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Pistil-Specific and Ethylene-Regulated Expression of 1-Aminocyclopropane-1-Carboxylate Oxidase Genes in Petunia Flowers

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The differential expression of the petunia 1-aminocyclopropane-1-carboxylate (ACC) oxidase gene family during flower development and senescence was investigated. ACC oxidase catalyzes the conversion of ACC to ethylene. The increase in ethylene production by petunia corollas during senescence was preceded by increased ACC oxidase mRNA and enzyme activity. Treatment of flowers with ethylene led to an increase in ethylene production, ACC oxidase mRNA, and ACC oxidase activity in corollas. In contrast, leaves did not exhibit increased ethylene production or ACC oxidase expression in response to ethylene. Gene-specific probes revealed that the *ACO1* gene was expressed specifically in senescing corollas and in other floral organs following exposure to ethylene. The *ACO3* and *ACO4* genes were specifically expressed in developing pistil tissue. In situ hybridization experiments revealed that ACC oxidase mRNAs were specifically localized to the secretory cells of the stigma and the connective tissue of the receptacle, including the nectaries. Treatment of flower buds with ethylene led to patterns of ACC oxidase gene expression spatially distinct from the patterns observed during development. The timing and tissue specificity of ACC oxidase expression during pistil development were paralleled by physiological processes associated with reproduction, including nectar secretion, accumulation of stigmatic exudate, and development of the self-incompatible response.

INTRODUCTION

Reproductive development in angiosperms culminates in the abscission and/or senescence of specific floral organs. This developmentally programmed organ death is thought to contribute to the success of sexual reproduction. For example, given their role as visual cues, the senesced or abscised flower petals would serve to deter continued visits by scarce pollinators. The ripening and senescence of fruit lead to seed dispersal, thus ensuring continued success of a species. In many cases, increased production of the phytohormone ethylene has been shown to play a regulatory role in the senescence of floral organs (Mattoo and Suttle, 1991). Ethylene's role in regulating the ripening process in climacteric fruit has been clearly defined by reverse genetics (Oeller et al., 1991; Picton et al., 1993) and through the identification and characterization of mutants altered in their response to ethylene (Lanahan et al., 1994). The senescence of flower petals in several plants is associated with increased production of ethylene (Hoekstra and Weges, 1986; Borochov and Woodson, 1989; Woodson et al., 1992; O'Neill et al., 1993). This increased ethylene serves to coordinate the biochemical processes of petal senescence, including the transcriptional activation of several senescence-related genes (Lawton et al., 1990; Woodson et al., 1993).

The regulation of ethylene biosynthesis has been the subject of intense investigation in recent years (for review, see Kende, 1993). The ethylene biosynthetic pathway has been completely elucidated and the novel amino acid 1-aminocyclopropane-1-carboxylate (ACC) shown to serve as the immediate precursor to ethylene. The conversion of *S*-adenosyl methionine to ACC is catalyzed by the pyridoxal phosphate-requiring enzyme ACC synthase and represents the rate-limiting step in ethylene biosynthesis in many tissues (Kende, 1989, 1993). The final step in the ethylene biosynthetic pathway is catalyzed by ACC oxidase, formerly referred to as the ethylene-forming enzyme (Kende, 1989, 1993). This enzyme also plays a role in the regulation of ethylene biosynthesis.

Increased ethylene production is often associated with increased activity of ACC oxidase as measured by the capacity of tissue to convert saturating levels of ACC to ethylene in vivo (Kende, 1993). Similar to ACC synthase, ACC oxidase activity increases in response to diverse stimuli, including ethylene (Dong et al., 1992), and elicitor treatment (Felix et al., 1991), and during the developmental processes of fruit ripening and flower senescence (Woodson et al., 1992). The nature of this reaction was unclear for several years following the identification of ACC as the ethylene precursor as a result of the lack of a reliable in vitro assay. Recently, significant progress has been made in the understanding of ACC oxidase following

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the report that an antisense ripening-related gene (pTOM13) inhibited ACC oxidase activity and ethylene production in transgenic tomatoes (Hamilton et al., 1990). Subsequently, pTOM13 and a highly related gene (pHTOM5) were shown to encode ACC oxidase following expression and recovery of authentic ACC oxidase activity in yeast (Hamilton et al., 1991) and Xenopus oocytes (Spanu et al., 1991), respectively. ACC oxidase cDNA clones have been isolated and used to characterize the expression of homologous transcripts in a number of species (Holdsworth et al., 1988; Wang and Woodson, 1991, 1992; Dong et al., 1992; Woodson et al., 1992; Balagué et al., 1993; Peck et al., 1993). In tomato, ACC oxidase is encoded by a small family of three genes (Holdsworth et al., 1988; Bouzayen et al., 1993). These genes were shown to exhibit differential expression during fruit ripening and wounding (Holdsworth et al., 1988).

We have been studying the regulation of ethylene biosynthesis in flowers and its role in petal senescence and reproductive physiology. In this study, we examined the differential regulation of ACC oxidase genes in petunia during flower development and in response to ethylene. The petunia ACC oxidase gene family consists of four members arranged in two unlinked clusters of tandemly arranged genes (Tang et al., 1993). Three members of this gene family have been shown to be expressed, and cDNAs representing their mRNAs have been isolated (Tang et al., 1993). Utilizing gene-specific probes, we show that expression of the *ACO1* gene leads to increased ACC oxidase activity during petal senescence and that this gene is also expressed in floral organs following treatment with ethylene. Furthermore, we report that two ACC oxidase genes (*ACO3* and *ACO4*) are specifically expressed in pistils during flower development. The accumulation of ACC oxidase transcripts during pistil development was found to be localized specifically to secretory tissues of the stigma and nectary, suggesting a role for ethylene in the reproductive physiology of flowers.

RESULTS

Expression of ACC Oxidase during Corolla Senescence

The relationship between ethylene production and ACC oxidase activity was determined during development of petunia corollas after anthesis. As shown in Figure 1A, corolla senescence was associated with increased ethylene production beginning 8 days after anthesis in the absence of pollination. This increased ethylene production reached a maximum rate 9 days after anthesis and subsequently declined concomitant with petal wilting and death. The increase in ethylene production was preceded by increased ACC oxidase activity (Figure 1A).

We examined the expression of ACC oxidase mRNAs in corolla tissue during the transient increase in ethylene associated with senescence. This analysis revealed low levels of ACC oxidase mRNA detected by the full-length cDNA clone pPHEFE (Wang and Woodson, 1992) in corollas during the first 6 days after anthesis, as shown in Figure 1B. A dramatic increase in ACC oxidase transcripts occurred in corollas

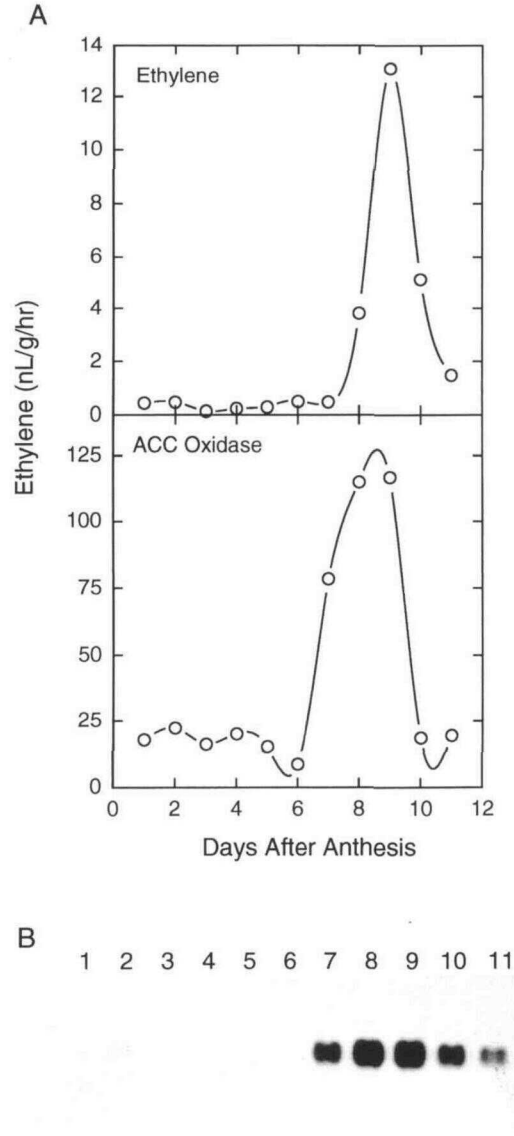

Figure 1. Ethylene Production, ACC Oxidase Activity, and ACC Oxidase mRNA Levels in Petunia Corollas during Senescence.

Flowers were tagged at anthesis and harvested at various times (days) after anthesis and during senescence.

(A) Corolla tissue was isolated and ethylene production and ACC oxidase were measured as described in Methods.

(B) Gel blot analysis of total RNA extracted from corollas at various times (1 to 11 days) after anthesis and probed with pPHEFE. Each lane contains 10 μg of total RNA and was stained with ethidium bromide prior to blotting to ensure equal loading of samples.

beginning 7 days after anthesis. The level of ACC oxidase mRNA remained elevated through 9 days after anthesis, after which a decline in mRNA abundance was associated with the advanced stages of corolla senescence. The accumulation of ACC oxidase mRNAs in corollas before the detectable increase in ethylene production suggests the increase in ethylene associated with corolla senescence was a result of both increased ACC oxidase activity and the availability of ACC. Furthermore, the temporal relationship between ACC oxidase mRNA levels and enzyme activity suggests the protein is synthesized de novo during corolla senescence.

ACC Oxidase Genes Are Induced by Ethylene in a Tissue-Specific Manner

Ethylene has been shown to stimulate the activity of ACC oxidase in a number of plants and tissues (Kende, 1993). We examined the effects of exogenous ethylene on ACC oxidase activity and gene expression in petunia corollas and leaves. Figure 2A shows that treatment of flowers with 10 μL/L ethylene for 24 hr led to a fivefold increase in corolla ethylene production and a 40-fold increase in ACC oxidase activity. In striking contrast, ethylene exposure resulted in decreased ethylene production by leaves and had no effect on the activity of ACC oxidase. As shown in Figure 2B, the levels of ACC oxidase mRNAs increased dramatically in corollas in response to ethylene treatment of flowers. Leaves exhibited a low level of ACC oxidase mRNAs in both air and ethylene, which is consistent with the lack of stimulation of enzyme activity by ethylene treatment.

To determine whether ethylene-responsive expression of ACC oxidase was corolla specific, we examined the abundance of transcripts in all the floral organs following exposure of intact flowers to ethylene. Figure 3A shows that ACC oxidase mRNAs detected by the full-length cDNA clone pPHEFE increased in response to ethylene in all floral organs, including sepals, corollas, anthers, stigmas/styles, and ovaries. The increase in ACC oxidase mRNAs in response to ethylene exposure was most dramatic in corolla tissue. In contrast to sepals, corollas, and anthers, the tissues of the pistil (stigma, style, and ovary) contained abundant levels of ACC oxidase transcripts in the absence of exogenous ethylene.

ACC Oxidase Genes Are Differentially Regulated

Previously, we reported that ACC oxidase is encoded by a small gene family in petunia, three members of which are actively transcribed (Tang et al., 1993). These genes are highly conserved in the coding region; therefore, the full-length pPHEFE cDNA, which represents the *ACO1* gene, does not differentiate between the transcripts from different ACC oxidase genes. To examine the expression of this gene family in more detail, we developed gene-specific probes based largely on the 3′ untranslated regions of these genes where they diverge significantly

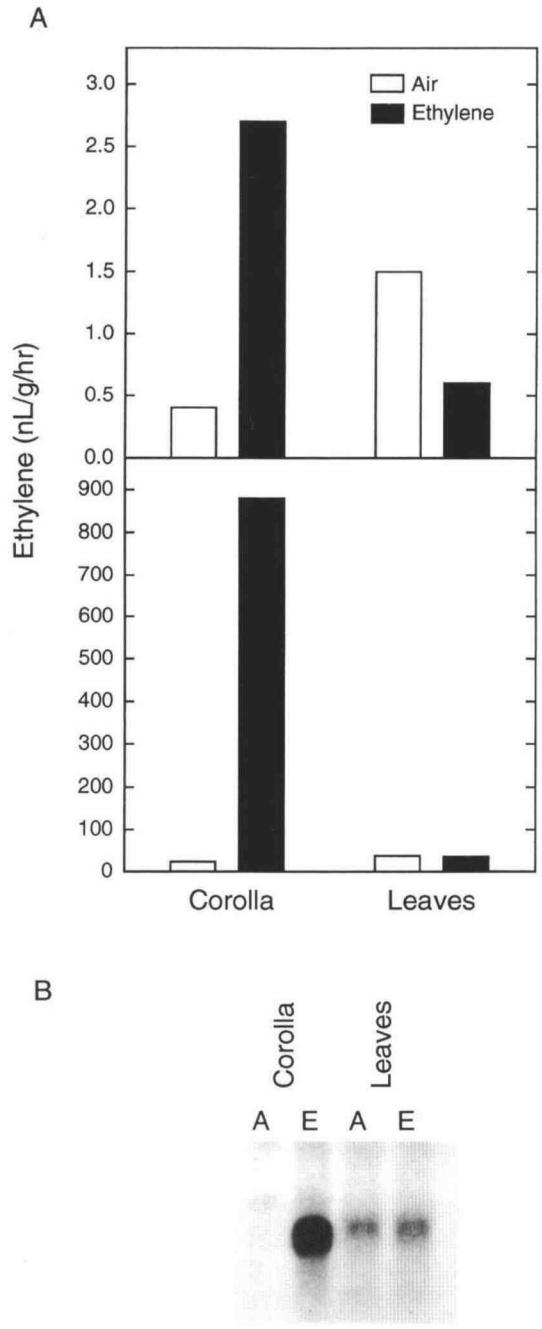

Figure 2. Ethylene Production, ACC Oxidase Activity, and ACC Oxidase mRNA Levels in Response to Ethylene Treatment.

(A) Ethylene production (top) and ACC oxidase activity (bottom) by corollas and leaves were determined following exposure to air or 10 μL/L ethylene for 24 hr.

(B) Gel blot analysis of RNA extracted from corollas and leaves following exposure to air (lanes A) or 10 μL/L ethylene (lanes E) for 24 hr and probed with pPHEFE. Each lane contains 10 μg of total RNA and was stained with ethidium bromide prior to blotting to ensure equal loading of samples.

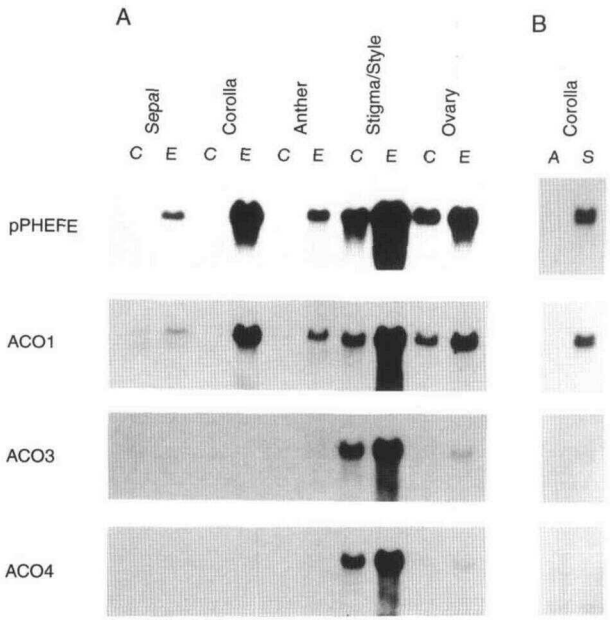

Figure 3. Differential Expression of ACC Oxidase Genes in Petunia Flowers.

(A) RNA gel blot analysis of ACC oxidase mRNA levels in floral organs from flowers harvested the day before anthesis and held for 24 hr in air (lanes C) or 10 μL/L ethylene prior to extraction of total RNA (lanes E). **(B)** ACC oxidase mRNA levels in corolla tissue at anthesis (lane A) and during senescence (lane S).

Each lane contains 10 μg of total RNA and was stained with ethidium bromide prior to blotting to ensure equal loading of samples. The blots were hybridized with the full-length pPHEFE cDNA or gene-specific probes for *ACO1*, *ACO3*, or *ACO4*.

(Tang et al., 1993). As shown in Figure 4, these probes do not cross-hybridize under the stringency conditions used for RNA gel blot analysis. Using these gene-specific probes, we analyzed the expression of ACC oxidase mRNAs in floral organs following ethylene treatment and during petal senescence. The results of this analysis are shown in Figure 3A. Transcripts detected by the *ACO1* probe increased in abundance in all floral organs in response to ethylene and were also detected in pistil tissue in the absence of ethylene. In constrast, ACC oxidase mRNAs encoded by the *ACO3* and *ACO4* genes were only detected in pistil tissue where their levels increased to a limited extent in response to ethylene. In addition, we examined the differential expression of ACC oxidase genes during corolla senescence. The senescence of corolla tissue was associated with the specific expression of the *ACO1* gene, as shown in Figure 3B. Taken together with the results shown in Figure 1, which indicate that ACC oxidase gene expression precedes the dramatic increase in ethylene associated with petal senescence, these data suggest that *ACO1* expression is under both developmental and ethylene regulation. Further, the data indicate that *ACO3* and *ACO4* may represent pistil-specific ACC oxidase genes.

ACC Oxidase Genes Are Temporally Regulated during Pistil Development

The specific accumulation of ACC oxidase transcripts in pistil tissue led us to examine the temporal expression of these genes during gynoecia development. To address their temporal expression, pistils were isolated from flowers at five stages of development leading up to anthesis. These stages are illustrated in Figure 5A. Stage 1 flowers are defined by a bud length of 18 mm from the base of the receptacle to the tip of the corolla. Stage 2 flowers are 30 mm and are defined by a lack of anthocyanin in the style and corolla. Stage 3 flowers are 45 mm with a fully elongated style and contain anthocyanin in both the corolla and pistil. Stage 4 represents flowers the day before anthesis. Stage 5 flowers are at anthesis and exhibit anther dehiscence and stigmatic secretion. RNA gel blot analysis revealed ACC oxidase transcripts accumulate in the stigma/style and the ovary in a developmental manner (Figure 5B). ACC oxidase mRNA was first detected by the full-length pPHEFE cDNA probe in both stigma/style and ovary tissue at stage 3 and remained abundant through anthesis (stage 5). All three ACC oxidase genes were expressed in a coordinate fashion during stigma/style development. The accumulation of ACC oxidase transcripts in developing ovary tissue was primarily a result of the expression of *ACO1*. An increase in *ACO3* and *ACO4* transcripts was also detected in

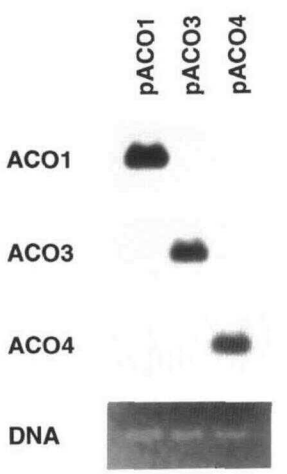

Figure 4. Specificity of ACC Oxidase Probes.

Each lane contains 100 ng of DNA representing the 3′ untranslated region of pACO1, pACO3, and pACO4 cDNA clones. The gel was stained with ethidium bromide prior to blotting. The blots were hybridized with ^{32}P-labeled probes as indicated. The conditions for hybridization and washing were identical to those used for RNA gel blot analysis as described in Methods.

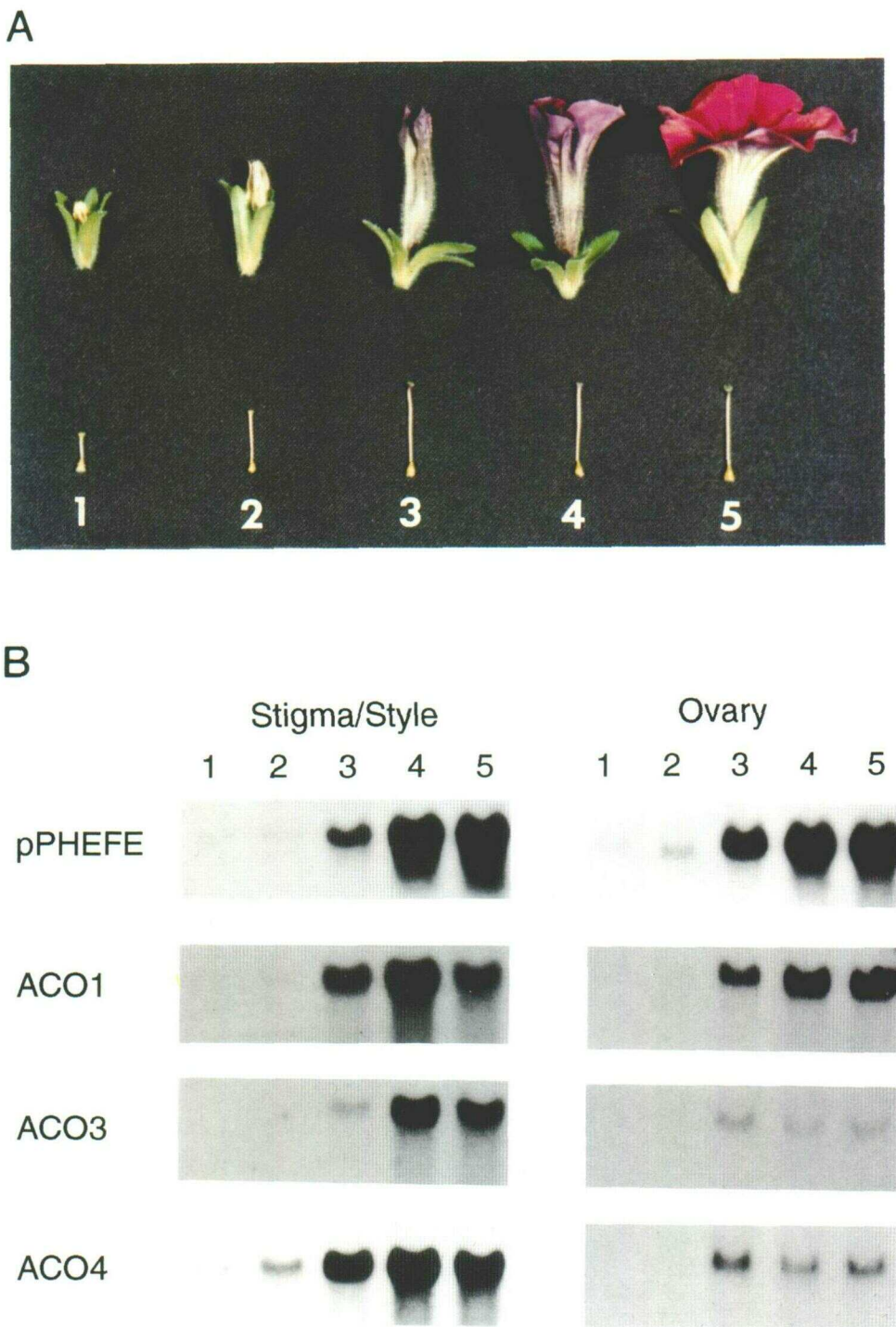

Figure 5. Expression of ACC Oxidase Genes during Pistil Development.

(A) Petunia flower development. Whole flowers and isolated pistils are shown at five stages of development through anthesis (stage 5).
(B) ACC oxidase mRNA levels in stigma/style and ovary tissue at the five stages (lanes 1 to 5) of flower development. Each lane contains 10 μg of total RNA and was stained with ethidium bromide prior to blotting to ensure equal loading of samples. The blots were hybridized with the full-length pPHEFE cDNA or gene-specific probes for *ACO1*, *ACO3*, or *ACO4*.

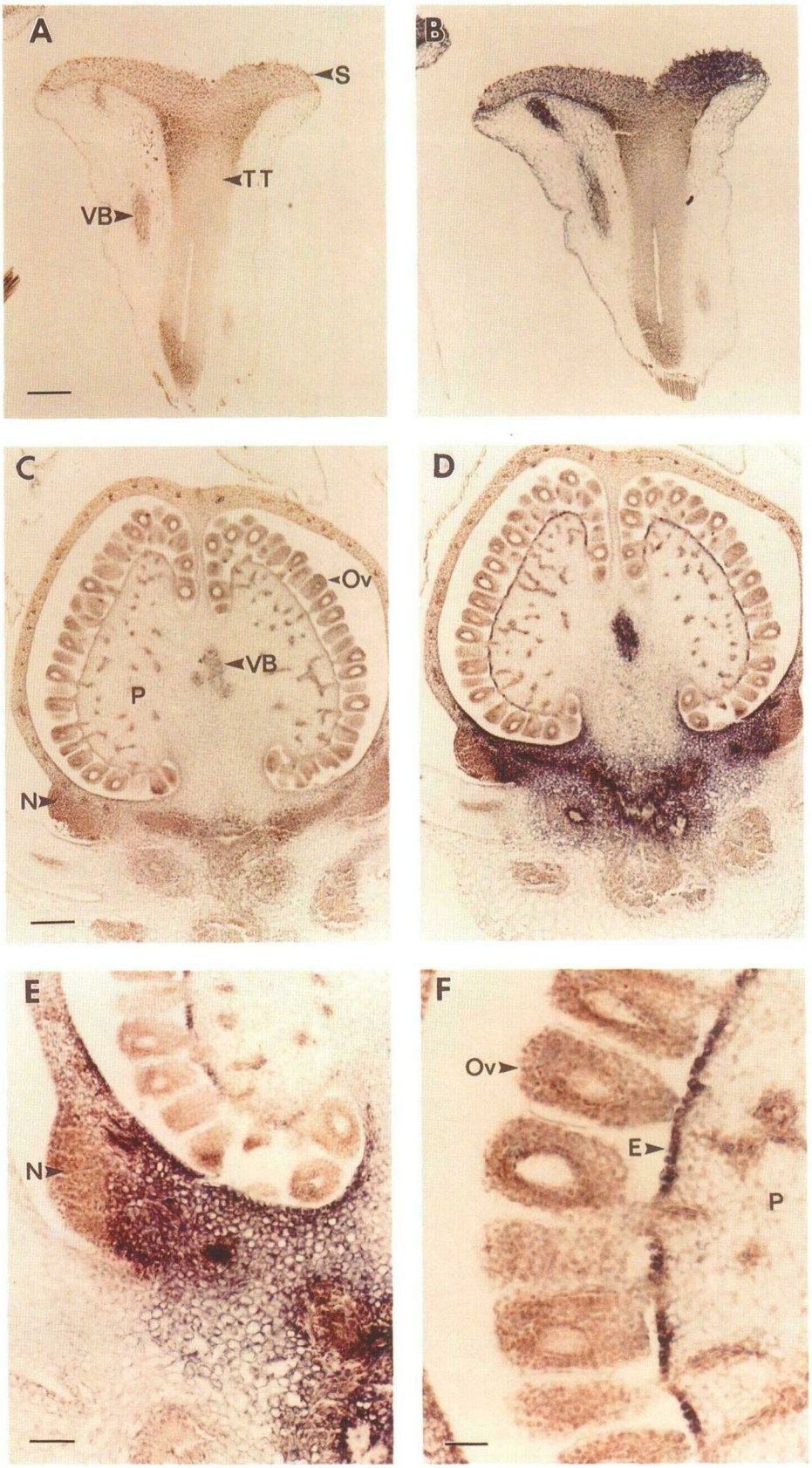

Figure 6. Localization of ACC Oxidase Transcripts in Pistil Tissue by in Situ Hybridization.

developing ovaries at stage 3 but to a lesser extent than *ACO1*. The development of petunia pistils was previously shown to be associated with increased ACC oxidase activity and ethylene production (Pech et al., 1987), indicating that ACC oxidase mRNAs are translated into functional protein. The temporal regulation of ACC oxidase genes during pistil development indicates that ethylene may play a role in the reproductive physiology of petunia. Further, the coordinate expression of all three functional ACC oxidase genes in developing pistils suggests these genes respond to common tissue-specific cellular factors.

Pistil-Specific ACC Oxidase mRNAs Are Localized in Secretory Tissue

We examined the spatial distribution of ACC oxidase transcripts in pistil tissue at anthesis by in situ hybridization using full-length pPHEFE sense and antisense RNA probes. The results of this analysis are shown in Figure 6. ACC oxidase mRNAs were specifically localized to the stigmatic region of the stigma/style (Figures 6A and 6B). ACC oxidase transcripts were detected in cells of the subepidermal secretory zone. In addition, ACC oxidase mRNAs were detected in vascular tissue beneath the stigmatic surface. No hybridization was apparent in transmitting tissue or cortical cells of the style. In situ hybridization of longitudinal sections of ovary/receptacle tissue with pPHEFE antisense RNA revealed abundant ACC oxidase mRNA in cells associated with the nectary and connective tissue of the receptacle, just beneath the ovary (Figures 6C to 6E). In addition, ACC oxidase transcripts were detected in epidermal cells and vascular bundles of the placenta (Figure 6F). No hybridization of the pPHEFE antisense probe was apparent in the ovules at anthesis (Figure 6F). These results indicate that expression of ACC oxidase genes in developing pistils is largely confined to the secretory tissue. Taken together with previous research on the developmental timing of secretory activity of these tissues (Herrero and Dickinson, 1980; Fahn, 1988), the temporal and spatial expression of ACC oxidase genes suggest a role for ethylene in the secretion of the "liquid cuticle" of the stigma and nectar from the nectary.

Spatial Expression of ACC Oxidase in Response to Ethylene Differs from Developmental Expression in Floral Organs

The results presented in Figure 3A clearly show the capacity for ethylene to induce the expression of *ACO1* in all floral organs. To examine the spatial distribution of ACC oxidase mRNAs in floral organs following ethylene treatment, flowers were harvested at stage 1 and exposed to 10 μL/L ethylene for 24 hr. We had previously shown that flowers at this stage accumulate ACC oxidase mRNAs in response to ethylene (data not shown). This allowed us to assess the effects of ethylene on ACC oxidase expression in the absence of developmentally regulated ACC oxidase genes in pistil tissue. Figure 7 shows the spatial distribution of ACC oxidase mRNAs in reproductive floral organs following exposure to ethylene. In situ hybridization of the pPHEFE sense and antisense probes to transverse sections of anthers from ethylene-treated flowers revealed ACC oxidase mRNAs were localized primarily in the vascular bundle (Figures 7A and 7B). In contrast, ACC oxidase transcripts were detected throughout the stigma/style after ethylene treatment and were particularly abundant in the transmitting tract, vascular bundle, and secretory cells of the stigma (Figures 7C and 7D). Hybidization of longitudinal ovary sections from ethylene-treated flowers with the sense and antisense ACC oxidase probes revealed the presence of message throughout the placenta extending into the receptacle tissue (Figures 7E and 7F). In contrast to the pattern of ACC oxidase expression in pistils during development (Figures 6C and 6D), ethylene treatment of stage 1 flowers led to the accumulation of ACC oxidase transcripts in the cells surrounding the embryo sac of the ovules (Figure 7F). This pattern of expression was also apparent in transverse sections of an ovary (Figure 7H). No hybridization of the antisense ACC oxidase probe was detected in the nectary tissue of ethylene-treated flower buds, in striking contrast to the pattern of expression at anthesis (Figures 6D and 6E). Ethylene treatment of stage 1 flowers did not lead to the expression of ACC oxidase in all cell types. For example, no detectable expression was seen in the cells of the ovary wall (Figure 7H) or cells of the anther endothecium

Figure 6. (continued).

Stigma/style and ovary tissue isolated from flowers at anthesis were fixed, embedded in paraffin, cut into 10-μm longitudinal sections, and hybridized with digoxigenin-labeled pPHEFE RNA probes. Hybridization was detected with anti-digoxigenin antibodies conjugated to alkaline phosphate and was visualized as blue color following development.

(A) Stigma/style longitudinal section hybridized with the sense probe. S, TT, and VB indicate the stigmatic surface, transmitting tract, and vascular bundle, respectively.

(B) Stigma/style longitudinal section hybridized with the antisense probe.

(C) Ovary/receptacle longitudinal section hybridized with the sense probe. N, Ov, P, and VB indicate the nectary, ovule, placenta, and vascular bundle, respectively.

(D) Ovary/receptacle longitudinal section hybridized with the antisense probe.

(E) Nectary and receptacle longitudinal section hybridized with the antisense probe. N indicates the nectary.

(F) Ovary longitudinal section hybridized with the antisense probe. E, Ov, and P indicate the placenta epidermis, ovule, and placenta, respectively.

Bars in **(A)** and **(C)** = 1 mm; bar in **(E)** = 400 μm; bar in **(F)** = 200 μm.

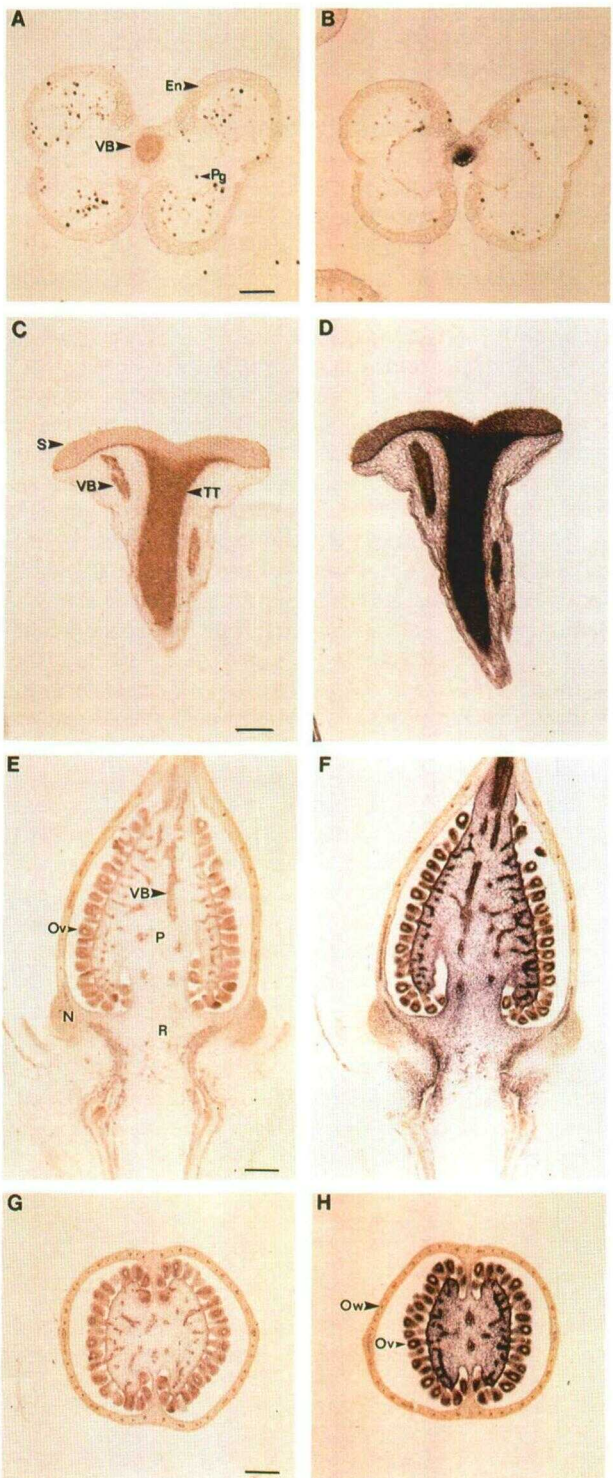

Figure 7. Localization of ACC Oxidase Transcripts in Reproductive Organs in Response to Ethylene.

Stage 1 flowers were treated with 10 μL/L ethylene in air for 24 hr. Floral organs were removed, fixed, embedded in paraffin, cut into 10-μm sections, and hybridized with digoxigenin-labeled pPHEFE RNA probes. Hybridization was detected with anti-digoxigenin antibodies conjugated to alkaline phosphate and was visualized as blue color following development.

(A) Transverse section of an anther hybridized with the sense probe. En, Pg, and VB designate the endothecium, pollen grain, and vascular bundle, respectively.

(B) Transverse section of an anther hybridized with the antisense probe.

(C) Stigma/style longitudinal section hybridized with the sense probe. S, TT, and VB indicate the stigma, transmitting tract, and vascular bundle, respectively.

(D) Stigma/style longitudinal section hybridized with the antisense probe.

(E) Ovary longitudinal section hybridized with the sense probe. N, Ov, P, R, and VB indicate the nectary, ovule, placenta, receptacle, and vascular bundle, respectively.

(F) Ovary longitudinal section hybridized with the antisense probe.

(G) Transverse section of an ovary hybridized with the sense probe.

(H) Transverse section of an ovary hybridized with the antisense probe. Ov and Ow designate the ovule and outer wall of the ovary, respectively. Bars in **(A)**, **(C)**, **(E)**, and **(G)** = 1 mm.

(Figure 7B). Together, these results indicate ethylene induces patterns of ACC oxidase expression unique from that of developmental signals.

The pattern of expression of ACC oxidase in corolla tissue is shown in Figure 8. Hybridization of transverse sections of the corolla limb (Figures 8A and 8B) and tube (Figures 8C and 8D) with ACC oxidase sense and antisense probes revealed a general pattern of expression in all living cells. The level of mRNA in these cells was considerably less as compared with other floral organs, and this is in striking contrast to the results of RNA gel blot analysis (Figure 3A). This is most likely a result of the limited RNA content of these highly vacuolated cells; this low content is reflected in the relatively low yield of RNA from corolla tissue as compared to other floral organs (data not shown).

DISCUSSION

In this study, we have examined the expression of ACC oxidase genes in petunia flowers during development and senescence and in response to ethylene. The senescence of petunia corollas is associated with increased ethylene production, and this ethylene plays an essential role in regulating the processes of cell death (Hoekstra and Weges, 1986). Here, we show that the increase in ethylene production by corollas was preceded by increased ACC oxidase activity as measured by the capacity of tissue to convert saturating levels of ACC to ethylene. The increase in enzyme activity was associated with a dramatic increase in ACC oxidase mRNA abundance. The strict correlation between enzyme activity and mRNA level suggests that ACC oxidase activity in senescing corollas

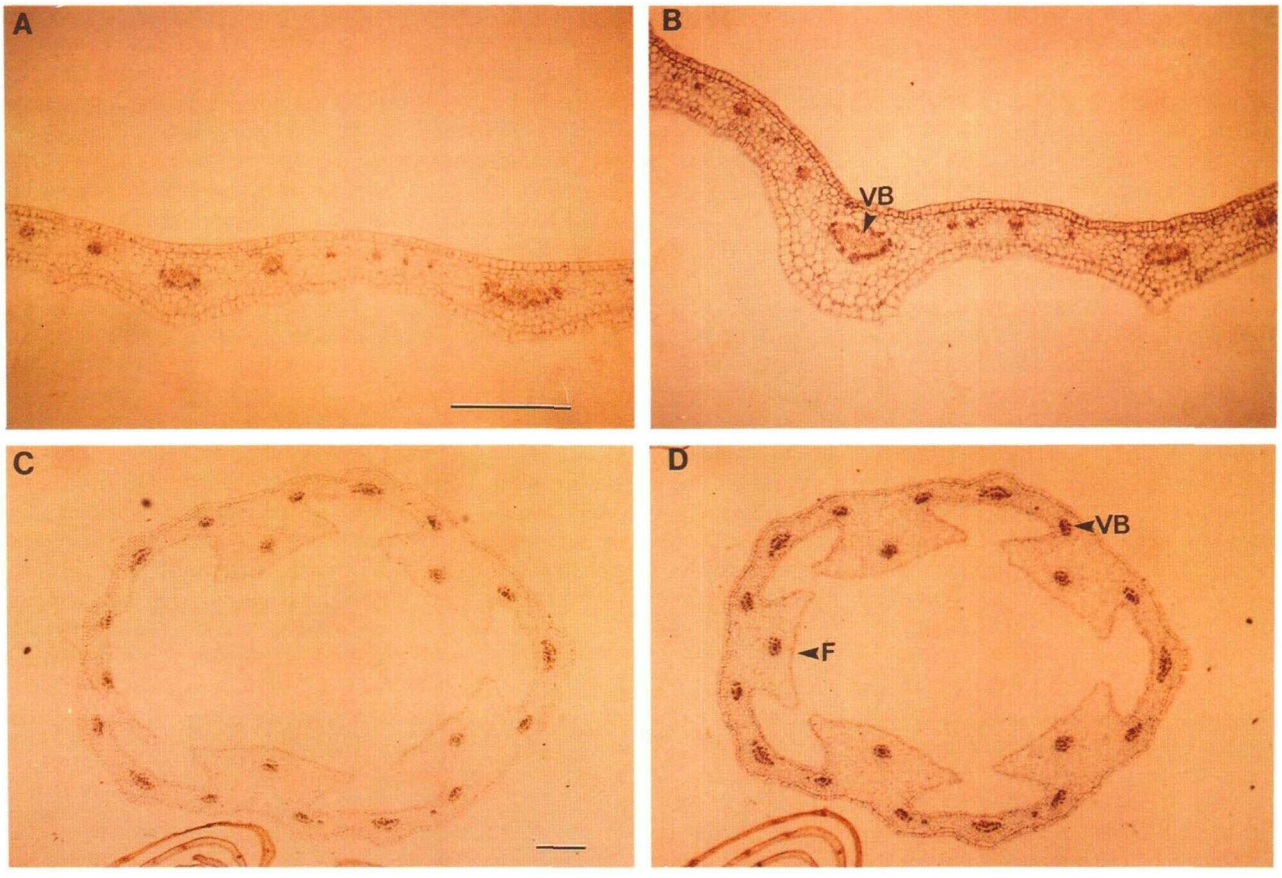

Figure 8. Localization of ACC Oxidase mRNA within Corolla Tissue in Response to Ethylene.

Stage 2 flowers were treated with 10 μL/L ethylene in air for 24 hr. Corollas were removed, separated into the limb and tube, fixed, embedded in paraffin, cut into 10-μm sections, and hybridized with digoxigenin-labeled pPHEFE RNA probes. Hybridization was detected with anti-digoxigenin antibodies conjugated to alkaline phosphate and was visualized as blue color following development.

(A) Transverse section of a corolla limb hybridized with the sense probe.

(B) Transverse section of a corolla limb hybridized with the antisense probe. VB indicates the vascular bundle.

(C) Transverse section of the corolla tube hybridized with the sense probe.

(D) Transverse section of the corolla tube hybridized with the antisense probe. F and VB designate the anther filament and vascular bundle, respectively. Bars in **(A)** and **(C)** = 1 mm.

is regulated primarily at the level of transcription. In many cases, the synthesis of ACC, catalyzed by ACC synthase, is considered the rate-limiting step in ethylene biosynthesis (Yang and Hoffman, 1984; Kende, 1993). This is most likely the case in senescing petunia corollas because an increase in ACC oxidase activity was not sufficient to lead to increased ethylene production, indicating the availability of ACC is rate limiting. However, the data do suggest an essential role for increased ACC oxidase activity in ethylene biosynthesis during senescence.

Similar patterns of ACC oxidase expression have been observed in other flowers that exhibit increased ethylene production during petal senescence (Woodson et al., 1992; Nadeau et al., 1993; O'Neill et al., 1993). In these cases, increased ethylene is implicated as the signal leading to ACC oxidase gene expression. Consistent with a role for ethylene in the regulation of ACC oxidase, we observed a dramatic increase in ACC oxidase mRNA and enzyme activity in corollas from presenescent flowers after ethylene treatment. In petunia flowers, increased ethylene cannot account for the initial stimulation of ACC oxidase gene expression because the level of mRNA increased prior to a measurable increase in ethylene production. However, the increase in ethylene during initial stages of corolla senescence most likely plays a role in further stimulation of ACC oxidase gene expression. Recent work on the catalytic mechanism of ACC oxidase indicates that this enzyme has low intrinsic catalytic power, which necessitates high levels of the transcript and protein to maintain elevated

levels of ethylene production (Dong et al., 1992; Pirrung et al., 1993). Therefore, the stimulation of ACC oxidase expression by ethylene is of central importance to the overall regulation of autocatalytic ethylene production during petunia corolla senescence. In keeping with this role, we observed that the stimulatory effect of ethylene on ACC oxidase gene expression was specific to floral tissue, which exhibits increased ethylene production, but does not occur in leaves where ethylene exposure reduced ethylene production through an autoinhibitory mechanism (Yang and Hoffman, 1984).

The petunia (cultivar V/R) ACC oxidase gene family is represented by three actively transcribed genes (*ACO1*, *ACO3*, and *ACO4*) and a fourth (*ACO2*) transcriptionally inactive pseudogene (Tang et al., 1993). The tomato ACC oxidase gene family also consists of three members that exhibit differential expression during fruit ripening and in response to wounding (Holdsworth et al., 1988). In this investigation, we showed that petunia ACC oxidase genes are subject to differential regulation. The accumulation of ACC oxidase transcripts in floral organs following ethylene treatment was largely a result of the expression of *ACO1*. The *ACO1* gene was also expressed specifically in senescing corollas during development, and this expression preceded the dramatic increase in ethylene production. Transcripts encoded by *ACO3* and *ACO4* were only detected in gynoecia tissues, indicating their transcription is regulated by tissue-specific factors associated with the developing pistil. In addition, the *ACO1* gene was expressed in developing pistil tissue coordinately with *ACO3* and *ACO4*. Whereas these genes share little overall nucleotide sequence homology in their 5′ flanking regions, they do exhibit a high degree of similarity in the flanking sequences immediately upstream of the transcriptional start site and including the TATA box (Tang et al., 1993). Current experiments are focusing on the identification of sequences responsible for ethylene and pistil-specific expression of ACC oxidase genes.

Expression of ACC Oxidase Genes in Pistils Is Both Developmentally and Spatially Regulated

The development of petunia pistils was associated with the accumulation of ACC oxidase mRNAs beginning 1 to 2 days before anthesis (stage 3). The expression of ACC oxidase genes was localized specifically to secretory cells of the pistil and receptacle including the stigma, placenta epidermis, and nectary. The temporal and spatial pattern of ACC oxidase expression is very similar to the self-incompatibility–related glycoproteins of tobacco (Cornish et al., 1987) and petunia (Ai et al., 1990; Clark et al., 1990). For example, Cornish et al. (1987) showed that S_2 mRNA accumulated in the secretory cells of the stigma, transmitting tract, and epidermal cells of the placenta. The cells of these tissues are all derived from a common progenitor in the L2 layer (Satina, 1944) and are positioned along the pathway taken by compatible pollen tubes to the ovary (Cornish et al., 1987). In contrast to the expression of S_2 alleles, ACC oxidase does not accumulate to high levels in transmitting tract tissue during pistil development. In this regard, it is interesting to note that the expression of a self-incompatibility–related gene in tobacco stigmas was found to precede the expression in transmitting tract tissue (Cornish et al., 1987), suggesting other factors may be important for this regulated expression.

In addition to the S_2 alleles, several other pistil-specific genes from solanaceous plants have been described. These include the extensin-like proline-rich proteins from tobacco (Chen et al., 1992; Goldman et al., 1992; Wang et al., 1993) and a protein of unknown function from tomato encoded by the cDNA pMON9612 (Budelier et al., 1990). All of these genes are specifically expressed in the transmitting tract and are secreted to the cell wall. ACC oxidases, on the other hand, are not thought to be part of the extracellular matrix, and the lack of a clear signal peptide is consistent with an intracellular localization of the protein. The coordinated expression of ACC oxidase genes during pistil development would appear to be a response to tissue-specific cellular factors, which are independent of ethylene. This is evidenced by the fact that treatment of flower buds (stage 1) with ethylene led to patterns of ACC oxidase expression unique from that of development. Taken together with the results of differential expression experiments, it is clear that ACC oxidase genes are regulated in a complex manner involving developmental, hormonal, and tissue-specific signals.

Possible Function of ACC Oxidase and Ethylene in Reproductive Physiology

The fact that ACC oxidase expression is spatially confined to specific cells of the pistil and receptacle and reaches a maximum at anthesis is suggestive of a role for ethylene biosynthesis in reproductive processes. Several physiological processes related to reproduction are developmentally regulated and correspond temporally to the expression of ACC oxidase. These physiological processes include increased ethylene production and ACC oxidase activity by the stigma (Pech et al., 1987), the self-incompatible response (Herrero and Dickinson, 1979; Clark et al., 1990), and the accumulation and secretion of cellular exudates by the stigma (Herrero and Dickinson, 1980; Shivanna and Sastri, 1981) and nectary (Fahn, 1988). Pollination of many flowers, including petunia, leads to a rapid increase in ethylene production by the pistil, and this ethylene is thought to play a role in postpollination development (Hoekstra and Weges, 1986; Singh et al., 1992; O'Neill et al., 1993; Zhang and O'Neill, 1993). Because of the high content of ACC in petunia pollen, it has been proposed that the conversion of pollen-held ACC to ethylene by the stigma is at least partially responsible for the early increase in ethylene following pollination (Singh et al., 1992). Developmentally regulated expression of ACC oxidase in the stigma could provide the second component of a two-component system necessary to produce ethylene, with the first component, ACC, coming from the pollen.

A further role for ACC oxidase and/or ethylene in pollen–pistil interactions is indicated by the specific expression in the epidermal cells of the placenta. The S_2 gene associated with self-incompatibility in ornamental tobacco was found to be expressed in the same cell layer with the proposed function of arresting growth of any self-pollen tubes that escape the normal inhibition zone of the transmitting tract (Cornish et al., 1987). At anthesis, the petunia stigma accumulates several droplets of lipid-rich exudate on the surface and thus is regarded as a "wet" stigma type (Konar and Linskens, 1966). This visible stigmatic secretion is preceded by the degeneration of cells in the secretory zone, where droplets of cellular-derived exudate coalesce in cavities generated through localized cell death (Herrero and Dickinson, 1979). The eventual secretion of this exudate is a result of cuticle rupture on the stigmatic surface (Shivanna and Sastri, 1981). The exudation of nectar from the secretory cells of the nectary occurs in a similar manner and developmental time frame (Fahn, 1988).

To our knowledge, a role for ACC oxidase or ethylene in the secretory processes of these cells has not been reported previously. Given ethylene's clear role in the regulation of cell death, one possible function for ethylene in secretion may be in the formation of cavities for storage and accumulation of exudate. Recently, a reverse genetic approach has proven useful in studying the role of ethylene in plant growth and development (Oeller et al., 1991; Picton et al., 1993). Transgenic tomatoes expressing antisense ACC synthase or ACC oxidase RNAs were shown to be dramatically inhibited in their capacity to produce ethylene. This led to an inhibition of fruit ripening, but apparently did not significantly alter fruit size or seed set. In both of these cases, the cauliflower mosaic virus 35S promoter was used to drive the expression of antisense ACC oxidase and ACC synthase transcripts. Given the low level of expression of genes in anther tissue and pollen under the control of this promoter, a role for ethylene in pollen development or pollen tube growth cannot be ruled out. In addition to pollination and fertilization, our results suggest that ethylene may play a role in other physiological processes associated with reproduction. Reverse genetic approaches, when care is taken to regulate the transgene in the appropriate tissue, may be useful in elucidating the role of ACC oxidase and ethylene in reproduction, including the secretory processes of the pistil and nectary.

METHODS

Plant Material

Petunia (*Petunia hybrida*) seeds of cultivars Pink Flash and V/R were obtained from Ball Seed Company (West Chicago, IL) and R. Koes (Free University, Amsterdam), respectively. Plants were grown under greenhouse conditions with a day/night temperature regime of 22°C/18°C. For senescence studies, flowers were emasculated the day before anthesis and allowed to senesce on the plant. For exposure to ethylene, flowers were harvested the day before anthesis, and their pedicles were placed in vials of distilled water in the laboratory. Recently matured and fully expanded leaves were harvested from plants and placed in Petri dishes on filter paper wetted with distilled water. Flowers and leaves were placed in an airtight 24-L chamber, and ethylene was injected to yield a final concentration of 10 μL/L. The concentration of ethylene was confirmed by gas chromatography both at the beginning and end of the 24-hr exposure.

Ethylene and 1-Aminocyclopropane-1-Carboxylate Oxidase Measurements

Ethylene production was measured by enclosing flower corollas or leaf tissue in 50-mL gas-tight containers for 0.5 to 1 hr, after which the headspace gas was assayed for ethylene by gas chromatography. The gas chromatograph was equipped with an activated alumina column and a flame ionization detector. The oven temperature was 80°C and the detector was 150°C. An authentic ethylene standard was used for calibration of concentration and retention time. In vivo 1-aminocyclopropane-1-carboxylate (ACC) oxidase activity was measured by placing intact corollas in a 50-mL side-arm flask containing 1 mM ACC. A vacuum was applied and released, infiltrating the tissue with ACC. Tissue was transferred to a 50-mL gas-tight container and sealed after 0.5 hr. A gas sample was removed after 0.5 hr and analyzed for ethylene.

RNA Gel Blot Analysis

Flower or leaf tissue was frozen in liquid N_2 and stored at −80°C before extraction of RNA. Total cellular RNA was isolated from tissue as previously described (Lawton et al., 1990), and the concentration and purity were determined by spectrophotometry. Ten-microgram total RNA samples were separated by electrophoresis through 1% (w/v) agarose gels containing 2.2 M formaldehyde. Gels were stained with ethidium bromide to visualize ribosomal subunit RNA and to ensure equal loading of samples. The separated RNAs were transferred to Nytran membranes (Schleicher & Schuell) and cross-linked with a controlled UV light source. Membranes were prehybridized at 42°C for 4 hr in a solution containing 50% (v/v) formamide, 5 × Denhardt's reagent (1 × Denhardt's solution is 0.02% each of Ficoll, PVP, and BSA), 0.1% SDS, 6 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM NaH_2PO_4, pH 7.4, and 1 mM EDTA), and 100 μg/mL denatured herring sperm DNA. Hybridization was performed in identical buffer solution containing 5×10^{-5} cpm denatured ^{32}P-labeled cDNA probe for 18 hr. cDNA probes were labeled by random priming with ^{32}P-dCTP at >3000 Ci/mmol (Du Pont–New England Nuclear). Following hydridization, membranes were washed three times at 42°C in 1 × SSPE and 0.1% SDS and subsequently exposed to Kodak XAR-5 film with an intensifying screen at −70°C.

Gene-Specific Probes

Previously, we described the isolation of a full-length petunia ACC oxidase cDNA clone designated pPHEFE (Wang and Woodson, 1992) and three partial cDNAs representing the 3′ region of transcripts encoded by the ACC oxidase genes *ACO1*, *ACO3*, and *ACO4* (Tang et al., 1993). The full-length cDNA clone pPHEFE was used as a nondiscriminating probe, and it hybridized to ACC oxidase transcripts from each of the three genes. We previously reported that the ACC oxidase

genes share less than 60% sequence homology within the 3′ untranslated region. Probes representing these regions were generated. The *ACO1* 3′ probe was 298 bp and was generated by digesting pPHEFE in pGEM7Zf(+) with BamHI (nucleotide position 994; Wang and Woodson, 1992) and EcoRI. A 3′ untranslated *ACO4* probe of 192 bp was generated by digesting pACO4 with ApoI just at the translational stop site and XhoI. Finally, the 3′ *ACO3*-specific probe was generated by polymerase chain reaction amplification using pACO3 as a template, T7 as the antisense primer, and the synthetic oligonucleotide 5′-CAACTGCTTAGGATTGCAA-3′ as the sense primer. Thirty-five cycles of denaturation (94°C, 1 min), annealing (60°C, 2 min), and *Taq* polymerase–mediated extension (72°C, 2 min) were used to amplify cDNA. The specificity of these probes was determined by DNA gel blot analysis. One hundred nanograms of each DNA probe was separated by electrophoresis through 1% agarose gels, denatured, transferred to Nytran membranes, and cross-linked with a controlled UV light source. Membranes were hybridized with ^{32}P-labeled gene-specific probes as described above for RNA gel blot analysis.

In Situ Hybridization

Floral organs were isolated from harvested flowers at various stages of development and following exposure to 10 μL/L ethylene for 24 hr. In situ hybridizations were carried out essentially as described by Cox and Goldberg (1988). Styles were fixed in 1% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.0, and other floral organs were fixed in FAA (50% ethanol, 5% acetic acid, and 10% formalin) at room temperature for 3 hr. Following dehydration, clearing, and embedding, tissue samples were cut into 10-μm sections and placed on replicate slides coated with poly-L-lysine. The tissue sections were dewaxed, hydrated, and blocked in 1% BSA and treated with HCl, proteinase K, and acetic anhydride. Digoxigenin-labeled sense and antisense riboprobes were synthesized by in vitro transcription from pBluescript SK– template containing the PHEFE cDNA insert. Plasmids were linearized, and digoxigenin-11-dUTP was incorporated using either T7 or T3 polymerase according to the manufacturer's instructions (Boehringer Mannheim). RNA transcripts were sheared to 150 bp by alkaline hydrolysis prior to hybridization. Slides were prehybridized for 1 hr at room temperature in 50% formamide, 30 mM NaCl, 10 mM Tris, pH 7.5, 1 mM EDTA, 1 × Denhardt's solution, 10% dextran sulfate, 100 mM DTT, 500 μg/mL denatured salmon sperm DNA, and 150 μg/mL yeast tRNA. Tissue sections were hybridized in the same solution containing 0.2 μg/mL digoxigenin-labeled probe for 30 hr at 45°C. Following hybridization, slides were treated with RNase A to remove nonhybridized probe and subsequently washed once at room temperature in 2 × SSC for 1 hr, once at room temperature in 1 × SSC for 1 hr, once at 37°C in 0.5 × SSC for 0.5 hr, and finally at room temperature in 0.5 × SSC for 0.5 hr. Hybridization of the riboprobes was detected with anti-digoxigenin antibodies conjugated to alkaline phosphatase and visualized by color development according to the manufacturer's instructions (Boehringer Mannheim).

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