Hormonal Changes after Compatible and Incompatible Pollination in *Theobroma cacao* L.

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Abstract. In order to characterize the self-incompatibility system in *Theobroma cacao*, the levels of ethylene, indole-3-acetic acid (IAA), and abscisic acid (ABA) were determined after pollination with compatible and incompatible pollen and in unpollinated flowers. Pollen tube growth rates after incompatible and compatible pollinations were identical, and the majority of the pollen tubes reached the ovules between 12 and 20 hours after pollination. ABA levels rose in incompatibly pollinated flowers, and fell in compatibly pollinated flowers, prior to pollen tube-ovule contact. Ethylene evolution remained stable in compatibly pollinated flowers and rose in incompatibly pollinated flowers. IAA concentrations increased in compatibly pollinated flowers, and remained stable in incompatibly pollinated flowers after pollination and subsequent to pollen tube-ovule contact.

Self-incompatibility (SI) in flowering plants is a biochemical recognition and rejection process that prevents self-fertilization. This process usually involves interactions between the male gametophyte (pollen grain) and the female sporophyte (stigma or style). Growth of a pollen tube can be inhibited on the stigma or in the style. Self-incompatibility in *Theobroma cacao* L. was first reported by Pound (1932). Investigations into the mechanism of SI in cacao by Knight and Rogers (1953, 1955) and Cope (1958, 1959, 1962) indicated that an incompatible pollination did not result in inhibition of pollen germination or pollen tube growth. Pollen tube growth rates in cacao are similar after compatible and incompatible pollination (Bouharmont, 1960; Cope, 1962; Knight and Rogers, 1955). Cope (1958) observed that sperm and egg failed to fuse in a percentage of ovules after an incompatible pollination, and flower abortion occurred. The *Theobroma* type of SI is unique in two ways: 1) the expression of the s-gene (recognition) apparently occurs only after the pollen tube and the ovules have come into contact, and 2) the rejection reaction results in

the abscission of the entire flower, not the deposition of callose in the pollen tube (de Nettancourt, 1977). Recent studies suggest recognition of self-pollen may occur earlier than previously reported. Aneja et al. (1994) found that pollen grains of the self-incompatible clone IMC 30 did not germinate after self-pollination unless the concentration of CO₂ was increased, and that SI could be overcome by the application of CO₂ to self-pollinated flowers of IMC 30. In nontreated, self-pollinated flowers, no fusion of the gametes was observed. Aneja et al. (1994) hypothesized that SI in *Theobroma* may be a result of two processes, one occurring during pollen germination (pollen-stigma interaction) and the second at the stage of gametic fusion. Warren (1994) and Warren et al. (1995) reported that isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH), and acid phosphatase (AP) activity were suitable predictors for self-incompatible *Theobroma* clones. These workers also noted that despite the initially higher pod-set of self-compatible clones, the number of pods at maturity was similar to that in self-incompatible clones.

Lanaud et al. (1987), using allozyme markers [IDH, MDH, and phosphoglucosyltransferase (PGMT)] and a recessive morphological marker (red axil spot) found that self-incompatibility in *Theobroma* could be overcome by mixing compatible with incompatible (self) pollen. This mentor effect implies that there is some substance on/in the compatible pollen grains that provides an acceptance message to the flower and masks the incompatible pollen. The mentor effect has also been reported by Glendinning (1960) and Opeke and Jacob (1967).

The incompatibility system in the closely related genus *Cola* behaves like a one-locus multiallelic sporophytic system acting on the

stigma (Jacob, 1980). With few exceptions, all members of a family have the same incompatibility system (Franklin et al., 1995), suggesting that recognition of self in *Theobroma* may be stigmatic. The pollen morphology also favors an early-acting sporophytic system (Zavada, 1984; Zavada and Taylor, 1986). Because of the conflicting results of mentor effects and sporophytic characteristics of SI in *Theobroma*, it is unclear when the recognition of self and the initiation of floral abscission occurs. The consequence of the recognition or the S-gene activation and rejection is detectable, e.g., by callose deposition in the pollen tubes. Callose can be easily detected using the fluorescent dye aniline blue (Williams et al., 1982). Callose may be considered a consequence of the rejection reaction rather than the primary cause of the arrest of pollen tube growth. Thus, recognition was assumed to occur prior to callose deposition. Floral abscission could be a consequence of the rejection reaction in *Theobroma*, with recognition preceding the first detectable changes in the hormonal environment that may lead to floral abscission. Abscission is usually a correlative, hormonally regulated process that involves the plant organ and the subtending abscission zone. Several plant hormones are thought to participate directly in the control of abscission, especially auxin (IAA), ethylene, and abscisic acid (ABA). Auxin and ethylene have had primary roles in the control of abscission (Addicott, 1983; Morgan, 1984; Osborne, 1991). Ethylene is the principal promoter of abscission, and IAA can inhibit or augment ethylene production and prevent or accelerate the abscission reaction. ABA typically increases in abscising tissues and may also participate in the abscission process. Suttle and Hulstran (1993) were able to show the dependence of ethylene-induced abscission in cotton on the presence of ABA. The purpose of the present study was to determine the endogenous levels of ethylene, IAA, and ABA at various times after compatible and incompatible pollination to detect the timing of self-recognition and initiation of the incompatibility reaction (floral abscission) in *Theobroma cacao*.

Materials and Methods

Research was carried out on clones at the *Theobroma cacao* germplasm collection of the U.S. Dept. of Agriculture Tropical Agricultural Research Station, Mayaguez, Puerto Rico. Flowers were hand-pollinated in the field on the first day of anthesis. Flower buds begin to swell the night prior to anthesis. The flowers open just prior to or after sunrise and the anthers dehisc shortly thereafter (Aneja et al., 1992; Vos, 1948). Immediately after pollination, short nylon stockings were placed over the flower cushions and taped to the trunk of the tree to prevent pollinator access. For the analysis of pollen tube growth rates, compatibly and incompatibly pollinated flowers were collected at 4-h intervals for 32 h after pollination. Flowers were fixed in 1 glacial acetic acid : 3 ethanol and stored in 70% ethanol.

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Pollen tube growth was monitored by aniline blue epifluorescence microscopy of whole pistil squashes (Williams et al., 1982).

Hormone analysis. Flowers were collected 0, 8, 24, 32, and 48 h after pollination for the quantitative analysis of IAA, ethylene, and ABA. Because of the limited material, flowers from different genotypes were pooled together. Since *Theobroma* is cauliflorous (the flowers are borne in tight clusters or cushions on the stem), the enclosure of individual flowers under field conditions for ethylene determinations is not practical. Therefore, 10 flowers per treatment were collected at each of the five time intervals and immediately placed into 13-mL gastight vials. After 8 h, a 5-mL gas sample was taken from the vial and transferred to an evacuated 75 × 13-mm vacutainer (Becton Dickinson). The ethylene content of these gas samples was analyzed with an SRI 8610 gas chromatograph (1-m Porapak Q column, flame ionization detector) calibrated with a 12.8-ppm ethylene standard.

Flowers collected for analysis of IAA and ABA were immediately frozen and lyophilized at the Univ. of Puerto Rico, Mayaguez. The extraction and purification method for IAA and ABA and their quantification by GC-SIM-MS were modified after Chen et al. (1988). Internal standards in the form $^{13}\text{C}_6$ -IAA (Cambridge Isotope Labs, 100 ng per sample) or $^2\text{H}_5$ -ABA [prepared according to Neill and Horgan (1987), 200 ng per sample] were added to 20–50 mg of dry material at the time of extraction. Tritiated IAA or ABA (25 nCi) was added as tracer. Extraction of tissue (3 × 2 mL, 30 mM imidazole/HCl pH 7.0 in 70% isopropanol) was followed by solid phase purification on 3 mL solid phase extraction columns (NH_2 for IAA and C_{18} for ABA). The eluate was dried in vacuo, taken up in 50% methanol (3 × 40 μL), and fractionated by reverse phase HPLC (Phenomenex, 00B-0351-EO) under isocratic conditions (mobile phase = 1% acetic acid, 35% methanol, and 64% water, 1 mL·min $^{-1}$). Fractions were collected at 1-min intervals, aliquots (10 μL) were counted, and the two fractions with the highest activity were pooled (typically 10–11 min for IAA and 22–24 min for ABA), dried in vacuo, taken up in methanol, and methylated with ethereal diazomethane (Cohen, 1984). Then the samples were dried under N_2 and taken up in 20 μL ethyl acetate for injection into the GC-MS.

Samples were injected into a Perkin-Elmer Autosystem GC with Qmass 910 equipped with a 15-m × 0.25-mm capillary column (J&W DB-1701) at 160 °C and ramped after 1 min 20 °C·min $^{-1}$ to 280 °C. The retention times for IAA and ABA were 4.1 and 5.2 min, respectively. Data were based on the mass fragments m/z 130 and 136 for IAA and m/z 190 and 193 for ABA. Corrections were made for nonlabeled molecules in the standards. Quantities were determined as amounts per 100 mg lyophilized material. Analyses were replicated three times for each sampling time, pollination condition, and hormone and averaged from several self-incompatible (2R, ICS 39, ICS 60) and self-compatible (SIAL 98) clones. Additional crosses were obtained from

clones of unknown compatibility status (6R, 15R). The results are reported as the mean of the three replicates $\pm$ SE.

Results

The gynoecium of *Theobroma cacao* is small (mean pistil length 3.69 $\pm$ 0.35 mm, n = 331). The ovary is superior and composed of five fused carpels. The style is hollow and is partially divided into five stigmatic lobes that usually adhere to one another. The mean length of the style is 2.27 $\pm$ 0.28 mm (n = 331). Pollen tube germination and growth was similar for compatible and incompatible pollen (Fig. 1A). Pollen grains started to germinate after 1.5 h in vitro (1% agar pollen germination medium, prepared according to Brewmaker and Kwack, 1963), and most pollen grains had germinated by 4 h. Pollen tubes in compatible and incompatible pollinations had grown to the base of the style within 8 h after pollination (Fig. 1A). The pollen tubes had engaged most ovules in the carpels by 16 h, and the delivery of the sperm cells and fertilization was completed between 16 and 24 h.

Abscission was greater in unpollinated than in incompatibly pollinated flowers. After 32 h, the abscission rate was significantly higher in incompatibly than in compatibly pollinated flowers (Fig. 1B).

Ethylene evolution at anthesis was below the detection limits of the system (<0.1 nL·L $^{-1}$) (Fig. 2A). Ethylene increased in all three treatments after 8 h. At 24 h, the levels of ethylene were significantly different in each of the treatments, unpollinated and incompatibly pollinated flowers exhibiting the highest levels. By 48 h, levels had dropped to those observed at 8 h. Compatibly pollinated flowers maintained the pre-24-h level throughout the experiment.

The endogenous level of IAA in unpollinated flowers was 26.2 $\pm$ 4.3 ng·100 mg $^{-1}$ dry mass. Little change was apparent at 8 h except for a small increase in the compatibly pollinated flowers (Fig. 2B), but after 24 h unpol-

linated flowers had the lowest level, incompatibly pollinated flowers were intermediate, and compatibly pollinated flowers contained the most IAA. Levels declined thereafter.

The content of ABA was much higher than that of IAA (μg vs. ng quantities), being 1.90 $\pm$ 0.25 μg ·100 mg $^{-1}$ dry mass at anthesis. After 8 h, the level in compatibly pollinated flowers decreased, while the levels in unpollinated and incompatibly pollinated flowers remained similar to the initial levels. After 24 h, ABA in compatibly pollinated and unpollinated flowers remained at the initial level, but the level in the incompatibly pollinated flowers rose substantially and peaked at 32 h. However, after 32 h the levels of ABA in the unpollinated flowers approached those in the incompatibly pollinated flowers (Fig. 2C).

Discussion

The data reveal a complex pattern in the changes of ethylene, ABA, and IAA after pollination of *Theobroma cacao* (Fig. 2). Based on the time course of these changes, one can distinguish a rapid response that occurred after pollination but before pollen–ovule contact and a second, slow response after pollen–ovule contact. In the first case, ABA rose in incompatibly pollinated flowers but not in unpollinated and compatibly pollinated flowers. The slow response was characterized by a dramatic increase in ABA in unpollinated flowers after 24 h and a smaller increase in compatibly pollinated flowers after 32 h. This increase occurred after a substantial lag phase but was still within the time in which abscission of unpollinated flowers occurred. The ABA response in unpollinated flowers may represent a slow response. The profiles of ethylene and IAA also indicated a delayed response, i.e., changes did not occur until 24 h, when the pollen tubes had engaged the ovules. Then ethylene increased dramatically in unpollinated and incompatibly pollinated flowers, while IAA increased substantially in compatibly pollinated flowers. Since the ethylene

Fig. 1. (A) Pollen tube growth in *Theobroma cacao* styles after compatible and incompatible pollinations. The 95% confidence interval of the regression line fit to the data is shown ($r^2 = 0.994$). (B) Abscission kinetics of *Theobroma cacao* for compatibly and incompatibly pollinated and for unpollinated flowers. Error bars represent the standard errors for five individually sampled replicates, each with $10 < n < 25$.

Fig. 2. (A) Total ethylene evolution from unpollinated flowers of *Theobroma cacao* styles after compatible and incompatible pollinations. Error bars represent the standard error of three replicates. The times refer to the collection times, but the data reflect the ethylene production in the vial for 8 h after collection. (B) Endogenous levels of free IAA in flowers of *Theobroma cacao* after compatible and incompatible pollinations and in unpollinated flowers. Error bars are the standard errors for three replicates. (C) Endogenous levels of free ABA in flowers of *Theobroma cacao* after compatible and incompatible pollinations and in unpollinated flowers. Error bars represent the standard errors for three replicates.

response occurred in unpollinated flowers, this peak may be an event initiated by aging rather than by pollination.

Because abscission is typically hormonally controlled, the fate of the flower may not be irreversibly determined until the pollen tube engages the ovule (12–20 h after pollination). The differential response of ABA to the type of pollination (compatible or incompatible) suggests that ABA is involved in the incompatibility reaction. In a compatibly pollinated flower with suppressed levels of ABA, an increase in IAA may delay senescence and inhibit abscission (Addicott, 1983; Addicott et al., 1955). Suppressed levels of ABA, elevated IAA concentrations, and unchanged, low levels of ethylene typically result in successful fruit development that is evident as early as 72 h after compatible pollination. After incompatible pollination, elevated levels of ABA are accompanied by high levels of ethylene and a small increase in IAA. This is indicative of a hormonal profile characteristic of impending floral abscission, which usually takes place within 48 h of pollination (Murray, 1975; Fig. 1B). However, the increase in IAA after self-incompatible pollination might be the cause of the transient growth and, compared with unpollinated flowers, delayed abscission.

Our data suggest that the incompatibility reaction in *Theobroma cacao* occurs in two steps. An early increase in ABA as a result of pollen–stigma interaction may be required for the subsequent abscission. A second response, after the pollen tube has contacted the ovule, affects the levels of IAA and ethylene to establish the hormonal conditions that determine the fate of the flower. Based on the correlative interactions of phytohormones, one would expect that reduced ethylene levels in incompatibly pollinated flowers could prevent ovule and flower abortion. This concept is validated by earlier reports in which application of the ethylene antagonist CO_2 (Burg and Burg, 1965)

after incompatible pollination resulted in seed set (Aneja et al., 1992). Based on the CO_2 effect on compatibly and incompatibly pollinated flowers, Aneja et al. (1994) also suggested that the incompatibility response in *Theobroma* is a two-step process, the first stage at pollination and the second stage at gametic fusion, as previously reported (Cope, 1962). Our data suggest a similar pattern in the hormonal response to compatible and incompatible pollination. The two-step incompatibility system may also account for the mentor effects observed by Glendinning (1960) and Lanaud et al. (1987). Based on the number of compatible pollinations and subsequent fusion events, sufficient auxin may be provided or induced by compatible pollen to counteract the increased ethylene levels due to “incompatible” pollen, and fruit development can progress. The dependence of ethylene-induced abscission on the presence of ABA was demonstrated by Suttle and Halstrand (1993) in cotton but may also apply for *Theobroma*. Thus, abscission is most likely to occur if high levels of both ethylene and ABA are present, which is what we observed in incompatibly pollinated flowers. In unpollinated flowers, the early ABA signal is missing and the increase in ethylene is not balanced by increased auxin levels. This hormonal condition also results in abscission. Thus, once the compatibility status is determined, the hormone changes can promote either fruit set or abscission.

If the concept developed so far is correct, it should be possible to delay abscission not only by application of CO_2 but also by application of synthetic auxins. The success of breeding programs to overcome self-incompatibility in *Theobroma* may be enhanced by the application of compounds that enhance levels of auxins or decrease ABA and/or ethylene. Such conditions may inhibit floral abscission and promote fruit development.

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