Brassinosteroid-induced Femaleness in Cucumber and Relationship to Ethylene Production

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Abstract. The Cucurbitaceae family is noted for a diversity of sex expression phenotypes. Typically, a phase of male flowers precedes either female or bisexual flower production. Sex determination of individual flowers is regulated by a combination of genetic, environmental, and hormonal factors. Ethylene, auxins, and gibberellins have all been shown to influence flower sex expression in cucurbits. Ethylene, which promotes femaleness, plays a predominant role. In this study, we tested whether brassinosteroids (BR), a more recently identified class of plant hormones, also influences cucurbit sex expression. Applied epi-brassinolide (epi-BL) caused a significant decrease in time of appearance of the first female flower on monoecious cucumber plants, and increased total female flowers on the main stem. Increasing concentrations had a stronger effect. Of the three species tested, cucumber, melon and zucchini, cucumber was the most responsive to BR. Application of epi-BL also caused an increase in ethylene production by cucumber and zucchini seedlings, suggesting that the BR effect may be mediated by ethylene. To investigate the possible relationship between BR and ethylene on sex expression, we identified the concentration of ethephon (5 ppm) that caused an increase in ethylene production comparable to that induced by 10 μ M epi-BL (approximately two-fold). Treatment with 5 ppm ethephon was sufficient to increase femaleness of cucumber plants, but not zucchini plants, suggesting that the difference in response to epi-BL treatment may reflect differences in sensitivity to ethylene. Collectively, our results indicate that application of brassinosteroids to cucumber cause earlier and increased female flower production, and that the effects may be mediated, at least in part, by brassinosteroid-induced production of ethylene.

Flower development is a critical factor influencing plant reproduction and crop yield. While most species produce bisexual flowers possessing both male (stamen) and female (carpel) organs, various species throughout the plant kingdom have evolved mechanisms to produce unisexual flowers (Ainsworth et al., 1998; Barrett, 1998; Tanurdzic and Banks, 2004). The Cucurbitaceae family is noted for a diversity of sex expression types (Perl-Treves, 1999; Roy and Saran, 1990). Monoecious species, such as cucumber (*Cucumis sativus* L.) and squash (*Cucurbita pepo* L.), bear separate male and female flowers on the same plant, while andromonoecious species, such as many melon genotypes (*Cucumis melo* L.), produce male and bisexual flowers. In both cases, male flowers are produced early in plant development, followed by a mixture of either female and male, or bisexual and male flowers.

Sex determination of individual flower buds is regulated by a combination of genetic, environmental, and hormonal factors. At the initial stages of floral primordium development, male and female buds are morphologically indistinguishable; all four whorls (sepals, petals, stamens, and carpels) are initiated, and subsequently, either male or female organs develop (Goffinet, 1990). Studies with homeotic mutants of cucumber indicate that position

within the developing flower bud (i.e., third or fourth whorl) is critical for determining whether the specific floral organ will develop (Kater et al., 2001).

Several plant hormones, including ethylene, auxins, and gibberellins (GAs) have been shown to influence flower sex expression in cucurbits (Rudich, 1990). Application of exogenous hormones at the appropriate time can cause floral primordia destined to become unisexual flowers to be converted to bisexual flowers, or completely change to the opposite sex. The extent of conversion can be affected by genotype, stage of floral development at the time of application, and amount of applied hormone.

Application of ethylene or ethylene releasing compounds have been shown to increase pistillate flower production in cucumber, squash and muskmelon (Augustine et al., 1973; Hume and Lovell, 1983; Karchi, 1970; McMurray and Miller, 1968; Owens, 1980; Robinson et al., 1969; Rudich et al., 1969, 1972). Consistent with exogenous effects, excised apices and flower buds of gynoecious (all female) cucumber plants produced 2- to 3- fold higher ethylene levels compared to monoecious genotypes, suggesting a correlation between sex expression and endogenous ethylene production (Byers et al., 1972a; Rudich et al., 1972, 1976; Trebitsh et al., 1987; Yamasaki et al., 2001). Although such a difference was not observed in melon, hypobaric conditions reducing ethylene levels increased maleness (Byers et al., 1972b) and transgenic melon plants constitutively expressing ACS (1-amino-cyclopropane-1-carboxylic acid syn-

thase), a key enzyme in ethylene biosynthesis, showed increased femaleness (Papadopoulou et al., 2005). Conversely, inhibitors of ethylene biosynthesis and action promote staminate flower development, and have been used by breeders to facilitate seed production on gynoecious genotypes (Byers et al., 1972; Den Nijs and Visser, 1980; Owens et al., 1980; Takahashi and Jaffe, 1984; Tolla and Peterson, 1979). Transgenic melon constitutively expressing the Arabidopsis mutant ethylene perception gene, *etr1-1*, failed to produce female flowers (Papadopoulou et al., 2002).

Application of auxin to cucumber flower buds at the pre-sexual stage also can increase female flower formation (Galun et al., 1963; Trebitsh et al., 1987). However, findings that endogenous IAA levels were lower in gynoecious than monoecious cucumber and squash genotypes (Chrominski and Kapcewicz, 1972; Trebitsh et al., 1987), and that treatment of gynoecious plant with anti-auxin compounds did not induce staminate flower production (Trebitsh et al., 1987), suggested a secondary role for auxins. Instead, it has been proposed that the effect of auxin is mediated by increased ethylene production that occurs in response to auxin application (Takahashi and Jaffe, 1984; Trebitsh et al., 1987).

Exogenous GAs exhibit the opposite effect of ethylene, and have been shown to induce formation of male flowers on leaf axils normally occupied by female flowers (Pike and Peterson, 1969; Tolla and Peterson, 1979). Higher levels and activity of GAs were reported in monoecious compared to gynoecious lines (Atsmon et al., 1968; Freidlander et al., 1977). However, GA effects also appear to be secondary to ethylene. Studies by Yin and Quinn (1995), comparing the effects of ethylene and GA concluded that ethylene is the predominant sex determining hormone, and Tolla and Peterson (1979) observed that inhibition of ethylene action was more effective in inducing male flower production in cucumber than was application of gibberellins.

In the past decade, a new group of plant hormones, the brassinosteroids, which can promote cell division and expansion, vascular differentiation, reproductive development, senescence, photomorphogenesis, and plant tolerance to stress, has received increasing attention (Clouse and Sasse, 1998; Sasse, 1997). A definitive role for endogenous brassinosteroids in plant growth and development was demonstrated by the identification of developmental mutants of Arabidopsis that are defective in brassinosteroid biosynthesis and response (Altmann, 1999; Clouse et al., 1996; Clouse, 1997; Kauschmann et al., 1996; Li and Chory, 1999).

Given the range of activities of brassinosteroids, and the role of several hormones in influencing sex determination in cucurbits, we were interested in testing whether exogenous brassinosteroid would affect sex expression in cucurbits. Our results demonstrate that application of brassinosteroid to cucumber caused earlier and increased female flower production and increased ethylene production, and suggest that the effects of brassinosteroid

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may be mediated, at least in part, via increased production of ethylene.

Materials and Methods

Plant materials. The genotypes used were monoecious cucumber cultivar Straight 8 (S8) (Hollar Seeds, Rocky Ford, Colo.), the gynoeious cucumber breeding line GY14 (kindly provided by Greg Tolla, Seminis Seeds), the monoecious zucchini cultivar Black Beauty (Willhite, Poolville, Texas), and the andromonoecious melon cultivar Hale's Best Jumbo (Hollar Seeds). For greenhouse trials, seeds were imbibed overnight at 30 °C before planting in 23-cm-diameter pots containing Baccto soil mix (70% to 80% sphagnum peat, pH 5.5 to 6.5; Baccto, Michigan Peat Co., Houston, Texas). Plants were grown in the greenhouse throughout the year under natural photoperiods at average temperatures of 25 °C, and fertilized once a week with 300 ppm 20–20–20 (Peter's Special).

Brassinosteroid experiments. The synthetic brassinosteroid, epibrassinolide (epi-BL; 22R, 23R, 24R2 α , 3 α , 22,23-tetrahydroxy-B-homo-7-oxa-5 α -ergostan-6-one) (Sigma Chemical Co., St Louis, Mo.) was applied by pipetting 250 μ L of 0.1, 1, or 10 μ M epiBL onto the apical meristem and developing leaf. The first

application was made at the first true leaf stage (mean diameter about 5 cm); two subsequent treatments were applied at three day intervals. Plants were scored for presence of pistillate buds on the main stem. The experiments were arranged in a randomized complete block design with 5 to 10 replications per treatment, as specified in figure legends. Each experiment was repeated three or four times.

Ethylene experiments. Cucumber (*Cucumis sativus* L. 'Straight 8') or zucchini (*C. pepo* 'Black Beauty') seeds were de-coated, sterilized in LD solution (Alcide LD, Alcide Corporation, Norwalk, Conn.) for 10 min, rinsed with distilled water three times, and transferred to 250-mL plastic containers (Magenta boxes, Magenta Corp., Chicago Ill.) containing medium with Murashige and Skoog basal salts (Sigma), 3% sucrose, 0.8% agar, adjusted to pH 5.7 to 5.8, and placed under 16 h light at 25 °C. Each box contained six seedlings. At 8 to 10 d old (cotyledons fully expanded, first true leaf just emerging), seedlings were treated with 20 μ L of 0, 1, and 10 μ M epi-BL. Headspace gas samples were taken with an airtight syringe at 0, 6, 12, 24, 48, and 72 h postapplication. The samples were analyzed in a gas chromatographer (HACH CARLE series 100 AGC, Linear 1200 recorder, with an alumina column and flame ionization

detector). The same experimental procedure was used to determine the concentration of ethephon (2-chloroethyl phosphonic acid) that releases the same amount of ethylene as was caused by application of 10 μ M epi-BL. Ethephon was applied at concentrations of 5, 50, and 500 ppm. Numbers of replications per experiment are indicated in the figure legend. Each experiment was repeated three times. The greenhouse experiments were performed as described above for epi-BL, using 5 ppm ethephon; each experiment was repeated four times.

Results

Applied epi-BL caused a significant decrease in node position of the first female flower on monoecious cucumber plants and a significant increase in the number of female flowers formed on the first 25 flowering nodes from the base of the plant (ANOVA, $P < 0.01$; Fig. 1A and B). There was a progressive effect on both time to appearance of the first female flower and number of female flowers as epi-BL concentration increased up to 10 μ M. Preliminary experiments showed that 100 μ M epi-BL had a stronger effect on femaleness, but it also occasionally caused damage to the growing points and distortion of vein development in

Fig. 1. Effect of brassinosteroid treatment on sex expression in cucumber (A–D) and zucchini (E). (A and B) Appearance of the first female bud and number of female buds formed along the first 25 nodes of the main stem of monoecious cucumber plants. (C and D) Appearance of the first female bud and number of female buds formed along the first 25 nodes of the main stem of gynoeious cucumber plants. (E) Number of female buds formed along 25 nodes of the main stem of monoecious zucchini. Each value for monoecious cucumber is the mean of four experiments ($\pm$ SE) with 9 replicates per experiment. Values for the gynoeious cucumbers are the mean of three experiments ($\pm$ SE) with 5, 9 and 9 replicates, respectively. Values for zucchini are the mean of three experiments, 9 replicates per experiment. Experiments were performed in spring and fall; equivalent trends were observed in each experiment. Bars marked with different letters are significantly different (ANOVA, $P < 0.01$).

leaf tissue and so was not included in subsequent experiments. As might be expected for gynoeious cucumbers that already produce female flowers at the first flowering node (usually node 4), epi-BL treatment did not influence the earliness or number of female buds on the gynoeious plants (Fig. 1C and D).

Of the three species tested, cucumber, melon, and zucchini, cucumber appeared to be the most responsive to brassinosteroid application. Application of epi-BL did not significantly alter the sex expression patterns, earliness, or number of female flowers of melon plants (data not shown) and did not increase femaleness in monoecious zucchini plants (Fig. 1E).

Exogenous brassinosteroid has been shown to increase ethylene production in several systems (Arteca et al., 1988; Vardhini and Rao, 2002; Woeste et al., 1999), therefore we sought to determine if brassinosteroid could increase ethylene production in cucumber seedlings, and if so, whether it might be related to the observed effect on sex expression. Application of epi-BL to cucumber seedlings at the time when the cotyledons were fully expanded and the first leaf was developing (about 8 to 10 d old), resulted in increased ethylene evolution compared to the water treated control for both monoecious and gynoeious cucumber genotypes (Fig. 2A and B). Ethylene evolution increased, and then leveled off approximately 24 h post-treatment. In both monoecious and gynoeious cucumber cultivars, higher levels

of epi-BL result in higher levels of ethylene production; 1.4- and 1.7-fold for 1 μ M, and 2- and 3-fold for 10 μ M respectively, at 24 h. Increased ethylene production of 2- to 3-fold also was observed for zucchini and melon seedlings following the application of epi-BL (data not shown).

To further investigate the possible relationship between brassinosteroid and ethylene on sex expression, we identified the concentration of ethephon that induces ethylene production comparable to levels induced by 10 μ M epi-BL. A range of concentrations, 5, 50, and 500 ppm ethephon was tested. The level applied for sex conversion of cucumber has generally been in the range of 50 to 250 ppm (e.g., Augustine et al., 1973; Freytag et al., 1970; Karchi and Grovers, 1972; McMurray and Miller, 1968). For both cucumber and zucchini, application of 5 ppm ethephon was found to give a 2-fold increase in ethylene production comparable to that observed with 10 μ M epi-BL (Fig. 2C and D).

A subsequent set of experiments was conducted to compare the effect of brassinosteroid and ethephon applied at concentrations that release comparable amounts of ethylene, on sex expression of cucumber and zucchini plants. Treatment with 5 ppm ethephon caused a significant decrease in the position of the first pistillate node of monoecious cucumber, and increase in the number of pistillate flowers compared to control monoecious cucumbers

(Fig. 3A and B; ANOVA, $P < 0.01$), indicating that the low level of ethylene produced by epi-BL could be sufficient to increase femaleness in monoecious cucumber. Consistent with the failure of epi-BL to increase female flower production on monoecious zucchini plants, the low ethylene treatment (5 ppm) also was not sufficient to induce earlier formation of female buds or increase the number of female buds in zucchini (Figure 3 B and D). Although the higher ethephon treatment (500 ppm) did not significantly affect the time of appearance of the first female zucchini flower, it did result in an increased number of female flowers on the first 20 nodes (ANOVA, $P < 0.01$).

Discussion

The influence of brassinosteroids on floral development of higher plants has not been extensively studied and has included mixed results. Exogenous brassinosteroids inhibited floral induction in *Pharbitis nil* (Kesy et al., 2003), did not influence flower induction when applied to the short-day plant, *Perilla frutescens*, under noninductive conditions, and caused slight enhancement of flowering of nonvernalized long-day *Raphanus sativus* plants (Suge, 1986). Effects of brassinosteroids on sex organs, have been implicated in *Arabidopsis*, maize, and the cucurbit, *Luffa cylindrica*. The *Arabidopsis* brassinosteroid mutants, *det2* (de-etiolated) and *cpd* (constitu-

Fig. 2. Ethylene evolution by cucumber or zucchini seedlings in response to brassinosteroid or ethephon treatment. (A and B) Ethylene evolution of monoecious (A) or gynoeious (B) seedlings treated with 1 or 10 μ M pt epi-BL. Each point is the mean of three experiments ($\pm$ SE) with 5, 4, and 4 replicate containers per treatment, respectively, and six seedlings per container. Similar trends were observed in all three experiments. Epi-BL treatment caused a significant increase in ethylene evolution for both monoecious and gynoeious cucumber seedlings (ANOVA). (C and D) Ethylene evolution of monoecious cucumber (C) or zucchini (D) seedlings treated with 10 μ M pt epi-BL or 5 ppm ethephon. (C) Each point is the mean of three experiments ($\pm$ SE) with 5, 4, and 5 replicate containers per treatment, and six seedlings per container. Similar trends were observed in all three experiments. (D) Each point is the mean of three experiments ($\pm$ SE) with five replicate containers per treatment per experiment, and six seedlings per container. Similar trends were observed in all three experiments. Epi-BL and ethephon caused significant increases in ethylene evolution relative to the untreated control seedlings; the effects of epi-BL and ethephon were not significantly different from each other (ANOVA).

Fig. 3. Effect of ethephon treatment on sex expression in cucumber and zucchini plants. (A and B). Appearance of the first female bud and number of female buds formed along the first 25 nodes of the main stem of monoecious cucumber plants. (C and D). Appearance of the first female bud and number of female buds formed along the first 20 nodes of the main stem of monoecious zucchini plants. Each value is the mean of four experiments ($\pm$ SE) with 9, 10, 8, and 7 replications per treatment for cucumber and 10 replications per treatment for zucchini. Experiments were performed in August, October, December, and March; similar trends were observed in all experiments. Bars marked with different letters are significantly different (ANOVA).

live photomorphogenesis and dwarfism), show reduced male fertility due to inhibition of pollen elongation during germination (Szekeres et al., 1996). In maize, brassinosteroids have been postulated as possible substrates of the maize *TASSELSEED2* (*TS2*) gene, which encodes a putative short chain alcohol hydroxysteroid dehydrogenase (Lebel-Hardenack et al., 1997; Delong et al., 1993). *TS2* is essential for male sex determination in maize via abortion of pistil primordia (Calderon-Urrea and Delaporta, 1999). Application of BR's to male inflorescences of *Luffa cylindrica* resulted in formation of bisexual and female flowers (Suge, 1986).

This work demonstrated that application of epi-BL to monoecious cucumber plants caused earlier and increased production of female buds, indicating a possible involvement of brassinosteroids in floral sex differentiation in cucumber. We did not, however, observe an increase in femaleness in zucchini or melon in response to applied epi-BL. The observed differences among the cucurbit species may reflect differences in responsiveness or sensitivity to brassinosteroids among the different species.

A possible explanation for the effect of brassinosteroid, is that it acts indirectly, via increased ethylene production. Brassinosteroids have been reported to cause increased ethylene biosynthesis in excised mung bean hypocotyls, *Arabidopsis* seedlings, and tomato pericarp discs (Arteca et al., 1988; Vardhini and Rao, 2002; Woeste et al., 1999). The experiments presented here demonstrated the ability of

brassinosteroid to induce ethylene production in cucumber and zucchini seedlings, and suggest that the increase in femaleness in cucumber may be attributed to increased ethylene availability.

A similar relationship to ethylene has been shown for the effect of auxin on increased femaleness in cucurbits (Takahashi and Jaffe, 1984; Trebitsh et al., 1987). Endogenous IAA levels were not correlated with gynoecey in cucumber and squash, and application of auxin competitors did not alter female flower formation (Chrominski and Kapcewicz, 1972; Trebitsh et al., 1987). However, auxin treatment increased ethylene evolution by cucumber plants, suggesting that the observed effect of auxin on sex expression is ethylene mediated (Trebitsh et al., 1987). Indeed, several cucumber ethylene biosynthetic genes also have been shown to be auxin regulated and promoter sequence analysis suggests the presence of auxin responsive elements (Little, 2005; Milbus and Tatlioglu, 2004).

Although melon and zucchini did not show increased femaleness in response to epi-BL treatment, they showed a similar increase in ethylene production as did cucumber. The different species, however, showed different sensitivity to ethylene consistent with their responsiveness to epi-BL. The 5 ppm ethephon treatment, which caused an equivalent increase in ethylene production to that caused by the 10 μ M epi-BL treatment, was sufficient to cause earlier and increased female flower formation in cucumber, but was not sufficient to significantly increase femaleness in zucchini. Thus a plau-

sible explanation for the different responses of cucumber and zucchini to epi-BL, is due to their differential sensitivity to ethylene. Greater sensitivity of sex expression in cucumber relative to melon or zucchini has been observed previously (Rudich et al., 1969).

In summary, our results show that exogenous brassinosteroid increased femaleness in cucumber and also caused increased ethylene production. Application of ethephon at a level that releases equivalent amounts of ethylene to the epi-BL caused similar effects on cucumber sex expression, and supports the hypothesis that the observed effect of exogenous epi-BL on sex expression is ethylene mediated. Difference in brassinosteroid response among the three cucurbit species is consistent with the possibility that brassinosteroid acts indirectly via increased ethylene production, and that the level of ethylene production is sufficient to increase femaleness in monoecious cucumber, but not in zucchini or melon.

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