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Temporal and Spatial Regulation of 1-Aminocyclopropane-1-Carboxylate Oxidase in the Pollination-Induced Senescence of Orchid Flowers[1]

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Pollination of many flowers initiates a sequence of precisely regulated developmental events that include senescence of the perianth and development of the ovary. The plant hormone ethylene is known to play a key role in regulating the biochemical and anatomical changes that constitute the postpollination syndrome. For this reason, we have studied the pollination syndrome in *Phalaenopsis* orchids by examining the spatial and temporal location of ethylene biosynthesis within the orchid flower, and how this biosynthesis is regulated by factors that influence expression of genes that encode key enzymes in the ethylene biosynthetic pathway. In particular, we examined the role in the postpollination syndrome of the expression of the gene for 1-aminocyclopropane-1-carboxylate (ACC) oxidase, which catalyzes the conversion of ACC to ethylene. In vivo incubation of tissues with the ethylene precursor ACC demonstrated that ACC oxidase activity increases after pollination in the stigma, contrary to the observation that activity is constitutive in petunia and carnation gynoecia. RNA blot hybridization of floral tissues indicates that the increase in ACC oxidase activity is due to de novo synthesis of mRNA and presumably protein, which is induced after pollination. Furthermore, the pattern of induction is consistent with a model of coordinate regulation of gene expression in which the pollination signal travels to other organs of the flower to induce their ethylene production. We have also used in situ hybridization to define further the temporal and spatial expression of ACC oxidase within the floral organs, showing that expression, and, by inference, the capability to oxidize ACC to ethylene, is induced in all living cells of the tissues examined after pollination. These findings contrast with work in petunia that suggests that ACC oxidase is localized to the stigmatic surface.

It has been recognized for many years that pollination of flowers greatly accelerates senescence of nonessential floral organs such as the petals and sepals (Borochov and Woodson, 1989). Pollination of the flower results in an increase in ethylene production, which is thought to coordinate the senescence process and which may play a role in other aspects of postpollination developmental processes such as ovary development (Zhang and O'Neill, 1993). Treatment of flowers with ethylene accelerates senescence, but senescence of the perianth can be reversibly inhibited by the competitive inhibitor of ethylene action norbornadiene (Borochov and Woodson, 1989; Wang and Woodson, 1989). This suggests that ethylene is both sufficient and necessary for normal pollination-induced senescence of the perianth.

The postpollination syndrome is fundamentally different in several important ways from age-related senescence, although both processes are mediated through ethylene. In orchid flowers, cells of the column (a specialized organ consisting of fused gynoecium and androecium in the orchid flower) swell so that the organ becomes enlarged and the stigmatic cavity encloses the pollen-bearing pollinia, ovule development is stimulated, and the ovary begins to enlarge and differentiate (Curtis, 1943). Treatment of flowers with ethylene does not stimulate these events, although treatment of pollinated flowers with norbornadiene does inhibit them (O'Neill et al., 1993). This suggests that ethylene is necessary but not sufficient to initiate these processes.

Because of the obvious importance of ethylene synthesis in coordinating postpollination events, we have chosen to examine the role played by regulation of enzymes involved in ethylene biosynthesis, and in particular the enzyme ACC oxidase. Ethylene is synthesized by plants through the conversion of S-adenosyl-L-Met to ACC, which is then oxidized to ethylene (Adams and Yang, 1979). The former reaction is catalyzed by the enzyme ACC synthase, and the latter reaction is catalyzed by the enzyme ACC oxidase (formerly known as the ethylene-forming enzyme). Although ACC synthase has been the topic of much recent research, relatively little is known about the role of ACC oxidase in regulating ethylene biosynthesis and subsequent ethylene-dependent developmental events. ACC oxidase was isolated initially from tomato as a cDNA clone, pTOM13, that was developmentally regulated during tomato fruit ripening (Holdsworth et al., 1987). It was later demonstrated that tomato plants containing an antisense copy of this cDNA produce greatly reduced levels of ethylene, and based on these findings it was proposed that pTOM13 encoded the ACC oxidase enzyme (Hamilton et al., 1990). This was conclusively demonstrated by functional expression of similar

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Abbreviation: ACC, 1-aminocyclopropane-1-carboxylate.

cDNA clones in *Saccharomyces cerevisiae* and *Xenopus laevis* oocytes (Hamilton et al., 1991; Spanu et al., 1991).

In this paper, we present data demonstrating that ACC oxidase activity is induced after pollination in *Phalaenopsis* orchid flowers, and that this induction is accomplished primarily at the level of gene expression. We also present the first evidence demonstrating that ACC oxidase mRNA is expressed in all living cells of floral tissues during the pollination-induced increase in ethylene biosynthesis, which suggests that all cells participate in the production of ethylene.

MATERIALS AND METHODS

Ethylene Biosynthesis

Ethylene production by whole, freshly harvested flowers of a clonal population of *Phalaenopsis* cv SM9108 (Stewart Orchids) was measured by enclosing the flowers in air-tight chambers for 1 h prior to the appropriate time point. Ethylene was sampled in duplicate with 1-mL syringes, and the concentration was determined by GC with an activated alumina column (Carle Analytical Gas Chromatograph 211) equipped with a flame-ionization detector and an SP4270 integrator (Spectra-Physics, San Jose, CA). Ethylene production (nL g^{-1} h^{-1}) was calculated on the basis of fresh weight determined prior to enclosure.

ACC Oxidase in Vivo Activity

Floral organs were dissected from cut *Phalaenopsis* flowers (Rod McLellan Orchids) and floated in 2 mL of 10 mM ACC, 50 mM Mes (pH 6.5) in 25-mL scintillation vials with gentle rotation for 2 h at room temperature. Vials were then sealed with serum vial septa and incubated for 1 h, after which the headspace was sampled in duplicate using 2-mL syringes. Ethylene production by the tissues was measured as for whole flowers. Petal and sepal tissue was combined and incubated as 1-cm discs, whereas the column (fused gynoecium and androecium of the orchid flower) was incubated as a whole unit. Preliminary experiments showed that these were the optimal conditions for achieving maximal ACC oxidase activity before stress-induced ethylene production by the tissues (data not shown).

ACC Oxidase Cloning and Sequencing

A degenerate oligonucleotide was constructed based on conserved regions of the ACC oxidase clones from tomato (Holdsworth et al., 1987) and avocado (McGarvey et al., 1990) and used to screen gynoecium- and perianth-specific cDNA libraries. The oligonucleotide was end labeled using [γ-^{32}P]dATP (Szostak et al., 1979), and hybridization was carried out at 42°C in 50% formamide (v/v), 6× SSC, 1× Denhardt's solution, 0.5% SDS (w/v), 100 μg/mL of sheared salmon sperm DNA, 0.05% NaPPi (w/v). Washing was performed at 42°C in 6× SSC, 0.05% NaPPi.

Approximately 60 cDNA clones were isolated, and an internal portion of each clone was sequenced using the screening primer. Candidates for ACC oxidase cDNAs were selected based on predicted amino acid sequence homology to tomato and avocado ACC oxidases. Orchid ACC oxidase cDNA clone number 1 (OAO1), which is presented in this paper, was isolated from a 24-h postpollination gynoecium cDNA library. The clone was subcloned into the plasmid vector pBluescriptII KS (Stratagene), and nested deletions were constructed using the Erase-a-Base system (Promega). Both strands were sequenced by the dideoxynucleotide chain-termination method (Sanger et al., 1977) using Sequenase enzyme (United States Biochemical). Sequence analysis and multiple sequence alignment (CLUSTAL) was accomplished using PC/Gene sequence analysis software (IntelliGenetics, Inc.).

RNA Extraction and Hybridization

RNA was extracted from *Phalaenopsis* cv SM9108 floral tissues at intervals after self-pollination as described by Cathala et al. (1983). Flowers were divided into floral organ units for this analysis: ovary and pedicel, column, petals combined with sepals, and labellum. The labellum, which is a specialized petal, was analyzed separately from the other perianth organs because of its morphological and behavioral uniqueness.

After RNA extraction, the poly(A)$^+$ fraction was purified using oligo(dT) bound to magnetic beads (Dynabeads, Dynal) (Jakobsen et al., 1990). Poly(A)$^+$ RNA was separated electrophoretically on formaldehyde agarose gels (Nevins and Wilson, 1981) and transferred to Nytran membranes (Schleicher and Schuell). Filters were UV-crosslinked for 2 min, then baked for 1 h at 80°C under vacuum.

RNA hybridization was carried out at 42°C in 50% formamide (v/v), 5× SSC, 1× Denhardt's solution, 50 mM phosphate buffer (pH 6.5), 0.2% SDS (w/v), 0.2 mg/mL of sheared salmon sperm DNA for 48 h. Filters were washed in 0.2× SSC, 1 mM EDTA, once for 10 min at room temperature, twice for 20 min at 55°C, and once for 20 min at 63°C. Insert from the cDNA clone OAO1 was labeled with [^{32}P]dCTP to high specific activity by random priming with the Klenow fragment of DNA polymerase (Promega) (Feinberg and Vogelstein, 1983) and used as the probe in all hybridizations.

In Situ Hybridization

In situ hybridization was carried out as described by Cox et al. (1984) and modified by Dietrich et al. (1989). *Doritaenopsis* flowers were used due to their easily managed size. Previous work in this laboratory has demonstrated that this closely related species responds similarly to *Phalaenopsis* (our unpublished results). Briefly, tissues were collected at intervals after pollination and fixed in formaldehyde, alcohol, and acetic acid (FAA), then dehydrated and embedded in Paraplast (Oxford Labware). Four to five tissue sections representing each time point were placed on replicate slides coated with poly-L-Lys. Several of these slides were reserved for conventional staining with toluidine blue to more clearly illustrate cellular detail by light microscopy. Prior to hybridization of the remaining slides, paraplast was removed and sections were blocked with 1% BSA (w/v) and treated with HCl, proteinase K, and acetic anhydride. Linearized pBluescriptII KS plasmid (Stratagene) containing OAO1 cDNA was used as template for [^{35}S]UTP-labeled asymmetric

RNA transcription using T3 and T7 RNA polymerases (Promega). Sense and antisense RNA probes were sheared to 0.2 kb by alkaline hydrolysis prior to hybridization. Hybridization to tissue sections was carried out for 16 h at 42°C in 50% formamide (v/v), 10% dextran sulfate (w/v), 300 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1× Denhardt's solution, 100 mM DTT, and 25 units/mL of RNA Guard (Pharmacia). Sections were treated with RNase H (Pharmacia) to remove nonhybridized probe, then washed once at room temperature in 2× SSC for 60 min and once at 55°C in 0.1× SSC for 60 min. Sections were coated with Kodak NTB-2 photographic emulsion and exposed at 4°C for 9 d. Sections were stained with fast green after development and photographed using a Nikon Optiphot-2 microscope. Silver grains appear as green particles under these conditions.

RESULTS

ACC Oxidase Activity Increases after Pollination

Pollination of *Phalaenopsis* flowers results in increased ethylene production first detectable at 6 h and reaching maximum levels by 36 h after self-pollination (Fig. 1). Unpollinated flowers do not produce measurable quantities of ethylene at any time point. The first physical symptoms of pollination are not apparent until 12 h after pollination, when cells of the column begin to swell. The labellum exhibits hyponastic behavior at approximately 24 h, and degradation of the perianth is not visually apparent until 36 h after pollination (O'Neill et al., 1993).

To examine whether increased ability of floral tissues to convert ACC to ethylene plays a role in the upsurge in ethylene production, we examined the in vivo activity of ACC oxidase in both the stigma, where pollination takes place, and the perianth, where senescence ultimately occurs (Fig. 2). In both the stigma and combined petal and sepal tissue of the *Phalaenopsis* flower, ACC oxidase activity was initially extremely low but increased following pollination. Increased levels of enzyme activity were first detected in the stigma of the flower, where activity attained peak levels of approximately 380 nL g^{-1} h^{-1} (Fig. 2A). Delayed increases in activity were observed in petal/sepal tissue, where peak activities of approximately 80 nL g^{-1} h^{-1} were obtained at 48 h after pollination (Fig. 2B). Within the time frame of the experiment, unpollinated flowers did not show significant enzyme activity, suggesting that the stress of harvest had not yet induced ethylene production. From this information, we conclude that ACC oxidase activity is not present constitutively in the orchid flower but is induced by perception of the pollination signal.

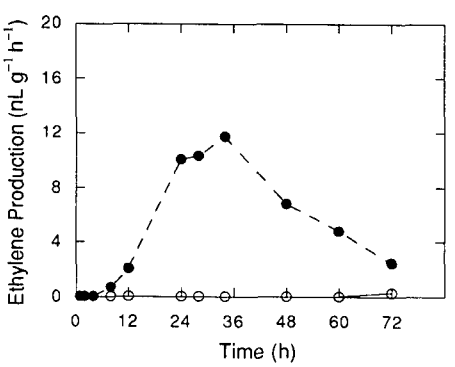

Figure 1. Ethylene production of *Phalaenopsis* cv SM9108 flowers. Self-pollinated (●) and unpollinated (○) flowers.

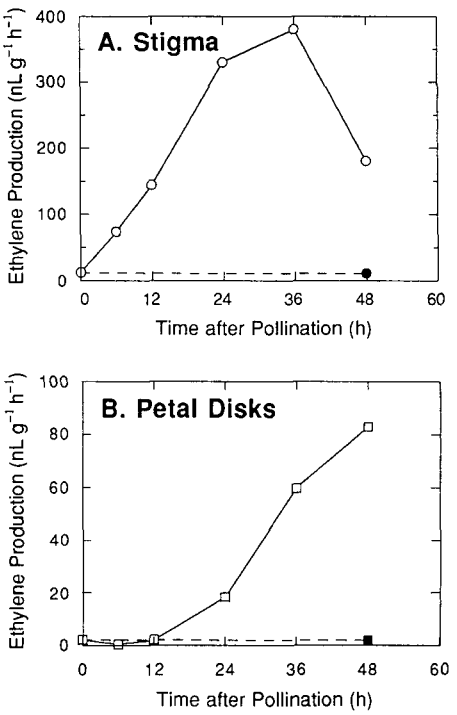

Figure 2. Ethylene produced by tissues floated in 10 mM ACC, 50 mM Mes (pH 6.5) for 3 h prior to measurement. A, Stigma tissue (column). B, Petal (and sepal) tissue discs. Pollinated flowers (○, □) and unpollinated flowers (●, ■).

Cloning of an ACC Oxidase Protein from Orchids

ACC oxidase is clearly induced after pollination, and there are several basic mechanisms by which this could be accomplished. Potentially, gene expression might be regulated by the pollination event, or alternatively, ACC oxidase activity might be limited by availability of the substrate or by modification of the protein. The expression of ACC oxidase mRNA was examined in *Phalaenopsis* orchids in order to understand the role of gene regulation in the pollination-induced increases in enzyme activity observed. A cDNA clone (OAO1) of approximately 1.4 kb in length was identified in the gynoecium library (Fig. 3). The sequence of OAO1 predicts a protein of an approximate molecular mass of 37 kD and shows approximately 70% identity at the amino acid sequence level with ACC oxidases cloned from tomato (Holdsworth et al., 1987), avocado (McGarvey et al., 1990), carnation (Wang and Woodson, 1991), apple (Dong et al., 1992), and petunia (Wang and Woodson, 1992) when compared individually (Fig. 4).

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AACAAACAGCTTAAGAGCTTTCTCTCTCTCAATAAGCTTTCCTCTTGAAACAGTGAGATCTAGTGAGAGAAA

CAGAGAGATGGAGAGCGGAAGCTTCCCTGTAATTAACATGGAGCTTCTCCAGGGTTCCCAGCGCCCGGCCGC
                                  M  E  L  L  Q  G  S  Q  R  P  A  A

CATGGCTCTTCTCCGAGACGCCTGTGAGAACTGGGGTTTGTACGAGTTACTGAACCACGGAATCTCCCACGA
  M  A  L  L  R  D  A  C  E  N  W  G  L  Y  E  L  L  N  H  G  I  S  H  E

GCTAATGAACCGGGTGGAGACGGTAAACAAAGAACATTACCGGCGGTTCCGGGAACAGCGCTTCAAAGAATT
  L  M  N  R  V  E  T  V  N  K  E  H  Y  R  R  F  R  E  Q  R  F  K  E  F

CGCGTCCAAAACCCTAGATACCGTGGAGAACGTCGAGCCGGAAAATCTGGACTGGGAGAGCACCTTCTTCCT
  A  S  K  T  L  D  T  V  E  N  V  E  P  E  N  L  D  W  E  S  T  F  F  L

CCGCCATCTCCCCACCTCCAACATCTCCCAAATCCCCGATCTGGATGACGATTGCCGGTCAACCATGAAGGA
  R  H  L  P  T  S  N  I  S  Q  I  P  D  L  D  D  D  C  R  S  T  M  K  E

ATTCGCGCTGGAGCTAGAGAACCTCGCGGAGAGACTGCTGGATCTGTTGTGCGAAGATTTGGGGCTGGAGAA
  F  A  L  E  L  E  N  L  A  E  R  L  L  D  L  L  C  E  D  L  G  L  E  K

AGGGTATTTGAAAAAGGTTTTCTGCGGGGGATCGGATGGTTTGCCGACGTTCGGGACGAAGGTGAGTAATTA
  G  Y  L  K  K  V  F  C  G  G  S  D  G  L  P  T  F  G  T  K  V  S  N  Y

TCCGCCATGTCCGAAGCCGGAGCTGATAAAGGGATTGAGAGCTCATACGGATGCAGGGGGGATCATTCTGTT
  P  P  C  P  K  P  E  L  I  K  G  L  R  A  H  T  D  A  G  G  I  I  L  L

GTTTCAGGATGATAAGGTTAGCGGGCTTCAGTTGCTCAAGGACGGGGAATGGATCGATGTTCCTCCCGTGCG
  F  Q  D  D  K  V  S  G  L  Q  L  L  K  D  G  E  W  I  D  V  P  P  V  R

CCATTCCATTGTCGTCAATATTGGAGATCAGCTGGAGGTGATAACGAATGGAAAATACAAGAGTGTGTTGCA
  H  S  I  V  V  N  I  G  D  Q  L  E  V  I  T  N  G  K  Y  K  S  V  L  H

TAGGGTGGTGGCCCAAACCGATGGAAACCGCATGTCGATCGCGTCGTTCTACAACCCTGGCAGCGACGCCGT
  R  V  V  A  Q  T  D  G  N  R  M  S  I  A  S  F  Y  N  P  G  S  D  A  V

GATCTTTCCGGCGCCGGCGCTGGTGGAGAAAGAGGCGGAGGAGAAGGAGGAGAAGAAGAAGGAAATTTATCC
  I  F  P  A  P  A  L  V  E  K  E  A  E  E  K  E  E  K  K  K  E  I  Y  P

AAAGTTTGTGTTCCAGGATTACATGAATCTGTACATTCGGAAGAAATTTGAGGCGAAGGAGCCGAGATTTGA
  K  F  V  F  Q  D  Y  M  N  L  Y  I  R  K  K  F  E  A  K  E  P  R  F  E

GGCGATGAAGAGTATGGAGATTGTTATGAGCTCTCAGCCGATACCCACTGCTTGATTGGTTCATTAATGGCT
  A  M  K  S  M  E  I  V  M  S  S  Q  P  I  P  T  A

ATTGTTTGTGAAGAATTTAGGGCATAGGGTTTAATCTATGTGTTTGTGGTTTGCTTGAGTCTAGTGGTTTGA

TTTATGTGAATTGTTTGGGGGCTTATCATTTATATATTTATATGTGTAGTTGTTATGTATGGATAGTTTGCT

ATTAAATTTGTTCCTTGGGGTTTGGGCGTAATTGAAGTGTGAGCTGATGTAGTCTTTGTTGTTCATCCCTTG

AATATTTATTGGCCACTAAATTTATTATTTA
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Figure 3. Nucleotide sequence of orchid ACC oxidase 1 (OAO1) and deduced amino acid sequence of OAO1 protein.

ACC Oxidase Gene Expression Is Induced by Pollination

As can be seen from Figure 5, orchid ACC oxidase transcripts accumulate dramatically after pollination in the stigma (column), petal, sepal, and labellum tissue of the flower. The most rapid induction is seen in the stigma tissue, in which the gene is induced to very high levels by 6 h after pollination. A faint band can be detected at 2 h after pollination under longer autoradiography exposures (20 h). Accumulation of mRNA transcript is delayed in the petal/sepal and labellum tissues, although mRNA levels at the peak of expression in the labellum are greater than in the stigma. Combined petal and sepal tissue exhibits lower levels of gene expression, which is consistent with the lower enzyme activity found in this tissue. Additionally, by 48 h the levels of ACC oxidase mRNA in the petal/sepal tissue is reduced, although enzyme activity has not yet declined, probably due to the more advanced state of degradation of this tissue. ACC oxidase mRNA accumulates in ovary and pedicel tissue as well, at levels at least 100-fold lower than in petal or sepal tissues. This pattern of accumulation is closely correlated with levels of enzyme activity, which suggests that ACC oxidase activity is controlled primarily through the regulation of gene expression.

ACC Oxidase Is Expressed in All Cells of Complex Tissues

To gain further insight into the role of ACC oxidase gene regulation in pollination-induced ethylene biosynthesis, we carried out in situ hybridization to transverse sections of column, petal, and labellum tissues. Figures 6 and 7 confirm the RNA blot hybridization analysis showing increased levels of expression after pollination with kinetics similar to those for enzyme activity and mRNA accumulation in all three

Figure 4. Comparison of the deduced amino acid sequence of OAO1 with ACC oxidases from tomato (PTOM13), avocado (AVOE3), carnation (SR120), apple (PAE12), and petunia (PETEFE). Asterisks (*) indicate identity; dots (•) indicate conservative substitutions.

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OAO1     MES-GSFPVINMELLQ---GSQRPAAMALLRDACENWGLYELLNHGISHELMNRVETVNKEHYRRFREQRFKEFA
PTOM13   ME---NFPIINLEKLN---GDERANTM--IKDACENWGFFELVNHGIPHEVMDTVEKMTKGHYKKCMEQRFKELV
AVOE3    MDS---FPVINMEKLE---GQERAATMKLINDACENWGFFELVNHISPVELMDEVERLTKEHYKKCMEQRFKELM
SR120    MANIVNFPIIDMEKLNNYNGVERSLVLDQIKDACHNWGFFQVVNHSLSHELMDKVERMTKEHYKKFREQKFKDMV
PAE12    MA---TFPVVDLSLVN---GEERAATLEKINDACENWGFFELVNHGMSTELLDTVEKMTKDHYKKTMEQRFKEMV
PETEFE   ME---NFPIISLDKVN---GVERAATMEMIKDACENWGFFELVNHGIPREVMDTVEKMTKGHYKKCMEQRFKELV
         *.    **..... ..   * .*. ..  ..***.***.....**  . *... ** ..*.**.. .**.**..

OAO1     SKTLDTVENVEPENLDWESTFFLRHLPTSNISQIPDLDDDCRSTMKEFALELENLAERLLDLLCEDLGLEKGYLK
PTOM13   ASKGLEAVQAEVTDLDWESTFFLRHLPTSNISQVPDLDEEYREVMRDFAKRLEKLAEELLDLLCENLGLEKGYLK
AVOE3    ASKVEGAV-VDANDMDWESTFFIRHLPVSNLSEIPDLTDEHRKVMKEFAEKLEKLAEQVLDLLCENLGLEKGYLK
SR120    QTKGLVSAESQVNDIDWESTFYLRHRPTSNISEVPDLDDQYRKLMKEFAAQIERLSEQLLDLLCENLGLEKAYLK
PAE12    AAKGLDDVQSEIHDLDWESTFFLRHLPSSNISEIPDLEEEYRKTMKEFAVELEKLAEKLLDLLCENLGLEKGYLK
PETEFE   ASKALEGVQAEVTDMDWESTFFLKHLPISNISEVPDLDEEYREVMRDFAKRLEKLAEELLDLLCENLGLEKGYLK
           .       . ..******...* * **.*..***... *. *..**  .*.*.* .******.*****.***

OAO1     KVFCGGSDGLPTFGTKVSNYPPCPKPELIKGLRAHTDAGGIILLFQDDKVSGLQLLKDGEWIDVPPVRHSIVVNI
PTOM13   NAFYGSKG--PNFGTKVSNYPPCPKPDLIKGLRAHDDAGGIILLFQDDFVSGLQLLKDEQWIDVPPMRHSIVVNL
AVOE3    MAFAGTTTGLPTFGTKVSNYPPCPRPELFKGLRAHTDAG-LILLFQDDRVAGLQLLKDGEWVDVPPMNHSIVINL
SR120    NAFYGANG--PTFGTKVSNYPPCPKPDLIKGLRAHTDAGGIILLFQDDKVSGLQLLKDGHWVDVPPMKHSIVVNL
PAE12    KVFYGSKG--PNFGTKVSNYPPCPKPDLIKGLRAHSDAGGIILLFQDDKVSGLQLLKDGEWVDVPPMHHSIVINL
PETEFE   NAFYGSKG--PNFGTKVSNLPPCPKPDLIKGLRAHTDAGGIILLFQDVKVSGLQLLKDGQWIDVPPMRHSIVVNL
          .* *...  *.******* ****.*.*.******.*** .******  *.*******..*.****..****.*.

OAO1     GDQLEVITNGKYKSVLHRVVAQTDGNRMSIASFYNPGSDAVIFPAPALVEKEAEEKEEKKKEIYPKFVFQDYMNL
PTOM12   GDQLEVITNGKYKSVLHRVIAQTDGTRMSLASFYNPGSDAVIYPAKTLVEKEAEE----STQVYPKFVFEDYMKL
AVOE3    GDQVEVITNGKYKSVMHRVVAQTDGNRMSLASFYNPGSDAVIFPAPALVEKEAEE----KKEVYPKFVFEDYMNL
SR120    GDQLEVITNGKYKSVMHRVIAQTDGNRMSLASFYNPGSDAVIFPAPALVEKE-EE----KCRAYPKFVFEDYMNL
PAE12    GDQIEVITNGKYKSVMHRVIAQSDGTRMSIASFYNPGNDSFISPAPAVLEKKTEDA---PT--YPKFVFEDYMKL
PETEFE   GDQLEVITNGKYKSVMHRVIAQKDGARMSLASFYNPGSDAVIYPAPALVEKEEAEK---NKQVYPKFVFEDYMKL
         ***.**********.***.**.**.***.*******.*. * ** ...**. ..        ******.***.*

OAO1     YIRKKFEAKEPRFEAMKSMEIVMSSQPIPTA
PTOM13   YAGLKFQAKEPRFEAMKAMESD----PIASA
AVOE3    YAGLKFQAKEPRFEVMKMKAVETANLSPITT
SR120    YLKLKFQEKEPRFEAMKAMETT---GPIPTA
PAE12    YSGLKFQAKEPRFEAMKAKEST----PVATA
PETEFE   YAGLKFQAKEPRFEAMKAMETDVKMDPIATV
         *   **..******.** ..      . ..
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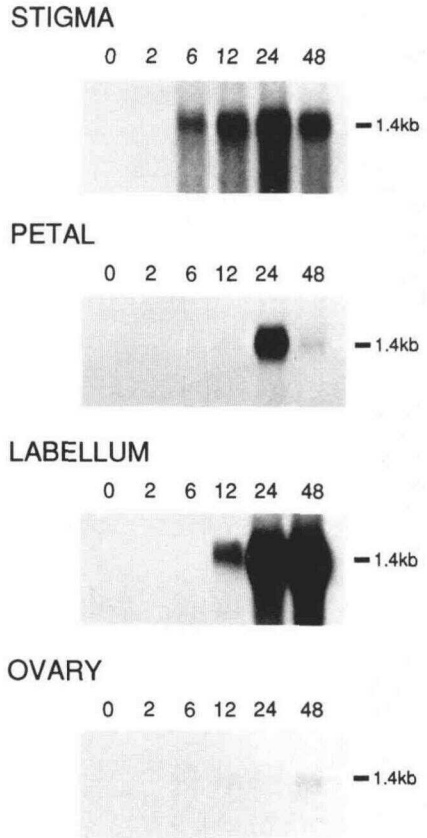

Figure 5. RNA gel blot analysis of the expression of ACC oxidase in the stigma, petals/sepals, labellum, and ovary tissues of the flower at various time points after pollination. Each lane contains 2 μg of poly(A)$^+$ RNA, and OAO1 insert was used as the hybridization probe.

tissues considered. Figure 6 shows changes in ACC oxidase gene expression in transverse sections of the column. This is a complex tissue consisting of many cell types, such as the cells of the stigmatic surface, transmitting tract cells, parenchymatous cells, vascular tissues, and epidermal papillae on the outer surface. Moreover, the orchid column is a fusion of the androecium and gynoecium of the flower, so that it is likely that some of the vascular and parenchymatous cells in these sections are derived from non-stylar origins. At 12 h after pollination, the earliest time point assayed, it is clear that there is no local distribution of mRNA encoding ACC oxidase or homologous sequences. In situ hybridization with ACC oxidase probe demonstrates that all living cells of the column express ACC oxidase message after induction by pollination and are thus likely to be competent to produce ethylene. Additionally, comparison of sense controls (Fig. 6b) and antisense-probed unpollinated tissue (Fig. 6c) suggests that there may be low levels of constitutive gene expression in the stigma that are not detectable by RNA blot hybridization analysis.

Figure 7 illustrates changes in gene expression in the petal (a-d) and labellum (e-h) tissues of the orchid flower. In both tissues, ACC oxidase message is also expressed in all living cells. Petal tissue, however, displays quantitatively less mRNA than the column, which corresponds to RNA blot hybridization data. In some sections, it appears that cells surrounding the vascular bundle may show more hybridization; however, we feel that this probably represents the greater structural integrity of these tissues at late stages of petal senescence and not tissue-specific gene expression. Quantitatively, a stronger hybridization signal is observed in labellum sections, which correlates well with RNA blot hybridization data showing stronger induction of ACC oxidase gene expression in this organ.

DISCUSSION

In this study, we have examined the role of ACC oxidase in the induction of ethylene biosynthesis that occurs after pollination of most flowers (Halevy and Mayak, 1981). The increase in ethylene production by *Phalaenopsis* orchid flowers correlates with an increase in ACC oxidase activity, as determined by in vivo incubation of tissues with the ethylene precursor ACC. After pollination, ACC oxidase activity levels rise rapidly in the column, where the pollination event is initially perceived (Arditti, 1979), but increases are delayed in the petals and sepals. This pattern of tissue-specific activity is consistent with the hypothesis that the gynoecium senses pollination of the flower and then propagates this information throughout the flower by synthesizing a translocated signal that must travel to other floral organs to induce ethylene biosynthesis in distal regions (Gilissen and Hoekstra, 1984; Hoekstra and Weges, 1986). Ethylene synthesis then results in increased expression of genes involved in the senescence program that causes petal degradation (Lawton et al., 1989, 1990). Additionally, we have observed that unpollinated *Phalaenopsis* flower tissue is not capable of oxidizing ACC to ethylene, which demonstrates that ethylene synthesis is not regulated solely by availability of the substrate ACC. This observation is contrary to the observation that in carnation and petunia styles as well as *Dendrobium* column tips, ACC oxidase activity is constitutive (Manning, 1985; Pech et al., 1987; Nair et al., 1991).

Similar results have been obtained in the emasculation response of *Cymbidium* orchid flowers (Woltering, 1990). Emasculation can induce ethylene production, which causes a change in pigmentation of the labellum, and this increase in ethylene production is associated with increases in ACC oxidase activity in the column of the flower. Because emasculation is an integral part of visitation of the flower by an insect pollinator, it is often associated directly with pollination and, therefore, may play a role in the normal induction of ACC oxidase expression during this process. It has also been observed that an increase in ACC oxidase enzyme activity is associated with age-related senescence of many flowers (Borochov and Woodson, 1989; Woodson et al., 1992), so it is not surprising that pollination-induced senescence also involves increased levels of enzyme activity.

Our data demonstrate that ACC oxidase gene expression increases in response to pollination in all organs of the flower, coordinate with increases in enzyme activity and ethylene production. This suggests that increased ACC oxidase activity and resultant ethylene production is primarily a result of de

(*Text continues on p. 38*)

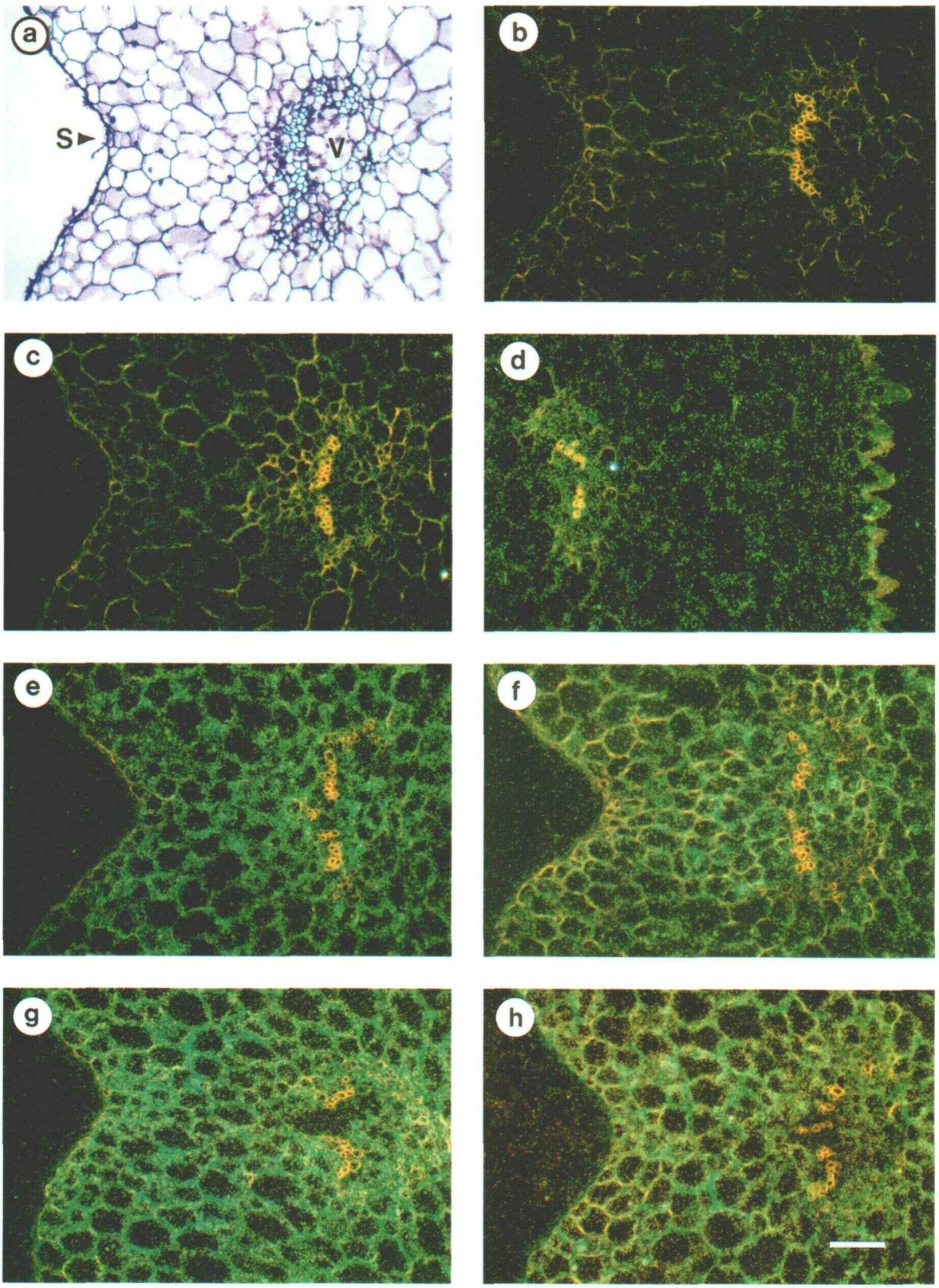

Figure 6. In situ hybridization to transverse sections of column tissue using RNA asymmetrically transcribed from OAO1 cDNA as probe. Scale bar = 100 μm (a-h). a, Light-field photograph of lower, stigmatic surface of toluidine blue-stained section; stigmatic surface (S) and vascular bundle (V) are indicated. b, Dark-field photograph of 72-h postpollination column probed with sense strand (control). c-h, Probed with antisense probe: c, unpollinated column, lower side; d, upper side of same unpollinated section; e, 12-h postpollination; f, 24-h postpollination; g, 48-h postpollination; h, 72-h postpollination.

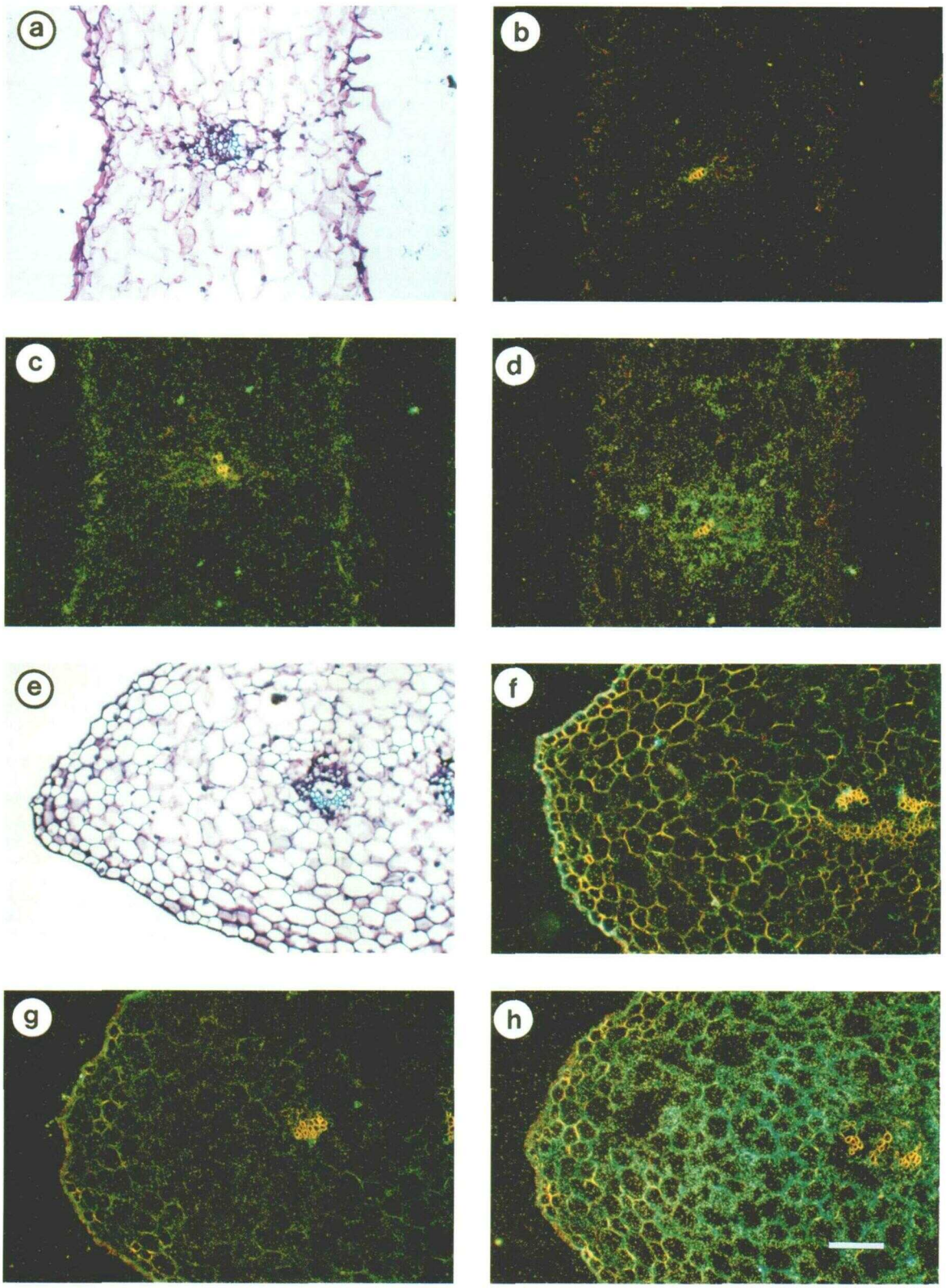

Figure 7. In situ hybridization to transverse sections of petal (a-d) and labellum (e-h) tissue using OAO1 RNA probe. Scale bar = 100 μm (a-h). a, Light-field photograph of petal section. b, Dark-field photograph of 72-h postpollination petal probed with sense strand. c, Unpollinated petal and d, 72-h postpollination petal probed with antisense strand. e, Light-field photograph of labellum section. f, Dark-field photograph of 72-h postpollination labellum probed with sense strand. g, Unpollinated labellum and h, 72-h postpollination labellum probed with antisense strand.

novo synthesis of protein. Furthermore, induction in the stigma occurs within 2 h of pollination, and by 12 h mRNA is present in all cells of the column. Because orchid pollen does not germinate for several days after contacting the stigmatic surface (Zhang and O'Neill, 1993), this rules out a role for wounding due to pollen tube growth in the stigma and style in triggering the pollination response, as has been suggested for other systems (Gilissen, 1977). Rather, in situ hybridization experiments show a pattern of diffuse and nonlocalized induction of ACC oxidase mRNA in the column, which implies that all cells are capable of ethylene production. This pattern suggests the involvement of a rapidly diffusable factor that induces ACC oxidase gene expression in the column. This observation contrasts with that made by Pech et al. (1987), who observed that ACC oxidase activity in unpollinated petunia stigmas was localized primarily in the uppermost tip, at the stigmatic surface.

ACC oxidase mRNA first appears in the perianth 12 h after pollination, preceding noticeable signs of petal and sepal degradation by at least 12 h. This suggests that ethylene production by the senescing organ plays a significant role in the induction of the senescence program in these organs. This hypothesis is supported by physiological experiments demonstrating that in *Petunia hybrida*, wilting of the perianth is not caused by stylar ethylene, but instead results from ethylene produced by the perianth itself (Hoekstra and Weges, 1986). This does not, however, rule out a role for stylar ethylene in inducing ethylene synthesis in other organs.

These observations are in accord with previous work demonstrating that age-related ethylene production by carnation flowers results from increased expression of genes encoding enzymes in the ethylene biosynthetic pathway (Woodson et al., 1992). In carnation flowers, however, there is considerable mRNA and ACC oxidase activity present prior to pollination in the style of the flower, whereas there is none present in the orchid prior to pollination. We speculate that this difference in expression pattern in unpollinated flowers may be the basis for the longevity of certain flowers, since expression of ACC oxidase in the style might ultimately lead to enough "leaky" ethylene production to stimulate full-scale autocatalytic ethylene production. Therefore, an extremely low level of ACC oxidase expression in the stigma and style of orchid flowers might provide the mechanism for the extraordinary lifespan of these flowers, since *Phalaenopsis* flowers last at least 3 months, compared with 2 weeks or less for many other flowers.

Moreover, the similarity in the pattern of induction of an age-related increase in ethylene biosynthesis and that of the pollination-induced ethylene peak leads to questions about the mechanism of induction of these genes. In carnation flowers, ACC oxidase gene(s) respond to developmental cues that establish expression in the style at anthesis (Woodson et al., 1992). ACC oxidase is also induced in the senescence program of cut carnation flowers, and physiological investigations suggest that in certain orchid flowers this mechanism of induction of gene expression may also operate (Goh et al., 1985; Nair and Tung, 1987). In nature, however, *Phalaenopsis* orchid flowers respond almost exclusively to a signal associated with pollination or emasculation associated with the pollination event during the extended period of time in which the flower remains receptive to pollination on the plant prior to senescence. Further research will serve to dissect the nature of the pollination signal that induces ACC oxidase gene expression and subsequent ethylene biosynthesis in *Phalaenopsis* orchids.

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