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Research article

Involvement of ethylene in sex expression and female flower development in watermelon (*Citrullus lanatus*)

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ABSTRACT

Although it is known that ethylene has a masculinizing effect on watermelon, the specific role of this hormone in sex expression and flower development has not been analyzed in depth. By using different approaches the present work demonstrates that ethylene regulates differentially two sex-related developmental processes: sexual expression, i.e. the earliness and the number of female flowers per plant, and the development of individual floral buds. Ethylene production in the shoot apex as well as in male, female and bisexual flowers demonstrated that the female flower requires much more ethylene than the male one to develop, and that bisexual flowers result from a decrease in ethylene production in the female floral bud. The occurrence of bisexual flowers was found to be associated with elevated temperatures in the greenhouse, concomitantly with a reduction of ethylene production in the shoot apex. External treatments with ethephon and AVG, and the use of *Cucurbita* rootstocks with different ethylene production and sensitivity, confirmed that, as occurs in other cucurbit species, ethylene is required to arrest the development of stamens in the female flower. Nevertheless, in watermelon ethylene inhibits the transition from male to female flowering and reduces the number of pistillate flowers per plant, which runs contrary to findings in other cucurbit species. The use of *Cucurbita* rootstocks with elevated ethylene production delayed the production of female flowers but reduced the number of bisexual flowers, which is associated with a reduced fruit set and altered fruit shape.

1. Introduction

In the *Cucurbitaceae* family sex expression is known to be controlled by different genetic, environmental and hormonal factors. In the most studied species, *Cucumis sativus* and *Cucumis melo*, sex is controlled by three major independent genes, the combination of which explains the main sex phenotypes found in these two species (Perl-Treves, 1999; Pierce and Wehner, 1990; Kenigsbuch and Cohen, 1990; Kubicki, 1969; Rudich, 1990). Sex expression in this family of plants can be modified by environmental factors, including light intensity, photoperiod and temperature. Winter conditions with short days, low light intensity and low night temperatures promote the production of female flowers, while summer conditions increase the production of male flowers (Peñaranda et al., 2007; Wien, 1997). In *Cucurbita pepo*, low temperature inhibits the development of male flowers and increases the number of female flower per plant, while high temperature induces a partial or complete transformation of female into bisexual flowers (Peñaranda et al., 2007).

Phytohormones are the main modulators of sex expression in this family. GAs promote the production of male flowers, while auxins and brassinosteroids promote the production of female ones (Rudich et al., 1972a,b, Trebitsh et al., 1987; Wien, 1997), although their functions appear to be mediated by ethylene. Undoubtedly, ethylene is the principal hormone regulating sex expression in the *Cucurbitaceae* family. The level of ethylene in flower buds seems to be essential for both sex determination and female flower development (Manzano et al., 2013). Thus, ethylene treatments promote the production of female flowers, while treatments with inhibitors of ethylene biosynthesis or perception, such as aminoethoxyvinylglycine (AVG) or silver thiosulphate (STS), increase the number of male flowers per plant (Byers et al., 1972; DenNijs and Visser, 1980; Manzano et al., 2011; Owens et al., 1980; Payán et al., 2006; Rudich, 1990; Rudich et al., 1969). It has been demonstrated that the monoecious phenotype of melon and

Abbreviations: ACC, aminocyclopropane carboxylic acid; *ACS, 1-aminocyclopropane-1-carboxylate synthase*; AVG, aminoethoxyvinylglycine; *Bog, Bolognese*; *CpWEI, Cucurbita pepo WEAK ETHYLENE INSENSITIVE*; Gas, Gibberellins; STS, Silver Thiosulphate; *Veg, Vegetable Spaghetti.*

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cucumber is controlled by a *1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE* (*ACS*) gene that is specifically expressed in carpels and is involved in the arrest of stamen development in female flowers. Andromonoecious varieties are indeed mutants for this gene, which results in an inhibition of the arrest of stamen development in the ovary-bearing flowers, leading to the formation of bisexual flowers instead of female ones (Boualem et al., 2008, 2009). Moreover, cucumber and melon gynoecious lines produce more ethylene than monoecious or andromonoecious ones (Atsmon and Tabbak, 1979; Manzano et al., 2008; Owens et al., 1980; Rudich et al., 1972a,b, Trebitsh et al., 1987, Yamasaki et al., 2001). In fact, gynoecious cucumber varieties have an additional *ACS* gene (Knopf and Trebitsh, 2006; Mibus and Tatlioglu, 2004; Trebitsh et al., 1997). Nevertheless, the gynoecious phenotype of melon results from a transposon in the promoter of the transcription factor *CmWIP1*, a gene that controls carpel abortion and indirectly promotes the development of stamens by repressing the expression of the monoecious gene *CmACS7* (Martin et al., 2009).

The sex phenotype of watermelon cultivars can be either monoecious (male and female flowers) or andromonoecious (male and bisexual flowers) (Rudich and Zamski, 1985), although most of the current cultivars are of the former type. In watermelon cultivars the number of pistillate flowers is low, with a phenological interval of 4–15 male flowers followed by a pistillate flower, depending on the genotype. Treatment with ethylene-releasing agents resulted mostly in the suppression of ovary development (Rudich and Zamski, 1985), while inhibitors of its biosynthesis and perception, hastened the appearance of the first pistillate flower and increased the pistillate to male flower ratio (Rudich and Zamski, 1985; Sugiyama et al., 1998). The masculinizing effect of ethylene in watermelon is in stark contrast to the feminizing effect of this same hormone in other cucurbit species such as cucumber, melon and squash (Manzano et al., 2011).

Given this inconsistency between species of the same family, in this paper we used different approaches to determine the true role of ethylene in sexual expression and flower development in watermelon. The results of ethylene production in the apical shoots and flowers, the effects of treatments with etephon and AVG, and the results of grafting watermelon on *C. pepo* rootstocks with different production and sensitivity to ethylene, have shown that as regards the control of sexual expression, the role of this hormone is contrary to that in *Cucumis* or *Cucurbita*. However, in the control of sex determination and development of individual floral buds, ethylene is required to arrest stamen development in female flowers, in a similar manner as occurs in its relatives.

2. Materials and methods

2.1. Plant material, growing conditions and evaluation of sexual expression

All the experiments in this paper were carried out on the commercial cultivars *Fashion* and *Premium*. *Fashion* is a seedless triploid hybrid, while *Premium* is a diploid cultivar that is used as a pollinator of triploids. The plants were grown in the spring-summer season of 2009/2010, 2010/2011 and 2011/2012 under standard greenhouses conditions in the area of Almería. Some of the experiments were carried out in climate-controlled chambers at different temperatures, but with 75% relative humidity and with a long photoperiod regime of 16 h light/8 h night. Watering and nutrition of the plants were carried out automatically following standard practices.

Sex expression in each plant was evaluated as both the number of initial nodes with male flowers before the production of the first female flower, and the percentage of female and bisexual flowers per plant in the first 30 nodes of both the main and the secondary shoots. At least 10 plants were tested to assess the sexual expression of each genotype, and the effects of both hormone treatments and *Cucurbita* rootstocks on the sexual expression of watermelon.

2.2. Hormonal treatments and measurements of ethylene production

To study the involvement of ethylene in both sexual expression and flower development, watermelon plants were treated with the ethylene releasing agent ethephon, and with the ethylene biosynthesis inhibitor aminoethoxyvinylglycine (AVG). To evaluate the effect on female flowering transition, in a first round of experiments the shoot apices of plants having one true leaf were treated once with either ethephon or AVG. For ethephon treatments the shoot apices were sprayed with 0.1% tween 20 and 500 or 1000 ppm of ethephon. Ethylene inhibiting treatments were carried out with solutions containing 0.1% tween 20 and 1 or 1.5 mM of AVG. The involvement of ethylene in female flower development and the conversion of female into bisexual flowers was investigated in successive experiments by treating watermelon plants having about 10 true leaves with 0.1% tween 20 and either 50 ppm of ethephon or 1 mM of AVG. These treatments were repeated three times a week for four weeks. Control plants were sprayed with only 0.1% tween 20. 10 plants were evaluated per treatment.

The production of ethylene was measured in the main shoot apices as well as in flower buds throughout five different stages of floral development (F1–F5). The different developmental stages were separated on the basis of the corolla length (F1 = 4 ± 1 mm, F2 = 8 ± 2 mm, F3: 12 ± 2 mm, F4: 15 ± 2 mm, F5: 22 ± 2 mm). In floral buds ethylene was determined in three biological replicates for each developmental stage, each one containing five female, male or bisexual flowers at the same stage of development. For main shoot apices, ethylene was determined in four biological replicates, each one containing eight apexes of plants in the same stage of development. The plant organs were excised from the plant and incubated at room temperature for 6 h in hermetic glass containers in the dark. Ethylene production was determined by analyzing 1 ml of gas from the *head*space on a Varian 3900 gas chromatograph apparatus, fitted with a flame ionization detector. The instrument was calibrated with standard ethylene gas.

Statistical differences between genotypes or treatments were determined by analysis of variance (ANOVA), followed by Tukey's multiple comparison test using the STATISTIX 8.0 software package.

2.3. Grafting experiments

For grafting experiments, the commercial interspecific hybrid between *Cucurbita moschata* x *Cucurbita maxima*, *Ercole* F1 (Nunhems), and two inbred lines of *C. pepo* subspecies *pepo* of the Vegetable Marrow Group, *Vegetable Spaghetti* (*Veg*) and *Bolognese* (*Bog*), were used. The *Veg* and *Bog* lines were selected because of their contrasting sex expression and ethylene production and sensitivity. While *Bog* develops the first female flowers between nodes 2–4, and produces a mean of 69–75% female flowers, the sex phenotype of *Veg* is strongly male, delaying the development of the first female flower to nodes 15–20, and producing less than 25% female flowers, with some of the plants producing less than 5% (Manzano et al., 2010). The extreme male phenotype of *Veg* is determined by a major gene that confers reduced ethylene production and sensitivity, while *Bog* produces much more ethylene and has a higher sensitivity to the hormone (Manzano et al., 2010, 2011). Grafting was carried out when the first true leaf developed in