethylene perception than CpETR2B (subfamily II). In Arabidopsis, both *etr1-1* and *etr1-4* mutants are insensitive to ethylene, but *etr1-2*, *etr2-1*, and *ein4-3* maintain a reduced response to ethylene (Hall et al., 1999).

A whole-genome duplication occurred just before speciation of the genus *Cucurbita*, which explains why only the species of the genus *Cucurbita* have two subgenomes (A and B) (Sun et al., 2009). Nevertheless, given that some of the ETR genes were not down-regulated in all *etr1a* or *etr2b* tissues, it is also likely that reduction in gene expression reflects a differential regulation of the analyzed ethylene receptor genes.

**Mutations in CpETR1A and CpETR2B alter sex determination and expression**

Sex determination in individual floral buds of monoecious and dioecious species is controlled by diverse mechanisms that suppress the development of either stamen or carpel primordia in floral buds that will result in female or male flowers, respectively (Fig. 8; Kater et al., 2001; Bai et al., 2004). In the Cucurbitaceae, the main sex regulator is ethylene, but the genetic network controlling sex determination in these species is still poorly understood. With the exception of WIP1, all major genes controlling sex determination encode key enzymes involved in ethylene biosynthesis (ACS and ACO; Fig. 8). The gene ACS2/ACS7 controls monoeoy, and loss-of-function mutations lead to plants with male and hermaphrodite flowers (andromonoecy) (Boualem et al., 2008; Boualem et al., 2009; Martínez et al., 2014; Ji et al., 2016; Manzano et al., 2016). Mutations in this gene do not affect sexual expression, that is, the ratio between male and female flowers in the plant (Martínez et al., 2014; Manzano et al., 2016). On the other hand, the genes ACS11 and ACO2 are required for the female flower development pathway, and mutations in these genes result in plants with only male flowers (androecy) (Boualem et al., 2015; Chen et al., 2016). Finally, the transcription factor WIP1 is required for male flower development and is negatively regulated by ACS11, and its dysfunction results in plants with only female flowers (gynoecy) (Boualem et al., 2015; Hu et al., 2017).

So far, no ethylene receptor has been positioned in the genetic network controlling sex determination in this group of species (Fig. 8), although there was some evidence indicating their participation. Thus, the transcription level of ETR2 and ERS1 is higher in gynoecious than monoecious apical shoots of *C. sativus* (Yamazaki et al., 2000), and the down-regulation of GsETR1 in the stamens of female cucumber flowers appears to be required for the arrest of stamen development (Wang et al., 2010). Moreover, transgenic *C. melo* plants overexpressing the Arabidopsis ethylene-insensitive allele *etr1-1* are altered in sex determination and sex expression (Little et al., 2007; Switzenberg et al., 2015). Given that *etr1a* and *etr2b* not only disrupt female flower development (converting monoecy into andromonoecy) but also significantly increase the number of male flowers in the plant, it is likely that ETR1 and ETR2 integrate the two ethylene biosynthesis pathways that result in the determination of male and female flowers, perceiving and signaling the ethylene produced by ACS2/7 as well as that produced by ACS11 and ACO2 (Fig. 8).

The mechanisms triggering the sex of each specific floral meristem must be regulated by the level of ethylene sensitivity conferred by ethylene receptors (Fig. 9). In fact, the degree of conversion of female flowers into bisexual and hermaphrodite flowers, the delay in pistil flowering transition, and the increase in the number of male flowers per plant are all correlated with the level of ethylene sensitivity in *etr1a* and *etr2b* single and double mutants. The final level of ethylene sensitivity in the tissue will be affected by the strength of each mutation, but
also by the number of WT and mutant ethylene receptors in the tissue (Hall et al., 1999), and by the cooperation between them for repressing ethylene signaling (Fig. 9).

The ethylene-insensitive mutations also alter the growth rate of petals and carpels, making mutant bisexual and hermaphrodite flowers reach anthesis later than WT female flowers. This delay in anthesis time could be associated with the development of stamens, since the anthesis time of the mutant hermaphrodite flower was similar to that of the male flower. Moreover, the mutant ovary continues to grow as long as the petals remain green and do not reach anthesis (Fig. 4). These data suggest that fruit set in C. pepo is not triggered by pollination, but is a default developmental program, which has a checking point at anthesis. If the flower is pollinated, development of the ovary continues and the fruit sets. In the absence of pollination and fertilization, growth of the ovary is aborted. Fruit set is known to be regulated positively by hormones such as auxins and gibberellins, and negatively by ethylene (Martínez et al., 2013; Shinozaki et al., 2018; Snaider et al., 2018). Therefore, the reduction of ethylene sensitivity in the *etr1a* and *etr2b* mutants could trigger parthenocarpy by delaying flower anthesis and consequently the checking point for fruit set.

**Supplementary data**

Supplementary data are available at *JXB* online.

Table S1. Sequence and coverage statistics.

Table S2. Primers and TaqMan probes used for genotyping *etr1a* and *etr2b* mutations.

Table S3. Primers for qRT–PCR analysis.

Table S4. Proteins used to perform the phylogenetic analysis.

Table S5. Segregation of ethylene-sensitive, -intermediate and -insensitive plants in the offspring of backcrossed (BC 1 and BC2) and selfed (BC1S1 and BC2S1) generations.

Fig. S1. Distribution of staminate and pistillate flowers in the 40 first nodes of the plant.

Fig. S2. Alignment of the *CpETR1A* amino acid sequence with homologous sequences from diverse species.

Fig. S3. Alignment of the *CpETR2B* amino acid sequence with homologous sequences from diverse species.

**Acknowledgements**

This work was supported by grants AGL2014-54598-C2-1-R and AGL2017-82885-C2-1-R, which were funded partly by the European Regional Development Fund and partly by the Spanish Ministry of Science and Innovation, and grant P12-AGR-1423, funded by Junta de Andalucía, Spain.

**Conflict of interest**

The authors state that no conflict of interest exists regarding this publication.

**References**

Bai SL, Peng YB, Cui JX, Gu HT, Xu LY, Li YQ, Xu ZH, Bai SN. 2004. Developmental analyses reveal early arrests of the spore-bearing parts of...
reproductive organs in unisexual flowers of cucumber (Cucumis sativus L.). Planta 220, 230–240.

Binder BM, Bleecker AB. 2003. A model for ethylene receptor function and 1-methylcyclopropene action. Acta Horticulturae 628, 177–187.

Bleecker AB, Estelle MA, Somerville C, Kende H. 1988. Insensitivity to ethylene conferred by a dominant mutation in Arabidopsis thaliana. Science 241, 1096–1098.

Boualem A, Fergany M, Fernandez R, et al. 2008. A conserved mutation in an ethylene biosynthesis enzyme leads to andromonoecy in melons. Science 321, 836–838.

Boualem A, Troade C, Campos C, et al. 2015. A curcubit androecy gene reveals how unisexual flowers develop and dioecy emerges. Science 350, 688–691.

Boualem A, Troade C, Kovalski I, Sari MA, Perle-Treves R, Bendahmane A. 2009. A conserved ethylene biosynthesis enzyme leads to andromonoecy in two Cucumis species. PLoS ONE 4, e6144.

Byers RE, Baker LR, Sell HM, Herner RC, Dilley DR. 1972. Ethylene: a natural regulator of sex expression of Cucumis melo L. Proceedings of the National Academy of Sciences, USA 69, 717–20.

Chang C, Kwok SF, Bleecker AB, Meyerowitz EM. 1993. Arabidopsis ethylene-response gene ETR1: similarity of product to two-component regulators. Science 262, 539–544.

Che G, Zhang X. 2019. Molecular basis of cucumber fruit domestication. Current Opinion in Plant Biology 47, 38–46.

Chen H, Sun J, Li S, et al. 2016. An ACC oxidase gene essential for cucumber carpel development. Molecular Plant 9, 1315–1327.

Chen YF, Shackle SN, Bowers J, Zhao XC, Etheridge N, Schaller GE. 2007. Ligand-induced degradation of the ethylene receptor ETR2 through a proteasome-dependent pathway in Arabidopsis. Journal of Biological Chemistry 282, 24752–24758.

Dellaporta SL, Calderon-Urrea A. 1993. Sex determination in flowering plants. The Plant Cell 5, 1241–1251.

Den Nijs APM, Visser DL. 1980. Induction of male flowering in gynoecious cucumbers (Cucumis sativus L.) by silver ions. Euphytica 29, 273–280.

Gao Z, Schaller GE. 2009. The role of receptor interactions in regulating ethylene signal transduction. Plant Signaling & Behavior 4, 261–271.

Gao Z, Wen CK, Binder BM, Chen YF, Chang J, Chiang YH, Kerris RJ. 2008. Heteromeric interactions among ethylene receptors mediate signaling in Arabidopsis. Journal of Biological Chemistry 283, 23801–23810.

Garcia A, Aguado E, Parra G, et al. 2018. Phenomic and genomic characterization of a mutant platform in Cucurbita pepo. Frontiers in Plant Science 9, 1049.

Grefen C, Städele K, Růžicka K, Obrdlik P, Harter K, Horák J. 2008. Subcellular localization and in vivo interactions of the Arabidopsis thaliana ethylene receptor family members. Molecular Plant 1, 308–320.

Guzmán P, Ecker JR. 1990. Exploiting the triple response of Arabidopsis to identify ethylene-related mutants. The Plant Cell 2, 513–523.

Hall AE, Chen QG, Findell JL, Schaller GE, Bleecker AB. 1999. The relationship between ethylene binding and dominant insensitivity conferred by mutant forms of the ETR1 ethylene receptor. Plant Physiology 121, 291–300.

Harkey AF, Watkins JM, Olex AL, DiNapoli KT, Lewis DR, Fetrow JS, Binder BM, Muday GK. 2018. Identification of transcriptional and receptor networks that control root responses to ethylene. Plant Physiology 176, 2005–2118.

Hu B, Li D, Liu X, Qi J, Gao D, Zhao S, Huang S, Sun J, Yang L. 2017. Engineering non-transgenic gynoecious cucumber using an improved transformation protocol and optimized CRISPR/Cas9 system. Molecular Plant 10, 1575–1578.

Hua J, Chang C, Sun Q, Meyerowitz EM. 1995. Ethylene insensitivity conferred by Arabidopsis ERS gene. Science 269, 1712–1714.

Hua J, Meyerowitz EM. 1998. Ethylene responses are negatively regulated by a receptor gene family in Arabidopsis thaliana. Cell 94, 261–271.

Hua J, Sakai H, Nourizadeh S, Chen QG, Bleecker AB, Ecker JR, Meyerowitz EM. 1998. EIN4 and ERS2 are members of the putative ethylene receptor gene family in Arabidopsis. The Plant Cell 10, 1321–1332.

Jamilena M, Mariotti B, Manzano S. 2008. Plant sex chromosomes: molecular structure and function. Cytogenetic and Genome Research 120, 265–266.

Ji G, Zhang J, Zhang H, et al. 2016. Mutation in the gene encoding 1-aminocyclopropane-1-carboxylic synthase 4 (CitACS4) led to andromonoecy in watermelon. Journal of Integrative Plant Biology 58, 762–765.

Kater MM, Franken J, Carney KJ, Colombo L, Angenent GC. 2001. Sex determination in the monocious species cucumber is confined to specific floral whorls. The Plant Cell 13, 481–493.

Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33, 1870–1874.

Li Z, Huang S, Liu S, et al. 2009. Molecular isolation of the M gene suggests that a conserved-residue conversion induces the formation of bisexual flowers in cucumber plants. Genetics 182, 1381–1385.

Little HA, Papadopoulou E, Hammar SA, Grumet R. 2007. The influence of ethylene perception on sex expression in melon (Cucumis melo L.) as assessed by expression of the mutant ethylene receptor, At-etr1-1, under the control of constitutive and floral targeted promoters. Sexual Plant Reproduction 20, 123–136.

Malepszy S, Niemirowicz-Szczyt K. 1991. Sex determination in cucumber (Cucumis sativus) as a model system for molecular biology. Plant Science 80, 39–47.

Manzano S, Aguado E, Martínez C, Megías Z, García A, Jamilena M. 2016. The ethylene biosynthesis gene CitACS4 regulates monoecy/ andromonoecy in watermelon (Citrullus lanatus). PLOS ONE 11, e0154362.

Manzano S, Martínez C, García JM, Megías Z, Jamilena M. 2014. Involvement of ethylene in sex expression and female flower development in watermelon (Citrullus lanatus). Plant Physiology and Biochemistry 85, 96–104.

Manzano S, Martínez C, Megías Z, Gómez P, Garrido D, Jamilena M. 2011. The role of ethylene and brassinosteroids in the control of sex expression and flower development in Cucurbita pepo. Plant Growth Regulation 65, 213–221.

Marco-Sola S, Sammeth M, Guigó R, Ribeca P. 2012. The GEM mapper: fast, accurate and versatile alignment by filtration. Nature Methods 9, 1185–1188.

Martin A, Troade C, Boualem A, Rajab M, Fernandez R, Morin H, Pitrat M, Dogimont C, Bendahmane A. 2009. A transposon-induced epigenetic change leads to sex determination in melon. Nature 461, 1135–1139.

Martínez C, Manzano S, Megías Z, Barrera A, Boualem A, Garrido D, Bendahmane A, Jamilena M. 2014. Molecular and functional characterization of CppCSC27A gene reveals its involvement in monoecy instability, and other associated traits in squash (Cucurbita pepo L.). Planta 239, 1201–1215.

Martínez C, Manzano S, Megías Z, Garrido D, Picó B, Jamilena M. 2013. Involvement of ethylene biosynthesis and signalling in fruit set and early fruit development in zucchini squash (Cucurbita pepo L.). BMC Plant Biology 13, 139.

McKenna A, Hanna M, Banks E, et al. 2010. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Research 20, 1297–1303.

Montero-Pau J, Blanca J, Bombarely A, et al. 2018. De novo assembly of the zucchini genome reveals a whole-genome duplication associated with the origin of the Cucurbita genus. Plant Biotechnology Journal 16, 1161–1171.

Pannell JR. 2017. Plant sex determination. Current Biology 27, R191–R197.

Perle-Treves R. 2004. Male to female conversion along the cucumber shoot: approaches to studying sex genes and floral development in Cucumis sativus. In: Ainsworth CC, ed. Sex determination in plants. London: Garland Science, 193–221.

Resnick JS, Rivasola M, Chang C. 2008. Involvement of RTEL1 in conformational changes promoting ETR1 ethylene receptor signaling in Arabidopsis. The Plant Journal 56, 423–431.

Rudich J, Haley AH, Kedar N. 1969. Increase in femaleness of three cucurbits by treatment with Ethrel, an ethylene releasing compound. Planta 69, 68–76.
Role of ethylene perception in Cucurbita sex determination

Shinozaki Y, Nicolas P, Fernandez-Pozo N, et al. 2018. High-resolution spatiotemporal transcriptome mapping of tomato fruit development and ripening. Nature Communications 9, 364.

Shnaider Y, Mitra D, Miller G, Daniel A, Doniger T, Kuhalskaya A, Scozza F, Fernie AR, Brotman Y, Perl-Treves R. 2018. Cucumber ovaries inhibited by dominant fruit express a dynamic developmental program, distinct from either senescence-determined or fruit-setting ovaries. The Plant Journal 96, 651–669.

Sun H, Wu S, Zhang G, et al. 2017. Karyotype stability and unbiased fractionation in the paleo-allotetraploid Cucurbita genomes. Molecular Plant 10, 1293–1306.

Switzenberg JA, Beaudry RM, Grumet R. 2015. Effect of CRC::etr1-1 transgene expression on ethylene production, sex expression, fruit set and fruit ripening in transgenic melon (Cucumis melo L.). Transgenic Research 24, 497–507.

Tieman DM, Taylor MG, Ciardi JA, Klee HJ. 2000. The tomato ethylene receptors NR and LeETR4 are negative regulators of ethylene response and exhibit functional compensation within a multigene family. Proceedings of the National Academy of Sciences, USA 97, 5663–5668.

Wang W, Esch JJ, Shiu SH, Agula H, Binder BM, Chang C, Patterson SE, Bleecker AB. 2006. Identification of important regions for ethylene binding and signaling in the transmembrane domain of the ETR1 ethylene receptor of Arabidopsis. The Plant Cell 18, 3429–3442.

Wang DH, Li F, Duan QH, Han T, Xu ZH, Bai SN. 2010. Ethylene perception is involved in female cucumber flower development. The Plant Journal 61, 862–872.

Wuriyanghan H, Zhang B, Cao WH, et al. 2009. The ethylene receptor ETR2 delays floral transition and affects starch accumulation in rice. The Plant Cell 21, 1473–1494.

Xie F, Liu Q, Wen CK. 2006. Receptor signal output mediated by the ETR1 N terminus is primarily subfamily I receptor dependent. Plant Physiology 142, 492–508.

Yamasaki S, Fujii N, Takahashi H. 2000. The ethylene-regulated expression of Cs-ETR2 and Cs-ERS genes in cucumber plants and their possible involvement with sex expression in flowers. Plant and Cell Physiology 41, 608–616.

Zhang J, Shi J, Ji G, Zhang H, Gong G, Guo S, Ren Y, Fan J, Tian S, Xu Y. 2017. Modulation of sex expression in four forms of watermelon by gibberellin, ethephone and silver nitrate. Horticultural Plant Journal 3, 91–100.

Zhao XC, Qu X, Mathews DE, Schaller GE. 2002. Effect of ethylene pathway mutations upon expression of the ethylene receptor ETR1 from Arabidopsis. Plant Physiology 130, 1983–1991.

Zuckerkandl E, Pauling L. 1965. Evolutionary divergence and convergence in proteins. In: Bryson V, Vogel HJ, eds. Evolving genes and proteins. New York: Academic Press, 97–166.