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Temporal and Spatial Expression of 1-Aminocyclopropane-1-Carboxylate Oxidase mRNA following Pollination of Immature and Mature Petunia Flowers[1]

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Pollination of petunia (*Petunia hybrida*) flowers induces a rapid increase in ethylene production by styles, which subsequently leads to increased ethylene production by the corolla, inducing senescence. We have investigated the temporal and spatial expression of 1-aminocyclopropane-1-carboxylate (ACC) oxidase transcripts in petunia styles in an attempt to elucidate its role in increased ethylene biosynthesis following pollination. Previously, we reported that the development of petunia flowers was associated with increased ACC oxidase mRNA localized specifically in the stigmatic regions of the style (X. Tang, A.M.T. Gomes, A. Bhatia, W.R. Woodson [1994] Plant Cell 6: 1227–1239). The rapid increase in ethylene production by styles within the 1st h following pollination was correlated with the expression of ACC oxidase mRNAs during development. Pollination of petunia flowers prior to anthesis and the expression of ACC oxidase mRNA led to a substantial increase in ethylene production, but this was delayed by several hours in comparison with flowers at anthesis. This delayed increase in ethylene production by pollinated styles from immature flowers was associated with an increased ACC oxidase transcript abundance. Treatment with the ethylene action inhibitor 2,5-norbornadiene did not affect the early increase in ethylene production or the expression of ACC oxidase mRNAs. No differences in the rate of pollen germination or tube growth were detected when applied to stigmas from immature or mature flowers, indicating that the delay in ethylene production was likely the result of limited ACC oxidase activity. Localization of ACC oxidase mRNAs following pollination by in situ hybridization revealed an abundance of transcripts in transmitting tract tissue within 4 h of pollination of both immature and mature styles, in contrast to their localization in stigmatic cells during development.

Pollination leads to a rapid increase in ethylene production by the pistil and a subsequent wave of increased ethylene by other floral organs in several plant species, including petunia (*Petunia hybrida*) (Nichols, 1977; Pech et al., 1987; Larsen et al., 1993; O'Neill et al., 1993). It has been suggested that ethylene plays a regulatory role in postpollination developmental events, including ovary growth and development (O'Neill et al., 1993; Zhang and O'Neill, 1993), pigmentation changes (Woltering and Somhorst, 1990), corolla abscission (Stead, 1992), and petal senescence (Nichols, 1977; Nichols et al., 1983; Pech et al., 1987). Ethylene is synthesized from *S*-adenosylmethione by the enzymes ACC synthase and ACC oxidase (Kende, 1993). The development of petunia styles is associated with an increase in the activity of ACC oxidase (Pech et al., 1987) and the expression of ACC oxidase mRNAs (Tang et al., 1994) in the stigmatic tissue. At anthesis, the petunia stigma is capable of converting applied ACC to ethylene but does not produce significant ethylene until pollinated (Pech et al., 1987). This indicates that the increase in ethylene following pollination is likely due to an increased synthesis of ACC or the delivery of ACC to the stigma by the pollen. Petunia pollen contains significant levels of ACC (Whitehead et al., 1983), which upon delivery to the stigma is converted by the action of ACC oxidase to ethylene. However, the role for pollen-held ACC in pollination-induced ethylene has been questioned. Treatment of stigmas with aminoethoxyvinylglycine, an inhibitor of ACC synthase, prevents the increase in ethylene following pollination (Hoekstra and Weges, 1986), indicating that de novo synthesis of ACC is necessary for the increase in ethylene. In addition, pollination leads to a rapid increase in ACC synthase activity in pollinated petunia styles (Pech et al., 1987). These results suggest that the elicitation of ethylene biosynthesis by pollen involves an increase in the synthesis of ACC but do not necessarily rule out a role for pollen-held ACC in this response.

We are interested in the regulation of ethylene biosynthesis in flowers, particularly following pollination and during senescence. In this regard we have initiated a study of the ACC oxidase gene family from petunia and its regulation (Wang and Woodson, 1992; Tang et al., 1993, 1994). The petunia ACC oxidase gene family consists of four members, three of which are actively transcribed. The *ACO1* gene is expressed primarily in corolla tissue during senescence and is induced by ethylene in all floral organs. Pistil development is associated with increased expression of all active ACC oxidase genes, and *ACO3* and *ACO4* are specifically localized to this tissue. Here we have investigated the temporal and spatial expression of ACC oxidase mRNAs in styles following pollination. We have exploited the ability of petunia to be pollinated in

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Abbreviation: NBD, 2,5-norbornadiene.

the bud stage (Herrero and Dickinson, 1980) prior to the expression of ACC oxidase mRNA to investigate the patterns of ethylene production and ACC oxidase expression in the absence of developmentally regulated ACC oxidase activity.

MATERIALS AND METHODS

Petunia hybrida cv V/R plants were grown to flowering under greenhouse conditions. Developmental stages of flowers were previously described (Tang et al., 1994) with stage 5 representing anthesis. Flowers at stages 4 and 5 were emasculated prior to anther dehiscence. A small window was cut out of the corolla tissue from younger flowers to facilitate pollination, and similar treatments were imposed on control flowers. All pollinations were performed on flowers left on the plant.

Ethylene Measurement

Ethylene production was measured by enclosing five styles in a 5-mL gas-tight container for 10 min, after which the head-space gas was analyzed for accumulated ethylene by GC. The chromatograph was equipped with an activated alumina column and a flame ionization detector with an oven temperature of 80°C and a detector temperature of 100°C. Nitrogen was used as a carrier gas. The concentration of ethylene was determined by comparing the peak with a 1-μL/L certified standard (Matheson Gas Products, Secaucus, NJ).

RNA Gel Blot Analysis

Stylar tissue was frozen in liquid N_2 and stored at −80°C prior to extraction of RNA. Total cellular RNA was isolated from frozen tissue as previously described (Lawton et al., 1990), and the concentration was determined by spectrophotometry. Ten-microgram samples of total RNA were separated by electrophoresis through a 1% (w/v) agarose gel containing 2.2 M formaldehyde. The separated RNAs were transferred to supported nitrocellulose membrane filters (Schleicher & Schuell) and cross-linked with a Stratalinker (Stratagene) controlled UV light source. The full-length ACC oxidase cDNA pACO1 (Wang and Woodson, 1992) was used as a hybridization probe following labeling with [^{32}P]dATP by random priming. Prehybridization, hybridization, and washing conditions were carried out as previously described (Tang et al., 1994). The hybridized membranes were exposed to XAR-5 film (Kodak) with an intensifying screen at −70°C.

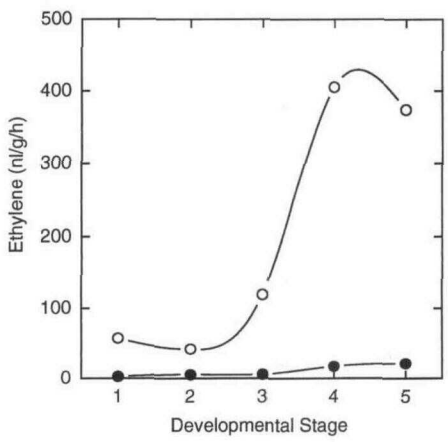

Figure 1. Ethylene production by pollinated stigma/styles at different stages of floral development. Flowers were pollinated at various stages of development, and stigma/styles were removed from flowers 1 h after pollination. Stage 1 flowers (1) are defined by a bud length of 18 mm from the base of the receptacle to the tip of the corolla. Stage 2 flowers (2) are 30 mm in length. Stage 3 flowers (3) are 45 mm with a fully elongated style. Stage 4 (4) represents flowers the day before anthesis, and stage 5 flowers (5) are at anthesis, exhibiting anther dehiscence and stigmatic secretion. Ten stigma/styles from each stage were enclosed in a 6-mL vial and capped and ethylene was measured after 10 min. ○, Pollinated; ●, unpollinated.

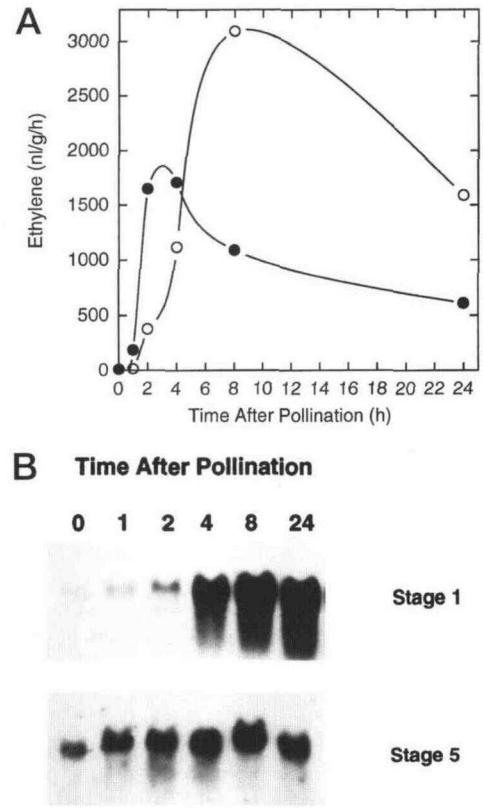

Figure 2. A, Ethylene production by stigma/styles following pollination of immature (stage 1, ○) and mature (stage 5, ●) flowers. Flowers were pollinated on the plant and harvested for ethylene measurement at the various times indicated. Ten stigma/styles were enclosed in a 6-mL vial and capped, and ethylene was measured after 10 min. B, Expression of ACC oxidase mRNA in stigma/styles following pollination of immature (Stage 1) and mature (Stage 5) flowers. Total RNA was extracted from stigma/styles at various times following pollination and subjected to RNA gel blot analysis using pACO1 as a hybridization probe.

In Situ Hybridization

Pollinated styles were fixed in 2% glutaraldehyde in 0.05 M KPO_4 buffer (pH 7.0) at room temperature for 3 h. Tissues were dehydrated and embedded as previously de-

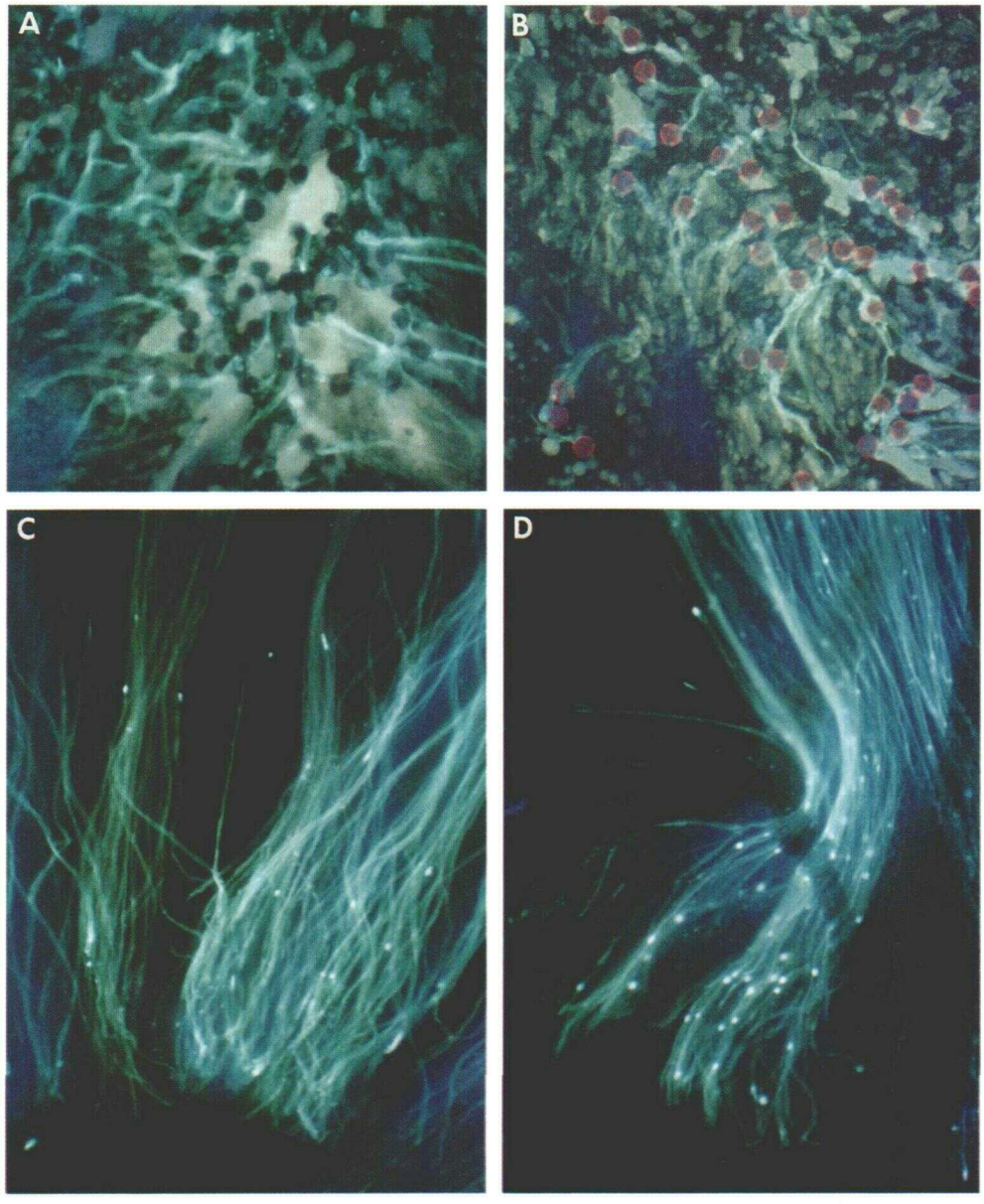

Figure 3. Germination and growth of pollen tubes in petunia stigma/styles following pollination of immature (stage 1) and mature (stage 5) flowers. Pistils from flowers at stage 1 (A and C) or stage 5 (B and D) were self-pollinated, fixed, and stained with aniline blue as described in "Materials and Methods." Stained pollen tubes were visualized by fluorescent microscopy. Stigmas were fixed and stained 1 h after pollination (A and B) or the lower half of the style was stained 24 h after pollination (C and D).

scribed (Cox and Goldberg, 1988), and the sections were hybridized to digoxigenin-11-UTP-labeled pACO1 sense and antisense probes as previously described (Tang et al., 1994).

Callose Staining of Pollen Tubes

Pollinated styles were fixed in ethanol:acetate (3:1, v/v) overnight, rinsed with distilled water, and incubated in 1 N NaOH at room temperature for 8 h. Tissue samples were rinsed in 0.1 M KPO_4 (pH 9.0), stained in 0.1% aniline blue in 0.1 M KPO_4 (pH 9.0), and squashed on a slide with a coverslip. Pollen tubes were visualized under a fluorescence microscope and photographed with Ektachrome 400 ASA film (Kodak)

RESULTS

Flower Development and Pollination-Induced Ethylene

The development of petunia flowers is associated with the increased expression of ACC oxidase genes a few days prior to anthesis (Tang et al., 1994). These ACC oxidase transcripts are localized in the stigmatic cells of the pistil (Tang et al., 1994), which exhibits high ACC oxidase enzyme activity at anthesis (Pech et al., 1987). Petunia stigmas

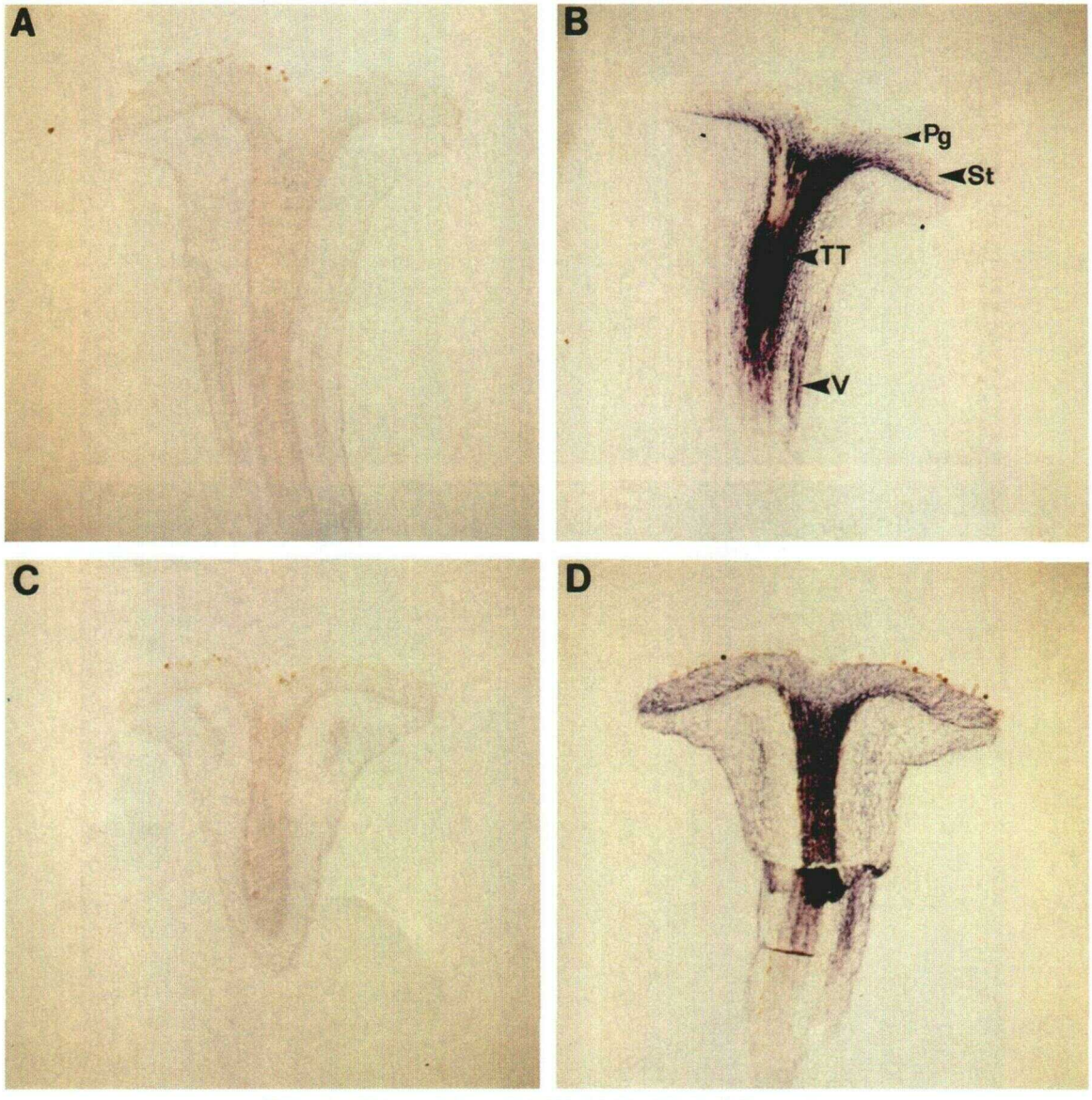

Figure 4. (Figure continues and legend appears on facing page.)

are receptive to pollination in the bud stage (Herrero and Dickinson, 1980) prior to the accumulation of ACC oxidase mRNA (Tang et al., 1994) or activity (Pech et al., 1987). To determine whether the capacity for pollen to elicit a rapid increase in ethylene was correlated with this developmental regulation of ACC oxidase expression, flowers were pollinated at various stages leading up to anthesis (stage 5). The results of this analysis are shown in Figure 1. Pollination led to an increase in ethylene production within 1 h at all stages. The rapid elicitation of ethylene increased with flower development beginning at stage 3, reaching its highest levels by stage 4 just prior to anthesis. This result indicates that the early increase in ethylene production following pollination correlates with the developmental expression of ACC oxidase mRNA, which begins at stage 3 (Tang et al., 1994).

To further investigate the regulation of pollination-induced ethylene, stage 1 and stage 5 flowers were pollinated and stigma/style ethylene production was followed for 24 h; these results are shown in Figure 2A. Pollination of flowers at anthesis (stage 5) led to a rapid increase in ethylene production, reaching a maximum rate 4 to 5 h after pollination and subsequently declining. In contrast, pollination-induced ethylene production by stigma/styles of stage 1 flowers was delayed, reaching maximum rates 8 h after pollination. The peak rate of ethylene production in stage 1 stigma/styles was substantially greater than that in flowers pollinated at anthesis. The level of ACC oxidase mRNA in stage 1 and stage 5 stigma/styles following pollination was assessed by RNA gel blot analysis using the pACO1 cDNA as a hybridization probe, which detects transcripts from the *ACO1*, *ACO3*, and *ACO4* genes (Tang et al., 1994). Consistent with previous results (Tang et al., 1994), ACC oxidase transcripts were more abundant in stigma/styles from stage 5 flowers as compared with stigma/styles from flowers at stage 1 (Fig. 2B). Pollination of stage 1 flowers led to a dramatic increase

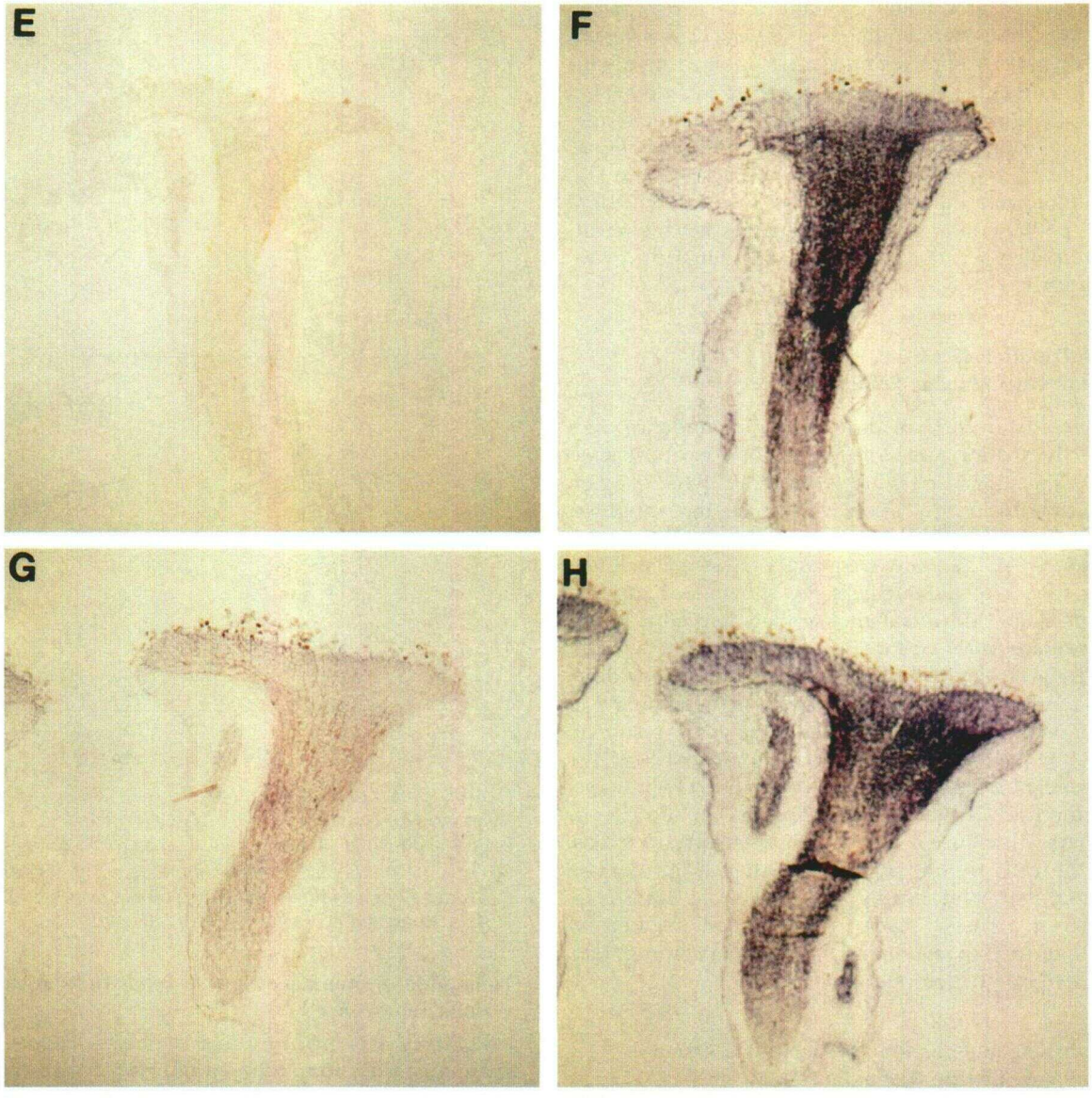

Figure 4. (Continued from facing page.) In situ localization of ACC oxidase mRNA in sigma/style tissue of petunia flowers following pollination. Stigma/style tissue isolated from stage 1 or stage 5 flowers after self-pollination were fixed, embedded in paraffin, cut into 10-μm longitudinal sections, and hybridized with digoxigenin-labeled pACO1 RNA probes. Hybridization was detected with anti-digoxigenin antibodies conjugated to alkaline phosphatase and was visualized as a blue color following development. Pg, St, TT, and V indicate pollen grain, stigma, transmitting tissue, and vascular tissue, respectively. A, Stigma/style tissue from stage 1 flowers hybridized with the sense pACO1 probe 4 h after pollination. B, Stigma/style tissue from stage 1 flowers hybridized with the antisense pACO1 probe 4 h after pollination. C, Stigma/style tissue from stage 1 flowers hybridized with the sense pACO1 probe 24 h after pollination. D, Stigma/style tissue from stage 1 flowers hybridized with the antisense pACO1 probe 24 h after pollination. E, Stigma/style tissue from stage 5 flowers hybridized with the sense pACO1 probe 4 h after pollination. F, Stigma/style tissue from stage 5 flowers hybridized with the antisense pACO1 probe 4 h after pollination. G, Stigma/style tissue from stage 5 flowers hybridized with the sense pACO1 probe 24 h after pollination. H, Stigma/style tissue from stage 5 flowers hybridized with the antisense pACO1 probe 24 h after pollination.

in ACC oxidase mRNA beginning 4 h after pollination. This increase in ACC oxidase mRNA was associated with the increased production of ethylene, indicating that the delay in ethylene production following pollination of stage 1 stigma/styles was due in part to the low level of ACC oxidase expression. In contrast to immature flowers, pollination of stage 5 flowers led to a moderate increase in ACC oxidase transcript abundance.

Pollen Germination and Tube Growth

It has been suggested that germination and growth of pollen tubes play a role in the elicitation of ethylene production by petunia styles (Hoekstra and Weges, 1986). We examined the germination and growth of pollen tubes on stigmas of stage 1 and stage 5 flowers to determine whether differences could account for the delay in pollination-

induced ethylene production. Staining of callose deposits within pollen tubes with aniline blue revealed that pollen germination was evident within the 1st h following application to the stigma of both stage 1 and stage 5 flowers (Fig. 3, A and B). Furthermore, pollen tubes penetrated the length of the style within the first 24 h following pollination of both stage 1 and stage 5 flowers (Fig. 3, C and D). These data indicate that the delay in ethylene production following pollination of stage 1 flowers was not the result of delayed pollen germination or differences in the growth of pollen tubes.

Spatial Distribution of ACC Oxidase mRNAs following Pollination

The increase in ACC oxidase mRNA abundance and enzyme activity during pistil development is localized specifically to the stigmatic region (Pech et al., 1987; Tang et al., 1994). In situ hybridization with sense and antisense pACO1 probes was used in an attempt to assess the spatial distribution of ACC oxidase transcripts following pollination. The results of this analysis are shown in Figure 4. Styles from stage 1 flowers do not contain detectable levels of ACC oxidase mRNA as determined by in situ hybridization (Tang et al., 1994). The increase in ACC oxidase mRNA abundance in stage 1 stigma/styles beginning 4 h following pollination (Fig. 2B) was largely the result of expression by the transmitting tract tissue of the styles (Fig. 4B). The level of ACC oxidase mRNA remained high in the transmitting tract 24 h after pollination, but in addition, a more general distribution of ACC oxidase expression was seen in the cells of the stigma and style (Fig. 4D). In contrast to the strict localization of ACC oxidase mRNAs to the stigma at anthesis (stage 5), pollination resulted in expression of ACC oxidase in cells of the transmitting tract within 4 h of pollination (Fig. 4, F and H).

Role of Ethylene in Pollination-Induced ACC Oxidase Expression and Ethylene Production

Petunia pollen contains a significant amount of ACC that may be converted to ethylene by stigmatic ACC oxidase (Whitehead et al., 1983; Singh et al., 1992; Tang et al., 1994). We reasoned that this early increase in ethylene may serve as an initial inducer, enhancing the expression of ethylene biosynthetic pathway genes and thus the production of ethylene. To investigate this possibility, flowers were treated with NBD, a competitive inhibitor of ethylene action (Wang and Woodson, 1989), immediately after pollination, and the production of ethylene and expression of ACC oxidase was monitored. In both immature and mature flowers, ethylene production by stigma/styles was not affected by NBD treatment during the first 8 h following pollination (Fig. 5); however, it was inhibited by NBD treatment 24 h after pollination of both immature and mature stigma/styles. Consistent with the failure of NBD to inhibit the early increase in ethylene production following pollination, the expression of ACC oxidase mRNA was not reduced by this treatment during the first 8 h following pollination (Fig. 6).

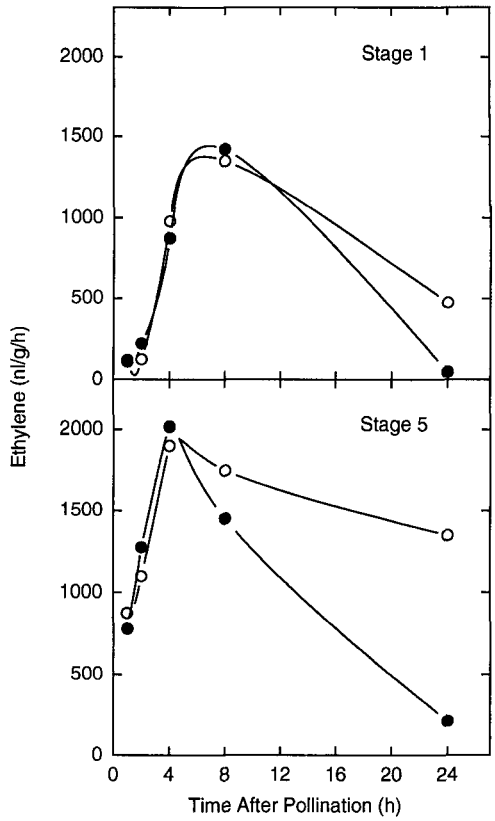

Figure 5. Effect of NBD on ethylene production by petunia stigma/styles following pollination. Flowers were pollinated at stage 1 or stage 5 and held in an atmosphere of 2500 parts per million of NBD (●) or air (control, ○). Ethylene production by stigma/styles was measured at various times following pollination and treatment with NBD as described in "Materials and Methods."

Pollination of Immature Flowers Leads to Premature Corolla Senescence

Pollination of mature petunia flowers reduces the life span of corollas from 6 to 7 d to less than 2 d (Pech et al., 1987; Singh et al., 1992); therefore, we examined the effects of pollination on corolla development and senescence of immature flowers. Flowers were pollinated at stage 1 and left on the plant to monitor their development. Whereas unpollinated flowers developed normally, opening within 3 to 4 d, pollinated flowers exhibited limited corolla growth, showing signs of senescence (wilting and drying) within 2 d of pollination (Fig. 7).

DISCUSSION

Pollination initiates a series of developmental processes essential for the successful reproduction of many angiosperm species. These processes include localized cell death in the stylar transmitting tissue (Herrero and Dickinson, 1980), continued ovary and ovule development (Zhang and O'Neill, 1993), and corolla senescence (Stead, 1992). One of the earliest detectable biochemical events following pollination is the increased production of ethylene by the pistil (Pech et al., 1987; O'Neill et al., 1993; Larsen et al., 1995). In several cases this increased ethylene serves to coordinate

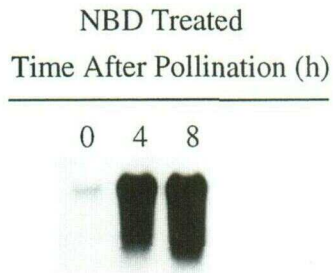

Figure 6. Expression of ACC oxidase mRNAs in stigma/styles from immature (stage 1) flowers following pollination and treatment with NBD. Total RNA was extracted from pollinated stigma/styles incubated in an atmosphere of 2500 parts per million of NBD at various times following pollination and subjected to RNA gel blot analysis using pACO1 as a hybridization probe.

postpollination development (Zhang and O'Neill, 1993; Larsen et al., 1993). The nature of the pollen-pistil interactions leading to the induction of ethylene synthesis is largely unknown. We previously reported that petunia flower development was associated with the accumulation of ACC oxidase transcripts specifically in the stigma, such that at anthesis pistils were capable of converting ACC to ethylene (Tang et al., 1994). In this study we have further investigated the role of ACC oxidase in pollination-induced ethylene by examining the temporal and spatial patterns of ACC oxidase expression following pollination of flowers in the bud stage, prior to the developmental expression of ACC oxidase. Other researchers have clearly shown that immature petunia flowers are capable of receiving pollen (Herrero and Dickinson, 1980; Sivanna and Sastri, 1981). Whereas pollination of mature flowers led to a rapid increase in ethylene production by stigma/styles, ethylene production was delayed in immature flowers. Timing of the increase in ethylene production was associated with increased abundance of ACC oxidase mRNAs. Taken together with the previous finding that stigmas from immature flowers exhibit low ACC oxidase activity (Pech et al., 1987), these results clearly demonstrate a role for increased ACC oxidase gene expression in the rapid production of ethylene following pollination.

In many tissues ethylene is under autocatalytic regulation, i.e. ethylene stimulates its own synthesis (Yang and Hoffman, 1984; Kende, 1993). Consistent with this, ethylene treatment has been shown to lead to increased levels of both ACC synthase (Park et al., 1992) and ACC oxidase (Holdsworth et al., 1987; Woodson et al., 1992; Tang et al., 1994; Peck and Kende, 1995). Treatment of immature petunia flowers with ethylene was shown to lead to increased ACC oxidase mRNAs in pistil tissue (Tang et al., 1994). Furthermore, the localization of ACC oxidase mRNAs was not limited to the stigma as during flower development but, rather, was distributed throughout the stigma and stylar tissues. These results indicate that most of the cells in the pistil are able to respond to ethylene by increased expression of ACC oxidase mRNA. In this study we have shown that the expression of ACC oxidase in stigma/styles following pollination of immature flowers is not the result of increased ethylene. Treatment with the ethylene action inhibitor NBD did not reduce the level of ethylene production or the expression of ACC oxidase mRNA during the first 8 h following pollination. Similarly, O'Neill et al. (1993) reported that pollination of orchids led to increased ACC oxidase mRNAs in the stigma and that their accumulation was not prevented by treatment with NBD. The maintenance of elevated ethylene appears to involve an autoenhancement of ethylene synthesis, which is evident by inhibition of ethylene by NBD 24 h following pollination of immature petunia pistils. These results indicate that increased ethylene by pollinated petunia pistils involves both ethylene-independent factors derived from the pollen and ethylene-dependent factors in the pollinated pistil.

Currently, the nature of the pollen-derived factors that are capable of eliciting increased ACC oxidase expression and ethylene production by pollinated pistils is not clear. It

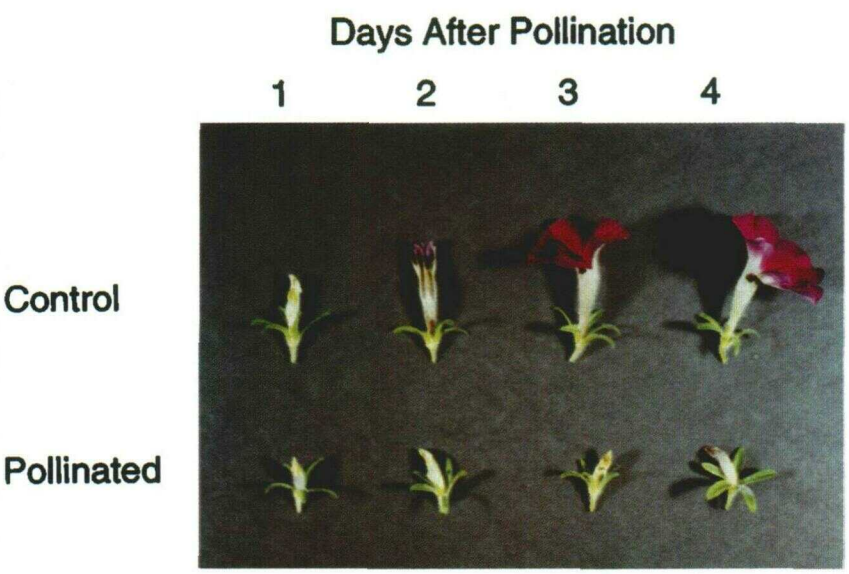

Figure 7. Effect of pollination of immature (stage 1) flowers on corolla development and senescence. The top series of flowers were left unpollinated and the bottom series of flowers were pollinated at stage 1.

has been suggested that auxin from the pollinia in orchids plays a role in the stimulation of ethylene production by pistils (Zhang and O'Neill, 1993). In contrast, petunia pollen contains little auxin (Stead, 1992), and auxin applied to petunia stigmas failed to elicit an increase in ethylene production (Pech et al., 1987). The penetration of the stigma by germinating pollen tubes and further growth through the transmitting tissue of the style also has been suggested as a mechanism by which ethylene synthesis is stimulated. The timing of ACC oxidase expression and localization in the transmitting tissue found in this study is consistent with a role for pollen tubes in the elicitation of ethylene production. Germination and growth of pollen tubes are associated with the release of cell-wall-degrading enzymes (Brown and Crouch, 1990), the products of which have been shown to function as elicitors of ethylene biosynthesis in many tissues (Felix et al., 1991).

In other species, a role for growing pollen tubes in the elicitation of ethylene production has been questioned. Pollination-induced ethylene by orchid pistils occurs within 6 h of pollination, well before the germination and growth of pollen tubes (O'Neill et al., 1993). In carnation, the growth of pollen tubes was found to be insufficient to elicit a sustained increase in ethylene production by pistils (Larsen et al., 1995). Rather, reactions associated with the perception of a compatible pollen appear to be involved in the elicitation of ethylene production by pistil tissue (Larsen et al., 1995). Petunia pollen contains significant levels of ACC, which has been suggested to play a role in pollination-induced ethylene (Whitehead et al., 1983; Singh et al., 1992). The role of ACC has been questioned, since ACC synthase inhibitors prevent pollination-induced ethylene (Hoekstra and Weges, 1986). Our results with immature flowers clearly show that pollen is capable of eliciting an increase in the expression of ACC oxidase mRNAs independently of ethylene action. Therefore, the rapid stimulation of ethylene production by pollinated pistils likely involves pollen-borne elicitors capable of inducing both ACC synthase and ACC oxidase. A similar rapid stimulation of ACC synthase and ACC oxidase has been described for elicitor-treated tomato cell cultures (Felix et al., 1991). An intriguing possibility is that pollen serves as a source of similar elicitor molecules that act in a chemosensory perception system, which results in the stimulation of ethylene biosynthesis.

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