77

BARD

FINAL REPORT

PROJECT NO. US-1867-90

**Molecular Genetic Analysis of Pollination-Induced
Senescence in *Phalaenopsis* Orchids**

S.D. O'Neill, A.H. Halevy, A. Borochoy

1996

Standard BARD Cover Page for Scientific Reports

DATE: 1/2/96

BARD
P.O. BOX 6
Bet Dagan, ISRAEL

BARD project No. US-1867-90R

Title:

Molecular Genetic Analysis of Pollination-induced Senescence in
Phalaenopsis orchids

Investigators' Names
(Principal listed first)

S. D. O'Neill

A. H. Halevy

A. Borochoy

Investigators' Institutions

University of California, Davis

The Hebrew University, Rehovot

The Hebrew University, Rehovot

Project's starting date: 9/1/91

Type of Report: 1st Annual ☐ 2nd Annual ☐ Final ☒

Shahman O'Neill
Signature Shahman O'Neill
Principal Investigator

Louise Ivey **JAN 5 1996**
Signature
Institution's Authorizing Official
Louise Ivey
Contracts & Grants Officer

TABLE OF CONTENTS

COVER PAGE	1
I. TABLE OF CONTENTS	2
II. ABSTRACT	3
III. PUBLICATIONS FROM BARD GRANT #US-1867-90R	4
IV. PROJECT OBJECTIVES	9
V. FINAL SCIENTIFIC REPORT	10
V.1. INTERORGAN REGULATION OF GENE EXPRESSION AND ETHYLENE BIOSYNTHESIS IN POLLINATED <i>PHALAENOPSIS</i> FLOWERS	10
V.2. SIGNAL TRANSDUCTION IN POSTPOLLINATION REGULATION IN <i>PHALAENOPSIS</i> FLOWERS	34
VI. FIRST AND SECOND ANNUAL REPORTS	54

IV. PROJECT OBJECTIVES

The original grant proposed to test three hypotheses:

- 1) The induction and progression of flower senescence is regulated in part at the level of gene expression.
- 2) Senescence of climacteric flowers share common underlying regulatory mechanisms and common regulatory signals.
- 3) Altered sensitivity of the perianth to ethylene is related to modification of the signal transduction mechanism via the phosphoinositide cycle.

We proposed to test these hypotheses by accomplishing the following specific objectives:

- 1) Clone genes that show altered patterns of expression in the orchid gynoecium and/or perianth associated with pollination-induced orchid flower senescence.
- 2) Determine if genes that have already been cloned from orchid flower cDNA libraries (for example, those encoding ACC synthase, ACC oxidase, and cellulase) are differentially expressed both spatially and temporally following pollination.
- 3) Analyze the content and kinetic parameters of the different components of the phosphoinositide pathway following pollination in relation to the sensitization of the perianth to ethylene.
- 4) Analyze the regulation of gene expression in relation to ethylene to determine if the pollination-induced physiological enhancement in ethylene sensitivity is manifested at the level of gene expression.
- 5) To identify the sensitivity factor of orchid flowers and compare this with factors previously identified in petunia.

V. FINAL SCIENTIFIC REPORT

V.1. INTERORGAN REGULATION OF GENE EXPRESSION AND ETHYLENE BIOSYNTHESIS IN POLLINATED PHALAENOPSIS FLOWERS

United States Team: Dr. Sharman D. O'Neill

A flower is a complex structure composed of different organs. Each of them plays a vital role in successful plant reproduction, which is controlled by pollination. Following pollination, major developmental changes occur in all floral organs. These changes include perianth senescence and abscission, pigmentation changes, ovary growth, ovule differentiation (in most orchids and several other species), and female gametophyte development.

In many species, pollination simply accelerates changes already proceeding, although at a much slower rate, in the ovary and perianth of unpollinated flowers. However, the postpollination changes in flowers of most orchid species are precisely and completely triggered by pollination. Pigmentation change in perianth is one of the most obvious signs of all pollination-induced changes. Depending on species or genus of orchids, pigmentation change can be fading, greening, or anthocyanin formation in some or all parts of the perianth. Color change is also observed in petals of *Lupinus albus* and many other angiosperm species. The other obvious change in perianth is wilting and senescence. The above changes indicate that pollination results in rapid changes in floral organs other than the stigma (the site of action), suggesting that translocatable factors are involved in the postpollination changes. The objectives of this research were to identify the signaling mechanisms and responses that regulate coordinate senescence in the multiple floral organs. Because we determined that ethylene played a major role in coordinating many aspects of postpollination development, the primary research focus was on the interorgan regulation of genes encoding ethylene biosynthetic enzymes.

V.1.A. REGULATION OF AN ETHYLENE-INDUCED ACC SYNTHASE GENE IN POLLINATED PHALAENOPSIS FLOWERS

The ethylene biosynthetic pathway has been elucidated and involves the conversion of S-adenosylmethionine (AdoMet) to 1-aminocyclopropane-1-carboxylic acid (ACC), which is then rapidly converted to ethylene. In the past several years, genes encoding ACC synthase have been isolated from zucchini, tomato, winter squash, apple, carnation, arabidopsis, and mung bean. In general, these studies have shown that ACC synthase is encoded by a multigene family, which suggests that the expression of the different gene members is spatially and temporally regulated in a tissue-specific manner during plant growth and development.

Pollination causes an increase in ethylene production in all floral organs of *Phalaenopsis* flowers (O'Neill et al., 1993). To determine whether or not this increase was due to the result of *de novo* synthesis of ACC in the four organs or to the translocation of ACC from one organ to others, crude extracts from all organs were assayed for their levels of ACC synthase activity. Figure 1 shows that levels of enzyme activity in the stigma (column), labellum, and ovary were initially very low but increased to peak levels at 24 h after pollination. At 12 h after pollination, the level of enzyme activity in the stigma was approximately 6-fold higher than those in the labellum and ovary, indicating that it increased first and earlier than 12 h in the stigma. In contrast to the stigma, labellum, and ovary, the perianth had an extremely low level of enzyme activity throughout a 72-h period of pollination, demonstrating that ACC was not produced in this organ (Figure 1D). Taken together, the above results indicate that ACC synthase is clearly induced by pollination in *Phalaenopsis* flowers.

Isolation of orchid ACC synthase cDNA clones.

To examine whether expression patterns of ACC synthase mRNA expression were correlated with patterns of ACC synthase activity in floral organs of pollinated orchid flowers, an orchid gynoeceium cDNA library was screened for cDNA clones encoding ACC synthase. Figure 2 shows the nucleotide and deduced amino acid sequences of ACS1A and ACS1B cDNAs. Their sequences share 99.1% and 98.1% identity at the nucleotide and amino acid levels, respectively. A major difference between two cDNAs is a stretch of 60 bp which is absent in the ACS1A cDNA. Each cDNA contains a large open reading frame that encodes proteins of 425 and 444 amino acids, respectively. Deduced amino acid sequences of orchid ACC synthase share 50-67% identity with those from other plant species when compared individually (data not shown). Based on this comparative analysis, the orchid ACC synthase cDNAs were most similar to the sequences from mung bean VR-ACS4 and VR-ACS5 and least similar to arabidopsis AT-ACS2.

Expression of ACC synthase in *E. coli*

The function of the ACS1A and ACS1B cDNAs was determined by expression in *E. coli*. ACC synthase activity was observed in pTAS2F and pGAS2F transformants in the absence of IPTG, illustrating incomplete suppression of the *tac* promoter (Table 1). Under IPTG induction, the level of enzyme activity in the pTAS2F transformant increased 12.5-fold. Table 1 shows that ACC synthase activity was not detected in pTAS1F and pGAS1F extracts, demonstrating that ACC synthase polypeptide encoded by the ACS1A cDNA was nonfunctional in this assay system. As expected, cells harboring pTrc99A, pGEX-3X, pTAS1R, pTAS2R, or pGAS2R as negative controls had no enzyme activity (Table 1). Together, the above data indicate that the ACS1B

cDNA encodes a functional ACC synthase whereas the ACS1A cDNA may encode a nonfunctional protein.

Table 1: Expression of ACC synthase in *Escherichia coli*.

Construct	Orientation of the insert	ACC synthase activity (nmol/ mg / h)	
		- IPTG	+ IPTG
pTrc99A	-----	0	0
pTAS1F	Forward	0	0
pTAS1R	Reverse	0	0
pTAS2F	Forward	0.83	10.4
pTAS2R	Reverse	0	0
pGEX-3X	-----	0	0
pGAS1F	Forward	0	0
pGAS2F	Forward	2.72	2.14
pGAS2R	Reverse	0	0

Transformants were cultured in the presence (+) or absence (-) of IPTG. Controls (pTrc99A and pGEX-3X) contained no insert.

ACC synthase mRNA accumulation is induced by pollination

Figure 3 shows that a 1.65 kb ACC synthase (ACS1) mRNA accumulated in the stigma and labellum but not in the ovary and perianth of pollinated flowers. Furthermore, this mRNA was not detected in young leaves and roots of *Phalaenopsis* (data not shown). ACS1 represents either ACS1A or ACS1B or both because their nucleotide sequences are 99.1% identity. In the stigma, the level of mRNA transcript was low at 6 h but increased dramatically with its peak at 24 h after pollination, correlating with the level of enzyme activity found in this organ. Although the accumulation of ACS1 mRNA was delayed in the labellum, its peak level occurred at 24 h after pollination, which is consistent with the pattern of enzyme activity in this organ. In contrast to the stigma and labellum, the ovary exhibited no detectable level of ACS1 transcripts even though enzyme activity was observed in this organ, indicating that a highly divergent ACC synthase

mRNA was expressed in the ovary following pollination (see below). ACC synthase mRNA was not detected in the perianth following pollination, which is consistent with results exhibiting that the enzyme activity was absent in this organ (Fig. 1).

Pollination-associated factors regulate ACC synthase gene expression in orchids

We demonstrated that pollination-associated factors such as emasculation, ACC, and auxin (NAA), induced ACS1 mRNA accumulation in the stigma, ovary, and labellum of detached flowers at 24 h and 48 h after treatments (O'Neill et al., 1993). Consistent with these results, ACS1 mRNA accumulated at 24 h in the stigma, ovary, and labellum of ACC-treated flowers that were still attached to the plant (Figs. 4 and 5A). Moreover, a low level of ACS1 transcript was detected at 12 h in the stigma, and this mRNA was not detected at 6 h although 30 μ g of total RNA was used (data not shown). These data illustrates that ACC synthase mRNA accumulated in the stigma earlier than it did in the ovary and labellum. Figure 4B shows that ACS1 mRNA was detected at 24 h in the stigma but not in the ovary of emasculated flowers. Similar to results of studies on detached flowers, exogenous NAA induced the accumulation of ACC synthase in the stigma and labellum of attached flowers (Figs. 4A and 5A). Furthermore, a substantial level of ACS1 mRNA was detected in the stigma at 8 h but not at 4 h (Fig. 4A). Since a very low level of ACC synthase mRNA was detected at 6 h following pollination, it was speculated this mRNA might also be induced by exogenous NAA at 6 h. In addition, to investigate whether NAA levels might play a role in the induction of ACS1 mRNA accumulation, different concentrations of NAA were applied to the stigma of flowers for 6 h. ACS1 mRNA accumulation was not detected in the stigma of flowers *in planta* but was detected in the stigma of detached flowers after 6 h of NAA application (Fig. 7). These results suggest that during the first six hours following the treatment, stress (e.g., detachment) sensitizes the stigma for a later response to NAA.

ACS1 gene expression is regulated by ethylene

In this study, the detection of ACS1 mRNA transcripts in the stigma at 6 h after pollination and at 8 h after NAA treatment coincided with a low level of ethylene measured in this organ at 6 h following pollination (Nadeau et al., 1993). Moreover, this mRNA was not detected in floral organs of flowers treated with ACC or NAA in the presence of AVG, an inhibitor of ethylene production (O'Neill et al., 1993). These data suggest that ACS1 gene expression is regulated by ethylene. To further validate this speculation, flowers were treated with ACC, ethylene, or NAA in conjunction with NBD, a competitive inhibitor of ethylene action. Figures 6 and 7 show that ACC synthase mRNA was not detected in any floral organs of flowers treated with ACC, NAA, or pollination in the presence of NBD. The negative results were not due to cell death caused by toxic levels of NBD. But, it was rather due to the complete inhibition of ethylene action by NBD

because the application of ethylene to flowers pretreated with NBD for 24 h restored ACS1 mRNA accumulation in the stigma, ovary, and labellum (Fig. 6). Taken together, all of the above results illustrate that ACS1 gene expression is regulated by ethylene in pollinated orchid flowers.

Because ethylene affects diverse post-pollination developmental processes including pollen germination and ovary development in orchids (Zhang and O'Neill, 1993), it was speculated that ethylene production might be confined to specific cell types, leading to locally high ethylene concentrations. To determine the cell-specific accumulation of ACC synthase mRNA, *in situ* hybridization analysis was performed in the stigma tissue. Because ACS1 transcripts were detected at 6 h after pollination, it was speculated that these transcripts would be localized in the stigma at 4 h. However, results showed that levels of ACS1 mRNA were relatively the same as at 0 h, and that they gradually increased after 8 h following pollination in all cells, including parenchyma cells surrounding the vascular bundles (Fig. 8). The same result was also observed in all cells of the stigma 24 h of ethylene-treated flowers (Fig. 8O and 8P). These results illustrate that the accumulation of ACC synthase transcripts was not cell-specific because all cells were competent to make ethylene.

These results demonstrated that in the ovary of pollinated flowers, the high level of ACC synthase activity observed following pollination was not accompanied by detectable ACS1 mRNA. However, ACC and ethylene, but not auxin, induced this mRNA accumulation in the ovary. These data suggest that pollination factor(s) and auxin appear to inhibit ACC and ethylene from inducing this gene expression in this organ. Thus, determining the regulatory sequence of this gene may provide clues on how it is regulated by ethylene, auxin, and pollination factor(s). The above results also suggest the existence of at least one other ACC synthase gene whose expression is responsible for enzyme activity in the ovary. This gene is speculated to be highly divergent from the ACS1 genes with which there was no cross-hybridization. This speculation is plausible because in winter squash the auxin-induced ACC synthase cDNA did not cross-hybridize with the wound-induced ACC synthase cDNA although both cDNAs share 56.1% nucleotide similarity.

Consistent with our results in this flower system, the induction of ACS1 mRNA accumulation by ACC, NAA, and emasculation was mediated by ethylene (O'Neill et al., 1993). Since the increased level of this mRNA was concomitant with the level of ethylene production in the stigma and labellum, it is proposed that the expression of the ACS1 genes is induced by ethylene in a dose-dependent manner. On the other hand, auxin is likely to be the primary pollination factor inducing ACC synthase gene expression for the following reasons. First, a substantial amount of auxin is found in the pollinia of orchid species. Second, auxin is the known factor that can mimic pollination effects such as column swelling and stigma closure in orchids. Third, auxin induced growth of hair cell whereas ethylene caused cell death during ovule development in the ovary of *Phalaenopsis* (Zhang and O'Neill, 1993). Last, auxin can induced

expression of auxin-induced genes within minutes after its application. Therefore, it is speculated that auxin in the orchid pollinia stimulates the accumulation of an auxin-regulated ACC synthase gene in the stigma upon pollination, resulting in ethylene production. Ethylene then induces ACS1 mRNA accumulation for further production of ACC and ethylene.

V.1.B. INDUCTION OF AN AUXIN-REGULATED ACC SYNTHASE GENE IN POLLINATED ORCHID FLOWERS

Results described above showed that ACC synthase activity was not accompanied by ACS1 mRNA accumulation in the ovary of the pollinated flower. Also, a low level of ACS1 mRNA accumulation was detected in the stigma at 6 h following pollination. However, ACC synthase activity was detected as early as 1.5 h in the stigma, and its level increased following pollination. These results suggest that additional distinct ACC synthase genes are expressed in the stigma and ovary of pollinated orchids. The next goal was to isolate additional ACC synthase cDNAs to determine whether a previously undetected and divergent ACC synthase was responsible for early pollination and auxin-regulated ethylene production in the stigma and ovary. A stigma-specific pACS2 cDNA represented a *PS-ACS2* gene encoding an auxin-induced ACC synthase isozyme whose mRNA accumulation increased rapidly in the stigma by the induction of auxin and pollination was identified.

Two distinct ACC synthase genes are expressed in the stigma and ovary of pollinated orchids

Total RNA isolated from stigmas and ovaries of *Phalaenopsis* flowers at 2 h or 4 h after pollination was subjected to RT-PCR using degenerate primers to conserved domains of ACC synthases. Complementary DNAs, pACS2 and pACS3, were isolated from the stigma and ovary, respectively. These cDNAs share 63% and 54.1% identity at the nucleotide and amino acid levels, respectively (Fig. 9). In addition, pACS2 and pACS3 share 74% and 62% nucleotide identity with pACS1B, respectively, indicating that these new cDNAs encode distinct ACC synthase isozymes (Fig. 9.2A). The differences are sufficient to account for the failure to previously detect these ACC synthase transcripts in the stigma and ovary of pollinated flowers. Genes corresponding to pACS2 and pACS3 were named as *PS-ACS2* and *PS-ACS3* (*PS* for *Phalaenopsis* sp.), respectively.

To examine whether patterns of *PS-ACS2* and *PS-ACS3* gene expression were correlated with patterns of ACC synthase activity in the stigma and ovary, RNA blot hybridization analysis was performed. Figure 10A shows that a pACS2 cDNA probe hybridized to a 1.9-kb ACC synthase mRNA (*PS-ACS2*) in the stigma. Also, the level of this transcript was low at 2 h but increased to a peak at 24 h. A detectable level of *PS-ACS2* mRNA was also observed at 1 h (data

not shown). The pattern of PS-ACS2 mRNA accumulation correlated well with the pattern of ACC synthase activity in the stigma of pollinated flowers.

Similarly, a pACS3 cDNA probe hybridized to a 2.2-kb *PS-ACS3* transcript in the ovary (Fig. 10B). The mRNA accumulation level was initial low at 2 h but increased to a peak at 24 h after pollination. It appears that the expression of *PS-ACS3* resulted in the increase in ACC synthase activity detected in the ovary of pollinated *Phalaenopsis*.

Auxin induces ACC synthase gene expression in the stigma of orchids

A recent review reports that the pollinia of several orchid species contain a substantial amount of auxin. To determine whether auxin was responsible for the expression of the *PS-ACS2* gene as well as the increase in ACC synthase activity in the stigma of *Phalaenopsis*, flowers were treated with NAA and subjected to RNA blot hybridization analysis and enzyme activity assays. Since the expression level of *PS-ACS2* was relatively high at 6 h after pollination, this time point was chosen for NAA treatment. Also, because the auxin content in the *Phalaenopsis* pollinia has not been determined, different arbitrary amounts of NAA solution were applied to the stigma of flowers for 6 h. Figure 11A shows that levels of PS-ACS2 mRNA accumulation corresponded to amounts of NAA solution. Since the level of accumulation of PS-ACS2 mRNA at 1 μ g NAA was approximately equivalent to that at 6 h after pollination, this amount was used for subsequent experiments.

Figure 11A illustrates that a high level of PS-ACS2 mRNA transcript was observed in the stigma during 24 h of NAA treatment. Its expression pattern was similar to that of pollination. However, a decline in the level of mRNA after 8 h of NAA treatment suggested that a continuous supply of auxin would be required for the further induction of PS-ACS2 mRNA. The expression of the *PS-ACS2* gene was directly induced by auxin but not via ethylene because its mRNA accumulation was induced by NAA treatment and pollination in the presence of NBD, an ethylene action inhibitor (Fig. 11A). Additionally, the application of 10 nmol of ACC did not stimulate the accumulation of PS-ACS2 mRNA in the stigma (data not shown). Consistent with these mRNA results, NAA but not ACC induced ACC synthase activity in the stigma tissue 2 h after the treatments. It is noteworthy that emasculation and gibberellic acid did not induce the accumulation of PS-ACS2 mRNA (data not shown). On the other hand, CaCl_2 treatment in the presence or absence of EGTA (a chelator of calcium ions) induced a slight increase in the level of PS-ACS2 mRNA (Fig. 12A). The same result was also observed in the stigma of flowers treated with MgCl_2 as a control. Surprisingly, EGTA also stimulated a small increase in PS-ACS2 mRNA accumulation (Fig. 12A). These results demonstrate that this induction is likely due to something associated with treatment by CaCl_2 , MgCl_2 , and EGTA, possibly a stress reaction, indicating that calcium ions are not the primary pollination factor. Taken together, the above data indicate that

auxin, but not ACC, is likely to be the primary pollination signal that induces the expression of the *PS-ACS2* gene in the stigma of pollinated orchids.

Auxin also induces the expression of *PS-ACS3* mRNA in the ovary of *Phalaenopsis*

We previously showed that auxin played a crucial role in ovule development in pollinated *Phalaenopsis*. (Zhang and O'Neill 1993) Therefore, it was important to investigate whether auxin (NAA) induced the accumulation of *PS-ACS3* mRNA in the ovary tissue. Figure 10B shows that levels of *PS-ACS3* mRNA accumulation in the ovary increased over a 24-h period following the application of 1 μ g of NAA solution to the stigma tissue. Furthermore, this mRNA was also induced by NAA in the presence of NBD. These results indicate that *PS-ACS3* gene expression was indeed induced by auxin. However, these levels were much lower and occurred later than those in the ovary of pollinated flowers when comparing them at appropriate time points (Figs. 10B and 11B). These results might be due to a small amount of NAA applied to the flowers or additional effects of pollination factors other than auxin. Figure 11B demonstrates that the amount of exogenous NAA was not the factor influencing the level of *PS-ACS3* mRNA accumulation because as much as 10 μ g of NAA solution resulted in a detectable level this mRNA level at 6 h after the treatment. Thus, it was likely that pollination factor(s) other than auxin also induced the *PS-ACS3* mRNA accumulation because the higher level of message was found in the ovary of the pollinated flower as compared to the NAA-treated flower (Figs. 10B and 11B). Emasculation was found to have no effects on this mRNA accumulation (data not shown). Gibberellic acids are found in petunia pollen, and GA_3 has been reported to activate expression of a chalcone synthase gene in the corolla of *Petunia hybrida*. To examine whether gibberellic acids might be the unknown pollination factor, flowers were treated with GA_3 solution. RNA blot hybridization analysis showed no detectable *PS-ACS3* mRNA in the ovary of GA_3 -treated flowers (data not shown). It was interesting to investigate whether Ca^{2+} might be the unknown pollination factor because Ca^{2+} is crucial for germination and growth of pollen tubes. RNA hybridization analysis showed that an extremely low level of this message was detected in the ovary tissue of flowers treated with $CaCl_2$, $MgCl_2$, or EGTA, illustrating that Ca^{2+} is not the pollination factor (Fig. 12B). Taken together, all of the above results indicate that the expression of the *PS-ACS3* gene in the ovary of pollinated *Phalaenopsis* was partially induced by auxin but mainly by other unknown pollination factor(s).

ACC synthase transcript and enzyme activity are spatially expressed in the ovary/pedicel of pollinated orchid flowers

Because we showed that auxin and ethylene coordinately regulate ovary growth and ovule development in the pollinated *Phalaenopsis* flowers (Zhang and O'Neill 1993), it was important to examine the patterns of ACC synthase activity and ACC synthase transcript in different portions of the ovary/pedicel structure. The structure was divided into three portions (ovary, transition zone, and pedicel) as shown in Figure 13A. Twenty four hours after pollination, these parts were collected separately for RNA blot hybridization analysis and ACC synthase activity assays. The results showed that levels of ACC synthase transcript and activity were lowest in the ovary portion and highest in the transition zone portion (Figs. 13B and 13C), but that the *PS-ACS3* gene was expressed throughout the ovary/pedicel structure of the pollinated flower.

V.1.C. A MODEL OF INTERORGAN REGULATION OF ACC SYNTHASE GENES IN POLLINATED *PHALAENOPSIS* FLOWERS

The postpollination syndrome in the *Phalaenopsis* orchid system was dissected by investigating the temporal and spatial regulation of ACC synthase gene expression. The results from this project are depicted by the model shown in Figure 14. In the stigma, pollen-borne auxin induces the expression of the auxin-regulated ACC synthase (*PS-ACS2*) gene, resulting in ACC synthesis. This gene expression occurs within 1 h following pollination. Newly formed ACC is oxidized by basal constitutive ACC oxidase to ethylene, which then induces the expression of the ethylene-regulated ACC synthase (*PS-ACS1*) and oxidase (*ACO1*) genes for further autocatalytic production of ethylene. It is speculated that during the 6-h period following pollination, emasculation leads to the production or release of a sensitivity factor that sensitizes the cells of the stigma to ethylene. Both auxin and ethylene are responsible for column swelling, stigma closure, and column senescence. Furthermore, ethylene is required for male gametophyte development.

ACC and ethylene molecules are translocated from the stigma to the labellum and perianth. In the labellum, ethylene induces the expression of *PS-ACS1* and *ACO1* resulting an increased production of ACC and ethylene. Ethylene is responsible for inrolling and senescence of the labellum. Again, ACC and ethylene are translocated to the perianth, where ACC is converted to ethylene by ACC oxidase. Ethylene is responsible for wilting and senescence of the perianth.

In the ovary, pollen-borne auxin induces the expression of the other auxin-induced ACC synthase (*PS-ACS3*) gene. It is speculative but possible that in the presence of auxin, pollen-held brassinosteroids may activate synergistically the expression of *PS-ACS3* for ACC synthesis. Most of the ACCs is translocated to the perianth and perhaps to the labellum for ethylene synthesis. However, a small amount of ACC is converted to ethylene by ACC oxidase so that a small amount

of ethylene together with auxin coordinately regulates ovule differentiation. The low level of this enzyme is the result of the late expression of the *ACO1* gene. In the ovary, auxin inhibits the expression of the *PS-ACS1* gene by an unknown mechanism. This inhibition is crucial for ovule development and fertilization because too much ethylene results in cell death in the ovary and abortion of the flower.

V.2. SIGNAL TRANSDUCTION IN POSTPOLLINATION REGULATION IN *PHALAENOPSIS*

Israeli team: Drs. Abraham Halevy and Amihud Borochoy

The longevity of cut *Phalaenopsis* (*Phalaenopsis* hybrid, cv. Herbert Hager) and *Dendrobium* (*Dendrobium* hybrid, cv. Jaquelyn Thomas) flowers is normally 2 to 3 weeks. After pollination however, there is a rapid acceleration of the wilting process, beginning after only 25 h (Fig. 15). Enhancement of senescence in several *Phalaenopsis* cultivars as well as in *Doritaenopsis*, *Dendrobium* and *Cymbidium*, was induced by successful pollination and only slightly or not at all by emasculation (Tab. 2).

Following pollination there was an increase in ethylene production and in sensitivity to ethylene. The increase in ethylene production began about 12 to 14 h after pollination and peaked after 30 h (Fig. 16). The increase in ethylene sensitivity appeared in both the flower column and the perianth, and was detected as early as 4 h after pollination, peaking after 10 h (Fig. 17). The increase in ethylene sensitivity following pollination was not dependent on endogenous ethylene production as it occurred also in flowers treated with AOA, an inhibitor of ethylene biosynthesis (Figs. 17 and 18). The time course of the changes in ethylene production and sensitivity to ethylene following pollination are shown in Fig. 19.

Since pollination occurs at the stigma and the senescence processes are observed at the corolla, it is obvious that an interorgan signaling mechanism transduces the pollination stimulus from the stigma to the other parts of the flower, where it induces the increases in ethylene production, sensitivity to ethylene and the enhancement of senescence. In this work we examined the possible involvement of lipoxygenase activity, jasmonates and short-chain saturated fatty acids in the regulation of pollination-induced flower senescence.

Application of linoleic and linolenic acids to *Phalaenopsis* and *Dendrobium* flowers enhanced their senescence and promoted ethylene production. These effects were specific to unsaturated fatty acids which serve as substrates for lipoxygenase action, and did not occur following similar treatments with saturated fatty acids (Figs. 20 and 21). Several major lipoxygenase pathway products including JA-Me, traumatic acid, *trans*-2-hexenal and *cis*-3-hexenol, also enhanced flower senescence (Tab. 3). JA-Me induced increases in ethylene production and ACC content (Fig. 22). In contrast, treating flowers with the lipoxygenase inhibitors SHAM and n-PG, which inhibited lipoxygenase activity in-vitro (Fig. 23), had no effect on pollination-induced senescence of the flowers (Tab. 4). Furthermore, during the 50 h period following pollination, there was no increase in lipoxygenase activity in the flowers (Fig. 24). During the 10 h period from pollination of *Dendrobium* flowers until the initiation of ethylene production, there was no effect of pollination on jasmonate levels in either the perianth or the

columns (Fig. 25). These results suggest that in spite of our findings regarding JA-Me enhanced ethylene production and flower senescence, lipoxygenase is not directly involved in the endogenous regulation of pollination-induced *Phalaenopsis* and *Dendrobium* flower senescence.

Application of short-chain saturated fatty acids, ranging in chain length from 7 to 10 carbons, onto stigmas of *Phalaenopsis* flowers increased their sensitivity to ethylene in the same way as pollination (Tab. 5). Following pollination, there was a significant increase in the endogenous content of these fatty acids in the flower's column and perianth, with octanoic acid (C₈) being the main fatty acid observed (Figs. 26 and 27). The increase in short-chain saturated fatty acid content was observed as early as 6 h after pollination and began to decline 6 h later. The time course of the changes in the short-chain saturated fatty acids was similar to that of the changes in ethylene sensitivity following pollination. Incorporation of octanoic acid into liposomes or microsomal membranes isolated from *Phalaenopsis* petals resulted in a decrease in lipid order that was detected by fluorescence polarization of DNSP but not of DPH (Figs. 28 and 29). At the peak of ethylene sensitivity, 10 h after pollination, there was a significant decrease in the lipid order of microsomal membranes isolated from *Phalaenopsis* columns and perianths, detected by DNSP but not by DPH. Application of octanoic acid to the stigma imitated the effect of pollination on membrane lipid order (Tab. 6). We suggest that short-chain saturated fatty acids may be the ethylene "sensitivity factors" produced following pollination, and that their mode of action involves a decrease in the order of specific regions in the membrane lipid bilayer, consequently altering ethylene action.

Following pollination there is also a stimulation of a cellular signal transduction mechanism that transduces the response to ethylene. We examined the possible involvement of GTP-binding proteins, calcium ions and protein phosphorylation in the cellular response to ethylene following pollination of *Phalaenopsis* flowers.

In a previous study we found that treating flowers with compounds that modulate GTP-binding protein activity, such as cholera toxin and GTP γ S, increased the sensitivity of the flowers to ethylene, and that following pollination there was a significant increase in their activity. In the present work we found, using Western blot analysis of microsomal proteins, that a peptide with a molecular mass of 42 kD cross-reacts with antibodies against a well conserved amino acid sequence (G α -common peptide) of mammalian G-proteins (Fig. 30).

Calcium ions are also involved in the ethylene signal transduction pathway. Application of calcium ions, together with their ionophor A23187, increased the sensitivity of pollinated flowers to ethylene, while EGTA, a chelator of calcium, decreased it (Tab. 7). The main effect of calcium ions was on promoting protein phosphorylation, as detected following incorporation of [³²P] orthophosphate into the proteins (Fig. 31).

Protein phosphorylation in the flower column increased significantly following pollination, with a peptide of ca. 30 kD most heavily phosphorylated (Figs. 31 and 32). The increase in protein phosphorylation was a direct response to the increase in ethylene production following pollination, since treatments of the flowers with inhibitors of ethylene biosynthesis or action also inhibited the increase in protein phosphorylation (Fig. 31).

These results indicate a direct involvement of GTP-binding proteins, calcium ions and protein phosphorylation in the cellular signal transduction response to ethylene following pollination. Modulations of cytosolic calcium levels allowed us to modify the flowers responsiveness to ethylene.

Premature senescence of the corolla is a common response to pollination and is thought to be controlled by the enhancement of ethylene production. In the following experiments we examined the possibility that the pollination-induced increase in ethylene sensitivity makes the flower responsive to the constant basal levels of ethylene produced by the flower, thus triggering autocatalytic production of ethylene to its climacteric peak.

1-Methylcyclopropene (1-MCP) is a new ethylene action inhibitor. This compound is a nontoxic odorless gas, and was found to be very effective in inhibiting ethylene responses in petunia and phlox flowers. Treatment of *Phalaenopsis* flowers with STS or 1-MCP, both inhibitors of ethylene action, completely inhibited the pollination-induced increase in ethylene production and the enhanced senescence of the flowers (Fig. 33, Tab. 8). This indicates that the pollination-induced increase in ethylene production is a response to existing ethylene. Treatment of flowers with calcium and its ionophore A23187, which increased ethylene sensitivity, also promoted ethylene production and senescence of unpollinated flowers (Tab. 7, Fig. 34). EGTA, a calcium chelator, decreased the sensitivity of pollinated flowers to ethylene and delayed and decreased the pollination-induced ethylene production (Tab. 7, Fig. 35). These results, together with the observation that the pollination-induced increase in ethylene sensitivity appears earlier than the increase in ethylene production (Fig. 19), support the hypothesis that the increase in ethylene sensitivity is the initial pollination-induced event triggering the enhancement of ethylene production, which leads to enhanced flower senescence.

We propose that changes in ethylene sensitivity might also be involved in the regulation of unpollinated flowers longevity. In *Phalaenopsis* flowers, there was a decline in ethylene sensitivity with age, which might explain the long life span of the flowers. However, after 3 weeks, at the onset of senescence, there was a marked increase in ethylene sensitivity (Tab. 9). In two genotypes of petunia (*Petunia hybrida*, L.) having significantly different life spans, we found that differential sensitivity to ethylene, independent of ethylene production, governs flower longevity of the two lines (Fig. 36).

Table 2. Effects of pollination and emasculation on the longevity of intact and cut flowers of several orchid cultivars. Flowers were pollinated or emasculated and the time to the first visible sign of wilting was measured. Values are means of 5 replications.

Cultivar	Intact			Cut		
	C ¹	E ²	P ³	C ¹	E ²	P ³
	(days)					
<i>Phalaenopsis</i> hyb.						
White Magentic North	>40	>40	3.0	21.0	12.0	1.2
Striped Fireball	>40	16	4.0	27.0	19.0	1.5
Pink Herbert Hager	>40	>40	3.0	23.0	13.0	1.0
<i>Doritaenopsis</i>						
Red Beard	>40	>40	3.0	32.0	18.0	1.0
<i>Dendrobium</i>						
Jaquelyn Thomas	>40	>40	2.0	28.0	11.0	1.3
<i>Cymbidium</i>						
Rosarita	13.0	6.0	3.0	7.0	4.0	2.0

¹C - Control

²E - Emasculation

³P - Pollination

Table 3. Effects of the LOX pathway substances JA-Me, traumatic acid, *trans*-2-hexenal and *cis*-3-hexenol on the longevity of cut *Phalaenopsis* flowers. Values are means of 3 experiments $\pm$ SE.

Treatment	Time to 50% wilting (days)	Percent of control (%)
<u>JA-Me</u>		
0	15.0 $\pm$ 2.7	100
25 nmol flower ⁻¹	7.9 $\pm$ 0.9	53
50 nmol flower ⁻¹	5.3 $\pm$ 4.0	35
100 nmol flower ⁻¹	4.0 $\pm$ 0.3	27
200 nmol flower ⁻¹	3.6 $\pm$ 0.2	24
<u>Traumatic acid</u>		
0	15.0 $\pm$ 1.8	100
50 nmol flower ⁻¹	15.0 $\pm$ 1.8	100
200 nmol flower ⁻¹	6.0 $\pm$ 0.7	40
1000 nmol flower ⁻¹	2.4 $\pm$ 0.2	16
<u><i>trans</i>-2-Hexenal</u>		
0	17.0 $\pm$ 3.0	100
20 μ l l ⁻¹	4.8 $\pm$ 0.5	28
40 μ l l ⁻¹	2.3 $\pm$ 0.3	14
<u><i>cis</i>-3-Hexenol</u>		
0	17.0 $\pm$ 3.0	100
20 μ l l ⁻¹	5.5 $\pm$ 0.8	32
40 μ l l ⁻¹	4.8 $\pm$ 0.7	28

Table 4. Effects of LOX inhibitors, SHAM and n-PG, on pollination-induced senescence of *Phalaenopsis* flowers. Values are means of 3 experiments $\pm$ SE.

Treatment	Time to 50% wilting (days)
Non-pollinated	16.0 $\pm$ 2.5
Non-pollinated + 10 mM SHAM	16.0 $\pm$ 2.5
Non-pollinated + 10 mM n-PG	16.0 $\pm$ 2.5
Pollinated	1.5 $\pm$ 0.4
Pollinated + 10 mM SHAM	1.5 $\pm$ 0.4
Pollinated + 10 mM n-PG	1.5 $\pm$ 0.4

Table 5. Effects of pollination and short-chain saturated fatty acids on the sensitivity of *Phalaenopsis* flowers to ethylene. Flowers were held in 0.5 mM AOA, and 50 nmole of heptanoic acid (C₇), octanoic acid (C₈), nonanoic acid (C₉) or decanoic acid (C₁₀) were applied to the stigma. Following treatments, flowers were exposed to 0.5 $\mu\text{l l}^{-1}$ ethylene for 10 h and the time to 50% wilting was measured. Values are means of 3 experiments $\pm$ SE.

Treatment	Time to 50% wilting (days)	Percent of control (%)
Unpollinated	3.3 $\pm$ 0.4	100
Unpollinated + C ₇	2.4 $\pm$ 0.3	73
Unpollinated + C ₈	2.3 $\pm$ 0.3	70
Unpollinated + C ₉	2.6 $\pm$ 0.3	79
Unpollinated + C ₁₀	2.3 $\pm$ 0.3	70
Pollinated	2.5 $\pm$ 0.3	76

Table 6. Effects of pollination and octanoic acid on the polarization value of microsomal membranes isolated from *Phalaenopsis* flowers. Flowers were unpollinated, pollinated or had 50 nmole octanoic acid applied on their stigma, and after 10 h, microsomal membranes were prepared from the columns and perianths separately. The P value of the microsomal membranes, labeled with DPH or DNSP, was measured by fluorescence polarization. Values are means of 3 experiments $\pm$ SE.

Treatment	P value	
	DPH	DNSP
<u>Column</u>		
Unpollinated	0.267 $\pm$ 0.002	0.219 $\pm$ 0.002
Unpollinated + octanoic acid	0.267 $\pm$ 0.002	0.188 $\pm$ 0.001
Pollinated	0.267 $\pm$ 0.002	0.167 $\pm$ 0.001
<u>Perianth</u>		
Unpollinated	0.277 $\pm$ 0.002	0.236 $\pm$ 0.002
Unpollinated + octanoic acid	0.277 $\pm$ 0.002	0.211 $\pm$ 0.002
Pollinated	0.277 $\pm$ 0.002	0.194 $\pm$ 0.001

Table 7. Effects of calcium on the sensitivity of pollinated *Phalaenopsis* flowers to ethylene. Flowers were held in 0.5 mM AOA together with either 1 mM CaCl_2 and 2 μM A23187 or 10 mM EGTA or 10 mM LaCl_3 and then pollinated and exposed to 0.5 $\mu\text{l l}^{-1}$ ethylene for 10 h. Values are means of 4 replications $\pm$ SE.

Treatment	Time to 50% wilting (h)	Percent of control (%)
Unpollinated	76 $\pm$ 4	-
Pollinated	66 $\pm$ 4	100
+ Ca^{2+} and A23187	40 $\pm$ 4	66
+ EGTA	84 $\pm$ 6	127
+ LaCl_3	84 $\pm$ 6	127

Table 8. Effects of ethylene action inhibitors on the on the longevity of unpollinated and pollinated *Phalaenopsis* flowers. Flowers were treated for 6 h with 250 nl l^{-1} 1-MCP or held continuously in 0.5 mM STS. Values are means of 3 experiments $\pm$ SE.

Treatment	Time to 50% wilting (days)
Unpollinated	13.0 $\pm$ 1.3
+ 1-MCP	15.9 $\pm$ 0.9
+ STS	9.5 $\pm$ 0.4
Pollinated	2.0 $\pm$ 0.0
+ 1-MCP	13.7 $\pm$ 0.8
+ STS	8.9 $\pm$ 1.5

Table 9. Effect of *Phalaenopsis* flower age on the sensitivity to ethylene. Unpollinated flowers were harvested at different times after anthesis and were exposed to 0.5 $\mu\text{l l}^{-1}$ ethylene for 12 h. Values are means of 4 replications $\pm$ SE.

Flower age (days from anthesis)	Time to 50% wilting (h)
1	30 $\pm$ 3
7	54 $\pm$ 7
14	54 $\pm$ 7
21	33 $\pm$ 3

Figure 15. Time course of pollination-induced senescence of *Phalaenopsis* flowers.

Figure 16. Ethylene production by cut *Phalaenopsis* flowers. (□) Control, (○) pollinated flowers treated with AOA, (●) emasculated flowers, (Δ) pollinated flowers, (▲) pollinated and emasculated flowers. Values are means of 4 replications $\pm$ SE.

Figure 17. Time course of the changes in sensitivity to ethylene following pollination of *Phalaenopsis* flowers. A. Flowers in water were exposed to $4 \mu\text{l l}^{-1}$ ethylene for 4 h at different periods after pollination. Values are the increase in wilting grades 12 h after ethylene treatment over those of pollinated flowers not exposed to ethylene. B. Flowers held in 0.5 mM AOA and exposed to $10 \mu\text{l l}^{-1}$ ethylene for 4 h at different periods after pollination. Values are the increase in wilting grades 4 days after ethylene treatment over those of pollinated flowers not exposed to ethylene. Letters on top of bars indicate separation of means ($P < 0.05$, $n = 5$) by Duncan's multiple test.

Figure 18. Effects of ethylene concentration on the time to 50% wilting in *Phalaenopsis* flowers held in 0.5 mM AOA. Flowers were exposed to ethylene for 12 h. (o) Control, (●) pollinated. Values are means of 5 replications $\pm$ SE.

Figure 19. Time course of changes in ethylene production and sensitivity to ethylene following pollination of *Phalaenopsis* flowers. The changes in ethylene sensitivity are represented by the increase in wilting grades following exposure to $4 \mu\text{l l}^{-1}$ ethylene for 4 h at different periods after pollination as compared to pollinated flowers not exposed to ethylene. The values were measured 12 h after the ethylene treatment and are means of 4 replications. The values of ethylene production are means of 4 replications $\pm$ SE.

Figure 20. Effects of linoleic acid on the longevity of cut *Phalaenopsis* flowers. Values are means of 4 replications $\pm$ SE.

Figure 21. Effects of fatty acids on the longevity (A) and ethylene production (B) of cut *Phalaenopsis* flowers. 5 μmole of palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) or linolenic acid (C18:3) were tipped onto the stigma. Ethylene production was measured after 48 h. Values are means of 4 replications $\pm$ SE.

Figure 22. Effects of JA-Me on ethylene production (A) and ACC content (B) in cut *Dendrobium* flowers. 50 nmole JA-Me were applied to the stigma. (○) Control, (●) JA-Me. Values are means of 3 replications $\pm$ SE.

Figure 23. Effects of SHAM and n-PG on LOX activity in *Phalaenopsis* columns. Different concentrations of SHAM (○) or n-PG (●) were added to the reaction mixture. Values are means of 3 experiments $\pm$ SE.

Figure 24. Effects of pollination on LOX activity in *Phalaenopsis* flowers. Proteins were extracted immediately or 10, 25 or 50 h after pollination, and LOX activity was measured in the column (o) or perianth (●). Values are means of 3 experiments $\pm$ SE.

Figure 25. Effects of pollination on jasmonate content in *Dendrobium* columns. Flowers were kept non-pollinated (o) or were pollinated (●). At different times after pollination, columns were separated for jasmonate analysis. Values are of samples taken from 6 different flowers.

Figure 26. Effects of pollination on the amounts of octanoic acid (C₈) and total C₇ to C₁₀ saturated fatty acids in *Phalaenopsis* columns. Flowers were unpollinated (Δ, o) or pollinated (▲, ●), and the amounts of octanoic acid (Δ, ▲) or total C₇ to C₁₀ fatty acids (o, ●) were measured. Values are means of three separate experiments $\pm$ SE.

Figure 27. Effects of pollination on the amounts of octanoic acid (C₈) and total C₇ to C₁₀ saturated fatty acids in *Phalaenopsis* perianths. Flowers were unpollinated (Δ,○) or pollinated (▲,●), and the amounts of octanoic acid (Δ,▲) or total C₇ to C₁₀ fatty acids (○,●) were measured. Values are means of three separate experiments $\pm$ SE.

Figure 28. Effects of different ratios of octanoic acid on the polarization values of egg lecithin liposomes (o) or *Phalaenopsis* petal microsomal membranes (●) as determined with DPH. Values are means of three separate experiments $\pm$ SE (the SE are smaller than the symbols and therefore cannot be seen).

Figure 29. Effects of different ratios of octanoic acid on the polarization values of egg lecithin liposomes (o) or *Phalaenopsis* petal microsomal membranes (●) as determined with DNSP. Values are means of three separate experiments $\pm$ SE (the SE are smaller than the symbols and therefore cannot be seen).

Figure 30. Western blot analysis of microsomal membranes isolated from *Phalaenopsis* petals. Proteins (60 μ g) were resolved on 12% SDS-PAGE, blotted and reacted with antibodies against $G\alpha$ -common. The blots were labeled with [125 I] protein A. The arrow points to the main labeled peptide.

Figure 31. Effects of CaCl_2 , pollination, AOA and 1-MCP on *in vivo* protein phosphorylation in *Phalaenopsis* columns. Pollinated or unpollinated flowers were incubated for 25 h with 3.7 MBq [32 P]orthophosphoric acid and 1 mM CaCl_2 and 2 μ M A23187, 0.5 mM AOA or pretreated for 6 h with 250 nl l⁻¹ 1-MCP. Following treatments, columns were homogenized and incorporation of 32 P into the proteins was measured. Values are means of 3 independent experiments $\pm$ SE.

Figure 32. Effect of pollination on *in vivo* protein phosphorylation. Control (lanes 1,3) and pollinated (lanes 2,4) flowers were incubated with 3.7 MBq [^{32}P] orthophosphate and homogenized. Proteins were precipitated in TCA and 50 μg aliquots were loaded on 12% SDS-PAGE, developed and autoradiographed. Lanes 1,2 are the comassie blue staining and lanes 3,4 are the autoradiograph developed from the gel. The arrow on the right indicates the main phosphorylated peptide following pollination.

Figure 33. Effects of ethylene action inhibitors STS and 1-MCP on ethylene production following pollination of *Phalaenopsis* flowers. A. Flowers were unpollinated (○), pollinated (●), pollinated after pretreatment with 250 nl l^{-1} 1-MCP for 6 h (Δ) or pollinated and held in 0.5 mM STS (▲). B. Details of the effect of 1-MCP on the initial increase in ethylene production. Values are means of 4 replications $\pm$ SE.

Figure 34. Effects of calcium ions on ethylene production of unpollinated *Phalaenopsis* flowers. (○) Control, (●) flowers treated with 1 mM CaCl₂ and 500 μM A23187. Values are means of 4 replications ± SE.

Figure 35. Effects of calcium ions and EGTA on ethylene production following pollination of *Phalaenopsis* flowers. (○) Unpollinated, (●) pollinated, (Δ) pollinated flowers treated with 1 mM CaCl₂ and 2 μM A23187, (▲) pollinated flowers treated with 10 mM EGTA. Values are means of 4 replications ± SE.

Figure 36. Effect of exposure to ethylene on the wilting of short-life (A,C) and long-life (B,D) petunia flowers held in water (A,B) or in 0.5 mM AOA (C,D). Flowers were harvested at anthesis and exposed for 10 h to $2 \mu\text{l l}^{-1}$ ethylene. (o) Control flowers, (●) ethylene-treated flowers. Values are means of 6 replications SE.

Cover Page.

Date: 12/16/92

BARD
P. O. BOX 6
Bet Dagen, ISRAEL

Bard Project No. US-1867-90R

Title:

Molecular Genetic Analysis of Pollination-induced Senescence in Orchids.

Investigators' Names
(Principal listed first)

Investigators' Institutions

Sharman D. O'Neill

Section of Botany
University of California, Davis

Abraham H. Halevy

Faculty of Agriculture
The Hebrew University of Jerusalem

Amihud Borochoy

Faculty of Agriculture
The Hebrew University of Jerusalem

Project's starting date: 8/30/91

Type of Report: 1st Annual

Signature
Principal Investigator

Signature
Institution's Authorizing Official

Robert N. Shelton
Vice Chancellor-Research

DEC 16 1992

b. Description of the Research Activities and Results.

United States Group

GENERAL APPROACH:

Orchid flowers provide an excellent model system for studying the molecular events that regulate post-pollination developmental events in flowering plants. Although the most obvious response to pollination is senescence of the perianth, other responses include changes in pigmentation, ovary maturation and ovule differentiation which must precede fertilization. Each of these processes plays a significant role in successful fertilization and reproduction and are coordinately regulated by hormonal cues delivered with the pollen (auxin) and produced endogenously (ethylene) in the gynoecium and perianth. The approach we have taken is to focus on those genes encoding ethylene biosynthetic genes as a means to identify the molecular mechanisms regulating ethylene production, to study their interorgan regulation following pollination, and to study the developmental events that are in turn regulated by ethylene. This research relies primarily on the cloning of specific orchid genes encoding ethylene biosynthetic enzymes and assessing their patterns of expression in response to hormonal and physical cues at both the tissue- and cell-specific level.

The major accomplishments of this research project to date are:

- 1.) Construction of cDNA libraries to mRNA isolated from either the gynoecium or perianth of pollinated *Phalaenopsis* flowers.
- 2.) Isolation and sequencing of cDNA clones encoding ACC synthase.
- 3.) Isolation and sequencing of cDNA clones encoding ACC oxidase (formerly referred to as "Ethylene Forming Enzyme" or EFE).
- 4.) Characterization of expression of ACC synthase and EFE genes in the gynoecium and perianth in response to pollination and other pollination-associated factors and events, for example, auxin, ethylene, emasculation, and wounding.
- 5.) Isolation and characterization of a cDNA clone encoding endo- β -1,4-glucanase, believed to be the first monocot gene identified as well as the first cellulase gene that is negatively regulated by ethylene.
- 6.) SEM studies of two series of events: (1) the effect of pollination vs. emasculation on the surface and structure of the stigmatic cavity of the gynoecium, and (2) the effect of auxin over the same time course.

7.) Detailed anatomical studies of the effect of various factors and events on initial stigma responses and ovary development. These studies provide important background for all other work, be it physiological, biochemical or molecular in nature.

Together these accomplishments provide the basis for a detailed analysis of the molecular genetic regulation of orchid flower senescence. In addition, we have identified coordinated developmental events associated with ovary and ovule maturation following pollination. Because this developmental process is not coordinated with pollination in most other plants, it has been difficult to study at the molecular level. Orchid flowers provide a unique opportunity to study this critically important process in plant reproduction.

DETAILED DESCRIPTION OF THE RESULTS:

ACC synthase gene structures: We have isolated three different ACC synthase cDNA clones from the gynoecium cDNA library representing distinct members of a multigene family expressed in the stigma tissue of orchid flower. These genes have been sequenced by a graduate student supported by BARD funding of the project, Mr. Anhthu Bui, and their deduced amino acid sequences indicate these cDNAs represent closely related structural genes encoding the ACC synthase enzyme. The three ACC synthase cDNAs are designated as OAS (Orchid ACC Synthase) cDNA clones OAS1, OAS2 and OAS3 (Bui and O'Neill, unpublished results) We have included the nucleotide (nt) sequence of two of these orchid ACC synthase cDNAs in an alignment with one another (Figure 1). The sequences of these genes are nearly identical; however, as with the OAS2 sequence, there is an additional 60 nucleotides present in the OAS3 sequence making OAS3 more similar to OAS2, encoding a protein with an additional 20 amino acids. The absent region, which we have casually named the "missing link" for the sake of discussion, is also present in all other ACC synthase sequences from a diversity of plants (tomato, squash) as shown in Figure 2 at the level of the amino acid sequence. The absence of the "missing link" in OAS1 and other deletions in the coding sequence make this ACC synthase gene product the smallest identified to date and of special interest because of its uniqueness. A comparison of the predicted properties of two orchid ACC synthase polypeptides (OAS1 and OAS3) with several other recently characterized polypeptides is presented in Table 1.

All three OAS sequences contain most of the invariant amino acids (11/13) that are found in animal, bacterial, and yeast aminotransferase enzymes; remarkably

these amino acid residues occur in the corresponding correct positions within the orchid amino acid sequences (Fig. 2), with OAS1 having the best alignment.

ACC synthase gene expression: Our efforts have partially resolved the question of what factor(s) regulate the expression of the OAS genes. Previous work with the ethylene antagonist, norbornadiene (NBD) indicated that at least one ACC synthase gene is positively regulated by ethylene in orchid gynoecia, consistent with a mechanism of autocatalytic ethylene production as observed in climacteric fruit in orchid gynoecia. No ACC synthase mRNA accumulated in perianth tissue in spite of the fact that this tissue produces ethylene. This suggests that ACC produced in the gynoecium may be translocated to the perianth. Other data (discussed below) indicates that an ACC oxidase gene is expressed in both gynoecium and perianth and could account for conversion of ACC to ethylene in the perianth.

Treatment of the flower with ethylene or auxin (NAA) indicated that mRNA transcript for an ACC synthase gene is positively regulated by auxin alone, and by ethylene alone, and that the mRNA accumulates predominantly in the stigma (Figure 3, top panel). The effects of auxin and ethylene together are not additive because the mRNA abundance is lower in the treatment suggesting a complex interaction between auxin and ethylene in controlling ACC synthase gene expression. Because auxin stimulates ethylene production, it is difficult to separate the effects of auxin and ethylene on ACC synthase gene expression. However, through the use of the inhibitor of ACC synthase activity, AVG, we have determined that the auxin effect on gene expression that we are seeing is indirect because auxin is probably operating through its effect on ethylene production (Figure 4). This is shown in Figure 4 whereby AVG inhibits the appearance of mRNA transcript even in the presence of NAA. The inhibition can be overcome by adding back ACC (overcoming the inhibitor block) or by treatment with AVG in the presence of ethylene, which directly induces mRNA transcription. These molecular results confirm our physiological findings with ethylene production by the flower in that, in the presence of AVG plus NAA, ethylene production is blocked; however, this inhibition can be overcome transiently by applying ACC, and to a greater extent, by treatment with exogenous ethylene (Figure 5)

From Figures 3 and 4, it can be concluded that the auxin effect on promoting high levels of ACC synthase mRNA is via its effect on ethylene production, i.e., not direct. Nonetheless, we have to presume that auxin must be directly promoting *imperceptibly* low levels of ACC synthase gene expression to *initiate* the whole

process, which cannot be accounted for by ethylene alone. We have not resolved yet the important question of whether the same gene is variably regulated by auxin and ethylene or whether there is a separate auxin-regulated gene, expressed at a very low level, that we have not been able to detect by Northern analysis. We are applying PCR (Polymerase Chain Reaction) procedures, which can overcome the technical limitations of Northern analysis, to answer this major question.

Based on our research findings to date, we have developed a working model for interorgan regulation of ethylene biosynthetic genes in orchid flowers (Figure 6). By this model:

- 1) Pollination or an associated factor is a primary signal that initiates expression of an ACC synthase gene in the stigma.
- 2) This may or may not be one of the ACC synthase genes we have isolated and may occur at levels below our present level of sensitivity.
- 3) ACC oxidase is also induced leading to ethylene production.
- 4) Ethylene gas permeates all the floral organs and directly promotes high levels of ACC synthase and ACC oxidase gene expression in all organs-- except for ACC synthase in the perianth.
- 5) Given that ethylene is produced by the perianth, we propose based on our *molecular analysis* that ACC is translocated from the gynoecium to the perianth where it is converted by the activity of a perianth-localized ACC oxidase to ethylene.

The combination of different ACC synthase genes that are regulated by different pollination-associated factors with similarly complex ACC oxidase genes in conjunction with their *coordinated* expression in distinct floral organs leads to a rather impressive interorgan regulation of the post-pollination response in the orchid flower. By the end of the third year, we hope to have pinned down the auxin-ACC synthase gene question. My prediction is that it is one of the genes we have already characterized.

ACC Oxidase gene structure: An integral part of the research program on orchid flowers is the second gene in the ethylene biosynthetic pathway, the ACC

oxidase gene, which converts the ACC made by ACC synthase to ethylene. This project is now the Ph.D. thesis project of Ms. Jan Nadeau. Since Ms. Nadeau is supported by a graduate fellowship from the American Orchid Society, this component of the research project is supported by the American Orchid Society as well as by BARD.

ACC Content and ACC oxidase activity of orchid flowers: Ms. Nadeau has determined the ACC content and ACC oxidase activity of various parts of the orchid flower (Figure 7). Both ACC content and ACC oxidase activity increase in parallel, and these patterns are well correlated with both ethylene production in the pollinated flower (Figure 5) as well as with ACC oxidase mRNA expression (Figure 8). In Figure 8, it can be seen that ACC oxidase mRNA is detectable at 18 hours post-pollination in the stigma and labellum tissue, peaking at 24 and 48 hours. The transcript is not detectable in the petal/sepal tissue until 48 hours after pollination, suggesting that the perianth gene is not directly stimulated by the pollination event. As it turns out, we hypothesize that the petal/sepal transcript is dependent for its induction on a supply of ACC translocated from the gynoecium.

ACC oxidase genes: Ms. Nadeau has isolated a number of cDNA clones whose nucleotide sequences share identity with pTOM13, the ethylene forming enzyme of tomato fruit, i.e., ACC oxidase of tomato. The first ACC oxidase that she has sequenced is Orchid ACC Oxidase 1 (OAO1). This sequence was isolated from the gynoecium cDNA library. We present this sequence in an amino acid alignment with TOM13 (pTOM13), and with AVOE3, a putative ACC oxidase from avocado (Figure 9). Clearly the numerous conserved domains and shared identity is convincing that the OAO1 clone is an authentic ACC oxidase. Several other putative ACC oxidase cDNAs have been identified, one of these representing a second gene, OAO2, which may be perianth-specific. This question, as well as the determination of the sequences of other candidate ACC oxidases, will be addressed by Ms. Nadeau in the second year of the project.

ACC oxidase gene expression is regulated by ethylene: We have examined the pattern of expression of both ACC synthase and ACC oxidase in pollinated flowers in the absence and presence of 2,5-norbornadiene (NBD), an inhibitor of ethylene action and therefore autocatalytic ethylene effects. Data is presented showing the results for the ACC oxidase gene, OAO1 (Figure 10). In contrast to the results with

ACC synthase (data not shown), ACC oxidase gene expression is induced early in unpollinated flowers, probably as a response to the necessary harvest for experimental manipulation (a wounding response), is further induced by pollination, and this latter increase is inhibited by NBD. The inhibition by NBD suggests that the pollination-induced increase reflects an ethylene stimulated increase via the autocatalysis feedback system (ethylene stimulates more ethylene production following binding to ethylene receptors). The second important finding is that an ACC oxidase gene is expressed in the petals/sepals (perianth in Figure 10). This is in sharp contrast to our results with the ACC synthase gene in which there was no expression detectable in the perianth tissue (data not shown). This expression is completely inhibited by NBD, suggesting that its expression is dependent on continued ethylene perception through the autocatalysis mechanism.

Several conclusions can be drawn from Figure 10.

(1) There are at least two different ACC oxidases in the gynoecium: one that is induced by wounding/stress associated with flower harvest, and a second that is induced by pollination.

(2) The pollination-induced gene is inhibited by NBD, and is probably an ethylene-regulated gene.

(3) Both of these genes requires the prior production of ACC, hence they are dependent on the activity of an ACC synthase.

(4) The "wound-induced" expression may represent the activation of an ACC oxidase gene by cell wall fragments that may be released upon wounding.

(5) The perianth ACC oxidase resembles the pollination induced gene of the gynoecium in its response to NBD. It is entirely dependent on continued ethylene perception for its expression. This leads to the conclusion that the pollination-related ACC oxidases are ethylene-regulated genes setting them apart from those that are induced by other events such as wounding.

These conclusions are supported by the results presented in Figures 3 and 4 (lower panels) whereby treatment with both ACC and ethylene can stimulate expression of OAO1 in the presence of AVG. As with ACC synthase, the auxin effect is probably

an indirect one, operating through its stimulatory effect on ethylene production. Similar results were found for the gene expressed in the petals (Figure 11).

ISRAEL GROUP

GENERAL APPROACH:

The Israeli team consisting of Drs. Abraham Halevy and Amihud Borochoy have taken two approaches in their investigation of the mechanism of pollination-induced flower senescence. Both of these investigations have focused on the increase in sensitivity to ethylene following pollination. Two reports have been prepared for publication. The first body of work reports on the process of pollination-induced senescence in *Phalaenopsis* orchid flowers. In this study, it was found that there was an increase in sensitivity to ethylene in less than four hours after pollination and that this sensitivity reached its peak at approximately 10 hours after pollination. Ethylene production started at a later time by several hours reaching its peak by 30 hours after pollination. The sensitivity to ethylene was determined to be independent of ethylene production because it is induced by pollination also in the absence of ethylene production in flowers treated with AOA, an inhibitor of ethylene biosynthesis. The mechanism by which the sensitivity to ethylene is made operative is still unknown; however, in another flower system, petunia, short chain fatty acids have been implicated as "sensitivity factors" which seem to function as transmissible signals to induce ethylene sensitivity. The mechanism by which the tissue becomes sensitive to ethylene though is still unknown.

The second approach to elucidating the mechanism of increased sensitivity involves an analysis of a cellular signal transduction pathway involving GTP-binding proteins (G-proteins) and membrane protein phosphorylation. In their analysis of the signal transduction pathway, Drs. Halevy and Borochoy have examined the relationship between the increase in ethylene sensitivity following pollination in *Phalaenopsis* petals and the activities of GTP-binding proteins and protein phosphorylation, two major components in the cellular signal transduction pathway. This has been done to examine the possible involvement of this signal transduction mechanism in the pollination-induced increase in sensitivity to ethylene. It was found that chemicals which enhance the activity of G-proteins and protein kinases also increased the sensitivity of cut *Phalaenopsis* flowers to ethylene. The results are shown in Figures 12 to 14. Figure 12 shows that treatment of flowers with cholera toxin increased their sensitivity to ethylene by inducing rapid wilting, and had no effect on control flowers which were held in ethylene-free air. Similarly, GTP S, a nonhydrolyzable analog of GTP, greatly increased the sensitivity of cut flowers to

ethylene, and had no effect on control flowers held in ethylene-free air (Fig. 13). Moreover, addition of Ca^{2+} with a specific ionophore (A23187) to enhance its uptake into the cells, increased ethylene sensitivity of cut flowers but had no effect on control flowers held in air (Fig. 14). These results showed that chemicals which enhance the activity of G-proteins and protein kinases increased the sensitivity of cut flowers.

Based on these preliminary findings, the possible participation of G-proteins and protein phosphorylation in the process of pollination-induced increase in sensitivity to ethylene was examined. This was accomplished by Western blot analysis of G-proteins that cross-reacted with G-protein antiserum. Two major protein bands were detected. These putative GTP-binding proteins showed higher binding activity of radiolabelled GTP S as compared to denatured membranes (Figure 15). The binding of the radioligand was very specific. GTP and GTP S were competitive and reduced the binding of radiolabelled GTP S, while GDP S was without effect (Fig. 16). It was also found that following pollination, there was an increase of 20% in the binding of radiolabelled GTP S, and that a similar increase was obtained also when flowers were treated with AOA to inhibit ethylene biosynthesis. Additional data not shown support the hypothesis that a signal transduction pathway involving G-proteins and protein phosphorylation is part of the mechanism of increase sensitivity following pollination. Work is ongoing to further elucidate the role of these signal transduction components in the process of pollination-induced senescence.

FIGURE LEGENDS

Figure 1. Nucleotide sequence alignment of two ACC synthase cDNA clones, OAS1 and OAS3.

Figure 2. Amino acid sequence alignment of orchid ACC synthase (OAS1) with the ACC synthases of squash and tomato. Stars (*) indicate identical amino acids and periods (.) indicate conservative replacements (i.e., similar amino acids). The colored ball indicate the the location of 11 invariant amino acid residues found in all aminotransferase-type enzymes.

Figure 3. RNA gel blot (Northern blot) analysis of expression of ACC synthase mRNA (upper panel) and ACC oxidase mRNA (lower panel) in the stigma (S) or ovary (O) of flowers treated with NAA, ethylene, or NAA & ethylene as compared to untreated flowers. The size (kb) of the single detectable transcript is shown on the left.

Figure 4. RNA gel blot (Northern blot) analysis of expression of ACC synthase mRNA (upper panel) and ACC oxidase mRNA (lower panel) in the stigma (S) or ovary (O) of flowers treated with AVG alone or in combination with NAA, ACC, or ethylene as compared to untreated flowers. The size (kb) of the single detectable transcript is shown on the left.

Figure 5. Time course of ethylene production by orchid flowers following pollination or treatment of the stigma with ACC, NAA, or AVG in combination with ACC, NAA, or pollination. Pollination, NAA and ACC all induce ethylene production. AVG essentially inhibits this production except during the initial hours following treatment when a non-pollination related ACC oxidase activity may be operative in producing ethylene.

Figure 6. Model for the interorgan regulation of ethylene biosynthetic genes in orchid flowers.

Figure 7. Time course of increase in ACC content and ACC oxidase activity in various parts of the *Phalaenopsis* orchid flower following pollination.

Figure 8. Northern blot hybridization analysis of the pattern of expression of the ACC oxidase gene, OAO1, in the separately analyzed parts of the orchid flower following pollination. A single transcript is detectable at approximately 1.4 kb.

Figure 9. Amino acid sequence alignment of orchid ACC oxidase (OAO1) with the ACC oxidases of tomato and avocado. Stars (*) indicate identical amino acids and periods (.) indicate conservative replacements (i.e., similar amino acids).

Figure 10. RNA gel blot (Northern blot) analysis of expression of the ACC oxidase mRNA corresponding to OAO1 in the gynoecium (upper panel) and perianth (lower panel) of unpollinated, pollinated and pollinated + NBD treated flowers.

Figure 11. RNA gel blot (Northern blot) analysis of expression of ACC oxidase mRNA corresponding to OAO1 in the petal (P) or labellum (L) tissue of flowers treated NAA, ethylene or NAA & ethylene as compared to untreated flowers. The size (1.4 kb) of the single detectable transcript is shown on the left.

Figure 12. Effect of cholera toxin (6 $\mu\text{g/ml}$) on the sensitivity of cut *Phalaenopsis* flowers to ethylene (0.5 $\mu\text{l/L}$) exposure for 12 hours. () cholera toxin and ethylene, () ethylene, (), cholera toxin, () control. Values are means of 5 replications $\pm$ S.E..

Figure 13. Effect of GTP S on the sensitivity of cut *Phalaenopsis* flowers to ethylene. GTP S concentration was 50 μ M, and the flowers were exposed to 0.5 μ l/L ethylene for 12 hours. () GTP S and ethylene, () ethylene, () GTP S and control without ethylene. Values are means of 5 replications $\pm$ S.E..

Figure 14. Effect of calcium and ionophore A23187 on the sensitivity of cut *Phalaenopsis* flowers to ethylene. Flowers were exposed to 0.5 μ l/L ethylene for 12 hours. () calcium + A23187 and ethylene, () ethylene, () calcium + A23187 and control without ethylene. Values are means of 5 replications $\pm$ S.E..

Figure 15. Effect of GTP S concentrations on the binding of 10^{-8} M [35 S] GTP S to *Phalaenopsis* petals microsomal membranes. () nonboiled membranes, () boiled membranes. Values are means of 3 replications $\pm$ S.E..

Figure 16. Effect of putative competitors on the binding of [35 S] GTP S to *Phalaenopsis* petals microsomal membranes. Values are means of 3 replications after subtracting the means of boiled controls $\pm$ S.E..

C. EVALUATION OF RESEARCH PROGRESS.

Our research project on the molecular biology of pollinated orchid flowers and the interorgan regulation of ethylene biosynthetic genes is now well under way. We have isolated a number of key genes involved in the post-pollination developmental syndrome including perianth senescence, and these provide us with the tools to dissect the process at all levels and to understand specifically the molecular basis of the response. Understanding the coordinated regulation of the post-pollination phenomenon in orchid flowers will provide important insight into plant reproduction in general and may provide the foundation for designing molecular genetic strategies to enhance the longevity of short-lived orchid flowers in the future.

D. DESCRIPTION OF THE COOPERATION.

The U.S. laboratory will continue analysis of the molecular basis of pollination-induced changes in the flower. Dr. O'Neill will visit the Israeli laboratories in March of 1993. The Israeli investigators will continue their analysis of signal transduction and the pollination-induced change in sensitivity to ethylene. Dr. Halevy will visit the U.S. group in the summer of 1993 as he did in July of 1992 during the first year of the project.

E. CONCLUSIONS AND PLANS FOR THE NEXT YEAR.

The major goal of this research project is to understand the molecular genetic basis for the post-pollination developmental syndrome of orchid flowers. The post-pollination syndrome includes perianth senescence, floral color change, ovary maturation and ovule development. We have been developing the biochemical and molecular genetic tools to address the role of altered gene expression in the post-pollination changes in orchid flowers. Initially, the research focused specifically on the regulation of pollination-induced perianth senescence. However, in the course of research it became clear that several development events in addition to perianth senescence, including pollen tube growth, ovary maturation and ovule differentiation are also regulated by the same or closely-coordinated signals. This has led to a broadening of the initial research objectives to include a better understanding of the coordination of hormonal signals that regulate gene expression during post-pollination development. In the first year of the project, the research in the U.S. laboratory has focused on cloning and characterizing a number of orchid genes encoding ACC synthase and ACC oxidase, the key enzymes regulating ethylene biosynthesis in plants. Objectives for the next year of this project are to:

- (1) Continue analysis of the factors (auxin, ethylene, ACC, other pollen-derived factors) associated with the specific induction of each of the ACC synthase and ACC oxidase genes.
- (2) Characterize cell-specific expression of ethylene biosynthetic genes in relation to the coordination of multiple pollination-induced developmental processes, including perianth senescence and ovule maturation.
- (3) Construct an orchid genomic library from which the corresponding genes for each ACC synthase and ACC oxidase will be isolated.
- (4) Sequence the promoter regions of these genomic counterparts and analyse the regulatory sequences present in each gene that contributes to its spatial and temporal regulation following pollination.

(5) Continue biochemical and physiological analyses of the factors associated with pollination (auxin, ACC, ethylene, emasculation, wounding, etc,) that are involved in gene regulation.

(7) continue RNA blot hybridization analysis of the expression of each gene following pollination, emasculation, and other pollination-associated treatments, some of which will be done in the presence of various inhibitors of ethylene biosynthesis or action (AVG, NBD).

(7) Continue analysis of the signal transduction mechanism involved in pollination-induced changes in tissue sensitivity to ethylene, specifically the involvement of G-proteins and membrane protein phosphorylation.

(8) express each cDNA clone of the ACC synthases and ACC oxidases in *E. coli* to test whether these genes encode functional polypeptides. Several plant proteins have been critically assessed by expression in *E. coli*.

(9) prepare antibodies to the expressed protein for later analysis of proteins *in vivo* by Western blot analysis (protein blots).

(10) Localization of ACC synthase and ACC oxidase gene expression. We have worked out the procedures for *in situ* localization of ACC synthase and ACC oxidase. This is a technically challenging procedure requiring a combination of skills, for example, good tissue sectioning skills as well as advanced skills in molecular procedures. Mr. Xiansheng Zhang, a highly skilled embryologist from China visiting the U.S. laboratory and supported by the BARD grant, has worked out the procedures for *in situ* hybridization of orchid flower tissue. Thus, a major effort in the next year will be to localize expression of ethylene biosynthetic genes by *in situ* hybridization within the pollinated ovary or following treatment with auxin, ethylene, and other pollination-associated factors.

These are some of the immediate goals of the overall orchid project. The achievement of each one of these goals requires a major concerted research effort as well as the successful execution of numerous technically challenging procedures.

ANTICIPATED PROCEDURES FOR THE SECOND AND THIRD YEARS:

PCR amplification, to characterize the complete family of genes encoding ethylene biosynthetic enzymes.

DNA sequencing, to characterize the gene sequences in detail.

RNA blot hybridization analysis, to analyze specific gene expression in flower tissues following pollination, emasculation or treatment with auxin.

***In situ* hybridization**, to characterize specific gene expression in a cell-specific manner. This procedure is technically difficult, and it is anticipated that application of this technique to orchid flowers will be the primary focus of research in the next year.

Genomic DNA isolation, to isolate high molecular weight DNA for genomic cloning and DNA analysis.

DNA blot hybridization analysis to understand the organization of the gene families encoding ACC synthase and ACC oxidase. This procedure is technically difficult because of the polyploid nature of the orchid nuclear genome and the possibility that at least some of these genes may be linked or arrayed in tandem and difficult to distinguish.

Genomic cloning to isolate the genomic counterparts of each cDNA that we have isolated.

The above procedures will be used as needed to accomplish each specific research goal of this BARD-funded project.

F. LIST OF PUBLICATIONS.

No publications have resulted thus far from the first year's work although two publications have been prepared by the Israeli team and one by the U.S. team. The latter manuscript has been submitted to *The Plant Cell* for consideration for publication.

*Properties of ACC synthase polypeptides encoded by various
monocot and dicot genes*

Species	Amino acid no.	Predicted molecular mass (Da)	Isoelectric Point
Orchid (OAS1)	425	47,553	5.45
Orchid (OAS3)	444	49,876	5.85
Carnation	516	58,063	8.79
Winter Squash	493	55,895	6.81
Tomato	482	54,192	5.65
Zucchini	475	53,481	8.20

AMINO ACID SEQUENCE ALIGNMENT OF OAS1 AND OAS3

```

OAS1PRO  - MSKMFGKEVPLSKMAVSKAHGEGSPYFAGWKAYEENPYDVVGNPDGVIQMGLAEN -55
          ***** ** *****
OAS3PRO  - MSKMFGKEVPLSKMAVSKAHGEGSPYFAGWKAYEENRYDAVGNPDGVIQMGLAEN -55

OAS1PRO  - QLSFDLLEEYLELHPEAFSWASDSSSFRENALFQDYHGLQTLRQALASFMEKIRG -110
          *****
OAS3PRO  - QLSFDLLEEYLELHPEAFSWASDSSSFRENALFQDYHGLQTLRQALASFMEKIRG -110

OAS1PRO  - GRSKFDANRIVLTAGATAANEILTFILADRGDALLVPTPYYPG----- -153
          *****
OAS3PRO  - GRSKFDANRIVLTAGATAANEILTFILADRGDALLVPTPYYPGFLRDLQWRTGVT -165

OAS1PRO  - -----SNGFQLTLSSLEKAYAEAKASNFNVRGLLMTNPCNPLGTSASLSLLQ -200
          *****
OAS3PRO  - IFPVHCYSSNGFQLTLSSLEKAYAEAKASNFNVRGLLMTNPCNPLGTSASLSLLQ -220

OAS1PRO  - DIIHFISDKNIHLISDEIYSGSVFSSTNLFSISDLITDAISEQVHIVYSLSKDLG -255
          *****
OAS3PRO  - DIIHFISDKTIHLISDEIYSGSVFSSTNLFSISDLITDAISEQVHIVYSLSKDLG -275

OAS1PRO  - LPGFRVGALYSYNDRVVKTARRMSSFSLVSSQTQKLLSFMLSDEEFTVRYIEKNR -310
          *****
OAS3PRO  - LPGFRVGALYSYNDRVVKTARRMSSFSLVSSQTQRLLSFMLSDEEFTVRYIEKNR -330

OAS1PRO  - ERLRERYELVVNGLKEAGIECLKGEAGLFCWVNMEKLMEEETKEGEAELWKVIID -365
          *****
OAS3PRO  - ERLRERYELVVNGLKEAGIECLKGEAGLFCWVNMEKLMEEETKEGEAEPWKVIID -385

OAS1PRO  - DLKLNISPGSSCCCAEPGWFRLCFANMSRETLEVALKRLKDFAQKKVAAKKKKMN -420
          *****
OAS3PRO  - DLKLNISPGSSCCCAEPGWFRLCFANMSRETLE-GVKRLKDFAQKKVAAKKKKMN -439

OAS1PRO  - NRYFC -425
          *****
OAS3PRO  - NRYFC -444

```

AMINO ACID SEQUENCE ALIGNMENT OF ORCHID, SQUASH AND TOMATO ACC SYNTHASE

OAS1PRO	VLIKIMSKM-----FGKEVPLSKMAVSKAHGEGSPYFAGWKAYEENPYDVVGN	48
SQUASHPRO	-----MEFHQIDE-RNQALLSKIAVDDGHGENSPYFDGWKAYDNDPFHPEDN	46
TOMAS2PRO	KLGNKMDLETSEISNYKSSVLSKLASNEQHGENSEPYFDGWKAYDNDPFHLVNN	55
	 * * * * *	
OAS1PRO	PDGVIQMGLAENQLSFDLLEEYLELHPEA-FSWASDSSSFRENALFQDYHGLQTL	102
SQUASHPRO	PLGVIQMGLAENQLSFDMIVDWIRKHPEASICTPKGLERFKSIANFQDYHGLPEF	101
TOMAS2PRO	LNGVIQMGLAENQLSVDLIEEWIKRNPKASICTNDGIESFRRIANFQDYHGLPEF	110
	***** * * * * *	
OAS1PRO	RQALASFM EKIRGGRSKFDANRIVLTAGATAANEILTFILADRGDALLVPTPYYP	157
SQUASHPRO	RNGIASFMGKVRGGRVQFDP SRIVMGGGATGASET VIFCLADPGDAFLVPSPIYA	156
TOMAS2PRO	TNAIAKFM EKTRGGKVKF DAKRVVMAGGATGANETLILCLADPGDAFLVPTPYYP	165
	 * * * * *	
OAS1PRO	G-----SNGFQTLTSSLEKAYAEAKASNFNVRGLLMTNPC	192
SQUASHPRO	AFDRDLKWRTRAQIIRVHCNSSNNFQVTKAALEIAYKKAQEANIKVKGVIITNPS	211
TOMAS2PRO	GFNRDLRWRSGVQLLPISCKSCNNFKITIEAIEEAYEKQQANVKIKGLILTNPC	220
	 * * * * *	
OAS1PRO	NPLGTSASLSLLQDIHFISDKNIHLISDEIYSGSVFSSTNLFSISDLITDAIS-	246
SQUASHPRO	NPLGTTYDRDTLKTLLVTFVNQHDIHLICDEIYSATVFKAPT FISISIAQIVEEMEHC	266
TOMAS2PRO	NPLGTILDRDTLKKISTFTNEHNIHLVCDEIYAATVFN SPKFVSIAEIINE-DNC	274
	***** * * * *	
OAS1PRO	---EQVHIVYSLSKDLGLPGFRVGALYSYNDRVVKTARRMSSFSLVSSQTQKLLS	298
SQUASHPRO	-KKELIHILYSLSKDMGLPGFRVGIIYSYN DVVRRARQMSSFGLVSSQTQHLLA	320
TOMAS2PRO	INKDLVHIVSSLSKDLGFPFGFRVGIVYSFNDDV VNCARKMSSFGLVSTQTQHLLA	329
	 * * * * *	
OAS1PRO	FMLSDEEFTVRYIEKNRERLRERYELVVNGLKEAGIECLKGEAGLFCWVNMEKLM	353
SQUASHPRO	AMLSDEDFVDKFLAENSKRLAERHARFTKELDKMGITCLNSNAGVFVWMDLRRL	375
TOMAS2PRO	FMLSDEDFVEEFLIESAKRLRERYEKFTRGLEEIGIKCLESNAGVYCWM DLRSLL	384
	***** * * * *	
OAS1PRO	EEETKEGEAELWKVIIDDLKLNISPGSSCCCAEPGWFR LCFANMSRETLEVALKR	408
SQUASHPRO	KDQTFKAEMELWRVIINEVKLVN SPGSSFHVTEPGWFRVCFANMDDNTVDVALNR	430
TOMAS2PRO	KEATLDAEMSLWKLIINEVKLVN SPGSSFNCSEVGWFRVCFANIDDQTMEI ALAR	439
	 * * * * *	
OAS1PRO	LKDFAQ-----KKVAAKKKKMNNVY-FC-----	430
SQUASHPRO	IHSFVENIDKKEDNTVAMPSKTRRRENKLRLSFSFSGRRYDEGNVLNSPHTMSPH	485
TOMAS2PRO	IRMFMDAYNNVNKNGV-MKNKHNGR-----GTTYDLTPQMGSTMKM---	479
	 * * * *	
OAS1PRO	----- 430	
SQUASHPRO	SPLVIAKN 493	
TOMAS2PRO	--LL---A 482	

FIGURE 3

ACC SYNTHASE:

ACC OXIDASE:

FIGURE 4

FIGURE 5

FIGURE 6

MODEL FOR INTERORGAN REGULATION OF ETHYLENE
BIOSYNTHETIC GENES IN FLOWERS

ACC Content and ACC Oxidase Activity of *Phalaenopsis* Orchids

Stigma

Petals/Sepals

FIGURE 8

OA01	MESGSPVINNMELQSGSRPAAMALXRDACENWGLXHELLNHGISHELMNRVETVN	55
TOM13	MEN--FPIINLEKLNGDERATM--IKDACENWGFFELNVHGI PHEVMdTVEKMT	51
AVOE3	MDS--FPVINMEKLEGQERAATMKLINDACENWGFFELNVHISPVELMDVEERLT	53
	*.. **.*.* *.* ..** .*****. **.*** .*. *.** ..	
OA01	KEHYRRFREQRKFKEF-ASKTLDTVENVEPENLDWESTFFLRHLPTSNISQIPDL	109
TOM13	KGHYKKCMEQRFKELVASKGLEAVQA-EVTDLWESTFFLRHLPTSNISQVPDL	105
AVOE3	KEHYKKCMEQRFKELMASKVEGAV--VDANDMDWESTFFIRHLPVSNLSEIPT	106
	*.***. *****. *** ..******.*****.***.***.	
OA01	DDCRSTMKEFALELENLAERLLDLLCEDLGLEKGYLKKVF CGGS DGLPTFGTKVS	164
TOM13	EYREV MRDFAKRLEKLA EELL DLLCEN LGLEKG YLKNAFY GSKG --PNFGTKVS	158
AVOE3	DEHRKVMKEFAEKLEKLA EQVL DLLCEN LGLEKG YLKMA FAGTT TGLPTFGTKVS	161
	.. *..*..** **.*.*.*****.*****.***.***. *	
OA01	NYP PCPKPELIKGLRAHTDAGGI ILL FQDDKV SGLQL LKDGEWIDVPXL RHSIVV	219
TOM13	NYP PCPKPDLIKGLRAHDDAGGI ILL FQDDFV SGLQL LKDEQWIDVPPMRHSIVV	213
AVOE3	NYP PCPRPELFKGLRAHTDAG-LILL FQDDR VAGLQL LKDGEWDVPPMNHSIVI	215
	*****.*.*.*****.***.*****.*****.***.*****.*	
OA01	NIGDQLEVITNGKYKSVLHRVVAQT DGNRMSIASFY NPGSDA VIFPX PALVEKEA	274
TOM13	NIGDQLEVITNGKYKSVLHRVIAQT DGTRMSLASFY NPGSDA VIYPA KTLVEKEA	268
AVOE3	NIGDQVEVITNGKYKSMHRVVAQT DGNRMSLASFY NPGSDA VIFPA PALVEKEA	270
	*.***.*****.***.*****.***.*****.*****.***.*****	
OA01	EEKEEKKKEIYPKFVFDYMNLYIRKKFEAKEPRFEAMKSMEIVMSSQPIPTA	327
TOM13	EE----STQVYPKFVFDYMKLYAGLKFOAKEPRFEAMKAME----SDPIASA	313
AVOE3	EEK----KEVYPKFVFEDYMNLYAGLKFOAKEPRFEVMKMKAVETANLSPITT	319
	*******.***.* **.*.....** ..

FIGURE 11

Figure 12

Figure 13

Figure 14

Figure 15

Figure 16

Standard BARD Cover Page for Scientific Reports

DATE: 9/21/93

BARD
P.O. BOX 6
Bet Dagan, ISRAEL

BARD project No. US-1867-90R

Title:

Molecular Genetic Analysis of Pollination-induced Senescence in
Phalaenopsis orchids

Investigators' Names
(Principal listed first)

S. D. O'Neill

A. H. Halevy

A. Borochoy

Investigators' Institutions

University of California, Davis

The Hebrew University, Rehovot

The Hebrew University, Rehovot

Project's starting date: 9/1/91

Type of Report: 1st Annual 2nd Annual x Final

Sharon D O'Neill

Signature
Principal Investigator

Signature
Institution's Authorizing Official

b. DESCRIPTION OF THE RESEARCH ACTIVITIES AND RESULTS.

UNITED STATES

GENERAL APPROACH:

In the second year of the project, we have continued our efforts to elucidate the molecular basis of postpollination development in orchid flowers. In *Phalaenopsis* orchids, pollination initiates a suite of developmental changes including perianth senescence, stigma closure, and ovary and gametophyte development. Because ethylene is thought to play a key role in coordinating these developmental changes, we have continued to study the temporal and spatial patterns of expression of genes encoding 1-aminocyclopropane-1-carboxylate (ACC) synthase and ACC oxidase following pollination and treatment with pollination-associated factors.

MOLECULAR ANALYSIS OF ACC SYNTHASE AND ACC OXIDASE GENE EXPRESSION IN POLLINATED FLOWERS.

Over the past two years we have developed the physiological, anatomical and molecular tools to address the role of altered gene expression in the postpollination response of orchid flowers. Towards this goal, we carried out a detailed molecular analysis of the expression of orchid ACC synthase and ACC oxidase genes and their interorgan regulation following pollination. This research was conducted partly in year two of the granting period, and recently has resulted in two publications that are appended in Appendix I of this report (O'Neill et al., 1993; Nadeau et al., 1993). In the second year of the project, we have focused on examining the temporal and spatial patterns of expression of the ACC oxidase and ACC synthase genes, induced by pollination and associated factors. A brief summary of our results is presented below.

ACC Oxidase *In Situ* Hybridization.

In order 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 using OAO1 RNA probe. Figure 6 and 7 of the appended paper by Nadeau et al. (1993, Appendix I) demonstrate that ACC oxidase expression increases after pollination in the column, labellum and petals, as was shown previously by us through RNA blot hybridization analysis and enzyme activity studies. Moreover, *in situ* hybridization analysis demonstrates that all living cells of each organ express ACC oxidase mRNA, despite the complex nature of these tissues. In fact, the column contains cells of vascular tissues, parenchymatous cells, stigmatic cells and epidermal papillae, tissues which are derived ancestrally not only from stigmatic tissues but also from anther tissue. Petal and labellum tissues express ACC oxidase mRNA in all living cells also, including cells surrounding vascular bundles and epidermal cells. The diffuse nature of gene expression in the stigmatic tissues argues in favor of the involvement of a rapidly diffusible factor in stimulating gene expression, as would be expected for a gaseous signal such as ethylene. Because all living cells express ACC oxidase mRNA, it is likely that they are capable of converting ACC to ethylene and are involved in the biosynthesis of ethylene during postpollination events.

ACC Synthase *In Situ* Hybridization.

Because the stigma is the initial site of ACC synthase gene expression as indicated by our previous RNA blot hybridization analysis (see O'Neill et al., 1993), we were interested in determining the spatial pattern of ACC synthase transcripts in this organ using *in situ* hybridization procedure. Our results show that as with ACC oxidase, ACC synthase mRNA accumulated in all living cells of the stigma at 24 and 48 hours after pollination (data not shown). A similar localization pattern was also observed in the stigma of ethylene-treated flowers (data not shown). This result indicates that the accumulation of ACC synthase transcripts was not cell-specific at these time points, being approximately 18 hours or longer

after the initial detection by RNA blot hybridization analysis of ACC synthase mRNA in the stigma (Figure 1). Thus, it is possible that cell-specific accumulation of ACC synthase mRNA would be observed in the stigma at 6 hours or at even earlier time points following pollination. Experiments are in progress to examine this possibility.

ACC As The Primary Signal Molecule.

We have ruled out the possibility that ACC itself acts as a signal that induces ethylene biosynthesis by treating flowers with the inhibitor of ethylene action, norbornadiene (NBD) in combination with ethylene, and examining ACC synthase and ACC oxidase gene expression (O'Neill et al., 1993). Figure 2A and 2B demonstrate that control flowers do not express ACC synthase, and ACC oxidase is expressed only in the ovary, presumably in response to harvest. When ACC is applied to the stigma, it is converted to ethylene and induces senescence and gene expression that results in autocatalytic ethylene production. When flowers are treated with NBD, however, the induction of both genes that normally would be triggered by ethylene derived from the applied ACC is inhibited. This indicates that ACC alone is not sufficient to induce ethylene biosynthetic gene expression.

Heterologous Expression Studies of ACC Synthase.

One of our research goals for the second year was to express our ACC synthase and ACC oxidase cDNA clones in *E. coli* to test whether these genes encode functional polypeptides. This would be especially important for our work on ACC synthases since our OAS1 and OAS2 cDNA clones contain the smallest coding regions reported to date for plant ACC synthases. To determine if OAS1 and OAS2 (described in O'Neill et al, 1993; Bui and O'Neill, unpublished results) encode functional ACC synthase enzymes, we attempted to express the coding regions of our two orchid clones using an *E. coli* expression vector system. Protein secretion in this system is induced by IPTG. ACC synthase activity was assayed by measuring the conversion of SAM to ACC. *E. coli* cells transformed with expression plasmids harboring OAS2 (pTAS2F, pGAS2F) produced ACC (Table 1; Bui and O'Neill, unpublished results). Moreover, ACC synthase activity was recovered from protein extracts of these cultures. These results demonstrate that OAS2 encodes a functional ACC synthase protein. In contrast, neither *E. coli* cells transformed with control plasmids (pTrc99A, pGEX-3X, pTAS1R, pTAS2R, pGAS2R) nor those harboring OAS1 (pTAS1F, pGAS1F) produced ACC (Table 1). Furthermore, ACC synthase activity was not recovered from protein extracts of these cultures. Failure of OAS1 to produce an active ACC synthase when expressed in *E. coli* suggests that the cDNA does not encode a functional protein or that it is recalcitrant to functional expression in this heterologous host.

Construction of a Genomic Library and Genomic Clone Isolation.

One of the goals for the second year involved construction of an orchid genomic DNA library from which genomic clones could be isolated and sequenced. To complete this goal, a genomic DNA library was constructed in the phage cloning vector lambda-DASH (Stratagene) and packaged in Gigapack II gold packaging extract (Stratagene). The genomic library was amplified to 10^{10} phages/mL.

To obtain genomic clones containing ACC synthase, about 2×10^5 recombinant phage were screened with OAS2 cDNA probe. Following sequence analysis, a positive clone was identified as containing the genomic sequence corresponding to the ethylene-regulated ACC synthase cDNA clone, OAS2. A partial sequence of the genomic clone is shown in Figure 3. Additional sequence analysis of the promoter region is in progress to identify regulatory sequences that may contribute to the transcriptional regulation of ACC synthase genes expressed in response to pollination-induced ethylene.

ORCHID FLOWER SENESENCE:

Another specific objective of our research is to clone genes that are involved in pollination-induced orchid flower senescence. Because flower senescence involves coordinated alterations in metabolism, followed by tissue death, we chose to investigate the possible involvement of proteins such as *cx-cellulase* and ubiquitin in the flower senescence program. *Cx-cellulase* is a well-characterized ethylene-regulated gene involved in fruit ripening and organ abscission. Ubiquitin is a highly conserved protein involved in protein turnover and degradation. Our progress to date in characterizing the expression of the genes encoding both cellulase and ubiquitin in response to pollination is described below.

1. UBIQUITIN

Ubiquitin is a highly conserved protein found in animals, yeast and plants that is involved in proteolytic degradation of proteins through the ubiquitin pathway. The ubiquitin protein is a small protein comprised of 76 amino acid residues and is generally synthesized from polyubiquitin genes consisting of a varied number of head-to-tail repeats of the ubiquitin coding region. The final translation product is then cleaved into individual ubiquitin units, which are then covalently attached to proteins in a multi-step enzymatic process. Ubiquitination of proteins serves as a marker for degradation of its conjugate by specific proteases, though it may also serve as a regulatory mechanism by which the function of proteins to which it is conjugated is altered.

Cloning and Characterization of Orchid Ubiquitin cDNA.

Because senescence of the unnecessary organs of the flower following pollination or harvest for cut flowers involves the rapid degradation of proteins, we investigated the possible role of ubiquitin in this process. A 1.5 kb cDNA clone was isolated from the 24-hour postpollination perianth cDNA library. The amino acid sequence of one ubiquitin coding region unit is shown in Figure 4 (Zheng and O'Neill, unpublished results). The entire cDNA clone represents a transcript from a polyubiquitin gene containing six repeats of the ubiquitin coding sequence. A comparison of the amino acid sequence of this clone with other known ubiquitin sequences is illustrated in Figure 4.

Expression of Orchid Ubiquitin Following Pollination.

In order to assess whether ubiquitin might play a role in flower senescence, especially perianth senescence which involves rapid degradation, we examined its expression in all organs of the flower in response to pollination. RNA blot hybridization analysis of the abundance of orchid ubiquitin mRNA is shown in Figure 5. It is clear from this analysis that there are no dramatic changes in ubiquitin expression during pollination-induced senescence of the perianth, though this analysis suggests that there is a small increase in expression after pollination, which reaches a maximum at 24 hours after pollination. This increase in expression may reflect the shift in these tissues from quiescence to an active program of senescence, but does not seem adequate to propose that the ubiquitin pathway is switched on to accomplish the wholesale degradation of protein during senescence of these tissues.

This analysis also indicates that there is a slight increase in ubiquitin transcripts in both the stigma and ovary at 24 and 48 hours after pollination, but then expression appears to be lower at one and two weeks after pollination. It is possible that this increase in ubiquitin expression allows the ovary to exchange a set of proteins involved in quiescence for a set of proteins expressed during the development of the ovary after pollination by specific degradation. If this is the case, then this represents a novel function for ubiquitin in floral tissue.

Our future work will focus on confirming these results, as well as on examining the expression of ubiquitin at later timepoints during perianth senescence when it might be expected that more protein degradation will take place.

2. CELLULASE

Pollination of orchids leads to a syndrome of developmental changes, only one of which is regarded as degradative. For example, pollination in orchid flowers results in growth of the stigma and ovary. The growth of the stigma, which involves tissue swelling and closure of the stigmatic cavity around the pollen masses, is observed by 24 hours after pollination, a time prior to visible symptoms of perianth senescence. Later pollination results in pronounced degradation of the perianth, visible after 24 hours, and increasing to an obvious levels by 48 hours. These opposite developmental processes are coordinately regulated by the hormone, ethylene, as previously described (O'Neill et al., 1993). It is well known that ethylene induces a number of genes during fruit ripening and degradation. One of these ethylene-regulated genes encodes endo- β -1,4-glucanase or cx-cellulase. The substrate of this enzyme is not known with certainty but it has been proposed to be involved in cleaving xyloglucan linkages and has also been reported to be auxin-induced in growing tissues (Verma et al., 1975; Hoson and Nevins, 1989). In orchid flowers, cellulase activity could be associated with the cell wall loosening that accompanies cell expansion or in perianth degradative processes. Thus, we were interested in exploring the possibility that the induction of cellulase at the level of gene expression was also a component of the postpollination response.

Cloning and Characterization of Orchid Cellulase cDNA.

To investigate the possible involvement of cellulase at the level of gene expression in the postpollination response of orchid flowers, we screened our cDNA libraries with a degenerate oligonucleotide probe for dicot cx-cellulase. A large number of clones were obtained; however, these cDNA clones all appeared to belong to a single class of cx-cellulase. From this group of cross-hybridizing clones, we selected the longest cDNA clone of approximately 1.7 kb. This clone had been designated Orchid Cellulase (OC), and the sequence has been determined from both strands. Based on a comparison with several other plant cellulases we have concluded that our OC clone contains a nearly full length cellulase coding region. An alignment of the orchid cellulase coding region with that of bean, tomato, and avocado cellulase is presented in Figure 6. This analysis indicates that orchid cDNA lacks 90 amino acids at the amino terminus and thus is not a full-length clone for cellulase. This conclusion is supported by RNA blot hybridization analysis which indicates that the cellulase transcript is 2.3 kb (data not shown). Thus, our cellulase clone lacks about 0.6 kb of 5'-end sequence. Efforts to isolate the full-length clone are in progress.

Analysis of Cellulase Expression in Pollinated Flowers.

In order to determine the pattern of expression of the cellulase gene in orchid flowers, we examined the abundance of the cellulase transcript corresponding to OC in the various parts of the flower by RNA blot hybridization analysis (data not shown). Our initial results indicate that this gene is expressed in all organs of unpollinated flowers. Following pollination, we observed that the cellulase mRNA decreased rather than increased in abundance, especially in the perianth, except in the case of the stigma where a slight increase in abundance was observed. This decrease in cellulase transcript occurred in parallel with the rise in ethylene production and the increase in expression of the ethylene biosynthetic genes encoding ACC synthase and ACC oxidase.

Pollination-associated Factors affecting Cellulase Gene Expression.

Because we observed a decrease in cellulase mRNA levels in tissues that are producing ethylene, we decided to assess possible regulation by ethylene. Treatment of flowers with norbornadiene, an inhibitor of ethylene action, resulted in an increase in abundance of cellulase suggesting that ethylene has an inhibitory effect on the expression of the OC gene. In addition, the expression of this gene in stigma tissue may be regulated by some other factor, for example, auxin which causes an increase in mRNA abundance when applied to the stigma tissue. Thus it is possible that the orchid cellulase we have cloned and

called OC represents an auxin-stimulated cellulase. If so, this cellulase may function as a growth cellulase rather than one associated with senescence processes of degradation of tissues. Further work is in progress to address this possibility.

Genomic DNA Analysis.

Southern blot hybridization analysis was performed to determine if the OC cDNA clone corresponded to a single copy gene or was a member of a multigene family. Nuclear DNA was digested with restriction enzymes, fractionated in an agarose gel, then transferred to a nylon membrane for blot hybridization analysis. The results are shown in Figure 7, which shows a genomic DNA blot probed with the OC coding region. Under hybridization conditions of moderate stringency, the OC probe hybridized to two or three restriction fragments. However, under conditions of lower stringency, several additional DNA fragments were detected (data not shown). These results indicate that, in orchids, cellulase is encoded by at least two different genes.

Table 1. Expression of ACC Synthase in *E. coli*

Construct	Orientation	ACC synthase activity (nmol/ mg / h)		ACC content in 50 ml medium (nmol)	
		- IPTG	+ IPTG	- IPTG	+ IPTG
pTrc99a	----	0	0	0	0
pTAS1F	Correct	0	0	0	0
pTAS1R	Opposite	0	0	0	0
pTAS2F	Correct	0.83	10.4	24.8	309.3
pTAS2R	Opposite	0	0	0	0
pGEX-3X	----	0	0	0	0
pGAS1F	Correct	0	0	0	0
pGAS2F	Correct	2.72	2.14	25.7	118.6
pGAS2R	Opposite	0	0	0	0

Figure 1. Accumulation of ACC synthase mRNA in various organs of the orchid flowers at the indicated times following pollination.

The RNA blot was hybridized to ^{32}P -labelled OAS1 probe. Each lane contained 2.0 μg poly(A)⁺RNA.

A. ACC Synthase

B. ACC Oxidase

Figure 2. RNA blot hybridization illustrating the abundance of mRNAs in the stigma (S), ovary (O), petal (P) and labellum (L) tissues of *Phalaenopsis* orchid flowers 24 hours after treatment. **A)** ACC synthase gene expression. **B)** ACC oxidase gene expression. Petals and labellum, 2 μ g poly (A)⁺ RNA/lane; stigma and ovary, 1 μ g poly (A)⁺ RNA/lane.

OAS	5'-gagatcatcaaaccatgaaaatatctaactgtgctgcagctctgaaaaca	-193
OAS	gctgtcctaaacactctgcagataacactctccattaaattaaaaaaaaa	-143
OAS	gaaaattgacagaaaaaaattagaaccaatcattagcttaattaaatggt	-93
OAS	aatataattaatttcatgcaaatttatagtcccatgcatgaccagcttgg	-43
OAS	gtttggagccctataaaatagagcaatgtatggcagacaatCAATTCCTT	8
OAS2	G*****	8
OAS	GTAGCCTTCTCCTTCTTTTTTCTAAGTCTTCTCCAATTAATACTTGTCT	58
OAS2	*****	58
OAS	CAATCTAATCTCTGCTATCAGTAAGTACTAATTAAAATCATGTCTAAAT	108
OAS2	*****	108
OAS	GTTTGGGAAAGAGGTGCCATTGTCAAAAATGGCGGTGTCTAAAGCTCATG	158
OAS2	*****	158
OAS	GAGAGGGCTCTCCATACTTCGCTGGCTGGAAAGCTT-3'	194
OAS2	*****	194

Figure 3. The nucleotide alignment of a genomic DNA sequence (OAS) and a cDNA sequence (OAS2) of orchid ACC synthase.

Partial DNA sequences are shown. Upper-case letters represent the transcribed sequence. Lower-case letters represent the 5'-flanking region of the genomic sequence. The putative TATA and CAAT boxes are underlined. Negative numbers are assigned to nucleotides in the 5'-flanking region of the genomic sequence based on +1 as the transcription initiation start site. Asterisks represent identical nucleotides.


```

1
|
UBIQ$ORCH  MQIFVKTLTGKTTITLEVES$DTIDNVKAKIQDKEGIPP
UBIQ$SOYBN MQIFVKTLTGKTTITLEVES$DTIDNVKAKIQDKEGIPP
UBIQ$CHLRE MQIFVKTLTGKTTITLEVES$DTIENVKAKIQDKEGIPP
UBIQ$YEAST MQIFVKTLTGKTTITLEVES$DTIDNVKSKIQDKEGIPP
UBIQ$HUMAN MQIFVKTLTGKTTITLEVEPSDTIENVKAKIQDKEGIPP
*****

```

```

76
|
UBIQ$ORCH  DQQRLLIFAGKQLEDGRTLFDYNIQESTLHLVLRRLRGG
UBIQ$SOYBN DQQRLLIFAGKQLEDGRTLADYNIQESTLHLVLRRLRGG
UBIQ$CHLRE DQQRLLIFAGKQLEDGRTLADYNIQESTLHLVLRRLRGG
UBIQ$YEAST DQQRLLIFAGKQLEDGRTLSDYNIQESTLHLVLRRLRGG
UBIQ$HUMAN DQQRLLIFAGKQLEDGRTLSDYNIQESTLHLVLRRLRGG
*****

```

Figure 4. Comparison of the amino acid sequences of ubiquitin from *Doritaenopsis* orchid, soybean (UBIQ\$SOYBN), *chlamydomonas reinhardtii* (UBIQ\$CHLRE), yeast (UBIQ\$YEAST) and human (UBIQ\$HUMAN). (*) indicates identity, (·) indicates similarity.

Figure 5. RNA blot hybridization illustrating the abundance of ubiquitin mRNAs following pollination in the stigma, ovary and perianth of *Doritaenopsis* flowers. 30 μ g total RNA/lane.

OC	-----	0
BEANCELL	MGYHSVF--IAVFLWSSMVCHNGLAMDDGKLTSSSGPPNYDYADALAKA	48
TOMCELL	MACSKNIWVIVIFF----LCILAGPIIAQ-----DYNDSLGKA	34
AVOCELL1	MDCSSPLSLFHLL----VCTV-----MVKCCSASDLHYSDALEKS	37
OC	-----	0
BEANCELL	ILFFEGQSRSGKLPSSQRVKWREDSALSDGKIQNVNLMGGYYDAGDNVKFG	98
TOMCELL	ILFFEGQFSGKLPVSQRVKWRGDSALIDGII EHVNLIGGYYDAGDNVKFG	84
AVOCELL1	ILFFEGQREGKLPTNQRLTWRGDSGLSDGSSYHVDLVGGYYDAGDNVKFG	87
OC	-----YSAKYEAIGELAHIREIIKWGADYLLRTFNSSSD	34
BEANCELL	WPMFSTSLLSWAAVEYESEISSVNQLGYLQSAIRWGADFMLRAHTSPT-	147
TOMCELL	WPMAYSLTLLSWAAIEYQTQISSNQLVHLQNAIRWGTNPLIRAHSTST-	133
AVOCELL1	LPMFTTTMLAWGIIIEFGCLMP--EQVENARAALRWSTOYLLKASTATSN	135
OC	-----	81
BEANCELL	NIDRIAAQVGVDGTSGGPMPNDHYCWRPEDMDYPRPVFECHG---CSDL	187
TOMCELL	TL---YTQVGDGNA-----DHNCWERPEDMDTPRTVYKIDANS PGTEV	173
AVOCELL1	TL---YTQVGDGNA-----DHQCWERPEDMDTPRTLYKITSNSPGSEV	175
OC	SL---YVQVGEPNA-----DHRCWERPEDMDTPRNVYKVSTQNP GSDV	..
BEANCELL	..***..	..
TOMCELL	..***..	..
AVOCELL1	..***..	..
OC	AAEMSAALASASIVFKD-NKIYSQKLVHGAATLWKPARLQRRARYSAG-GS	129
BEANCELL	AAEYAAAALSAASIVFKKIDAKYSSSTLLSHSKSLFDPADKNRGSYSGS---	234
TOMCELL	AADVAAAFAAASIVFKNIDSNYSTKLLKRSRSLFAPADKYRGSYQAS---	220
AVOCELL1	AAETAALAAASIVFGDSDSYSTKLLHTAVKVPEFADQYRGSYSDSLGS	225
OC	..***..	..
BEANCELL	DPAKFYNS-TSYWDEFVWGGSWMYLATGNSSYLQ LATHPKMAKHAGAFWG	178
TOMCELL	--CPFYCSYSGYQDELLWAAAWLYKASGESKYL SYII---SNQ---GWS	275
AVOCELL1	--CPFYCSYSGYKDELLWAAAWLYKAGGGNNYLN YAS---INQ---GWS	261
OC	VVCPFYCSYSGYNDELLWGASWLHRASQNASYMTYIQ---SNGHTLGAD	271
BEANCELL	..***..	..
TOMCELL	..***..	..
AVOCELL1	..***..	..
OC	GPDYGVLSWDNKLPGAQVLLSRLRLFLSPGYPYEEILRTFHNQTNIVMCS	228
BEANCELL	QTVSE-FSWDNKFVGAQTLLTEE--FYGGK---KDLAKIKTDAESFICA	318
TOMCELL	QVASE-FSWDDKFAGAQTLLAKE--YLNCK---SNLEKFKKDADS FICG	304
AVOCELL1	DDDIS-FSWDDKRVGTVLLSKG--FLQDRI---EELQLYKVHTDNYICS	315
OC	..***..	..
BEANCELL	FLPVFKSFNR--TKGGLIQLNHGRPOPLQYVVNAAF LAAVYS DYLEAADT	276
TOMCELL	VMPGSNSRQIKTTPGGL--LFTRDSSNLQYTTSS THVLFIFSRILNRNHI	366
AVOCELL1	LMPSSSIQIKTTPGGL--LYYRDSSNLQYVNGATM VLFMYTKVLEAAGI	352
OC	LIPGTSSFQAQYTPGGL--LYKGSASNLQYVTSTAF LLLTYANYLNSSG-	362
BEANCELL	..***..	..
TOMCELL	..***..	..
AVOCELL1	..***..	..
OC	PGWYCGPNFFPTSVLNFAKTQIDYILGKNPEKMSY VVGFGNRFPKHVHH	426
BEANCELL	NGINCGSSHFTASQIRGFAKTQVEYILGKNPMKMS YMVGFGSKYPKQLHH	416
TOMCELL	GGVTCGSVNFSTSKIKAFKLOVDYILGNNPLKMSY MVGFGNKYPTKLHH	402
AVOCELL1	GHASCGTTTVAKNLISLAKKQVDYILGQNPAMKMSY MVGFGERYPOHVHH	412
OC	..***..	..
BEANCELL	RGASIPK---NGTKYDCKGGWK-WRDTKKPNPNTI VGAMVAGPDRHDGFH	472
TOMCELL	RGSSIPSIVKHPAKVGCNAGLSDYNSANPNPNTHV GAIVGGPIQMT-FH	465
AVOCELL1	RTSSLSPIYNHPTRVGCNDGYSSWYNSNPNPNTHV GAIVGGPNSGDQFI	452
OC	RGSSLPVQVHPNSIPCNAQFQYLYSSP-PNPNILV GAILGGPDNRDSFS	461
BEANCELL	..***..	..
TOMCELL	..***..	..
AVOCELL1	..***..	..
OC	DVRTNYNYTEPTLAGNAGLVAALIALSGEGNSVDK NTIPSAVPPMPFSPPP	422
BEANCELL	DARSDYSHAEPPTYINAA FVASISALLAKT-----	495
TOMCELL	DSRSDYSHSEPTTYMNAAFIGSVAALIDQTKEGEH---YGEINSQFNKTG	499
AVOCELL1	DDRNNYQQSEPATYINAPLVGALAFFAANPVTE-----	494
OC	..***..	..
BEANCELL	PPPAFWKP	430
TOMCELL	-----	495
AVOCELL1	FM-----	501
OC	-----	494

Figure 6. Comparison of cellulase amino acid sequences.

Cellulase sequences are from orchid (OC), bean (BEANCELL), avocado (AVOCELL1), and tomato (TOMCELL). Asterisks represent conserved residues whereas dots represent conservative substitutions. Gaps were introduced to maximum matching.

Figure 7. Genomic Southern blot hybridization analysis.

Orchid genomic DNA was digested with *Bam* HI, *Eco* RI, *Hind* III, and *Pst* I. Each lane contained 10 µg of DNA. The blot was hybridized to a ³²P-labelled OC probe.

ISRAEL

GENERAL APPROACH:

Research in the second year has focused on further evaluation of the regulation of ethylene sensitivity in promoting postpollination developmental responses. The findings related to regulation of ethylene sensitivity complement the USA research on regulation of ethylene biosynthesis in pollinated flowers.

THE ROLE OF ETHYLENE SENSITIVITY IN FLOWER LONGEVITY

Petunia lines differing in longevity were analyzed to determine whether altered ethylene production or sensitivity was responsible for delayed senescence in the long-lived genotype. Although ethylene production was delayed in the long-lived genotype as compared to the short-lived genotype, experiments to test the sensitivity of each genotype to ethylene indicated that the short-lived genotype responded much more rapidly to exogenous ethylene. These data suggest that the differences in flower longevity result from altered ethylene sensitivity, indicating that this ethylene sensitivity is genetically determined. This data is accepted for publication in *Physiologia Plantarum* (Appendix I).

ROLE OF ENDOGENOUS SIGNAL MOLECULES ON FLOWER SENESCENCE

Endogenous signal molecules and hormones, such as auxin, are well known to regulate flower senescence. Because flower senescence is associated with membrane degradation, potential products of lipid degradation and oxidation were examined as possible regulators of the senescence program. Jasmonic acid is known plant signal molecule that is derived from linoleic acid that can be released from membrane lipids by phospholipase A.

Methyl jasmonate treatment of flowers promoted flower senescence and ethylene production (Table 1, p. 13 of Porat, Borochoy, Halevy manuscript, Appendix I). Treatment of the flowers with AOA prior to methyl jasmonate treatment completely prevented methyl jasmonate-stimulated flower senescence (Table 3, p. 15 of Porat, Borochoy, Halevy manuscript, Appendix I). This result indicates that methyl jasmonate does not directly promote flower senescence but acts by promoting ethylene biosynthesis. These data provide clear evidence for regulation of flower senescence by endogenous signal molecules and support the possibility that methyl jasmonate may provide a chemical signal that links membrane degradation and flower senescence.

c. EVALUATION OF RESEARCH PROGRESS.

In the past year, the majority of the specific research goals proposed in the first progress report have been completed. In addition, other research on the interorgan regulation of the ethylene biosynthetic genes, and the signals involved in their expression, has also been underway. Progress in these areas will be presented in the final report. In general, we have accomplished a great deal and have made major contributions to understanding the molecular basis of postpollination flower development and perianth senescence.

d. DESCRIPTION OF THE COOPERATION.

During the past year, the U. S. and Israeli teams have been in communication about their respective laboratory results. Both labs have been successful in completing the objectives of the proposed cooperative research, and both groups have produced a number of publications and manuscripts. No visits were made during the second year of the project.

e. CONCLUSIONS AND PLANS FOR NEXT YEAR.

In the final year of the project, the work described in this report will be completed for publication. Specific objectives for the final year will include:

1. Characterize cell-specific expression of ACC synthase and ACC oxidase at early time points after pollination to detect the most initial response to pollination and associated factors such as auxin. To achieve this objective, we will perform *in situ* hybridization analysis of the stigma tissue of pollinated, AVG-treated, and AVG/pollinated orchid flowers. The time points after pollination will be 1-6 hours at 1 hour intervals, 12 hours, and 24 hours. Inserts from OAS2 and an auxin-regulated ACC synthase cDNA clone will be used to synthesize RNA probes.
2. Determine the role of auxin as a primary signal of pollination. This and the following three objectives will complement our work on ethylene regulation of ACC synthase and ACC oxidase gene expression in flowers.
3. Clone and characterize an auxin-regulated ACC synthase gene induced in the stigma by pollen-borne auxin. This may require construction of a new cDNA library to an earlier timepoint than we currently have. We plan to examine the pattern of expression of the auxin-regulated ACC synthase in floral parts of pollinated, auxin-treated, auxin- and ethylene-treated flowers in the absence and presence of inhibitors of ethylene biosynthesis (AVG) and action (NBD or DACP).
4. Complete an analysis of the response of the ethylene biosynthetic genes to emasculation, a component of the pollination of orchids in nature.
5. Determine the fate and translocation within the orchid flower of radiolabelled ^{14}C -ACC applied to the stigma.
6. Isolate full-length α -cellulase cDNA and complete sequence analysis. Characterize the expression of α -cellulase in relation to pollination-induced phenomena including stigma and ovary growth and perianth senescence.
7. Complete our analysis of ubiquitin gene expression during postpollination flower development.

In addition to these specific objectives, Dr. O'Neill of the United States team will visit the laboratories of Professors Halevy and Borochoy of the Israeli team in the spring of 1994.

f. LIST OF PUBLICATIONS

UNITED STATES

1. O'Neill, S.D. 1992. Interorgan regulation of postpollination development in orchid flowers. *In: Proceedings of the Nagoya International Orchid Congress*. Nagoya, Japan. pp. 67-74.
2. Nadeau, J.A., Bui, A.Q., Zhang, X., and O'Neill, S.D. 1993. Interorgan regulation of post-pollination events in orchid flowers. *In: Cellular and Molecular Aspects of the Plant Hormone Ethylene*. J. C. Pech, A. Latche, and C. Balague, Eds.. Kluwer Academic Publishers. Dordrecht, The Netherlands. pp. 304-309.
3. O'Neill, S.D. 1993. Changes in gene expression associated with floral induction and evocation. Chapter 4 in: *The Molecular Biology of Flowering*. B.R. Jordan (ed.). C.A.B. International of Wallingford, Oxon, United Kingdom. pp. 69-92.
4. O'Neill, S.D., Nadeau, J.A., Zhang, X.S., Bui, A.Q., and Halevy, A.H. 1993. Interorgan regulation of ethylene biosynthetic genes by pollination. *The Plant Cell* 5:419-432.
5. Zhang, X.S., and O'Neill, S.D. 1993. Ovary and gametophyte development are coordinately regulated by auxin and ethylene following pollination. *The Plant Cell* 5:403-418.
6. O'Neill, S.D., Nadeau, J.A., Bui, A.Q., Zhang, X.S., Li, J., Van Tassel, D. and Zheng, C.C. 1993. Photoperiodic floral induction and postpollination flower development. *Flowering Newsletter* 15:22-29.
7. Nadeau, J.A., Zhang, X.S., Nair, H., and O'Neill, S.D. 1993. Temporal and spatial regulation of ACC oxidase in the pollination-induced senescence of orchid flowers. *Plant Physiology* (in press for November, 1993).

ISRAEL

1. Porat, R., Reuveny, Y., Borochoy, A., and Halevy, A.H. 1993. Petunia flower longevity: the role of sensitivity to ethylene. *Physiologia Plantarum* (in press).
2. Porat, R., Borochoy, A., and Halevy, A.H. 1993. Enhancement of petunia and dendrobium flower senescence by jasmonic acid methyl ester is via the promotion of ethylene production. (Accepted for publication).