The Plant Cell, Vol. 11, 1061–1071, June 1999, www.plantcell.org

Silencing Gene Expression of the Ethylene-Forming Enzyme Results in a Reversible Inhibition of Ovule Development in Transgenic Tobacco Plants

Domenico De Martinis[1] and Celestina Mariani[2]

Department of Experimental Botany, Graduate School Experimental Plant Sciences, Catholic University of Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands

To study the role of ethylene in plant reproduction, we constructed transgenic tobacco plants in which the expression of a pistil-specific gene coding for the ethylene-forming enzyme 1-aminocyclopropane-1-carboxylate oxidase was inhibited. Flowers from transgenic plants showed female sterility due to an arrest in ovule development. Megasporogenesis did not occur, and ovules did not reach maturity. When pollinated, pollen tubes were able to reach the ovary but did not penetrate into the immature ovule in transgenic plants. Flower treatment with an ethylene source resulted in a functional recovery of ovule development and restored guidance of the pollen tube tip into the ovule micropyle that resulted in seed set. The recovery was abolished if inhibitors of ethylene action were present. These results demonstrate that the plant hormone ethylene is required during the very early stages of female sporogenesis and ultimately to enable fertilization.

INTRODUCTION

Many physiological and developmental processes in higher plants, including ripening of fruits, abscission, senescence, and response to wounding, are controlled by the plant hormone ethylene (Abeles et al., 1992; Theologis, 1992). Although the role of this hormone in plant reproduction has been studied in a number of flower types with regard to pollen tube–style interactions (Wang et al., 1996), pollination-induced flower senescence (Tang et al., 1994), and fruit ripening (Lincoln et al., 1987; Hamilton et al., 1990), little is known about the role of ethylene in early pistil development. Tova et al. (1997) reported the isolation of an auxin-inducible gene encoding 1-aminocyclopropane-1-carboxylic acid (ACC) synthase that is tightly associated with the *F* locus, which determines female sex expression in cucumber, thereby supporting the hypothesis that ethylene plays a pivotal role in the determination of sex in cucumber flowers. In monocots, namely, orchid, Zhang and O'Neill (1993) have shown that pollination and auxin regulate ethylene production and ovary development. When inhibitors of ethylene were used, pollination- or auxin-induced ovary development was inhibited. More recently, Bui and O'Neill (1998) hypothesized that an unknown pollination factor and auxin have a synergistic effect in the stimulation of ethylene biosynthesis and, consequently, ovary development in orchid.

In petunia flowers, the expression of the gene family encoding ACC oxidase (ACO), the enzyme involved in the last step of ethylene biosynthesis, is temporally regulated during pistil development (Tang et al., 1994). These authors suggested that ethylene plays a role in reproductive physiology by regulating the maturation of the secretory tissues of the pistil. In Arabidopsis, the *ETR2* gene encoding an ethylene receptor was recently cloned (Hua et al., 1998; Sakai et al., 1998). Because its expression pattern was found to be ubiquitous, but definitely higher in the inflorescence, floral meristems, and developing petals and ovules, the authors suggested a possible tissue-specific role for *ETR2.* However, to date, a clear demonstration that ethylene controls ovule development has not been provided.

We recently isolated and characterized a tobacco pistil-specific cDNA encoding ACO. To understand the role of *ACO* gene expression during pistil development, we used a transgenic gene silencing approach that has already been proven to be effective in elucidating the role of ethylene production in fruit ripening in tomato (Hamilton et al., 1990; Oeller et al., 1991).

Here, we report the characterization of *ACO* gene expression in the tobacco ovary during development, and we describe the ovule morphology of transgenic plants in which *ACO* mRNA accumulation was greatly reduced. We show that *ACO* downregulation influences ovule development and

[1] Current address: Ente per le Nuove Tecnologie, l'Energia e l'Ambiente, C.R. Casaccia, Divisione Biotecnologie ed Agricoltura, CP 2400, Rome, Italy.

[2] To whom correspondence should be addressed. E-mail mariani@sci.kun.nl; fax 31-24-3652490.

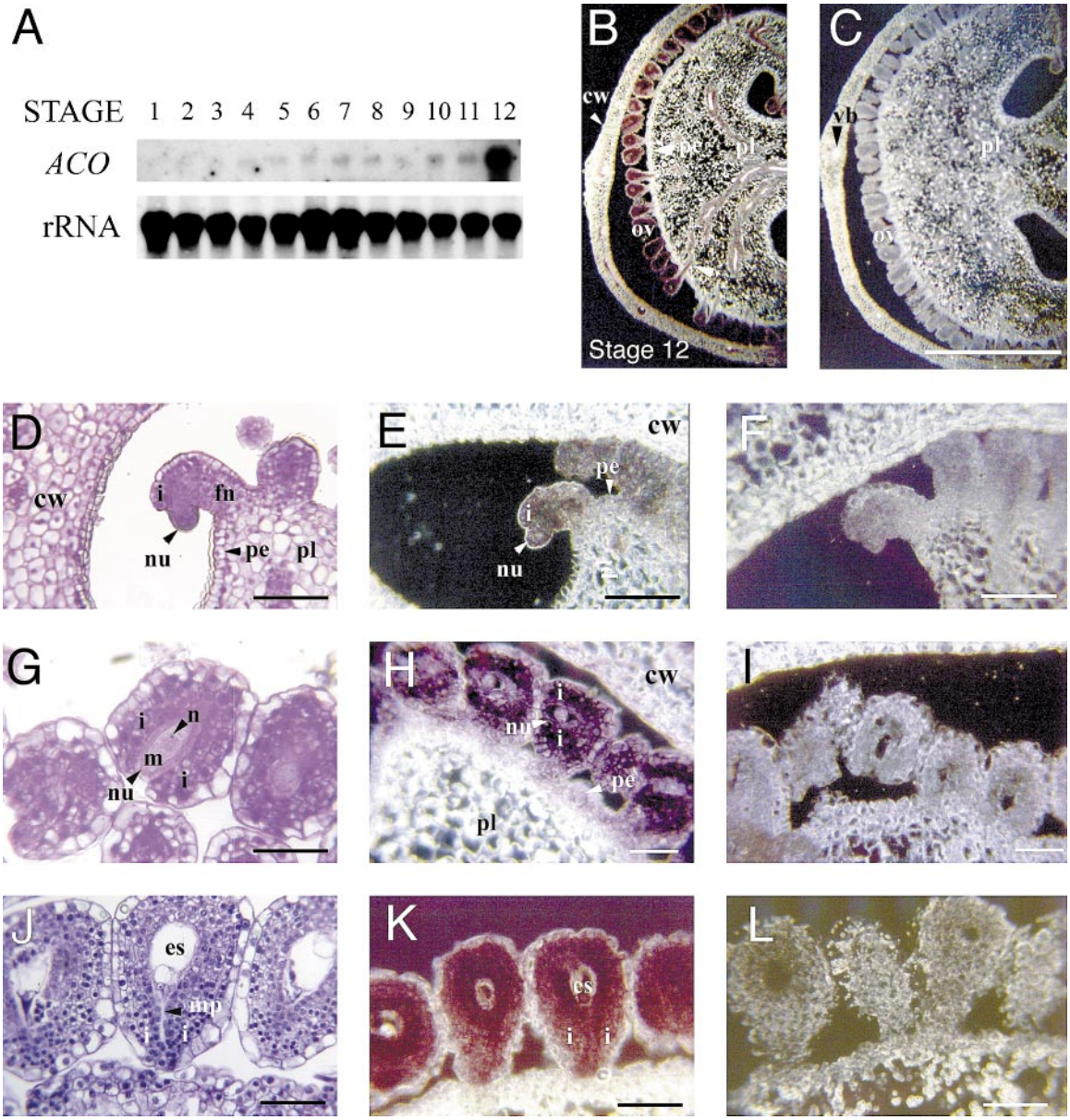

Figure 1. Temporal and Spatial Characterization of *ACO* Gene Expression in the Ovary during Tobacco Flower Development.

We monitored *ACO* gene expression in the ovary during the 12 stages of tobacco flower development (Goldberg, 1988; Koltunow et al., 1990). For in situ hybridization, the ovaries from tobacco flowers at different developmental stages were used. Hybridization was visualized as a red/purple color after development and was photographed by using dark-field microscopy. For cytological analysis of ovule morphogenesis, the same ovary samples were stained with toluidine blue and photographed using bright-field microscopy.

(A) RNA gel blots were hybridized with labeled full-length *ACO* cDNA. The filters were stripped and rehybridized with labeled tobacco ribosomal cDNA (rRNA). Ten micrograms of total RNA from ovaries of flowers at different stages (1 to 12) of flower development was loaded in each lane.

(B) Cross-section of ovary from wild-type tobacco flower at anthesis (stage 12) hybridized with the antisense *ACO* probe. Hybridization is visible as a dark-red color on the ovules.

(C) Same stage as shown in **(B)** hybridized with the sense *ACO* probe.

(D) Developing ovule at flower stages 1 to 4. The single integument is still a primordium and does not envelop the nucellus.

(E) In situ hybridization with the antisense *ACO* probe of a developing ovule at the same stage as shown in **(D)**.

(F) Same stage as shown in **(E)** hybridized with the sense *ACO* probe.

(G) Ovule at the beginning of megasporogenesis (flower stages 4 and 5). The nucellus harbors a mononucleate megasporocyte and becomes surrounded by the single integument. Within the megasporocyte, the nucleus and the nucleolus are visible.

the process of fertilization. The phenotype obtained is reversible if an ethylene source becomes available to the flower, thereby demonstrating direct involvement of the hormone in ovule development.

RESULTS

ACO Gene Expression during Ovule Development in Tobacco

We previously have characterized the spatial and temporal expression of the *ACO* gene during pistil development and pollination in tobacco (K. Weterings, M. Pezzotti, M. Cornelissen, and C. Mariani, unpublished data). The *ACO* gene is mainly expressed in the pistil—in the stigma, in the transmitting tract of the style, and in the ovary—and expression is not detectable in the pollen or in the anther. Figure 1A shows *ACO* gene expression in the ovary during flower development. Expression is first detectable in the ovaries of young flower buds at stages 4 or 5 (Goldberg, 1988; Koltunow et al., 1990). In situ hybridization in wild-type tobacco shows that *ACO* transcript accumulation within the ovary occurs preferentially in the ovules (Figures 1B and 1C). During flower development, the ovules originate from the placenta as fingerlike structures (Figure 1D) consisting of the funiculus, which attaches the ovule to the placenta, the integument primordia, and the nucellus, which harbors the megasporocyte. The single integument gradually surrounds the nucellus that contains the mononucleate megasporocyte (Figure 1G). During these early developmental stages, *ACO* expression occurs preferentially on the funiculus, on the integument primordia, and on the nucellus (Figures 1E, 1F, 1H and 1I). During megasporogenesis, the integument grows to define the micropyle; the nucellus is clearly visible and will disappear when the functional megaspore is formed.

Later in ovule development, the functional megaspore divides to form the embryo sac (megagametogenesis); before pollination occurs, all of the ovules will have reached maturity. Figure 1J shows a detailed view of ovules toward the end of megagametogenesis (flower stage 12) with a formed embryo sac and an integument that defines the micropyle. At this stage, *ACO* expression is clearly restricted to the ovule integument and embryo sac and is no longer detectable on any other tissue of the ovary (Figures 1B, 1K, and 1L). After pollination, the ovule undergoes further developmental changes related to calcium distribution in the synergids, in the egg cell, in the embryo sac, and in the micropylar canal (Tian and Russel, 1997). Table 1 summarizes the various developmental stages of the tobacco ovule based on morphological criteria. Taken together, our results demonstrate that

Table 1. Ovule Development in Wild-Type Tobacco[a]

Flower Stage[b]	Description	Bud Length (mm)
1 to 3	Megasporocyte and nucellus are formed and become surrounded by a single integument.	8 to 14
4 to 5	Beginning of megasporogenesis (meiosis I).	16 to 20
6 to 7	During second round of meiosis, large callose accumulations are visible around the nucellus. The integument grows to completely surround the nucellus.	22 to 28
8 to 9	At the end of megasporogenesis, the degenerated megaspores are surrounded by callose accumulations, and the functional megaspore is formed.	39 to 43
10	All of the ovules contain the functional megaspore that will produce the embryo sac. Megagametogenesis begins.	45
11 to 12	Four/eight-nucleate embryo sac.	46

[a] The tobacco ovary contains ~2000 ovules. Ovule differentiation is not synchronized and follows the Polygonum-type model.
[b] Flower stages and bud length as defined by Koltunow et al. (1990).

Figure 1. (continued).

(H) In situ hybridization with the antisense *ACO* probe of a developing ovule at the same stage as shown in **(G)**.
(I) Same stage as shown in **(H)** hybridized with the sense *ACO* probe.
(J) Ovules at flower stages 10 to 12. All the ovules are at the end of megagametogenesis, the integument clearly defines the micropyle, the nucellus is no longer present, and the embryo sac is formed.
(K) In situ hybridization with the antisense *ACO* probe of a developing ovule at the same stage as shown in **(J)**.
(L) Same stage as shown in **(K)** hybridized with the sense *ACO* probe.
cw, carpel wall; es, embryo sac; fn, funiculus; i, integument; m, megasporocyte; mp, micropyle; n, nucleolus; nu, nucellus; ov, ovules; pe, placenta epidermis; pl, placenta; vb, vascular bundle. Bar in **(C)** = 1 mm for **(B)** and **(C)**; bars in **(D)** to **(L)** = 100 μm.

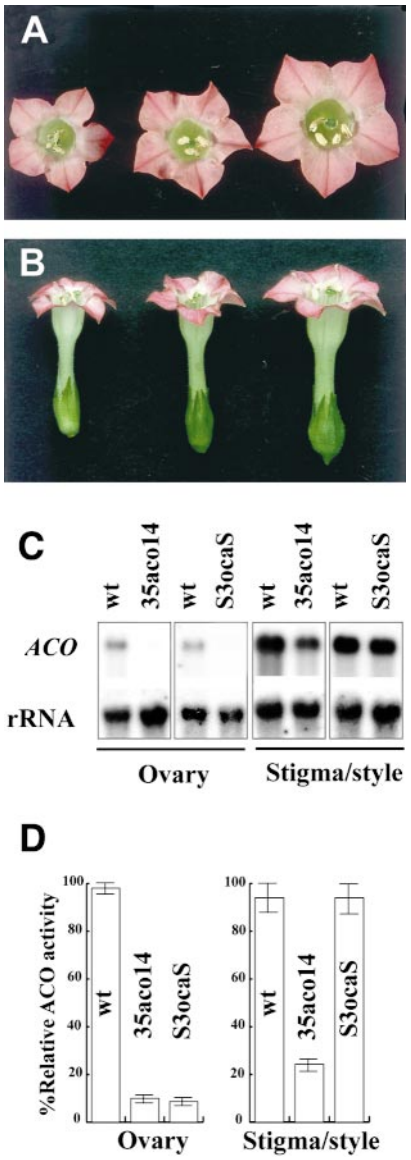

Figure 2. Flower Morphology, *ACO* Gene Expression, and ACO Activity in Wild-Type and Transgenic Tobacco Plants at Anthesis.

(A) and **(B)** The wild-type flower (right), the flower of the antisense transformant S3ocaS (center), and the flower of the cosuppression transformant 35aco14 (left).

(C) RNA gel blot analysis of the transformants. RNA was isolated from dissected ovaries and stigma/style portions of wild-type, 35aco14, and S3ocaS pistils at anthesis. Ten micrograms of total ovary RNA and 5 μg of total stigma/style RNA were loaded per lane. The RNA gel blots were hybridized with labeled full-length *ACO* cDNA. The filters were stripped and rehybridized with labeled tobacco ribosomal cDNA (rRNA) to ensure equal loading and transfer of RNA from each tissue.

(D) ACO activity assay. ACO activity was assayed by measuring the conversion of exogenous ACC to ethylene, as described in Methods. Error bars indicate ±SE.

wt, wild type.

ACO gene expression in the tobacco ovary is present during ovule development from the very early stages until flower anthesis, when the ovules are ready to be fertilized.

ACO Gene Silencing in Transgenic Tobacco

Because the product of the *ACO* gene is the last enzyme in the biosynthetic pathway of ethylene, *ACO* expression in plant cells suggests the production of ethylene. To understand the possible role of ethylene in ovule development, we produced transgenic tobacco plants in which *ACO* gene expression was silenced by two different approaches. In one approach, we constructed a chimeric antisense gene consisting of the 690-bp 3′ end fragment of the *ACO* cDNA, cloned in reverse orientation under control of the pistil-specific *Petunia inflata* promoter S3 (Lee et al., 1994) into plant expression vector pBIN19 (Bevan, 1984). Transgenic tobacco plants were regenerated after Agrobacterium-mediated transformation, essentially as described previously (Tavazza et al., 1988). We also generated transgenic plants expressing the *ACO* full-length cDNA in the sense orientation, under control of the 35S cauliflower mosaic virus promoter, to inhibit the gene by cosuppression (Meyer and Saedler, 1996). Both promoters confer pistil expression of the gene encoding β-glucuronidase in transgenic tobacco plants (D. De Martinis and C. Mariani, unpublished observation).

Transgenic plants were selected on the basis of kanamycin resistance, transferred into the greenhouse, and analyzed for flower phenotype. We selected the transgenic plants at the flowering stage on the basis of lack of seed setting and flower morphology (Figures 2A and 2B, small flowers). We selected five of 40 independent regenerants from transformations with the antisense construct and six of 37 independent regenerants from transformations with the sense construct. Apart from the flower phenotype and the failure to produce fruit capsules and seeds, these plants were not different from wild-type plants. DNA gel blot analysis confirmed the presence of the heterologous gene constructs in the selected lines (data not shown). When transgenic flowers were either self-pollinated or pollinated with wild-type pollen, they failed to produce seeds and abscissed exactly as did unpollinated wild-type flowers. We could produce heterozygous T_1 generations by backcrossing wild-type flowers with transgenic pollen. The progenies resulting from these crosses segregated for the same floral phenotype as was observed in the primary transformants (see Methods). Because the transgenic pollen was capable of fertilizing and transmitting the transgene, our results indicate that the lack of seed set in transgenic plants was not caused by male sterility but had to be related to a pistil defect. Because there were no variations in flower phenotype between transgenic plants harboring the two different chimeric genes, we chose one plant line from each set of T_1 generations for further analysis. We designate as line S3ocaS the plants carrying the *ACO* gene in the antisense orienta-

tion and as line 35aco14 the plants carrying the cosuppression construct.

RNA gel blot analysis of the transgenic plants revealed the level of *ACO* transcript accumulation in the wild-type and transgenic tobacco (Figure 2C). In the ovaries of the transformants S3ocaS and 35aco14, the presence of the *ACO* mRNA was no longer detectable. Interestingly, we could not obtain *ACO* downregulation in the stigma and style of the plants harboring the antisense construct, whereas we obtained a clear reduction using the cosuppression approach. Similar results were obtained when we tested for ACO enzymatic activity in the ovary of the transgenic plants. Figure 2D shows that the decrease in *ACO* mRNA level in the ovary corresponds to a proportional decrease in ACO enzymatic activity.

Ovules in Transgenic Plants Fail to Guide Pollen Tubes

To understand the causes of the sterility that we observed in the transgenic plants, we monitored wild-type pollen tube growth into the pistils of their T_1 progeny. In wild-type plants, the pollen tubes emerging from the transmitting tract into the ovary diverge 90° to penetrate the micropyle of a selected ovule (Figure 3A). In the transgenic plants, pollen tubes germinated and penetrated all of the tissues of the pistil normally, just as in wild-type flowers. However, once in the ovary, these pollen tubes did not turn toward micropyles (Figures 3B and 3C) but kept growing along the placenta. Similar behavior was observed in pollinated wild-type flowers at stage 6, when the pistil is already receptive and able to sustain pollen tube growth but the ovules are still immature (D. De Martinis and C. Mariani, unpublished observation). Furthermore, many ovules in the transgenic flowers were found to have a large accumulation of callose (Figure 3D), which is usually considered to be a marker of the early stages of ovule development (Bouman, 1984; Angenent and Colombo, 1996) before the embryo sac is formed. Hülskamp et al. (1995) clearly demonstrated that proper embryo sac formation in Arabidopsis is a requisite for pollen tube penetration to proceed correctly into the ovule. Therefore, the failure of the pollen tube to penetrate the micropylar structures in the transgenic plants suggests the absence of the appropriate guiding system, which may result from an inappropriate developmental process.

Megasporogenesis Is Arrested in Ovules of Sterile Plants

Cytological analysis revealed that the ovules of the female-sterile transgenic plants were arrested in development. Figure 4 shows that at flower anthesis (stage 12), the morphology of the ovules in plants of both the 35aco14 and S3ocaS lines corresponded to that of the wild-type ovule at the megasporocyte stage (stages 4 and 5 in wild-type flowers; cf. Figures 1D and 1G). Furthermore, differential interference contrast microscopy (Schneitz et al., 1995) revealed that ovules from S3ocaS lines could also produce binucleate megasporocytes (data not shown), indicating a different degree of developmental arrest if compared with the 35aco14 lines. In any case, at anthesis, the ovules of all transgenic plants of lines S3ocaS and 35aco14 always showed the main traits of early ovule morphogenesis: presence of the nucellus

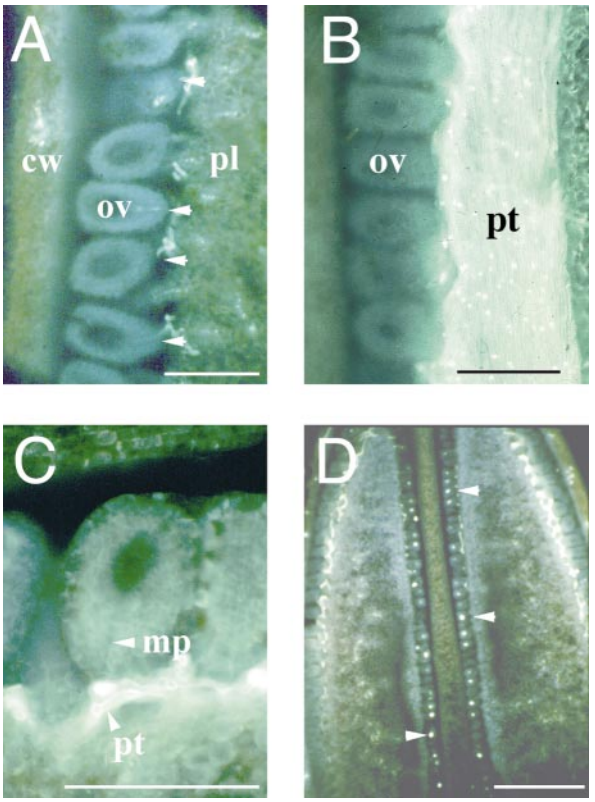

Figure 3. Pollen Tube Behavior in Wild-Type and Transgenic Ovaries.

Ovaries from pollinated pistils were sectioned, stained with aniline blue, and analyzed using fluorescence microscopy. The paths of the pollen tubes are detectable as a yellow/green fluorescence. In the transgenic plants, the pollen tubes grow normally into the ovaries but do not change their growth direction toward the micropyle, as they do in the wild-type ovaries.

(A) Pollen tubes penetrate the micropyle of wild-type ovules and reach the embryo sac. The arrowheads indicate the fertilized ovules.

(B) 35aco14 ovary. A large number of pollen tubes penetrate the ovary; no penetration of the micropyle is visible.

(C) Ovule from a 35aco14 plant. The arrowheads indicate the empty micropyle and a pollen tube.

(D) An overview of a pollinated 35aco14 ovary. Ovules with large callose accumulation are clearly visible (arrowheads). The S3ocaS plants show the same callose accumulation as well as the same pollen tube behavior in the ovary as 35aco14 plants.

cw, carpel wall; mp, micropyle; ov, ovule; pl, placenta; pt, pollen tubes. Bar in **(A)** = 300 μm; bars in **(B)** and **(C)** = 200 μm; bar in **(D)** = 750 μm.

(Figures 4A and 4B), callose accumulation within the nucellus (Figure 3D), and absence of the embryo sac. The arrest in development was also clearly visualized by using scanning electron microscopy (Figures 4C to 4F). Ovules from transgenic plants at anthesis often had a short integument and a naked nucellus (Figures 4C to 4E), a situation comparable to ovules from young wild-type flower buds (Figure 4F).

These results clearly show that *ACO* downregulation in the tobacco ovary inhibited integument growth and megasporogenesis, suggesting that ethylene controls ovule development.

Ethylene Restores Ovule Development in the Transformed Plants

To demonstrate the direct involvement of ethylene in regulating ovule development, we provided an ethylene source to the transgenic flowers. However, certain factors had to be taken into consideration. The carpel wall may act as a barrier for the delivery of ethylene specifically to the ovule tissues. Furthermore, the application of ethylene in the form of an exogenous gas to the flower is known to cause cell deterioration in the transmitting tissue (Wang et al., 1996). To overcome these problems and to deliver ethylene directly to the ovary, we used ethephon (2-chloroethylphosphonic acid), the hydrolytic breakdown of which leads to evolution of ethylene (Yang, 1969). Although it has been shown that some ethephon effects are not due to ethylene release (Lawton et al., 1994), ethephon in general closely mimics the ethylene-related physiological changes and induces gene expression in different plants, including Arabidopsis (Lawton et al., 1994) and tobacco (Ohme-Takagi and Shinshi, 1995; Wang et al., 1996). Thus, we treated transgenic flowers either with ethephon or with silver thiosulfate (STS), an inhibitor of ethylene action, to study their effect on ovule development and pollen tube guidance.

Figure 5A shows that ovules recovered their functionality after ethephon treatment and "attracted" the pollen tubes to the micropyle. Moreover, ethephon-treated flowers were able to produce fully developed ovules with a normal embryo sac (Figure 5B). These results demonstrate that the action of ethylene is necessary to tobacco plants to produce mature and functional ovules. In contrast, if the transgenic flowers at anthesis were treated with a combination of ethephon and STS (Figure 5C), ovules remained immature and showed callose accumulation, and the pollen tubes failed to target the micropyle, which is the situation in transgenic untreated flowers (Figure 3). Furthermore, Figure 5D shows clearly that these ovules did not complete megasporogenesis and still had a short integument and a pronounced nucellus. Together, these experiments enabled us to discriminate between the effect of pollination and that of ethylene, because when ethylene action was inhibited by the application of STS, pollination alone did not induce ovule development (Figures 5C and 5D).

To quantify the percentage of transgenic ovules that could complete development after ethephon treatment, we allowed pollinated ethephon-treated flowers to set seeds. The results, shown in Figure 5E, indicate clearly that only when an ethylene source becomes available to the flower can the fertility be restored. To confirm that the effects of ethephon we observed were due to ethylene release and not to hydrochloric and phosphonic acids that coevolved with ethylene after ethephon treatment, we also treated transgenic flowers with an equimolar solution of hydrochloric acid in phosphate buffer as a control for every experiment with ethephon and did not observe any improvement in fertility compared with water-treated or untreated flowers.

These results indicate that the induction of fertility in transgenic flowers is due to ethylene release and not to the effects of the acids. However, from our observations, we could not discriminate whether ethylene directly influenced pollen tube guidance into the micropyle or whether it controlled ovule development, which consequently determined pollen tube attraction into the micropyle. To further investigate this aspect, we treated wild-type flowers at anthesis (with fully developed embryo sacs) with STS to inhibit the ethylene produced endogenously by the flowers and then pollinated them. Although ethylene action was inhibited, the pollen tube could target and enter the micropyle, and the treatment did not influence seed setting in wild-type flowers (D. De Martinis and C. Mariani, unpublished data). Furthermore, ethylene does not seem to promote the reorientation of pollen tube growth in vitro (R. Malhó, personal communication), thus excluding a direct involvement of ethylene in pollen tube guidance. Taken together, all our results consistently demonstrated that ethylene controls ovule development and that only mature ovules are competent to attract pollen tubes.

DISCUSSION

The plant hormone ethylene induces a number of responses in plants (Abeles et al., 1992). With the exception of orchid, little is known about the role of ethylene in ovule development (Zhang and O'Neill, 1993). We have analyzed the role of this hormone during the reproductive processes of tobacco. Unlike in orchid, in tobacco, megasporogenesis has already been completed before pollination occurs. At anthesis, the ovules are in the terminal phases of megagametogenesis. The embryo sac is present, and cellularization and early differentiation of the egg cell and the synergids have occurred (Tian and Russel, 1997). In young flower buds (stage 6), pollination does not induce embryo sac formation and seed production. Thus, in this system we can exclude the presence of a pollination-induced signal that initiates maturation of the ovule.

We isolated a clone from a tobacco pistil cDNA library that corresponds to the mRNA encoding the ethylene-forming

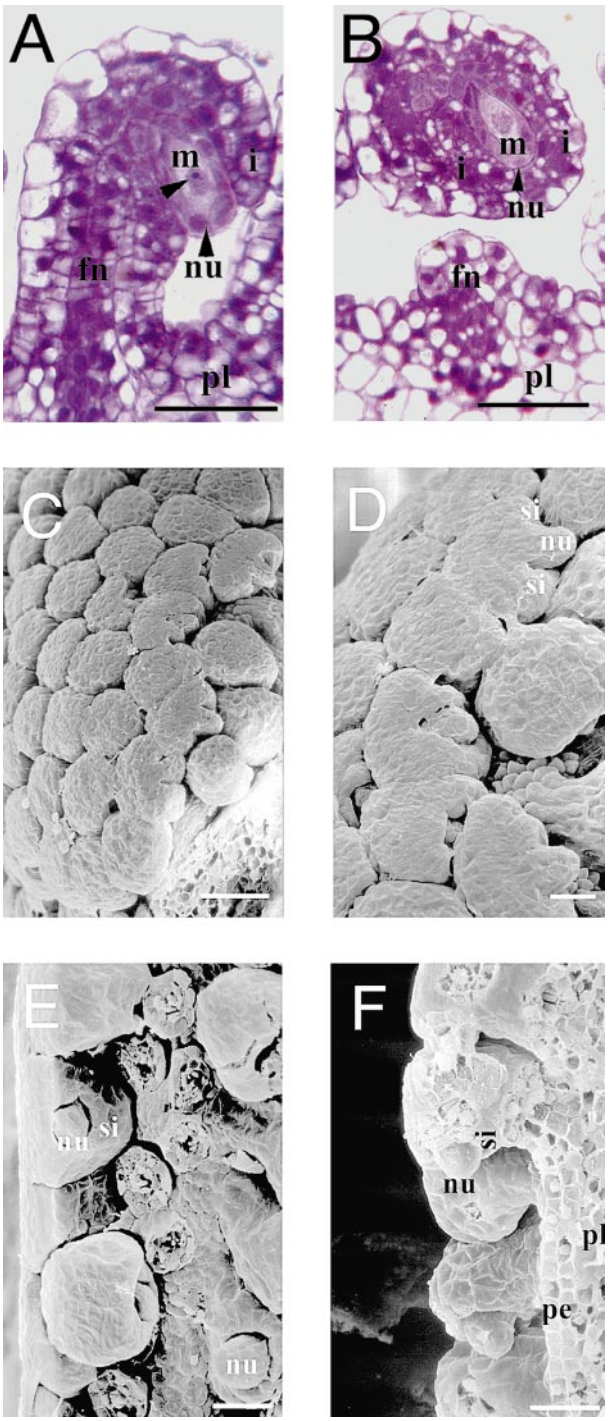

Figure 4. Microscopic Analysis of the Most Observed Ovule Phenotypes from Transgenic Flowers at Anthesis.

Ovaries from flowers at anthesis (stage 12) were stained with toluidine blue and photographed using bright-field microscopy or coated with gold for observation with a scanning electron microscope.

(A) Ovules from flowers at anthesis (stage 12) of 35aco14 transgenic plants show a short integument and naked nucellus that surrounds the densely stained megasporocyte as in ovules from wild-type flowers at stages 1 to 4 (Figures 1D and 1G). Within the megasporocyte, the nucleus and the nucleolus are visible.

(B) A transgenic ovule from flowers at anthesis of S3ocaS plants is completely surrounded by the integument. The nucellus is still present, and the nucleolus within the nucleus is not visible, suggesting that the nucleus is undergoing meiosis I (prophase).

(C) to **(E)** Scanning electron microscopy of the most observed phenotypes from transgenic plants with flowers at anthesis. **(C)** and **(D)** show ovules from 35aco14 transgenic plants. **(E)** shows ovules from S3ocaS flowers at anthesis. Several ovules have a naked nucellus and short integument.

(F) Scanning electron microscopy of the ovules from a wild-type flower at stage 3.

fn, funiculus; i, integument; m, megasporocyte; nu, nucellus; pe, placenta epidermis; pl, placenta; si, short integument. Bars in **(A)** to **(F)** = 50 μm.

enzyme ACO. ACO is encoded by a gene family whose pattern of expression has also been carefully described in petunia (Tang et al., 1994), in which ovule development (Angenent et al., 1995) is similar to that in tobacco. It is noteworthy that in petunia, *ACO* expression in developing pistils is largely confined to the secretory tissue and is not detectable in the ovules at anthesis, although it is induced here after treating the flower with ethylene. Conversely, in tobacco, *ACO* gene expression was detectable in the ovary in the period during which the first events of megasporogenesis occur. *ACO* expression was first detectable in the placenta epidermis and in the ovule, becoming ovule specific at later stages of development. These results indicate that similar or identical genes can be differently regulated even in closely related species, such as petunia and tobacco. However, because the pattern of expression of *ACO* in petunia at the very early stages of ovule development is not known, we cannot exclude the possibility that ethylene also plays a role in ovule development in this species.

The pattern of expression of the *ACO* gene that we isolated is specifically linked to the reproductive tissues of the pistil and suggests a specific role of this member of the *ACO* gene family in the reproductive physiology of the tobacco flower. Using a transgenic approach, we successfully reduced *ACO* gene expression within the ovary. We used an antisense and a cosuppression strategy to downregulate *ACO* mRNA accumulation and the relative ACO enzyme activity in the ovary. The cosuppression strategy also resulted in a strong decrease in mRNA accumulation in the stigma and style with a proportional reduction in ACO activity in these tissues. The two sets of transformants showed a similar flower phenotype, with reduced size and, importantly, female sterility. This particular phenotype suggests an interorgan regulation (O'Neill et al., 1993) in which the ovary influences the size of all the flower parts. The absence of seed setting is consistent with the sporophytic expression pattern

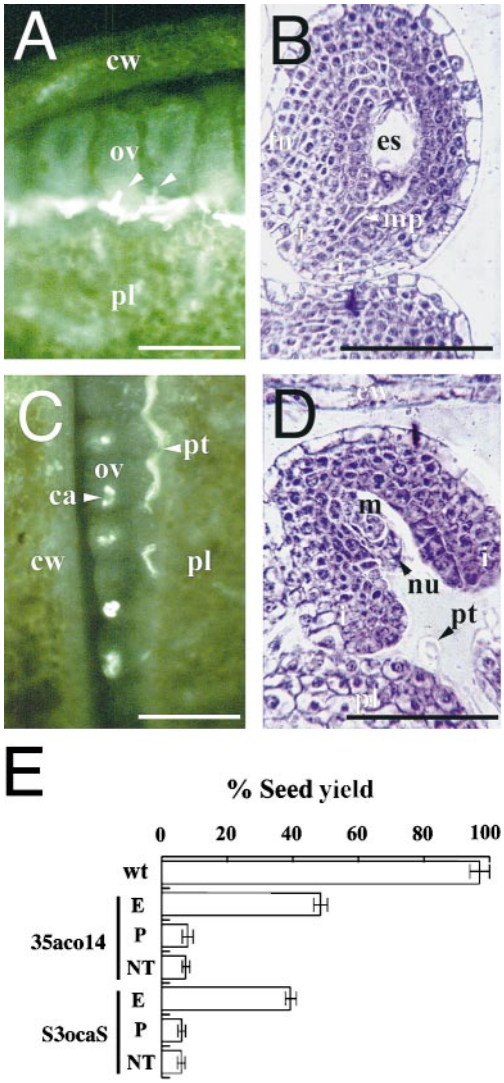

Figure 5. Pollen Tube Behavior, Ovule Morphology, and Percentage of Seed Set in Ethephon-Treated Flowers from Transgenic Plants.

After treatment and pollination (see Methods), ovaries from pollinated pistils were sectioned, stained with aniline blue, and analyzed by fluorescence microscopy or fixed, dehydrated, and resin-embedded for analysis of sections using a bright-field microscope.

(A) Pollen tubes do recover the ability to target the micropyle (arrowheads) in ethephon-treated transgenic flowers.

(B) A fully developed ovule with a normal embryo sac from a transgenic flower treated as given for **(A)**.

(C) Pollen tubes do not recover the ability to target the micropyle if the transgenic flowers are treated with a combination of ethephon and STS, an inhibitor of ethylene action (see Methods). Ovules show callose accumulation within the nucellus.

(D) Ovules from pollinated flowers treated as given for **(C)**.

(E) Percentage of seed production in transgenic flowers after ethephon treatment. Error bars indicate ±SE. wt, wild type; E, ethephon treatment; P, hydrochloride and phosphonic acids treatment; NT, not treated.

of the *ACO* gene. Both these characteristics are inheritable by the progeny.

Cytological analysis revealed that in the transgenic plants, ovules did not complete megasporogenesis and did not produce an embryo sac. Consequently, pollen tubes penetrating the ovary did not grow toward the immature ovule, perhaps because they lacked the appropriate guidance system (Hülskamp et al., 1995). Our results indicate that one factor, the downregulation of a pistil-specific gene encoding the ethylene-forming enzyme, leads to an arrest in female gametophyte development during the very early moments of differentiation. We provide direct evidence for a role of ethylene in ovule development. Moreover, the fact that the supply of an ethylene source was sufficient in itself to stimulate the recovery of fully developed and functional ovules clearly demonstrates that ethylene alone induces ovule maturation at this stage in transgenic plants with reduced ACO activity.

The reproductive biology of mutants affected in ethylene perception and transduction pathways has not been described. It has been demonstrated that a dominant mutant receptor from Arabidopsis is able to confer ethylene insensitivity to petunia and tomato plants. A thorough characterization is in progress to reveal any alteration in the transgenic plants produced, including infertility (Wilkinson et al., 1997).

Recent advances in the studies on mechanisms of ethylene perception and transduction in Arabidopsis and tomato (Hua and Meyerowitz, 1998; Hua et al., 1998; Lashbrook et al., 1998; Sakai et al., 1998) have shown that the ethylene receptors are encoded by a gene family that in Arabidopsis is composed of at least five members that may possibly possess different ethylene-binding affinities and signaling activities. It is possible that the redundancy of these genes masks the effect of ethylene on flower development and fertility. One of these gene family members, the gene encoding the ethylene receptor ETR2, shows an enhanced expression pattern in developing carpels, especially in the funiculi and in the ovules, after the early stages of megasporogenesis (Arabidopsis flower stages 9 to 11, as described by Smyth et al. [1990] and Schneitz et al. [1995]). Although the flower phenotype of these ethylene-insensitive mutants has not been described, these observations and our findings suggest that ethylene plays a role in flower development in several plant species, presumably through a phosphorylation– dephosphorylation cascade leading to altered gene expression (Chang et al., 1993; Kieber et al., 1993; Raz and Fluhr, 1993; Hua et al., 1995; Sessa et al., 1996; Chao et al., 1997).

ca, callose; cw, carpel wall; es, embryo sac; fn, funiculus; i, integument; m, megasporocyte; mp, micropyle; nu, nucellus; ov, ovule; pl, placenta; pt, pollen tubes. Bars in **(A)** and **(C)** = 300 μm; bars in **(B)** and **(D)** = 100 μm.

Targets of this cascade include DNA binding factors, namely, ethylene-responsive element binding proteins (EREBPs), the genes for which have been cloned from tobacco (Ohme-Takagi and Shinshi, 1995) and Arabidopsis (Buttner and Singh, 1997). It has been shown that genes necessary for ovule and female gametophyte development, such as *AINTEGUMENTA* (*ANT*), encode putative transcription factors that belong to the same class of EREBPs as those mentioned above (Elliott et al., 1996; Klucher et al., 1996). However, these authors did not examine whether ethylene plays a role in ovule/flower morphogenesis.

Taken together, these observations suggest the presence of flower-specific, ethylene-inducible transcription factors that may regulate the expression of genes necessary for ovule development. As yet, we have not been able to detect any expression of *ANT* within the tobacco ovary by using an *ANT*-specific Arabidopsis probe. However, we cannot exclude the presence of flower-specific EREBPs. The cloning of those factors and the characterization of their expression in the tissues of the ovules will elucidate whether ethylene controls ovule morphogenesis by a direct function on the megasporocyte or at the sporophytic level.

In summary, we have shown that in transgenic plants in which a pistil-specific *ACO* gene was silenced, the ovules did not complete megasporogenesis. The reversibility of the phenotype suggests that exogenous ethylene can reactivate the "machinery" necessary for megasporogenesis and embryo sac formation, probably activating the proper ethylene-related transcription factors. These results definitively demonstrate that ethylene is an essential hormone for ovule development.

METHODS

Plant Material

Tobacco (*Nicotiana tabacum*) SR1 plants were grown under standard greenhouse conditions.

RNA Gel Blot Analysis in Wild-Type and Transgenic Plants

Total RNA was isolated as described by van Eldik et al. (1995) and separated on a 1.2% agarose gel containing 0.4 M formaldehyde and 0.1 μg/mL ethidium bromide. After electrophoresis, the RNA was transferred overnight to a nylon membrane by capillary transfer in 20 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate). RNA was fixed to the membrane, as described in the Hybond-N nylon membrane (Amersham) handbook. Filters were prehybridized, hybridized, and washed essentially as described by Sambrook et al. (1989). ^{32}P-labeled probes were prepared from the full-length *ACO* and ribosomal tobacco cDNAs (kindly provided by M. Pezzotti, Plant Genetic Systems, Gent, Belgium, and K. Weterings, University of Nijmegen). Membranes were stripped before rehybridization with a new probe.

In Situ Hybridization

Ovaries isolated from flowers at different developmental stages from young flower bud to anthesis were fixed, embedded in paraffin, cut into 10-μm longitudinal sections, and hybridized with digoxigenin-labeled, single-stranded *ACO* sense and antisense RNA probes, essentially as described by Canas et al. (1994). Hybridization was detected with antidigoxigenin antibodies conjugated to alkaline phosphatase and was visualized as a red/purple color after development.

Sense and Antisense Gene Constructs and Plant Transformation

In the antisense construct, the 690-bp 3′ end fragment of the *ACO* cDNA (1-amino cyclopropane-1-carboxylic acid [ACC] oxidase; EMBL accession number X98493) was cloned in reverse orientation under the control of the pistil-specific *Petunia inflata* promoter *S3* (Lee et al., 1994). For the sense construct, the *ACO* full-length cDNA in sense orientation was cloned under the control of the 35S cauliflower mosaic virus promoter (Angenent et al., 1994). Both constructs were cloned in the binary vector pBIN19 for plant transformation and transferred to *Agrobacterium tumefaciens* LBA4404. The recombinant Agrobacterium strains were used to transform tobacco SR1 by using a standard leaf disc transformation and regeneration method (Tavazza et al., 1988). Transgenic plants were selected in vitro on the basis of kanamycin resistance and allowed to grow in the greenhouse. Five independent regenerants carrying the antisense construct and six carrying the cosuppression construct were selected on the basis of flower morphology and female sterility. These plants were backcrossed to the wild type to obtain a T_1 generation. Segregation analysis on the basis of kanamycin resistance showed that all kanamycin-resistant plants were also female sterile as the male parent. The segregation analysis for 35aco14 gave 40 kanamycin-resistant to 82 kanamycin-sensitive plants; for S3ocaS, it was 90 kanamycin-resistant to 112 kanamycin-sensitive plants.

ACO Activity Assay

ACO activity was assayed by measuring the conversion of exogenous amounts of ACC to ethylene in a manner similar to that described by Hamilton et al. (1990). Ovaries or stigmas and styles were cut and immediately infiltrated under vacuum for 10 min with a solution of 10 mM 2-aminoethoxyvinylglycine (Sigma), 10 mM ACC (Sigma), and 100 mM sodium phosphate, pH 6.5. The samples were sealed in 10-mL aliquots 1 hr later, and a gas sample was removed after 2 hr to measure the ethylene concentration by gas chromatography.

Microscopy

Freshly collected ovaries were fixed for 24 hr in a fixing solution composed of absolute ethanol, acetic acid, 37% formaldehyde, and distilled water (50:5:10:35 [v/v/v/v]) under vacuum.

For scanning electron microscopy, after dehydration to 70% ethanol, the samples were partially dissected and the pericarp was removed from the ovary to expose the ovules. The samples were then

dehydrated in a graded series to 100% ethanol. Critical-point drying was conducted in liquid carbon dioxide. Samples were mounted on stubs, sputter-coated with gold, and examined with a scanning electron microscope.

For light microscopy, samples were dehydrated to 100% ethanol and embedded in Technovit 8100 embedding resin (Kulzer and Co., Wehrheim, Germany). Sections 8 μm thick were cut with glass knives and stained with a 0.1% toluidine blue solution for observation with a bright-field light microscope.

For differential interference contrast microscopy, the samples were dehydrated to 70% ethanol, and the ovules were dissected from the ovary on a microslide and incubated in Herr's clearing fluid (Herr, 1971) composed of 80% lactic acid, chloral hydrate, phenol, clove oil, and xylene (2:2:2:2:1 [w/w/w/w]) for 24 hr at 4°C. After clearing, the samples were examined with a microscope equipped with Nomarski optics.

Fluorescent staining of callose with aniline blue was used to visualize the pollen tube penetration into the ovule. Fresh slices (100 μm thick) of ovaries from pollinated flowers were stained on a microslide for 1 hr with 0.1% aniline blue in 0.2 M phosphate buffer, pH 10. Fluorescence was visualized using a microscope equipped for UV epifluorescence.

Chemical Treatment of Flowers

Similar experiments were conducted simultaneously using flowers attached to the plant or detached. For experiments using detached flowers, flowers were excised at the pedicel and immediately transferred into tubes containing the desired solution. Otherwise, flowers were treated while attached to the plant by injecting 10 μL of the desired solution into the ovary by using a Hamilton syringe. To restore ovule development, we used 100 mg/L ethephon (2-chloroethylphosphonic acid). Transgenic flowers were emasculated 1 day before anthesis. At anthesis, the flowers were pollinated, excised from the plant, and placed in a solution of ethephon for 72 hr.

To eliminate the possibility that the observed phenotype was induced from hydrochloride and phosphonic acids, which coevolve with ethylene after ethephon treatment (Lawton et al., 1994), we also placed excised transgenic flowers in an equimolar solution of NaH_2PO_4-HCl. Under these conditions, ovule morphology is identical to that of untreated transgenic flowers. To inhibit ethylene action in ethephon-treated transgenic flowers or in wild-type flowers, we used a solution of silver thiosulfate (STS; 1 mM $AgNO_3$ plus 4 mM $Na_2S_2O_3$), essentially as described by Hoekstra and van Roekel (1988). Under these conditions, the postpollination senescence of the flower is completely inhibited. Flowers were emasculated, excised, and placed in a solution of STS 1 day before pollination. At anthesis, the transgenic flowers were pollinated and placed in a solution of ethephon–STS for 72 hr. The wild-type flowers were pollinated and kept in the same solution of STS for an additional 72 hr. To obtain seed setting, we treated attached transgenic flowers 1 day before anthesis and pollinated them at anthesis.

ACKNOWLEDGMENTS

We thank Hyun-Sook Lee for stimulating suggestions and discussions; Teh-hui Kao for kindly providing the *S3* promoter used for the antisense gene silencing approach; Gerco Angenent for kindly providing the plasmid for cosuppression; and Jan Derksen, Jacques van Went, Elisabeth Pierson, Sander van der Krol, Sharman O'Neill, David Twell, and Bob Fischer for fruitful discussions and suggestions. Photographs and prints were prepared by Gerard Dekkers and Huub Spruijt of the photography department at the Catholic University of Nijmegen and processed by Boudewijn van Veen of the media service department at the Agricultural University of Wageningen, The Netherlands. D.M. was supported by a training grant from the European Community.

Received October 26, 1998; accepted March 28, 1999.

REFERENCES

Abeles, F.B., Morgan, P.W., and Saltveit, M.E., Jr. (1992). Ethylene in Plant Biology, 2nd ed. (San Diego, CA: Academic Press).

Angenent, G.C., and Colombo, L. (1996). Molecular control of ovule development. Trends Plant Sci. **1,** 228–232.

Angenent, G.C., Franken, J., Busscher, M., Weiss, D., and van Tunen, A.J. (1994). Co-suppression of the *Petunia* homeotic gene *fbp2* affects the identity of the generative meristem. Plant J. **5,** 33–44.

Angenent, G.C., Franken, J., Busscher, M., van Dijken, A., van Went, J.L., Dons, H.J.M., and van Tunen, A.J. (1995). A novel class of MADS box genes is involved in ovule development in *Petunia*. Plant Cell **7,** 1569–1582.

Bevan, M.W. (1984). Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res. **12,** 8711–8722.

Bouman, F. (1984). The ovule. In Embryology of Angiosperms, B.M. Johri, ed (Berlin: Springer-Verlag), pp. 123–157.

Bui, A.Q., and O'Neill, S.D. (1998). Three 1-aminocyclopropane-1-carboxylate synthase genes regulated by primary and secondary pollination signals in orchid flowers. Plant Physiol. **116,** 419–428.

Buttner, M., and Singh, K.B. (1997). *Arabidopsis thaliana* ethylene-responsive element binding protein (AtEBP), an ethylene-inducible, GCC box DNA-binding protein interacts with an ocs element binding protein. Proc. Natl. Acad. Sci. USA. **94,** 5961–5966.

Canas, L.A., Busscher, M., Angenent, G.C., Beltran, J.P, and van Tunen, A.J. (1994). Nuclear localisation of the *Petunia* MADS box protein FBP1. Plant J. **6,** 597–604.

Chang, C., Kwok, S.F., Bleecker, A.B., and Meyerowitz, E.M. (1993). *Arabidopsis* ethylene-response gene *ETR1*: Similarity of product to two component regulators. Science **262,** 539–544.

Chao, Q., Rothenberg, M., Solano, R., Roman, G., Terzaghi, W., and Ecker, J.R. (1997). Activation of the ethylene gas response pathway in Arabidopsis by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. Cell **89,** 1133–1144.

Elliott, R.C., Betzner, A.S., Huttner, E., Oakes, M.P., Tucker, W.Q.J., Gerentes, D., Perez, P., and Smyth, D.R. (1996). *AINTEGUMENTA*, an *APETALA2*-like gene of Arabidopsis with pleiotropic roles in ovule development and floral organ growth. Plant Cell **8,** 155–168.

Goldberg, R.B. (1988). Plants: Novel developmental processes. Science **240,** 1460–1467.

Hamilton, A.J., Lycett, G.W., and Grierson, D. (1990). Antisense

gene that inhibits synthesis of the hormone ethylene in transgenic plants. Nature **346,** 284–287.

Herr, J.M. (1971). A new clearing-squash technique for the study of ovule development in angiosperms. Am. J. Bot. **33,** 54–57.

Hoekstra, F.A., and van Roekel, T. (1988). Effect of previous pollination and stylar ethylene on pollen tube growth in *Petunia hybrida* styles. Plant Physiol. **86,** 4–6.

Hua, J., and Meyerowitz, E.M. (1998). Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana*. Cell **94,** 261–271.

Hua, J., Chang, C., Sun, Q., and Meyerowitz, E.M. (1995). Ethylene insensitivity conferred by *Arabidopsis ERS* gene. Science **269,** 1712–1714.

Hua, J., Sakai, H., Nourizadeh, S., Chen, Q.J., Bleecker, A.B., Ecker, J.R., and Meyerowitz, E.M. (1998). *EIN4* and *ERS2* are members of the putative ethylene receptor gene family in Arabidopsis. Plant Cell **10,** 1321–1332.

Hülskamp, M., Schneitz, K., and Pruitt, R.E. (1995). Genetic evidence for a long-range activity that directs pollen tube guidance in Arabidopsis. Plant Cell **7,** 57–64.

Kieber, J.J., Rothenberg, M., Roman, G., Feldmann, K.A., and Ecker, J.R. (1993). *CTR1*, a negative regulator of the ethylene response pathway in Arabidopsis, encodes a member of the Raf family of protein kinases. Cell **72,** 427–441.

Klucher, K.M., Chow, H., Reiser, L., and Fischer, R.L. (1996). The *AINTEGUMENTA* gene of Arabidopsis required for ovule and female gametophyte development is related to the floral homeotic gene *APETALA2*. Plant Cell **8,** 137–153.

Koltunow, A.M., Truettner, J., Cox, K.H., Wallroth, M., and Goldberg, R.B. (1990). Different temporal and spatial gene expression patterns occur during anther development. Plant Cell **2,** 1201–1224.

Lashbrook, C.C., Tieman, D.M., and Klee, H.J. (1998). Differential regulation of the tomato *ETR* gene family throughout plant development. Plant J. **15,** 243–252.

Lawton, K.A., Potter, S.L., Uknes, S., and Ryals, J. (1994). Acquired resistance signal transduction in Arabidopsis is ethylene independent. Plant Cell **6,** 581–588.

Lee, H.S., Huang, S., and Kao, T.-h. (1994). S proteins control rejection of incompatible pollen in *Petunia inflata.* Nature **367,** 560–563.

Lincoln, J.E., Cordes, S., Read, E., and Fischer, R.L. (1987). Regulation of gene expression by ethylene during *Lycopersicum esculentum* (tomato) fruit development. Proc. Natl. Acad. Sci. USA **84,** 2793–2797.

Meyer, P., and Saedler, H. (1996). Homology dependent gene silencing in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **47,** 23–48.

Oeller, P.W., Lu, M.W., Taylor, L.P., Pike, D.A., and Theologis, A. (1991). Reversible inhibition of tomato fruit senescence by antisense RNA. Science **254,** 437–439.

Ohme-Takagi, M., and Shinshi, H. (1995). Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element. Plant Cell **7,** 173–182.

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. Plant Cell **5,** 419–432.

Raz, V., and Fluhr, R. (1993). Ethylene signal is transduced via protein phosphorylation events in plants. Plant Cell **5,** 523–530.

Sakai, H., Hua, J., Chen, Q.J., Chang, C., Medrano, L.J., Bleecker, A.B., and Meyerowitz, E.M. (1998). *ETR2* is an *ETR1*-like gene involved in ethylene signaling in *Arabidopsis.* Proc. Natl. Acad. Sci. USA **95,** 5812–5817.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Schneitz, K., Hülskamp, M., and Pruitt, R.E. (1995). Wild-type ovule development in *Arabidopsis thaliana*: A light microscope study of cleared whole-mount tissue. Plant J. **7,** 731–749.

Sessa, G., Raz, V., Savaldi, S., and Fluhr, R. (1996). PK12, a plant dual-specificity protein kinase of the LAMMER family, is regulated by the hormone ethylene. Plant Cell **8,** 2223–2234.

Smyth, D.R., Bowman, J.L., and Meyerowitz, E.M. (1990). Early flower development in *Arabidopsis*. Plant Cell **2,** 755–767.

Tang, X., Gomes, A.M.T.R., Bhatia, A., and Woodson, W.R. (1994). Pistil-specific and ethylene-regulated expression of 1-aminocyclopropane-1-carboxylate oxidase genes in petunia flowers. Plant Cell **6,** 1227–1239.

Tavazza, R., Ordas, R.J., Tavazza, M., Ancora, G., and Benvenuto, E. (1988). Genetic transformation of *Nicotiana clevelandii* using a Ti plasmid derived vector. J. Plant Physiol. **133,** 640–644.

Theologis, A. (1992). One rotten apple spoils the whole bushel: The role of ethylene in fruit ripening. Cell **70,** 181–184.

Tian, H.Q., and Russel, S.D. (1997). Calcium distribution in fertilized and unfertilized ovules and embryo sacs of *Nicotiana tabacum* L. Planta **202,** 93–105.

Tova, T., Staub, J.K.E., and O'Neill, S.D. (1997). Identification of a 1-aminocyclopropane-1-carboxylic acid synthase gene linked to the female (*F*) locus that enhances female sex expression in cucumber. Plant Physiol. **113,** 987–995.

van Eldik, G.J., Vriezen, W.H., Wingens, M., Ruiter, R.K, van Herpen, M.M.A, Schrauwen, J.A.M., and Wullems, G.J. (1995). A pistil-specific gene of *Solanum tuberosum* is predominantly expressed in the stylar cortex. Sex. Plant Reprod. **8,** 173–179.

Wang, H., Wu, H.M., and Cheung, A.Y. (1996). Pollination induces mRNA poly(A) tail-shortening and cell deterioration in flower transmitting tissue. Plant J. **9,** 715–727.

Wilkinson, J.Q., Lanahan, M.B., Clark, D.G., Bleecker, A.B., Chang, C., Meyerowitz, E.M., and Klee, H.J. (1997). A dominant mutant receptor from *Arabidopsis* confers ethylene insensitivity in heterologous plants. Nature Biotechnol. **15,** 444–447.

Yang, S.F. (1969). Ethylene evolution from 2-chloroethylphosphonic acid. Plant Physiol. **44,** 1203–1204.

Zhang, X.S., and O'Neill, S.D. (1993). Ovary and gametophyte development are coordinately regulated by auxin and ethylene following pollination. Plant Cell **5,** 403–418.