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Interorgan Regulation of Ethylene Biosynthetic Genes by Pollination

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Pollination initiates a syndrome of developmental events that contribute to successful reproduction, including perianth senescence, changes in pigmentation, and ovule differentiation in preparation for impending fertilization. In orchid flowers, initiation of each of these processes in distinct floral organs is strictly and coordinately controlled by pollination, thus providing a unique opportunity to study the signals that coordinate interorgan postpollination development. Because ethylene has been implicated in contributing to regulation of several aspects of postpollination development, we focused on determining the expression of its biosynthetic genes and their possible role in regulation. The abundance of mRNA encoding both 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase in the stigma, ovary, and labellum was found to be coordinately regulated by emasculation, auxin, and ethylene. Although petals contribute up to 26% of total flower ethylene and accumulate high levels of ACC oxidase mRNA and activity following pollination, no ACC synthase mRNA or activity was detected in this tissue. Together, these results support a model of interorgan regulation of postpollination development that depends on pollination-stimulated accumulation of mRNA encoding ethylene biosynthetic enzymes in a developmentally regulated and tissue-specific manner. This model relies on the translocation of a soluble hormone precursor, ACC, rather than on the translocation of the hormone itself. In this way, ACC serves to actuate the response already initiated by ethylene perceived by other parts of the flower. Thus, ACC may function as a secondary transmissible signal that coordinates postpollination development in diverse floral organs.

INTRODUCTION

Pollination of flowers is a key regulatory event in plant reproduction. Following pollination, a major developmental switch occurs involving an interorgan signaling within the flower. Pollination-induced signals originating in the stigma are translocated to other organs of the flower, namely, the ovary and perianth segments, where they initiate a suite of developmental changes comprised of a number of developmental processes including perianth senescence, pigmentation changes, ovary maturation, ovule differentiation, and female gametophyte development, components of which occur in all flowering plant species. This postpollination syndrome of developmental events is central to the basic mechanism of sexual reproduction ensuring successful fertilization and embryogenesis.

In many flowers, pollination serves to accelerate rather than induce developmental changes already occurring in the ovary and perianth, but which nevertheless proceed, although at a much slower rate, in unpollinated flowers. In contrast, postpollination development in orchid flowers is precisely and completely triggered by pollination. This postpollination developmental syndrome includes the induction and coordination of ovary and ovule development in preparation for fertilization and the remobilization of nutrients out of the perianth to these developing structures, resulting in perianth senescence. In addition, changes in pigmentation of floral organs, most notably the perianth, occur presumably to signal pollinators that a flower has already been visited and thus no longer offers a nectar reward (Van de Pijl and Dodson, 1966; Dressler, 1982). In the absence of pollination, the orchid flower will not exhibit these developmental changes as it ages. A corollary to this absolute requirement for pollination to induce postpollination senescence is that, in the absence of pollination, many orchid flowers are extremely long lived (Goh and Arditti, 1985), with some individual flowers having a life span up to 6 months. Darwin proposed that the extraordinary longevity of orchid flowers was an adaptation to pollination by highly specific insect vectors, with a longer life span increasing the likelihood that a specific pollinator would eventually find a particular flower with which it has coevolved (Darwin, 1862).

Pollination responses have been studied in a number of flowers mainly with regard to pollen tube/style interactions (Nasrallah et al., 1985, 1988; Anderson et al., 1986; Bernatsky et al., 1987; Cornish et al., 1987; Sanders and Lord, 1989; Haring et al., 1990), with only a few focusing on pollination-induced perianth senescence (reviewed by Halevy, 1986; Stead, 1992). Because pollen germinates on the stigma but subsequent developmental responses occur in the ovary and perianth, a

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pollination signal must move from the stigma to other floral organs, thus implicating the involvement of an interorgan messenger of pollination. The identity of the pollination signal remains in doubt but has been proposed to be auxin (Fitting, 1909; Müller, 1953; Burg and Dijkman, 1967) or 1-aminocyclopropane-1-carboxylic acid (ACC) (Whitehead et al., 1983, 1984; Pech et al., 1987), both of which are deposited by the pollen, or ethylene (Burg and Dijkman, 1967; Dijkman and Burg, 1970; Arditti et al., 1973; Arditti, 1979).

Both auxin and ACC may be present in pollen making these substances likely candidates for the pollination signal, but studies with radiolabeled auxin applied to the stigmatic surface indicate that auxin translocation is too slow to be the pollination signal in orchid and carnation flowers (Strauss and Arditti, 1982). On the other hand, a model of auxin as the initial pollination signal has been proposed by Burg and Dijkman (1967) in which auxin from the pollen diffuses through the stigma and stylar tissue of the column where it stimulates ethylene production as it moves, with the ethylene in turn directly triggering perianth senescence. Similarly, exogenous application of ACC results in a burst of ethylene production in many flowers, but this alone is not sufficient to elicit the full syndrome of postpollination development (Hoekstra and Weges, 1986) and instead leads to rapid cellular degradation throughout the flower (Zhang and O'Neill, 1993). Although ethylene itself clearly plays an important role in coordinating interorgan postpollination development, ethylene alone is not sufficient to trigger initiation of ovary development and ovule differentiation in orchid flowers. The development of the ovary as well as other aspects of the postpollination syndrome have an absolute requirement for the participatory action of auxin (Zhang and O'Neill, 1993).

Taken together, these findings indicate that auxin and ethylene are important factors in the initiation and coordination of postpollination development; however, the failure of exogenous application of these compounds to mimic pollination suggests that temporal and spatial regulation of the processes that regulate hormone levels in vivo may be important in the coordination of hormone levels that orchestrate the full complement of postpollination developmental responses. In this study, we describe the regulation of genes encoding ethylene biosynthetic enzymes in orchid flowers following pollination. The results of our studies suggest a model of interorgan regulation of postpollination flower development by spatial separation of expression of ethylene biosynthetic genes and translocation of the hormone precursor ACC.

RESULTS

Orchids Have a Complex Floral Structure

Based on the comparative morphology of their reproductive structures, orchids are viewed as the most evolutionarily advanced group of flowering plants (Van de Pijl and Dodson, 1966; Withner et al., 1974; Dressler, 1982). The specialized structure of the Phalaenopsis orchid flower used in our investigations is shown in Figure 1. The Phalaenopsis flower has a highly modified monocot flower structure typical of the orchid family. These modified structures are evolutionary specializations that have enabled the orchid family to coadapt with a variety of insect and animal pollinators. The male and female reproductive organs are fused into a compound structure known as the column. Male reproductive organs, including the anthers, pollen, and anther cap, are formed at the tip of the column. Individual pollen grains occur in tetrads and are organized in a pair of granular pollen masses, called pollinia. Just below this region, the column contains the modified stigma and the recessed stigmatic cavity, which create a chamber for the pollinia within the column. Part of the stigma includes a specialized structure called the rostellum, which is connected to the pollinia by two thin layers of tissue. Subtending the column is the perianth, which is composed of a whorl of sepals and a whorl of petals. In the orchid, the median petal has been modified into a specialized petalloid structure called the labellum, contributing to the bilateral symmetry of the orchid flower. The ovary

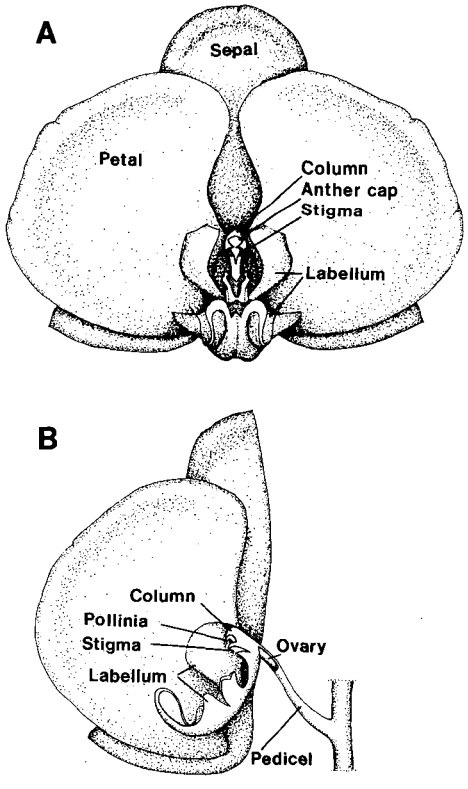

Figure 1. Structure of the Phalaenopsis Orchid Flower.

(A) Diagram of the front view of the Phalaenopsis flower showing organs.
(B) Diagram of a longitudinal view through the same flower showing inferior ovary.

of the orchid is inferior and has a compound structure typical of monocots with three carpels, but in Phalaenopsis, as in many other species of orchid, the ovary is not mature at the time of anthesis (Zhang and O'Neill, 1993). The ovary is a short segment ~5 mm in length at the time of pollination and is directly connected to the pedicel of the flower.

Because we were interested in understanding the basis of interorgan regulation of postpollination development, the parts of the orchid flower shown in Figure 1 were collected and analyzed separately or in groups of similar organs, as described in Methods. For example, the stigma, ovary, and labellum were separated while the remaining petals and sepals were grouped together representing the composite perianth. Although technically part of the perianth, the labellum was analyzed as a separate organ because of its structural uniqueness. This experimental approach later proved valid in terms of organ-specific gene expression that differentiated the labellum from other perianth tissues.

Pollination of orchid flowers is naturally mediated by insects that serve as pollen vectors bringing about cross-pollination. Before pollination, the pollen is separated from the stigmatic cavity at the tip of the column by the rostellum, which is protected beneath the anther cap. Thus, the first step in pollination involves emasculation of the flower wherein the anther cap and pollinia are removed by the pollinator. During this process, projecting parts of the rostellum that are also connected to the pollinia are disrupted; thus, this region of the column may be wounded as the pollinia are removed. This is followed by deposition of the pollinia on the stigmatic surface of another flower.

Two points in the pollination process are noteworthy with regard to our investigation. First, the initial emasculation step may involve wounding the tip of the column, which may be a facet of the stimulus that induces pollination-associated events, including changes in gene expression. Second, pollination is initially perceived at the stigmatic surface, but postpollination developmental events are initiated in the ovary and perianth, indicating that signals must move between organs to coordinate the process. These signals move rapidly, preceding pollen germination and growth of the pollen tubes into the style by at least 4 days in orchids (Zhang and O'Neill, 1993). In view of the possibility that both pollination and emasculation contribute to the postpollination response, we have analyzed these processes independently.

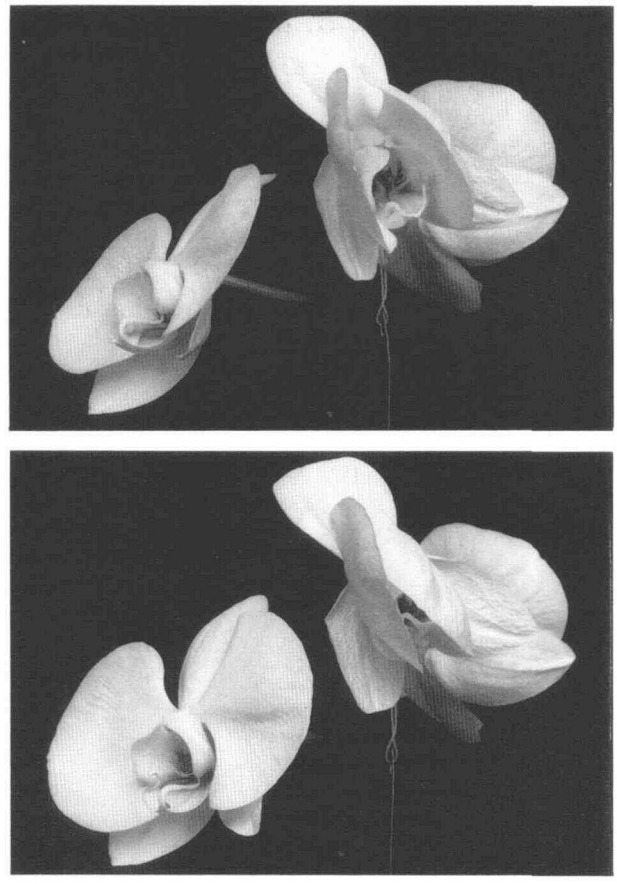

Figure 2. Effect of Pollination on the Phalaenopsis Orchid Flower.

Upper Panel: A flower 48 hr after pollination (right). The control flower (left) was not pollinated.

Lower Panel: The same flowers 72 hr after pollination.

Physiology of the Pollination Response and the Involvement of Ethylene

Experiments were conducted to examine the physiology of the pollination response in long-lived Phalaenopsis orchid flowers to determine whether the response in these flowers is similar to that previously observed for pollination and senescence-associated responses in carnation (Nichols, 1968; Bufler et al., 1980; Woodson et al., 1992), petunia (Gilissen, 1977; Gilissen and Hoekstra, 1984; Whitehead et al., 1984; Nichols and Frost, 1985; Pech et al., 1987), and other flowers (reviewed by Borochov and Woodson, 1989). To understand the hormonal and molecular basis for the postpollination response, it was necessary to establish the time course of the onset of the postpollination response as indicated by the rise in ethylene production by the flower. Figure 2 shows Phalaenopsis flowers at 48 and 72 hr after pollination and the effect of pollination in promoting perianth senescence, which is the most obvious symptom of the pollination response. Pollination resulted in visible wilting symptoms within 48 hr (upper section); wilting was more pronounced after 72 hr (lower section) when the perianth had become noticeably degraded. Other morphological changes associated with pollination included hyponastic closure of the petals, swelling of the column leading to enclosure of the pollinia within the stigmatic chamber, and growth of the ovary as reported previously for this species (Curtis, 1943).

Phalaenopsis flowers were also examined for pollination-induced ethylene production, as shown in Figure 3A. Following pollination, there was a dramatic increase in ethylene

production by the detached whole flower that was first detectable ~8 hr after pollination, with maximal levels of ethylene production occurring between 24 and 36 hr after pollination. The time course and extent of ethylene production following pollination were similar for flowers treated and maintained on the plant (data not shown). In contrast, there was a difference in ethylene production of unpollinated flowers that was associated with detachment from the plant. Unpollinated control flowers maintained as detached flowers during the experiment began to produce a substantial amount of ethylene by ~72 hr after harvest, at which time the detached flowers showed symptoms of water stress and other symptoms unrelated to pollination (Figure 3A). On the other hand, unpollinated control flowers attached to the plant until just prior to determination of ethylene production did not produce detectable levels of ethylene during the course of the experiment (Zhang and O'Neill, 1993). Emasculation also induced ethylene production by detached flowers similar in magnitude but somewhat delayed relative to that produced in response to pollination (Figure 3A). Unlike pollination, emasculation treatment did not induce similar levels of ethylene production in flowers that were still attached to the plant or in a subset of detached flowers (data not shown).

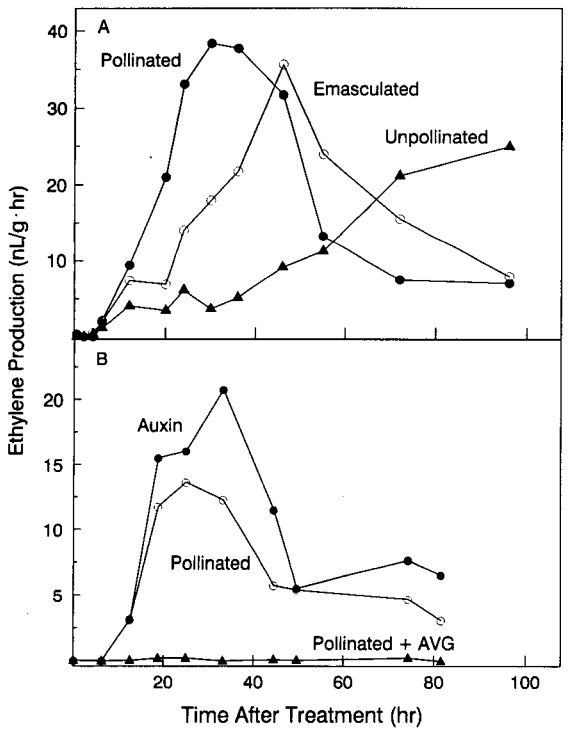

Figure 3. Time Course of Ethylene Production by Phalaenopsis Orchid Flowers.

(A) Ethylene production of pollinated (●), emasculated (○), and unpollinated control (▲) flowers.

(B) Ethylene production of pollinated (○), AVG-treated, pollinated (▲), and auxin-treated, unpollinated (●) flowers.

In preliminary experiments, we determined that a variety of different treatments to the stigma or whole flower resulted in ethylene production. Because many treatments to flowers resulted in ethylene production, it was necessary to establish treatment conditions that would eliminate its production and allow for the independent assessment of treatment and ethylene effects. Aminoethoxyvinylglycine (AVG) is a potent inhibitor of ACC synthase activity and, therefore, ethylene biosynthesis (Yu and Yang, 1979). The use of this inhibitor was tested as a means to inhibit ethylene production when applied to the orchid stigma prior to other treatments. Figure 3B illustrates that AVG completely blocked ethylene biosynthesis in pollinated flowers.

It has long been proposed that the causative agent in the postpollination response is auxin deposited on the stigma with the pollinia (Curtis, 1943; Burg and Dijkman, 1967). Indeed, there are a number of reports that auxin is a natural component of orchid pollen (Müller, 1953; Arditti, 1979; Stead, 1992). In light of this association between auxin and pollination in orchids, we also examined the effect of auxin on the stimulation of ethylene produced by detached flowers. Figure 3B shows that auxin, applied as naphthaleneacetic acid (NAA), is at least as effective as pollination in stimulating ethylene production in orchid flowers.

Ethylene Production by the Organs of the Orchid Flower

As part of our investigation of interorgan regulation of postpollination development, it was necessary to determine the contribution of individual floral organs to the total amount of ethylene produced by the whole flower and how this contribution might change with time after pollination. Thus, we examined ethylene production of the detached organs of the orchid flower following pollination. Figure 4 shows the amount of ethylene produced by the excised stigma, ovary, labellum, and perianth over a 72-hr period following pollination. The overall pattern of ethylene production by the organs of the flower suggests that ethylene is produced initially in the stigma and labellum. After 36 hr, when ethylene production by the whole flower is roughly at its peak (Figures 3A and 4), the perianth tissue, excluding the labellum, begins to make a more significant contribution to whole flower ethylene production, which accounts for 13.6% ethylene at 48 hr and 26% at 72 hr after pollination. The increase in ethylene production by the perianth was correlated with its progressive senescence, as shown in Figure 2. It is significant to note that ethylene produced by the intact flower was significantly higher than that of the isolated parts, especially at 36 and 48 hr after pollination, suggesting that separating the flower parts leads to a reduced capacity by these tissues to produce ethylene. Ethylene production by the perianth may have been only apparently low in our

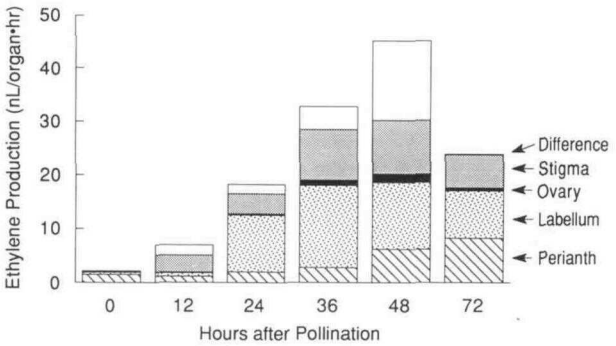

Figure 4. Contribution of Floral Organs to Total Ethylene Production by the Flower.

At each time point, whole flower ethylene production was determined; flowers were then dissected and the amount of ethylene produced by each organ was determined separately. The difference represents the intact flower production minus the sum of ethylene produced by the individual, dissected organs.

assays, because it was measured over a 2-hr period using excised flower parts that were detached from the stigma, a potential source of translocatable stimulus. Thus in the plant, the perianth may contribute more ethylene than these data indicate. Because ethylene production is lower in excised flower parts as compared to the intact flower, we concluded that significant wound-induced ethylene production did not occur within the time frame of the assay.

Genes Encoding Ethylene Biosynthetic Enzymes Are Regulated by Pollination in a Tissue-Specific Manner

To examine the spatial separation of ethylene biosynthetic activities as a mechanism of interorgan developmental regulation, we determined the temporal and organ-specific patterns of accumulation of mRNAs encoding two key ethylene biosynthetic enzymes, ACC synthase and ACC oxidase, by RNA gel blot hybridization analysis, as shown in Figure 5. ACC synthase mRNA accumulation was induced in the gynoecium (the stigma and ovary) and labellum tissue by pollination. A single diffuse polyadenylated RNA of ~1.6 kb was apparent under both high and low hybridization stringency. This size corresponds to the size of the two full-length ACC synthase cDNA clones of orchid, ACC synthases 1 and 2 (OAS1 and OAS2, respectively), which we have characterized previously (A. Q. Bui and S. D. O'Neill, unpublished results; GenBank accession numbers L07882 for OAS1 and L07883 for OAS2), and with other ACC synthase mRNAs (Sato and Theologis, 1989; Nakajima et al., 1990; Van der Straeten et al., 1990; Huang et al., 1991; Nakagawa et al., 1991; Olson et al., 1991; Sato et al., 1991; Dong et al., 1992; Park et al., 1992). ACC synthase mRNA reached its highest level of abundance between 24 and 48 hr after pollination, approximately coincident with the maximum levels of ethylene produced by the flower (Figure 3A). Figure 5 also illustrates that ACC synthase mRNA did not accumulate in perianth tissue, an unexpected result in view of the finding that this tissue makes substantial amounts of ethylene following pollination (Figure 4).

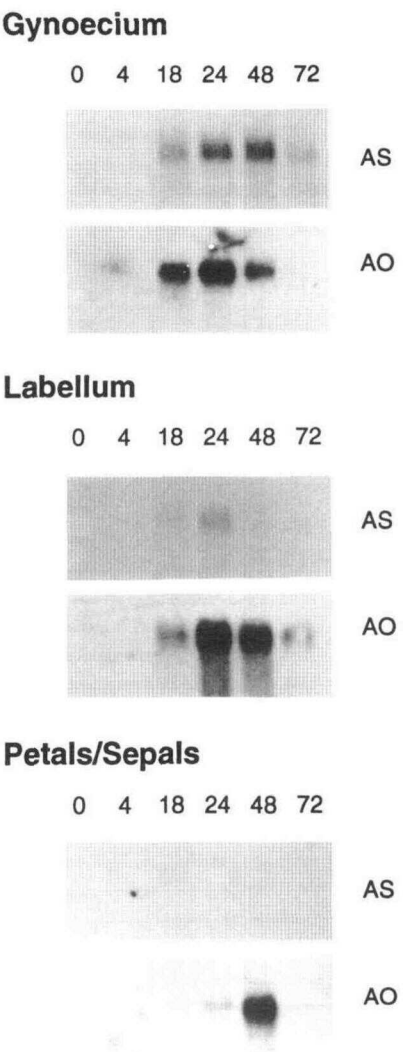

Figure 5. RNA Gel Blot Hybridization Analysis of ACC Synthase and ACC Oxidase mRNA Abundance in Organs of the Orchid Flower after Pollination.

ACC synthase mRNA (AS) abundance was analyzed using OAS1 cDNA to probe RNA gel blots, and ACC oxidase (AO) mRNA abundance was analyzed using an OAO1 cDNA probe. Each lane contains 2.5 μg of poly(A)$^+$ RNA (gynoecium) or 5.0 μg of poly(A)$^+$ RNA (labellum and perianth). The flowers in this experiment were pollinated and maintained on the plant. Numbers represent hours after pollination.

Several cDNA clones related to ACC oxidases from tomato (Smith et al., 1986) and avocado (Christoffersen et al., 1982) were isolated from orchid gynoecium and perianth cDNA libraries (J. A. Nadeau and S. D. O'Neill, unpublished results). The first of these cDNAs that we have characterized is orchid ACC oxidase 1 (OAO1, GenBank accession number L07912); it corresponds to an mRNA whose abundance increases dramatically in all organs of the flower following pollination, as shown in Figure 5. The temporal pattern of ACC oxidase mRNA accumulation was also similar to that of ACC synthase in that its abundance peaked at ~24 hr after pollination. However, ACC oxidase mRNA accumulation differed from that of ACC synthase in two important ways. First, after induction ACC oxidase mRNA was 100- to 1000-fold more abundant than that of ACC synthase. Second, ACC oxidase mRNA accumulated in the perianth. The accumulation of ACC oxidase mRNA in perianth tissue reached a maximum ~48 hr after pollination, coinciding with the onset of senescence of this floral tissue. ACC oxidase mRNA accumulation in the petals and sepals was delayed relative to the time course of its accumulation in the gynoecium.

Pollination Factors Regulating Expression of Genes Encoding Ethylene Biosynthetic Enzymes

To investigate and elucidate the role of specific pollination-associated factors that may regulate the observed changes in ACC synthase and ACC oxidase mRNA levels in Phalaenopsis flowers, we considered several major factors that are thought to be associated with pollination: emasculation, auxin and/or ACC deposited by the pollinia, and ethylene itself. We examined the effects of emasculation on the accumulation of ACC synthase and ACC oxidase mRNAs to determine if emasculation itself may be an important signal in promoting the postpollination syndrome. Figure 6 illustrates the levels of ACC synthase and ACC oxidase mRNA transcripts in orchid flower tissues. Tissue samples from the gynoecium (stigma or ovary) and perianth (labellum or petals plus sepals) of detached flowers were isolated 24 hr after emasculation with or without AVG, as shown in Figure 6. Both ACC synthase and ACC oxidase mRNA levels increased dramatically in the gynoecium and labellum in response to emasculation of detached flowers. Consistent with all of our other observations, ACC synthase mRNA was not detected in petal and sepal tissues. In nearly all cases, emasculation-induced accumulation of both ACC synthase and ACC oxidase mRNA was completely blocked by the presence of AVG, an inhibitor of ethylene biosynthesis that was described previously for Figure 3, suggesting that the effects of emasculation on mRNA accumulation are mediated by ethylene.

To investigate the roles of auxin and ethylene as pollination-associated signal factors, we examined the effects of auxin, when applied directly to the stigma of individual flowers, and exogenous ethylene, when applied to detached whole flowers, on levels of ACC synthase and ACC oxidase mRNA accumulation. Figures 7A and 7B illustrate that NAA induced the accumulation of ACC synthase mRNA in the stigma and labellum but not in the ovary or petals. ACC oxidase mRNA levels were elevated above levels induced by harvest of the flower in response to auxin treatment in the stigma, labellum, and petals but not in the ovary. Following auxin treatment, ACC oxidase mRNA levels declined in the ovary to levels almost undetectable 48 hr after pollination, as compared to levels present in the ovary of control flowers harvested 24 hr previously. Figures 7A and 7B show that exogenous ethylene application also stimulated ACC synthase mRNA accumulation in the

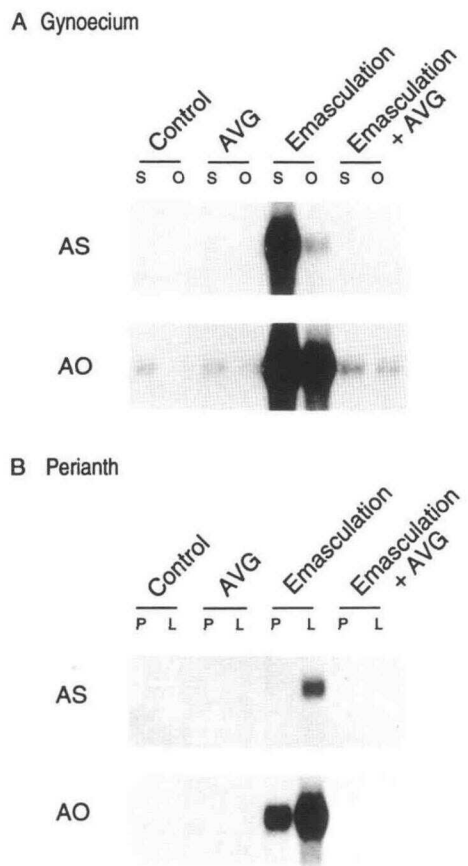

Figure 6. RNA Gel Blot Hybridization Analysis of the Role of Emasculation in ACC Synthase and ACC Oxidase Gene Expression in the Orchid Flower.

(A) mRNA abundance in the gynoecium 24 hr after pollination. S, stigma; O, ovary.

(B) mRNA abundance in the perianth 24 hr after pollination. P, petals and sepals; L, labellum.

The treatments in this experiment were performed on detached flowers. Each lane contains 2.0 μg of poly(A)$^+$ RNA. AS, ACC synthase; AO, ACC oxidase.

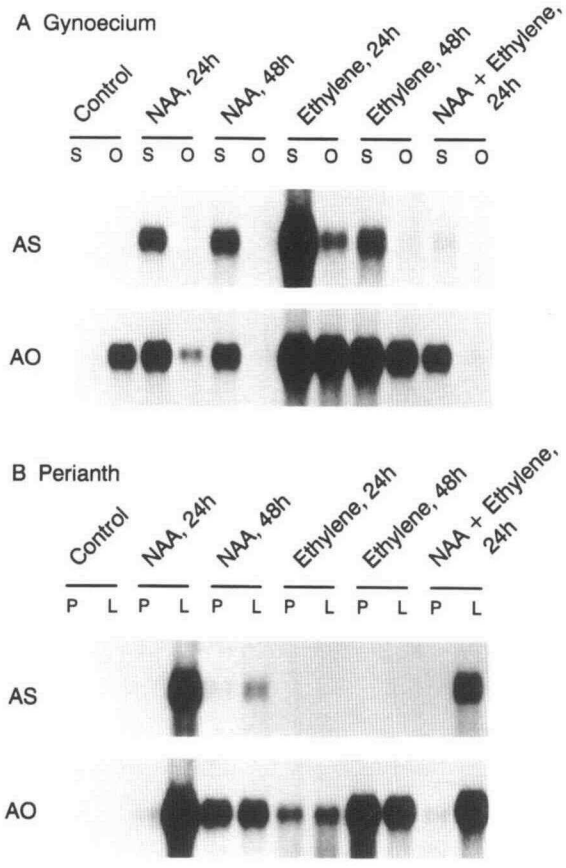

Figure 7. RNA Gel Blot Analysis of ACC Synthase and ACC Oxidase mRNA Abundance in Response to Auxin and Ethylene.

(A) mRNA abundance in stigma (S) and ovary (O) of flowers treated with NAA, ethylene, or NAA and ethylene as compared to untreated flowers.

(B) mRNA abundance in petals and sepals (P) and labellum (L) from the same flowers as given in **(A).**

Each lane contains 2.0 μg of poly(A)$^+$ RNA. AS, ACC synthase; AO, ACC oxidase. Times given indicate hours after treatment.

stigma and ovary, but again no ACC synthase mRNA was detected in the petals, even though the entire flower was exposed to a high concentration of ethylene. Ethylene failed to stimulate the appearance of an ACC synthase mRNA transcript in the labellum, even though a transcript was detectable in auxin-treated tissue, suggesting that in the labellum, an ACC synthase gene that is directly induced by auxin is present.

ACC oxidase mRNA accumulation was stimulated by ethylene in all floral tissues examined, as shown in Figures 7A and 7B. When auxin and ethylene were applied simultaneously, the presence of auxin appeared to partially repress the stimulation of both ACC synthase and ACC oxidase mRNA accumulation observed in most organs in the presence of ethylene alone. The labellum was an exception, because in this tissue, ethylene did not induce ACC synthase mRNA accumulation and more weakly induced ACC oxidase accumulation relative to that induced by auxin. Therefore, in tissue isolated from the labellum, it appears that auxin alone stimulates the expression of these genes.

To determine whether ACC synthase and ACC oxidase mRNA accumulation resulted directly from treatment with auxin or was mediated through ethylene, similar experiments were repeated in the absence and presence of AVG. Figure 8 shows the results obtained when orchid flowers were treated in the presence of AVG. Ethylene promoted high levels of accumulation of both ACC synthase and ACC oxidase mRNAs in the presence of AVG, but the accumulation of both mRNAs in response to auxin was completely abolished by AVG. In short-term experiments (6 hr), exogenous application of ACC promoted the accumulation of both mRNAs, presumably by the ability of ACC to bypass the AVG-induced block on ethylene biosynthesis and provide a short-lived burst of ethylene production (Zhang and O'Neill, 1993). In longer term experiments (24 hr), ACC application did not promote sustained accumulation of either mRNA. Because ACC treatment induces only short-lived and relatively low levels of ethylene from direct conversion of the applied ACC, this result suggests that continuous ethylene is required to promote accumulation of ACC synthase

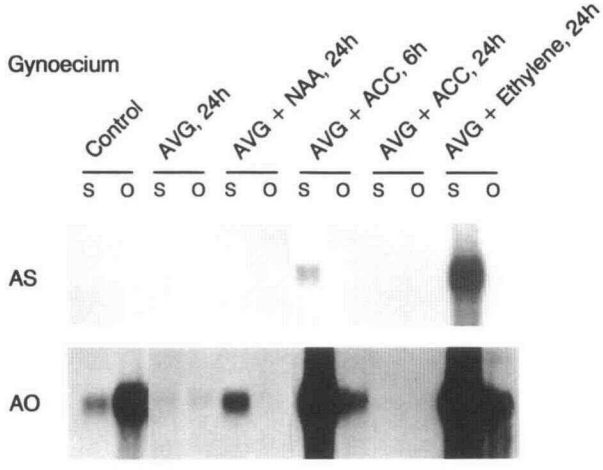

Figure 8. RNA Gel Blot Hybridization Analysis of the Role of Auxin, ACC, and Ethylene in ACC Synthase and ACC Oxidase Gene Expression in the Orchid Flower.

The mRNA abundance in the stigma (S) and ovary (O) of flowers treated with AVG alone or in combination with NAA, ACC, or ethylene as compared to untreated (control) flowers. AS, ACC synthase; AO, ACC oxidase. Treatments in this experiment were performed on detached flowers. Each lane contains 2.5 μg of poly(A)$^+$ RNA. The lanes marked control indicate no treatment; lanes labeled AVG, 24 hr are the same as the control lanes but contain tissue treated with AVG; subsequent lanes contain tissue treated with AVG in combination with NAA, ACC, or ethylene and harvested at the times indicated.

and ACC oxidase mRNAs in the gynoecium, which is consistent with observations previously made in tomato fruit and carnation flowers (Oeller et al., 1991; Woodson et al., 1992).

Together, the results examining the role of pollination-associated factors on accumulation of ACC synthase and ACC oxidase mRNAs suggested that ethylene itself is the most important factor in stimulating accumulation of these transcripts and led us to further investigate the role of ethylene in post-pollination events. To test whether exposure to ethylene alone is sufficient to promote the pattern of ethylene production observed in pollinated flowers, we utilized the ethylene analog propylene. Propylene is thought to interact with the hypothetical ethylene receptor in a manner similar to ethylene, but with a much lower affinity. At 100-fold higher concentrations than ethylene, propylene can be used to induce ethylene responses in plant tissue (Burg and Burg, 1967; McMurchie et al., 1972). This allows measurement of autocatalytically induced ethylene production that would be precluded by treatment with ethylene itself. As shown in Figure 9, exogenous propylene (1000 μL/L) promoted ethylene production that peaked at 36 hr after treatment, which is a pattern very similar to that observed for both pollinated or auxin-treated flowers (Figures 3A and 3B). Propylene-treated flowers exhibited symptoms of perianth senescence similar to those induced by exogenous ethylene treatment or following pollination. However, postpollination changes in the gynoecium associated with ovary development and inducible by pollination or auxin treatment were not induced by propylene treatment (data not shown). These data demonstrated that the observed pattern of ethylene production resulting from pollination can be mimicked by the exogenous application of propylene, suggesting that ethylene alone, or its analog, is sufficient to promote the pattern of ethylene production and senescence-related changes observed in pollinated flowers, but is not sufficient to promote the complete postpollination syndrome.

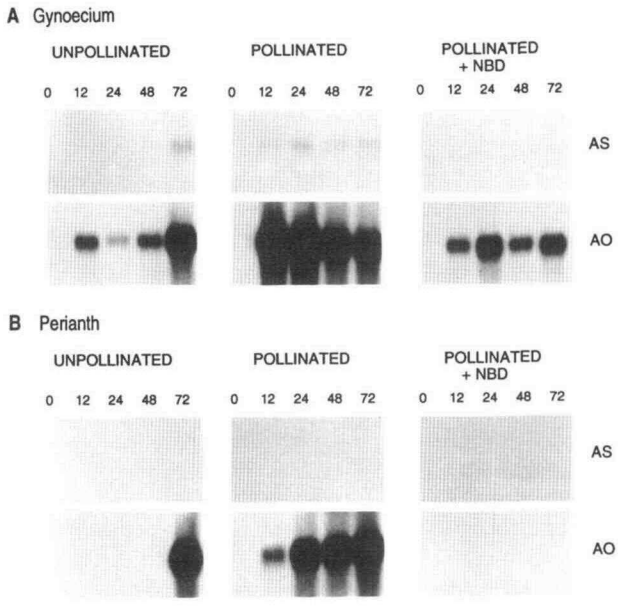

Figure 10. RNA Gel Blot Hybridization Analysis of the Involvement of Ethylene Perception in the Induction of ACC Synthase and ACC Oxidase mRNA following Pollination.

(A) mRNA abundance in gynoecium tissue. Each lane contains 1.0 μg of poly(A)$^+$ RNA.
(B) mRNA abundance in perianth tissue. Each lane contains 3.0 μg of poly(A)$^+$ RNA.
Unpollinated, pollinated, and NBD-treated, pollinated flowers were dissected into gynoecium and perianth at intervals (hours) after pollination. Treatments were performed on detached flowers. AS, ACC synthase; AO, ACC oxidase.

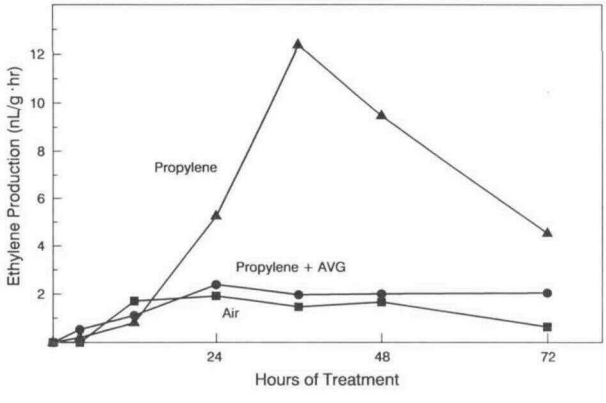

Figure 9. Effect of Propylene on Ethylene Production of Orchid Flowers.

Ethylene production of flowers held in air (■) or propylene with (●) or without (▲) AVG pretreatment. Flowers were pretreated with AVG on the stigma in AVG treatments and then enclosed in gas-tight flow-through chambers circulating either air or 1000 μL/L propylene. Gas samples were removed from the chambers at intervals (hours) for analysis.

ACC Synthase and ACC Oxidase Gene Expression Is Regulated Directly by Ethylene

We also used the inhibitor of ethylene production, 2,5-norbornadiene (NBD), a gaseous compound that competes with ethylene for binding to the ethylene receptor and thereby blocks ethylene action (Sisler and Yang, 1984), to examine the role of ethylene in gene expression. NBD had a pronounced effect on the physiological response to pollination. NBD treatment of flowers at the time of pollination inhibited gynoecium swelling and closure, ovary growth, and perianth senescence, suggesting the active involvement of ethylene in these processes (data not shown). Analysis of mRNA extracted from NBD-treated flowers demonstrated that pollination-induced accumulation of ACC synthase mRNA in the gynoecium was completely blocked by NBD, as shown in Figure 10. The

absence again of detectable levels of ACC synthase mRNA transcript in the perianth tissue confirms our earlier finding that the induction of this gene in the perianth is not a major component of the mechanism of the postpollination response (also see Figure 5). A small amount of ACC synthase transcript appeared in gynoecium tissue mRNA from unpollinated control flowers after 72 hr, which is coincident with a small amount of measurable ethylene production by these flowers. We considered this to be a result of the stress associated with the necessary harvest for experimental manipulation.

A parallel analysis was conducted to determine the amount of ACC oxidase mRNA accumulation following pollination and in the presence of NBD, as shown in Figure 10. In contrast to the previous results obtained with ACC synthase mRNA, ACC oxidase mRNA was detectable in gynoecium tissue 12 hr after the start of experimental treatment in unpollinated flowers. We believe that this amount of mRNA is attributable to an induction of ACC oxidase gene expression by wounding associated with flower harvest. Following pollination, a pronounced increase in ACC oxidase mRNA levels occurred in both the gynoecium and perianth, with maximum mRNA transcript abundance between 12 to 24 hr and 24 to 72 hr, respectively. The component of ACC oxidase gene expression induced following pollination was completely abolished by NBD, suggesting that induction is dependent on ethylene perception in both tissues. The other component of ACC oxidase gene expression that was induced by harvest was not sensitive to NBD, suggesting that its induction is independent of ethylene production by the flower. The latter component may instead be induced directly by other factors, such as cell wall fragments, salicylic acid, jasmonic acid, or a peptide inducer (Anderson et al., 1982; Parthier, 1990; Pearce et al., 1991; Ryan and Farmer, 1991; Raskin, 1992), known to be involved in wound or stress responses in other systems.

DISCUSSION

Pollination is a key regulatory process in flower development. Following pollination, a major developmental switch occurs involving interorgan signaling within the flower. Specifically, signals originating in the stigma, the site of pollination, are transduced to other organs of the flower, especially the ovary and perianth, where they trigger ovary development and senescence, respectively. Orchid flowers are particularly well suited for studying this developmental transition because the response is entirely dependent on pollination and so can be distinguished from aging responses that accompany postpollination events in most other flowers (Halevy, 1986). In this report, we focused on the regulation of genes encoding ethylene biosynthetic enzymes as components of the interorgan signaling process. In a previous study, our focus was on the major developmental response to pollination-associated signals, namely, ovary maturation, ovule differentiation, and gametophyte development (Zhang and O'Neill, 1993).

Ethylene plays an important role in coordinating postpollination development. To identify pollination-associated factors contributing to the regulation of ethylene biosynthetic capacity in the flower, we assayed levels of ACC synthase and ACC oxidase mRNAs in response to emasculation, ACC, and auxin. Emasculation constitutes a physical perturbation that accompanies pollination, whereas ACC and auxin are two substances reported to be a component of orchid pollen (Fitting, 1909; Müller, 1953; Arditti, 1979; Stead, 1992). The role of emasculation as an important signal in the postpollination syndrome was investigated because it has been previously reported that emasculation leads to pigmentation changes, perianth senescence, and the induction of ACC synthase activity in the column, but not the perianth, of Cymbidium orchid flowers (Arditti, 1979; Woltering, 1989, 1990; Woltering and Harren, 1989; Woltering and Somhorst, 1990). In our experiments, emasculation of flowers detached from the plant resulted in both ethylene production and changes in ACC synthase and ACC oxidase mRNA abundance in most organs. In all our experiments, however, emasculation-induced ethylene production lagged behind that induced by pollination. Furthermore, emasculation of flowers maintained on the plant did not induce the production of ethylene (Zhang and O'Neill, 1993). Thus, it is likely that pollination signals precede and are distinct from those resulting from emasculation. In addition, our results suggest that under natural conditions (e.g., in attached flowers) emasculation does not promote significant levels of ethylene production, and only pollination initiates the postpollination developmental syndrome.

The role of auxin as a hormonal signal in the postpollination development of orchid flowers was of particular interest because of previous reports that orchid pollen contains substantial amounts of auxin (Arditti, 1979; Stead, 1992). Burg and Dijkman (1967) proposed that pollen-borne auxin is the primary signal initiating postpollination development in orchids. This proposal relies on the transport of auxin from the site of pollination to other parts of the flower, although reports concerning the movement of stigma-applied auxin within the orchid flower are conflicting (Burg and Dijkman, 1967; Strauss and Arditti, 1982). Nevertheless, when we applied auxin to the stigma of Phalaenopsis flowers, ovary development was initiated and sustained for up to 5 days, thus mimicking the effect of pollination and suggesting that auxin or auxin-dependent signals move to the ovary (Zhang and O'Neill, 1993).

In the experiments reported here, we applied NAA to determine its effect on ethylene production and gene expression in the various organs of the flower. Auxin treatment stimulated ethylene production by the flower in a manner similar to that of pollination. Auxin treatment also promoted the accumulation of ACC synthase and ACC oxidase mRNAs similar to pollination. When flowers were treated with ethylene, ACC synthase and ACC oxidase mRNAs accumulated to much higher levels than with auxin. Surprisingly, treatment of flowers with both auxin and ethylene together resulted in decreased accumulation of ACC oxidase and ACC synthase mRNAs relative to either treatment alone. This observation is of potential

interest because only treatment with both ethylene and auxin leads to the initiation of ovary development (Zhang and O'Neill, 1993), suggesting that partial repression by auxin of ethylene-induced ACC synthase and ACC oxidase expression in the ovary is a normal prerequisite for further development of its tissue. The exact nature of how auxin and ethylene interact to coordinate the expression of genes encoding ethylene biosynthetic enzymes is unknown.

Previous investigation of carnation flowers has demonstrated that during age-related senescence both ACC synthase and ACC oxidase gene expression are coordinately induced in all organs of the flower (Woodson et al., 1992). In orchid flowers, there is evidence that ACC synthase and ACC oxidase activities are not uniformly expressed in all organs. ACC synthase activity has been reported to be induced in response to emasculation in the gynoecium, but not the perianth, of Cymbidium flowers (Woltering, 1989; Woltering and Somhorst, 1990). Based on results with orchids and other flowers, it has been demonstrated that ACC, synthesized in the gynoecium, is translocated to the perianth where it is converted to ethylene by ACC oxidase (Whitehead et al., 1983, 1984; Reid et al., 1984; Woltering, 1990). Such a spatial separation of ethylene biosynthetic enzyme activities could provide the basis for interorgan coordination of the postpollination syndrome of developmental events.

Our results indicate that although the perianth, excluding the labellum, contributes substantially to total ethylene production by the flower, ACC synthase mRNA accumulation and ACC synthase activity (A. Q. Bui and S. D. O'Neill, unpublished results) are not detectable in this organ. In addition, ethylene production in perianth tissue is reduced when separated from other flower organs, suggesting that ethylene production must be supported by ACC translocation from its site of synthesis in the stigma, ovary, and/or labellum to the petals. We have recently demonstrated that ^{14}C-ACC applied to the stigma of Phalaenopsis flowers was converted to ^{14}C-ethylene in the petals (J. A. Nadeau and S. D. O'Neill, unpublished results). These data are consistent with a model wherein the ethylene precursor, ACC, must be translocated to a major site of its conversion, the perianth. Figure 11 illustrates a general model of interorgan regulation of ethylene production in pollinated orchid flowers. In this model, pollination provides the primary signal that elicits the accumulation of ACC synthase mRNA and ACC synthase activity in the stigma. This activity leads to the accumulation of ACC, which can be converted to ethylene by a low basal level of ACC oxidase in the stigma. This ethylene acts autocatalytically to further stimulate the accumulation of ACC synthase and ACC oxidase mRNAs in the stigma, ovary, and labellum. Translocation of ACC as a mechanism of interorgan signaling has also been proposed for interorgan regulation of plant responses to flooding stress (Bradford and Yang, 1980). This mechanism is interesting because it relies on a soluble and translocatable hormone precursor rather than transport of the hormone itself, which in this case is gaseous and therefore, presumably, not amenable to targeted translocation processes.

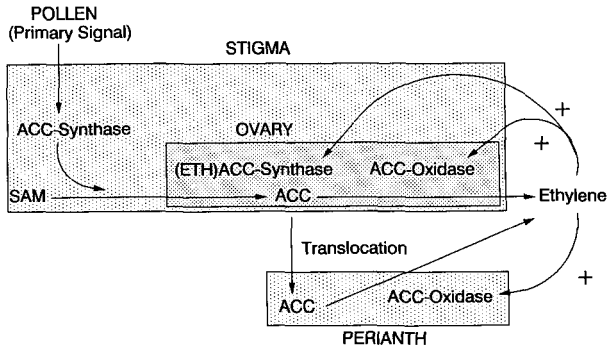

Figure 11. Model for the Interorgan Regulation of Ethylene Biosynthetic Genes in Pollinated Orchid Flowers.

ACC is 1-aminocyclopropane-1-carboxylic acid; ETH, ethylene stimulated; SAM, *S*-adenosyl-L-methionine.

Based on our experiments, pollination-induced accumulation of ACC oxidase mRNA in the perianth is ethylene dependent. Thus, translocated ACC acts only to actuate a response that is induced by ethylene in the perianth. In this sense, ACC should not be thought of as the transmissible signal that communicates to other parts of the flower that pollination has occurred, but instead as the actuator of the response. The primary signal perceived by the various organs of the flower is ethylene produced by the stigma.

We found that several pollination-associated treatments, such as emasculation, auxin, and ACC, promoted ACC synthase and ACC oxidase mRNA accumulation. Inhibitors of ethylene production (AVG) or action (NBD) completely reversed the effects of pollination and pollination-associated treatments on mRNA accumulation, suggesting that the observed response to each treatment was mediated by ethylene. These data are consistent with a model of autocatalytic ethylene production in the flower being initiated by pollination signals that first activate expression of ethylene biosynthetic genes in the stigma. It is important to note, therefore, that the genes we have studied do not show a pattern of regulation indicating that they are responsible for the initial induction of ethylene production, although such genes must exist to initiate the process. We believe that the patterns of ACC synthase and ACC oxidase mRNA accumulation described here can account for transduction and amplification of the pollination signal but do not reflect the primary responses to pollination and do not allow conclusions regarding the nature of the primary pollen-derived signal that initiates the process. We presume that an additional divergent ACC synthase mRNA accumulates in response to direct pollination signals, but that it must be present at very low levels because it has not been detected in our assays using degenerate oligonucleotide probes to conserved domains of ACC synthases. Resolution of this problem is likely to require highly sensitive methods to detect the earliest responses to pollination.

METHODS

Plant Material

Orchid plants of the genus *Phalaenopsis* (cultivar SM9108, Stewart Orchids, Carpinteria, CA) were obtained as a clonal population of genetically identical, mature individual plants and maintained under optimal growth conditions in a greenhouse at the University of California, Davis, Section of Botany. Approximately 100 individual plants were used in this investigation. Each mature plant yielded 8 to 30 flowers per plant, and each flower weighed ~7.5 g. For physiological experiments, flowers were either (1) treated and maintained on the plant until harvest for measurement of ethylene production, or (2) they were harvested by excision at the pedicel abscission zone, placed in water tubes, treated in the laboratory, and stored in a lighted, temperature-controlled growth chamber maintained at 22°C and 80% relative humidity throughout experimental treatment. Following treatment, flowers were harvested directly into liquid N_2 and stored at −80°C for later use. For RNA isolation, the stigma, ovary, labellum, and perianth parts (three sepals and two remaining petals) were collected separately in liquid N_2, pulverized to a fine powder, and stored at −80°C until used.

Experimental Manipulation

Pollination/Emasculation Studies

Flowers were hand pollinated by removing the anther cap and pollinia with a blunt forceps and then placing the pollinia on the stigma. For 0 hr time points, flowers were pollinated immediately prior to collecting dissected floral tissue in liquid N_2. Emasculation was achieved by removal of the androecium without subsequent pollination. For experiments comparing pollination or emasculation with control flowers (untreated), individual flowers were either treated or left untreated (controls) alternately along the raceme to compare the response of flowers of approximately equal age. Each flower was individually tagged with the date of anthesis and treatment. Flowers were left undisturbed on the plant for longevity studies in the absence of pollination. In general, flowers had an approximate life span of 4 months.

Treatments

Parallel sets of experiments were conducted as follows. For experiments using detached flowers, flowers were harvested by excision at the pedicel abscission zone and immediately inserted into floral tubes containing water. Otherwise, flowers were treated and maintained on the plant. In both cases, whole flowers were pollinated, emasculated, or treated by applying a 15-μL volume of either distilled H_2O as a control or naphthaleneacetic acid (NAA, 20 μg per flower), 1-aminocyclopropane-1-carboxylic acid (ACC, 10 nmol per flower), aminoethoxyvinylglycine (AVG, 0.5 mmol per flower), or combinations thereof to the stigma. Concentrations of NAA were chosen based on preliminary experiments and concentrations used for previous postpollination studies of orchids (Arditti and Knauft, 1969). For experiments involving the use of AVG, flowers were pretreated with AVG for 12 hr to ensure penetration of the inhibitor prior to treatment with NAA, ACC, or ethylene. Flowers were also pretreated with AVG prior to pollination or emasculation. For treatments with ethylene, detached flowers were used of necessity. Freshly harvested flowers were placed in a sealed chamber, and pure ethylene gas was applied at a concentration of 10 μL/L. These treatments were applied 24 hr prior to collecting tissue in liquid N_2 for later use in RNA isolation and enzyme assays. All chemicals described above were obtained from Sigma Chemical Co.

Propylene Studies

Detached flowers in water tubes were treated either with double-distilled H_2O (control) or AVG (0.5 nmol per flower) by application of a 15-μL volume directly to the stigma prior to the onset of the propylene experiment. Flowers were then sealed in 20-L glass tanks equipped with continuous air flow systems. Either air or propylene (1000 μL/L) was applied to separate tanks at a constant flow rate. Because the affinity of propylene for the ethylene receptor is ~100-fold less than that of ethylene, a 100-fold higher concentration of propylene was used in our experiment (Burg and Burg, 1967; McMurchie et al., 1972). Ethylene production by the flowers was monitored by withdrawing air samples from the tanks at appropriate time intervals for analysis of ethylene concentration by gas chromatography as described below. Six or more flowers were used for each treatment (air, propylene, air and AVG, and propylene and AVG), and all determinations of ethylene production (nanoliters per gram per hour) were based on the mean value of samples collected in triplicate.

Norbornadiene Studies

Detached flowers in water tubes were either pollinated or left untreated and placed into sealed 9-L tanks for 12, 24, 48, or 72 hr of treatment with either air or 2,5-norbornadiene (NBD) (Aldrich Chemical Co.). NBD was applied by pipetting 86 μL of a concentrated stock solution onto Whatman filter paper, which upon placement in the sealed jar volatilized to give a calculated concentration of 2000 μL/L. Sets of flowers were harvested at intervals from separate jars for RNA isolations. Ethylene production was monitored by withdrawing 2 mL of air from each tank at regular intervals. Ethylene concentration in the samples was determined by gas chromatography as described below. Visual observations of perianth senescence and gynoecium changes were monitored throughout the experiment. At least six flowers were used for each time point.

Gas Measurements

Ethylene production of individual flowers was determined by enclosing whole flowers in small gas-tight containers equipped with septa for up to 2 hr followed by sampling of a fixed volume of gas in the container headspace with the aid of a syringe. Ethylene concentration (microliters per liter) in the sample was determined by comparison with standard ethylene gas (1 μL/L) by gas chromatography using a Carle Analytical Gas Chromatograph 211 equipped with a flame ionization detector and an SP4270 integrator (Spectra-Physics Inc., San Jose, CA). Ethylene production (nanoliters per gram per hour) was calculated on the basis of the initial fresh weight of the flower or as described for individual experiments.

RNA Isolation

RNA was isolated from separately harvested organs of the flower using the method of Cathala et al. (1983). Briefly, frozen pulverized tissue (0.5 to 10.0 g, depending on the organ) was homogenized in 4 M guanidine isothiocyanate, 10 mM EDTA, 10 mM Tris-HCl, pH 8.0, and 8%

2-mercaptoethanol using a polytron tissue homogenizer. After centrifugation to remove insoluble material, total RNA was precipitated with 4 M LiCl at 4°C overnight, pelleted, and washed, and the final RNA precipitate was resuspended in RNA solubilization buffer. The resuspended RNA was extracted extensively with phenol/chloroform/isoamyl alcohol (24:23:1, v/v/v) and finally precipitated with 3 M NaOAc and absolute ethanol. Poly(A)$^+$ RNA was subsequently isolated by oligo(dT)-cellulose chromatography (Pharmacia LKB Biotechnology Inc.) as originally described by Aviv and Leder (1972) and stored as a precipitate at −80°C for later use. Typical total RNA yields from flower tissue were 100 μg/g fresh weight of stigma and ovary and 60 μg/g fresh weight for perianth, with a poly(A)$^+$ RNA yield of ~1.5% of total RNA for all organs. Reagents used for RNA isolation were obtained from Fisher or Sigma unless noted otherwise.

RNA Gel Blot Hybridization Analysis

Poly(A)$^+$ RNA was used for all RNA gel blot hybridizations. Poly(A)$^+$ RNA was fractionated by electrophoresis in formaldehyde agarose gels (Nevins and Wilson, 1981) with constant buffer circulation. RNA was transferred from agarose gels to nitrocellulose or nylon membrane (Schleicher & Schuell) as described by Thomas (1983). Blots were baked under vacuum for 2 hr at 80°C. Following prehybridization, RNA gel blots were probed with ^{32}P-labeled cDNA inserts representing the coding sequences of orchid ACC synthases (OAS1 and OAS2, with similar results) or ACC oxidase (OAO1). Hybridization was performed at 42°C for 48 hr in 50% formamide, 5 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.05 M phosphate buffer, pH 7.2, 1 × Denhardt's solution (1 × Denhardt's is 0.02% Ficoll, 0.02% PVP, 0.02% BSA), 0.2 mg/mL denatured sonicated salmon sperm DNA (Sigma, Type III), and 0.2% SDS. Filters were washed in 0.2 × SSC, 0.05 M phosphate buffer, pH 7.2, and 0.1% SDS beginning at 55°C and washing up to 65°C (melting temperature is −3°C) (Wahl et al., 1987).

Autoradiography was performed at −80°C using Kodak XAR-5 film and a single intensifying screen (Cronex Lightning Plus, Du Pont). Exposure times for blots probed with the ACC synthase cDNA were on the order of 3 or more days, whereas those using ACC oxidase cDNA as probe required far less exposure, usually ~12 hr. The cDNA inserts used as probes were isolated from vector sequences by electrophoresis in low-melting-temperature agarose (Sambrook et al., 1989) and labeled to high specific activity with ^{32}P-dCTP by random priming (Feinberg and Vogelstein, 1983). The cDNA inserts representing full-length cDNAs of ACC synthase and ACC oxidase are ~1.6 and 1.4 kb, respectively (A. Q. Bui, J. A. Nadeau, and S. D. O'Neill, unpublished results). The complete nucleotide sequences for these clones can be obtained from GenBank under the accession numbers L07882 for OAS1, L07883 for OAS2, and L07912 for OAO1.

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