The sex-determining gene *CitACS4* is a pleiotropic regulator of flower and fruit development in watermelon (*Citrullus lanatus*)

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Abstract

In the species of the *Cucurbitaceae* family, the occurrence of separate male and female flowers in the same plant (monoecy) is controlled by an ethylene biosynthesis *ACS* gene, which specifically suppresses the development of stamen in the femaleflower. In watermelon, a mutation of loss of function in *CitACS4* promotes the conversion of female into hermaphrodite flow- ers, and of monoecious into andromonoecious plants. We have studied whether the ethylene produced by CitACS4 enzymecould also be involved in other ethylene-regulated traits, including pistillate flowering transition and the number of female flowers per plant, the development of floral organs other than stamens, as well as fruit and seed set, and fruit development. A linkage analysis approach was performed in three independent F2 populations segregating for the two alleles of the gene(*M*, monoecious; *m*, andromonoecious), and the different traits under study. The *CitACS4m* allele not only cosegregated with andromonoecy, but also with earlier pistillate transition, an increased number of pistillate flowers per plant, and a slower growth and maturation of petals and carpels, which delayed anthesis time in hermaphrodite flowers. The *m* allele was also found to be linked to a reduced fruit set, which was not caused by a deficiency in pollination or fertilization. The gene also affected the longitudinal and transverse growth rates of the ovary and fruit, which means that fruits from andromonoeciousplants (*mm*) were rounder than those from monoecious (*MM*) ones. Taken together, these data indicate that the locus definedby the ethylene biosynthesis and sex-determining gene *CitACS4* acts as a pleiotropic regulator of the complete development of the pistillate flower and the earlier development of the fruit.

Keywords Watermelon · Monoecious · Andromonoecious · *CitACS4* · Fruit set · Fruit shape

Introduction

Watermelon (Citrullus lanatus) is a major horticultural crop worldwide, with a production of over 111 million tons in 2014 (FAOSTAT 2017). Production-related traits, including pollination efficiency and fruit set, are quite dependent on the sexual expression of the cultivar. The flowering pattern of watermelon Citrullus spp. is either monoecious (male and female flowers in the same plant), andromonoecious (male and hermaphrodite flowers in the same plant) or trimonoe- cious (female, hermaphrodite and male flowers in the same plant) (Rudich and Zamski 1985; Ji et al. 2015). Andromo- noecy and trimonoecy are undesirable traits in cucurbits, since hermaphrodite flowers need to be emasculated when acting as female parents in the production of hybrid seed (Prothro et al. 2013), and also because the trait is usually associated with a reduction in fruit set and fruit quality (Monforte et al. 2005; Abdelmohsin and Pitrat 2008; Mar- tínez et al. 2014).

Sex expression and flower development in watermelon are known to be regulated by several environmental factors and phytohormones such as ethylene and gibberellins. External treatments with ethylene and GA3 inhibit the transition from male to female flowering and reduce the production of pis- tillate flowers, while treatments with the ethylene inhibitors AVG promote female flowering transition and increase the number of pistillate flowers per plant (Manzano et al. 2014; Zhang et al. 2017). High temperatures, and the concomitant reduction in ethylene production, are also responsible for the conversion of monoecious into partially andromonoecious plants. Treatments with silver sulphate, an inhibitor of eth- ylene action, also produce a total or partial transformation of female into hermaphrodite flowers (Zhang et al. 2017), indicating that ethylene, as occurs in other cucurbit species, is responsible for the arrest of stamen growth during female flower development (Manzano et al. 2014, 2016). Studies on the inheritance of watermelon sex morphotypes have indi- cated that monoecy is dominant to andromonoecy and con- trolled by a single gene with two alleles (Rosa 1928; Poole and Grimball 1945;

Rudich and Zamski 1985; Salman- Minkov et al. 2008). It has recently been demonstrated that monoecy is actually controlled by a single semi-dominant gene called CitACS4 (Boualem et al. 2016; Manzano et al. 2016; Ji et al. 2016). The gene encodes for a flower-specific ACS enzyme involved in the biosynthesis of the ethylene required for stamen arrest in the female flowers. A single missense mutation in the coding region of this gene pro- duces an amino acid substitution of cysteine to tryptophan in residue 364 of the CitACS4 protein (C364 W), reducing the production of ethylene in pistillate floral buds and pro- moting a complete conversion of female into hermaphro- dite flowers, and therefore of monoecy into andromonoecy (Boualem et al. 2016; Manzano et al. 2016; Ji et al. 2016). The andromonoecious trait in other cucurbit species, includ- ing cucumber, melon and zucchini squash, also results from mutations in the orthologous ethylene biosynthesis genes CmACS7, CsACS2, and CpACS27A, respectively (Boualem et al. 2008, 2009; Martínez et al. 2014).

Besides sex determination, ethylene regulates several developmental processes associated with flower and fruit development. After pollination, the induction of ethylene production in the ovaries and petals appears to be respon- sible for coordinating ovary growth and petal senescence (Larsen et al. 1993; Balbi and Lomax 2003; Wang et al. 2005; Stepnova et al. 2008). Recent studies have shown an interconnection between early ovule abortion and the size of the silique in Arabidopsis ethylene mutants (Carbonell-Bejerano et al. 2011). In squash, Martínez et al. (2013) found that a reduction in ethylene production or signalling in the flower induces fruit set and early fruit development. Similarly, pollination and gibberellin treatments downregu- late ethylene biosynthesis and signalling genes in tomato immediately after fruit set (Pandolfini et al. 2007; Stepnova et al. 2008). Fruit set in watermelon is unstable at low tem- peratures and under cloudy or rainy weather, as the activity of flower-visiting insects is sluggish and the dehiscence of anthers is hindered (Tsukahara 1988). Whether this fruit setis dependent on ethylene is unknown, but there are some data suggesting that fruit set improves in monoecious cul- tivars (those producing more ethylene in the female flower)in comparison with andromonoecious ones (those produc- ing less ethylene in the female flower) (Wechter et al. 2008;Manzano et al. 2014).

Fruit shape is also related to sex expression in the spe- cies of *Cucurbitaceae*, which also suggests the potential involvement of ethylene in this developmental process. In cucumber and melon, the fruits developed from hermaph- rodite flowers on andromonoecious plants are rounder thanthose derived from female flowers (Loy 2006; Abdelmohsinand Pitrat 2008; Sakata et al. 2013; Díaz et al. 2014). In watermelon, Rosa (1928) also reported that andromonoe- cious plants produced fruit that was rounder, and Poole andGrimball (1945) detected a genetic linkage between round fruits and andromonoecy, and between oval-shaped fruits and monoecy.

In the present study, we used watermelon populations that segregate for two alleles of the *CitACS4* gene, and there- fore for monoecy and andromonoecy, to study whether *Cit-ACS4*, and consequently the production of ethylene in the female flower, not only controls sex determination, but is also responsible for the regulation of the following traits: number of male and female flowers per plant, floral organ maturation, fruit and seed set, growth rate and shape of the watermelon ovary and fruit.

Materials and methods

Plant material and growing conditions

Four inbred lines of watermelon (*C. lanatus*), three monoe-cious lines (P84, P85 and P86) and one andromonoecious line (P87), as well as the F2 generations derived from crossesbetween monoecious and andromonoecious lines (P84XP87, P85XP87 and P86XP87), were characterized. The number of phenotyped plants in the parent lines and in the plants genotyped as *MM*, *Mm* and *mm* of the F2 generations is shown in Table S1. Sex determination and sex expression inthe crosses P85XP87 and P86XP87 were previously studiedby Manzano et al. (2016). In this paper, we analysed the sexexpression and sex determination in a new cross between themonoecy unstable line P84 and the andromonoecious line P87, and studied the floral and fruit traits detailed below in the three crosses.

Seeds of the different lines were simultaneously germi- nated in seed trays in both spring/summer and autumn/win-ter seasons, and seedlings transplanted in a greenhouse at theexperimental station of the University of Almería (Spain), and grown under the same standard crop management of the region. Phenotypic evaluations were performed in the spring–summer and autumn–winter seasons of 2014, 2015

and 2016.

Genotyping for *CitACS4* alleles

The F2 seedlings from the three independent crosses (P84XP87, P85XP87 and P86XP87) were genotyped for *CitACS4* alleles before being transplanted to the green- house. The specific primer pairs CitACS4MF/CitACS4gen-R1 or CitACS4S-F/CitACS4M-R, designed to specifically amplify the *M* allele, and CitACS4A-F/CitACS4gen-R1or CitACS4S-F/CitACS4A-R, which only amplified the *m* allele, were used for genotyping. These primers pair resultedin a 253- or 271-bp PCR fragment of the *CitACS4* gene, respectively. Plant DNA was extracted from frozen young leaves using the CTAB method (Manzano et al. 2016), and the PCRs were performed in the GeneAmp PCR System 2700 (Applied Biosystems). PCRs consisted of 35 cycles of30 s at 95 °C, 30 s at 60 °C and 90 s at 72 °C. PCR fragments were resolved in agarose gels at 1% and plants classified in*MM*, *Mm* and *mm*. At least 15 seedlings of each *CitACS4* genotype were transplanted to the greenhouse for phenotyp-ing (Table S1).

Phenotyping for monoecy stability and sexexpression

To assess the level of monoecy in the different inbred linesand populations, the so-called Andromonoecy Index (AI; Martínez et al. 2014) was defined for each flower, plant andpopulation. Pistillate flowers were scored from 1 to 3 accord-ing to their degree of stamen development. Female flowers with no stamen were scored as AI = 1, while hermaphroditeflowers with complete stamens and anthers able to produce pollen were scored as AI = 3. A score of 2 was assigned to bisexual flowers with medium-sized stamens and anthers (Fig. 1). Based on the flower scores, the AI of each plant was assessed as the average AI of at least five pistillate flow-ers. The average AI in inbred lines was estimated from at least 15 plants. Plants with an AI = 1–1.2 were considered monoecious, those with AI = 1.2–2.7 were considered par-tially andromonoecious, and those with AI ≥ 2.7 were phe- notyped as andromonoecious (Manzano et al. 2016).

Sex expression in each plant was assessed by both the number of initial nodes with male flowers before the produc-tion of the first pistillate flower in the main shoot (female flowering transition), and the percentage of pistillate flow- ers per plant in the first 20 nodes of the main shoot. At least 15 plants were phenotyped to assess the AI and the sexual expression of both parental lines, and *MM*, *Mm* and *mm* plants of three F2 populations (Table S1).

Phenotyping for floral and fruit traits

To assess floral organ development, the growth rates of ova-ries and petals of each *CitACS4* genotype were determined by measuring the length of these floral organs every 2 daysuntil anthesis, starting with flower buds of about 2 mm in length.

The evaluation of fruit set and early fruit development was conducted in 15 pistillate flowers for each genotype (*MM*, *Mm* or *mm*) in each of the three analysed F2 popula- tions. Plants were hand-pollinated with fresh pollen of the same plant for a total of 12 consecutive days, when the envi-ronmental conditions were similar, and always at the same time of the day (9:00–10:00 in spring and 10:00–11:00 in fall). Pollination was done on the day of anthesis for both pistillate and male flowers. To prevent flower damage and abortion, the hermaphrodite and bisexual flowers in *Mm* and mm plants were not emasculated before pollination. After hand pollination, the length and diameter of at least 15 ova-ries/fruitlets were measured from anthesis to 14 days post- anthesis (DPA). The ratio between the number of fruits thatcontinued growing and the number of fruits whose growth aborted over this period of time was used to calculate the percentage of fruit set. When the number of abortions was very high, many more flowers were pollinated to reach a minimum of 10 fruits for seed set analyses (Table S2).

The ovary/fruitlet shape (FS) throughout development was assessed by calculating the ratio of fruit length (FL) over maximum fruit diameter (FD) at anthesis, at 14 DPA and in mature fruit (Díaz et al. 2014).

In at least 10 fully mature fruits for each *CitACS4* gen- otype, harvested 60 days after pollination, the number of viable and non-viable seeds in 1/4 of each fruit was assessed,after which the number of seeds

per kilogram of fresh fruit was calculated. The viability of seeds was determined usingthe floatation test. When newly extracted seeds were placedin a container with water, the submerged and floating seedswere classified as viable and non-viable, respectively. We verified that the floating seeds contained no embryo and did not germinate, while the submerged seeds contained embryos and most of them germinated under our conditions.

Fig. 1 Phenotypes of watermelon hermaphrodite, bisexual and female flowers

Evaluation of pollination and fertilization

Pollen–pistil interaction was analysed in female and her- maphrodite flowers of P84, P86 and P87 lines, determin- ing the best fertility period for setting fruits in each *Cit- ACS4* genotype, but also the possible failure associated withreduced fruit set in P87. Pistillate or hermaphrodite flower was hand-pollinated with its own fresh pollen at anthesis and at − 1, − 2, + 1 and + 2 days from anthesis, always at the same time of day. Hermaphrodite flowers from P87 linewere previously emasculated to avoid self-pollination beforescheduled date. Flowers were fixed in FAE (formalin/glacialacetic acid/ethanol 70%, in a ratio 1:2:17 v/v) 24 h after pollination. Fixed flowers were processed as explained in Cuevas et al. (1994) and stained using 0.1% aniline bluein phosphate buffer for observations under fluorescence microscopy (Martin 1959) in a Nikon Labophot epifluores-cence microscope. Pollen adhesion, germination, pollen tube growth, fertilization levels were determined in each flower and the results averaged for each pollination date. Pollen adhesion was estimated by counting the number of pollen grains in three different areas of the stigma (3.8 mm^2 each).Pollen germination was expressed as the ratio between pol-len grains adhered and those germinated forming a pollen tube and penetrating the stigma. Pollen tubes in the style were observed, and an approximate number range was indi-cated: 0–5 (very few pollen tubes), 5–25 (scarce number ofpollen tubes) or > 25 (high number of pollen tubes). In order to estimate fertilization, ovules were extracted from a sectionof the ovary of flowers pollinated at anthesis. Fertilization rates were calculated as the percentage of fertilized ovules. An ovule was considered fertilized if a pollen tube was pre-sent at the micropyle (Fig. 5). The presence of pollen tubes in the ovary was also observed.

Linkage and statistical analysis

As the *CitACS4* gene is involved in ethylene production, wehave studied whether this gene might also regulate other ethylene-regulated processes and traits in flowers and fruits.The expression of each trait was compared in *MM* and *mm* parental lines, as well as in the *MM*, *Mm* and *mm* plantsof the three segregating F2 generations. When differences between parental lines are maintained between *MM* and *mm*genotypes in the F2 generation, we conclude that *CitACS4* is cosegregating with the trait and therefore that the gene islikely involved in its regulation.

For statistical comparison, simple and factorial analyses of variance (ANOVA) at $p < 0.05$ were

performed by the Statistix 8.0 software package, and each two means were compared by Fisher's least significant difference (LSD) test. The Tukey's multiple comparison test was mainly used when the number of samples per comparing group was the same (only in the comparisons of pollination and fertiliza- tion events).

Results

Involvement of the *CitACS4* gene in sexdetermination and sex expression

Four types of flowers can be found in watermelon: female flowers, which develop carpels but no stamens, male flow- ers, developing stamens but no carpels, and hermaphrodite and bisexual flowers which are flowers producing both com-plete carpels and stamens, or complete carpels and partially developed stamens, respectively (Fig. 1). To assess the sex phenotype of watermelon plants, we defined the andromo- noecious index (AI). AI ranges from 1 to 3 and assesses the degree of development of stamens in each pistillate flower and therefore the level of monoecy–andromonoecy per plant and population. Plants and lines with AI = 1–1.19 produced predominantly female flowers and were considered monoe-cious, those with AI = 1.2–2.69 produced female, bisexual and hermaphrodite flowers and were considered partially andromonoecious, and those with AI $\geq$ 2.7 were considered andromonoecious because they produced predominantly hermaphrodite flowers.

Table 1 summarizes AI and other sex-related traits in the four parental lines, as well as in F2 generations derived from crosses between monoecious (P86, P85 and P84) and andromonoecious (P87) lines. The P87 line has a very sta- ble andromonoecy, producing only male and hermaphro- dite flowers with complete stamens and pollen (AI = 3). The monoecious P85 and P86 lines produced predominantly female flowers, although they also produced some bisexual flowers (AI = 1.16). The sexual phenotype of the two F1 hybrids P85XP87 and P86XP87 had an intermediate phe- notype (AI = 1.52 and AI = 1.35) and were therefore clas- sified as partially andromonoecious. The monoecious line P84 showed a higher andromonoecious index (AI = 1.26), suggesting that its monoecy is less stable than that of P85 and P86 lines. The AI of the F1 from P84XP87 (AI = 1.94) was also intermediate, although more biased to andromo- noecy (Table 1). As previously demonstrated for P85 and P86 (Manzano et al. 2016), the monoecy of P84 was also controlled by a single semi-dominant gene (Table S3). Among the 137 phenotyped F2 plants (P84XP87), 31 were monoecious, 81 were partially andromonoecious and 25 were andromonoecious, which fits the segregation ratio 1:2:1($\chi^2 = 5.087$, $p = 0.078$), expected for a single semi-dominant gene controlling the trait (Table S3).

Segregation data from P84XP87 confirmed that *Cit-ACS4* regulates monoecy/andromonoecy in watermelon. Indeed, the *M* and *m* alleles of the gene cosegregated with either monoecious or andromonoecious phenotypes in all analysed F2 plants. Homozygous *MM* and *mm* plants were monoecious and andromonoecious, respectively, while het- erozygous *Mm* plants had a partially andromonoecious phe-notype (Table 1).

A linkage analysis was also performed for two other sex expression traits that are known to be regulated by ethylene: the number of nodes before the production of the first pistil-late flower (pistillate flowering transition) and the number of pistillate flowers per plant (Table 1). For pistillate flow- ering transition, no difference was detected among the four parental lines. For the number of pistillate flowers per plant, however, monoecious lines P85 and P86 showed less number of female flowers than the andromonoecious P87, and in F2 populations derived from crosses between these lines, *MM* plants also produced fewer female flowers than *mm* plants (Table 1).

Involvement of the *CitACS4* in floral organ maturation

The anthesis time was measured as the number of days it takes a floral bud of 2 mm in length to reach anthesis in a minimum of 10 flowers for each *CitACS4* genotype. Female and male flowers in monoecious (*MM*) plants dif- fered in the time to reach complete maturation at anthe-sis, but no statistical difference was found between her- maphrodite and male flowers in andromonoecious plants

Table 1 Comparison of andromonoecious index (AI), pistillate flowering transition and percentage of pistillate flowers per plant in monoeciousand andromonoecious plants from parental, F1 and F2 generations

Generation	*CitACS4* genotype	AI	Sex phenotype	Pistillate flowering transition	Percentage pistil-late flowers/plant
P86	*MM*	1.16 cd	Mono	4.70ab	20.0 ab
P87	*mm*	3a	Andro	5.58a	25.42a
F1	*Mm*	1.35bc	PA	1.56c	19.16b
F2	*MM*	1.11d	Mono	4.65ab	15.00c
	Mm	1.51b	PA	3.83b	16.78bc
	mm	2.76a	Andro	4.20ab	18.33bc
P85	*MM*	1.16c	Mono	4.77ab	13.84c
P87	*mm*	3a	Andro	5.58a	25.42a
F1	*Mm*	1.52b	PA	3.64b	22.05ab
F2	*MM*	1.11c	Mono	5.18ab	16.09c
	Mm	1.67b	PA	4.04ab	16.85c
	mm	2.87a	Andro	4.26ab	20.00b
P84	*MM*	1.26c	Mono	6.78ab	22.78bc
P87	*mm*	3a	Andro	5.58b	25.42ab
F1	*Mm*	1.94b	PA	5.92b	22.92bc
F2	*MM*	1.22c	Mono	8.22a	21.22c
	Mm	1.8b	PA	7.46a	22.54bc
	mm	2.81a	Andro	6.52b	27.58a

The traits were assessed in monoecious (*MM*) and andromonoecious (*mm*) parental lines, and in *MM*, *Mm* and *mm* F2 plants derived from monoecious x andromonoecious crosses. Mono, monoecious; PA, partially andromonoecious; Andro, andromonoecious. Statistical analysis was performed using LSD test ($p \leq 0.05$), and the different letters indicate significant differences between genotypes of the same cross.

(Table 2), suggesting that the presence of stamens delayed the aperture of both male and hermaphrodite flowers.

Given that male and female flowers differ in their production of ethylene (Manzano et al. 2016), we stud-ied whether the ethylene derived from *CitACS4* expres-sion could also regulate anthesis time in pistillate and male flowers. The anthesis time of pistillate flowersin the andromonoecious line P87 was delayed (aver- age = 9.3 days) in comparison with that in the monoecious lines P86 (average = 6.0 days), P85 (average = 6.01 days) and P84 (average = 6.0 days) (Table 2). In the F2 gen- eration of P86XP87 and P85XP87, the hermaphrodite flowers of andromonoecious *mm* plants also delayed anthesis in comparison with female flowers of *MM* plants (Table 2).

Differences in anthesis times were also found in male flowers of the monoecious (P84, P85 and P86) and andromonoecious (P87) lines, but those differences were not maintained among the *MM*, *Mm* and *mm* genotypes in the F2 generations (Table 2), suggesting that *CitACS4* and ethylene could control the maturation time of the pistillate flower, but not that of the male flower.

Involvement of the *CitACS4* gene in ovary and fruitdevelopment

A linkage analysis was performed between the *CitACS4* gene and floral organ size throughout development, includ-ing ovary and fruit. At earlier stages of pistillate flower development, ovary growth rate in *MM*, *Mm* and *mm* flow- ers was very similar (Fig. 2; Table S4). Significant differ- ences in the petal and ovary size were detected, however, after 6 days, when the ovary and the corolla of *MM* flowers in both parental lines and F2 plants were larger than those of *mm* flowers (Fig. 2; Table S4). This higher growth ratein *MM* flowers was maintained up to anthesis, but given that *MM* flowers reached anthesis earlier than *mm* flowers, the size of the ovary at anthesis was smaller in the female flowers of monoecious *MM* plants than in the hermaph- rodite flowers of *mm* plants (Fig. 2, Table S4). The ovary growth rate of heterozygous *Mm* flowers was intermediate with respect to that of the two homozygous genotypes inthe three crosses (Fig. 2). These data demonstrated that the larger ovary size of *mm* flowers at anthesis is not due to a higher growth rate of the organ throughout development, but rather because the full

maturation of floral organs and anthesis is delayed in hermaphrodite flowers.

Table 2 Comparison of anthesis time (days) in pistillate and male flowers of monoecious (*MM*) and andromonoecious (*mm*) plants fromparental and F2 generations

Generation	*CitACS4* genotype	Anthesis time (days)	
		Female flowers	Male flowers
P86	*MM*	6.0d	7.8c*
P87	*mm*	9.3ab	9.8b
F2 (P86XP87)	*MM*	8.4c	11.0a*
	Mm	8.6bc	9.8b*
	mm	9.8a	11.0a
P85	*MM*	6.1c	7.8a*
P87	*mm*	9.3b	9.8b
F2 (P85XP87)	*MM*	7.2c	10.0b*
	Mm	8.9b	10.0b*
	mm	11.7a	11.2b
P84	*MM*	6.0c	8.5d*
P87	*mm*	9.3a	9.8bc
F2 (P84XP87)	*MM*	8.4b	10.4a*
	Mm	9.0ab	9.4c
	mm	9.0ab	10.0ab

The trait was assessed in monoecious (*MM*) and andromonoecious (*mm*) parental lines, and in F2 plants (*MM*, *Mm* and *mm*) derivedfrom monoecious x andromonoecious crosses. Statistical analysis wasperformed using the LSD test ($p \leq 0.05$). Different letters specify sig- nificant differences between genotypes within the same cross; * indi- cates significant differences between male and pistillate flowers of thesame genotype and generation

Immediately after anthesis, pollinated fruits of monoe-cious *MM* lines (P86, P85 and P84) also grew at a higher rate than those of the andromonoecious *mm* line, but these differences were not detected between *MM* and *mm* fruits in the F2 populations of the three crosses (Fig. 3, Table S4). At 14 DPA, *MM* fruits were larger than *mm* fruits, but in the F2 generations significant differences between *MM* and *mm* fruits were only detected in the P84XP87 cross (Fig. 3, Table S4).

We also found a close linkage between the gene andfruit shape, in that *mm* fruits were rounder than *MM* ones. The fruit shape, estimated as the ratio between fruit length and width (FS), did not change between the two experi- mental seasons (Table 3). The P87 line produced round- shaped fruits at anthesis, at 14 DPA and at the maturestage, while the monoecious lines P85 and P86 displayed oval-shaped fruits at anthesis (Fig. 3; Table 3) which became rounded 14 DPA and at maturation (Table 3). Inthe F2 generations derived from P85XP87 and P86XP87, *MM* fruits at anthesis were also more elongated than *mm* fruits, but no significant difference was detected between *MM* and *mm* fruits at 14 DPA or at the mature stage. Fruits of the monoecious line P84, on the other hand, displayed a more elongated shape at anthesis, becoming oval shape at 14 DPA and at maturation, in both experimental seasons (Table 3), and in the F2 from cross P84XP87, *MM* plants produced a more elongated fruit than *mm* plants (Table 3), suggesting that the elongated fruit shape of P84 is linked to *CitACS4*.

Involvement of the *CitACS4* in fruit and seed set

Fruit and seed set were determined by hand-pollinating a minimum of 15 flowers for each *CitACS4* genotype and thenassessing the number of setting fruits and viable seeds in atleast 10 mature fruits. Since emasculation could decrease fruit and seed set, none of the bisexual or hermaphrodite flowers were emasculated before hand pollination.

Fruit set varied between monoecious and andromo- noecious lines under the two studied conditions (spring/ summer and autumn/winter), with P85 and P86 showing significantly higher fruit set than P84 and P87. We have determined whether these differences cosegregated with*CitACS4* alleles in the F2 generations. As expected, no dif- ference was detected between *MM* and *mm* plants in the F2generation

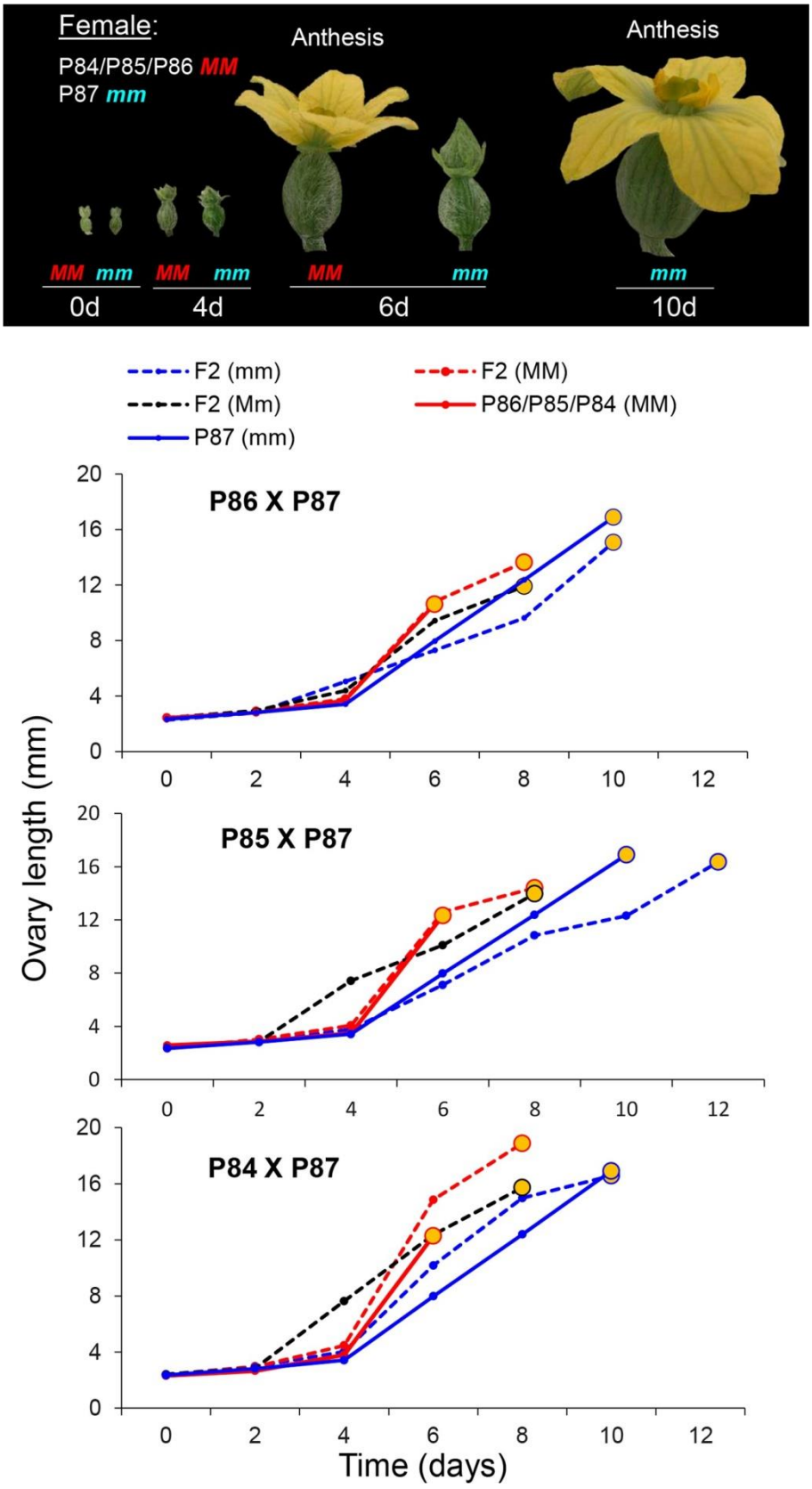

Fig. 2 Ovary growth rate in monoecious MM (P84, P85 and P86) and andromonoecious mm (P87) lines, and in MM, Mm and mm plants of three F2 gen- erations derived from crosses between monoecious and andromonoecious lines. Flowers were labelled when they were 2 mm in length, and ovaries were measured every 2 days until anthesis. Average of 10–15 flowers and fruits for each Cit- ACS4 genotype. Yellow circles show the average anthesis day. Significant differences between genotypes on each sampled day are shown in Table S4

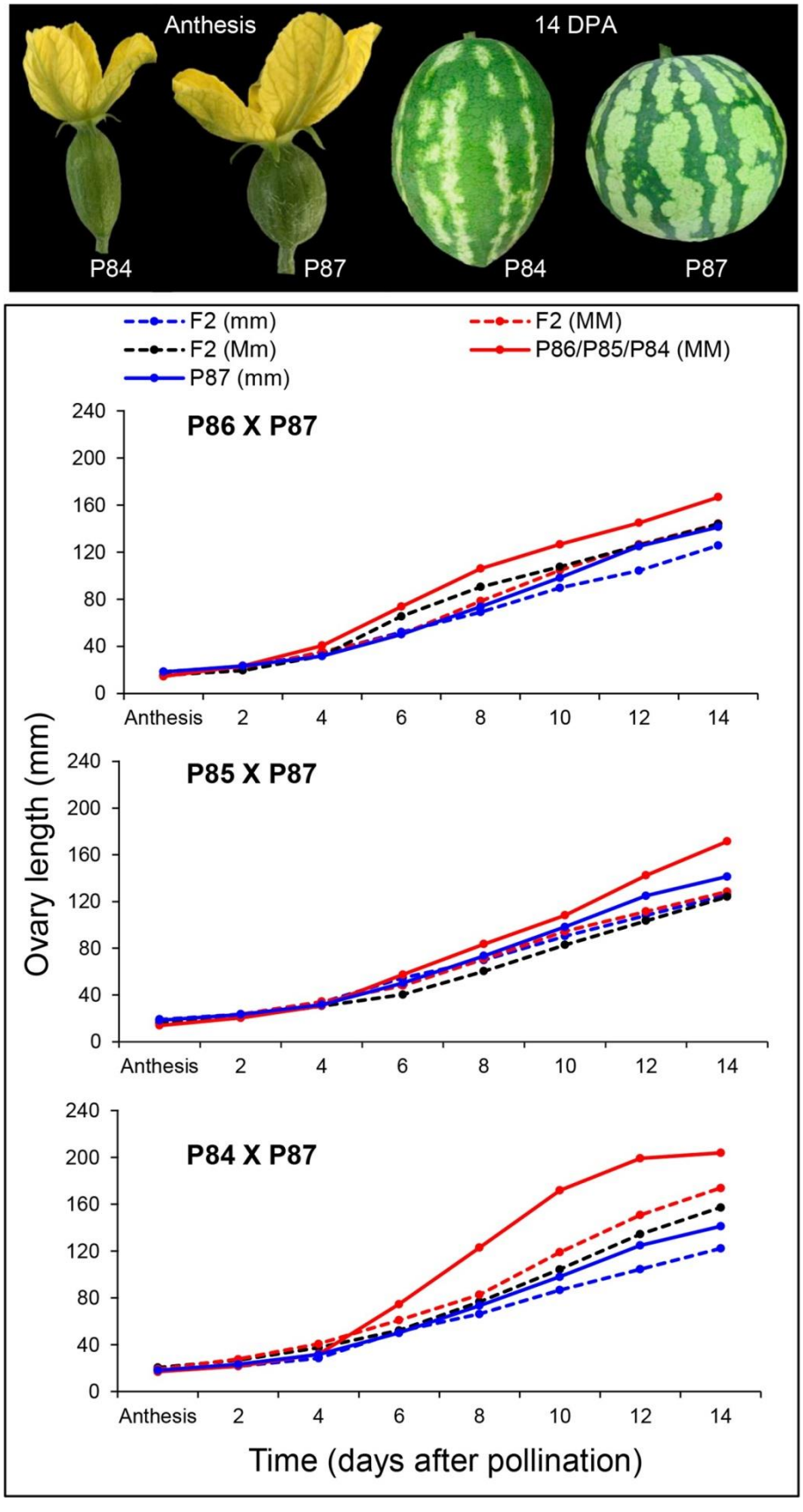

Fig. 3 Fruit growth rate in monoecious MM (P84, P85 and P86) and andromonoecious mm (P87) lines, and in MM, Mm and mm plants of three F2 generations derived from crosses between monoecious and andromonoecious lines. Fruit size was recorded every 2 days from anthesis up to 14 DPA. Average of 10–15 flowers and fruits for each MM, Mm and mm genotypes. Significant differences between genotypes on each sampled day are shown in Table S4.

of the cross P84XP87 (Fig. 4). In the P85XP87 and P86XP87 crosses, however, the higher fruit set of the monoecious parental lines was also observed in the monoe-cious *MM* plants of the segregating F2 generations (Fig. 4).Heterozygous *Mm* plants showed an intermediate percentageof fruit set (Fig. 4).
Table 4 compares the production of viable seeds in *MM*,*Mm* and *mm* fruits in parental lines and F2 generations of plants growing under autumn/winter conditions. The higher number of seeds in the monoecious *MM* lines (P84, P85 andP86) cosegregated with *MM* plants only in the F2 populationof the cross P86XP87 (Table 4). In the crosses P84XP87 and P85XP87, however, both *MM* and *mm* F2 fruits produced avery low number of seeds, displaying no significant differ- ence in seed set (Table 4).

Pollination and fertilization in femaleand hermaphrodite flowers

To investigate the possible factors accounting for the dif- ferences in fruit and seed set between female and hermaph-rodite flowers, we compared pollen–stigma interaction, pollen tube germination and growth, and ovule fertiliza- tion in pistillate flowers of monoecious (P86 and P84) and andromonoecious (P87) lines at anthesis, and at −2, −1, +1and + 2 days post-anthesis (DPA). The results are shown inTable 5 and Fig. 5. In female flowers of P84 and P86, pollenadhesion and germination occur similarly between flowers at different phenological stages of the flowers, reaching a maximum around anthesis. In hermaphrodite flowers of P87,however, pollen adhesion was clearly reduced at −1 and −2DPA, and pollen germination was almost nil in floral buds at − 2 DPA (Table 5). The dynamic of pollen tube growth followed the same trend in female and hermaphrodite flow-ers of P86, P84 and P87, with a maximum number of pollentubes in styles of flowers that were pollinated at anthesis (Table 5; Fig. 5). Nor were any differences found in pol- len tube penetration and fertilization between female and hermaphrodite flowers at any of the floral stages at which they were pollinated (Table 5; Fig. 5). In the ovary of flow-ers pollinated at anthesis and + 1 DPA, pollen tubes were frequently observed close to the ovules. When flowers were pollinated at anthesis, fertilization rates were similar in the three *CitACS4* genotypes, although slightly higher in P87 flowers (Table 5).

Table 3 Fruit shape index (FS) in *MM*, *Mm* and *mm* plants of parental lines and F2 generations

Generation	*CitACS4* Genotype	FS (length/width ratio)					
		Spring/summer			Autumn/winter		
		Anthesis	14dpa	Mature	Anthesis	14dpa	Mature
P86	*MM*	1.46b	1.17a	1.15abc	1.33a	1.16a	1.06a
P87	*mm*	1.26c	1.12a	1.07c	1.22b	1.06b	0.99b
F2 (P86XP87)	*MM*	1.63a	1.15a	1.21a	1.28a	1.16a	1.09a
	Mm	1.41b	1.18a	1.17ab	1.30a	1.13ab	1.15a
	mm	1.26c	1.09a	1.11abc	1.23b	1.13ab	1.05ab
P85	*MM*	1.39a	1.15a	1.13ab	1.30a	1.14b	1.03bc
P87	*mm*	1.26b	1.12a	1.07b	1.22b	1.06c	0.99c
F2 (P85XP87)	*MM*	1.42a	1.18a	1.14ab	1.31a	1.20ab	1.13a
	Mm	1.42a	1.14a	1.19a	1.26ab	1.22a	1.14a
	mm	1.22b	1.10a	1.09ab	1.24ab	1.15ab	1.07ab
P84	*MM*	1.48bc	1.21ab	ND	1.59b	1.35b	1.32b
P87	*mm*	1.26d	1.12c		1.22d	1.06c	0.99c
F2 (P84XP87)	*MM*	1.82a	1.34a		1.82a	1.58a	1.69a
	Mm	1.50b	1.17bc		1.45c	1.45ab	1.31b
	mm	1.31 cd	0.94c		1.17d	1.05c	0.96c

The trait was assessed in parental monoecious (MM) and andromonoecious (mm) lines, and in F2 plants (MM, Mm and mm) derived from monoecious x andromonoecious crosses. FS was calculated as the ratio of fruit length to width. Data are the average of a minimum of 10 fruits per genotype. Statistical analysis was performed using the LSD method ($p \leq 0.05$), and the different letters indicate significant differences between CitACS4 genotypes. ND, non-determined

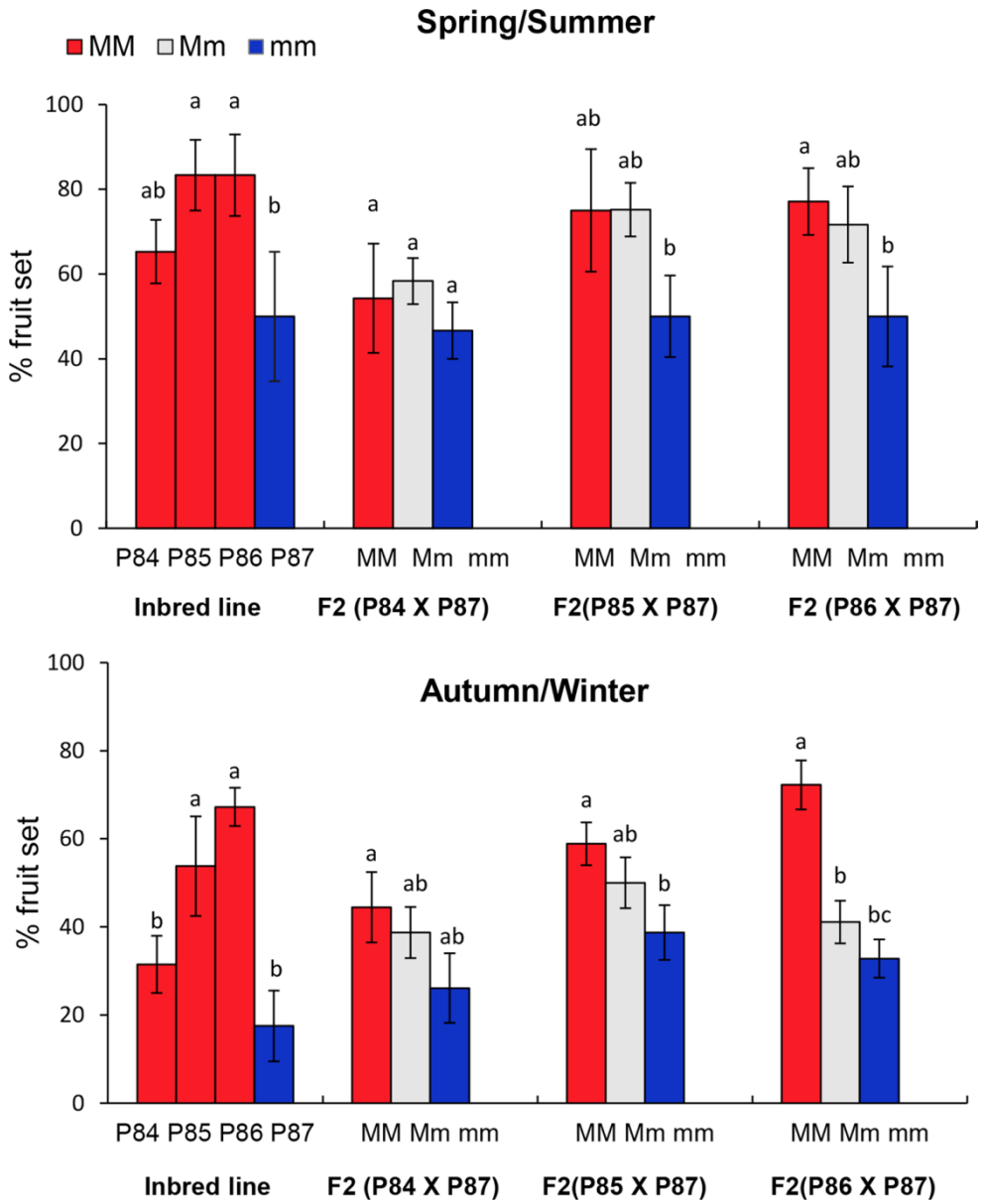

Fig. 4 Percentage of fruit set in monoecious (P84, P85 and P86)and andromonoecious (P87) lines, and in *MM*, *Mm* and *mm* plants of three F2 generations derived from crosses P84XP87,P85XP87 and P86XP87. Bars represent SE of at least 15 fruits. Different letters indicatesignificant differences betweengenotypes ($p \leq 0.05$)

Discussion

In the monoecious species of the *Cucurbitaceae* family,sex determination, i.e. the conversion of a putative her- maphrodite floral meristem into a female or a male flower, is known to be regulated by ethylene (Manzano et al. 2014). The key regulator is an ACS enzyme encoded by the orthologous genes *CmACS7*, *CsACS2*, *CpACS2/7* and *CitACS4* in melon, cucumber, zucchini and watermelon, respectively. These genes are specifically expressed in female floral buds at early stages of development, and theirfunction results in the arrest of stamen development during the formation of the female flower (Boualem et al. 2008, 2009; Li et al. 2009; Martínez et al. 2014; Manzano et al. 2016; Ji et al. 2016). Although sex determination seems to be the main function of these genes, they could also con- trol other developmental processes regulated by ethylene. In this paper, we have studied whether the watermelon

Cit-ACS4 gene could also be involved in sex expression, floral organ development, including petals, ovaries and fruits, as well as in fruit and seed set. Results demonstrated that the ethylene produced in earlier pistillate floral buds is enough to control the entire development of pistillate flowers and fruits.

In *CitACS4* loss of function mutants (*mm*), female flowersare converted into hermaphrodite flowers, and monoecious into andromonoecious plants (Manzano et al. 2016; Boualemet al. 2016; Ji et al. 2016). Crosses indicated that the monoe-cious allele *M* of P84, P85 and P86 is semi-dominant to the andromonoecious allele *m* of P87. The stability of sex determination, however, varied among monoecious lines (*MM*), showing P85 and P86 a more stable monoecy than P84. Consequently, the P84 line not only produced male and female flowers, but also several bisexual flowers with stamens at different developmental stages.

Regulation of sex expression

Sex expression, i.e. the transition from male to femalephases of development (pistillate flowering transition), andthe female-to-male flower ratio are regulated by ethylene inwatermelon and other cucurbit species, although in a com- pletely opposite way. In *Cucumis* and *Cucurbita* species, ethylene promotes femaleness (Rudich 1990; Perl-Treves 1999; Manzano et al. 2011, 2013), while in watermelonit stimulates maleness, delaying female flowering transi- tion and reducing the number of pistillate flowers per plant (Sugimaya et al. 1998; Manzano et al. 2014; Zhang et al. 2017). Our data indicate that the reduced ethylene in *mm* pistillate floral buds could affect the number of pistillate flower per plant but not the pistillate flowering transition. Inthe previous data, we found that sex expression traits were not affected by *CitACS4* gene. The differences between theprevious and current results are likely due to the influence of grafts. In the previous experiments, watermelon plants were grafted on *Cucurbita* rootstocks, which surely altered their sexual expression (Manzano et al. 2014). In the present study, however, none of the plants were grafted, and monoe-cious and andromonoecious plants showed more noticeabledifferences in the number of pistillate and male flowers perplant. Even so, P86 and P87 parental lines showed no dif- ference in sex expression, which suggests the influence of the genetic background or the existence of other genetic fac- tors regulating this trait. Therefore, although sex determina- tion and sex expression are different mechanisms (Manzanoet al. 2014), our present data demonstrate that the ethylene required for sex determination in the pistillate flowers, con-verting the putative hermaphrodite floral buds into female flowers, can influence the number of female flower per plant.Given that sex expression mechanisms should be controlledfrom the apical meristem, it is likely that the ethylene pro- duced in pistillate floral buds (encoded by *CitACS4*) can influence the production and/or the action of ethylene inthe apical shoot, as proposed by Manzano et al. (2013) in zucchini squash.

Coordination of floral organ development

Our data also demonstrate that *CitACS4* is a coordinator of floral organ development, acting as a repressor of stamen development, but also as a promoter of ovary and corolla growth, and consequently of the maturation and aperture ofthe pistillate flower. The growth rates of both petals and ova-ries were significantly higher in female flowers of *MM* plants than in hermaphrodite flowers of *mm* plants. Consequently,flower maturation and anthesis time are delayed in hermaph-rodite flowers in comparison with female ones, which leadsto larger *mm* ovaries than *MM* ones at anthesis, despite theirreduced growth rates. The differences in flower maturation between *mm* and *MM* plants did not affect male flowers, which showed a significantly longer anthesis time thanfemale flowers in *MM* but not in *mm* plants (Table 2). Thesedata strongly suggest that the masculinization of the pis- tillate flower in the andromonoecious *mm* plants decreases the growth rate of the flower and delays its maturation and aperture for about 2 days, in a similar way as occurs in maleflowers. Similar results have been found in zucchini, wherethe delayed anthesis of bisexual and hermaphrodite flowersresulted in ovaries much larger than those of female flowers (Martínez et al. 2013). Since female flowering occurs at later stages of the plant development, it is likely that the accelera-tion of the anthesis in female flowers was a coevolutionary mechanism that ensured pollination during the evolution ofmonoecy in the *Cucurbitaceae* family.

The role of ethylene as a promoter of carpel development, but also as a repressor of stamen development has been found not only in the unisexual flowers of cucurbit

Table 4 Number of viable seeds per kg of fruit pulp in *MM*, *Mm*and *mm* plants of parental lines and F2 generations growing under autumn/winter conditions

Generation	*CitACS4* genotype	Number of seeds
P84	*MM*	53.34a
P87	*Mm*	0b
F2 (P84XP87)	*MM*	0.69b
	Mm	0.59b
	mm	0b
P85	*MM*	50.04a
P87	*Mm*	0b
F2 (P85XP87)	*MM*	12.48b
	Mm	1.35b
	mm	4.12b
P86	*MM*	70.32a
P87	*Mm*	0 c
F2 (P86XP87)	*MM*	50.73ab
	Mm	27.6bc
	mm	10.61c

SE of at least 10 fruits per line and generation. Statistical analysis wasperformed using the LSD method ($p \leq 0.05$), and the different letters indicate statistical differences between genotypes within the same cross.

species (Boualem et al. 2008, 2009; Li et al. 2009; Martínez et al. 2014; Manzano et al. 2016), but also in the hermaphrodite flowers of *Arabidopsis* and other species. Overexpression of the ethylene biosynthesis cucumber gene *CsACO2* represses stamen development in *Arabidopsis* (Duan et al. 2008), while downregulation of the ethylene receptor gene *ETR1* reduces the ethylene signalling repressor CTR1, resulting in the production of female flowers in *Arabidopsis* (Wang et al. 2010). Transgenic tobacco silencing an *ACO* gene has shown female sterility due to an arrest of megasporogenesis (De Martinis et al. 1999). Here we demonstrate that ethylene is also a positive regulator of petal development and matu- ration and therefore of corolla aperture and anthesis time.

Table 5 Pollen–pistil interaction 24 h after pollination at different dates in P84, P86 and P87 lines.

Line	Pollination date (days from anthesis	Pollen adhesion (grains/mm^2)	Pollen germi- nation (%)	Pollen tube number in style	Pollen tubes in ovary and fertilization[2] (%)
P84	−2	10.8b[1]	37.7b	5–25	Not observed
	−1	18.8ab	57.8ab	> 25	Some pollen tubes
	0	22.5a	69.0a	> 25	20 ± 1
	+ 1	11.4b	69.1a	> 25	Many pollen tubes
	+ 2	20.6ab	67.8a	> 25	Many pollen tubes
P86	−2	10.6b	40b	5–25/> 25	Very few pollen tubes
	−1	15.3b	42.8ab	> 25	Few pollen tubes
	0	11.0b	69.5a	> 25	27 ± 1
	+ 1	29.8a	37.8ab	> 25	Many tubes near ovules
	+ 2	17.1b	26.7b	< 25	Some pollen tubes
P87	−2	7.1ab	0.76b	5–25/> 25	Not observed
	−1	5.8b	60.3a	> 25	Few pollen tubes
	0	25.4a	60.2a	> 25	33 ± 8
	+ 1	23.2ab	62.1a	> 25	P.t. near ovules
	+ 2	16.9ab	49.8a	< 25	P.t. near ovules

[1]Mean values ($n = 10$) followed by different letters in each line and column indicate means significant dif-ferences at $p < 0.05$ by Tukey test [2]Fertilization expressed as mean value ± standard error (%)

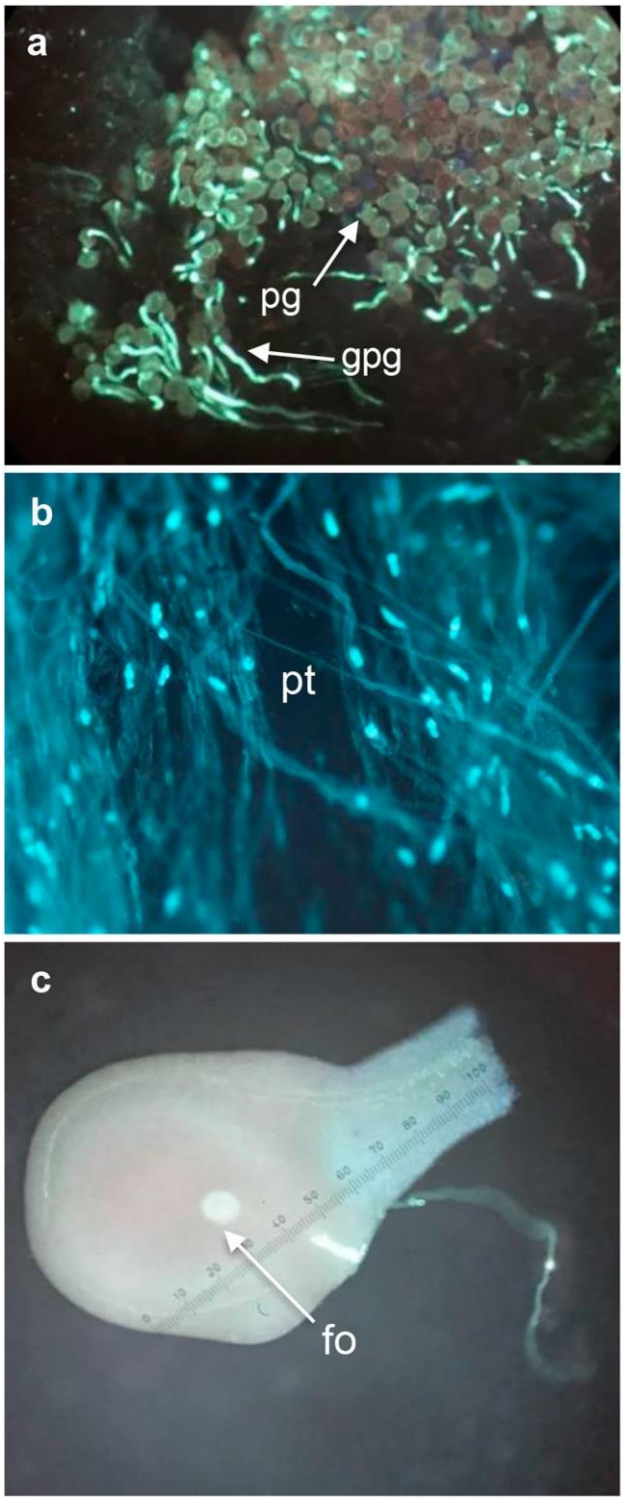

Fig. 5 Pollination and fertilization in andromonoecious line P87. **a** Pollen adhesion and germination in the stigma. **b** Pollen tubes grow- ing on the style. **c** Fertilized ovule. pg, pollen grain; gpg, germinated pollen grain; pt, pollen tubes; fo, fertilized ovule

Regulation of fruit set and development

Data indicated that *CitACS4* affects developmental events occurring before or at anthesis, including fruit shape and fruit setting, but not those occurring after anthesis andpollination such as fruit growth rate and

final size. In the P84XP87 cross, the monoecious *M* allele was linked to elon-gated fruits, while the andromonoecious *m* allele was ratherlinked to round-shaped fruits. Since the final shape of a fruitdepends on ovary shape at anthesis (Perin et al. 2002), it is not difficult to realize that a gene like *CitACS4*, which is spe-cifically expressed in floral buds at earlier stages of develop-ment, can regulate the final shape of the fruit. The associa- tion of hermaphrodite flowers with round-shaped fruits wasfirst reported by Rosa (1928) and later by Kubicki (1962) andWall (1967) in melon. Among QTLs controlling fruit shapein melon, that in LGII seems to be a pleiotropic effect ofthe sex-determining gene *CmACS7* (Perin et al. 2002; Díaz et al. 2014). The monoecious (*M*) and andromonoecious (*m*)alleles of the cucumber gene *CsACS2* also cosegregate withelongated and round fruits, respectively, although a novel allele of the gene (*m1*), encoding for a truncated protein,is responsible for elongated fruit shape and andromonoecy (Tan et al. 2015). In zucchini, hermaphrodite flowers of monoecious unstable cultivars produce larger ovaries and fruits, but fruit shape is not altered (Martínez et al. 2014).

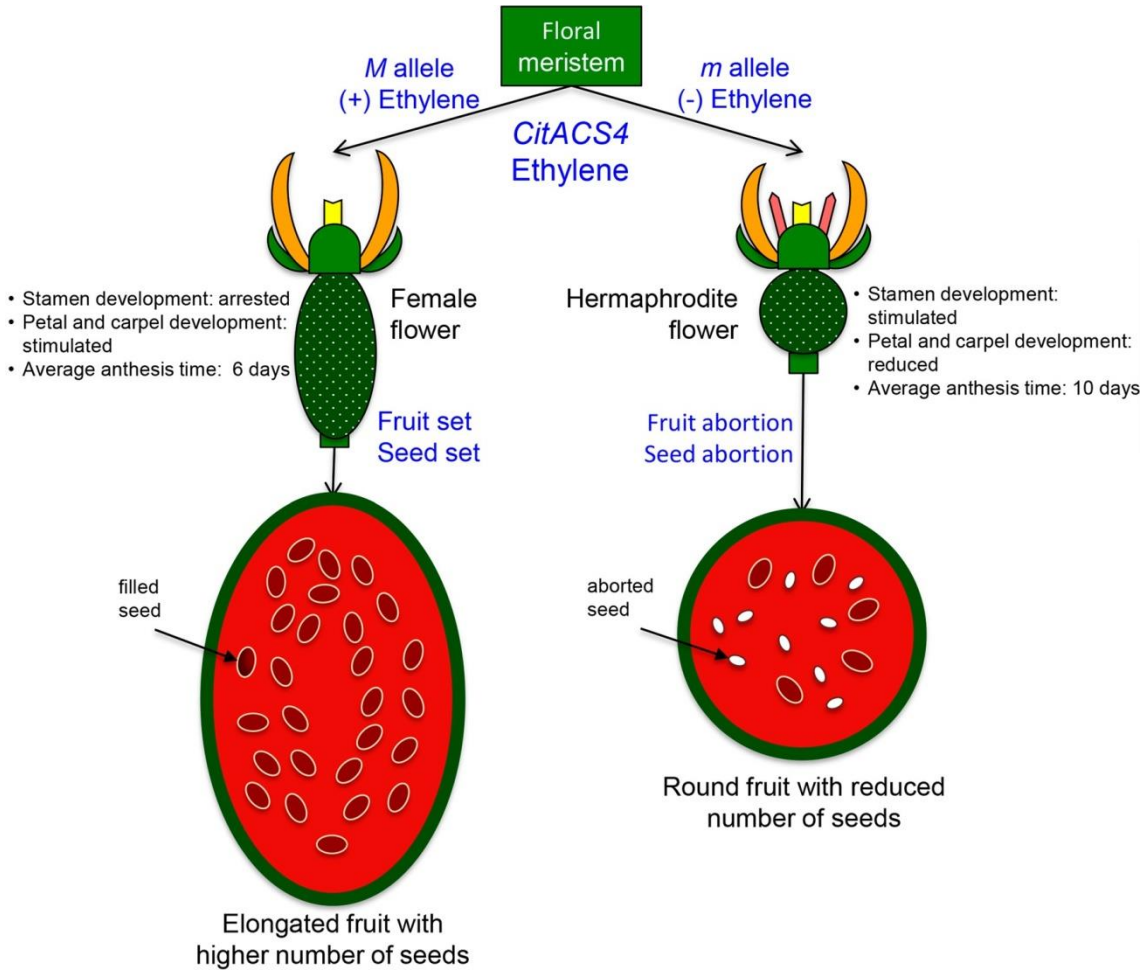

Fig. 6 Involvement of the ethylene biosynthesis gene *CitACS4* in watermelon flower and fruit development. In the monoecious *MM* plants, the production of ethylene in the floral meristem arrests the development of stamens, but stimulates the growth of petals and car- pels, which results in a female flower with an elongated ovary. In the *mm* plants, the lack of ethylene production prevents stamen arrest and reduces the growth rate of petals and carpels, which results in a her- maphrodite flower with a round-shaped ovary. After pollination, the reduced ethylene production in the *mm* flowers could be also respon- sible of fruit and seed abortion observed in the andromonoecious mm line.

The cosegregation between m allele and a reduced fruitset in P85XP87 and P86XP87 crosses could be the result of CitACS4, but the existence of other linked genes cannot be ruled out. Moreover, the fact that the monoecious line P84 does not differ in fruit set with respect to the andromonoe- cious one P87 also

suggests that the trait is influenced by other unlinked loci. The role of ethylene in fruit setting has not been studied in depth. A downregulation of ethylene biosynthesis and signalling genes has been observed imme- diately after anthesis in pollinated, GA3-treated and par- thenocarpic fruits of tomato (Vriezen et al. 2008) and zuc- chini (Martínez et al. 2013). Ethylene produced in the ovules appears to be responsible for both the ovule lifespan and the fate of the ovary/fruit in tomato (Olimpieri et al. 2007) and Arabidopsis (Carbonell-Bejerano et al. 2010, 2011), control- ling fruit set in response to GA in Arabidopsis-unfertilized ovaries. In zucchini, the inhibition of ethylene biosynthesis or response is sufficient to induce the set and early develop- ment of the fruit in the absence of pollination, demonstrating a direct involvement of ethylene in fruit set (Martínez et al. 2013). This post-anthesis ethylene, which could be involved in ovule senescence and fruit abortion, does not appear to be the same as the one responsible for mm fruit abortion in watermelon. Although this interesting finding requires more research, it seems that fruit set requires higher ethyl- ene production in the immature flower buds, probably for a coordinated development and maturation of floral organs at anthesis, but lower ethylene production in ovules and fruits immediately after anthesis, because at this later stage ethyl- ene could trigger ovule senescence and consequently fruit abortion.

Under unfavourable environmental conditions of autumn/winter, the correct set of seeds in fruits was found to be linked to the M/m locus only in one of the analysed F2 popu-lations (P86XP87), where monoecious *MM* fruits had higherseed yield than andromonoecious *mm* ones. This trait is veryinfluenced by environmental conditions, especially tempera-ture. The lack of linkage between seed set and M/m locus inthe other two crosses could indicate the existence of other major loci regulating this trait in watermelon. Therefore,the role of the M/m locus in the regulation of watermelon seed set will require further experimental work. Compari- son of pollen adhesion, pollen tube growth and ovule fer- tilization in monoecious and andromonoecious lines shed also no light on seed abortive mechanisms in fruits derived from hermaphroditic *mm* flowers. The pollination window at which pollen adheres to the stigma is slightly delayed in *mm* flowers, but pollen adhesion and pollen tubes observedat anthesis and + 1 and + 2 DPA ensured fertilization in both*MM* and *mm* flowers. Therefore, it is likely that the loss of seeds in the P87 line is not due to a lack of pollination or fertilization events, but rather to a premature abortion of fertilized ovules. Ethylene plays a significant role in ovule development and female gametophyte fertility (Tsai et al. 2008; Clark et al. 2010). In *Arabidopsis*, the onset of ovule senescence and the time window for the pistil to respond toGA treatments is modulated by ethylene (Carbonell-Bejer- ano et al. 2011). Silencing of ethylene biosynthesis genesin transgenic tobacco plants results in a reversible inhibi- tion of ovule development (De Martinis and Mariani 1999).Moreover, ethylene biosynthesis and signalling genes have been found to be hypomethylated in the female sterile rice mutant *fsv*, in which the ethylene genes were upregulated and then downregulated during ovule development (Yanget al. 2016; Liu et al. 2017).

Taken together, the results presented in this paper indicate that, in addition to arresting stamen growth and developmentand determining the sex identity of the female flower, the ethylene biosynthesis gene *CitACS4* is capable of regulating several developmental processes that occur in the pistillate flower and in the early development of the fruit (Fig. 6). Thedecrease in the production of ethylene associated with the loss of function *m* allele prevents stamen arrest, but inhibitsthe development of the petals and carpels, making the flowerreach anthesis about 4 days later. The result at anthesis isa hermaphrodite flower with a rounder and larger ovary than that of the female flower. The lack of ethylene dur-ing the development of the hermaphrodite flower could alsoexplain the reduced fruit set found in the andromonoecious*mm* plants (Fig. 6).

Author contribution statement MJ and EA conceived and designed the experiments. EA, AG, SM, VP and JC performed the experiments. EA and MJ analysed the data. MJ, EA, AG, JV and SM contributed reagents/materials/analysis tools. MJ and EA wrote the paper. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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