Plant Physiol. (1995) 108: 1405–1412

Pollination-Induced Ethylene in Carnation[1]

Role of Pollen Tube Growth and Sexual Compatibility

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The pollen-pistil interactions that result in the stimulation of ethylene production and petal senescence in carnation (*Dianthus caryophyllus* L.) flowers were investigated. Pollination of White Sim flowers with Starlight pollen elicited an increase in ethylene production by styles, leading to increased petal ethylene and premature petal senescence. In contrast, pollination with 87-29G pollen led to an early increase in ethylene production, but this was not sustained, and did not lead to petal senescence. Both Starlight and 87–29G pollen germinated on White Sim stigmas and their tubes grew at similar rates, penetrating the length of the style. Crosses between Starlight and White Sim led to the production of viable seeds, whereas 87–29G pollen was infertile on White Sim flowers. Pollination of other carnations with 87-29G elicited ethylene production and petal senescence and led to the production of viable seeds. These results suggest that physical growth of pollen tubes is insufficient to elicit a sustained increase in ethylene production or to lead to the production of signals necessary for elicitation of petal ethylene production and senescence. Rather, the cell-cell recognition reactions leading to sexual compatibility in *Dianthus* appear to play a role in this interorgan signaling after pollination.

Pollination of many angiosperm flowers sets in motion a series of developmental processes culminating in the ripening of fruit and dispersal of seeds. Several of these pollination-induced developmental events are initiated prior to fertilization and include processes such as ovary development and corolla senescence (Stead, 1992; O'Neill et al., 1993; Zhang and O'Neill, 1993). Given their role as visual cues, pollination-induced corolla senescence has likely evolved as a mechanism to deter further visits by pollinators. In addition, remobilization of cellular constituents from the senescing corolla to the developing ovary could provide an advantage to the survival of the species. The regulation of developmental events in the corolla by pollination requires a mechanism for interorgan communication. In this context the stigma acts as a sensing organ and communicates via a transmissible signal to the target tissue that a successful pollination has occurred (Larsen et al., 1993). In many species an increase in the production of ethylene is one of the earliest detectable biochemical events in the pollinated stigma, often occurring within the first few hours of pollination (Nichols 1977; Nichols et al., 1983; Hoekstra and Weges, 1986; Pech et al., 1987; Woodson et al., 1992; O'Neill et al., 1993). In the flowers of carnation (*Dianthus caryophyllus* L.) the early increase in ethylene by the stigma is followed by a sequential increase in ethylene production by the ovary and petals (Nichols et al., 1983; Woodson et al., 1992). There is considerable evidence that this "wave" of ethylene production serves to coordinate postpollination developmental events in various organs (Nichols et al., 1983; Hoekstra and Weges, 1986; Woodson et al., 1992; Zhang and O'Neill, 1993).

The nature of the pollen-pistil interactions that lead to increased ethylene biosynthesis is not fully understood. In orchid flowers, application of auxin to the stigma leads to an increase in ethylene production and stimulates ovary development in a manner similar to pollination (Zhang and O'Neill, 1993). Since orchid pollinia are known to contain auxin (Arditti, 1979), it has been suggested that pollen-derived auxin serves to induce ACC synthase activity, leading to increased production of ACC and ethylene (O'Neill et al., 1993). In striking contrast to orchids, carnations and petunia stigmas do not exhibit an increase in ethylene after application of auxin (Reid et al., 1984; Pech et al., 1987). Petunia pollen has been found to contain as much as 1500 nmol ACC g^{-1} (Hoekstra and Weges, 1986). It has been suggested that the diffusion of this ACC from the pollen onto the stigmatic surface could account for a portion of the ethylene released after pollination (Whitehead et al., 1983a; Hill et al., 1987; Singh et al., 1992). Consistent with this, the petunia stigma develops high ACC oxidase activity by anthesis and has the capacity to convert applied ACC to ethylene (Pech et al., 1987; Tang et al., 1994). The role of pollen-derived ACC in pollination-induced ethylene production remains unclear. Application of aminoethoxyvinylglycine, an inhibitor of ACC synthase, completely blocks pollination-induced ethylene in both petunia (Hoekstra and Weges, 1986) and carnation (Woltering et al., 1993) styles. Furthermore, pollination is associated with rapid induction of ACC synthase activity in the styles of both petunia (Pech et al., 1987) and carnation (Woodson et al., 1992). Taken together, these results suggest that increased synthesis of ACC is responsible for the increase in ethylene production after pollination.

[1] This work was supported by grants from the U.S. Department of Agriculture/National Research Initiative Competitive Grants Program (No. 92-37304-7867) and the American Floral Endowment. This is publication No. 14,582 of the Purdue University Office of Agricultural Research Programs.

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The germination and growth of pollen tubes through the styles of pollinated flowers may contribute to increased ethylene production. For example, γ-irradiated or foreign pollen, both of which fail to germinate, elicit early ethylene production by the stigma, but do not sustain this increased ethylene production or elicit postpollination responses in the corolla (Hoekstra and Weges, 1986). Self-incompatible pollination in *Petunia inflata* led to an early burst of ethylene, but this was not sustained as in compatible pollination (Singh et al., 1992). It has been proposed that the growing pollen tubes serve to mechanically wound the style, leading to sustained ethylene biosynthesis (Hoekstra and Weges, 1986). In addition, pollen and growing pollen tubes express a number of genes encoding peptides homologous with cell-wall-degrading enzymes, including polygalacturonase (Brown and Crouch, 1990) and pectate lyase (Wing et al., 1989). The action of these enzymes would be expected to release oligogalacturonide fragments that have been found to serve as elicitors of ethylene biosynthesis (Felix et al., 1991).

Although a role for pollen tube growth in the elicitation of ethylene production has been implicated by incompatible pollinations in petunia flowers (Singh et al., 1992), similar experiments have not been reported for carnation flowers. In contrast to the wet stigmas of petunia, the stigmatic surface of carnation is dry (Heslop-Harrison and Shivanna, 1977). In species with dry stigmas, incompatibility is often manifested by a failure of pollen to hydrate and germinate (Elleman et al., 1992). Self-incompatibility in *Dianthus* has not previously been described. In this paper we identify a carnation pollen source that exhibits a late-acting sexual incompatible response that is associated with normal pollen germination and tube growth, but a failure to set seed. Utilizing this pollen source, we show that the growth of pollen tubes through carnation styles is insufficient to elicit sustained ethylene production or the postpollination response of petal senescence. Our results suggest that reactions involved in the recognition of sexual compatibility are responsible for the stimulation of ethylene production.

MATERIALS AND METHODS

Plant Material

Plants of *Dianthus caryophyllus* L. were clonally propagated and grown under standard greenhouse conditions. White Sim and Starlight plants were originally obtained from Yoder Brothers (Barberton, OH). Rooted cuttings of 87–29G and 87–37G were obtained from Dr. Roger Uhlinger (University of Nebraska) and represent selections from a carnation breeding program. Flowers were harvested at anthesis and brought to the laboratory, and their cut stems were placed in deionized water. For pollination, stigmas were brushed with freshly dehisced anthers from either Starlight or 87–29G flowers. Stigmas from control flowers were brushed with a fine, camel hair brush to mimic the action of pollination.

Ethylene Measurement

The rate of ethylene production by styles and petals was measured by enclosing tissue isolated from intact flowers in gas-tight containers for 0.5 h. The headspace gas was sampled for ethylene using a gas chromatograph equipped with an activated alumina column and flame ionization detector. The column and detector temperatures were 80 and 120°C, respectively. The concentration of ethylene was determined by comparing the sample to an authentic ethylene standard.

Fluorescent and Scanning EM

For visualization of pollen tubes by light microscopy, pollinated styles were fixed in 10% formalin, 50% ethanol, and 5% glacial acetic acid for 4 h under vacuum. The tissue was subsequently treated with 5% NaOH for 16 h at 60°C, rinsed two times with deionized water, and placed in 50% bleach for 20 min. Pollen tubes were stained for deposited callose with 0.05% aniline blue (Eastman Kodak) at pH 9.0 and visualized using fluorescence microscopy (455-nm exciter filter, 515-nm barrier filter). For scanning EM, styles at different time points after pollination were fixed under vacuum for 16 h in 0.1 M $NaPO_4$ (pH 6.8) containing 2% glutaraldehyde and 2% paraformaldehyde. The samples were dehydrated in a graded ethanol series before being critical-point-dried and mounted on stubs. Specimens were sputter-coated with gold palladium and examined with a JEOL JSMj-840 scanning EM (Jeol Ltd., Tokyo, Japan) at an accelerating voltage of 7 kV.

Determination of Pollen ACC Content

ACC was extracted from pollen (25 mg) by grinding in 5 mL of 0.2 M TCA in a mortar and pestle. The extract was incubated on ice for 2 h and centrifuged to pellet insoluble debris. The supernatant was analyzed for ACC after conversion to ethylene according to the method of Lizada and Yang (1979).

RESULTS

Pollination-Induced Petal Senescence

Flowers of White Sim carnations lack functional anthers and are male sterile. We identified two clonal lines of

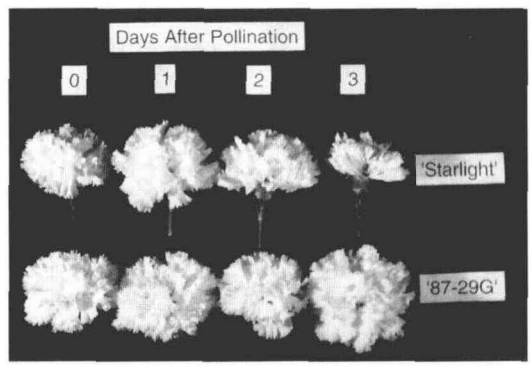

Figure 1. Senescence of White Sim carnation flowers after pollination with pollen from Starlight or 87–29G flowers.

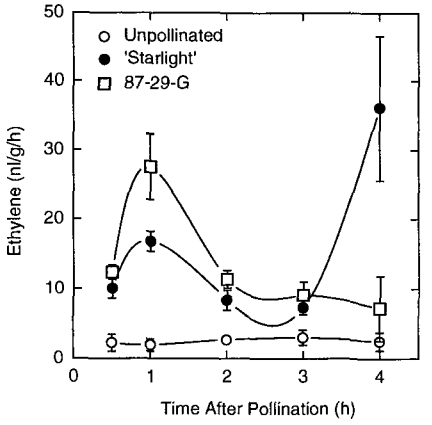

Figure 2. Ethylene production by White Sim styles at various times after pollination with pollen from Starlight or 87–29G flowers. The values are means ± SE of 10 flowers.

carnation (Starlight and 87–29G) maintained in our collection at Purdue University that produced sufficient pollen to serve as male parents in crosses with White Sim. Pollination with Starlight pollen elicited a typical response in White Sim flowers and led to the induction of petal senescence, as indicated by inrolling of petals 2 d after pollination (Fig. 1). In contrast, pollination with 87–29G pollen failed to elicit this response (Fig. 1), and flowers exhibited a vase-life similar to that of unpollinated flowers (data not shown).

Pollination-Induced Ethylene Production

An early response of carnation flowers to pollination is the induction of ethylene biosynthesis by styles (Nichols et al., 1984; Woodson et al., 1992). We examined the production of ethylene by White Sim styles at early time intervals after pollination with Starlight or 87–29G pollen. Styles of flowers pollinated with either Starlight or 87–29G produced elevated ethylene as early as 30 min after pollination (Fig. 2). This increased rate of ethylene production decreased 1 h after pollination, but subsequently increased dramatically only in styles of flowers pollinated with Starlight pollen. Subsequent analysis of ethylene production by both styles and petals over extended time intervals after pollination revealed that only Starlight pollen elicited a sustained increase in ethylene production (Fig. 3). Taken together with the early time point data presented in Figure 2, ethylene production by carnation styles after pollination with Starlight pollen is defined by three distinct peaks. The first peak (15–20 nL g^{-1} h^{-1}) occurs within the first 2 h after pollination. The second peak (150–200 nL g^{-1} h^{-1}) of ethylene production occurs approximately 12 h after pollination, and the third peak (300–350 nL g^{-1} h^{-1}) is sustained for at least 24 h, during which time the styles show visible signs of deterioration. Increased ethylene production by petals after pollination with Starlight pollen was apparent beginning 24 h after pollination and increased through 48 h (Fig. 3), at which time visible symptoms of senescence were apparent (Fig. 1). These results suggest that the early increase in ethylene production exhibited by styles pollinated with 87–29G pollen is not sufficient to sustain increased ethylene production by the style or to elicit a response of increased ethylene production by the petals.

Pollen ACC Content

We examined the ACC content of Starlight and 87–29G pollen in an attempt to determine if differences could account for the lower level of ethylene produced by 87–29G-pollinated styles. Starlight pollen contained 4.03 ± 0.18 nmol g^{-1} ACC and 87–29G pollen contained 3.01 ± 0.53 nmol g^{-1} ACC. Although somewhat lower in 87–29G, these differences in ACC content do not appear sufficient to account for the differences in ethylene production by pollinated styles.

Pollen Germination and Tube Growth

To begin to characterize the pollen-pistil interactions responsible for pollination-induced ethylene, we examined the germination of Starlight and 87–29G pollen on White Sim stigmas by scanning EM. *D. caryophyllus* flowers are characterized by having two to four separate styles with a long adaxial stigmatic surface. The receptive stigmatic surface of each style has many elongated unicellular papillae concentrated in distinct ridges along the entire length of the style (Fig. 4, A and B). This is a dry-type stigma, with no visible exudate (Heslop-Harrison and Shivanna, 1977).

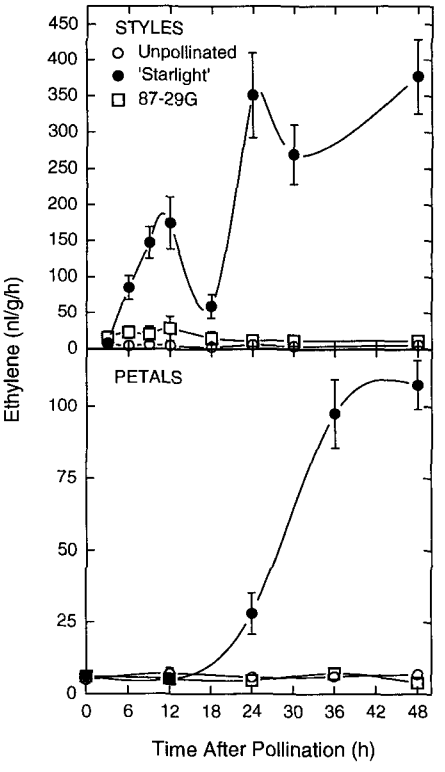

Figure 3. Ethylene production by White Sim styles and petals at various times after pollination, with pollen from Starlight or 87–29G flowers. The values are means ± SE of 10 flowers.

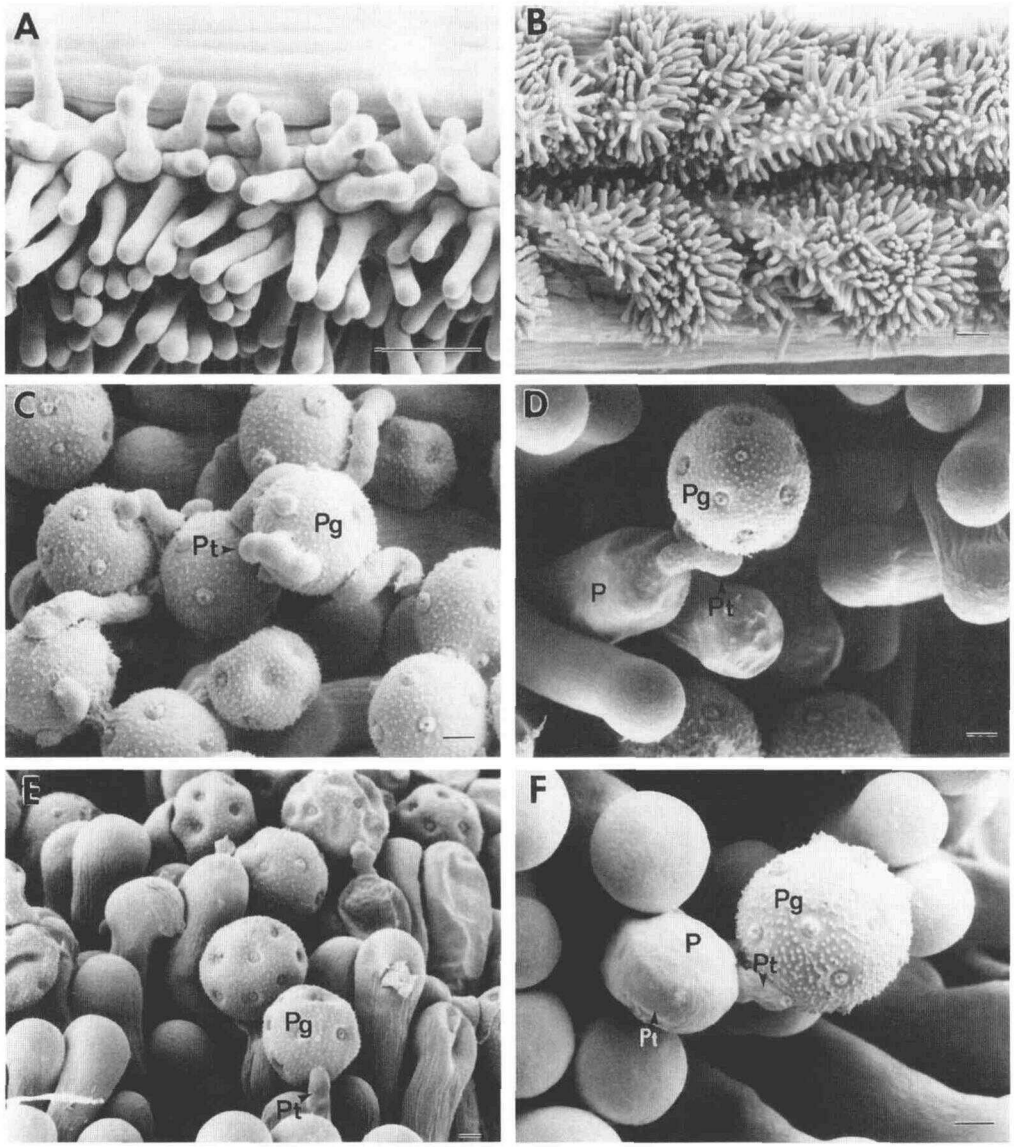

Figure 4. Germination and growth of pollen from Starlight or 87–29G flowers on White Sim styles. Styles were fixed and observed by scanning EM. P, Papilla; Pg, pollen grain; Pt, pollen tube. A, Receptive White Sim style showing unicellular papillae along the stigmatic surface. Bar = 100 μm. B, Receptive White Sim style showing the arrangement of papillae in ridges along the stigmatic surface. Bar = 100 μm. C, Germination of Starlight pollen 5 h after pollination of White Sim styles. Bar = 10 μm. D, Penetration of a White Sim papilla by a Starlight pollen tube. Bar = 10 μm. E, Germination of 87–29G pollen 5 h after pollination of White Sim styles. Bar = 10 μm. F, Penetration of a White Sim stigmatic papilla by a 87–29G pollen tube. Bar = 10 μm.

Examination of styles 5 h after pollination revealed that most Starlight pollen grains had germinated (Fig. 4C). Penetration of the papillae by Starlight pollen tubes was clearly visible at this time. The path taken by pollen tubes appeared similar to that of other dry-type stigmas exemplified by *Arabidopsis thaliana* (Elleman et al., 1992), in which the tube penetrates the stigmatic cuticle and enters the space between two cell wall layers (Fig. 4D). Pollen from 87–29G flowers germinated on the stigmatic surface of White Sim styles within 5 h of pollination (Fig. 4E) and appeared to penetrate papillae cells in a manner similar to Starlight pollen (Fig. 4F).

In an attempt to determine the growth characteristics of pollen tubes, pollinated White Sim styles were stained with analine blue and callose deposits in growing pollen tubes were visualized by fluorescence microscopy. The average length of Starlight and 87–29G pollen tubes at different times after pollination is shown is Figure 5. These data reveal that both Starlight and 87–29G pollen tubes grow at similar rates within White Sim styles, generally reaching the base of the style within 36 h after pollination. At this stage, pollinated White Sim styles were separated into the upper, middle, and lower thirds and photographed to reveal any differences in pollen tube morphology. No obvi-

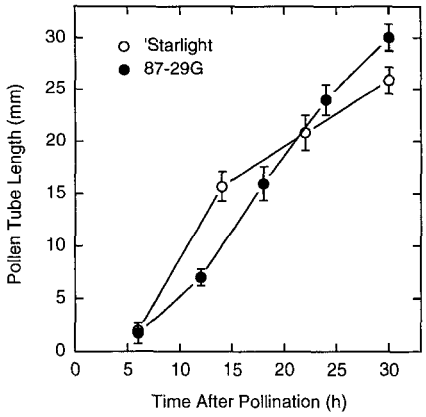

Figure 5. Pollen tube growth through White Sim styles at various times after pollination with pollen from Starlight or 87–29G flowers. Pollinated styles were fixed and stained with aniline blue, and pollen tubes were visualized by fluorescent microscopy. The values are means ± SE of five individual observations.

ous differences in pollen tube morphology, including the deposition of callose plugs along the length of the tubes, were noted (Fig. 6). Taken together, these data suggest that differences in pollination-induced ethylene production between styles pollinated with Starlight or 87–29G pollen are not the result of a failure of 87–29G pollen to germinate and grow through White Sim styles.

Sexual Compatibility

In a separate experiment, we examined the sexual compatibility of these crosses on plants maintained in the greenhouse. Ovaries from White Sim flowers pollinated with either Starlight or 87–29G pollen were harvested 30 d after pollination and their seed number was assessed (Table I). Starlight pollen was sexually compatible with White Sim pistils, as indicated by the production of seed. This seed was viable, exhibiting greater than 90% germination. In contrast, pollination of White Sim with 87–29G did not lead to the production of any seed. When 87–29G was used as the male parent in a cross with another selection (87–37G), viable seeds were produced (Table I). In addition, pollination of 87–37G plants with 87–29G pollen led to the induction of premature petal senescence, and this was associated with a substantial increase in stylar ethylene production (Fig. 7).

DISCUSSION

In this study we showed that pollen from Starlight flowers was capable of stimulating a rapid and sustained increase in stylar ethylene production, which in turn led to increased production of ethylene by petals, inducing senescence. These results are consistent with previous reports that pollination of carnation stigmas leads to increased stylar ethylene production (Nichols et al., 1983; Woodson et al., 1992). In dramatic contrast, pollen from 87–29G flowers elicited a rapid increase in stylar ethylene production, but this was not sustained and failed to lead to premature petal senescence. We exploited the differences in responses to pollination between these two clonally propagated carnation genotypes to study the pollen-pistil interactions that result in increased ethylene production and premature petal senescence in pollinated carnation flowers. The nature of the pollen-pistil interaction that results in increased ethylene production has been investigated in several species. In orchids, auxin contained within the pollinia is thought to play a role in the early stimulation of ethylene production by pistils (Arditti, 1979; Zhang and O'Neill, 1993). Although we cannot rule out differences in auxin levels between Starlight and 87–29G pollen, others have shown that application of auxin to carnation stigmas does not elicit an increase in ethylene (Reid et al., 1984). In petunia flowers, pollen has been shown to contain as much as 1500 nmol ACC g^{-1} (Whitehead et al., 1983a; Hoekstra and Weges, 1986; Singh et al., 1992). The diffusion of this pollen-held ACC onto the stigma surface could account for a portion of the ethylene released after pollination. In carnation, the amount of pollen-held ACC is insufficient (<10 nmol g^{-1}) to account for the initial amounts of ethylene produced. For example, assuming pollination with 0.1 mg of pollen and a pollen ACC content of 10 nmol g^{-1}, this would give rise to only 0.022 nL of ethylene. Pollinated styles generally produce 1 to 2 nL of ethylene within the first 2 h of pollination. Also, differences in ACC content between Starlight and 87–29G pollen were not sufficient to account for the differences in postpollination ethylene production. A role for pollen-held ACC is further questioned by the finding that aminoethoxyvinylglycine, an inhibitor of ACC synthase, completely blocks pollination-induced ethylene in both petunia styles (Hoekstra and Weges, 1986) and carnation styles (Woltering et al., 1993). Furthermore, pollination is associated with a rapid induction of ACC synthase activity in the styles of petunia and carnation (Pech et al., 1987; Woodson et al., 1992). Taken together, these results clearly point to a role for pollen-induced ACC synthase in regulating the increased ethylene production.

Pollination of flowers with foreign (Whitehead et al., 1983b; Hoekstra and Weges 1986), γ-irradiated (Hoekstra and Weges, 1986), or self-incompatible pollen (Singh et al., 1992) has been shown to elicit an increase in ethylene production by pistil tissue. However, as with the early increase in ethylene exhibited by styles pollinated with 87–29G pollen reported here, these flowers fail to maintain this increased ethylene production over time. These results led others to conclude that the initial increase in ethylene production likely reflects a nonspecific pollen-pistil interaction, whereas sustained ethylene production requires the germination and growth of pollen tubes (Whitehead et al., 1983b; Hoekstra and Weges, 1986; Singh et al., 1992). For example, the growing pollen tubes could serve to mechanically wound the stylar tissue and lead to increased ethylene through a mechanism similar to production of wound-induced ethylene in other tissues (Hoekstra and Weges, 1986). Consistent with this, mechanical wounding of the stigmatic surface of petunia gives rise to increased ethylene in a manner similar to that produced by pollination (Gilissen and Hoekstra, 1984; Lovell et al., 1987). In contrast, we have not detected increased ethylene production by

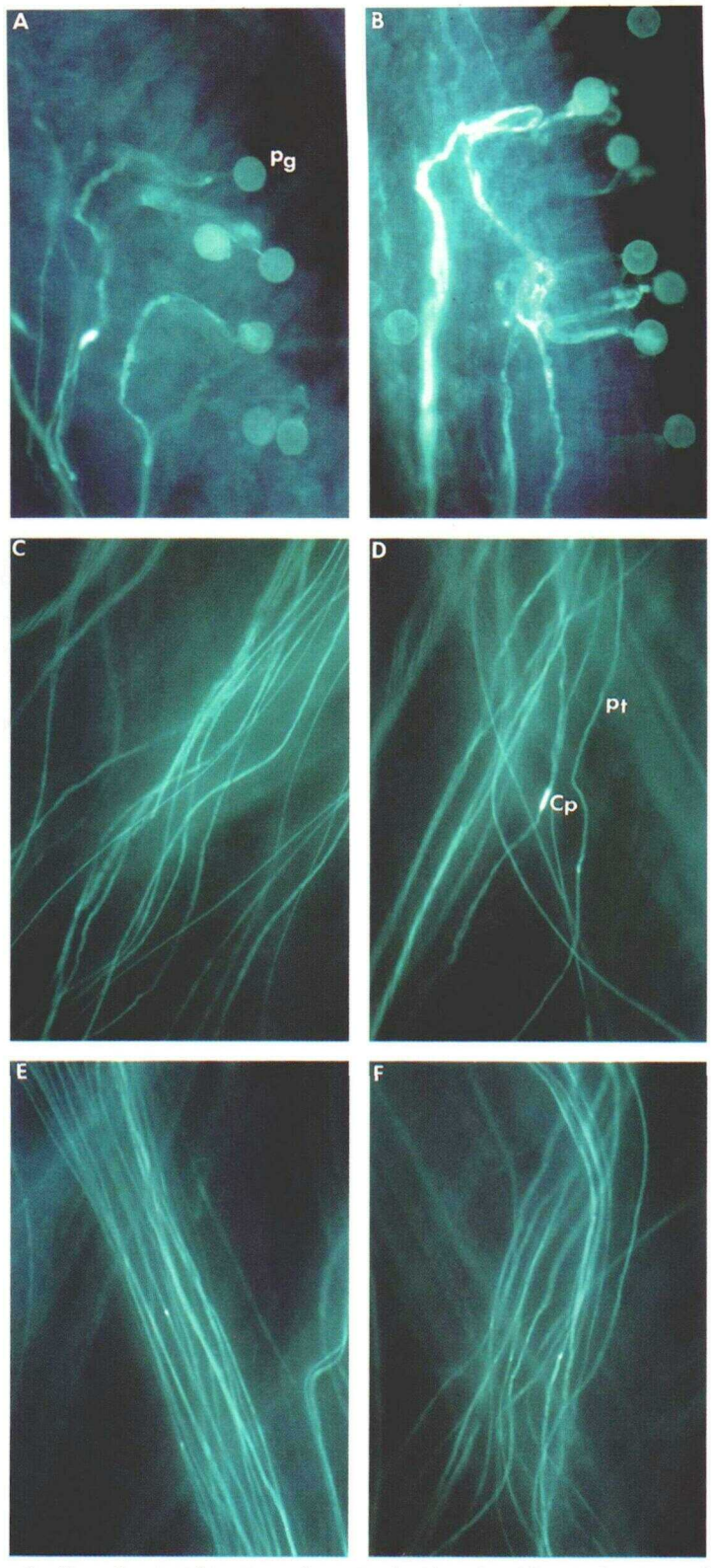

Figure 6. Visualization of pollen tubes in White Sim styles stained with aniline blue. Styles were pollinated with pollen from Starlight or 87–29G and observed 40 h later. Pg, Pollen grain; Pt, pollen tube; Cp, callose plug. A, Upper-third of White Sim style pollinated with Starlight pollen. B, Upper-third of White Sim style pollinated with 87–29G pollen. C, Middle-third of White Sim style pollinated with Starlight pollen. D, Middle-third of White Sim style pollinated with 87–29G pollen. E, Lower-third of White Sim style pollinated with Starlight pollen. F, Lower-third of White Sim style pollinated with 87–29G pollen.

Table I. *Seed number in carnation ovaries after pollination*

Female Parent × Male Parent	Seed Number[a]
White Sim × Starlight	18.6 ± 2.2
White Sim × 87–29G	0
87–37G × 87–29G	15.8 ± 3.6

[a] Data are the result of 10 individual crosses ± SE.

wounded carnation styles (Woodson et al., 1992), indicating that this may not be a universal response.

Recently, Singh et al. (1992) exploited the self-incompatible response of *Petunia inflata* to investigate pollination-induced ethylene. In this study, incompatible pollen elicited an early increase in ethylene, but this was not associated with a subsequent increase in ethylene production or petal senescence as it was in the compatible pollinations. They concluded that this differential response was likely due to differences in pollen tube growth between the two pollinations. Self-incompatible pollen tubes, in this case, cease growth approximately one-third down the length of the style. In an attempt to relate increased ethylene production to pollen germination and tube growth, we examined pollinated styles by scanning EM and found that the early increase in ethylene exhibited by both Starlight- and 87–29G-pollinated styles precedes the germination and growth of pollen tubes. Furthermore, we showed that the subsequent increase in ethylene between 6 and 18 h after pollination with Starlight was associated with the growth of pollen tubes through the stylar transmitting tract. Most surprising was the finding that the germination, growth, and morphology of 87–29G pollen tubes through the length of the White Sim style was not significantly different than those of the Starlight pollen, and therefore the lack of substantial ethylene cannot be attributed to limited pollen tube growth. This finding leads us to conclude that, in carnation, the physical growth of pollen tubes through the style is not sufficient to elicit a sustained increase in ethylene production or to lead to premature petal senescence.

The question raised by our results relates to the mechanism by which pollination leads to the sustained increase in ethylene production by styles and subsequent ethylene production by petals. Relative to this question is our finding that pollination of White Sim flowers with 87–29G pollen failed to elicit sustained ethylene production or lead to petal senescence, and this was associated with a failure to set seed. Therefore, 87–29G pollen is sexually incompatible with White Sim and leads to a similar response observed in incompatible crosses of *P. inflata* (Singh et al., 1992). *Dianthus* flowers are protandrous and typically outcross as a result of the temporal separation of anther dehiscence and pistil receptivity. To our knowledge a self-incompatible response has not been described for any members of the *Dianthus* genus. The incompatibility exhibited between White Sim and pollen from 87–29G is clearly not the result of a cessation of pollen tube growth, as it is in plants exhibiting both sporophytic and gametophytic incompatibility. Rather, the failure to set seed in this sexual cross likely results from a late-acting incompatible response. Late-acting incompatible responses have been described from a number of angiosperms (Seavey and Bawa, 1986) and are typically associated with ovular rejection of the male nuclei or early abortion of the zygote. In these cases pollen tubes grow normally through the styles but fail to set seed. In a cross with another carnation genotype, 87–29G pollen led to successful fertilization, suggesting a genetic component to this response. We have not been able to determine the parentage of 87–29G, as records are not available from the breeder. It will be interesting in future experiments to investigate the nature of this incompatible response in more detail.

The cross between 87–37G (female) and 87–29G (male) was associated with a dramatic and sustained increase in ethylene production by the styles and led to premature petal senescence. This result indicates that pollen factors necessary to elicit increased ethylene and petal senescence are not missing from 87–29G pollen, but rather it is the interaction between the pollen and pistil that is important in the regulation of this response. The nature of these interactions remains unclear; however, they are not likely to involve fertilization, since the increased ethylene production by both styles and petals precedes the arrival of the pollen tubes at the ovules. This observation has led others to propose that pollination is associated with the production of a transmissible substance in the style that moves down the pistil and into the petals, triggering a wave of ethylene production through these organs, leading ultimately to petal senescence (Nichols et al., 1983; Gilissen and Hoekstra, 1984; Hoekstra and Weges, 1986).

Our data suggest that the production of any ethylene-inducing transmissible signal is dependent on reactions associated with the perception of a compatible pollination. Linskens (1974) has previously reported that petunia ovaries exhibit increased sink strength within the first 24 h after a compatible pollination, which precedes the arrival of the pollen tubes. He further showed that this change in sink strength did not occur in incompatible crosses. In a search for the transmissible signal responsible for changes in ovary development after pollination, Linskens and Spanjers (1973) reported a change in voltage specifically in response to compatible pollination, suggesting that propa-

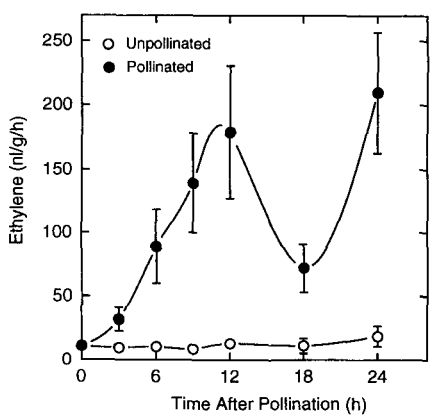

Figure 7. Ethylene production by 87–37G styles after pollination with pollen from 87–29G flowers. The values are means of 10 ± SE flowers.

gation of electrical signals may be involved in signaling a compatible pollination. Similar changes in electrical potential have been observed in pistils, specifically after compatible pollination in *Lilium* (Spanjers, 1981). Although the nature of the ethylene-inducible transmissible factor remains unclear, our data clearly show that pollen tube growth alone is insufficient to elicit the production of this signal.

Received December 27, 1994; accepted April 27, 1995.
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