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Flower Development, Reproduction and Fruit Ripening

the Role of Ethylene

Domenico De Martinis

Flower Development, Reproduction and Fruit Ripening

the Role of Ethylene

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op het gebied van Natuurwetenschappen, Wiskunde en Informatica

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This thesis is dedicated to my uncle
Perusino Perusini.
Man of wide culture, poetic imagination,
scientific sympathy and highest moral value.
He supported my dreams and enthusiasm
including my commitment for
working and studying in The Netherlands,
he has been an important mentor for me.

Dead in October 1996 while I was in Nijmegen.
I missed the opportunity to farewell.
For this, dear uncle, I apologise.

Citius! Altius! Fortius!

Contents

Chapter I

General Introduction

Chapter I.1

Modification of plant development by genetic manipulation of the ethylene biosynthetic pathway

D. De Martinis (2000) "Modification of plant development by genetic manipulation of the ethylene biosynthesis and action pathway". Developments in Plant Genetic and Breeding Series 6. G.E. de Vries and K. Metzlaff (Eds.). Elsevier Science B.V

Ethylene is one of the five "classical" naturally occurring plant hormones (Kende and Zeevaart 1997). Ethylene regulates a multitude of plant processes, ranging from seed germination to organ senescence. Of particular economic importance is the role of ethylene as an inducer of fruit ripening (Abeles 1992, Bleecker and Kende 2000). Moreover, ethylene is inducible in response to biotic and abiotic stress, such as pathogen attack, wounding, water-logging and drought (Yang and Hoffman 1984). The biosynthetic pathway of ethylene (C_2H_4) has been established and several steps of its perception and transduction pathway have been elucidated (Fig. I.1). Ethylene is synthesized in the "Yang" cycle (Abeles *et al.* 1992); from *S*-adenosyl-L-methionine via 1-aminocyclopropane-1-carboxylic acid (ACC). The enzymes catalyzing the two reactions in this pathway are ACC synthase and ACC oxidase (ACO) (Fig. I.1). Briefly, ethylene is perceived by receptor proteins and transduced through a phosphorylation cascade that leads to transcription factor activation and consequently ethylene-related gene expression (Chang 1996). Due to its role in the regulation of flower senescence and fruit ripening, the control of ethylene production and perception has been used as a major tool to prevent fruit/flower spoilage and prolong marketability. Plant physiologists (as well as fruit and flower producers and distributors) have been challenged to optimize harvesting and post-harvesting practices, to prevent the metabolic processes that are induced by ethylene. Plant biotechnology allowed plant scientists to study the control of ethylene biosynthesis and perception in plants. Mutants and transgenic plants altered in ethylene perception or production have been generated to elucidate ethylene function and the mode of action as well as to improve fruit and flower shelf-life. The aim of this Chapter is to illustrate how ethylene affects plant development by describing the plant phenotype of the transgenic plants and the mutants so far obtained.

Seed Germination, the "triple response" of *Arabidopsis thaliana*.
During seed germination, the terminal part of the shoot axis of certain dicotyledonous plants exhibits an apical arch-shaped structure, called "apical-hook". The apical hook may protect the apical meristem from injury while the stem is emerging from the soil into open air. During germination in the dark (etiolated seedlings), ethylene is produced locally in the apical hook region and is involved in determining the

shape of the plantlet. As soon as the etiolated plantlet tissues become photosynthetically competent, they loose sensitivity to ethylene. The application of ethylene to the etiolated seedlings results in the "triple response"; that consists of three distinct morphological changes in the shape of the seedling: inhibition of stem elongation, radial swelling of the stem and absence of the normal geotropic response (diageotropism). This effect was first described at the beginning of the XXth century by the Russian plant physiologist Neljubov in pea seedlings (Nelijubow 1901). The development of molecular genetic approaches in the model plant Arabidopsis enabled scientist to characterise many of the biochemical components of ethylene perception and signal transduction identified

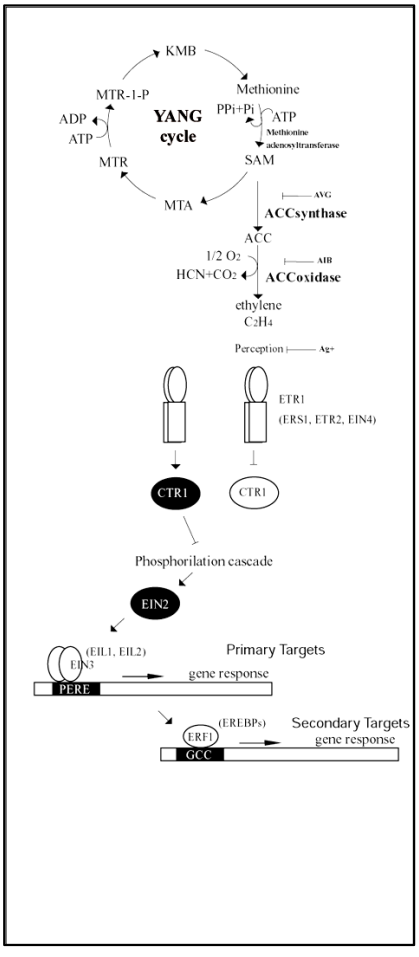

Figure I.1: ethylene biosynthesis and action.

ACC 1-aminocyclopropane-1-carboxylic acid;
ATP adenosine triphosphate;
KMB 2-keto-4-methylthiobutyrate;
MACC malonyl-ACC;
MTA 5'-methylthioadenosine;
MTR 5'-methylthioribose;
MTR-1-P MTR 1-phosphate,
SAM S-adenosylmethionine.
AVG aminoethoxyvinilglycine;
AIB a-aminoisobutyric acid.

In a proposed model of the phosphorilation cascade (Ouaked et al. 2003), CTR1 is a negative regulator of the MAPKK SIMKK and the MAPKs SIMK/MMK3 in *Medicago*, and MPK6/13 in *Arabidopsis*. In the presence of ethylene, ETR1 and CTR1 become inactivated, relieving SIMKK from inhibition. Subsequent activation of the MAPKs activates gene expression of ethylene-responsive genes via direct activation of EIN2 and EIN3 or through other factors.

by screening for altered triple response. In fact, in Arabidopsis the triple response is easily scored by visual inspection or with the aid of a dissecting microscope, and ethylene-insensitive mutants were identified by screening mutagenized populations of Arabidopsis seedlings growing in the dark and in the presence of applied ethylene (Bleecker et al. 1988, Guzmán & Ecker 1990, Ecker 1995). These studies have provided strong evidence that ethylene signalling is mediated by a family of copper-containing receptors that signal through a pathway that likely includes a MAP kinase cascade, a metal transporter intermediate, and a transcriptional cascade (Fig. I.1). One class of mutations, exemplified by etr1, led to the identification of the ethylene receptors, which turned out to be related to bacterial two-component signalling systems EIN4 (Chang et al. 1993, Hua et al.1995, 1998, Sakai et al. 1998, Hua and Meyerowitz 1998). Mutations that eliminate ethylene binding to the receptor yield a dominant, ethylene-insensitive phenotype. CTR1 encodes a Raf-like Ser/Thr protein kinase that acts downstream from the ethylene receptor and may be part of a MAP kinase cascade. Two-hybrid interaction assays suggest that CTR1 is directly regulated by ETR1 because CTR1 can physically interact with ETR1 in yeast (Clark et al., 1998). Mutants in CTR1 exhibit a constitutive ethylene-response phenotype. Both the ethylene receptors and CTR1 are negative regulators of ethylene responses. EIN2 and EIN3 are epistatic to CTR1, and mutations in either gene lead to ethylene insensitivity. Whereas the function of EIN2 in ethylene transduction is not known, EIN3 is assumed to activate a set of transcription factors called ERFs (ethylene response factors) or EREBPs (ethylene response element-binding proteins) either transcriptionally or post-transcriptionally, thereby regulating the expression of appropriate target genes in response to ethylene (Ohme-Takagi and Shinshi, 1995). recently, The structure and mode of action of the etr1 mutant (Chen et al., 2002) and the involvement of a MAPK pathway in ethylene signalling in plants have been described (Ouaked et al. 2003, Qu and Schaller 2004).

Plant Development

Ethylene does act to slow growth of many plant tissues. This can easily be observed in transgenic tobacco in which auxin overproduction and its concomitant ethylene overproduction causes a strong apical dominance and a large reduction in internodes

elongation (Romano et al. 1993). The introduction in those transgenic lines of an ACC deaminase gene, (that reduces ethylene production to wild-type levels) eliminates the reduction of internodes length, without affecting the auxin related apical dominance effect. This example shows that there are complex interactions between the plant hormones and sometimes it is difficult to distinguish the single hormone specific effect on plant growth and the effects consequent to the overall hormonal rate. In any case, due to its involvement in response to stress and in the control of ripening and senescence, plants of several species have been engineered for reduced production or sensitivity. The first breakthrough in the biotechnology of ethylene was the generation of transgenic tomato that was inhibited in ethylene synthesis by an antisense gene silencing approach (Hamilton et al. 1990). The introduction of a chimaeric gene consisting of the antisense TOM13 gene under the control of the 35S CaMV promoter resulted in reduced ethylene synthesis. Notably, that was the first demonstration that the antisense approach could lead to the identification of gene of unknown function, indicating that TOM13 encodes for an ACC-oxidase involved in the conversion of ACC to ethylene. Other examples of constitutive reduction of ethylene production and perception are also in other plants species, among which tobacco (Knoester et al. 1997, Knoester et al. 1998) tomato and petunia (Wilkinson et al. 1997). Although these plants were mainly characterised for fruit ripening (tomato), flower senescence (petunia) and biotic stress (tobacco) they provided an opportunity to study how reduced ethylene levels and perception affects the vegetative development of the plant. Transgenic tomato transformed either antisense ACS (Oeller et al. 1991) or ACO (Hamilton et al. 1990) transgenes evolved less ethylene. Silencing resulted in a reduction of ethylene production by 68% in wounded leaf and by 87-99.5% in ripening fruit. In transgenic “low ACO” tomatoes, leaf senescence, characterised by visible chlorophyll loss, was delayed by 10-14 days. Despite the decreased ethylene production levels, leaf senescence was not arrested although it was significantly delayed. Constitutive expression of ACS in transgenic tomato plants (Lanahan et al. 1994) resulted in high rates of ethylene production by many tissues of the plant and induced petiole epinasty, premature senescence and abscission of flowers, usually before anthesis. There were no obvious effects on senescence in leaves of ACS overexpressers. Taken

together, these comparisons between low and high ethylene producers in tomato suggest that, although ethylene may be important, it is not the only factor determining tomato leaf senescence. Thus, together with ethylene other signals must be involved. A second feature of low-ethylene producing transgenic tomatoes was a characteristic dark-green pigmentation, comparable to the pigmentation of the ethylene insensitive Arabidopsis mutants (see previous paragraph) which is correlated with a clear retention of photosynthetic activity. Molecular analysis (Isaac et al. 1995) demonstrated that the expression pattern of two photosynthetic-associated genes the chlorophyll a/b-binding protein (cab) and the ribulose biphosphate carboxylase/oxygenase small subunit (rbcs) was higher in transgenic "low-ACO" tomatoes than in wild-type leaves of the same age; cab and rbcs mRNAs decline during normal leaf senescence, thus indicating that in transgenic tomatoes the delay in leaf senescence was not only a visual characteristic but also reflected the molecular composition of the transgenic plant tissues. Moreover, the analysis of the photosynthetic activity by mean of chlorophyll content, oxygen evolution, and quantum yield of photosystem II in vivo (Fv/Fm, ref. Bjorkman and Demming 1989), indicated a clear retention of photosynthetic capacity in leaves of transgenic tomatoes, thus supporting the hypothesis that photosynthetic decline is coupled with the senescence syndrome (Hensel et al. 1993). In tobacco, transgenic plants were generated to express sense and antisense transcripts corresponding to ACS and/or ACO, but ethylene production was reduced with less efficiency than in tomato (by 74%), while plants overexpressing ACS produced the highest levels of ethylene (320% of wild type). High-ethylene producer tobacco plants had shorter internodes and resulted in a reduced size and/or internodal length if compared to wild type or low-ethylene producer plants. This result is similar to that described for auxin/ethylene overproducers previously mentioned, and for potato expressing antisense SAM-decarboxylase (Kumar et al. 1996), which resulted in high ethylene levels. Also in transgenic tobacco, low-ethylene production corresponded with a higher content of chlorophyll in leaves than wild type leaves of the same age. Another successful approach to study ethylene action has been the inhibition of ethylene perception by expressing the gene encoding for a mutated ethylene-receptor from Arabidopsis in heterologous plants (Knoester et al. 1998, Wilkinson et al. 1997). The introduction of the Arabidopsis etr

1-1 sequence encoding in tomato, petunia and tobacco conferred ethylene insensitivity to the transformed plants. In tobacco, the absence of ethylene sensitivity seemed to reduce their perception of neighbouring plants (Knoester et al. 1998). In general, when wild type seedlings were grown together, plant growth slowed before leaves of neighbouring plants overlapped. These plants remained relatively small and exhibited accelerated leaf senescence. In contrast ethylene-insensitive plants did not appear to perceive their neighbouring plants. Moreover, these plants did not show a reduction in growth, resulting in a "crowding effect" of interdigitating leaves which, moreover, remained fully green.

In addition to the regulation of plant aerial part development, ethylene markedly affects root morphogenesis. Numerous studies have indicated that ethylene is a positive regulator root epidermal development. Analysis of ethylene-related Arabidopsis mutants provided a versatile tool to observe root hairs development. In Arabidopsis, cells in the root epidermis are arranged in "hair" and "non-hair" forming files (Donald et al. 1994). Hair files are located over the anticlinal (radial) walls of underlying cortical cells while non-hair files are located over the outer periclinal (tangential) walls of cortical cells. The phenotypic characterisation of ctr1-1 mutant roots has implicated ethylene in the process of cell patterning and differentiation in the epidermis (Kieber et al. 1993, Takimoto et al. 1995); root-hair spacing in ctr 1-1 is abnormal and root hair cells differentiate in the position usually occupied by non-hair cells. Despite these observations, as the CTR 1 gene encodes for a protein kinase of the Raf family, the mutation may affects pathways controlling the specification of epidermal pattern independent of ethylene. Surprisingly, ethylene insensitive mutants, ein 2 and etr 1 do produce normal root hairs (Pitt et al. 1998); anyhow, it is possible that those mutants retain a residual ethylene response sufficient for some root hair development. To elucidate the role of ethylene in root development the study of mutants was elegantly complemented by the use of chemical inhibitors and inducers of ethylene production and perception. The combination of mutant analysis and pharmacological studies indicated that ethylene promote root air elongation. A model for the development of root hairs in relation to ethylene has been proposed by Takimoto et al. (1995). During normal development, non-hair forming cells are not exposed to ethylene, or are exposed to levels

below the threshold necessary to induce the differentiation of root hairs. Ethylene inhibition results in complete inhibition of root hairs (either in non-hair and hair-cells), while exogenous ethylene, delivery of ethylene precursor ACC, or ethylene constitutive-response (in ctr 1-1mutants) results in root-hair development also where non-hair cells are located. The authors suggested that, in normal conditions, root anatomy directs the delivery of ethylene or its precursor ACC only to the "hair-cell" files, thus determining the pattern of root hair formation.
Root formation was also studied in ethylene-insensitive plants (Clark et al. 1999). Experiments with ethylene-insensitive tomato (Never ripe –NR- Lycopersicon esculentum) and petunia (Petunia × hybrida) plants were conducted to determine if normal or adventitious root formation is affected by ethylene insensitivity. NR tomato plants produced more belowground root mass but fewer aboveground adventitious roots than wild-type Pearson plants. Applied auxin (indole-3-butyric acid) increased adventitious root formation on vegetative stem cuttings of wild-type plants but had little or no effect on rooting of NR plants. Reduced adventitious root formation was also observed in ethylene-insensitive transgenic petunia plants. Applied ACC increased adventitious root formation on vegetative stem cuttings from NR and wild-type plants, but NR cuttings produced fewer adventitious roots than wild-type cuttings. The data suggested that the promotive effect of auxin on adventitious rooting is influenced by ethylene responsiveness.

Flower development
Pollination-induced ethylene production and flower senescence have been the most widely studied ethylene-related physiological changes in the flower (O'Neill and Nadeau 1997).Ethylene may act also in flower induction and sex determination. The induction of flowering by ethylene is of considerable commercial importance in pineapple and in other tropical fruits (Reid 1988). Despite its economical importance, little information is yet available on the mechanism by which ethylene induces flowering in these species. Induction of flowering could be linked to the events that determines sex differentiation in the flower; it has been demonstrated that application of ethylene (or ethylene-releasing compounds) to seedlings dramatically changes the ratio of male to female flowers in members of the Cucurbitaceae (Abeles et al.

1992). More recently, it was reported that an auxin-inducible gene encoding for an ACC synthase was tightly associated with the F locus that determines female sex expression in cucumber, supporting the hypothesis that ethylene plays a pivotal role in the determination of sex in cucumber flowers (Tova et al. 1997). In orchids, (Zhang and O'Neill 1993) it was shown that pollination and auxin regulate ethylene production and ovary development. When inhibitors of ethylene were used, pollination-induced or auxin-induced ovary development, were inhibited. It was hypothesised that an unknown pollination factor has a synergistic effect with auxin in stimulating ethylene biosynthesis and subsequent ovary development in orchid (Bui and O'Neill 1998). In Petunia flowers, the expression of the ACO gene family is temporally regulated during pistil development (Tang et al. 1994), and it was suggested that ethylene plays a role in reproductive physiology by regulating the maturation of the secretory tissues of the pistil. Direct evidence that ethylene is necessary to induce female gametophyte development was provided in tobacco (De Martinis and Mariani 1999 and this thesis, Chapter V). The isolation and characterisation of a tobacco pistil-specific ACO gene revealed an increased expression in the tobacco ovary when the first events of megasporogenesis occur. The pattern of expression of this ACO gene was linked to the reproductive tissues of the pistil suggesting a specific role for this gene in the reproductive physiology of tobacco flower. Transgenic tobacco in which that pistil-specific ACO gene was silenced showed a flower phenotype with a reduced size and, importantly, female sterility. Cytological analysis revealed that in the transgenic plants, ovules did not complete megasporogenesis and did not produce the embryo sac. Moreover, the supply of an ethylene source was sufficient to recover fully developed and functional ovules, clearly demonstrating that ethylene alone induces ovule maturation in tobacco. Outside of orchids, in which ethylene-related ovary development was triggered by pollination, this was the first evidence of a direct role of ethylene in ovule development. Despite these findings, it is surprising that no clear data on the reproductive biology of ethylene-related Arabidopsis mutants or ethylene insensitive transgenic plants have been reported. Studies on the mechanism of ethylene perception and transduction in Arabidopsis (Hua et al. 1998, Sakai et al. 1998) have shown that the ethylene receptors are encoded by a gene family that in Arabidopsis is comprised of at least five

members that may possibly possess different ethylene binding affinities and signaling activities. It is possible that redundancy of these genes masks the effect of ethylene on flower development and fertility. One of these gene family members, the ethylene receptor gene ETR 2, shows an expression pattern enhanced in the developing carpels especially in the funiculi and in the ovules from the early stages of megasporogenesis. Arabidopsis double mutants ers1 etr1showed defect in flower development and fertility (Hall and Bleecker, 2003). Mutant floral buds were much smaller than wild-type buds and rarely opened normally. Instead, within most flowers, the inner floral organs remained enclosed by the sepals, and the bud eventually senesced without opening. Scanning electron microscopy of floral buds indicated that with the exception of the stamens, the floral organs in the double mutants appeared fairly normal but arrested in development. These observations together with the data provided on cucumber, orchids, and tobacco suggest that ethylene plays a role in female gametophyte development in several plant species.

Once the flower has been pollinated, major developmental changes occur, involving an interorgan signalling within the flower. Upon pollination a transient increase in ethylene production is induced in several flowers species such as, e.g., orchids, petunia and tobacco (Zhang and O'Neill 1993, Tang et al. 1994, Wang et al. 1996). This transient ethylene production is responsible for petal senescence, but it is also necessary to induce deterioration of pistil transmitting tissue that is thought to facilitate pollen tube growth toward the ovary. The senescence-related ethylene responses are very important for the flower industry, since many important commodities are extremely sensitive to ethylene. Genetic engineering to reduce the rate of ethylene biosynthesis may not be sufficient, as the presence of external ethylene may also induce flower senescence and drastically reduce marketability. To overcome this problem, genetic manipulation to reduce ethylene sensitivity rather than production happens to be a more successful strategy. Transgenic petunia plants containing the etr 1-1 cDNA have been characterised for the lack of ethylene sensitivity (Wilkinson et al. 1997). In particular, the senescence and abscission of flowers following pollination were monitored as indication of ethylene sensitivity. Corollas of pollinated flowers from transgenic petunia remained turgid and structurally intact for at least 5 days longer than the corresponding wild type control flowers. The molecular basis of

the extended flower-life and delayed abscission in the transgenic petunia was shown to be related to ethylene perception rather than ethylene synthesis, since the transgenic petunia retained the capability to respond to pollination by triggering a burst of ethylene.

Fruit Ripening

The stimulation of fruit ripening was one of the earliest reported effects of ethylene. The earliest record of human manipulation causing fruit ripening is in the Old Testament (see Theologis 1992 for full citation). Fruits are classified as climacteric or non-climacteric according to their respiratory output at the onset of the ripening process and the ability of ethylene to stimulate autocatalytic production of ethylene. In climacteric fruits such as bananas, apples, pears and tomatoes, a clear increase in respiration at the onset of ripening, concomitant with a dramatic increase in the rate of ethylene production occurs. In others as the melon, the substantial rise in ethylene production precedes the onset of ripening. Non-climacteric fruits, for example strawberries and those of the citrus family, do not show any increase neither in respiratory activity, nor in ethylene evolution at the onset of ripening. Ripening corresponds generally with the alteration of colour, flavour, aroma and texture, and the role of ethylene in ripening of climacteric fruits. Tomato has been the most often studied plant, not only for scientific interest, but also due to the economic importance as a major fruit crop. As already mentioned, the first evidence that the ethylene biosynthetic pathway could be manipulated in transgenic plants was provided in tomato (Hamilton et al. 1990, Oeller et al. 1991). The stable integration and expression of the ACO gene in antisense orientation resulted in a clear reduction of the rate of ethylene biosynthesis by 97%. Using the same approach, the expression of an antisense ACS gene resulted in an inhibition by 99.5% of ethylene production in fruits. These two examples of manipulation of the ethylene biosynthetic pathway opened the way to manipulate of ethylene production to achieve extended fruit storage life also in melon (Ayub et al. 1996). In these three examples of “low ethylene” transgenic plants (tomato ACO antisense, tomato ACS antisense and melon ACO antisense) reduction of ethylene biosynthesis resulted in increased resistance to over-ripening either on plants or detached and stored in air. In transgenic ACO antisense tomatoes, post-fertilisation development and colour changes at the

onset of ripening were normal if compared to wild-type tomatoes. However, although the timing of the onset of ripening was unaffected, the subsequent ripening process was moderated; reddening of the fruit was reduced and once fruits were stored at room temperature, fruits from ACO antisense transgenic tomatoes were noticeably more resistant to over-ripening and shrivelling than control fruits. The reduction of reddening was consistent with a clear reduction of carotenoid pigments, specifically lycopene, and a reduction of the expression of the mRNA encoding for the phytoene synthase, an enzyme involved in carotenoid biosynthesis. A similar phenotype was observed in transgenic ACS antisense tomato fruits that demonstrated a reduction of red coloration derived from lycopene accumulation and a progressive loss of chlorophyll that resulted in a yellow colour of the fruit. Fruits kept on air or on the plants for 90 up to 120 days eventually developed an orange colour but never turned red and softened or developed an aroma. Different behaviour was observed in fruits from transgenic ACO antisense melon; in those fruits, ethylene production was reduced by 99%. Chlorophyll degradation, fruit softening and activation of the peduncular abscission zone associated to ripening, resulted to be ethylene-dependent and were therefore reduced, while flesh pigmentation proved to be ethylene-independent. This is consistent with the observation that in melon the phytoene synthase gene is expressed before the onset of ripening. One remarkable feature of the transgenic melon was the absence of activation of the peduncular abscission zone. As a consequence fruit did not drop from the plant even at very late stage of development, thus resulting in higher sugar content in the fruits from transgenic plants. In general, all the three transgenic “low ethylene” plants produced fruits with longer shelf-life, but did not develop colour (tomato) and aroma (tomato and melon, Bauchot et al. 1998) normally. Fruit phenotype could be reverted upon exposure to exogenous ethylene, or its analogue propylene, both in tomato and in melon. Nevertheless but while ripening seemed to be completely reverted in transgenic ACS antisense tomato and transgenic ACO antisense melon, complete reversion did not occur in transgenic ACO antisense tomatoes. In fact, these transgenic fruits failed to accumulate lycopene to wild-type levels when detached from the plant. The authors (Picton et al. 1993) suggested that ethylene is not the only trigger of fruit ripening and suggested the involvement of another

"ripening-factor-X" associated with attachment to the plant which can modulate ripening in conjunction with ethylene. Also analysis of tomato (Solanum lycopersicum) mutants and inhibitor studies indicated that ethylene is necessary for full development of the ripening program of climacteric fruit such as tomato, yet ethylene alone is not sufficient. This suggests that an interaction between ethylene and non-ethylene (or developmental) pathways mediates ripening. (Barry et al. 2005)

Conclusions

The plant hormone ethylene is involved in a wide range of developmental processes, as well as in biotic and abiotic stress responses in plants. Its biosynthesis involves two key enzymes (ACS and ACO) each encoded by a multigene family and differentially expressed in response to different environmental or developmental stimuli. Ethylene perception and signal transduction are mediated by a family of receptors that activate a phosphorylation cascade that lead to the activation of transcription factor controlling ethylene-related gene-expression (Chang et al. 1993, Kieber et al. 1993, Ecker 1995, Hua et al. 1998, Sakai et al. 1998, Hua and Meyerowitz 1998, Ohme-Takagi and Shinshi 1995, Solano et al. 1998). The diverse roles that ethylene plays and the specificity of its action suggest a complex regulation of both biosynthetic and signal transduction pathways. In this Chapter, alterations of plant development upon manipulation of ethylene biosynthesis and action by mutagenesis or genetic engineering were reviewed. Several approaches demonstrated to be successful in the modification of the ethylene signal, and in generating fruits and flowers with a delayed ripening/senescence. The production of ethylene-insensitive transgenic plants is a complementary strategy to the alteration of ethylene biosynthesis. The latter is necessary in climacteric fruits in which an ethylene treatment is necessary to induce proper maturation when desired (before release on the market or before processing). Reduction of ethylene sensitivity could be of interest in floriculture (Wilkinson et al. 1997) and also in those vegetables in which ethylene causes post-harvest spoilage (e.g. lettuce and cucumbers). This review describes how constitutive modification of ethylene production/action in the plant results in several different alteration of plant behaviour. This is possibly due to the wide number of genes involved in ethylene biosynthesis and action in different plant

species, and to the changes in sensitivity to ethylene in different tissues and developmental stages. The analysis of the plants genetically altered in ethylene production and sensitivity will enable to understand the different responses to ethylene and distinguish between ethylene-related and ethylene-independent plant responses (Theologis 1993). In the future, it is conceivable that the dissection of the ethylene pathway of biosynthesis and action will enable us to generate plants with targeted modification of specific responses by the use of tissue-specific, developmental-regulated chimaeric genes.

Chapter I.2

Flower Development and Reproduction

The life cycle of a flowering plant

Plants undergo an alternation of spore- and gamete- forming generations. The sporophyte is the dominant generation in higher plants and produces gametes by meiotic division within the sexual organs (pistil and anther) to give rise to haploid megaspores and microspores, which undergo mitotic divisions to produce the gametophyte stages of the plant life cycle. The megaspore develops within the ovules into a female gametophyte, which typically contains seven cells; one becomes the egg, and the others aid in fertilisation or embryo development. The male gametophyte, or pollen grain, arises from a microspore produced within the anther locule and contains three cells at maturity; two sperm cells and a vegetative cell. Upon germination on a receptive stigma, the vegetative cell develops into a pollen tube that grows through the pistil to the ovule, where it delivers the two sperm cells to the embryo sac. The two sperm cells perform the "double fertilisation" (Lord and Russel 2002); one sperm cell fertilises the egg cell and the second the central cell that is usually binucleate, to generate a third life stage, the endosperm, distinct from the gametophyte and the sporophyte. The endosperm divides to become a terminally differentiated mass of tissue that provides nutrition to the growing embryo.

Pistil development

The female parts of an angiosperm flower are collectively referred as the gynoecium, which consists of one or more ovule-bearing unit structures, the carpels. The term pistil is also commonly used in describing the female part of a flower. Figure I.2 A-F illustrates the basic components of the tobacco pistil. In general, a completely developed pistil consists of three parts: the ovary at the base of the pistil which contains the ovules and which differentiates into the fruit following fertilisation; the style, an extension above the ovary, through which the pollen tubes grow toward the ovules and the stigma, at the top of the style, where pollen grains adhere and germinate (Fig. I.2 B). The tobacco pistil is formed by two carpels which fuse to form the ovary and develops the style and the bilobed stigma. The ovule, precursor of the seed, resides within the ovary (Fig. I.2 F), and is in itself a complex structure. A comprehensive description of tobacco ovule development at the cytological and molecular level will be provided in the following Chapters of this

thesis (Chapters IV-VI); briefly, the ovule consists of a central nucellus that contains the embryo sac (megagametophyte), one or two integuments, which enclose the nucellus and define the micropyle; and a supporting stalk, referred to as the funiculus. The pistil shares tissues with the vegetative plant body that are necessary for support, nutrition and protection. These tissues include ground tissue, vascular tissue and epidermis. Other tissues are unique to the style and therefore of direct interest in the study of the mechanisms of plant reproduction, such as the transmitting and stigmatic tissues. The transmitting tissue consists in elongated cells that are connected end to end through plasmodesmata; these cells are highly secretory and their secretion products accumulates in the stylar matrix, that is another characteristic histological feature of the differentiated transmitting tissue (Fig. I.2 E). The upper region of the style differentiates into the secretory structure of the stigma. Tobacco stigma is classified as “wet”, as at the receptive stage the surface is covered with a sticky exudate secreted by the cells of the secretory zone. Stigma development includes cell proliferation, formation of stigmatic papillae and secretion of compounds in the stigma exudate (Fig. I.2 C-D). Stigmaless transgenic plants were produced by expression of stigma-specific cytotoxic gene barnase (Goldman et al. 1994); in absence of the stigma, the top of the transmitting tract of the style presents a surface that resembles a dry stigma surface; tobacco pollen cannot germinate, nor penetrate this “novel” dry stigma surface, but it was shown that application of stigmatic exudate on the ablated surface of stigmaless plants restores the capacity of pollen tubes to penetrate the style. These stigmaless tobacco plants allowed understanding stigma development, in particular the differentiation of the secretory zone of the stigma and the transmitting tissue in the style (Wolters-Arts et al. 1996). Production and secretion of exudate seems to be the main function of the secretory zone, and fatty acids are the major component of the exudate in the pistil. Further studies demonstrated that lipids are the essential factor needed for pollen tube to penetrate the stigma and that in the presence of these lipids, pollen tube can even penetrate unrelated tissues, such as leaves (Wolters-Arts et al. 1998).

Pollination

During pollination, pollen grains germinate on the stigmatic surface, each extruding a pollen tube which penetrates the stigmatic tissue and elongates in the extracellular matrix of the transmitting tissue within the style (Kuboyama et al. 1994). Pollen tubes eventually reach the ovary; each of them enters an ovule to deposit the male gametes into the embryo sac for fertilisation (Marubashi and Nakajima 1981). Pollination induces cellular deterioration of the stigma and style that is considered to be meant to facilitate passage of pollen tubes from the stigma to the ovary. However, stigma and style do not only provide only a simple pathway for pollen tube growth but they are essential for proper pollen-pistil interactions and successful fertilisation. Studies of the mid-90's demonstrated that these tissues actually provide signal molecules necessary for acceptance or rejection and for directional pollen tube growth. Pollen-pistil interactions are a complex system that have been widely studied in self-incompatible plants (Kao and Tsukamoto 2004), but it was shown that pollination is mediated by different biochemical and molecular events also in self-compatible plants (Cheung 1995). In self-incompatible plants (e.g. petunia), the mechanism of acceptance/rejection of the pollen involves S allele-specific recognition between stigmatic and stylar S proteins and incompatible pollen (Lee et al. 1994, Sijacic et al. 2004); this response results in pollen inhibition. The self-compatible tobacco produces a floral transmitting tissue-specific glycoprotein (TTS) that attracts pollen tubes and stimulates their growth (Cheung et al. 1995). Since TTS protein are glycosylated, an increasing sugar concentration gradient is generated by the protein distribution through the style. It is possible that the sugar components of the protein provide nutrients to the pollen during its growth. At the molecular level, Wang and colleagues (1996) have shown that pollination induces accentuated poly (A) tail shortening of several stylar mRNAs including the mRNAs encoding for the TTS proteins. This process, which affects stability of mRNA, is mediated by ethylene via a protein phosphorylation-dependent signal transduction pathway. It is well known that pollination induces ethylene release from flowers and accelerates wilting, and the role of this hormone in plant reproduction has been studied in a number of flower types mainly with regards to pollen tube/style interaction and pollination-induced flower senescence. In tobacco, as in petunia and in other plant species

pollination induces ethylene release from the flower. A style-specific receptor-like protein kinase that may be involved in pollen recognition, and whose expression was increased by ethylene was described in tobacco (Li and Gray 1997). Together, these results suggest a possible involvement of ethylene in the mechanism of pollen recognition.

Tobacco produces self-compatible flowers and exhibits a characteristic "unilateral incompatibility", namely incongruity when pollinated with pollen of other Nicotiana species (Kostoff 1930). Kuboyama and colleagues (1994) described the unilateral incongruity of *Nicotiana repanda*, *Nicotiana rustica* and *Nicotiana trigonophylla* pollen in pollination on the tobacco pistil. Also pollen from *Petunia hybrida* (petunia) is able to germinate, and pollen tubes penetrate the pistil tissues of tobacco even if less efficiently than in self-pollinated tobacco. Together, the use of these different types of pollen sources (tobacco, repanda, rustica, trigonophylla and petunia) provided a tool to study the local responses of the pistil to pollen tube growth in the stigma, the style, and the ovary. Using this model, ethylene release in tobacco flowers after inter-specific crosses was studied. Results (described in Chapter III) show a correlation between ethylene evolution and pollen tube growth/arrest, suggesting that ethylene may be involved in the mechanism of pollen recognition in self-compatible plants.

Pollination also induces ovary development in orchids; in orchids the ovary is immature and lacks ovules until pollination triggers maturation of the ovary and differentiation of ovule within. This is the most extreme example of pollination-regulated ovary development and allowed the dissection of the regulation and progression of ovule and embryo sac development (Zhang and O'Neill 1993). The role of pollination-induced ethylene in early ovary development was underestimated probably because the growth response of this organ was attributed to a direct effect of auxin, but in orchids it was clearly demonstrated that ovary and gametophyte development are co-ordinately regulated by auxin and ethylene following pollination. In most flowers, the ovary matures during flower development and contains fully formed ovules prior to pollination, ready for fertilisation which generally occurs soon after pollination (Bouman 1984). Unlike in orchid, tobacco ovule development is completed before pollination

occurs (Chapter IV). Thus, in tobacco the presence of a pollination induced signal to initiate maturation of the ovule can be excluded.

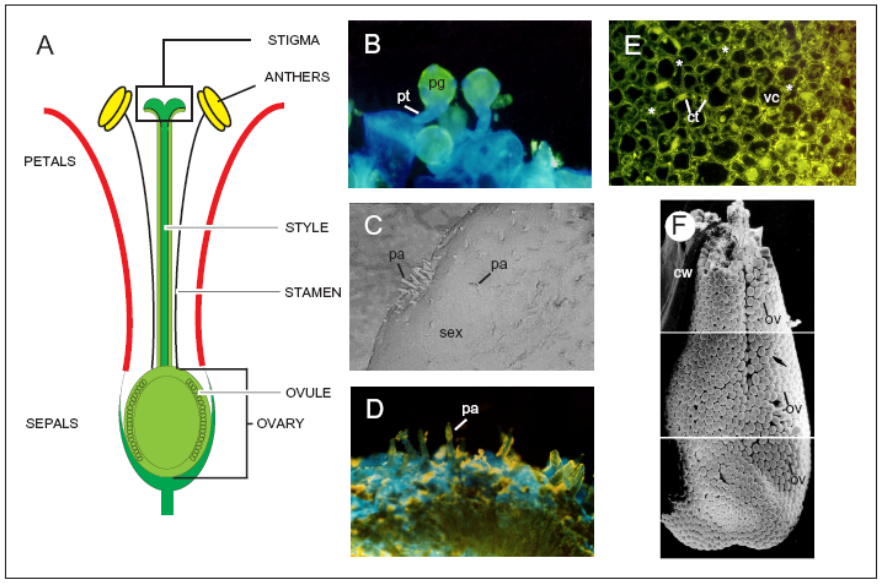

Figure I.2: tobacco flower overview.
(A) Schematic representation of a mature tobacco flower at anthesis (stage 12 of flower development, Goldberg 1988, Koltunow *et al.* 1990). **(B)** UV-Light microscope image of pollen grains germinating on stigma surface. Upon landing on the stigma surface, pollen grains hydrate, germinate and extrude a pollen tube which penetrates the stigmatic tissue and elongates in the extracellular matrix of the transmitting tissue. **(C)** SEM image of the tobacco stigma at anthesis. The stigma was isolated from dissected flowers and directly analysed by SEM, without preliminary fixation. This procedure allows the visualisation of the stigmatic exudate that covers the stigma papillae. **(D)** UV-light microscope image of the stigmatic surface. Stigma papillae protrude out from the stigmatic surface. At anthesis the stigmatic tissue becomes loose and enables secretion of stigmatic exudate (not shown). The yellow staining indicates the cuticle that covers the stigmatic surface until anthesis. Once maturation occurs, this cuticle disappears and the tissue beneath becomes visible (blue stain). **(E)** Transversal section of the stylar transmitting tract tissue at anthesis. Fluorescence (yellow) staining indicate the presence of secretion products in the extracellular matrix. **(F)** SEM image of the tobacco ovary. The tobacco ovary is composed of two carpels and harbours approximately 2000 ovules. A detailed analysis of tobacco ovary and ovule development is provided in Chapters IV and V of this thesis. Figure legend: (cw) carpel wall, (ct) cytoplasm, (*) extracellular matrix, (ov) ovule, (pg) pollen grain, (pa) papillae, (pt) pollen tube, (sex) stigmatic exudate, (vc) vacuole.

Chapter I.3

Photoacoustic Spectroscopy in Trace gas Monitoring: turning light into sound

The *photoacoustic* effect is based on the generation of acoustic waves as a consequence of light absorption (Zharov and Letokhov 1986, Harren and Reuss 1997). For a gas sample in a closed volume, light absorption results in temperature variation that is accompanied by a pressure variation. Turning the light source periodically on and off light absorption results in temperature variation that is accompanied by a pressure variation, which creates a sound wave that can be detected with a sensitive microphone.
For practical trace gas detection, the light source must satisfy two conditions. First, the radiation must be molecule specific, *e.g.* its wavelength should be well defined (narrow bandwidth) and tuneable. Within the infrared region, each molecular gas has its own absorption spectrum in which the absorption strength can vary strongly within a small wavelength interval. Second, it is advantageous to work with high intensities because the absorption signal is proportional to the intensity of the incoming light. An infrared laser provides high intensity, narrow band tuneable light and is therefore ideal to be used in PA detection techniques. Laser photoacoustic allows the detection of a large number of volatile molecules that have biological significance. When applied to the study of the gaseous molecule ethylene the system enabled researchers to measure ethylene release with a time-resolution and sensitivity unreachable with standard procedures.
We applied the LPA system to the study of fruit-fungus interactions, fruit ripening, pollen-pistil interactions, and flower senescence, to obtain an *in vivo* picture of ethylene evolution during both events. Results are described in Chapter II and III of this thesis. This part of the introduction provides some basic information about the LPA system.

History
The photoacoustic effect was already described by Alexander Graham Bell (1880), who reported the photoacoustic effect for the first time writing: *"thin disks of very many substances emitted sounds when exposed to the action of a rapidly interrupted beam of sunlight."* The sound production from those solid samples came from absorption in the infrared part of the solar energy spectrum. Further publications on this phenomenon followed this first work. However, due to the lack of a quantitative description and the lack of a sensitive microphone,

interest in the photoacoustic effect soon declined. In 1938 Viegerov refined the photoacoustic technique for the first spectroscopic gas analysis, thereafter Luft in 1943 measured trace gas absorption spectra with an infrared broadband light source down to the part per million level (ppm).
By the end of the 1960s, after the invention of the laser, scientific interest increased once again. Kerr and Atwood utilised laser photoacoustic detection to obtain the absorption spectrum of small gaseous molecules. Due to the improvement in the technology, they were able to measure low concentration of air pollutants. Later (Patel et al. 1974), the potential of the technique was demonstrated by measuring NO and H2O concentrations at an altitude of 28 km with a balloon-borne spin-flip Raman laser. After this, the photoacoustic effect was introduced into the field of trace gas detection with environmental, biological and medical applications.

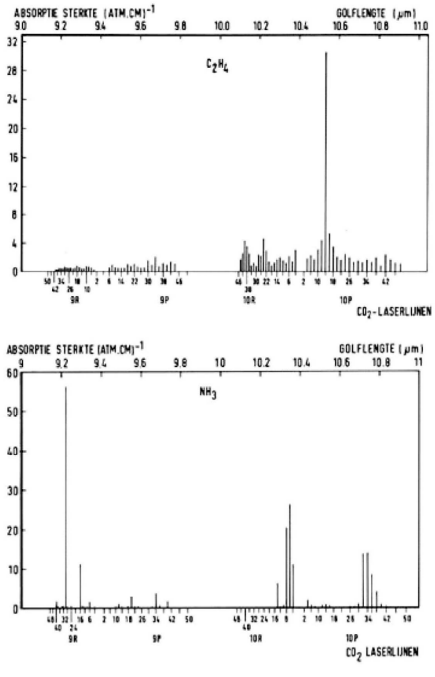

Figure I.3: LPA 'fingerprint-like' absorption pattern.

Each compound has its own set of absorption lines, the fingerprint spectrum. Light absorption capabilities (expressed in absorption cross section, or absorption constant) of a compound vary for each wavelength.

Ethylene (C2H4) has its highest absorption at 10.55 micron,

Ammonia (NH3) absorbes most effectively at 9.22 micron.

Devices and Equipment

In the photoacoustic effect, the pressure amplitude, and subsequently the microphone signal, is proportional to the number of absorbing molecules present in the gas (*e.g.* the trace gas concentration). It is also proportional to the absorption strength of the molecules at a specific light frequency; this results in a 'fingerprint-like' absorption pattern (Fig I.3) over the infrared region that allows the identification of a specific molecule. The microphone signal can be enhanced by increasing the laser power, the use of efficient acoustic amplifiers and by employing sensitive microphones.

The LPA system consists of a line-tunable CO_2 laser emitting radiation in the 9-11 μm infrared wavelength region and a photoacoustic cell, in which the gas is detected (see fig II.1 And III.1). The laser-based ethylene detector is able to distinguish between different gases by making use of their wavelength dependent fingerprint absorption. Trace gases released by the biological samples (*e.g.* flowers, plated fungi or infected fruits) are transported to the photoacoustic cell through a flow system using air as carrier gas. Gas mixtures are sensitively measured by the laser-based ethylene detector due to its distinct fingerprint-like spectrum in the CO_2 laser wavelength range (Brewer 1982). Inside the photoacoustic cell traces of a gas can absorb the laser radiation; the absorbed energy is released into heat, which will create a pressure increase inside a closed volume. By modulating the laser beam with a chopper, pressure waves are generated and detected with a sensitive miniature microphone. The amplitude of the acoustic waves is direct proportional to the concentration of each specific gas in the photoacoustic cell.

Each compound has its own set of absorption lines, the fingerprint spectrum. Light absorption capabilities (expressed in absorption cross section, or absorption constant) of a compound vary for each wavelength.

Ethylene (C_2H_4) has its highest absorption at 10.55 microns, Ammonia (NH_3) absorbs most effectively at 9.22 microns (Fig I.3). It is this property that enables spectroscopic methods to be very selective. Depending on the absorption strength of the gas molecules, the choice of laser frequencies and the experimental conditions, trace gas concentrations can be monitored at ppm, ppb and in some cases even ppt level (parts per million, billion and trillion, table 1).

Combinations of gases can also be measured using the same laser setup.

CO-laser driven photoacoustic spectrometer

CS_2	Carbon disulfide	10 pptv	CH_4	Methane	1 ppbv
CH_3CHO	Acetaldehyde	100 pptv	$S(CH_3)_2$	Dimethylsulfide (DMS)	1 ppbv
H_2O	Water (vapour)	100 pptv	NH_3	Ammonia	1 ppbv
NO_2	Nitrogen dioxide	100 pptv	$N(CH_3)_3$	Trimethylamine	1 ppbv
SO_2	Sulfur dioxide	100 pptv	CH_3CH_2OH	Ethanol	3 ppbv
N_2O	Nitrous oxide	1 ppbv	$CH_3(CH_2)_3CH_3$	Pentane	3 ppbv
NO	Nitric oxide	1 ppbv	CH_3SH	Methanethiol	10 ppbv
C_2H_2	Acetylene	1 ppbv	H_2S	Hydrogen sulfide	1 ppmv
C_2H_6	Ethane	1 ppbv	CO_2	Carbon dioxide	1 ppmv
C_2H_4	Ethylene	1 ppbv			

CO2-laser driven photoacoustic spectrometer

NH3	Ammonia	5 pptv
C2H4	Ethylene	10 pptv
O3	Ozone	20 pptv
H2S	Hydrogen Sulfide	40 ppbv

Table 1: Depending on the absorption strength of the gas molecules, the choise of laser frequencies and the experimental conditions, trace gas concentrations can be monitored at ppm, ppb and in some cases even ppt level (parts per million, billion and trillion). Combinations of gases can also be measured using the same laser setup. The detection limits indicated are best values and have been obtained in the absence of interfering gases.

The gas flow through the measuring system can be controlled using electrical three-way valves that switch a particular gas stream to the photoacoustic cell (on-position) or into the laboratory (off-position). In this way the gas emission from a number of cuvettes containing the biological samples can be measured, transporting the gas to the photoacoustic cell alternately and at controlled flow rates. The flow is adjusted by a flow controller and continuously monitored by a mass flow sensor. To eliminate other interfering gases which may influence the results due to the overlap between their spectral absorption and the CO_2 laser wavelengths, a number of filters and scrubbers must be introduced in the measuring system. With this setup (see also fig II.1 and III.1), the LPA system is operated fully automatically by programmed computer and it can be used to perform continuous measurements for periods up to several weeks.

Applications

Photoacoustic techniques show their full flavour in trace-gas detection for three main reasons: a) the spectral selectivity is exploited; many gases have clear infrared "fingerprint" spectra. b) photoacoustic and related techniques are fast. c) they are sensitive. Trace-gas detection techniques are important in environmental applications, but also for their possibilities in basic science (biology and agriculture). With their help it is possible to discover and control mechanisms such as, in plant physiology, those responsible for germination, blossoming, water household, stress reaction, respiration, senescence, ripening, wounding effects, post-anoxic injury, etc.

Some examples of such application and of the complementarily of laser physics with natural sciences are further described (Annex and Chapters II and III).

Annex

When physic meets biology: use of high-resolution laser-based techniques to study plant-microbe interactions

D. De Martinis. (2003) Mycological Research, 107 (8), 899-900.

Laser technologies were applied to the study of plant-pathogen interaction, in a EC project to monitor the evolution of plant natural compounds, such as the plant hormone ethylene and the plant natural antibiotic resveratrol (3,5,4'-trihydroxystilbene). The recently completed 'Fruta Fresca: improvement of natural resistance in fruit' project was funded in 1999 by a grant from the European Commission to Spain, Italy, The Netherlands, Israel and Switzerland (FAIR CT98-4211). The project allowed the collaboration between "hard-core" physic laboratories with molecular biology and agronomy research teams. The aim of the project was to develop laser techniques to allow fast, highly sensitive and possibly real-time detection of volatiles, such as C_2H_4, C_3H_6, higher alkales, acetaldehyde, hexenal, and non-volatiles, mainly resveratrol, in plant and fruits during fungus attack. Another aim of the project were to identify treatments that could potentially induce pathogen resistance in harvested fruit, possibly by inducing the production of natural compounds with antimicrobial activity, or by external treatment with such "natural antibiotics", in alternative to industrial fungicides. Two laser techniques were developed; a technique based on laser photoacoustic (LPA) spectroscopy that had already been demonstrated to be useful to study bacterial physiology (Staal *et al.* 2001), and a technique based on laser desorption (REMPI-TOF) that has been applied to the study of life sciences for the first time. The LPA system is based on the generation of acoustic waves as a consequence of light absorption. For a gas sample in a closed volume, light absorption results in temperature variation that is accompanied by a pressure variation, which creates a sound wave that can be detected with a sensitive microphone. The system allows the detection of a large number of volatile molecules that have biological significance. When applied to the study of the phytopathogenic fungus *Botrytis cinerea* in tomato fruit and *in vitro*, the sensitivity of the system allowed the measurement of fungus-produced ethylene, almost undetectable with standard procedures. The ethylene emission was correlated to the fungus growth and was compared with ethylene emission from infected fruits to correlate it to the progress of the disease (Cristescu *et al.* 2002). The REMPI-TOF system is based on the analysis of ions generated from a resonant multiphoton ionization system (REMPI) with a time-of-flight mass spectroscope (TOF). This methodology resulted in a highly sensitive system to analyse single molecules even from raw extracts (*e.g.* plant

extracts) without pre-purification steps. When coupled to the LPA system, it allowed the study of the effects of microbial flora on grape fruit (Montero *et al.* 2003), showing the relation between the presence of resveratrol, ethylene emissions and microflora colonisation of the fruit. The antifungal activity of resveratrol was eventually studied in detail also when applied to other fruit types (Jimenez *et al.*, 2005), indicating the potential broad application of such a molecule to control microbial flora growth on fruit. The ability to induce plant defence responses to prolong fruit shelf-life was also evaluated by pre-storage treatments that could result in decay reduction, without loss of quality. It was demonstrated how hot water rinsing and brushing technology, as well as an anoxia treatment resulted in a reduction of decay development in harvested tomato while maintaining fruit quality (Fallik *et al.* 2002, 2003). Although the components that function in the induction of antimicrobial activity in the fruit with these different treatments still needs to be identified, it seems clear that prolonged shelf life of the fruit can be obtained also without using antimicrobial compounds of industrial origin. In conclusion, the collaboration between physics and biology research teams provided new tools to approach the study of plant-microbe interaction, thus allowing the study of pathogen evolution with higher sensitivity and to define new methods to control microbial flora on fresh fruit. Applied laser technologies are a fast moving field that can be adapted to the need of life sciences, and a future evolution of those techniques in user-friendly tools will represent the opportunity to answer old questions with new approaches.

For a detailed description of the LPA system, please consult *http://www-tracegasfac.sci.kun.nl/*

For the complete list of publications and patents of FAIR CT98-4211, contact: Prof. A. Ureña, Universidad Complutense de Madrid, *laseres@eucmax.sim.ucm.es*

Scope of the Thesis

This thesis is focused on the role of ethylene in mediating plant responses, specific to the flower and the fruit, and ethylene signalling. Different aspects of the plant life cycle have been studied at physiological, biochemical and molecular level, from early flower development, to pollination to fruit spoilage. Since ethylene is involved in so many processes, it has been necessary to approach the scientific question using a broad spectrum of technologies that enabled us to monitor ethylene presence, describe the related developmental effect, quantify the effect, and even control ethylene production. To do this, photoacoustic spectroscopy, gas chromatography, microscopy, immunochemistry, molecular biology and genetic engineering were used. Among the events that result in ethylene synthesis are plant-pathogen interaction, fruit ripening, pollination and flower senescence. In chapters II and III two aspects of cell-cell interactions related to those events are studied; fruit pathogenesis by the grey mould *Botrytis cinerea*, and flower responses to pollination. Both events are described by means of laser photoacoustic spectroscopy that enabled us to describe the specific pattern of ethylene synthesis related to the different types of interactions.

Chapters IV to VI describe flower development with special attention to ovule development and the role of ethylene to affect megasporogenesis and subsequent fertilisation. This topic has been approached essentially by means of molecular biology and genetic engineering to characterise the pattern of expression of the gene encoding the ethylene-forming-enzyme ACC oxidase (ACO), and to produce transgenic plants in which ACO gene expression was greatly reduced. Results indicated that the plant hormone ethylene is required for proper ovule development, and suggested the presence of flower-specific, ethylene-inducible transcription factors that may regulate the expression of genes necessary for ovule development. This hypothesis is explored in chapter VII where the cloning of flower-related EREBPs and the characterisation of their pattern of transcription in different plant tissues and in the pistil during flower development and pollination are described.

Chapter II

Ethylene production by *Botrytis cinerea* in *vitro* and in tomato fruit

Cristescu *et al.* (2002). Applied and Environmental Microbiology, 68 (11) 5342-50.

ABSTRACT
A laser-based ethylene detector was used for on-line monitoring of ethylene released by the phytopathogenic fungus *Botrytis cinerea in vitro* and in tomato fruit. Ethylene data were combined with the results of a cytological analysis of germination of *B. cinerea* conidia and hyphal growth. We found that aminoethoxyvinylglycine and aminooxyacetic acid, which are competitive inhibitors of the 1-aminocyclopropane-1-carboxylic acid pathway, did not inhibit the ethylene emission by *B. cinerea* and that the fungus most likely produces ethylene via the 2-keto-4-methylthiobutyric acid pathway. *B. cinerea* is able to produce ethylene *in vitro*, and the emission of ethylene follow the pattern that is associated with hyphal growth rather than the germination of conidia. Ethylene production *in vitro* depended on the L-methionine concentration added to the plating medium. Higher values and higher emission rates were observed when the concentration of conidia was increased. Compared with the ethylene released by the fungus, the infection-related ethylene produced by two tomato cultivars (cultivars Money Maker and Daniela) followed a similar pattern, but the levels of emission were 100-fold higher. The time evolution of enhanced ethylene production by the infected tomatoes and the cytological observations indicate that ethylene emission by the tomato-fungus system is not triggered by the ethylene produced by *B. cinerea*, although it is strongly synchronized with the growth rate of the fungus inside the tomato.

Introduction
The gaseous plant hormone ethylene regulates various physiological processes ranging from seed germination to organ senescence and is involved in the reactions to abiotic and biotic stresses (Abeles *et al.* 1992, Mattoo and Suttle 1991, Pazout *et al.* 1982). An increase in ethylene production is frequently observed during the interaction between a host and a pathogen (Abeles *et al.* 1992). It has been suggested that ethylene released during infection represents an early response of plants to the perception of a pathogen attack and can be associated with induction of a defence reaction (Boller 1991). On the other hand, ethylene is considered to be very important in the development of disease symptoms (Mattoo and Suttle 1991). However, its role in pathogenesis and resistance is far from clear (Boller 1991, Dong 1998, Feys and Parker 2000).

Some microorganisms, including phytopathogenic fungi and bacteria, can synthesize ethylene themselves; therefore, analysis of ethylene emission from a host-pathogen system is a complex problem. Plants can produce ethylene via the 1-aminocyclopropane-1-carboxylic acid (ACC) pathway (Yang and Hoffman 1984). In microorganisms there are two known pathways for ethylene biosynthesis (Fukuda *et al.* 1993). Ethylene can be produced either via 2-keto-4-methylbutyric acid (KMBA), as it is in *Escherichia coli* (Ince and Knowles 1986) and *Cryptococcus albidus* (Fukuda *et al.* 1989b), or via 2-oxoglutarate, as it is, for example, in *Penicillium digitatum* (Fukuda *et al.* 1989a) and *Pseudomonas syringae* (Nagahama *et al.* 1991).

The fungus *Botrytis cinerea* is a plant necrotrophic pathogen that colonizes senescent or dead plant tissues and softening fruits, causing grey mould. Fungal hyphae can penetrate through wounds or natural openings of the plant tissue and spread from previously colonized dead tissues into healthy tissues. This fungus is a major cause of postharvest rot of perishable plant products, including tomatoes at harvest and in storage. Since it is also able to infect at low temperatures, it can result in important economic losses in either pre- or postharvest crops (Mansfield 1980).

The ability of *B. cinerea* to adapt to various environmental conditions has been well investigated, and different mechanisms for its action in attacked host tissue have been proposed (Barkai-Golan 1988, Elad 1997, ten Have *et al.* 2001, von Tiedemann 1997, Zuckermann *et al.* 1997). These mechanisms include hydrolytic enzymes secreted during germination (Elad 1997), other cell wall-degrading enzymes which have increased activity in infected ripening fruit (Barkai-Golan 1988), and active oxygen species that induce cell death caused by *B. cinerea* (von Tiedemann 1997).

Usually, grey mould development is associated with an increase in ethylene production from the infected tissues, which is most often attributed to the host plant (Elad and Eversen 1995). Previously, it was reported that *B. cinerea* grown on various nutrient sources did not produce ethylene (Elad 1990). This result contradicts the later finding (Qadir *et al.* 1997) that ethylene is released by *B. cinerea* when it is grown on a defined medium. The mechanism used by the fungus to synthesize ethylene is not known. There is limited information available about ethylene production by *B. cinerea*; the possible role of fungus-produced ethylene in *B. cinerea* has been debated over the

years (Barkai-Golan 1989, El-Kazzaz *et al.* 1983, Kepczynska 1989, Kepczynska 1993, Qadir *et al.* 1997), and controversial conclusions concerning the role of ethylene in spore germination and mycelium growth have been described. More recently, it has been reported that ethylene is a primary marker for fruit pathogenesis, and several other infection-related plant products have also been studied as early markers of pathogenesis (Polevaya *et al.* 2002).

In order to improve our knowledge about grey mould development in plant tissues and the role of ethylene during the host-pathogen interaction, we studied ethylene production by *B. cinerea in vitro* with regard to the effects of culture media and ethylene production by infected tomato fruits. The ability of *B. cinerea* to synthesize ethylene from L-methionine either via the ACC pathway or via the KMBA pathway was examined. The aim of this study was to determine the relationship between ethylene released by the fungus *in vitro* and the enhanced ethylene production in *B. cinerea*-infected tomatoes with respect to disease development.

To do this, we used a laser-based ethylene detector (Fig. II.1) which has high sensitivity and relatively high time resolution. This instrument allows on-line monitoring of ethylene emission in a flowthrough system with a detection limit of 10 parts per trillion (Bijnen 1996). To our knowledge, this is the first report of real-time monitoring of ethylene production by *B. cinerea* and infected tissue. Use of the laser-based ethylene detector in combination with a broad range of well-characterized biochemicals related to ethylene biosynthesis allowed us to produce a comprehensive description of the mode of ethylene formation and action by *B. cinerea* both *in vitro* and *in vivo*.

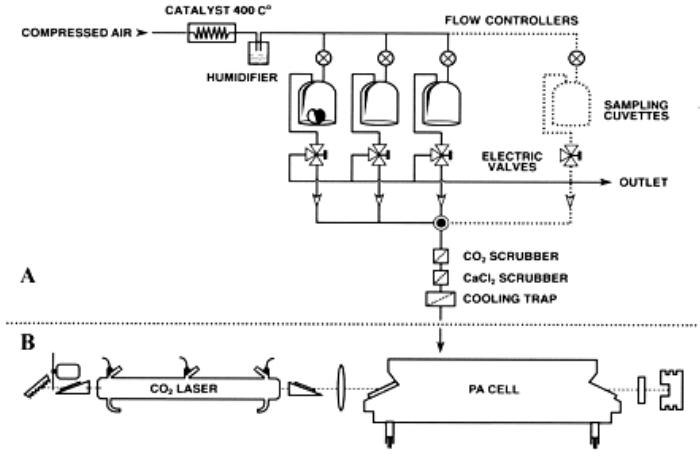

Figure II.1: The ethylene detection set-up. A) gas flow system. B) laser-based ethylene detector consisting of a photoacoustic (PA) cell placed inside the CO_2 laser.

Results

Ethylene production by *B. cinerea*.

We measured ethylene emission from *B. cinerea* grown on PDA and PDA supplemented with L-methionine and analyzed the morphology of fungal development *in vitro*. Previously, it was estimated that very little ethylene was produced by *B. cinerea* in the absence of L-methionine in basal media, and there was no quantification or description of ethylene evolution over time (Qadir *et al.* 1997). Our measurements proved that *B. cinerea* produced small amounts of C_2H_4 when it was grown *in vitro* on PDA without L-methionine. By using the laser-based ethylene detector, the C_2H_4 released by the plated conidia could be quantitatively monitored every 72 s. A typical pattern is shown in Fig. II.2A. Constant emission at a rate of 0.17 ± 0.04 nl h^{-1} was detected during the first 24 h for 160 µl of a suspension containing 2×10^7 conidia ml^{-1}. The rate of ethylene production increased to a peak of about 1 ± 0.05 nl h^{-1} 43 h after the conidia were plated on PDA, after which it decreased to 0.2 ± 0.04 nl h^{-1}. As controls we used 160 µl of an autoclaved conidial suspension and 160 µl of autoclaved hyphae plated on PDA (data not shown). In these cases, no increase in ethylene emission was observed over a 3-day

period. The equivalent rates of ethylene production by the controls, representing the non-enzymatically produced ethylene background, were constant and were 0.18 ± 0.05 nl h^{-1} for the autoclaved conidial suspension and 0.18 ± 0.04 nl h^{-1} for the autoclaved hyphae. When only PDA alone was monitored (with no conidia or autoclaved sample plated on it), the ethylene detector measured a constant background value of 0.17 ± 0.03 nl h^{-1}. Ethylene emission from plated conidia increased substantially if L-methionine was added to the growth medium. We monitored on-line ethylene release from 2 x 10^7 *B. cinerea* conidia ml^{-1} (160 µl of conidial suspension) plated on PDA containing different concentrations of L-methionine (between 0.05 and 70 mM). The pattern of ethylene production (Fig. II.2B) was similar to the pattern for the fungus grown on PDA without L-methionine. The rate of production started at low values, increased to a peak about 43 h after the conidia were plated, and then declined to the background level. Long-term experiments over more than 2 weeks showed that the background level also stayed constant during sporulation (data not shown). Ethylene production increased as the L-methionine concentration increased up to 10 mM, reached a plateau at 10 to 15 mM L-methionine, and decreased when more L-methionine was added to the growth medium (Fig. II.2C). However, some variability in ethylene production at L-methionine concentrations less than 20 mM was observed. To eliminate such variables in our study, all of the experiments described below were performed with L-methionine concentrations of 25 to 35 mM, at which low variability was found. Radial growth was slightly less for media containing L-methionine than for medium without L-methionine, although no significant variations were observed for media containing different L-methionine concentrations (Fig. II.2C). Ethylene emission by *B. cinerea* was also found to be dependent on the concentration of conidia plated on the growth medium.

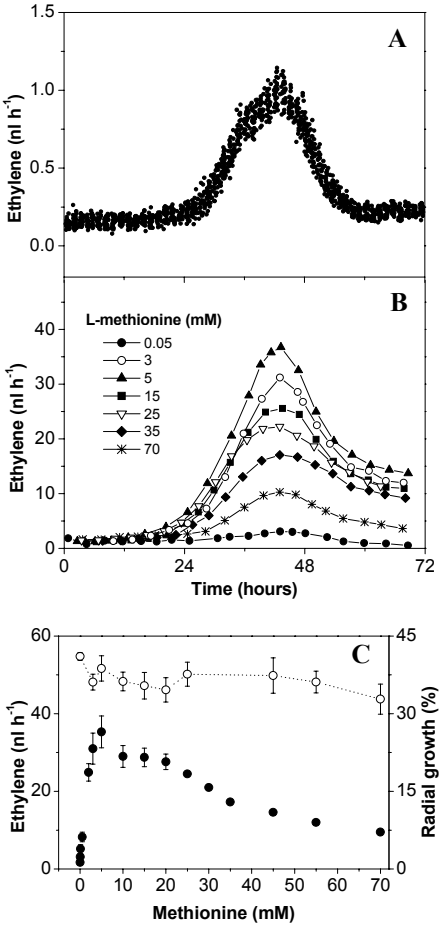

Figure II.2 Ethylene released by *B. cinerea* in vitro.
Fungus (160 µl of a conidial suspension containing 2 x 10^7 conidia/ml) was plated at zero time on PDA (only 75% of the data are displayed) (**A**) and PDA supplemented with L-methionine (**B**). For a better overview the results for only 7 of the 15 L-methionine concentrations used are displayed (0.05, 3, 5, 15, 25, 35, and 70 mM). (**C**) Maximum ethylene production (•) and radial growth (○) for different L-methionine concentrations. The values are means of the maximum amount of ethylene released at 43 h and the radial growth measured at 43 h; the error bars indicate standard deviations (for L-methionine concentrations greater than 25 mM the standard deviations of the ethylene values are smaller than the symbols).

Figure II.3 shows the amounts of ethylene produced after 160-µl suspensions containing 1.5 x 10^8, 2 x 10^7, and 2 x 10^5 conidia ml^{-1} were plated on PDA containing 25 mM L-methionine. The amount of ethylene released and the rate of production increased as the concentration of conidia increased. Light microscope analysis showed that under the conditions used in our experiments, germination of conidia occurred within the first 3 h after harvesting and plating (Fig. II.4A). At 18 h after plating, elongating hyphae were clearly visible (Fig. II.4B), and at 24 h after plating, before the concentration of ethylene reached the maximum value, the fungal hyphae extensively grew and branched (Fig. II.4C). There was no further differentiation of

the fungus, only continuous hyphal growth, up to 72 h after plating (Fig. II.4D).

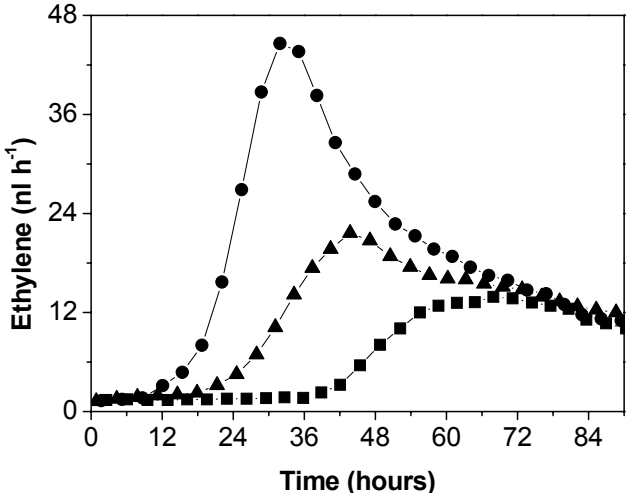

Figure II.3: Ethylene production by the fungus when different concentrations of conidia, (160 µl of a conidial suspension for each concentration) including 1.5 x 10^8 conidia ml-1 (•), 2 x 10^7 conidia ml^{-1} (▲), and 2 x 10^5 conidia ml^{-1} (■), were plated on PDA containing 25 mM L-methionine.

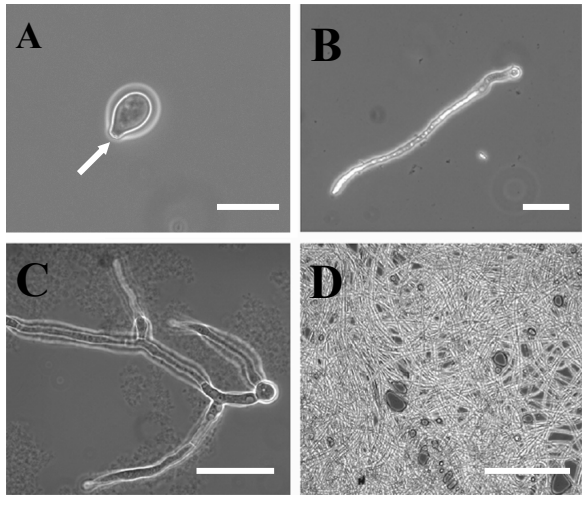

Figure II.4. Cytological observation of germination of B. cinerea conidia and hyphal growth in vitro. Conidia of *B. cinerea* at a concentration of 2 x 10^7conidia ml-1 were plated on PDA supplemented with 25 mM L-methionine. (A) Conidium at 3 h after plating. The conidia are oval, and germination occurs at one end (arrow). Bar = 10 µm. (B) Conidium at 18 h after plating. Hyphal elongation is clearly visible. Bar = 40 µm. (C) Conidium at 24 h after plating. The fungal hyphae are branching and starting to create a dense skein. Bar = 25 µm. (D) Hyphae at 72 h after plating. Fungal growth is proceeding randomly in all directions, and there is no further visible differentiation. Bar = 500 µm.

Ethylene biosynthesis by *B. cinerea*.

The high rate of emission of ethylene by the fungus grown on PDA with L-methionine led to the hypothesis that ethylene is biosynthesized via a pathway in which L-methionine is used as a precursor. Furthermore, addition of another compound, 2-oxoglutarate, to PDA did not increase the amount of ethylene released (data not shown). To investigate the ethylene biosynthesis pathway, AOA (1 and 10 mM) and AVG (0.25 and 5 mM), both of which are competitive inhibitors of ACC synthase, were applied along with the conidial suspension. The results show that although L-methionine stimulated the release of ethylene, no inhibitors of the ACC metabolic pathway were effective in reducing the rate of ethylene production by *B. cinerea* (Fig. II.5, left side). The ability of *B. cinerea* to convert externally applied ACC or KMBA to ethylene was tested by directly adding ACC (10 mM) or KMBA (10 mM) to a conidial suspension placed on PDA without L-methionine (Fig. II.5, right side). The effect of adding 10 mM ACC to PDA was the same as the effect of adding 1 mM ACC (data not shown). The presence of ACC did not stimulate ethylene production, while addition of KMBA resulted in a ~47% increase in ethylene emission compared to the emission of the control. The same results were obtained when these chemicals were added to unsolidified PDA after autoclaving (data not shown).

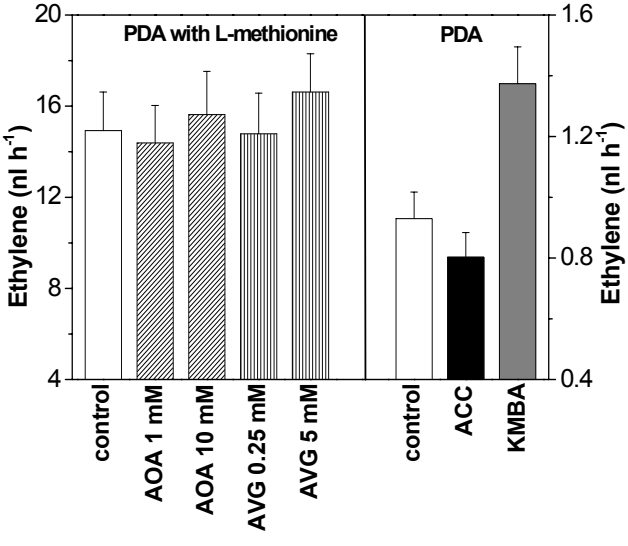

Figure II.5: Ethylene production by *B. cinerea* (concentration, 2 x 10^7 conidia ml^{-1}). At zero time suspensions of conidia containing AOA (1 or 10 mM) or AVG (0.25 or 5 mM) were plated on PDA supplemented with 35 mM L-methionine (left side). Suspensions of conidia containing ACC (10 mM) or KMBA (10 mM) were also plated at zero time on PDA without L-methionine (right side). The control samples contained no additional chemicals.

Ethylene release by *B. cinerea*-infected tomato fruit.

In order to evaluate the relationship between the ethylene produced by *B. cinerea in vitro* and the ethylene released by the fungus-host system, we considered the ethylene responses of artificially inoculated fruits of two tomato cultivars, one which is known to be fast ripening (cultivar Money Maker) and one which is known to be slow ripening (cultivar Daniela). Significant differences between the amounts of ethylene released by infected tomatoes and the amounts of ethylene released by mock-infected fruits of both cultivars were observed (Fig. II.6). When 160-µl portions of conidial suspensions (the same amount that was for *in vitro* ethylene measurements) containing 10^7 to 10^8 conidia ml^{-1} were inoculated inside tomatoes, no visible disease was noted during the first day after inoculation, while our measurements showed that elevated levels of ethylene were released after 6 h. When the signs of infection became visible, there were rapid increases in production, up to four- to sixfold increases; the maximum levels occurred after 36 to 43 h, depending on the concentration of the inoculums. Higher and faster ethylene emission was observed for the fast-ripening cultivar, cultivar Money Maker (Fig. II.6A), than for the slow-ripening cultivar, cultivar Daniela (Fig. II.6B). When a lower inoculum concentration (160 µl of a suspension containing 2×10^5 conidia ml^{-1}) was used, ethylene emission started to increase slowly 24 h after inoculation (Fig. II.6, insets) and reached a maximum level after 70 h. Decay (Fig. II.7) became visible after 48 h, when the rate of ethylene emission started to increase more than it increased during the first 24 h. Faster disease development was observed for cultivar Money Maker infected tomatoes than for cultivar Daniela infected tomatoes. Compared with the ethylene measurements obtained with the fungus *in vitro*, there are two aspects which have to be considered. First, the levels of ethylene produced from *B. cinerea*-infected tomatoes were much higher (more than 100-fold higher for typical 80- to 100-g [fresh weight] tomatoes) than the levels produced by the fungus *in vitro*. Second, the rate of infection-related ethylene production was higher than the corresponding rate for the fungus. For example, the ethylene emission *in vitro* from 160 µl of a suspension containing 2×10^5 conidia ml^{-1} started to increase after 36 h (Fig. II.3), while the ethylene emission from infected tomatoes (both cultivars) inoculated with the same amount and concentration of conidia started to increase in less than 24 h (Fig. II.6, insets). To discriminate

between the ethylene released by the host and the ethylene released by the fungus inside the host, slices of tomato pericarp were imbibed in a solution of AOA (2 mM) to block ethylene biosynthesis and then inoculated with 80 µl of solution containing 2×10^7 conidia ml^{-1} (Fig. II.8). The pattern of ethylene release from an infected slice was similar to the pattern of ethylene release from an infected fruit and showed a maximum about 43 h after inoculation. When the tomato slice was imbibed in AOA, the infection-related ethylene level was three times less than the level of ethylene released by the slice imbibed in distilled water. Very little ethylene was produced by the uninfected slice imbibed in AOA. At 12 h after inoculation, ethylene emission from the uninhibited sample started to increase at high rate, reaching about 9 nl h^{-1} after 24 h. The ethylene released by the AOA-treated and infected slice exhibited the same pattern as the ethylene released by the AOA-treated and uninfected slice, and for both samples a constant level of only 1.5 nl h^{-1} was observed during the first 24 h. This observation confirmed the inhibitory action of AOA on ethylene biosynthesis from an infected slice. Light microscope analysis of a *Botrytis*-infected tomato (data not shown) indicated that fungal growth inside the infected fruit tissue proceeds essentially as it does under *in vitro* conditions; germination of conidia occurred within the first 3 h after inoculation, and after 18 h elongating fungal hyphae were clearly visible. Growth continued with no further differentiation of the fungus up to 72 h after infection.

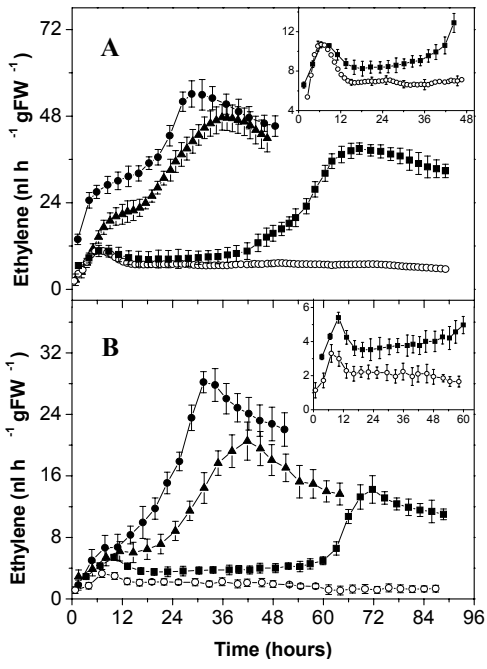

Figure II.6: Ethylene released by tomato cultivars. Money Maker (A) and Daniela (B) that were mock infected (○) or were inoculated with B. cinerea at a concentration of 1.5 x 10^8 conidia ml^{-1} (•), 2 x 10^7 conidia ml^{-1} (▲), or 2 x 10^5 conidia ml^{-1} (■). At zero time tomatoes were inoculated and immediately placed into cuvettes with a continuous airflow at a rate of 3 to 4 liters h^{-1}. The insets show the ethylene emission from mock-infected tomatoes (○) and tomatoes infected with 2 x 10^5 conidia ml^{-1} (■) for the first 2 to 3 days. Measurement was stopped when the fruits were completely deteriorated.

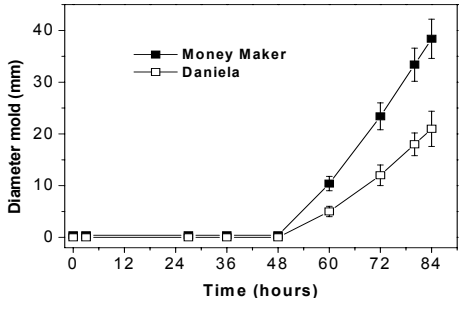

Figure II.7: Development of decay in cultivar. Money Maker (■) and Daniela (□) tomatoes infected with 2 x 10^5 B. cinerea conidia ml-1

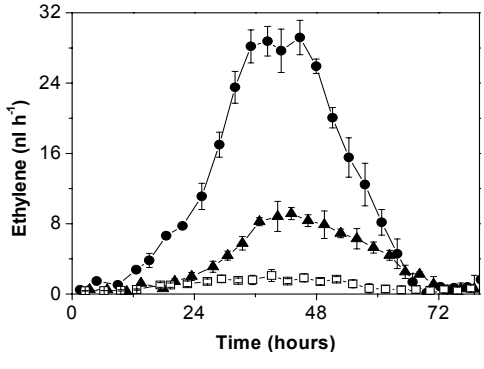

Figure II.8: Slices of tomato pericarp (22 g) imbibed in 20 ml of water (•) or 2 mM AOA (▲) and directly infected with 2 x 10^7 B. cinerea conidia ml^{-1} (two inoculations, 40 µl each). The effect of inhibition of ethylene production by AOA on tomato slices that were not infected was also determined (□).

Discussion

Ethylene production has been thoroughly studied in higher plants (Abeles *et al.* 1992), and it has been shown that ethylene is also released by algae, lichens, fungi, and even humans as a result of lipid peroxidation (Epstein *et al.* 1986, Fukuda *et al.* 1993, Harren *et al.* 1999, Lurie and Garty 1991, Zuckermann *et al.* 1997). In this work, we studied ethylene production and action related to *B. cinerea* and the associated development of grey mould. This fungus is well known for its extremely broad host spectrum; however, the factors which are important for the infection process are still not well understood. The study of fungal infection-related plant hormones is therefore of great interest. Our results showed that the fungus *B. cinerea* is able to produce ethylene *in vitro*; emission can be correlated with fungus growth. When *B. cinerea* grew on media without L-methionine, ethylene release was almost undetectable with standard procedures; use of the laser-based ethylene detector enabled us to detect it. Addition of L-methionine greatly enhanced ethylene production by *B. cinerea*. A small amount of L-methionine (0.05 mM L-methionine) in PDA resulted in up to a threefold increase in the amount of ethylene released compared with the amount of ethylene released by the fungus growing on PDA without L-methionine. The highest rate of ethylene production occurred when 3 to 15 mM L-methionine was added to PDA; ethylene production decreased when higher concentrations of L-methionine were added. Our results obtained by investigating 15 concentrations of L-methionine differ from those reported by Qadir *et al.* (1997), who examined the effects of adding 1, 5, 10, 35, and 50 mM L-methionine to PDA and found that the maximum amount of ethylene was produced by fungus grown on PDA supplemented with 35 mM L-methionine. This may have been due to the fact that large errors were introduced by the integration method over 7 days of ethylene production (Qadir *et al.* 1997). Additionally, Qadir *et al.* used only a few concentrations of L-methionine, and no measurements were obtained for concentrations between 10 and 35 mM or less than 1 mM.

Microorganisms and higher plants can biosynthesize ethylene by different pathways, and in two of these pathways L-methionine is a precursor (Fukuda *et al.* 1993). The most-studied pathway for ethylene biosynthesis is via ACC and occurs mainly in higher plants (Yang and Hoffman 1984), but there are two known pathways for

ethylene formation by microorganisms, namely, the 2-oxoglutarate and KMBA pathways (Fukuda *et al.* 1993). The 2-oxoglutarate pathway does not occur in *B. cinerea* according to our results (data not shown), which confirms the findings of Qadir *et al.* (1997). By using a pharmacological approach, we found that *B. cinerea* most likely uses the KMBA pathway; inhibitors of the ACC ethylene formation pathway had no effect on the emission of ethylene from this fungus, whereas addition of KMBA to the fungus resulted in an increase in ethylene emission compared to the emission in the control grown on PDA without L-methionine. The amount of ethylene released after KMBA is added to a conidial suspension or PDA is less than the amount released in the presence of L-methionine. This can be explained by taking into account the possibility that KMBA is not completely taken up by the fungus or the possibility that *B. cinerea* may use an additional pathway for ethylene formation. Using light microscopy, we observed that ethylene production by the fungus *in vitro* is associated primarily with the period when the most active growth of the fungus occurs. Cytological analyses indicated that germination of conidia occurred before the amount of ethylene released became substantial. Thus, the ethylene released by *B. cinerea* must be associated with hyphal growth rather than with germination of conidia. The production of ethylene *in vitro* depends on the L-methionine concentration added to the plating medium, and higher levels are observed when the concentration of conidia is higher. Note that at higher concentrations of conidia the ethylene emission rate is higher. This finding can be related to fungal neighbourhood sensing.

The level of ethylene production by *B. cinerea*-infected tomatoes was significantly higher than the level in mock-infected tomatoes and started to increase before there was visible decay. Our results demonstrate that ethylene can be considered a sensitive marker for early infection of harvested fresh products, in accordance with a recent study (Polevaya *et al.* 2002). In an earlier work it was shown that higher inoculum concentrations of *B. cinerea* increased infection both in flowers and in leaf removal wounds (Eden *et al.* 1996). We found that in the case of infected tomato fruit the development of decay depends on the inoculum concentration and that decay occurs more rapidly with higher concentrations of conidia. In particular, at higher inoculum concentrations (1.5×10^8 and 2×10^7 conidia ml^{-1}) we observed faster development of decay for cultivar Money Maker

infected tomatoes, which was accompanied by high levels of ethylene release, than for cultivar Daniela infected tomatoes. The difference can be explained by taking into account the fact that cultivar Money Maker is a fast-ripening cultivar and that it's ripening rate can be increased due to ethylene induced by conidia inoculated into the fruit.

The amount of infection-related ethylene produced by both tomato cultivars was much higher (>100-fold higher) than the amount of ethylene released by the fungus *in vitro*, although the evolution patterns were similar. Therefore, ethylene generation by infected tomatoes can be considered a likely response of the host to the stress caused by *B. cinerea* infection.

For the fungus *in vitro*, we found that exogenous application of ethylene by fumigation with 10 or 20 ppb or 1 ppm of C_2H_4 in air did not affect conidial germination or hyphal growth (data not shown). In this case, the ethylene produced by the fungus and its temporal evolution were similar to the results obtained for the fungus *in vitro* that was not fumigated (data not shown). Thus, the presence of ethylene may provide an advantage to the fungus indirectly, because it stimulates the softening of the plant tissue and therefore facilitates tissue penetration and fungal spread (Diaz *et al.* 2002).

However, in our conditions, the ethylene levels produced by the fungus *in vitro* were too low even compared with the levels of ethylene released by mock-infected fruits to be considered substantial in inducing fruit spoilage. Moreover, enhanced formation of ethylene by the infected tomatoes was observed before the amount of ethylene released by the fungus *in vitro* started to increase. Cytological analysis indicated that germination of conidia and fungal growth inside the fruit are similar to germination of conidia and fungal growth under vitro conditions and that equivalent dynamics of ethylene release correspond to similar fungal growth.

These results led us to the conclusion that ethylene production by *B. cinerea* does not trigger the emission of ethylene by the tomato-fungus system. Rather, the emission is synchronized with the growth rate of the fungus inside the tomato.

Experiments performed with slices of tomato pericarp showed that it is possible to use the laser-based ethylene detector to determine the contribution of ethylene by the fungus and, separately, the contribution of the infected fruit tissue. Inhibition of ethylene biosynthesis in *Botrytis*-infected tomato pericarp with AOA prior to inoculation

significantly decreased the ethylene emission. In this case, the ethylene released from the infected tissue could be produced by the fungus itself, as we demonstrated in the *in vitro* experiments, and could also be partially released by the tissue due to possible stimulation of ethylene production by the fungus. Our results demonstrate that the activity of the inhibitor was strong enough to inhibit the release of ethylene from the tomato after inoculation. Moreover, the ethylene evolution from an AOA-treated and infected slice showed a pattern similar to that of ethylene production by the fungus *in vitro* (Fig. II.2B). Additionally, the level of ethylene emission from a tomato slice imbibed with AOA is very low, and consequently, the ethylene produced from an AOA-inhibited and *B. cinerea*-infected slice should be attributed to the fungus more than to residual tissue activity. From these observations we concluded that the ethylene released by *Botrytis*-infected tomato pericarp treated with AOA was primarily due to emission by fungal hyphae.

The decrease in ethylene emission from an infected tomato after the peak was reached corresponded with an advanced stage of the fungal infection. It has been suggested that during the infection process the infected tissue gradually loses the capacity to convert ACC to ethylene and that in the last stages of decay the host ethylene-producing mechanism is suppressed (Achilea 1985).

In summary, our observations indicate that *B. cinerea* is able to produce ethylene autonomously *in vitro* and in tomato fruit and to induce ethylene synthesis by an infected tomato due to its own growth. We have shown that a laser-based ethylene detector is a suitable instrument for on-line measurement of ethylene released by a fungus *in vitro* and *in vivo*. Moreover, this instrument is a powerful tool for early detection of traces of ethylene released in vegetal tissue infected by microorganisms (fungi or bacteria) after long periods of incubation (weeks). Its high sensitivity and fast response should allow workers to investigate new aspects of the temporal and functional relationships between fungal and plant ethylene biosynthesis. This may lead to future applications in postharvest technologies based on alternative strategies for fresh plant produce protection, mainly focused on the activity of the fungus rather that on inhibition of plant-produced ethylene, which usually associate enhanced shelf life with decreased flavour and quality.

The role played by ethylene in the infection process remains to be clarified. The new molecular tools for studying *B. cinerea* molecular biology described in the late 1990s (ten Have *et al.* 2001, Wubben *et al.* 2000) should enable characterization of the role of fungus-produced ethylene in pathogenesis. Isolation of the genes involved in ethylene biosynthesis by the fungus and deletion of these genes should reveal whether ethylene production by the fungus plays a role in the fungus-plant interaction. In addition, ethylene perception by the fungus should also be investigated.

Materials and Methods

Growth of fungal strains and isolation of conidia: An isolate of *B. cinerea* (strain VTF1, kindly provided by E. Fallik, ARO-Volcani Centre, Bet-Dagan, Israel) was maintained on potato dextrose agar (PDA) plates for 14 days at 22°C before conidia were isolated. To prepare a conidial suspension, the fungus was removed from the cultured plates by gentle brushing of the plate surfaces with a sterile platinum loop and was suspended in distilled water. The fungal suspension was filtered through two layers of gauze to separate the conidia. The concentration of conidia was determined by using a Neubauer counter (0.0025 mm^2) and a light microscope. Three concentrations, 1.5 x 10^8, 2 x 10^7, and 2 x 10^5 conidia ml^{-1}, were prepared in sterilized water supplemented with 0.03% Tween 20 to ensure uniform distribution of the conidia.

***In vitro* fungal growth for ethylene measurement**: To investigate the ability of *B. cinerea* to produce ethylene *in vitro*, strain VTF1 was grown in both PDA and PDA supplemented with different concentrations of L-methionine, an ethylene precursor (15 concentrations between 0.05 and 70 mM were used). The precursor was added to PDA, and the pH of each medium was then adjusted to 3.8 before autoclaving. Portions (160 µl) of a suspension containing a specific concentration of conidia were uniformly plated by placing 40 4-µl droplets on 9-cm-diameter Petri dishes containing 25 ml of solidified PDA. The plates were then immediately placed inside a closed glass cuvette for ethylene measurement with the laser-based ethylene detector and flushed with humidified air at a continuous flow rate of 2 to 3 litres h^{-1} and at atmospheric pressure.

Chemical treatment of the fungus: To study inhibition of ethylene biosynthesis, we used two inhibitors of ethylene biosynthesis via the ACC pathway, aminoethoxyvinylglycine (AVG) at concentrations of 0.25, 2.5, and 5 mM and aminooxyacetic acid (AOA) at concentrations of 1 and 10 mM. The chemicals were added to suspensions containing 2×10^7 conidia ml^{-1} before they were placed on PDA supplemented with 25 to 35 mM L-methionine. Stimulation of ethylene production was examined by adding ACC or KMBA to a conidial suspension before it was placed on PDA without L-methionine. All the chemicals were provided by Sigma Aldrich. Along with ethylene monitoring with the laser-based ethylene detector, fungal radial growth was measured. A single drop of conidia (40 µl of a suspension containing 2×10^7 conidia ml^{-1}) was spotted onto a Petri dish, and the radial growth of the fungal colony was measured.

Plant material and tissue inoculation: Tomato (*Lycopersicon esculentum*) cultivars Money Maker and Daniela were grown in a greenhouse under natural light supplemented with artificial illumination provided by high-pressure metal halide lamps (Philips type HPI-T 400 W) at 70% relative humidity with 18-h photoperiods by using 25°C in the light period and 18°C in the dark period. Tomato fruits were harvested at the pink-light red stage of development (according to the U.S. Department of Agriculture, Agricultural Marketing Service, standard grading system for fresh tomatoes), and each fruit surface was gently cleaned with a wet pad before inoculation. Four injections, each consisting of 40 µl of a conidial suspension, were performed at an equatorial site with a needle head (diameter, 0.8 mm) at constant depth of 2 mm below the fruit skin. Fruits injected similarly with 40 µl of sterile distilled water per injection served as controls (mock-infected fruits). Immediately after inoculation the fruits were placed into a glass cuvette (0.7 litre) connected to the laser-based ethylene detector and flushed with humidified air at a continuous flow rate of 3 to 4 litres h^{-1} and at a total pressure of 1 atm. The development of decay was monitored, and the extent of decay was expressed as the lesion diameter (in millimetres). Experiments were also performed with single slices of tomato pericarp (cultivar Daniela; fresh weight, 22 g; thickness, 5 mm); two inoculations, each consisting of 40 µl of a suspension containing 2 x

10^7 conidia ml^{-1}, were used. The slices were then placed on Petri dishes and imbibed in 20 ml of distilled water or a solution of AOA (2 mM).

Real-time monitoring of ethylene production: Ethylene production was monitored in real time by using a sensitive laser-based ethylene detector in combination with a gas flowthrough system (Bijnen 1996). A schematic diagram of the setup is shown in Fig. II.1. A detailed description of the system has been given elsewhere (Harren and Reuss 1997 , te Lintel Hekkert *et al.* 1998). Briefly, the detector consists of a line-tuneable CO_2 laser emitting radiation in the 9- to 11-µm infrared wavelength region and a photoacoustic cell, in which the gas is detected. The laser-based ethylene detector is able to distinguish between different gases by making use of their wavelength-dependent fingerprint absorption characteristics. Trace gases released by the biological samples (plated fungi or infected fruits) were transported to the photoacoustic cell through a flow system by using air as the carrier gas. Ethylene gas mixtures are sensitively measured by the laser-based ethylene detector due to the distinct fingerprint-like spectrum of ethylene in the CO_2 laser wavelength range (Brewer *et al.* 1982). Inside the photoacoustic cell traces of ethylene can absorb the laser radiation; the absorbed energy is released into heat, which creates an increase in pressure inside a closed volume. By modulating the laser beam with a chopper, pressure waves (i.e., sound) are generated and detected with a sensitive miniature microphone. The amplitude of the acoustic waves is directly proportional to the concentration of ethylene in the photoacoustic cell. The gas flow through the measuring system can be controlled by using electrical three-way valves that route a particular gas stream to the photoacoustic cell (on position) or into the laboratory (off position). In this way the gases emitted from a number of cuvettes (up to six cuvettes per experiment) containing the biological samples were transported to the photoacoustic cell alternately and at controlled flow rates, which prevented accumulation-induced effects. Usually, in the off position the gas stream was vented to avoid interruption of the flow through the cuvettes. Switching between different cuvettes with a flow rate of 3 litres h^{-1} caused a delay of 4 min before the photoacoustic cell was completely refilled. The flow was adjusted by a flow controller and was continuously monitored with a mass flow sensor (Brooks

Instruments type 5850 S). The laser-based ethylene detector and the electric three-way valves were operated fully automatically by a computer program and could be used to obtain measurements continuously for periods of up to several weeks. To eliminate other interfering gases which may have influenced the results due to the overlap between their spectral absorption characteristics and the CO_2 laser wavelengths, a number of filters and scrubbers were introduced into the measuring system. A platinum-based catalyser (platinum on Al_2O_3), which operated at a minimum temperature of 400°C and was placed before the entrance of the cuvettes, provided airflow free of any traces of external ethylene (or other hydrocarbons). To measure low ethylene concentrations, it was necessary to reduce the CO_2 and water concentrations before the samples entered the photoacoustic cell. These compounds could introduce undesired supplementary absorption profiles. Even if these gases were not present in the original gas flow, they could be produced in the cuvettes. Consequently, a scrubber with KOH (moist pellets) was used to reduce the CO_2 concentration to less than 1 ppm, and a tube with $CaCl_2$ (granules) was placed directly after this scrubber in order to decrease the water content in the gas. To remove ethanol and some other heavier hydrocarbons, a cooling trap (-150°C) was inserted into the gas flow system just before the photoacoustic cell. The gas was filtered by passing it through 0.2-µm-pore-size Millipore filters placed at the inlet and outlet of the sampling cuvettes. The ethylene levels for an empty cuvette were subtracted from the emission rates obtained. All experiments were conducted under normally illuminated laboratory conditions at a constant temperature of 22°C. The ethylene production by the fungi was related to the emission rate by multiplying the measured value by the flow rate; the results were expressed in nanoliters per hour. For the tomato data the rate of ethylene production was expressed in nanoliters per hour per gram (fresh weight). To obtain a better overview of the ethylene emission rates, we displayed the results of the experiments as averages of the sampling rate every 3 h (the errors due to averaging were smaller than the symbol sizes). Higher sampling rates were used for the ethylene released by fungus grown on PDA without L-methionine. Each experiment was repeated at least six times, and representative data are shown below.

Microscopy: In separate experiments, samples of plated fungi or infected fruits kept under the conditions that were used during ethylene measurements were collected at specific times and analyzed by light microscopy to determine fungal morphology. Mycelium of a plated *B. cinerea* culture was gently scraped from the growth medium (PDA) with a sterile platinum loop. The sample was suspended in water on a glass microscope slide and covered with a glass coverslip. Mycelium growing on an infected fruit surface was also scraped from the fruit and analyzed on a microscope slide as described above. Tissue from the inoculation site was briefly softened in a 0.2 M NaOH solution at 60°C and then gently squashed on a glass microscope slide with a glass coverslip. The microscope slides were examined with a Zeiss AxioPlan 2 Imaging microscope and photographed with a digital camera (AxioCamera), and the images were processed with specialized software (AxioVision).

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Chapter III

Ethylene response to pollen tube growth in *Nicotiana tabacum* flower

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ABSTRACT
In Nicotiana tabacum flowers, pollination induces a transient increase of ethylene production by the pistil. This increase of ethylene is produced with a characteristic dynamic that corresponds to the main steps of the pollen tube journey into the pistil; penetration into the stigma, growth through the style, entrance into the ovary and fertilization. Ethylene is *de novo* synthesized in the pistil, and its production is reduced in the dark. We monitored ethylene production in tobacco flowers after pollination with incongruous pollen from thee different *Nicotiana* species, *N. rustica*, *N. repanda* and *N. trigonophylla* and with *Petunia hybrida.* Pollen from all those different sources can germinate onto the stigma surface but each pollen type shows a different behavior and efficiency in penetrating the pistil tissues. Thus, these different crosses provided a model to study the response of the pistil to pollination and fertilization. Ethylene evolution upon pollination in tobacco differed consistently in each cross, suggesting that ethylene is correlated with the response to pollen tube growth in the tobacco flower.

Introduction
Pollen recognition and acceptance by the pistil tissues are the first steps for a successful fertilisation. During the journey trough the stigma, the transmitting tract of the style and the ovary, the pollen tubes are surrounded by tissues that are believed to provide physical and chemical supports and directional guidance to the pollen tube growth process. Pollen-pistil interactions have been widely studied in self-incompatible flowers, and several mechanisms that allow discriminating among pollen types have been identified (Dickinson 1996, Kao and McCubbin, 1996). Much less is known about the signaling between the pollen and the pistil in self-compatible plants. In general, pollination induces many physiological responses within the flower, including deterioration and death of specific pistil tissues and ethylene evolution, and it is believed that pollination-related modification of the pistil tissues are meant to facilitate the growth of pollen tubes towards the ovary (Cheung, 1995). Wang *et al.* (1996), described the cytological and molecular modifications that occur in the self-compatible *Nicotiana tabacum* (tobacco) flower upon pollination, and suggested that the pollination-induced deterioration in the pistil transmitting tissue is a programmed process to facilitate the

passage of the pollen tubes from the stigma to the ovary, by weakening the transmitting tract of the style and releasing nutrients necessary to the pollen tube during its growth. It was also suggested that ethylene may take part into this programmed process, as ethylene application can itself mimic molecular and cytological modifications similar, but not identical, to that of pollination. To study the relationship between the events that determine pollen recognition and ethylene evolution in the pistil upon pollination we studied the dynamic of pollination using the flower of tobacco as a model system. Tobacco produces self-compatible flowers and exhibits a characteristic "unilateral incompatibility", namely incongruity when pollinated with pollen of other *Nicotiana* species (Kostoff, 1930). Kuboyama and colleagues (1994) described the unilateral incongruity of *Nicotiana repanda, Nicotiana rustica* and *Nicotiana trigonophylla* pollen in pollination on the tobacco pistil. Briefly, *N. repanda* (repanda) pollen tubes are arrested in the stigma after pollination and no growth can be observed 3h after pollination. *N. rustica* (rustica) pollen tubes keep growing faster than that of self-pollinated tobacco for 12 h after pollination and then loose their direction, swell and wound about in the middle of the style, to stop growing 24 h after pollination. *N. trigonophylla* (trigonophylla) pollen tubes grow at a slower rate than those of tobacco. It seems that pollen tubes continue growing gradually until flower abscission. Moreover, also pollen from *Petunia hybrida* (petunia) is able to germinate, and pollen tubes penetrate the pistil tissues of tobacco even if less efficiently than in self-pollinated tobacco. However, petunia pollen is able to induce similar cytological and molecular changes as those described for tobacco self-pollination (Wang *et al.* 1996). Together, the use of these different types of pollen tubes growth (tobacco, repanda, rustica, trigonophylla and petunia) provides a tool to study the local responses of the pistil to pollen tube growth in the stigma, the style, and the ovary. Using this model, we investigated the ethylene release in tobacco flowers after inter-specific crosses. At molecular level, a detailed description of the expression of ACCsynthase and ACCoxidase coding genes during pollen tube growth in tobacco styles was provided in 2002 (Sanchez and Mariani, 2002 and Woeterings *et al.* 2002) . Here, we directly detected ethylene in these different pollination experiments by mean of a laser photoacoustic system

(Woltering *et al.* 1993; Bijnen *et al.* 1996) that allows real-time and high sensitive ethylene measurement *in vivo*.
In this work, the results show a correlation between ethylene evolution and pollen tube growth/arrest, suggesting that ethylene may be involved in the mechanism of pollen recognition in self-compatible plants.

Results

Ethylene production upon self-pollination in *N. tabacum*

Pollen-pistil interactions can be divided in different phases: landing of the pollen on the stigma surface, pollen hydration and germination, pollen tube penetration into the stigmatic tissues, growth into the transmitting tract of the style, penetration into the ovary and fertilization. These events have been described by several authors (Marubashi and Nakajima 1981, Goldman *et al.* 1992, Kuboyama *et al.* 1994, Cheung *et al.* 1995). To determine the relationship between pollen-pistil interactions and ethylene, we measured ethylene release from the flower upon pollination. Pollinated flowers were sealed in a glass cuvette and flushed with air at a constant rate; the outflow was directed to the laser-based ethylene detection system (Fig. III.1). This experimental set-up enabled us to work with flowers attached to the plant, thus providing *in vivo* measurements that allowed us to preserve the natural physiology of the flower and to perform long-time measurement (up to five days and longer) that usually could not be achieved on cut flowers.
Figure III.2 A shows ethylene evolution from the tobacco flower upon self-pollination. We could distinguish a first peak 3 h after pollination when the pollen tubes are in the stigmatic tissue, and a second peak between 24 and 30 h after pollination, when the pollen tubes reach the end of the style. A third peak was visible at 60 h after pollination, presumably when most of the ovules have been fertilized. Ethylene production is affected by dark and light periods. When a dark period was produced, a sudden drop in ethylene levels was measured. Such reduction was not measured in continuous light conditions. In this situation ethylene levels were higher while the pollen tube grew in the transmitting tract of the style (between 6 and 24 h after pollination), but the main levels of ethylene production and the time of appearance of the peaks remained unchanged. This is not surprising, as a previous

report (Woltering *et al.* 1997) already showed low activity of the ethylene-forming enzyme (ACC-oxidase) in the dark. To eliminate the light as a variable in the study of pollination-induced ethylene evolution, all the experiment further described were performed in continuous light conditions.

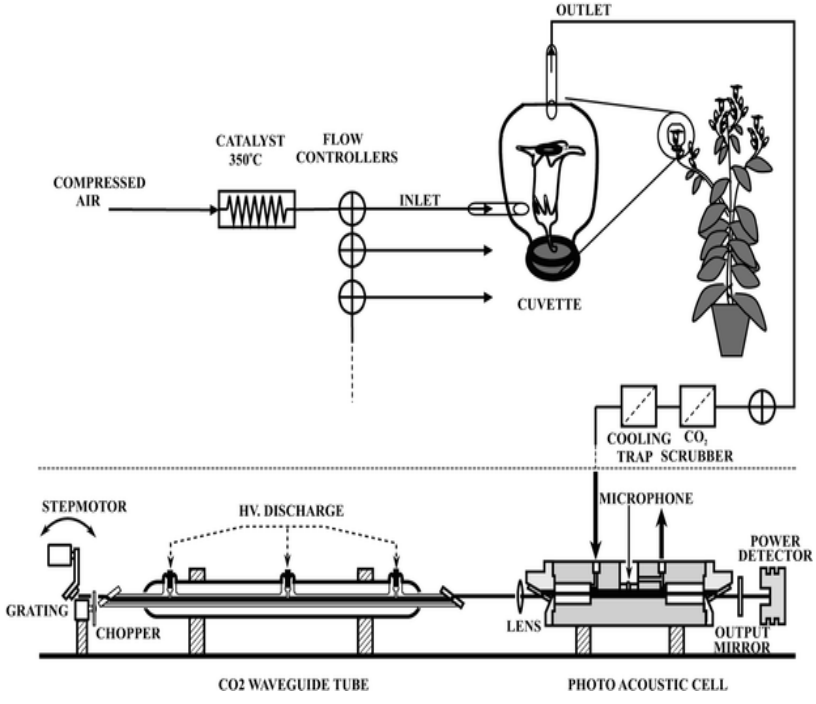


Figure III.1: Experimental set-up for the photoacoustic measurements of C_2H_4 emitted from tobacco flowers.
Flowers were self-pollinated and closed in a glass cuvette. The petiole that attaches the flower to the plant was sealed using soft rubber, terostat and parafilm. Gasses released from the flower were delivered to the photoacoustic cell though a flow-though system containing air as carrier gas. Before air enters the sample cuvette, the hydrocarbons present in air are dissociated into CO_2 and H_2O by a catalyst, and before air enters the photoacoustic cell, a KOH-based scrubber eliminated CO_2 to prevent interference of gas absorption signals with the C_2H_4 photoacoustic signal. Moreover an additional cooling trap was placed after the KOH scrubber to remove other volatile compounds that may cause interference (e.g. water vapor).

Effect of senescence, mock-pollination and stigma applied AVG on ethylene release in tobacco.
When tobacco flowers were emasculated and left unpollinated, produced ethylene at a constant basal level corresponding to 2-4 nl/h for more than 100h (Fig. III.2 B), when senescence occurred. Furthermore, if Aminoethoxy Vinyl Glycine (AVG), an inhibitor of the biosynthesis of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), was applied to the stigma prior to pollination, ethylene production was consistently reduced (Fig. III.2 C). Altogether, these results indicate that neither senescence, nor mock-pollination with heat killed pollen does elicit ethylene production and that upon pollination, ethylene is *de novo* synthesized from the tobacco pistil. Reduction of ethylene biosynthesis however, did not seemed to affect the process of fertilization, as the seed set of self-pollinated tobacco after AVG treatment was comparable to that of untreated flowers (not shown).

Ethylene production in *N. tabacum* x *N. repanda* pollination
In this cross most pollen tubes stopped are arrested in the stigma, and no further growth could be observed 3h after pollination (Kuboyama *et al.* 1994). Ethylene response in this cross-marked clearly pollen tube behavior; a first peak of ethylene raised reaching his maximum 3h after pollination, but decreased rapidly to basal levels. If AVG was applied to the stigma prior to pollination, ethylene production remained to basal levels, as in unpollinated flowers (Fig. III.3 A).

Ethylene production in *N. tabacum* x *N. rustica* pollination
For the first 12h after pollination, rustica pollen tubes grow into the style of tobacco slightly faster than that of self-pollinated tobacco. The growth rate eventually decreased and pollen tube arrested after approximately 24h at about 2/3 of the style (Kuboyama *et al.* 1994). In this pollination (Fig. III.3 B) ethylene evolved with a first peak comparable to that of tobacco self-pollination, which dropped 5h to 10h after pollination to levels of 6 nl/h. Ethylene release increased again between 15 to 25 h after pollination and decreased then gradually to basal levels. Also in this case the first peak of ethylene is produced by *de novo* synthesis; application of AVG prior to pollination decreased pollination-induced ethylene response to levels equal to unpollinated flowers.

Ethylene production in *N. tabacum* x *N. trigonophylla* pollination
Pollen tubes of trigonophylla grow slowly into the tobacco style. 50h after pollination pollen tubes have reached less than 2/3 of the style. Kuboyama and co-workers (1994) reported that pollen tubes seem to continue gradual growth up to 4 days (96 h) after pollination. We could usually follow ethylene emission after pollination up to 5 days (120 h) after pollination. Ethylene production reached a maximum peak of approximately 14-nl/h 3 h after pollination and then decreased to basal levels until the end of the experiment (Fig. III.3 C). It must be noted that sometimes the tobacco flowers were not able to sustain this inter-specific pollination up to 5 day and abscised. However, in 11 measurement that we could perform in this pollination experiments we found always the similar pattern of ethylene release described above, with the exception of 3 experiments in which a second peak at 72 h was produced, ranging around 6-10 nl/h in 2 experiments and 14 nl/h. We verified that in all experiments trigonophylla pollen was able to reach the ovary after 120 h (5 days) but any seed set was never achieved. Also in the case of trigonophylla pollen, the first peak was diminished if AVG was applied to the stigma prior to pollination. Despite the variability observed in ethylene release in this case, we could observe, as with rustica pollen, that in most of the experiments ethylene levels drops to basal levels after the first peak and did not increase although pollen tubes penetrated the style.

Ethylene production in *N. tabacum* x *P. hybrida* pollination
Pollen from petunia germinated efficiently on the tobacco stigma but pollen tube growth was slower than that of self-pollinated tobacco and the pollen tubes reached halfway the style 24 h after pollination (Wang *et al.* 1996 and our unpublished observation). As in all the other measurements we could detect a first peak 3 h after pollination, but significantly lower than in the other crosses (Fig. III.4). Cytological analysis (not shown) indicated that petunia pollen could reach the ovary approximately 48 h after pollination, at the time when also a second peak of ethylene was clearly measured. As in all the other crosses, also in this case the first peak of ethylene was reduced if AVG was applied to the stigma before pollination. As compared to pollination with trigonophylla pollen, pollination of tobacco with petunia pollen always resulted in a seed set comparable to that of self-

pollinated tobacco. Likewise tobacco, AVG treatment did not seemed to affect seed setting that which resulted comparable to that of untreated flowers. In all cases the seeds obtained from pollination of tobacco pistils with petunia pollen were not viable, thus indicating that the inter-specific barriers between tobacco and petunia must be effective during post-fertilization development.

Discussion

Pollen-pistil interaction is a complex system that has been widely studied in self-incompatible plants (Dickinson 1996, Kao and McCubbin, 1996). Self-incompatibility response involves S allele-specific recognition between stigmatic or stylar S proteins and incompatible pollen. This response results in pollen inhibition. However, a number of molecules were shown to have an effect also on compatible pollination. Flavonoids, lipids and some proteins have been observed to be essential for pollen germination and tube growth in the pistil in Petunia, Arabidopsis and Tobacco (reviewed by Cheung, 1995). In general, pollination induces tissue deterioration to facilitate passage of pollen tubes from the stigma to the ovary. It was shown that this process in tobacco maybe mediated by the plant hormone ethylene (Wang *et al.* 1996). In our study we analyzed the kinetic of ethylene response to pollination in tobacco. The use of the laser-based ethylene detection system directly on uncut flowers enabled us to produce a detailed *in vivo* profile of ethylene evolution upon pollination with high sensitivity and high time-resolution if compared to standard gas-chromatography or indirect biochemical approaches. Our results showed that ethylene release in the tobacco flower is a direct consequence of pollination and does not occur during flower senescence. In all the measurements we performed with unpollinated flowers, and also in the measurements with heat-killed pollen, ethylene release did not increase but remained constant despite the increasing petal senescence that was clearly visible, 3-4 days after anthesis. This is in contrast with other studies on flower senescence and pollination i.e. in petunia (Hoekstra and Weges 1986, Singh *et al.* 1992), but it must considered that in most of the previous cases flowers were excised from the plant, thus altering flower physiology and introducing the variables typical of post-harvest physiology. In this study, we distinguished different phases of ethylene production upon self-pollination in tobacco (Fig. III.2 A); a rapid production

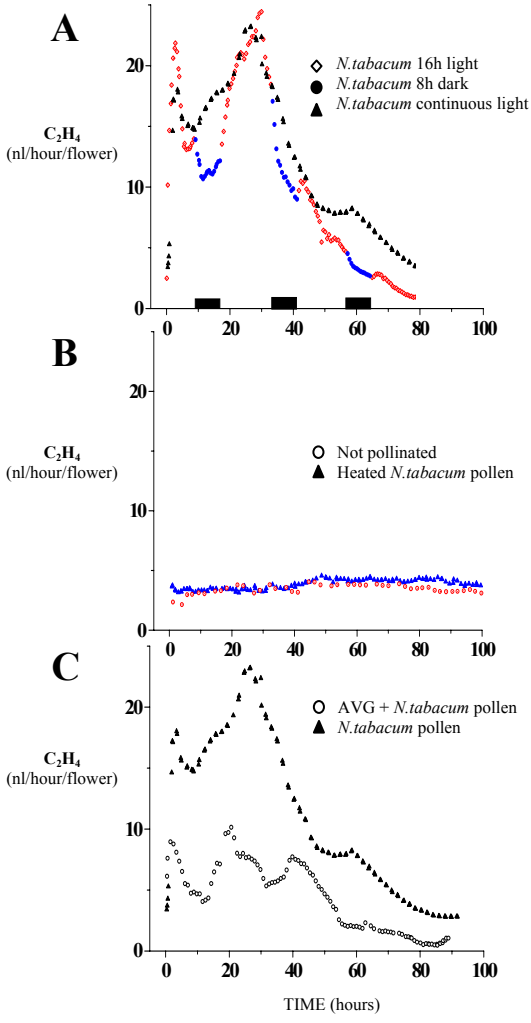
within the first 3h, when pollen tubes penetrate the stigmatic tissues, a subsequent gradual increase in the first 24 h, during pollen tube growth in the style, and a second major peak between 24-30 h after pollination, when pollen tubes reach the ovary. As pollen from many species was found to contain the ethylene precursor ACC, it was widely discussed in different studies whether the first peak of ethylene is a result of the conversion of pollen borne ACC or if it is a *de novo* synthesis by the pistil. Several authors (Hoekstra and Weges, 1986, Woltering *et al.* 1992, Woltering at al. 1997) indicated that the amount of ACC in pollen is usually not sufficient to explain the early ethylene peak and that treatment with AVG, inhibits ACC biosynthesis, can block pollination-induced ethylene production. In *Petunia inflata*, Singh *et al.* (1992) found a correlation between the ACC content in the pollen and the amount of ethylene produced. By a transgenic approach, Lei *et al.* (1996) showed that in genetic engineered Petunia in which pollen ACC content was greatly reduced, pollination-induced ethylene production by the flower was unaffected. Our results showed that if AVG was applied before pollination, the first ethylene peak was reduced. We obtained the same results using pollen from repanda, rustica, trigonophylla and petunia. Moreover if mock-pollination was performed using heat-inactivated pollen, no ethylene response could be monitored. These results prove that ethylene release upon pollination is *de novo* synthesized during the active penetration of the pollen tubes into the stigmatic tissues. Our data suggests that with respect to pollination-induced ethylene production, the stigma responds in the same way to different pollen sources, as the first ethylene peak was induced with the same kinetic and, with the exception of Petunia pollen, approximately to similar levels in all pollinations. In Petunia flowers it was shown how ACC-oxidase transcripts were most abundant in the outer cell layer of the stigma (Tang *et al.* 1994), suggesting that the ethylene produced by the stigma rapidly escapes to the outside air and plays no role in signaling trough the pistil (Woltering *et al.* 1997). Thus, we can conclude that the stigma functions in the pollination process by producing ethylene, but the process is not used by the pistil to discriminate between different pollen types.

After the first peak, ethylene was then produced at increasing amounts that reach the maximum 24-30 h after self-pollination in tobacco, during the period in which pollen tubes grow though the transmitting

tract of the style. Pollen-pistil interactions in the style seem in part to be mediated by style-specific proteins such as TTS 1 and 2 of tobacco that are involved in the nutrition and guidance of the pollen tube (Cheung *et al.* 1995). Pollination induces accentuated poly(A) tail shortening of several mRNAs including the mRNAs encoding for the TTS proteins. This process which affects stability of mRNA is mediated by ethylene via a protein phosphorilation-dependent signal transduction pathway (Wang *et al.* 1996). Moreover, recently it was described a tobacco style specific receptor-like protein kinase that may be involved in pollen recognition and whose expression can be increased by ethylene (Li and Gray, 1997). Our results showed that ethylene production rapidly decreases in pollination with rustica and trigonophylla pollen, despite their active growth into the style. In the case of rustica pollen, ethylene production decreased far before pollen tubes arrested and in the case of trigonophylla ethylene was reduced to background levels after the first peak although the pollen tubes could slowly grow into the style and reach the ovary. The increase of ethylene production after the first peak can be observed only in pollination with self-tobacco pollen or petunia pollen, and those were the only pollinations that resulted in seed set. Together, these results suggest that pollination-induced ethylene production represents a response of the flower to specific pollen recognition. This response may be part of a concerted action that controls pollen acceptance in the self-compatible tobacco.

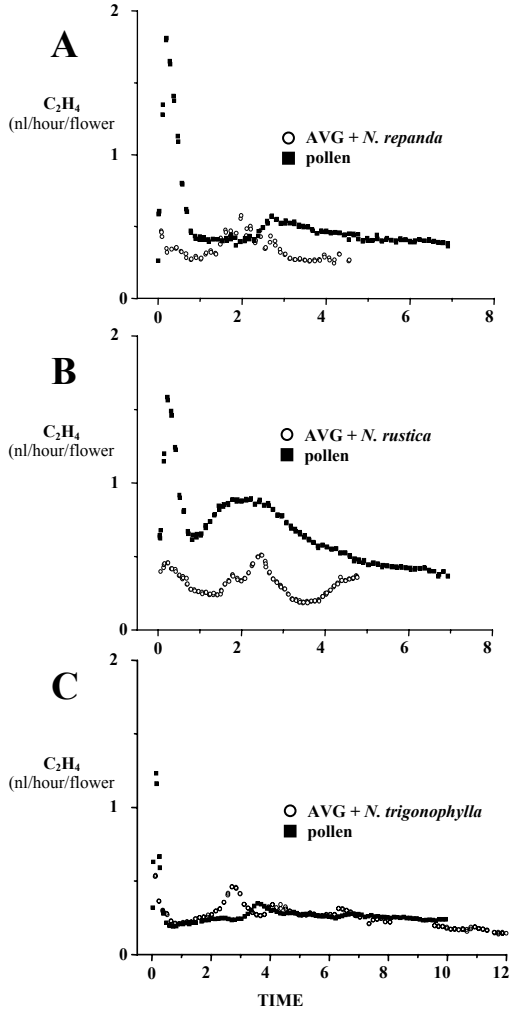
In summary, we described ethylene evolution upon pollination in the tobacco flower using the laser-based ethylene detection system that enabled us to perform *in vivo* measurement with high sensitivity and high time-resolution. Ethylene release by the flower was measured in unpollinated, mock-pollinated or self-pollinated tobacco flowers, or after inhibition of ethylene biosynthesis and in inter-specific crosses. Our results showed that ethylene is *de novo* synthesized upon penetration of the pollen tubes into the style but its production does not correlate with the rate of pollen tube growth into the style but it depends on the type of pollen used. Decreased ethylene production by AVG treatment of the stigma did not affect the process of fertilization self-pollination in tobacco, thus indicating that ethylene alone is not essential for pollen tube growth into the style.

Figure III.2: Ethylene production upon self-pollination in tobacco.



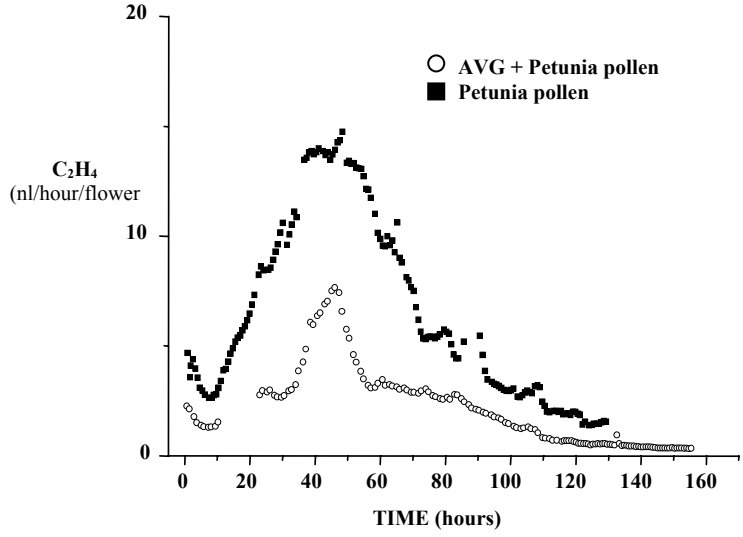
(A) ethylene production in self-pollinated tobacco flowers in continuous light conditions and alternate night and day-light conditions. The dark period (indicated by the black bars on the axis) corresponds to a clear decrease in ethylene release if compared to the ethylene release in continuous light conditions. **(B)** ethylene production in not pollinated and mock-pollinated flowers. If left unpollinated, tobacco flowers release ethylene at basal levels of approximately 4nl/hour; the same result was achieved if pistils were pollinated with heated pollen. Pollen loses completely viability by heating anthers at 60°C for two hours. **(C)** Effect of AVG treatment on pollination-induced ethylene release. 10 ml of 100mM AVG were placed onto the stigma surface 3h before pollination. In control flowers, 10 ml of H_2O was applied onto the stigma 3h before pollination.

Figure III.3: Ethylene production upon pollination of tobacco pistil with repanda, rustica and trigonophylla pollen and effect of AVG treatment.



Stigmas were treated as described in figure III.2 C to reduce ethylene production.
(A) Pollination with repanda pollen and effect of AVG treatment.
(B) Pollination with rustica pollen and effect of AVG treatment.
(C) Pollination with trigonophylla pollen and effect of AVG treatment.

Figure III.4: Ethylene production of tobacco pistil upon pollination with petunia pollen and effect of AVG treatment.



Tobacco stigmas were treated as described in figure III.2 C to reduce ethylene production.

Materials and Methods

Plant Material: We used the self-compatible *Nicotiana tabacum* "Petit Havana" SR1 plants as a pistillate parent and *Nicotiana repanda* Willd, *Nicotiana rustica* L. cv *Rustica, Nicotiana trigonophylla* Dun and the self-incompatible *Petunia hybrida* W166K as pollen parents. All plants were grown under standard greenhouse conditions.

Pollination: Pollination was carried out using mature flowers emasculated one day before anthesis. Immediately after being pollinated the flower was enclosed in a small glass cuvette; the petiole that attaches the flower to the plant was sealed to avoid gas dispersal using soft rubber, terostat and parafilm. A flow of air at 1.5 l/h was continuously passed though the cuvette during the whole experiment, thus avoiding accumulation of gasses in the cuvette itself.

Chemical treatment of the flowers: To inhibit ethylene biosynthesis, 10 µl of a 100mM AVG solution (amino ethoxy vinyl glycine, Sigma)

was applied to the stigma prior pollination. When AVG solution was placed onto the stigmas, a drying period of 2 h was practiced before pollination.

Ethylene measurement: *In vivo* ethylene production was monitored in a flow though system by means of laser photoacoustic spectroscopy (Woltering *et al.* 1988; Voesenek *et al.* 1993; Bijnen *et al.* 1996). Briefly, the detection system consists of a line-tunable infrared CO_2 laser, which emits radiation in the 9-11μm infrared wavelength region, and of a photoacoustic cell, in which the gas is detected (Fig. III.1). The gas absorption was measured at two different infrared laser wavelengths (one weak and one strong absorbing) which allowed calculation of the ethylene concentration in the sample thereby excluding interference from other gases. Selection of the desired wavelength is achieved by adjusting a diffraction grating at one end of the laser tube; a partially reflecting mirror is placed at the other end of the laser, after the photoacoustic cell. Gasses released from the biological samples were then delivered to the photoacoustic cell though a flow-though system (1.5 L/h) containing air as carrier gas. Inside the detection cell the trace gas will absorb the laser light; the absorbed energy is released into heat, which will create inside a fixed volume a pressure increase. By modulating the laser beam with a chopper a pressure wave (e.g. a sound) is generated with a frequency equal to the chopper frequency. A miniature microphone, located in the cell, detects the acoustic signal. The amplitude of the sound wave is proportional to the concentration of ethylene gas in the photoacoustic cell. The gas in the flow-though system is pretreated to improve the detection limit for ethylene and to avoid interference with other volatiles. Before entering the sampling cuvette containing the flower, the airflow was passed though a catalyst to remove hydrocarbon traces by converting them in CO_2 and H_2O. Gasses released from the biological samples were then delivered to the photoacoustic cell though the flow system, but between the sampling cuvette and the photoacoustic cell a KOH-based scrubber eliminated CO_2 without influencing the C_2H_4 concentration. Removal of CO_2 is necessary to avoid interference at the absorption wavelengths used to detect the presence of ethylene. Moreover an additional cooling trap was placed after the KOH scrubber to remove other volatile compound that may

cause interference (e.g. water vapor). With this set-up it was possible to reach a detection limit of 6 ppt for ethylene.

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Chapter IV

Wild-type ovule development in *Nicotiana tabacum*: a light microscope study of cleared whole-mount tissue.

With Celestina Mariani

ABSTRACT
Ovules play a central role in reproduction, generating the female gametophyte within sporophytic integuments. When fertilised, the integuments differentiate into the seed coat and support the development of the embryo and endosperm. The knowledge of ovule morphology and the understanding of the pattern of development is the first step in studying the mechanisms of ovule maturation and successful fertilisation. In this study, a comprehensive description of ovule development in *Nicotiana tabacum* (tobacco) is provided, from the emergence of the ovule primordia to fertilisation. The work is based on a light-microscopical analysis of cleared whole-mount ovules. This technique largely eliminates the need to section and allows the rapid evaluation of a large number of ovules. The study is supplemented with scanning electron microscopy of early developing ovules and with aniline staining to identify callose accumulation during megaspore degeneration and formation of the functional megaspore. Ovule differentiation starts in young flower buds and follows the polygonium-type model; development is constant during flower development and ovules are at the end of the megagametogenesis when the flower reaches anthesis, and pollination occurs. Differentiation and development of ovules in tobacco in relation to the whole flower development were examined. A classification scheme for ovule development is proposed, based on readily assessed flower developmental stages.

Introduction
The ovule, located within the ovary, is a major part of the female reproductive system and the precursor of the seed. It harbours the embryo sac with the egg cell and it is the place where fertilisation takes place. Subsequent embryogenesis and endosperm development occur during seed formation. Ovule morphology has been studied in a large number of plants species (see Bouman, 1984), however little is known about the forces that regulate its development. In the last years, considerable effort has been invested to identify the mechanisms that determine ovule identity, development and fertilisation (Zhang and O'Neill 1993, Hulskamp *et al.* 1995, for review see Angenent and Colombo, 1996, Drews *et al.* 1998, Skinner and Gasser, 2004). A systematic analysis of ovule development has been hampered due to the inaccessibility of the organ within the ovary and the thickness of

the ovules in many species. Therefore, tedious sectioning techniques and electron microscopy had to be performed to study morphology and development. This makes the morphological analysis of ovule-defective mutation cumbersome and time consuming. The emergence of *Arabidopsis thaliana* as a model system for a genetic and molecular approach to investigate developmental processes, enabled the identification of a number of mutants and genes involved in flower and pistil development (Sessions, 1999). In *Arabidopsis*, it was demonstrated that Light-microscopy analysis of cleared whole mount tissues allowed the description of ovule development by the morphological inspection of a large number of ovules, thereby largely eliminating the need for sectioning (Schneitz *et al.* 1995). In this study, a similar approach to describe ovule development in *Nicotiana tabacum* (tobacco) was applied. Although tobacco flower and male gametophyte development were described in detail (Goldberg, 1989, Koltunow *et al.* 1990), a description of ovule development to complement and complete the picture of the tobacco flower was still lacking. A prerequisite for an understanding of the mechanisms of ovule development is its dissection into distinct steps. This initially requires a morphological definition of developmental steps at a high resolution on which a subsequent systematic genetic and molecular analysis can be based. Here, description of the main stages of ovule development in tobacco is presented; from the emergence of the ovule primordia to the events of megasporogenesis, megagametogenesis and fertilisation. This work was based on a clearing technique and conventional light microscopy. It was complemented by scanning electron microscopy and by UV light microscopy of aniline blue stained ovules. Together these techniques allowed the dissection of ovule development and fertilisation and the creation of a classification scheme of ovule developmental stages. This classification is related to the tobacco flower developmental stages previously described (Goldberg, 1989, Koltunow *et al.* 1990). The scheme allows sampling of ovaries that harbour a majority of ovules at the desired specific developmental stage, using as a reference flower developmental stage that is easy-to-score by simple green-house visual inspection.

Results

Flower and pollen development in *N. tabacum* (tobacco) have been described previously (Goldberg, 1988, Koltunow *et al.* 1990).

Nineteen developmental stages, numbered from –7 to 12 were defined on the basis of the morphological and molecular markers. In our analysis of ovary development this classification system is utilised.

Early ovule development and megasporogenesis

The ovary of *N. tabacum* (tobacco) is composed of two carpels that arise from the floral apex in the center of the flower (Fig. I.3). Early developing ovules are composed of the funiculus, which attaches the ovule to the placenta, the integument primordia and the nucellus that harbours the megasporocyte. In young flower buds, ovules arise from the placenta as finger-like structures (Fig. IV.1 A-D). At stage -2 of flower development, the ovules are already visible as protuberances differentiated from the ovararian placenta (Fig. IV.1 A-B). Later on, at stage -1 the ovule protuberance is more clear and at stage 1 differentiation between the nucellus and the integument primordia becomes visible (Fig. IV.1 C-D). Light microscope analysis of cleared ovules revealed that at stage 1 the nucellus harbours a mononucleate megasporocyte (Fig. IV.2 A-B); this is the megaspore mother cell that will produce the haploid megaspores upon meiosis. The megasporocyte is a large cell (compared to the cells of the nucellus and integument) located just beneath the nucellar layer, and increases in size while it is surrounded by the integument (Fig. IV.2 C). During growing and differentiation, the ovule becomes anatropous. The first meiotic division of the megasporogenesis begins at approximately stage 4 of flower development, when the integument surrounds the nucellus almost completely and defines the micropyle. Megasporogenesis starts in young flower buds at stage 4-5; the megasporocyte undergoes meiosis to produce the haploid megaspore (Fig. IV.2 D-E); callose accumulates around the forming megaspores (Fig. IV.3 A) and at the end of the megasporogenesis the nucellar tissue disappears and only one functional megaspore is produced (Fig. IV.2 F). In tobacco, as in other plant species (Bouman, 1984, Angenent and Colombo 1996), the presence of callose accumulation within the nucellus, is a marker of megasporogenesis. Callose surrounds the forming megaspores and causes megaspore degeneration, disappearing when the single functional megaspore divides to form the embryo sac (Fig. IV.3 B-C). At stage 9 of flower development, all ovules present a functional megaspore.

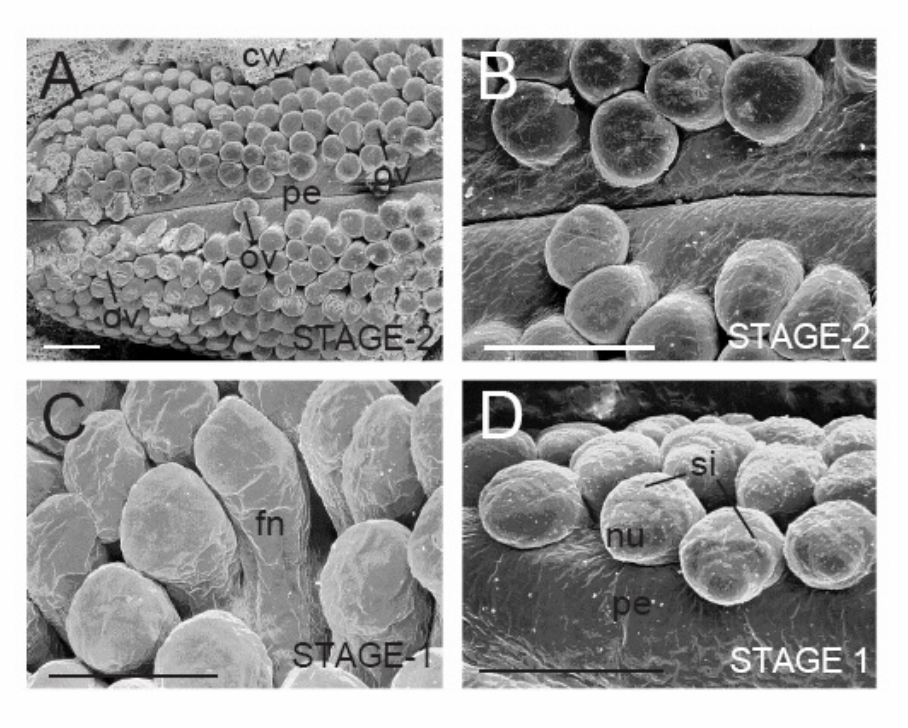


Figure IV.1: Scanning Electron Micrograph of the Developing Ovary.
(A) Overview of ovary at flower stage -2 **(B)** Enlarged view of (A) developing ovules emerging as placental protuberance. **(C)** Immature ovules at flower stage -1. **(D)** Immature ovules at flower stage 1, the developing integument becomes visible. (cw) carpel wall, (fn) funiculus, (nu) nucellus, (ov) ovule, (pe) placenta epidermis, (si) short integument. Size bars: (A) 50μm, (B-C) 40 μm, (D) 100 μm.

Megagametogenesis

Megagametogenesis and embryo sac development occur rapidly between stage 9 and stage 12 of flower development. Upon megasporogenesis, the megaspores at the micropylar end of the ovule degenerate and only one megaspore at the chalazal end divides to form the embryo sac. The embryo sac development is of the "polygonium type" (Reiser and Fisher, 1993). During the first mitotic division, a single, large vacuole is observed located between the two nuclei (Fig. IV.2 G). The second nuclear division results in the four nuclear stage embryo sac (Fig. IV.2 H). By stage 12 of flower development, after the third round of nuclear division and cellularization, the ovules reach the typical Polygonium-type embryo

sac (Fig. IV.2 I-J) consisting of seven cells and eight nuclei. The monitoring of the megagametogenesis in tobacco indicated the limits of exploitation of the light microscope analysis of cleared whole-mount ovules; the increase in size of the ovule is from less than 100µm in the mononucleate megasporocyte to 300µm in the eight-nucleate embryo sac. The rapid increase in size of ovule and embryo sac did not allow to identify on a single focus plan, all of the components of the embryo sac, and did not enabled us to identify clearly the cellularization stage of the eight nucleate stage.

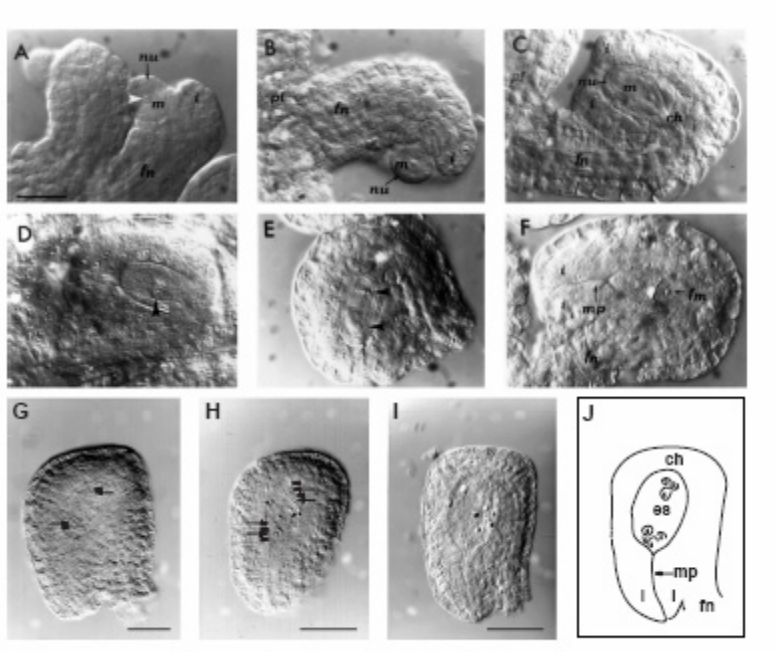


Figure IV.2: Wild-type tobacco ovule development. The ovule is comprised of the funiculus, a stalk that attaches the ovule to the placenta, the nucellus, which surrounds the megasporocyte and will disappear at maturity, and the integument, which envelops the nucellus and defines the micropyle. Ovaries from flowers at different developmental stages from 1 to 12 were fixed, dissected and cleared for observation with a differential interference contrast (DIC) microscope. **(A-C)** wild-type, flower stages 1-4: the mononucleate megasporocyte (m) becomes surrounded by the integument, the white arrow indicates the megasporocyte's nucleoli. **(D)** flower stages 5-8: the ovules undergo meiosis I. The dividing nucleus is indicated by the arrow. **(E)** flower stages 6-9: the ovules undergo meiosis II. The dividing nuclei are indicated by the arrows. **(F)** flower stages 9-10: all the ovules contain the functional megaspore (fm) that will produce the embryo sac. During megasporogenesis, and it disappears when the functional megaspore is formed. **(G)** 2-nuclei embryo sac. The arrows

indicate the 2 nuclei. A large vacuole between the two nuclei is visible. **(H)** 4-nuclei embryo sac. The arrows mark the two nuclei at the micropylar pole and at the chalazal end. **(I)** 8-nuclei embryo sac. Ovule size did not allowed to identify on a single focus plan, all of the components of the embryo sac, and did not enabled us to identify clearly the cellularisation at this stage. The arrangement of the embyo sac components is described in panel (J). **(J)** Schematic representation of the tobacco embryo sac at anthesis: 1) egg cell, 2) synergid, 3) antipodals, 4) polar nuclei. Size bars: (A-F) 50 μm, (G) 50 μm, (H-I) 100μm. (ch) chalaza, (es) embryo sac, (fm) functional megaspore, (fn) funiculus, (i) integument, (m) megasporocyte, (mp) micropyle (nu) nucellus, (pl) placenta.

Pollination/fertilisation

At stage 12 the flower is completely open, anther dehiscence occurs and the stigma is usually self-pollinated. As described in the previous chapter, pollen tubes grow through the stigma and style to reach the ovary and then target receptive ovules. At approximately 30h after pollination, the first pollen tubes enter the ovary and most of the pollen tubes enter the micropyle 48h after pollination. A pollen tube must change its direction of growing to enter a micropyle because the tobacco ovule is anatropous; the micropyle slightly turns in the direction of the funiculus and opens in the bottom side facing the placental tissue. Entering an ovule, the pollen tube growing on the placental tissue must twist itself 90° and grow vertically toward the micropyle (Fig. IV.4 A-B). Ovary receptivity seems to be essential to guide pollen tubes to the targets in various plants; several studies already in the mid 1960's suggested the presence of a chemotropic signal to attract and guide pollen tubes into the ovary (Welk *et al.* 1965). More recently, genetic studies in *Arabidopsis* provided evidences that ovules guide the pollen tubes from a distance (Hulskamp *et al.* 1995, Byzova *et al.* 1999). These results indicated that proper embryo sac formation in *Arabidopsis* is a requisite for correct pollen tube penetration into the ovule. Therefore, inappropriate embryo sac development would failure the pollen tube to penetrate the micropylar structures, due to the absence of the appropriate guiding system toward the ovule.

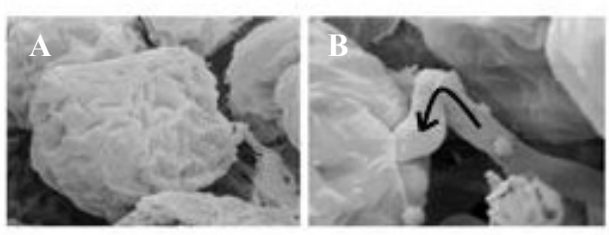


Figure IV.4: Ovule penetration by the pollen tubes. (A) Scanning Electron Micrographs of a pollen tube penetrating the ovule. **(B)** Enlargement of (A). Note the change in the pollen tube orientation in targeting the micropyle. (fn) funiculus, (mp) mycropile, (ov) ovule, (pt) pollen tube. Size bars: (A) 100 µm, (B) 10 µm.

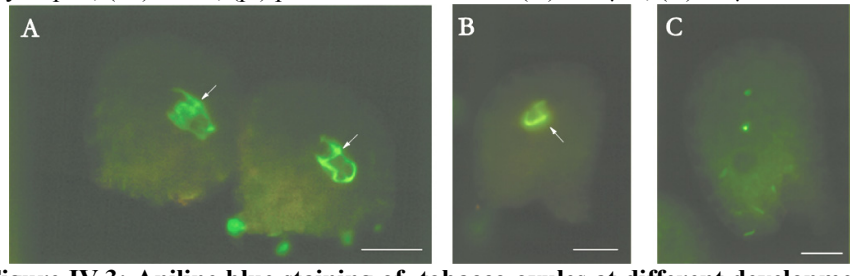


Figure IV.3: Aniline blue staining of tobacco ovules at different developmental stages. (A) Ovule at the beginning of megasporogenesis. Callose accumulates around the forming megaspores.**(B)** Ovule at the end of the megasporogenesis the integuments completely surround the fuctional megaspore. Callose surrounds the forming megaspores and cause megaspore degeneration.**(C)** 2 nuclei stage embryo sac. Callose disappears when the single functional megaspore divides to form the embryo sac.Size bars: 50µm

Table 2. Ovule development in wild-type Nicotiana tabacum[a]

Flower stage[b]	Description	Bud length (mm) [b]
1 to 3	Megasporocyte and nucellus are formed and become surrounded by the single integument	8 to 14
4 to 5	Beginning of megasporogenesis *meiosis I*)	16 to 20
6 to 7	Second round of meiosis, large callose accumulation are visible around	22 to 28
8 to 9	End of megasporogenesis. The degenerated megaspores are surrounded by callose accumulations and the functional megaspore is formed	39 to 43
10	All the ovules contains the functional megaspore (fm) that will produce the embryo sac. Megagametogenesis begins	45
11 to 12	Four/eigth-nucleate embryo sac	46

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

Discussion

In this study, the morphological characteristics of ovule development in *Nicotiana tabacum* from the appearance of ovule primordia until fertilisation, were described. Tobacco flower development and male gametophyte development were described in detail at morphological and molecular levels by Koltunow and colleagues (1990). Although the female gametophyte has been the focus of many investigations at molecular and cytological levels as well, in different plant types, a description of tobacco female gametophyte to complete the picture of flower development was still lacking. Table 2 summarises the events of megagametogenesis and megasporogenesis during flower development. The tobacco ovary is composed of almost 2000 ovules and their development is not completely synchronised. Ovules at the base of the ovary appeared to be delayed in development compared to the ovules located closer to the style (not shown). The techniques used allowed easy analysis of a large number of samples, although the final stages of ovule development could not be analysed in detail, revealing the limits of the technique. Tobacco ovule development follows the *polygonium* type model. Differentiation of ovule components starts between stage -1 and stage 1 of flower development, when the integument becomes visible. From our observations, it seems that gametophytic (megasporocyte) and sporophytic (nucellus, integument and funiculus) tissues develop synchronously; the megasporocyte growth corresponds with the integument growth and the anatropous orientation of the ovule (Fig. IV.2 C). Formation of the haploids megaspores by meiosis begins when the integument surrounds the nucellus and start to define the micropyle (Fig. IV.2 D-E). Thus, it is tempting to suggest that the two processes may be linked and perhaps dependent on each other, possibly through some kind of tissue interaction. In *Arabidopsis*, the ant-1 and ant-3 mutants lack integument development; this occurs alongside the absence of megasporocyte development, suggesting that correct sporophyte development somehow regulates embryo sac development. However this is contradicted by the observation that two ovule mutants 47H4 and 54D12 have apparently normal sporophyte development despite aberrant embryo sac development (Hulskamp *et al.* 1995, Byzova *et al.* 1999). Megasporogenesis is characterised in tobacco by two major cytological markers, the cell layer of the nucellus, that disappears when the functional megaspore is formed and the callose, which

disappears at the beginning of the megagametogenesis. As in many other plant species, callose accumulates around the developing megaspores, where it is thought to isolate the megaspores, inducing their degeneration. Callose is easy to identify by aniline blue fluorescent staining in squashed ovary, thus representing a very reliable cytological marker to identify the switch of the ovules from megasporogenesis to megagametogenesis. Embryo sac formation occurs rapidly between stage 9 and 12 of flower development. Our analysis revealed that at anthesis, ovules have completed megagametogenesis, but the thickness of the ovule however, did not allowed a detailed analysis at this stage. Further studies indicated that other developmental changes may occur before fertilisation; apparently ovule maturation is still not complete, the polar nuclei are still located closely to the apical ends of the ovule and have not yet moved to the center of the embryo sac (Tian and Russel, 1997). Ultrastructural analysis of tobacco ovules at anthesis using TEM (Transmission Electron Microscope) revealed that other maturation events must also occur in the ovule before fertilization. For example calcium (Ca^{2+}) accumulates in the synergids and in the micropyle before fertilisation occurs (Tian and Russel, 1997) and calcium accumulation is higher in the receptive synergid, especially in the micropylar region. The role of calcium in the ovule is potentially related to the chemotropic attraction of pollen tubes, which in many plants grow towards calcium gradients (Mascarenhas and Machis, 1962, Reger *et al.* 1992). Calcium accumulating in the micropyle may create a gradient which attracts the growing pollen tubes and induces the change in direction to enter the micropyle. Thus, although megagametogenesis appears to be completed at anthesis, further changes may still occur in the ovule before fertilisation.

In summary, the different stages of ovule development in relation to flower development have been dissected, and the key stages of ovule formation have been determined by easy-to-use techniques. The knowledge of ovule formation is a necessary prerequisite to study ovary-related gene expression and to analyse any related mutant or transgenic plant and finally to identify key developmental stages that may represent targets for genetic and molecular analysis (mutant screening, expression library construction). Our analysis makes possible the accurate staging of ovule development with the simple visual reference to the flower stage from which they were isolated. In

the following chapters of this thesis (Chapters V and VI) gene expression of a pistil specific gene and the phenotype of transgenic plants in which the relative gene expression was silenced will be described, using as reference the flower and ovule stages mentioned in this chapter.

Materials and Methods

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

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) 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 carried out in liquid carbon dioxide. Samples were mounted on stubs, sputter-coated with gold and examined with a scanning electron 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) 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 visualise the pollen-tube penetration into the ovule. Fresh slices (100 m 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.

Chapter V

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

De Martinis and Mariani (1999). The Plant Cell 11:1047-1060

ABSTRACT
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 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.

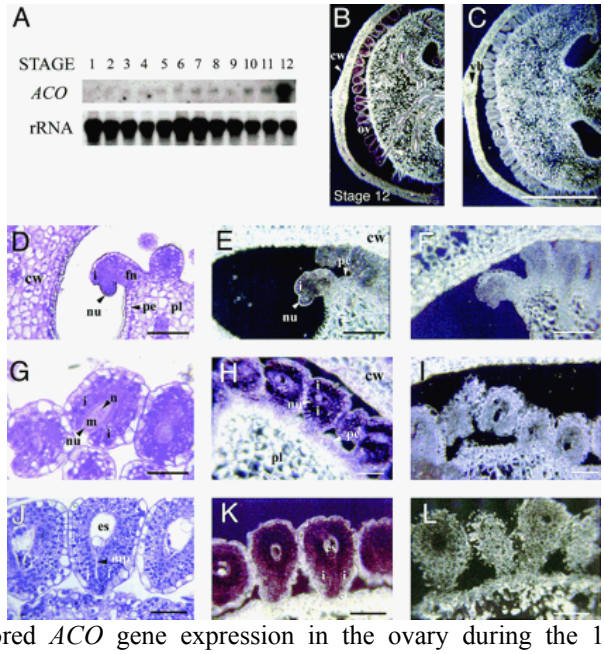
Results

ACO Gene Expression during Ovule Development in Tobacco

We have characterized the spatial and temporal expression of the *ACO* gene during pistil development and pollination in tobacco (Weterings, Pezzotti *et al.* 2002 and this Chapter). 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 V.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 (Fig.V.1 B and C). During flower development, the ovules originate from the placenta as finger-like structures (Fig.V.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 (Fig.V.1G). During these early developmental stages, *ACO* expression occurs preferentially on the funiculus, on the integument primordia, and on the nucellus (Fig.V.1E, V.1F, V.1H and V.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 V.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 (Fig.V.1B, V.1K, and V.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 2 (chapt. IV) summarizes the various developmental stages of the tobacco ovule based on morphological criteria. Taken together, our results demonstrate that *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.

Figure V.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 were 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.

***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 ß-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 (Fig.V.2 A and B, 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 orientation 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 (Fig.V.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 V.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 (Fig.V.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 (Fig. V.3 B and C) 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 (Fig.V.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. Hulskamp *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.

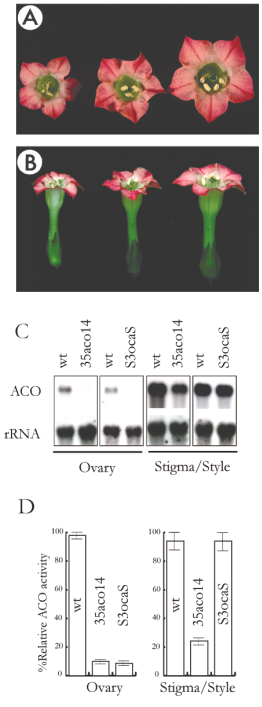
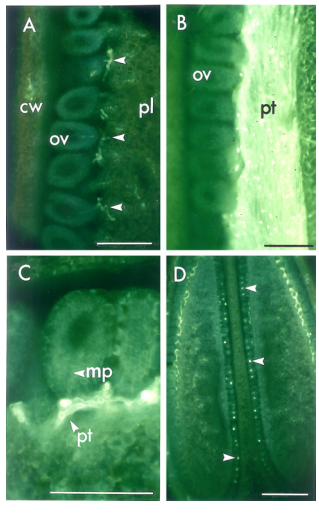


Figure V.2: Flower Morphology, *ACO* Gene Expression, and ACO Activity in Wild-Type and Transgenic Tobacco Plants at Anthesis.
(A) and **(B)** The wild-type flower (right), the flower of the antisense transformant S3ocaS (center), and the flower of the cosuppression transformant 35aco14 (left). **(C)** 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. t, wild type.

Figure V.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.

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 V.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. Fig.V.1D and V.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 (Fig.V.4 A and B), callose accumulation within the nucellus (Fig.V.3D), and absence of the embryo sac. The arrest in development was also clearly visualized by using scanning electron microscopy (Fig.V.4C to 4F). Ovules from transgenic plants at anthesis often had a short integument and a naked nucellus (Fig.V.4C to 4E), a situation comparable to ovules from young wild-type flower buds (Fig.V.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

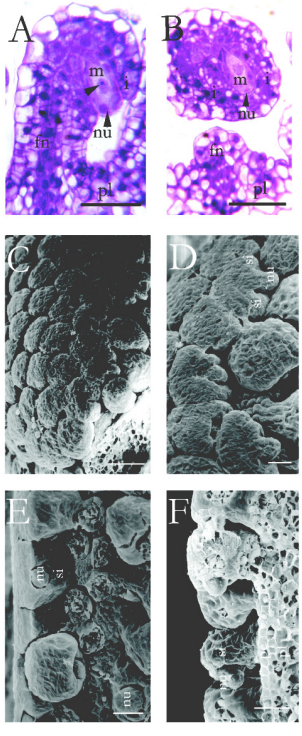
To demonstrate the direct involvement of ethylene in regulating ovule development, we provided an ethylene source to the transgenic flowers. However, certain factors had to be taken into consideration. The carpel wall may act as a barrier for the delivery of ethylene specifically to the ovule tissues. 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 V.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 (Fig.V.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 (Fig.V.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 (Fig.V.3). Furthermore, figureV.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 (Fig.V.5 C and D). 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 V.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

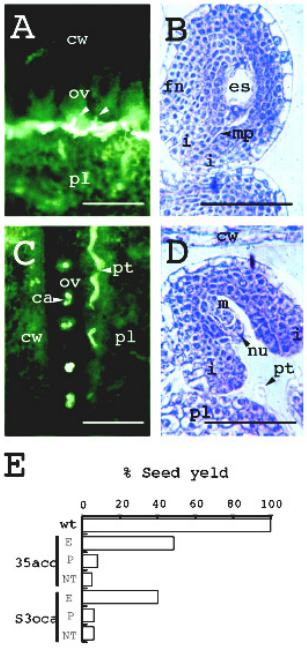
whether it controlled ovule development, which consequently determined pollen tube attraction into the micropyle. To further investigate this aspect, we treated wild-type flowers at anthesis (with fully developed embryo sacs) with STS to inhibit the ethylene produced endogenously by the flowers and then 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.

Figure V.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 (Figure V.1 D and G). 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.

Figure V.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. 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.

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 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 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 (Hulskamp *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 signalling 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).

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.

Materials and 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 x SSC (1 x 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 colour 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 co-evolve 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

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Chapter VI

Stigma and style development and the role of pollination in induction of ovule development in low ACO transgenic plants

With Maria Herrero and Celestina Mariani

ABSTRACT
We evaluated the phenotypic aspects of flower development and fertility in transgenic plants in which the ethylene biosynthetic pathway had been modified by silencing of the pistil-specific 1-aminocyclopropane-1-carboxylate oxidase (ACO) gene. Although the principal observed phenotype was related to megasporogenesis (see previous chapter), overall flower phenotype was altered in transgenic plants. The flowers produced were of reduced size, and cytological analysis indicated a delay in tissue development of the stigma and style of the transgenic plants although less dramatic than that previously observed in the ovary. The reversibility of fertility upon flower treatment with an ethylene source suggested that in different physiological conditions the induced female sterility could be overcome. To evaluate this, different pollination procedures were applied to evaluate increase seed yield and, the role of pollination in inducing ovule development in ACO-silenced transgenic plants.

Introduction
In the previous chapters, ovule development in transgenic plants that had modified ethylene biosynthesis due to silencing of a pistil specific gene encoding the ethylene-forming enzyme ACC oxidase (ACO) was described. Two different sets of transformants were generated ("low ACO" plants), that showed a similar flower phenotype, with reduced size and female sterility ("low ACO" flowers). Upon visual inspection, flowers from transgenic plants appeared smaller but identical in colour and shape to that produced in wild type tobacco plants. Thus, although the most evident phenotype was the alteration in megasporogenesis and the resulting female sterility, other alterations in the pistil tissue could not be excluded. Moreover, our data indicated that the absence of seed setting was not complete in transgenic plants, although the presence of an ethylene source produced a significant increase of seed setting (see fig V.5), demonstrating the direct role of this plant hormone in control of ovule development. The minimal seed set in transgenic plants could be explained by an "escape" of a number of ovules, e.g. by an unpredictable reduction of the ACO silencing effect ("leaky" expression), that may enable part of the ovules to reach maturity, or by an effect induced by the pollination event to "boost" ovule development also in tobacco, although with low efficiency. This could

not be excluded, as in other plant systems it has been postulated that an "unknown pollination factor" may induce ovary development (Bui and O'Neill 1998). Thus, to test this second hypothesis, different pollination procedures were utilised to try to overcome ovule sterility. This small study shows the main observations on stigma and style development and on seed yield in low ACO flowers, completing and concluding the work described in the previous chapter.

Results

Pollen hydration, stigma and style development in low ACO flowers.

Stigma/style anatomy and pollination has been previously described in this thesis (see chapter I, III-V and related references). Extended descriptions at cytological and molecular levels were also provided by de Graaf (1999) and Sanchez (2000). Briefly, early pollen-stigma interactions can be divided in different phases: landing of the pollen on the stigma surface, pollen hydration and germination, pollen tube penetration into the stigmatic tissues. These events occur within the first 3 hours of the pollen landing on the stigma. Then, pollen tubes grow into the transmitting tract of the style, and penetrate into the ovary approximately at 30 hours after pollination (hap).

The pollen grain contains a primary wall called the intine, which envelops the protoplast of the male gametophyte, and a rigid outer, secondary wall named the exine. The exine is thought to protect the male gametophyte between the shedding of the pollen and the landing on a stigma surface. Pollen rehydration in angiosperms occurs once the pollen grain lands on the stigma. The uptake of water results in pollen swelling, a restoration of its cytoplasmatic organisation and metabolism and the building up of osmotic pressure which leads to the exposure of the pollen grain aperture sites at that place which results in the protrusion of the pollen tube. The pollen tube protrudes towards the stigma and initiates penetration (fig VI.1 A). The same dynamic does not occur in wild type pollen grains that have landed on stigmas of low ACO flowers (Fig. VI.1 B and C); Light microscope observations showed that most of the pollen grains do hydrate, but pollen tube elongation towards the stigma surface does not occur immediately. Figures VI.1 B and C clearly show that pollen grains try to extrude pollen tubes in all directions before to continue in the

direction of the stigma surface at the best-orientated aperture. Stigma morphology in low ACO plants showed a morphology that may explain the dynamic of pollen germination; at maturity (Stage12), the stigma of wild type plants is composed of the stigma lobes and the so-called secretory zone. In species with a wet stigma such as tobacco, the secretory cells are highly active in the production and release of exudate during pistil maturation. Exudate accumulation on the stigma surface is visible in figure VI.1 D. The composition of the stigma exudate and pollen coat is thought to play a role during the decisive steps of pollen-stigma interaction after pollen landing, namely, pollen germination and pistil penetration by the pollen tube (see also Wolters-Arts *et al.* 1996, de Graaf, 1999 and this thesis, chapter I). The same morphology does not occur in low ACO plants; stigmas at stage 12 do not show extended secretion of stigma exudate as clearly visible from SEM (fig VI.1 E and F). Cross sections of the stigmas also showed that on the stigma surface of low ACO flowers there is a cuticle that covers the stigma's papillae and that is degenerated in wild type stigmas (compare Fig. VI.1 G, with Fig. VI.1 H and I). Presumably, the presence of this cuticle does not allow proper exudate secretion in low ACO plants. Moreover, other differences in the transmitting tract of the style were observed. In wild type plants, styles from unpollinated flowers at stage 12 were stained in order to enhance the presence of lipids in the cells and/or in the intercellular spaces. Figure VI.1 J, shows that most of the cells of the transmitting tract harbour mainly empty vacuoles, that have delivered their contents into the intercellular space, that appears loose and clearly stained. In contrast, styles from low ACO flowers display the opposite morphology; the transmitting tract seems to be more compact and most of the cells harbour vacuoles that still retain their content; although the intercellular spaces are also stained (Fig. VI.1 K and L).

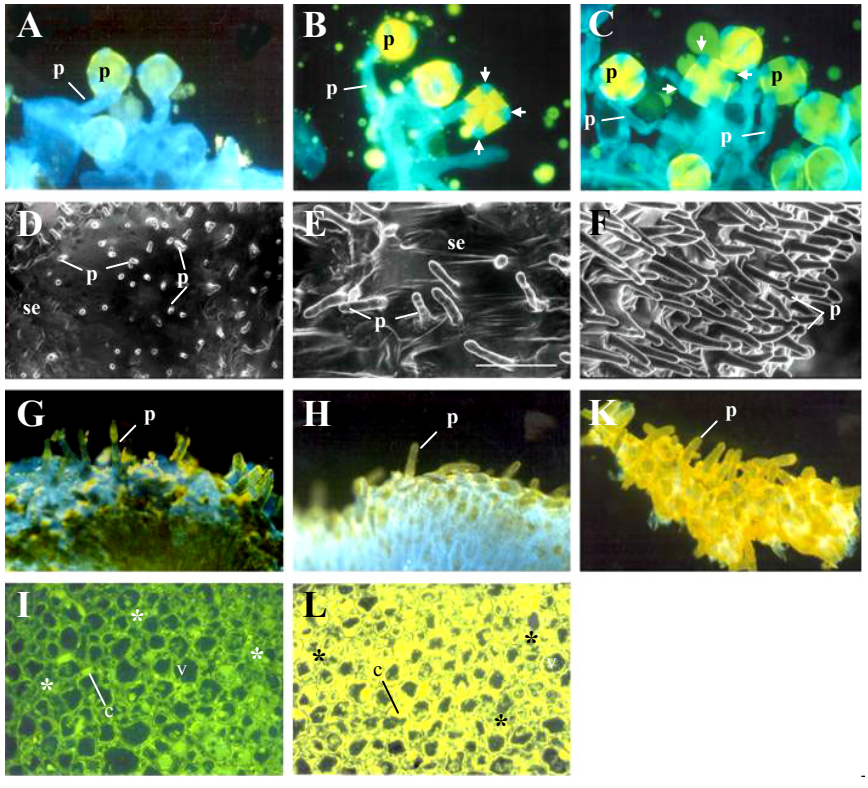


Figure VI.1: Pollination and stigma-style development in wild type and lowACO flowers at anthesis (stage 12). A-C; pollen tube germination on wild-type and low ACO stigmas. **(A)** UV-Light microscope image of wild type pollen grains germinating on stigma surface of wild type flowers. Upon landing on the stigma surface, pollen grains hydrate, germinate and extrude a pollen tube which penetrates the stigmatic tissue and elongates in the extracellular matrix of the transmitting tissue. **(B)** UV-Light microscope image of wild type pollen grains germinating on stigma surface of S3OCAS lowACO flowers and **(C)** 35ACO14 lowACO flowers. Upon landing on the stigma surface, pollen grains hydrate and germinate, but sometimes the pollen tube elongation does not occur immediately in the direction of the stigma surface but proceeds in all directions (arrows) before continuing in the direction of the stigma surface. D-I; Stigma morphology of wild-type and low ACO stigmas. (D-F) SEM image of the tobacco stigma at anthesis. The stigma was isolated from dissected flowers and directly analysed by SEM, without preliminary fixation. This procedure allows the visualisation of the stigmatic exudate that covers the stigma papillae. **(D)** In wild type flowers the stigma exudate covers almost completely the stigma papillae, and only few papillae's tips emerge from the exudate. **(E)** In S3OCAS lowACO flowers stigma exudate is lower and stigma papillae clearly emerge from the exudate. **(F)** In 35ACO14 lowACO flowers stigma

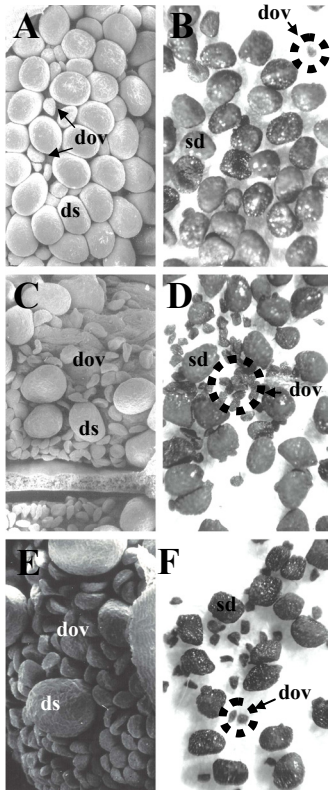
exudate is absent.(G-I) UV-light microscope image of the stigmatic surface. Stigma papillae protrude out from the stigmatic surface. **(G)** In wild type flowers at anthesis, the stigmatic tissue becomes loose and enables secretion of stigmatic exudate (not shown). The yellow staining indicates the cuticle that covers the stigmatic surface until anthesis. Once maturation occurs, this cuticle disappears and the tissue beneath becomes visible (blue stain). **(H)** In S3OCAS lowACO flowers the stigma papillae appears to be uniformly coated by the cuticle, fragmented in wt flowers. **(I)** In 35ACO14 lowACO flowers the stigma papillae are strongly coated by the cuticle. (J-K) Transversal section of the stylar transmitting tract tissue at anthesis. Fluorescence (yellow) staining indicates the presence of secretion products in the extracellular matrix. **(J)** In wild type flowers at anthesis, the transmitting tract tissue appears loose and the extracellular matrix harbour the majority of the secretion products. **(K)** In 35ACO14 lowACO flowers at anthesis, yellow staining is more intense, the transmitting tract tissue appears more compact than in wt flowers, cells seem to retain the product that will be eventually secreted. (ct) cytoplasm, (*) extracellular matrix, (pg) pollen grain, (pa) papillae, (pt) pollen tube, (sex) stigmatic exudate, (vc) vacuole. Size bars;

Pollination and seed yield in low ACO flowers

In the previous chapter, it was shown that partial seed set could be achieved in low ACO flowers upon pollination upon treatment of flowers with an ethylene source (approximately 50% of the normal seed set, Fig. V.5 E). Also untreated or hydrochloride and phosphonic acid treated plants, achieved a small seed set (less than 10% of the normal seed set, Fig. V.5 E). Here, ovaries developing into capsules in flowers from wild type tobacco and transgenic low ACO tobacco plants were compared. At 144hap the differences in seed development were observed. In wild type tobacco flowers the majority of the ovules developed into seeds (Fig. VI.2 A), but a portion of the ovules failed to develop into seeds and collapsed, squeezed between the developing seeds. Some other ovules did seem to increase in size, but appeared smaller than the majority of the seeds harboured in the ovary/capsule. Pollinated ovaries from low ACO flowers showed the opposite seed development (Fig. VI.2 C and E); the majority of the ovules appeared flattened and collapsed, although a few seeds seemed to develop. At capsule maturation (approx 45dap) mature seed morphology was evaluated. In wild type tobacco flowers (Fig. VI.2 B) most of the seeds developed normally appearing brown in colour and lignified. The original shape of the ovule, with the funiculus and the main body that comprised the integuments and the embryo sac was still recognisable, although modified. Some seed-like structures smaller in

size could also be observed. These seed-like structures also appeared brown and lignified and retained an ovule-like structure too. These were presumably formed from the ovules that had failed to develop into seed (observed at 144hap, fig. VI.1 A). As for early capsule development, low ACO flowers showed the opposite seed development; few seeds of normal appearance could be found, the majority being small seed-like structures similar to that just observed.
To study the hypothesis that the event of pollination could "boost" low efficiency ovule development in tobacco and therefore induce partial seed development even in low ACO flowers, seed setting in low ACO flowers by three different types of pollination were evaluated; the "standard" hand-pollination at stage 12 of flower development (as described in previous chapters), a "double" pollination, with flowers pollinated a second time 24h after the first pollination, a "late" pollination, with flowers pollinated 48h after anthesis (stage 12). In these conditions, if the pollination event induced signalling, double pollination should result in an increase in seed yield. In the case ovule development continue slowly in low ACO flowers, late pollination should result in an increase in seed yield. Results (table 3) do show an increase in seed yield compared to standard pollination, with both the pollination methods and in both the low ACO plant lines (S3ocaS and 35aco14). Plant line 35aco14 did not produce a consistent seed yield; standard pollination resulted in 13% of seed yield, double pollination produced a small increase of less that 4%, and late pollination resulted in a yield of approximately 24.5% compared to that observed in wild type flowers seed yield. The S3ocaS plant line could produce a higher seed set after standard pollination; equivalent to a 37.8% of the total seed yield reachable in wild type tobacco flowers. Double pollination produced a small increase of less that 10%, but late pollination resulted in a seed yield comparable to the 86% of the total yield reachable in wild type flowers (Fig. VI.3). It should be noted that, in general, seed yield in S3ocaS plants was higher than that described in the previous chapter. This may be due to improvement of growing conditions that facilitated overall plant development. In any case, for both the low ACO plants lines analysed, double pollination produced small increase in seed yield, between 4-10% of the total yield obtained with standard pollinations, whereas seed yield obtained with late pollination approximately doubled for both the transgenic plants lines analysed.

Figure VI.2: Seed development in wild type and lowACO flowers.



(A) In wild type tobacco flowers the majority of the ovules developed into seeds, but a small number of the ovules failed to develop into seeds and collapsed, squeezed between the developing seeds. (B) most of the seeds from pollinated wt flowers developed normally and appear brown in colour and lignified. Some seed-like structures smaller in size could also be found. These seed-like structures appeared brown and lignified as well and retain a ovule-like structure. Those derived presumably from the ovules that failed to develop into seed that we observed in VI.2A. (C) Ovaries from pollinated S3OCAS lowACO flowers. Ovules that appeared flattened and collapsed are more abundant than in wt flowers, although few seeds seemed to develop. (D) Seeds of S3OCAS lowACO flowers. Arrows indicate small seed-like structures. (E) Ovaries from pollinated 35ACO14 lowACO flowers. Arrows indicate collapsed ovules (D) Seeds of 35ACO14 lowACO flowers. Arrows indicates small seed-like structures. (dov) degenerated ovule, (ds) developing seed (sd) seed.

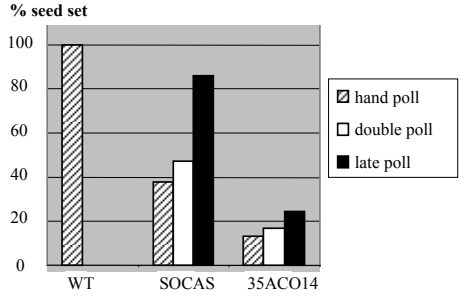


Figure VI.3: Percentage of seed production in lowACO flowers by three different types of pollinations.

Wild type	S3 OCA S	35 ACO 14
hand pollination	**hand pollination**	**hand pollination**
133,7	65,6	2
142,9	48,6	44,2
118,4	41	25,1
151,6	48,1	32,7
158	50,3	10,1
173,4	69,4	29,2
169,1	61	19,4
162,2	70,8	18,8
142	57,2	13,2
112	51	0
172,7		
average	**average**	**average**
148,7272727	56,3	19,47
	double poll	**double poll**
	89,7	25,9
	65,4	5,8
	82,2	23,3
	79,1	24,6
	85,5	36,6
	38,6	55,7
	50	44,5
	53,1	7,2
	73,7	21,4
	86,3	7,9
	average	**average**
	70,36	25,29
	late poll	**late poll**
	157,3	34,6
	154,3	44,1
	177,5	38,9
	134,6	78,8
	145	52,8
	126	65
	0	36,7
		0
	average	**average**
	127,8142857	43,8625

Table 3: seed production in lowACO flowers by three different types of pollinations. The seed yields (in mg) for every pollinated flower are listed. If we consider the average seed yield in wild type plants as 100%, the % of seed production for the lowACO plants for the different pollination are calculated (shown in Fig. VI.3)

Discussion

Silencing of the ACO gene in transgenic plants resulted in flowers with reduced ovule development (described in chapter V). Transgenic "low ACO" plants developed small flowers that were identical in colour and shape to that produced in wild type tobacco plants. Light and scanning electron microscopy observation of pollinated stigmas and styles at stage 12 of flower development indicated that other alterations occur in low ACO flowers; the stigma did not fully mature and did not secrete the stigma exudates in a normal fashion. This exudate is considered to be very important for correct pollen tube growth as demonstrated in stigmaless transgenic tobacco plants. In the absence of the stigma, the top of the transmitting tract of the style presents a surface that resembles a dry stigma surface. Tobacco pollen cannot germinate, nor penetrate this "novel" dry stigma surface, but it was shown that application of stigmatic exudate on the ablated surface of stigmaless plants was sufficient to restore the capacity of pollen tubes

to penetrate the style (Goldman *et al.* 1994, see chapter I for details). This may explain why wild type tobacco pollen on stigmas of low ACO flowers germinated differently than on wild type stigmas initially extruding the pollen tube tip in all directions before the elongation of the pollen tube tip was finally directed towards the stigma surface. The styles of these plants were also different to wild type tissues at the same stage. In this case the tissues appeared delayed in development.

The observed phenotype in the stigmas and styles of transgenic plants could be due to interorgan regulation (O'Neill *et al.* 1993) in which the delay in ovary development influenced the size of all the flower parts.

The "multidirectional" germination of the pollen, the absence of stigmatic exudate and the relative "firmness" of the transmitting tract may cause a delay in pollen penetration towards the ovary, although in wild type plants pollen germination and stigma/style penetration can occur also at earlier stages of flower development, from stage 6 when the flower is still immature (de Graaf 1999, Sanchez 2001). Thus, although stigma and style are believed to provide physical and chemical supports and directional guidance to the pollen tube growth process, their complete maturation does not seem to be essential to enable pollen tube germination and penetration towards the ovary. The effect of pollination procedures on seed yield has also been studied in the low ACO plants. The results exclude a clear role of the pollination event to induce ovule development in tobacco, as in both transgenic plant lines double pollination did not produce any substantial increase in seed set. Interestingly, late pollination clearly resulted in a higher seed set in S3ocaS plant lines. This is consistent with the previous observation of the two different plant lines; S3ocaS showed a less extreme plant phenotype, with an intermediate size and retained higher ACO mRNA accumulation and related ACO enzyme activity in the stigma and style, than the 35aco14 plant line. Furthermore, as previously indicated, 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 development if compared with the 35aco14 lines. The fact that late pollination resulted in increased seed set in the S3ocaS plant line suggests that ovules could develop further to maturity, although at a slower rate than ovules from wild type flowers. Thus, previous conclusions indicating that ovules from transgenic low

ACO plant lines are arrested in development should be revaluated; it would be more appropriate to indicate that the ACO downregulation corresponded to a delay in ovule development. These observations are consistent with the flower phenotypes and the biochemical and molecular characterisation produced for the transgenic plant lines. The more dramatic change in flower phenotype (35aco14 transgenic plant line, see previous chapter fig V.2 A and B) is associated with stronger ACO downregulation (see previous chapter fig V.2 C and D) and stronger female sterility (this chapter Fig. VI.2), whereas, the intermediate flower phenotype (S3ocaS transgenic plant line) correlated with intermediate values of ACO activity and reduced female sterility that could be partially overcome as shown here. Therefore, the analysis presented here confirms the conclusions about the putative role of ethylene in ovule development already discussed in the previous chapter.

Materials and Methods

Plant Material: Tobacco (*Nicotiana tabacum*) SR1 plants and transgenic "low ACO" plant lines 35aco14 and S3ocaS were grown under standard greenhouse conditions.

Pollination: Pollination was carried out using mature flowers emasculated one day before anthesis. Three types of hand-pollinations were performed; the "standard" hand-pollination at stage 12 of flower development (as described in previous chapters), a "double" pollination, with flowers pollinated a second time 24h after the first pollination, a "late" pollination, with flowers pollinated 48h after anthesis (stage 12). Seeds were harvested 45 dap and weighted to evaluate seed yield.

Microscopy:

For light microscopy, samples were dehydrated to 70% ethanol and subsequently analysed with light or scanning electron microscopy

For light microscopy samples were squashed (Fig. VI.1 B) or hand sectioned (Fig. VI.1 D and E), stained and analysed using a microscope equipped for UV epifluorescence.

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 developing seeds. The samples were then dehydrated; mounted on stubs and sputter-coated with gold, for examination with SEM essentially as described in chapter V.

Chapter VII

Ethylene-Responsive-Elements-Binding-Proteins (EREBPs) in the tobacco flower

With Imke Haenen and Celestina Mariani
EREBP V accession number: AY627865 18/05/2004
EREBP VI accession number: AY627866 18/05/2004

ABSTRACT
A tobacco pistil library was screened for the presence of ethylene-responsive element binding proteins (EREBPs). Sequence analysis identified two novel putative EREBPs, that harbour the highly conserved 60-amino-acid long domain found in all family members as well as the APETALA2 gene, which regulates meristem identity, floral organ specification and seed coat development in *Arabidopsis*. RT-PCR analysis revealed expression of the two putative EREBPs in several tissues of the plant, including the flower. Individual EREBPs exhibited different patterns of expression in the pistil during flower development and pollination.

Introduction
The plant hormone ethylene functions as a signal and can be perceived by all parts of the plant that respond via a phosphorylation cascade (Raz and Fluhr, 1993). Ethylene biosynthetic pathway has been elucidated (Yang and Hoffman 1984) and genes encoding the biosynthetic enzymes have been cloned from several plant species (see also chapter I). In the last decade genes encoding for ethylene receptors have also been characterised and the mechanism of ethylene perception and transduction began to be elucidated. In 1995 Ohme-Takagi and Shinshi identified a small family of tobacco proteins that bind to the ethylene-responsive elements of genes encoding pathogenesis-related proteins. The amino-acid sequences of the ethylene-responsive elements binding proteins (EREBPs) are rather divergent, except for a 60-amino-acid domain that is highly conserved among all family members. This conserved domain corresponds to the DNA binding domain of EREBPs. Sequence comparison shows that the EREBPs DNA binding domain is closely related to the APETALA2 (AP2) domain (Weigel, 1995, Riechnmann and Meyerowitz 1998). The AP2 gene regulates meristem identity, floral organ specification and seed coat specification in *Arabidopsis*. In contrast with the EREBPs however, AP2 appears to contain two DNA binding domains, and does not appear to be ethylene regulated (R.Fisher, personal communication). In previous studies on flower development and ovule formation the presence of flower-specific, ethylene-inducible transcription factors that may regulate the expression of genes necessary for ovule development was suggested (De Martinis and Mariani 1999 and this thesis chapter V). The cloning

of those factors and the characterisation of their expression in the reproductive tissues could elucidate how ethylene controls plant reproduction. In this work, the cloning of flower-related EREBPs and the characterisation of their pattern of expression in different plant tissues and in the pistil during flower development and pollination is described.

Results

Pistil cDNA library screening and isolation of putative novel EREBPs

We previously analysed EREBPs 1-4 expression in the tobacco flower by Northern blot (EREBPs 1-4 cDNA clones were kindly provided by M.Ohme-Takagi). Analysis was performed on developing and pollinated tobacco pistils. Results (not shown) indicated that there was no detectable expression of EREBPs in developing pistils, but there was expression, just detectable by Northern blot analysis, of EREBP 1, in stigmas and styles at 24 and 30 hours after pollination (hap), and in ovaries from 12 hap to 60 hap (not shown). These preliminary results indicated that EREBPs, if present, are most likely to be expressed at detectable levels in pollinated flowers. Thus, a cDNA library (previously constructed by M. Pezzotti) of mRNA purified from tobacco pistils 12h after pollination was screened. The library was probed with a mixture of EREBPs 1-4 probes that were allowed to hybridise at low stringency (see methods for details). A total of sixteen cDNA clones that hybridised with EREBPs 1-4 were isolated, and sequence analysis excluded clones that revealed complete sequence homology with EREBP 1-4. Of the sixteen putative EREBPs cDNA isolated, eight shared homology with EREBP1, one with EREBP2, four with EREBP3 and none with EREBP4. Three additional cDNA clones that showed no homology with any of the four EREBPs were also isolated.

Two of the clones, labelled as EREBP V and EREBP VI, which appeared distincts from EREBPs 1-4, but seemed to belong to the EREBPs class were further analysed in detail.

EREBP sequence analysis

EREBP V and VI presented an open reading frame that began with an ATG codon and coded for 259 and 244 amino-acid respectively, with

a similar predicted molecular mass of approximately 27 kD (Fig.VII.1, Fig.VII.2). BLASTA analysis of EREBP V revealed homology with *Stylosanthes hamata* EREBP-3, (Gardner *et al*., unpublished). EREBP VI showed homology mainly with a *Nicotiana tabacum* mRNA for ethylene responsive element binding factor (Ashida *et al.* 2000), which also has homology to EREBP 3. Comparison of the amino-acid sequences deduced from EREBP V and VI cDNA sequences revealed a highly homologous region of approximately 59 amino-acids in both. This corresponds to the characteristic DNA binding domain of EREBPs. No other significant sequence homology was found between the two deduced EREBPs. At the amino-acid level, the two EREBPs shared 29% identity, and 86% identity within the 60 amino-acid DNA binding domain. EREBP V shares 48% identity with EREBP 3 and 96% identity within the DNA binding domain. EREBP VI shares 29% identity with EREBP 3 and a 81% identity within the DNA binding domain (Fig. VII.3).

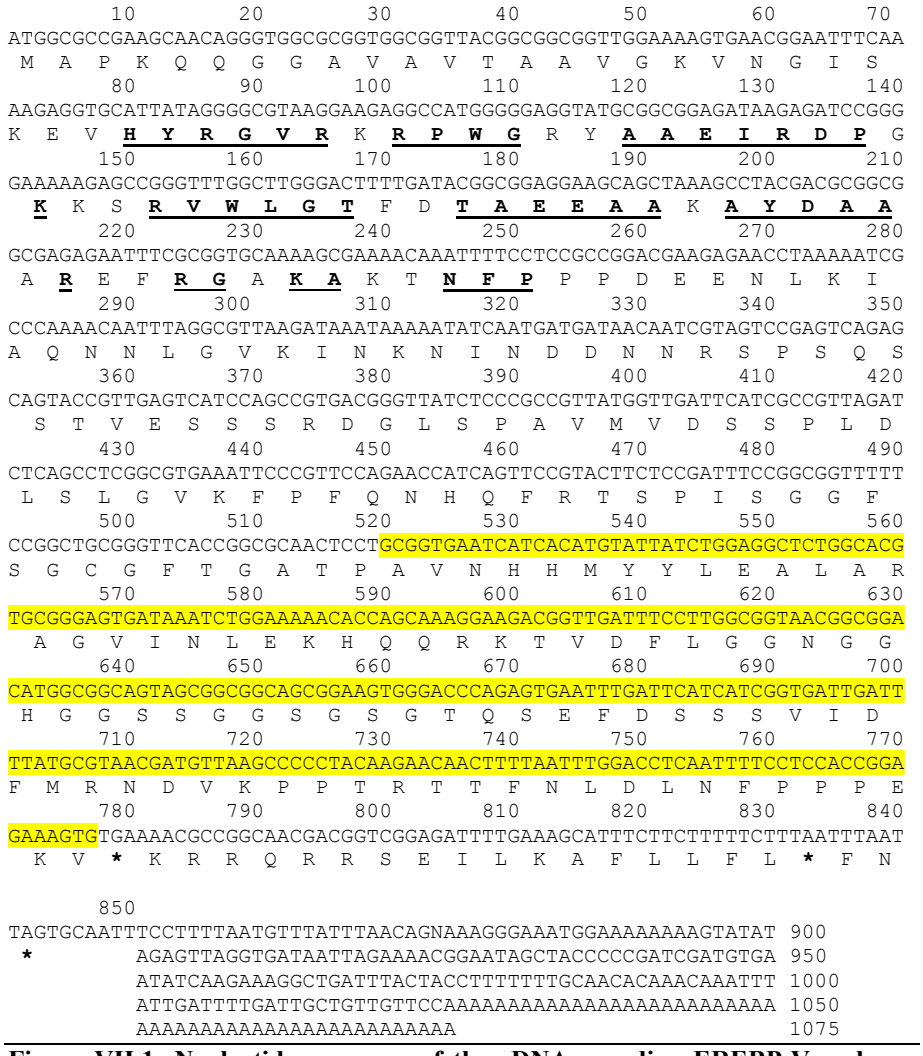


Figure VII.1: Nucleotide sequence of the cDNA encoding EREBP-V and the deduced amino acid sequence.

The nucleotide sequence derived from the cDNA for EREBP V starts in the 5' leader region and ends in a poly(A)-containing sequence down-stream from the termination codon. The 60-amino acid long domain coincident with the DNA binding domain of EREBPs that is highly conserved among all family members is indicated bold.underlined. Calculated molecular weigth of EREBP V is 27,8 kD, and estimated pI is 9,33. Region amplified by PCR for expression study is highlighted.

```
        10         20         30         40         50         60
ATGCGAAGAGGTAGAGCAACCGCGGCGGCGAAGCAAGCGGCGGAGGCTTCACCGGCGGCT
 M  R  R  G  R  A  T  A  A  A  K  Q  A  A  E  A  S  P  A  A
        70         80         90        100        110        120
GGATCTGGAGGATTGAAAGACATTAGGTTTCGTGGTGTCAGAAAACGGCAGTGGGGAAGA
 G  S  G  G  L  K  D  I  R  F  R  G  V  R  K  R  Q  W  G  R
       130        140        150        160        170        180
TTTGCGGCGGAGATTAGAGACCCGTGGAAAAAACTAGGTTGGCTTGGCACTTTCGATTCA
 F  A  A  E  I  R  D  P  W  K  K  L  G  W  L  G  T  F  D  S
       190        200        210        220        230        240
GCTGAAGAAGCCGCTAAAGCTTATGACGCTGCAGCTCGGACTCTTCGGGGACCTAAAGCA
 A  E  E  A  A  K  A  Y  D  A  A  A  R  T  L  R  G  P  K  A
       250        260        270        280        290        300
AAAACTAATTTCCCTTTACCTCCGTATTCTCACTTCAATCAAACCATAAACCCTAACGGC
 K  T  N  F  P  L  P  P  Y  S  H  F  N  Q  T  I  N  P  N  G
       310        320        330        340        350        360
CCGTTTATTGACCCGAGATTGTACTCTCAGGAAAACCATCCGATTGTTATTCAAAGACCT
 P  F  I  D  P  R  L  Y  S  Q  E  N  H  P  I  V  I  Q  R  P
       370        380        390        400        410        420
ACATCGAGCAGCATGAGTAGTACCGTAGAATCCTTCAGTGGGCCCAGGCCGCCGCCACGC
 T  S  S  S  M  S  S  T  V  E  S  F  S  G  P  R  P  P  P  R
       430        440        450        460        470        480
CAGCAAACGGCCGTGTTGCCTTCGAGAAAACATCCTCGATCGCCGCCGGTCGTGCCGGAC
 Q  Q  T  A  V  L  P  S  R  K  H  P  R  S  P  P  V  V  P  D
       490        500        510        520        530        540
GACTGCCGGAGTGATTGTGACTCGTCTCTTCTGTTGTTGAAGATGGCGATTGTGATAATG
 D  C  R  S  D  C  D  S  S  L  L  L  L  K  M  A  I  V  I  M
       550        560        570        580        590        600
ATAATGAAAATGATAATATCGTTTCATCAGCTTTTCGGAAACCATTGCCTTTCGATCTCA
 I  M  K  M  I  I  S  F  H  Q  L  F  G  N  H  C  L  S  I  S
       610        620        630        640        650        660
ACTTTCCTCCACCTATGGATGCTGATTCTGATGATCTTCACTGCACAGCATTATGTCTTT
 T  F  L  H  L  W  M  L  I  L  M  I  F  T  A  Q  H  Y  V  F
       670        680        690        700        710        720
GATGGAATATGGTTTCGAAATTTGGATCTGTTAATTATTCGAAAAAGCCCCATTTGGTTT
 D  G  I  W  F  R  N  L  D  L  L  I  I  R  K  S  P  I  W  F
       730        740        750        760        770        780
CCCTTCTTTATTTAATTATTGATCTCGAGAAAAAAGGGGGTGGAAAGAAAAAAAAGATCA
 P  F  F  I  *  L  L  I  S  R  K  K  G  V  E  R  K  K  R  S
       790        800        810        820        830        840
TAGCTTTAAGGCTATTGATATGAGTCTGTTGCTGGAAGAGATTTGAACTCTTCAATTTAC
 *  L  *  G  Y  *   850

TTGTCCCTATTTAATATCATGTTATTTCATGATGGAAAAAAAAAAAAAAAAA890
```

Figure VII.2: Nucleotide sequence of the cDNA encoding EREBP-VI and the deduced amino acid sequence.

The nucleotide sequence derived from the cDNA for EREBP VI starts in the 5' leader region and ends in a poly(A)-containing sequence down-stream from termination codon. The 60-amino acid long domain coincident with the DNA binding domain of EREBPs that is highly conserved among all family members is indicated bold underlined. Calculated molecular weigth of EREBP VI is 27,6 kD, and estimated pI is 10,66. Region amplified by PCR for expression study is highlighted.

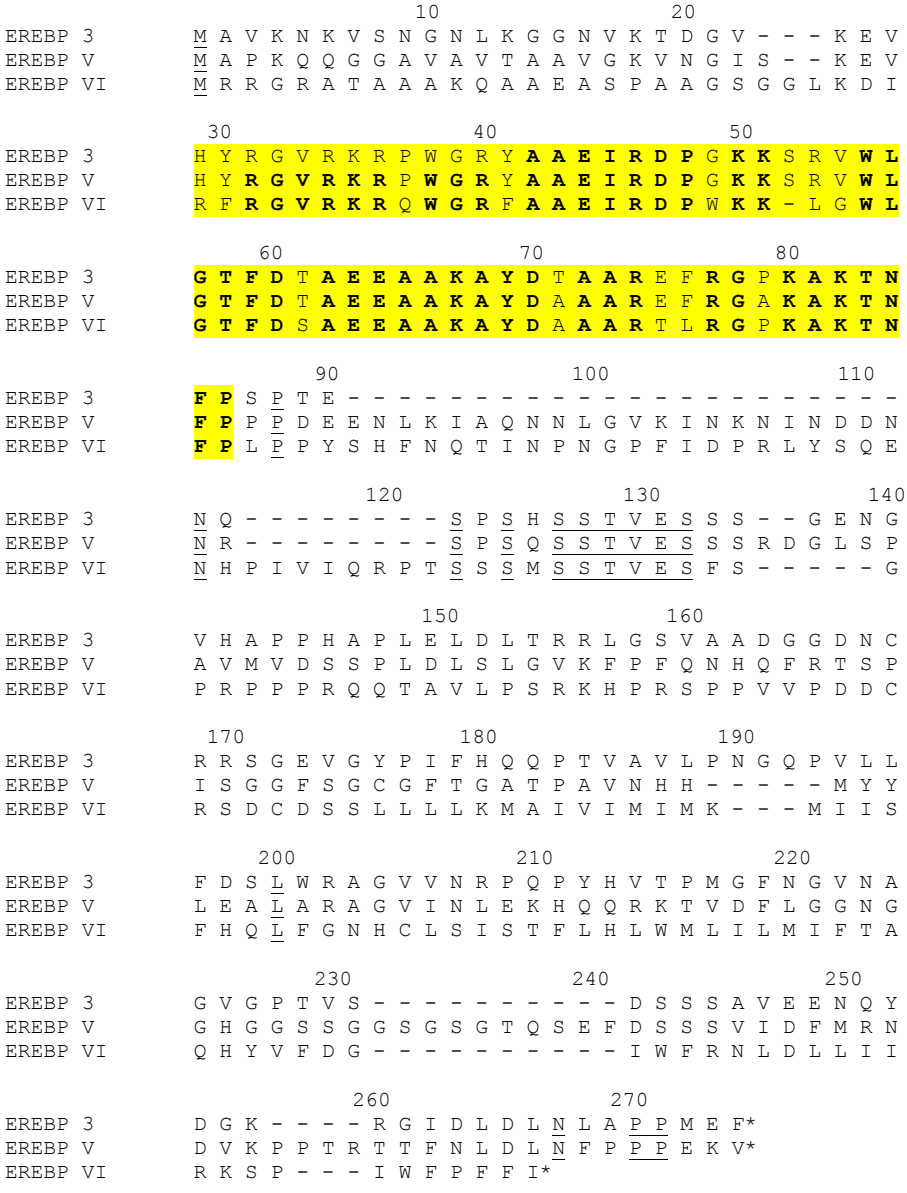


Figure VII.3: Deduced amino acid sequence of EREBP 3, EREBP V and EREBP VI.

Amino acid sequences of EREBP V and VI are aligned with EREBP 3. The DNA binding domain is highlighted. Within this domain the amino acids identical among the three sequences are indicated in bold. Identical aminoacids outside the DNA binding domain are underlined. Dashes indicate gaps in the aminoacid sequence used to optimize the alignment

EREBPs expression pattern

Accumulation of individual EREBPs mRNA was analysed by RT-PCR, and compared to the expression of housekeeping gene class elongation factor 1 (EF1). Total RNA was prepared from tobacco leaves, stems, roots, seedlings and anthers. Primers specific for the non-conserved regions of the two EREBPs were designed in order to exclusively amplify EREBP V or EREBP VI. The same procedure was used to study pistil-specific EREBP expression; total RNA was prepared from tobacco ovaries and stigmas/styles at flower developmental stages 1 to 12 and at different hours after pollination. Results showed that both EREBPs do not present a flower-specific expression; EREBP V showed an ubiquitous pattern of expression (Fig. VII.4), in the leaves, stem, roots, anthers and seedlings, whereas EREBP VI was expressed only in the leaves and stem and was barely detectable in seedlings (Fig. VII.4). Although the two EREBPs showed expression in non-reproductive tissues of the tobacco plant, their expression was modulated in the pistil during flower development and upon pollination (Fig.VII.5). EREBP V is expressed at low levels in ovaries from developing flowers at stage 1 to 4 and more prominently at stage 9 to 12 (Fig.VII.5 A). EREBP VI expression increased in ovaries from developing flowers at stage 2 to 4, and then increased again from stage 9 to 12 (Fig.VII.5 B). In stigmas/styles from developing flowers EREBP V expression at stage 1 and 2 was detected, but not any expression of EREBP VI (Fig.VII.5 D). During pollination, expression of both EREBPs appeared to be modulated; in the ovary EREBP V is still expressed at 3hap and its expression increased at 6hap to reduce again at 24hap and increase to steady state at 30hap until 60hap (Fig.VII.5 F). EREBP VI expression is low but detectable at 3, 30 and 60 hap and higher at 6, 24 and 48 hap (Fig.VII.5 G). In the pollinated stigmas/styles, EREBP V is detectable at 3hap and increases to steady state at 6hap to 48 hap. It ceased to be detectable at 60hap (Fig.VII.5 F). EREBP VI was detectable at 6 and 30 hap (Fig.VII.5 G).

Discussion

Little is known about the functional role of the plant hormone ethylene in plant reproduction. So far, most studies have focused on post-pollination flower senescence, although some studies indicate the role of ethylene in pollen recognition (see chapter III and related

references) and even in pre-pollination ovary development (see chapter V and related references). To function, ethylene signal needs to be transduced via a phosphorylation cascade that leads to a gene-induced response. EREBPs are among the factors involved in the last step of this response, binding the ethylene responsive genes. Tobacco flower cDNA library screening enabled us to isolate several cDNAs with homology with EREBPs, among which two cDNA labelled as EREBP V and VI were characterised more in detail. On the basis of sequence comparison two novel EREBPs that belong to the EREBP-3 class were isolated. As for EREBPs 1-4, these two novel EREBPs have sequence similarity restricted to the region of 59-60 aminoacids that correspond to the DNA binding domain. The limited similarity outside the conserved region suggests the possibility of a different mode of activation. EREBP V and VI exhibited a different pattern of expression. EREBP V showed an ubiquitous pattern of expression, in the stem, leaves, roots, anthers and seedlings, whereas EREBP VI was expressed only in the stem and leaves and was barely detectable in seedlings. In the pistil, both are expressed in the ovary at similar stages during flower development, but EREBP VI is not expressed in stigma/style from developing flower. Both EREBPs showed modulation during pollination.

Since the first description of EREBPs (Ohme-Takagi and Shinshi, 1995) several genes belonging to the same class have been found to be involved in biotic and abiotic stresses (Kizis and Pages 2002, Mazarei *et al*., 2002 for reviews see Kizis *et al.* 2001, Ohme-Takagi *et al.* 2000) and in development in different plant species, *e.g.* in *Arabidopsis* (Van Der Graaff *et al*. 2002), in maize (Chuck *et al.* 2002), and in rice (Yang *et al*. 2002). Two genes of the EREBP/AP2 family were isolated also from gymnosperm in *Picea abies* (Vahala *et al.*, 2001). These two *P. abies* genes are differentially expressed in different organs and it has been suggested that they may be important developmental regulators. The role of EREBPs in regulating plant reproduction has been indicated in cucumber (Ando and Sakai, 2002), where the authors reported the analysis of a gene designated as ERAF16. ERAF16 encodes a transcription factor that is expressed in correlation with the development of the female flower. It was found to be inducible by ethephon and its expression was more prominent in the gynoecious cultivar than in those of the monoecious cultivar. The authors suggested that ERAF16 is involved in the ethylene-induced

formation of female flowers. However, ERAF16 was expressed in opened flowers and also in vegetative tissues, therefore suggesting that the role of ERAF16 protein varies, depending on the tissue and developmental stage. From this perspective it could be speculated that EREBP V and VI may function similarly in tobacco, as they are expressed in vegetative parts as well as in reproductive organs of the plant. Data presented so far do not allow conclusions to be drawn yet, but future studies may clarify the role of EREBP V and VI in tobacco reproduction. The availability of the cDNA sequences should allow us to address these issues.

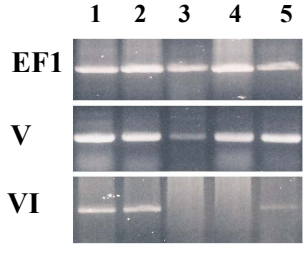


Figure VII.4: RT-PCR analysis of EREBP mRNA expression in tobacco plant. Total RNAs were prepared from different part of tobacco plant EREBP V and VI. Reverse transcription was performed using oligo dT and derived cDNA templates were evaluated upon amplification with primers for the house-keeping Elongation Factor 1 gene family (EF1). Equalised templates were then amplified with primer specific for EREBP V (V) or EREBP VI (VI). Plant tissues: 1) leaves, 2) stem, 3) roots, 4) anthers, 5) seedlings.

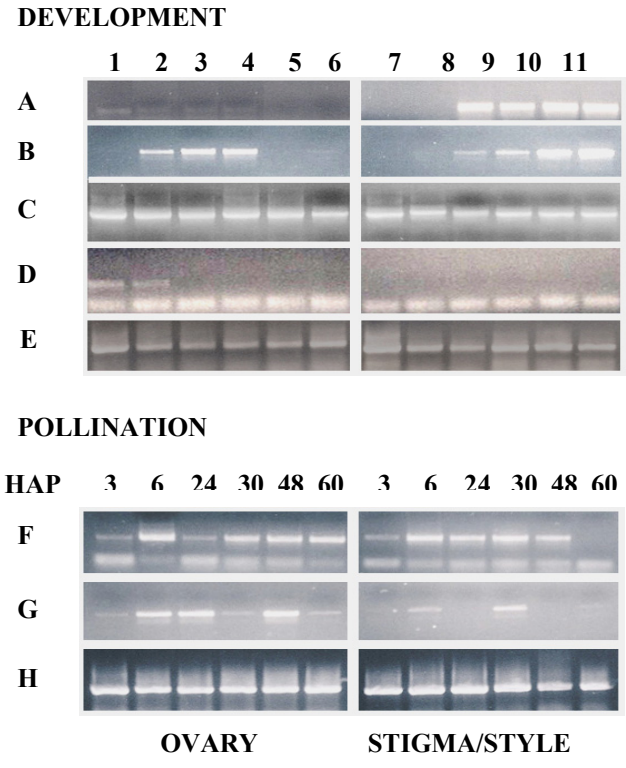


Figure VII.5: RT-PCR analysis of EREBP mRNA expression in tobacco flowers. Total RNAs were prepared from different part of tobacco plant. EREBP V and VI RT-PCR was performed as described in previous figure. (A-E) RT-PCR of mRNAs of ovaries and styles from developing flower stages 1 to 12. (F-G) RT-PCR analysis of mRNAs of ovaries and styles from developing flower stages 1 to 12.

(A) Ovary stage 1-12, primers for EREBP V.
(B) Ovary stage 1-12, primers for EREBP VI.
(C) Ovary stage 1-12, primers for EF 1.
(D) Style stage 1-12, primers for EREBP V.
(E) Style stage 1-12, primers for EF 1.
(F) primers for EREBP V.
(G) primers for EREBP VI.
(H) primers for EF1
HAP; hours after pollination

Materials and 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 and analysed essentially as already described in chapter V. ^{32}P-labeled probes were prepared from the full-length *EREBPs 1-4* 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.

Library screening: EREBPs V and VI were isolated by screening a tobacco-pollinated stigma-style cDNA library (Weterings et al., 2002) with a mixed ^{32}P-labeled probe of EREBPs 1-4, hybridised at low stringency.

RT-PCR Expression analysis: We used a RT-PCR approach to monitor EREBP V and VI gene expression in flower at different developmental stages and plant tissues. DNA primers were designed to obtain specific DNA sequence amplification by PCR. RNA was extracted from flowers and plant tissues as described above. PCR amplification products were visualised on agarose gels, using as a control the expression levels of the constitutively expressed gene Elongation Factor 1-a (EF1-a). EF1- a primers sequence utilised are (courtesy of C. Rosati):

EF1-ATTGTGGTCATTGGYCAYGT
EF2-CCAATCTTGTAVACATCCTG

Concluding remarks, future perspectives

Ethylene (C_2H_4) is a small molecule with a very broad range of effects on plant physiology. Development, senescence, fertilisation and the response to biotic and abiotic stresses, all appear to be modulated or at least influenced by ethylene evolution.

This thesis attempts to address some of the questions about the role of ethylene in flower development, pollination, fertilisation and finally fruit spoilage. The importance of ethylene in physiological events such as pollination-induced flower senescence or fruit spoilage has been known for decades, but for others such as pistil development, the role of ethylene was less evident. The use of state-of-the-art laser technologies has revealed novel aspects to pollen-pistil and pollen-pathogen interactions.

To understand the role of ethylene in tobacco flower development, we characterised the gene expression of the ethylene-forming enzyme, ACC oxidase (ACO), during pistil development. The characterisation of *ACO* gene expression in the tobacco ovary during development, and of the phenotype of transgenic plants in which the *ACO* mRNA accumulation was greatly reduced, demonstrated a direct involvement of the hormone in ovule development (De Martinis and Mariani, 1999). This corroborated previous studies on orchids that first suggested a possible role for ethylene in regulating ovule development (Zhang and O'Neill 1993). The present study (Chapter IV to VI), represents an extensive description of female development and ovule fertilisation in tobacco, and complements previous studies on tobacco flower and male organ development (Goldberg 1989, Koltunow *et al.* 1990). The transgenic approach employed, resulted in the downregulation of one member of the gene family encoding the ethylene forming enzyme, but our inability to directly measure ethylene levels in the ovary did not permit a direct study of the effects of the molecule in this organ. However, the evidence produced in tobacco, together with related observations in cucumber (Tova *et al.* 1997), petunia (Tang *et al.* 1994), *Arabidopsis* (Sakai *et al.* 1998) and very recently melon (Papadopulou, 2005), indicate that ethylene is involved in floral induction and ovary/ovule development, as was first indicated in the orchid study. More research is required and

presumably mutants impaired in ethylene biosynthesis and/or perception will be particularly useful in this regard.
The studies on pollination and pathogenesis in fruit described a plant response to an external stimulus. There is considerable commercial interest in fruit ripening and spoilage, as well as in flower senescence and in the mechanism of fertilisation. This has resulted in a substantial output of scientific literature in this area. The use of the Laser Photoacoustic technology, a powerful tool to study ethylene evolution, allowed these physiological events to be viewed from a different perspective with a sensitivity and a time resolution never before attained, so providing new insights into previously described models. The study at a molecular level of congruous and incongruous pollination in tobacco has been extensively described elsewhere (Sanchez, 2001. Ph.D thesis and related references). In this thesis, ethylene levels were directly measured on pollinated flowers still attached to the plant (De Martinis *et al.*, 2002, and Chapter III), in contrast to previous studies performed on detached flowers, or using indirect biochemical approaches. These studies indicated that ethylene release in the tobacco flower is a direct consequence of pollination and does not occur during flower senescence. Moreover, different phases of ethylene production upon self-pollination in tobacco were described. The use of a pharmaceutical approach, together with the study of cross-pollination with incongruous pollen types, demonstrated *de novo* ethylene synthesis during the active penetration of the pollen tubes into the stigmatic tissues, and specific pollen recognition by the pistil. This response may be part of a concerted action that controls pollen acceptance in the self-compatible tobacco, although ethylene is not essential for pollen tube growth into the style.
The same approach was used to study fruit-fungus interaction (Cristescu *et al.* 2002 and Chapter II), specifically with *Botrytis cinerea* grey mould. This fungus is well known for its extremely broad host range, however the factors which are important for the infection process are still not well understood. Notably, the use of laser-based technology, in contrast to previously employed standard procedures, demonstrated that *B. cinerea* is able to produce ethylene *in vitro*. Moreover, either *in vitro* or on the fruit, at higher conidia concentration not only is the ethylene level greater, but its evolution proceeds at a faster rate. The rate of ethylene emission by the fungus during fruit infection is very similar to that of the fungus grown alone

in vitro. During infection, ethylene emission is synchronized with the growth rate of the fungus inside the fruit. These observations could be related to fungal neighbourhood sensing. The presence of ethylene may provide an advantage to the fungus indirectly, because it stimulates the softening of the plant tissue and therefore facilitates tissue penetration and fungal spread. It is possible therefore that the fungus evolved the ability to produce ethylene, as shown, to accelerate fruit spoilage and facilitate tissue colonisation, and in addition evolved the ability to sense ethylene, in order to take advantage of nearby spoiling plant tissue. The acceleration of ethylene evolution *in vitro* could however be a consequence of the artificial culture conditions. Although these aspects of ethylene perception were not described in this thesis, the new molecular tools for studying *B. cinerea* molecular biology developed in the late 1990s (i.e., Wubben *et al.* 2000, ten Have *et al.* 2001) should allow characterization of ethylene production and perception in the fungus.

The timing of production and concentration of "stress ethylene" appear to be crucial for its effect on the plant response. Ethylene production has the ability to trigger exaggerated disease symptoms that appear to be deleterious to plant health. One model (Stearns and Glick, 2003) that describes the apparently paradoxical effect of ethylene on plants emphasized the fact that in stressed plant tissues there is an initial small peak of ethylene close in time to the onset of stress. This could be the beginning of infection (Figure II.6, within the first 12 h) or the beginning of pollination (Figures III.2-4 within the first 3 h). The first peak is only a small fraction of the magnitude of the second peak and is believed to initiate a response by the plant (i.e. acceptance or rejection). The second peak is so large, however, that processes such as senescence or spoilage are initiated, the overall effect of which may be deleterious to the plant. The emerging picture is that the plant response to ethylene depends on the concentration of the molecule and on the characteristics of the targeted tissue. The type of response to ethylene, including the induction of a signal cascade, will depend on the ability of the targeted tissue to perceive the molecule, and therefore varied responses will be observed in different tissues. Targets of the signal transduction 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). Since the first description of EREBPs, several genes belonging to the same class have been found to be involved in the response to biotic and abiotic stresses, and in development in different plant species such as maize, rice and even gymnosperms. It has also been suggested that these genes play a role in regulating plant reproduction (see chapter VI, Discussion). In Chapter V, the existence of flower-specific EREBPs were hypothesized. The cloning and characterisation of two EREBPs, named EREBP V and VI, was described in Chapter VII, along with their sequence analysis and expression profile in different tissues of tobacco, including the flower during development and pollination. The experimental data generated did not allow any conclusions to be drawn about their role in the flower, but the availability of the cDNA sequences should allow us to address these issues in future studies.

In conclusion, this thesis demonstrates the use of novel approaches to measure ethylene biosynthesis, release and signal transduction responses, providing an insight into the role of ethylene in flower development and the mechanism of reproduction, fruit ripening and spoilage.

Colour tables

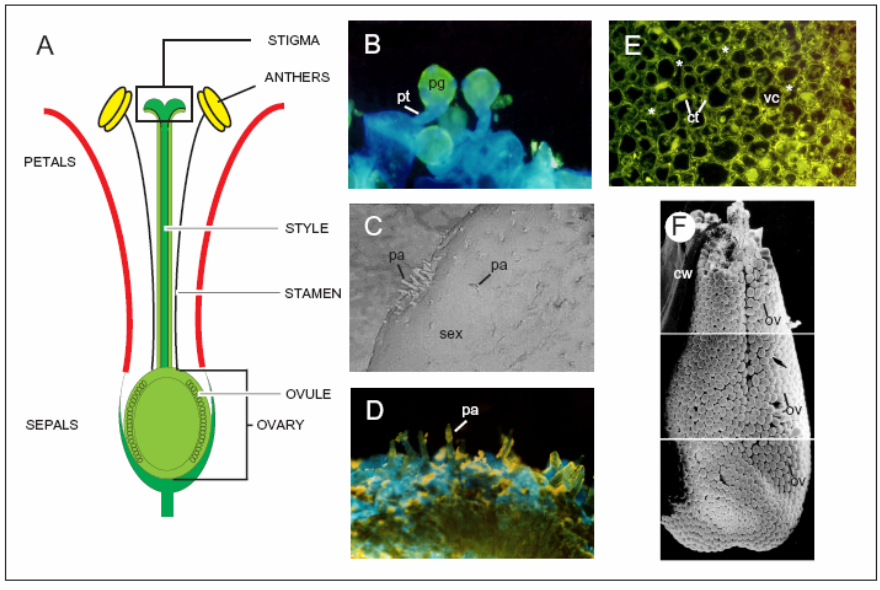


Figure I.2: tobacco flower overview.

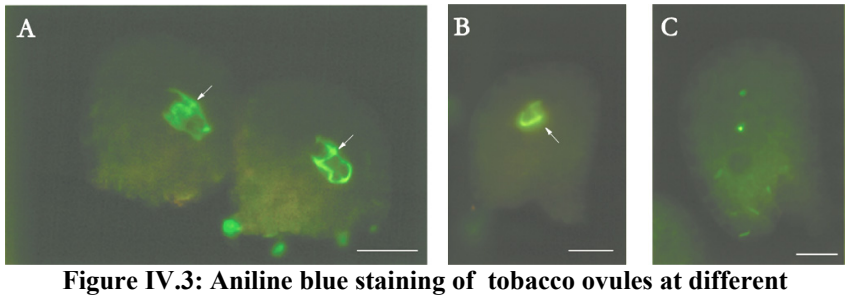


Figure IV.3: Aniline blue staining of tobacco ovules at different developmental stages.

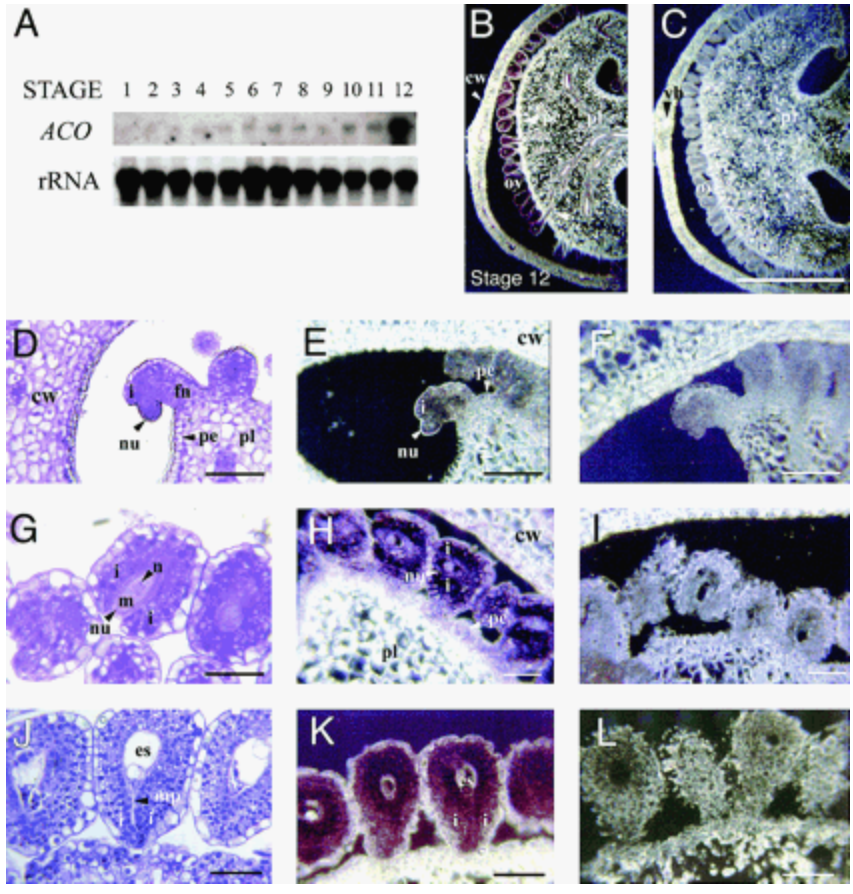


Figure V.1: Temporal and Spatial Characterization of ACO Gene Expression in the Ovary during Tobacco Flower Development.

NOTES

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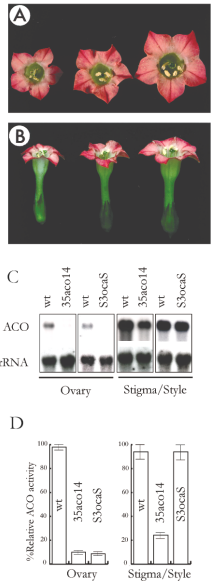

Figure V.2

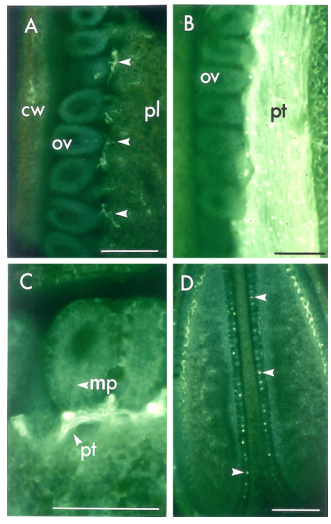

Figure V.3

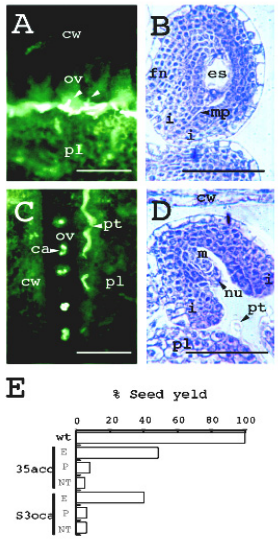

Figure V.4

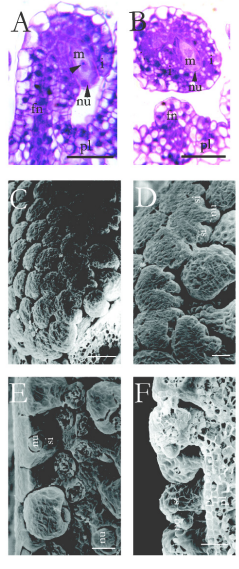

Figure V.5

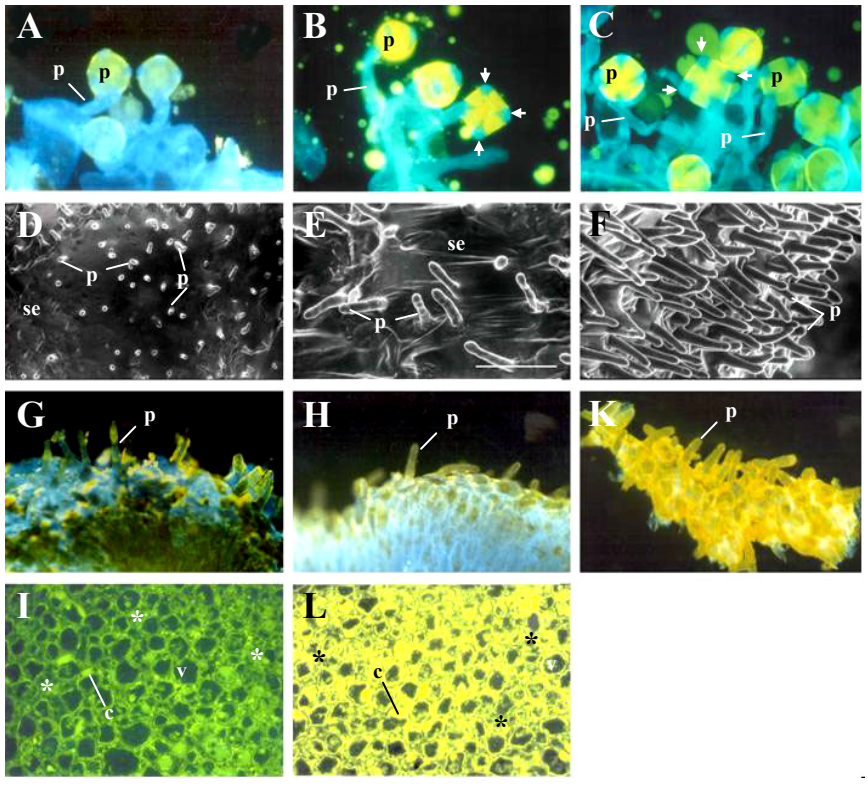

Figure VI.1: Pollination and stigma-style development in wild type and lowACO flowers at anthesis (stage 12)

Summary/ Samenvatting

This thesis approaches the study of the role of ethylene in different aspects of plant reproduction; flower development, pollination, fruit ripening and spoilage. These different aspects have been studied at physiological, biochemical and molecular level.

Chapter I, general introduction is divided in three subchapters and an annex. **Chapter I.1**[1] reviews plant development in relation to the ethylene biosynthesis, mainly indicating how the use of transgenic plants and mutants enabled scientist to better understand ethylene functions. **Chapter I.2** reviewes flower development and reproduction, describing both flower anatomy and the fertilisation mechanism. **Chapter I.3** describes the *Laser PhotoAcoustic* system (LPA), since LPA is a very specific system that allows high sensitivity/high time resolution of trace gas analysis, including ethylene. Such system is not yet common to laboratory use, it is therefore advisable to browse that chapter for a better understanding of the studies where this method has been used (Chapt. II and III of this thesis). The **ANNEX**[2], describes different laser-based approaches used to address biological questions mainly in the field of plant biology and microbiology. In chapters **II**[3] **and III**[4] two aspects of cell-cell interactions related to ethylene release have been studied; fruit pathogenesis by the grey mould *Botrytis cinerea* (Chapt. II), and flower responses to pollination (Chapt. III). **Chapters IV, V**[5] and **VI**

[1]De Martinis (2000) "Modification of plant development by genetic manipulation of the ethylene biosynthesis and action pathway". In Developments in Plant Genetic and Breeding Series 6. G.E. de Vries and K. Metzlaff (Eds.). Elsevier Science B.V

[2]De Martinis. (2003) When physic meets biology: use of high-resolution laser-based techniques to study plant-microbe interactions. Mycological Research, 107 (8), 899-900.

[3]Cristescu *et al.* (2002). Ethylene production by Botrytis cinerea in vitro and in tomato fruit Applied and Environmental Microbiology, 68 (11) 5342-50

[4]De Martinis, *et al.* (2002) "Ethylene response to pollen tube growth in *Nicotiana tabacum* flower". Planta, 214 (5) 806-12

[5]De Martinis and Mariani (1999). Silencing Gene Expression of the Ethylene-Forming Enzyme Results in a Reversible Inhibition of Ovule Development in Transgenic Tobacco Plants . The Plant Cell 11:1047-1060

describe flower development with special attention to ovule development and to the role of ethylene in megasporogenesis and subsequent fertilisation. This topic has been approached essentially by means of molecular biology and genetic engineering to characterise the pattern of expression of the gene encoding the ethylene-forming-enzyme ACC oxidase (ACO), and to produce transgenic plants in which ACO gene expression was greatly reduced. **Chapter VII** describes the cloning of flower-related EREBPs and the characterisation of their pattern of transcription in different plant tissues and in the pistil during flower development and pollination.

Dit proefschrift beschrijft de studie van de rol die ethyleen speelt in verschillende aspecten van plant reproductie, bloemontwikkeling, bevruchting en het rijpen en bederven van fruit. Deze verschillende aspecten zijn onderzocht op fysiologisch, biochemisch en moleculair niveau.

Hoofdstuk 1, de algemene introductie, is verdeeld in drie paragrafen en een bijlage. **Hoofdstuk I.1**[1]vat de plantontwikkeling in relatie tot de ethyleen biosynthese samen, hoofdzakelijk aanwijzend hoe het gebruik van transgene planten en mutanten het mogelijk maken voor onderzoekers om de functies van ethyleen beter te begrijpen. **Hoofdstuk I.2** recenseert de bloemontwikkeling en reproductie, en beschrijft zowel de anatomie van de bloem en de bevruchtingsmechanismen. **Hoofdstuk I.3** beschrijft het "Laser Photo Acoustic" systeem (LPA). LPA is een zeer specifiek systeem dat met hoge gevoeligheid en hoge tijdsresolutie sporen van gas detecteert, inclusief ethyleen. Dit systeem wordt nog niet veel gebruikt in laboratoria, en het is daarom aan te raden om dit hoofdstuk door te lezen om het onderzoek waarvoor dit systeem gebruikt is (hoofdstuk II en III van dit proefschrift) beter te begrijpen. **De bijlage**[2] beschrijft verschillende laser-gebaseeerde benaderingen, gebruikt om de biologische vragen te beantwoorden, hoofdzakelijk met betrekking to de plant biologie en microbiologie.

In de hoofdstukken **II**[3] en **III**[4] zijn twee aspecten van cell-cell interactie gerelateerde ethyleen uitstoot bestudeerd; de grijze schimmel *Botrytis cinerea* (hoofdstuk II), ziekteverwekker in fruit, en de reactie van bloemen op bevruchting (hoofdstuk III). Hoofdstukken **IV, V**[5] en **VI** beschrijven bloemontwikkeling met speciale attentie

voor de ontwikkeling van de eicel en de rol van ethyleen met betrekking tot de megasporogenisis en daaropvolgende bevruchting. Dit onderwerp is benaderd hoofdzakelijk met behulp van moleculaire biologie en genetische modificatie voor de bestudering van het expressie patroon van het gen dat codeert voor het ethyleen-vormende enzym ACC oxidase (ACO), en het produceren van transgene planten waarin de expressie van het ACO gen sterk gereduceerd was. Hoofdstuk VII beschrijft de klonering van bloem gerelateerde EREBPs en de karakterisering van hun expressiepatroon in verschillende plantweefsels en in de stamper gedurende de bloemontwikkeling en bevruchting.

Complete list of Publications

1. J.B. Jiménez, J.M. Orea, C. Montero, A. González Ureña, E. Navas, K. Slowing, M.P. Gómez-Serrranillos, E. Carretero and D. De Martinis. (2005) Use of *trans*-resveratrol (3, 5, 4'-thrydroxystilbene) to control microbial flora, prolong shelf-life and preserve nutritional quality of fruit. ***J. Agric Food Chem***. Mar 9;53(5):1526-1530
2. D. DeMartinis. (2003) "When physics meets biology: high-resolution laser-based techniques to study plant-microbe interactions". ***Mycological Research***, 107 (8), 899-900.
3. *D. De Martinis, and E. Benvenuto (2003)."*Botrytis cinerea* Infection Activates Ethylene and Jasmonic Acid-Related Gene Expression in Harvested Tomato Fruits." In:"Biology and Biotechnology of the Plant Hormone Ethylene" NATO Science Series: Life and Behavioural Sciences Vol. 349 M. Vendrell *et al.* eds IOS press.
4. S.M. Cristescu, D. De Martinis, S. te Lintel Hekkert, D. H. Parker, F.J..M. Harren (2002) "Ethylene production by Botrytis cinerea *in vitro* and in tomatoes". ***Applied and Environmental Microbiology***, 68 (11) 5342-50.
5. **D. De Martinis, (2002) "Raccolta e conservazione della frutta fresca:nuovi metodi per problemi antichi" ***EAI*** (Energia,Ambiente e Innovazione). 5; 33-40
6. D. Pashkoulov, I. Giannetti, E. Benvenuto, and D. De Martinis (2002) "Biochemical Characterisation of Polygalacturonases from five different isolates of *Botrytis cinerea*" ***Mycological Research***, 106 (7), 827-831.
7. D. De Martinis, G. Cotti, S. te Lintel Hekkert, F.J.M. Harren and C. Mariani. (2002) "Ethylene response to pollen tube growth in *Nicotiana tabacum* flower ". ***Planta***, 214(5)806-12
8. *D. De Martinis (2001) "Expression Profiling of the Metabolism of Ripening/Spoilage in Harvested Tomato Fruits" 11th Workshop: Molecular and physiological aspects of plant-pathogen interactions. ***PETRIA***, 11(2),123-124
9. *D. De Martinis (2000) "Modification of plant development by genetic manipulation of the ethylene biosynthesis and action pathway". Developments in Plant Genetic and Breeding Series 6. G.E. deVries and K. Metzlaff (Eds.). Elsevier Science B.V.
10. D. De Martinis and C. Mariani. (1999) "Silencing Gene Expression of the Ethylene-Forming Enzyme Results in a Reversible Inhibition of Ovule Development in Transgenic Tobacco Plants". ***the Plant Cell*** 11:1047-1060
11. *D. De Martinis, I. Haenen, M. Pezzotti, E. Benvenuto and C. Mariani. (1998) "Ethylene and flower development in tobacco plants". In:"Biology and Biotechnology of the Plant Hormone Ethylene II" A.K. Kanellis, et al. eds, NATO-ASI Series Kluwer Law International.
12. P. Tavladoraki, E. Benvenuto, S.Trinca, D. De Martinis , A.Cattaneo & P.Galeffi. (1993) "Transgenic plants expressing functional "Single Chain F_V" antibody are specifically protected from virus attack" ***Nature***, Vol. 366, 469-472.

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* book chapter, congress proceeding

** in italian

Acknowledgments

The work presented in this thesis started more than a decade ago, in January 1995 when I arrived in Nijmegen from south Italy, acutely aware of the cold but full of enthusiasm. The promising results obtained in the first two years prompted my supervisor Celestina Mariani (*Titti*) to invite me to stay for a third year and ultimately to present my research to the KUN as a PhD thesis. It was a charming invitation that I readily accepted with the same naïve enthusiasm that brought me to Nijmegen three years before, without even caring where Nijmegen actually was (I had indeed a look on a map after a couple of weeks I was there). Once back in Rome in January 1998, I spend the first months in getting organised in the laboratory and writing papers for publication. *Titti* while revising my work, initially believed that my data was not convincing enough anymore to support a PhD thesis, as my hypothesis on ethylene and ovule development in tobacco was only supported by studies in orchids, and there was even a time when I thought that my studies would never become a PhD thesis and that they would never be collected in this dissertation that represents, for every scientist, a milestone in his academic career.

However, further studies at ENEA (Rome), back in Nijmegen and in Madrid confirmed the earlier findings, and, in addition, results coming from other groups worldwide also indirectly confirmed the early studies on orchids and our studies on tobacco. Consequently, the significance of my studies was enhanced and in 2004 *Titti* decided that I should present my work as a PhD to KUN, that had in the meantime been renamed “Radboud University”.

Thus, as so much time has passed and also due to family bereavement it would be inappropriate to conclude this thesis with the traditional jokes detailing nights drinking beer with lab friends. Yet, I did share a few beers with some lab mates (actually very few, at that time I was a sober fellow). I also shared my fears and doubts with them as we worked in the lab - weekdays, Saturdays, Sundays, late at night. In those days we would spend more time in the lab than out.

Among them I particularly remember Wim Vriezen who started a PhD at the same time I arrived in Nijmegen, and also Jaap Kooiman who

didn't complete his PhD studies. A short time later Maurice Bosch arrived in the lab as well as the two flowers Chiara Nardi and Ana Sanchez. Within the lab they were the ones with whom I had the most in common. I should also mention Imke Haenen, the first student I ever supervised, who was particularly important for my personal and professional growth. I spent perhaps my happiest times with my friends from the Department of Physics - Fausto Piazza and his girlfriend Gina Cotti (who eventually became his wife) Sacco te Lintel-Hekkert and his girlfriend Simona Cristescu (who eventually become his wife). Going to the Physics laser facility on Monday morning was the best way to start the week. Once out of the laboratory three other scientists, Lucia Colombo, Francesca Mittempergher and later on Silvia Ferrario were the best friends you could ever meet. All those "old boys" now heading into their 40^{s}, deserve a big hug from me.

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BIBLIOGRAPHY

1. Abeles FB, Morgan PW, and Salveit ME, (1992).Ethylene in Plant Biology, 2nd edn, Academic Press.
2. Achilea, O, Fuchs, Y, Chalutz, E, and Rot, I, (1985). The contribution of host and pathogen to ethylene bisynthesis in *Penicillium digitatum*. Physiol. Plant Pathol. 27:55-63.
3. Alonso JM, Hirayama T, Roman G, Nourizadeh S and Ecker JR (1999) EIN2, a bifunctional transducer of ethylene and stress responses in *Arabidopsis*. *Science*, 284, 2148–2152.
4. Ando S, Sakai S, (2002). Isolation of an ethylene-responsive gene (ERAF16) for a putative methyltransferase and correlation of ERAF16 gene expression with female flower formation in cucumber plants (Cucumis sativus). Physiol Plant Oct;116(2):213-222
5. Angenent GC and Colombo L, Molecular control of ovule development, (1996). Trends Plant Sci. 1,228-232.
6. Angenent GC, Franken J, Busscher M, van Dijken A, van Went JL, Dons HJ, van Tunen AJ, (1995). A novel class of MADS box genes is involved in ovule development in petunia. Plant Cell. 7(10):1569-82.
7. Ashida Y, Yokobatake N, Kohci C, Shimoda K, and Hirata T, (2000). Cloning of cDNA encoding ethylene-responsive element binding protein –5 in the cultured cells of Nicotiana tabacum. DNA seq 11(1-2),125-129
8. Ayub R, Guis M, Ben Amor M, Gillot L, Roustan JP, Latche A, Bouzayen M, Pech JC, (1996). Expression of ACC oxidase antisense gene inhibits ripening of cantaloupe melon fruits.Nat Biotechnol Jul;14(7):862-6
9. Barkai-Golan R, Lavy-Meir, G, and Kopeliovitch E, (1988). Pectolytic and cellulolytic activity of *Botrytis cinerea*. *Pers*. related to infection of non-ripening tomato mutants. J. Phytopathol. 123:174-183.
10. Barry CS, McQuinn RP, Thompson AJ, Seymour GB, Grierson D, Giovannoni JJ. (2005) Ethylene Insensitivity Conferred by the Green-ripe and Never-ripe 2 Ripening Mutants of Tomato.*Plant Physiology*, Vol. 138, pp. 267-275.
11. Barkai-Golan R, Lavy-Meir, G, and Kopeliovitch E, (1989). Effects of ethylene on the susceptibility to *Botrytis cinerea* infection of different tomato genotypes. Ann. Appl. Biol. 114:391-396.
12. Bauchot AD, John, P Mottram DS, (1999). Role of ethylene on aroma formation in cantaloupe charentais melon. In AK Kanellis, (Eds.)Biology and biotechnology of the plant hormone ethylene II. NATO-ASI series, Kluwer Acad.Publishers.
13. Bell, AG (1880). Upon the production of sound by radiant energy, Philosophical Magazine and Journal of Science XI, 510-528.
14. Bevan MW, (1984). Binary Agrobacterium vectors for plant transformation. Nucleic acid research. 12, 8711-8722

15. Bijnen FGC, Reuss J, harren FJM, (1996). Geometrical optimization of a longitudinal resonant photoacoustic cell for sensitive and fast trace gas detection. Rev.Sci.Instrum. 67:2914-2923

16. Blázquez MA, Green R, Nilsson O, Sussman MR, and Weigel D, (1998). Gibberellins Promote Flowering of Arabidopsis by Activating the *LEAFY* Promoter Plant Cell 10: 791-800.

17. Bleecker AB, Estelle MA, Somerville C, Kende H. 1988. Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. *Science* 241:1086-89

18. Bleecker AB (2000) ETHYLENE: A Gaseous Signal Molecule in Plants. Ann. Rev. Cell and Dev. Biol.Vol. 16: 1-18

19. Bjorkaman O and Demming B, (1989). Photon yeld of O2 evolution and clorophyll fluorescence characteristic at 77K among vascular plants of diverse origin. Planta, 170,489-504.

20. Boller, T. 1991. Ethylene in pathogenesis and disease resistance, p. 293-324. *In* A. K. Mattoo and J. C. Suttle (ed.), The plant hormone ethylene. CRC Press, Boca Raton, Fla.

21. Bouman F, (1984). The ovule. In Embryology of Angiosperms. BM. Johri, ed. (Berlin: Springer), pp. 123-157.

22. Brewer, RJ, Bruce CW, and Mater JL, (1982). Optoacoustic spectroscopy of C_2H_4. Appl. Optics 21:4092-4100.

23. Bui AQ, and O'Neill SD, (1998). Three 1-aminocyclopropane-1-sarboxylate synthase genes regulated by primary, and secondary pollination signals in orchid flowers. Plant Physiol, 116, 419-428.

24. Buttner M, Singh KB., (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 U S A. May 27;94(11):5961-6.

25. Byzova MV, Franken J, Aarts MG, de Almeida-Engler J, Engler G, Mariani C, Van Lookeren Campagne MM, Angenent GC. (1999). Arabidopsis STERILE APETALA, a multifunctional gene regulating inflorescence, flower, and ovule development.Genes Dev. 1999 Apr 15;13(8):1002-14.

26. Canas LA, Busscher M Angenent GC, beltran JP and van Tunen AJ, (1994). Nuclear localisation of the Petunia MADS box protein FBP1. plant j.. 6, 597-604.

27. Chang C, Kwok SF, Bleecker AB, Meyerowitz EM, (1993). Arabidopsis ethylene-response gene ETR1: similarity of product to two-component regulators. Science. Oct 22;262(5133):539-44.

28. Chang C, (1996). The ethylene signal transduction pathway in Arabidopsis: an emerging paradigm? Trends Biol. Sci, 21, 129-133.

29. Chae HS, Faure F, Kieber JJ, (2003). The *eto1*, *eto2*, and *eto3* mutations and cytokinin treatment increase ethylene biosynthesis in *Arabidopsis* by increasing the stability of ACS protein. *Plant Cell* 15, 545–559

30. Chao Q, Rothenberg M, Solano R, Roman G, Terzaghi W, Ecker JR., (1997).Activation of the ethylene gas response pathway in Arabidopsis by

the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. Cell. Jun 27;89(7):1133-44.

31. Chen YF, Randlett MD, Findell JL and Schaller GE (2002) Localization of the ethylene receptor ETR1 to the endoplasmic reticulum of *Arabidopsis*. *J Biol Chem*, 277, 19861–19866.

32. Cheung AY, (1995). Pollen-pistil interaction in compatible pollination. Proc. Natl. Acad. Sci. USA 92: 3077-3080.

33. Cheung AY, Wang H, and Wu H, (1995). A floral transmitting tissue-specific glycoprotein attracts pollen tubes, and stimulate their growth. Cell, 82: 383-393.

34. Chuck G, Muszynski M, Kellogg E, Hake S, Schmidt RJ, (2002). The control of spikelet meristem identity by the branched silkless1 gene in maize. Science, Nov 8;298(5596):1238-41.

35. Clark DG, Gubrium EK, Barrett JE, Nell TA, and Klee HJ, (1999). Root Formation in Ethylene-Insensitive Plants. Plant Physiol. 121: 53-60.

36. Clark KL, Larsen PB, Wang X and Chang C (1998) Association of the *Arabidopsis* CTR1 Raf-like kinase with the ETR1 and ERS ethylene receptors. *Proc Natl Acad Sci USA*, 95, 5401–5406.

37. Cormack H, (1935). The development of root hair by Elodea Canadensis. New phytol. 34, 19-25.

38. Cristescu S., De Martinis D., te Lintel Hekkert S., Parker D.H., & Harren F.J.M., (2002) Ethylene production by Botrytis cinerea in vitro and in tomatoes. Appl. Environ. Microbiol. 68, No. 11, p. 5342-5350.

39. de Graaf BHJ, (1999). Pistil proline-rich proteins in *Nicotiana tabacum.*Their involvement in pollen-pistil interaction. Ph.D Thesis, Catholic University of Nijmegen (NL).

40. De Martinis D, Mariani C, (1999). Silencing gene expression of the ethylene-forming enzyme results in a reversible inhibition of ovule development in transgenic tobacco plants Plant Cell. Jun;11(6):1061-72.

41. Dickinson HG, (1996). Plant signalling comes on age: identification of self-pollen in Brassica involves a transmembrane receptor kinase. Trends in Plant Science 1:16-138.

42. Dong, X, (1998). SA, JA, ethylene, and disease resistance in plants. Curr. Opin. Plant Biol. 1:316-323.

43. Drews GN, Lee D, and Christensen CA, (1998). Genetic analysis of female gametophyte development and function. Plant Cell,10, 5-17.

44. Dolan L, Duckett CM, Grierson D, Linstead CP, Schneider K, Lawson E, Dean C, and Roberts K, (1994). Clonal relationship and cell patterning in the root epidermis of Arabidopsis. Development. 120, 2465-2474.

45. Drews GN, Lee D, Christensen CA .(1998). Genetic Analysis of Female Gametophyte Development and Function. Plant Cell 10: 5-18

46. Ecker JR, (1995). The ethylene signal transduction pathway in plants. Science. May 5;268(5211):667-75.

47. Eden, MA, Hill, RA, Beresford, R, and Stewart, A, (1996). The influence of inoculum concentration, relative humidity, and temperature of infection of greenhouse tomatoes by *Botrytis cinerea*. Plant Pathol. 45:795-806.

48. El-Kazzaz, MK, Sommer NF, and Kader, AA, (1983). Ethylene effects on *in vitro* and *in vivo* growth of certain postharvest fruit-infecting fungi. Phytopatology 73:998-1001.

49. Elad, Y, (1990). Production of ethylene by tissue of tomato, pepper, French-bean and cucumber in response to infection by *Botrytis cinerea.* Physiol. Mol. Plant Pathol. 36:277-287.

50. Elad, Y, (1997). Responses of plants to infection by *Botrytis cinerea* and novel means involved in reducing their susceptibility to infection. Biol. Rev. 72:381-422.

51. Elad, Y, and Eversen, K, (1995). Physiological aspects of resistance to *Botrytis cinerea*. Phytopathology 85:637-643.

52. Elliott RC, Betzner AS, Huttner E, Oakes MP, Tucker, WQ, Gerentes D, Perez P, Smyth DR, (1996). AINTEGUMENTA, an APETALA2-like gene of Arabidopsis with pleiotropic roles in ovule development, and floral organ growth. Plant Cell, 8, 155-168.

53. Epstein, E, Sagee, , O, Cohen, JD, and Garty, J, (1986). Endogenous auxin and ethylene in the Lichen *Ramalina duriaei.* Plant Physiol. 82:1122-1125.

54. Fallik, E, Ilic, Z, Tuvia-Alkalai, S, Copel, A & Polevaya, Y (2002). A short hot water rinsing and brushing reduces chilling injury and enhance resistance against Botrytis cinerea in fresh harvested tomato. *Adv. Hortic. Sci.* 16: 3-6.

55. Fallik, E, Polevaya, Y, Tuvia-Alkalai, S, Shalom, Y & Zuckermann, H (2003). A 24 h anoxia treatment reduces decay development while maintaining tomato fruit quality. *Postharvest Biol. Technol.* (in press).

56. Feys, BJ, and Parker, JE, (2000). Interplay of signalling pathways in plant disease resistance. Trends Genet. 16:449-455.

57. Fukuda, H, Kitajima, H, Fujii, T, Tazaki, M, and Ogawa, T, (1989). Purification and some properties of novel ethylene-forming enzyme produced by *Penicillium digitatum*. FEMS Microbiol. Lett. 59: 1–6.

58. Fukuda, H, Takahashi, M, Fujii, T, Tazaki, M, and Ogawa, T, (1989). An NADH: Fe(III) EDTA oxidoreductase from *Cryptococcus albidus*: an enzyme involved in ethylene production *in vivo*? FEMS Microbiol. Lett. 60: 107–112.

59. Fukuda, H, Ogawa, T, and Tanase, S, (1993). Ethylene production by micro-organisms, p. 275–306. *In* A. H. Rose (ed.), Advances in microbial physiology, vol. 35. Academic Press Inc., London.

60. Gardner RC, Richards KD, Bradbury LE, Delhaize E; "Aluminium Tolerance in Yeast Conferred by Over-expression of Stylosanthes Genes"; Unpublished. Gardner RC, Richards KD, Carrucan G, Bradbury LE. Submitted (05-MAR-1997) to the EMBL/GenBank/DDBJ databases.

61. Goldberg RB, (1989). Plants: novel developmental processes. Science, 240, 1460-1467

62. Goldman MH, Pezzotti M, Seurinck J, Mariani C, (1992).Developmental expression of tobacco pistil-specific genes encoding novel extensin-like proteins. Plant Cell. Sep; 4(9):1041-51.

63. Goldman MHS, Goldberg RB, and Mariani C, (1994). Female sterile tobacco plants are produced by stigma-specific cell ablation. The EMBO J 13 (13): 2976-2984.

64. Guzman P, Ecker JR., (1990). Exploiting the triple response of Arabidopsis to identify ethylene-related mutants. Plant Cell. Jun;2(6):513-23.

65. Halevy AH and Mayak S, (1981). Senescence and postharvest physiology of cut flowers. Hortic. Rev. 3,59-143.

66. Hall AE and Bleecker AB (2003). Analysis of Combinatorial Loss-of-Function Mutants in the Arabidopsis Ethylene Receptors Reveals That the *ers1 etr1* Double Mutant Has Severe Developmental Defects That Are EIN2 Dependent The Plant Cell, Vol. 15, 2032-2041,

67. Hamilton, AJ, Lycett, GW, and Grierson, D, (1990) Antisense gene that inhibits synthesis of the hormone ethylene in transgenic plants. Nature 346:284-287

68. Harren FJM, Berkelmans, R, Kuiper, K, te Lintel Hekkert, S, Scheepers, P, Denhuijzen, R, Hollander, P, and Parker, DH, (1999). On-line laser photoacoustic detection of ethene in exhaled air as biomarker of ultraviolet radiation damage of the human skin. Appl. Phys. Lett. 74:1761-1763.

69. Harren FJM, Bijnen FGC, Reuss J, Voeseneck LACJ, Blom CWPM, (1990). Sensitive intracavity photoacoustic measurement with a CO2 waveguide laser. Appl.Phys. B, 50,137-144.

70. Harren, F and Reuss J, R, (1997). Photoacoustic spectroscopy, in 'Encyclopedia of Applied Physics' Vol. 19 (Ed.) G.L. Trigg (VCH, Weinheim) 413-435

71. Hensel LL, Grbic V, Baumgarten DA, and Bleecker AB, (1993). Developmental and Age-Related Processes That Influence the Longevity and Senescence of Photosynthetic Tissues in Arabidopsis. Plant Cell 5: 553-564.

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

73. Hoekstra A and Weges R, (1986). Lack of control by early pistillate ethylene of the accelerated wilting in Petunia hybrida. Plant Phys. 80,403-408.

74. Hoekstra A and Van Roekel T, (1988). Effect of previous pollination and stylar ethylene on pollen tube growth in Petunia hybrida styles. Plant Phys. 86,4-6.

75. Hua, J, and Meyerowitz, EM, (1998) Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana*. Cell 94:261-271.

76. Hua, J, Chang, C, Sun, Q, and Meyerowitz, EM, (1995) Ethylene insensitivity conferred by *Arabidopsis ERS* gene. Science 269:1712-1714.

77. Hua J, Chang C, Sun Q, Meyerowitz EM. 1995. Ethylene insensitivity conferred by Arabidopsis *ERS* gene. *Science* 269:1712-14

78. Hua, J, Sakai, H, Nourizadeh, S, Chen, QJ, Bleecker, A.B, Ecker, JR, and Meyerowitz, EM, (1998) *EIN4* and *ERS2* are members of the putative ethylene receptor gene family in Arabidopsis. Plant Cell 10:1321-1332

79. Hulskamp M, Schneitz K, Pruitt RE, (1995). Genetic evidence for a long-range activity that directs pollen tube guidance in Arabidopsis. Plant Cell, 7, 57-64.

80. Ince, JE, and Knowles, CJ, (1986). Ethylene formation by cell-free extracts of *Escherichia coli*. Arch. Microbiol. 146:151-158.

81. Irish VF, (1999) Patterning the flower. Dev Biol 15;209(2):211-20

82. Isaac J, Drake R, Farrell A, Cooper W, Lee, L, Horton P, and Grierson D, (1995). Delay leaf senescence in deficient ACC-oxidase antisense tomato plants: molecular and physiological analysis. Plant J. 7(3),483-490.

83. Kao TH**,** and Tsukamoto T, (2004) The Molecular and Genetic Bases of S-RNase-Based Self-Incompatibility Plant Cell., 16 Suppl:S72-83

84. Kao TH, McCubbin AG., (1996).How flowering plants discriminate between self and non-self pollen to prevent inbreeding. Proc Natl Acad Sci U S A. Oct 29;93(22):12059-65.

85. Kende H, Zeevaart J, (1997).The Five "Classical" Plant Hormones. Plant Cell.Jul;9(7):1197-1210.

86. Kepczynska, E, (1989). Ethylene requirement during germination of *Botrytis cinerea* spores. Physiol. Plantarum 77:369-372.

87. Kepczynska, E, (1993). Involvement of ethylene in the regulation of growth and development of the fungus *Botrytis cinerea Pers. ex. Fr.* Plant Growth Regul. 13:65-69.

88. Kerr, EL, Atwood,JG, (1968). The laser illuminated absorptivity spectrophone: e method for measurement of weak absorptivity in gases at laser wavelength. Appl. Opt. 7, 915-921

89. Kieber, JJ, Rothenberg, M, Roman, G, Feldmann, KA, and Ecker, JR, (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

90. Kizis D, and Pages M, (2002). Maize DRE-binding proteins DBF1 and DBF2 are involved in rab17 regulation through the drought-responsive element in an ABA-dependent pathway. Plant J.Jun;30(6):679-89

91. Kizis D, Lumbreras V, Pages M, (2001). Role of AP2/EREBP transcription factors in gene regulation during abiotic stress. FEBS Lett. Jun 8;498(2-3):187-9.

92. Klucher KM, Chow H, Reiser L, Fischer RL, (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.

93. Knoester M, van Loon LC, van den Heuvel J, Hennig J, Bol JF, Linthorst HJM., (1998). Ethylene-insensitive tobacco lacks nonhost resistance against soil-borne fungi Proc Natl Acad Sci U S A. Feb 17;95(4):1933-7.

94. Knoester, M, Linthorst, H J M, Bol, J, & Van Loon, L C, (1997). Modulation of stress-inducible ethylene biosynthesis by sense and antisense gene expression in tobacco.Plant Sci. 126, 173-183

95. Koltunow AM, Truenettner J, Cox KH, Wallroth M, Goldberg RB, (1990). Different temporal, and spatial expression pattern occur during anther development. Plant Cell, 2, 1201-1224.

96. Kostoff D, (1930). Ontogeny, genetics and cytology of Nicotiana hybrids. Genetica 12: 133-139.

97. Kuboyama T, Chung CS and Takeda G, (1994). The diversity of interspecific pollen-pistil incongruity in Nicotiana. Sex. Plant Reproduction. 7:250-258.

98. Kumar, A, Taylor, MA, Mad Arif, SA. & Davis, HV, (1996). Antisense and sense expression of S-adenosylmethionine decarboxylase cDNA in transgenic potato plant and its effects on polyamines and ethylene biosynthesis. Plant Journal 9, 147-158.

99. Lanahan MB, Yen HC, Giovannoni JJ, and Klee HJ, (1994) The Never Ripe Mutation Blocks Ethylene Perception in Tomato. Plant Cell 6: 521-530.

100. Lashbrook CC, Tieman DM, Klee HJ., (1998). Differential regulation of the tomato ETR gene family throughout plant development. Plant J. 1998 Jul;15(2):243-52.

101. Lawton KA, Potter SL, Uknes S, Ryals J, (1994). Acquired Resistance Signal Transduction in Arabidopsis Is Ethylene Independent. Plant Cell. May;6(5):581-588.

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

103. Lei CH, Lindstrom JT, Woodson WR, (1996). Reduction of 1-aminocyclopropane-1-carboxylic acid (ACC) in pollen by expression of ACC deaminase in transgenic Petunias. Plant Phys. 111. Supplement 149.

104. Li HY, and Gray JE, (1997). Pollination enhanced expression of a receptor-like protein kinase related gene in tobacco styles. Plant Mol. Biol. 33(4):653-665.

105. Lincoln, JE, Cordes, S, Read, E, and Fischer, RL, (1987) Regulation of gene expression by ethylene during *Lycopersicum esculentum* (tomato) fruit development. Proc. Natl. Acad. Sci. USA 84:2793-2797

106. Lord EM, Russel SD, (2002). The mechanisms of pollination and fertilization in plants. Annu Rev Cell Dev Biol. 18:81-105.

107. Lurie, S, and Garty, J, (1991). Ethylene production by the lichen *Ramalina duriaei*. Ann. Bot. 68:317-319.

108. Mansfield, JW, (1980). Mechanism of resistance to *Botrytis*, p.181-218. *In* J. R. Coley-Smith, K. Verhoeff and W. R. Jarvis (ed.), The biology of *Botrytis*. Academic Press, New York/London.

109. Mariani C, de Bleuckeleer M, Truettner J, Leemans J, and Goldberg RB, (1990). Induction of male sterility in plants by a chimaeric ribonuclease gene. Nature 347:737-741.

110. Mariani C, Wolters-Arts M. (2000) Complex waxes. Plant Cell., Oct;12(10):1795-8.

111. Mattoo, AK, and Suttle, JC, (1991). The plant hormone ethylene. CRC Press, Boca Raton, Fla.

112. Marubashi W, and Nakajima T, (1981). Pollen Tube Behavior in the Ovary of Nicotiana tabacum L Japan J. Breed. 31(29): 133-140.

113. Mazarei M, Puthoff DP, Hart JK, Rodermel SR, Baum TJ, (2002). Identification and characterization of a soybean ethylene-responsive element-binding protein gene whose mRNA expression changes during soybean cyst nematode infection. Mol Plant Microbe Interact. Jun;15(6):577-86.

114. Meyer, P, and Saedler, H, (1996). Homology dependent gene silencing in plants. Annu. Rev. Plant Phys. Plant Mol. Biol. 47:23-48

115. Morrison DK and Cutler RE (1997) The complexity of Raf-1 regulation. *Curr Opin Cell Biol*, 9, 174–179.

116. Montero C, Cristescu SM, Jimenez JB, Orea JM, te Lintel Hekkert S, Harren FJM, & Gonzalez Ureña A, (2003). trans-Resveratrol and grape disease resistance. A dynamical study by high-resolution laser-based techniques. *Plant Physiol.* 131, No.1, p. 129-138

117. Nagahama, K, Ogawa, T, Fujii, T, Tazaki, M, Tanase, S, Morino, Y, and Fukuda, H, (1991). Purification and properties of an ethylene-forming enzyme from *Pseudomonas syringae* pv. *phaseolicola* PK2. J. Gen. Microbiol. 137: 2281–2286.

118. Nelijubow D, (1901). Ueber die horizontale nutation der stregel von Pisum sativum un einiger anderen. Pflanzen Beih. Bot. Zentralbl. 10, 128-139.

119. O'Neill, SD. and Nadeau, JA, (1996). Postpollination flower development. Hort. Reviews Vol. 19.

120. O'Neill SD, Nadeau JA, Zhang XS, Bui AQ, Halevy AH.,(1993). Interorgan regulation of ethylene biosynthetic genes by pollination. Plant Cell. Apr;5(4):419-32.

121. Oeller, PW, Lu, MW, Taylor, LP, Pike, DA, and Theologis, A, (1991). Reversible inhibition of tomato fruit senescence by antisense RNA. Science 254:437-439

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

123. Ohme-Takagi M, Suzuki K, Shinshi H, (2000). Regulation of ethylene-induced transcription of defense genes. Plant Cell Physiol. Nov;41(11):1187-92.

124. Ouaked F, Rozhon W, Lecourieux D, Hirt H (2003) A MAPK pathway mediates ethylene signaling in plants. EMBO J. 17;22(6):1282-8.

125. Papadopoulou, E, Little, HA, Hammar, SA, Grumet, R (2005) Effect of modified endogenous ethylene production on sex expression, bisexual flower development and fruit production in melon (*Cucumis melo* L.). Sex. Plant Rep. 18: 131-142.

126. Patel, CKN, Burkhardt,EG, Lambert, CA, (1974) Spectroscopic measurement of stratosferic Nitric Oxide and water vapour. Science, 184, 1173-1176.

127. Pazout, J, Pazoutova, S, and Vancura, V, (1982). Effects of light and oxygen on ethylene formation and conidiation in surface cultures of *Penicillium cyclopium*. Curr. Microbiol. 7:133-136.

128. Picton S, Barton SL, Bouzayen M, Hamilton AJ, and Grierson D, (1993). Altered fruit ripening and leaf senescence in tomatoes expressioçng an antisense ethylene-forming enzyme transgene. Plant J. 3(3), 469-481.

129. Pitts RJ, Cernac A, Estelle M, (1998).Auxin and ethylene promote root hair elongation in Arabidopsis. Plant J. Dec;16(5):553-60

130. Polevaya, Y, Alkalai-Tuvia, S, Copel, A, and Fallik, E, (2002). Early detection of grey mould development in tomato after harvest. Postharvest Biol. Technol., in press.

131. Qadir A, Hewett, EW , and Long, PG, (1997). Ethylene production by *Botrytis cinerea*. Postharvest Biol. Technol. 11:85-91.

132. Qu X and Schaller GE (2004). Requirement of the histidine kinase domain for signal transduction by the ethylene receptor ETR1. Plant Physiol.136(2):2961-70.

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

134. Reid MS, Ethylene in plant growth, development and senescense, in: PJ. Davies (Eds.) Plant Hormones, 2nd edition, Kluwer Acad. Publishers pp.486-508.

135. Reiser L, and Fischer RL, (1993). The ovule and the embryo sac. Plant Cell, 5, 1291-1301.

136. Riechmann JL, Meyerowitz EM. The AP2/EREBP family of plant transcription factors. Biol Chem. 1998 Jun;379(6):633-46.

137. Romano CP, Cooper ML, Klee HJ., (1993). Uncoupling Auxin and Ethylene Effects in Transgenic Tobacco and Arabidopsis Plants. Plant Cell. Feb;5(2):181-189.

138. Rosati C, Cadic A, Duron M, Renou JP, Simoneau P, (1997). Molecular cloning and expression analysis of dihydroflavonol 4-reductase gene in flower organs of Forsythia x intermedia. Plant Mol Biol. Oct;35(3):303-11

139. Sakai H, Hua J, Chen QJ, Chang C, Medrano LJ, Bleecker AB, Meyerowitz EM, (1998). ETR2 is an ETR1-like gene involved in ethylene signaling in Arabidopsis. Proc. Natl. Acad. Sci. USA, 95, 5812-5817

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

141. Sanchez, AM (2001). Pollen-pistil interaction in *Nicotiana*. Study of an incogruous cross. Ph.D Thesis, Catholic University of Nijmegen (NL)

142. Schaller GE and Bleecker AB (1995) Ethylene-binding sites generated in yeast expressing the *Arabidopsis ETR1* gene. Science, 270, 1809–1811.

143. Schneitz K, Hulskamp M, Pruitt RE, (1995). Wild-type ovule development in Arabidopsis thaliana: a light microscope study of cleared whole-mount tissue. The Plant J. ,7 (5), 731-749

144. 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

145. Skinner DJ, Hill TA, Gasser CS. (2004). Regulation of ovule development. Plant Cell;16 Suppl:S32-45.

146. Sijacic P, Wang X, Skirpan AL, Wang Y, Dowd PE, McCubbin AG, Huang S, Kao TH (2004) Identification of the pollen determinant of S-RNase-mediated self-incompatibility. Nature. 20;429(6989):302-5.

147. Singh A, Evensen KB, and Kao TH, (1992). Ethylene synthesis and floral senescence following compatible and incompatible pollinations in Petunia inflata. Plant Phys. 99:38-45

148. Smyth, DR, Bowman, JL, and Meyerowitz, EM, (1990) Early flower development in *Arabidopsis*. Plant Cell 2:755-767

149. Smyth DR., (1996) Plant genetics: fast flowering. Curr Biol. Feb 1;6(2):122-4

150. Solano R, Stepanova A, Chao Q, Ecker JR., (1998).Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. Genes Dev. Dec 1;12(23):3703-14

151. Staal M, Lintel-Hekkert ST, Harren F, & Stal L (2001). Nitrogenase activity in cyanobacteria measured by the acetylene reduction assay: a comparison between batch incubation and on-line monitoring. *Environ. Microbiol.* 3(5):343-51

152. Stearns JC, Glick BR (2003). Transgenic plants with altered ethylene biosynthesis or perception. Biotechnol Adv. May;21(3):193-210.

153. Tang X, Gomes AMTR, Bhatia A, Woodson WR, (1994). Pistil-specific, and ethylene-regulated expression of of 1-aminocyclopropane-1-carboxylate oxidase genes in petunia flowers. Plant Cell 6: 1227-1239

154. Tanimoto K, Roberts K and Dolan L, (1995). Ethylene is a positive regulator of root hair development in *Arabidopsis thaliana*. Plant J. 8(6), 943-948

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

156. te Lintel Hekkert, S, Staal MJ, Nabben, RHM, Zuckermann, H, Persijn, S, Staal, LJ , Voesenek, LACJ, Harren, FJM, Reuss, J, and Parker, DH, (1998). Laser photoacoustic trace gas detection, an extremely sensitive technique applied in biological research. Instrum. Sci. Technol. 26:157-175.

157. ten Have, A, Breuil, WO, Wubben, JP, Visser, J, and van Kan, JA, (2001). *Botrytis cinerea* endopoligalacturonase genes are differentially expressed in various plant tissues. Fungal Genet. Biol. 33:97-105.

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

159. Theologis, A, (1993) Use of a tomato mutant constructed with reverse genetics to study fruit ripening, a complex developmental process. Dev. Genet.14,282-295

160. Tian, HQ, and Russel, SD, (1997). Calcium distribution in fertilized, and unfertilized ovules, and embryo sacs of Nicotiana tabacum L Planta, 202: 93-105.

161. Tova, T, Staub, JK.E, O'Neill SD, (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 Phys., 113, 987-995.

162. Vahala T, Oxelman B, von Arnold S, (2001). Two APETALA2-like genes of Picea abies are differentially expressed during development.J Exp Bot. May;52(358):1111-5.

163. Van Der Graaff E, Hooykaas PJ, Keller B, (2002). Activation tagging of the two closely linked genes LEP and VAS independently affects vascular cell number. Plant J. Dec;32(5):819-30.

164. van Eldik GJ, Vriezen WH, Wingers M, Ruiter RK, Van Herpen MMA, Schrauwen JAM, Wullems GJ, (1995). A pistil-specific gene of *Solanum tuberosum* is predominantly expressed in the stylar cortex. Sex Plant Reprod 8: 173-179.

165. Vogel JP, Woeste KE, Theologis A, Kieber JJ(1998),.Recessive and dominant mutations in the ethylene biosynthetic gene *ACS5* of *Arabidopsis* confer cytokinin insensitivity and ethylene overproduction, respectively. *Proc. Natl Acad. Sci. USA* 95, 4766–4771

166. von Tiedemann, A, (1997). Evidence for a primary role of active oxygen species in induction of host cell death during infection of bean leaves with *Botrytis cinerea*. Physiol. Mol. Plant Pathol. 50:151-166.

167. Wang KL, Yoshida H, Lurin C, Ecker JR (2004)Regulation of ethylene gas biosynthesis by the Arabidopsis ETO1 protein. ETO mutations increase ethylene biosynthesis in *Arabidopsis* by increasing the stability of ACS protein Nature. 428(6986):945-50

168. Wang, H, Wu, HM, and Cheung, AY, (1996). Pollination induces mRNA poly(A) tail-shortening and cell deterioration in flower transmitting tissue. Plant J. 9:715-727

169. Weigel D, (1995) The APETALA2 domain is related to a novel type of DNA binding domain. Plant Cell 7,388-389.

170. Welk, Sr. M, Millington, F, and Rosen WG, (1965). Chemotropic activity and the pathway of the pollen tube in Lily. Amer. J. Bot. 52, 774-781

171. Weterings K, Pezzotti M, Cornelissen M, Mariani C.(2002) Dynamic 1-aminocyclopropane-1-carboxylate-synthase and -oxidase transcript accumulation patterns during pollen tube growth in tobacco styles.Plant Physiol. Nov;130(3):1190-200

172. Wilkinson, JQ, Lanahan, MB, Clark, DG, Bleecker, AB, Chang, C, Meyerowitz, EM, and Klee, HJ, (1997), A dominant mutant receptor from *Arabidopsis* confers ethylene insensitivity in heterologous plants. Nature Biotechnol. 15:444-447

173. Woltering EJ, van Hout M, Somhorst D and Harren F, (1992). Roles of pollination, short chain saturated fatty acids in flower senescence. Plant Griowth Regulation 12:1-10

174. Woltering EJ, de Vrije T, Harren F, and Hoekstra FA, (1997). Pollination and stigma wounding: same response, different signal? J. of Exp. Botany. 48 (310):1027-1033

175. Wolters-Arts, M, Derksen, J, Kooijman, J W. & Mariani, C, (1996). Stigma development in *Nicotiana tabacum*. Cell death in transgenic plants as a marker to follow cell fate at high resolution. Sex. Plant Rep. 9, 243-254

176. Wolters-Arts, M, Lush, WM, and Mariani, C, (1998). Lipids are required for directional pollen-tube growth. Nature 392:818-821

177. Yang, SF, (1969). Ethylene evolution from 2-chloroethylphosphonic acid. Plant Phys. 44:1203-1204

178. Yang, SF, Hoffman, NE, (1984). Ethylene biosynthesis and its regulation in higher plants. Ann. Rev. Plant Physiol. 35: 155-189

179. Yang HJ, Shen H, Chen L, Xing YY, Wang ZY, Zhang JL, and Hong MM, (2002). The OsEBP-89 gene of rice encodes a putative EREBP transcription factor and is temporally expressed in developing endosperm and intercalary meristem. Plant Mol Biol. Oct;50(3):379-91

180. Zharov VP and Letokhov VS (Eds., 1986). Laser Optoacoustic Spectroscopy (Springer Verlag, Berlin Heidelberg,)

181. Zhang, XS, and O' Neill, SD, (1993). Ovary, and gametophyte development are coordinately regulated by auxin, and ethylene following pollination. Plant Cell 5, 403-418

182. Zuckermann, H, Staal, M, Stal, LJ, Reuss, J, te Lintel Hekkert, S, Harren, FJM, and Parker, DH, (1997). On line monitoring of nitrogenase activity in cyanobacteria by sensitive laser photoacoustic detection of ethylene. Appl. Environ. Microbiol. 63:4243-425