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Expression of the ACC synthase and ACC oxidase coding genes after self-pollination and incongruous pollination of tobacco pistils

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Abstract

In tobacco, as in other species, ethylene is produced in response to pollination. Although tobacco is a self-compatible species, it displays unilateral incongruity with other *Nicotiana* plants. Incongruous pollination also results in ethylene production, but this production differs depending on the pollen used and is related to the extent to which pollen tubes grow in the tobacco style. In the investigation reported here we followed the expression of the ACC synthase- and ACC oxidase-coding genes upon pollination of tobacco pistils and compared self-pollination with incongruous pollination. The pattern of expression of these genes also correlated with pollen-tube growth, although wounding alone cannot explain the results obtained. We also examined the expression of these genes upon pollination of immature tobacco pistils, in which different pollen tubes grew indistinctly inside the tobacco style and reached the ovary at the same rate. In this situation no significant differences in gene expression could be observed between the different pollinations. Ethephon, a substance that produces ethylene, could, in some cases, minimize the arrest of incongruous pollen tubes inside the style.

Introduction

The gas ethylene is the simplest plant hormone produced either during plant development (e.g. during fruit ripening, senescence, seed development, flowering) or as a response to environmental cues, wounding, pathogenesis, and other stress-related responses (Kieber and Ecker, 1993). In reproduction, ethylene is involved in post-pollination responses that include corolla and style senescence, changes in petal colour, ovary enlargement and even ovule development in some species (O'Neill, 1997).

The synthesis of ethylene begins with the production of *S*-adenosylmethionine (SAM) from the amino acid methionine. SAM is then converted to 1-aminocyclopropane-1-carboxylic acid (ACC), a reaction catalysed by ACC synthase, which is further oxidized to ethylene in the presence of ACC oxidase. Both the ACC synthase- and the ACC oxidase-coding genes belong to multigene families. Tobacco pistil-specific ACC synthase and ACC oxidase cDNAs have been isolated (Weterings, Pezzotti, Cornelissen and Mariani, manuscript in preparation).

After production and release, ethylene is sensed by the plant, and the signal is transduced in a physiological response. The ethylene-signalling pathway has been elucidated in *Arabidopsis* through the isolation and characterization of several mutants deficient in the perception of ethylene or the response to it (Johnson and Ecker, 1998). Homologues for the genes cloned in *Arabidopsis* have also been identified in other species, suggesting that similar pathways might occur throughout the plant kingdom (Bleecker, 1999). The model currently accepted for ethylene action postulates the existence of a phosphorylation cascade that connects the ethylene membrane receptor (where ethylene is sensed upon binding) to the action of different ethylene-related transcription factors that lead to the phenotypes associated with exposure to ethylene (Woeste and Kieber, 1998).

In most species, pollination is accompanied by increased ethylene production in the stigma and style (O'Neill, 1997). Several changes, such as abscission and senescence of specific flower organs, take place in the flower upon pollination, and ethylene is believed to be involved in the inter-organ signalling that serves to co-ordinate those changes with pollen germination and tube growth (Tang *et al.*, 1994). Post-pollination responses and the involvement of ethylene have been a matter of study in several species including geranium (Clark *et al.*, 1997), *Petunia* (Hoekstra and Roekel, 1988; Tang *et al.*, 1994), carnation (Jones and Woodson, 1997) and orchids (Nadeau *et al.*, 1993).

Activation of the expression of the ethylene biosynthesis genes ACC synthase and ACC oxidase often occurs after pollination and seems to be the main route by which plants control the ethylene produced upon pollination. Following the expression of these genes is therefore one way to characterize the involvement of ethylene in post-pollination responses. The other way is to follow ethylene production *in planta* (De Martinis *et al.*, submitted).

Common tobacco, *Nicotiana tabacum*, like *Petunia hybrida* or *Petunia inflata*, belongs to the Solanaceae family. Tobacco is self-compatible and, consequently, upon self-pollination, the pollen tubes grow all the way down to the ovary where fertilization takes place. In contrast, in some inter-specific crosses between tobacco and other *Nicotiana* species pollen grains are able to germinate, and pollen tubes grow only to a certain extent in the tobacco style. As there is no involvement of the *S*-locus that could account for the differences between compatible and incompatible pollination, these incongruous crosses provide a unique tool for dissecting the relationship between pollen-tube growth and post-pollination responses.

Kuboyama *et al.* (l996) described how pollen grains from *Nicotiana repanda* and *N. rustica* behave when placed on the tobacco stigma. Immediately after germination the two kinds of pollen tubes are indistinguishable from self-pollen. A few hours after pollination, however, *N. repanda* pollen tubes arrest in the stigma, while *N. rustica* pollen tubes grow further at the same rate as tobacco, only stopping half the length of the style. Another *Nicotiana, N. maritima*, presents a similar growth pattern to that of *N. repanda*, but the pollen tubes emerge from the stigma and arrest as soon as they reach the transmitting tissue. In all these cases, the inability of the pollen tubes to reach the ovary of tobacco is only observed if fully mature tobacco pistils are pollinated. If immature tobacco pistils are used instead in the crosses described, there is no difference in pollen-tube growth, and all of the pollen tubes reach the bottom of the style at approximately the same rate.

In the investigation reported here we made use of the fact that different types of *Nicotiana* pollen tubes grow different lengths in the style of tobacco in order to compare the expression of the ACC synthase and ACC oxidase genes in the pistil after pollination. We decided to use *N. maritima* instead of *N. repanda* because this ensured that all of the pollen tubes grew at least a few millimetres into the transmitting tissue. We also made use of the fact that tobacco flowers can be pollinated at an immature stage to study gene expression in a situation in which there is no difference between self or incongruous pollen tubes.

Materials and methods

Plant material

Plants of *Nicotiana tabacum* L. (tobacco) cv. Petit Havana, *N. rustica* L. and *N. maritima* were grown under greenhouse conditions. Only tobacco pistils were used in these experiments, but the three types of *Nicotiana* flowers served as pollen donors.

Pollinations

Tobacco flowers were emasculated at stage 10 of flower development (according to Koltunow *et al.*, 1990) and pollinated at a time corresponding to anthesis with pollen of different origins. The pistils (excluding the ovary) were collected at several time points after pollination. For simplicity, the stigma/style used in all experiments, are referred to as style in Results and Discussion.

For bud pollination, the flowers were emasculated and immediately pollinated at stage 6 of flower development.

Pollinated mature styles were collected 72 h after pollination to allow the largest number of pollen tubes to grow. Immature styles were collected 24 h after pollination because at this stage all pollen tubes of the three species used have reached the bottom of the style, at this time.

Total RNA isolation

After collection, the tissue was frozen in liquid nitrogen and stored at $-80\ ^{\circ}C$ until RNA extraction.

The tissue was ground in phenol/extraction buffer (Goldberg, 1988) and extracted with phenol/chloroform. The nucleic acids were precipitated with sodium acetate and ethanol, resuspended, and the RNA precipitated with lithium chloride. Finally, the RNA was dissolved in water and the concentration measured spectrophotometrically.

Northern blot analysis

RNA (5–10 μg per sample) was separated on an agarose gel with 6% formaldehyde in MOPS buffer, and the gel was blotted overnight onto a Hybond-N membrane.

After baking for 2 h at 80 °C the membranes (Hybond-N) were hybridized according to the manufacturer's instructions with pistil-specific ACC oxidase or ACC synthase cDNA labelled with ^{32}P-dATP. For quantification, control hybridizations with a probe for 25S ribosomal RNA were also performed after stripping of the membrane.

A Kodak film was then exposed to the hybridized membrane in the presence of intensifier screens at −80 °C for variable periods of time. The films were scanned with a BioRad densitometer and the intensity of the signal measured with Molecular Analyst software (BioRad). For each blot the signal was measured for the transcripts of interest and also for the 25S transcript. All values for gene expression presented in the graphs were then corrected for the amount of rRNA present in the membrane by dividing the value obtained for ACC oxidase or ACC synthase by the corresponding value for 25S probe. In each chart 100% was attributed to the sample with the highest value for gene expression, and the other values were relative values within the same experiment.

Chemical treatments of the flowers

After emasculation tobacco flowers were placed into 1.5 ml tubes containing different solutions, i.e. 1 mM ethephon (2-chloro-ethylphosphonic acid), 0.1 mM AVG (aminoethoxyvinylglycine), or 0.5 mM STS (0.5 mM $AgNO_3$ and 2 mM $Na_2S_2O_3$). Then, 24 h after emasculation, the pistils were pollinated with different kinds of pollen. Controls were always performed by replacing the solutions by demineralized water. The flowers were collected 24 or 48 h after pollination and the pistils prepared as described below for observation of the pollen tubes. For RNA extraction, the styles were collected 24 h after pollination.

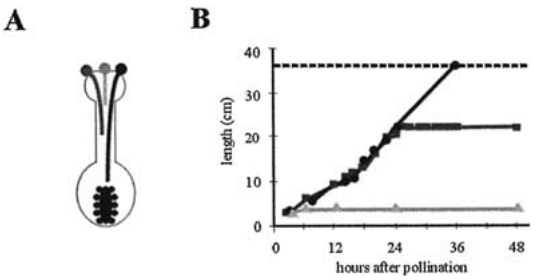

Figure 1. Pollen tube growth of different *Nicotiana* species in the tobacco style. Tobacco pollen (●), *N. rustica* pollen (■), *N. maritima* pollen (♧). A. Schematic representation of the different pollen tubes. B. Pollen tube length over time after pollination. Dashed line indicates total stylar length.

Histological procedures

The pollinated pistils were fixed in a solution of ethanol/acetic acid (3:1) at 4 °C overnight. The pistils were then rinsed with water and macerated by treatment with 2 M NaOH at 60 °C for 1 h. After several rinses the pistils were squashed between a glass slide and a cover slip in an aniline blue solution (0.1% in phosphate buffer). The slides were observed with a fluorescence microscope (UV light), and the length of the pollen-tube bunch was measured by marking the end of the bunch on the cover slip with a marker pen.

Results

Pollen-tube growth in tobacco pistils

After the pollination of mature tobacco pistils, all pollen grains of tobacco, *N. rustica* and *N. maritima* displayed similar germination and initial pollen-tube growth on the stigma, but only tobacco pollen tubes were able to reach the ovary. *N. maritima* pollen tubes arrested as soon as they penetrated the transmitting tissue of the style, 3 h after pollination. In the case of *N. rustica*, the pollen tubes grew normally until about half the length of the tobacco style but then no further growth was observed, 20 h after pollination (Figure 1). Pollen-tube arrest was accompanied by severe callose deposition in both species. In addition, *N. rustica* pollen tubes curled and wound, especially at the place of arrest.

Pollination with tobacco or *N. rustica* pollen accelerated tobacco flower senescence and the flowers abscised 4–5 days after pollination. Flowers pollinated with *N. maritima* pollen wilted later (6–7 days after pollination), almost at the same time as non-pollinated (only emasculated) flowers.

After the pollination of immature flowers, the pollen tubes grew normally all the way down to the ovary regardless of their source, although usually these flowers dropped after 2 days at most, showing little corolla development. If emasculation was performed 24 h before pollination, the flowers detached even earlier. Emasculation also altered the behaviour of *N. maritima* pollen tubes inside the tobacco style, since these were arrested, as in the fully mature pistil, a few hours after pollination. The emasculation of immature flowers did not affect *N. rustica* pollen-tube growth in tobacco, but winding and curling of the pollen tubes were observed at the bottom of the style.

Expression of the ACC synthase gene in mature and immature tobacco pistils after different kinds of pollination

The ACC synthase transcript was not detectable in total RNA extracted from non-pollinated mature tobacco styles. After pollination with tobacco pollen, however, the level of the transcript increased substantially, with a peak of expression at 36 h after pollination, followed by some decrease that did not reach the level of non-pollinated styles. Both *N. rustica* and *N. maritima* pollen led to an increase of ACC synthase gene expression, however the levels of transcript present were lower than those of self-pollinated styles.

N. rustica pollinations showed a plateau of expression between 12 and 24 h, but subsequently the expression of the ACC synthase gene decreased to levels similar to that of the *N. maritima* pollination (Figure 2A).

In immature non-pollinated styles ACC synthase mRNA was also not detectable. Pollination with any kind of pollen triggered a slight increase in ACC synthase expression that produced a similar pattern for the different pollen types. The level of expression was always very low, producing a very faint signal that could barely be quantified (Figure 2B).

Expression of the ACC oxidase gene in the mature and immature tobacco pistils after different kinds of pollination

Mature non-pollinated tobacco styles produced a basal level of ACC oxidase transcript. All different types of pollination caused this level to rise, but the increase was especially significant for tobacco and *N. rustica* pollen. The *N. maritima* pollination initially led to an increase, but 6 h after pollination the mRNA level had returned to non-pollinated levels. Tobacco and *N. rustica* pollinations had similar patterns of ACC oxidase gene expression (Figure 3A).

To examine the relationship between pollen-tube growth and ACC oxidase expression, we divided the tobacco style into three regions: stigma, upper part of the style (ca. 2 cm), and lower part of the style, and expression of ACC oxidase was measured in the different segments. Pistils were collected 48 h after pollination when the pollen tubes of the different *Nicotiana* species had reached the different areas: tobacco pollen tubes were at the bottom of the style, *N. rustica* pollen tubes were only in the upper part of the style, and *N. maritima* pollen tubes were only in the stigma region.

As already observed for the entire style, non-pollinated styles had a basal level of expression that was detectable in all three areas. The presence of pollen tubes in each area (regardless of their source) coincided with a higher amount of transcript. It is interesting to note that for the *N. rustica* pollination, the expression of ACC oxidase in the lower part of the style was higher than in the corresponding segment of the non-pollinated style, even though there were no pollen tubes present (Figure 3B).

In immature styles, even in the absence of pollination, there was an increase in the level of ACC oxidase mRNA over time. All the different kinds of pollen tubes were, however, able to produce a significant increase of the level of this transcript, and this did not differ much between the different pollinations (Figure 3C).

In the case in which emasculation was performed 24 h before pollination, the pattern observed for the *N. maritima* pollination was more similar to the non-pollinated situation. In contrast, in the case of *N. tabacum* and *N. rustica* pollination, the expression of ACC oxidase was very similar with or without previous emasculation (Figure 3D).

Treatment of tobacco flowers with ethephon

In order to ascertain if there was any effect of ethylene on pollen-tube growth, we carried out treatments with ethephon, an ethylene-producing substance, on excised flowers.

Ethephon treatment prior to pollination did not affect tobacco pollen-tube growth. It affected *N. rustica* pollen tubes very mildly in the sense that less winding was observed. Surprisingly, upon *N. maritima* pollination, instead of an arrest in growth immediately below

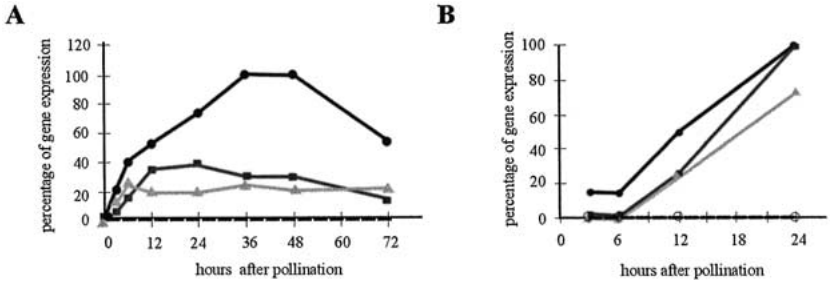

Figure 2. Level of ACC synthase expression after pollination with different kinds of pollen. Tobacco pollen (●), *N. rustica* pollen (■), *N. maritima* pollen (▲), non-pollinated (dashed line, ○). A. Pollination of mature tobacco pistils. B. Pollination of immature tobacco pistils. Charts represent relative values only within the same experiment; the highest value was set to 100%.

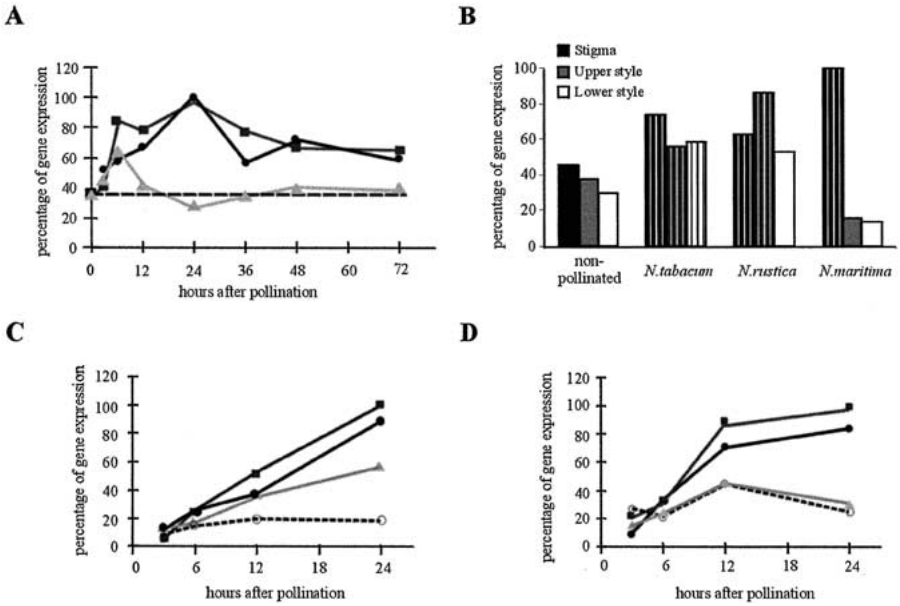

Figure 3. Level of ACC oxidase expression after pollination with different kinds of pollen. Tobacco pollen (●), *N. rustica* pollen (■), *N. maritima* pollen (▲), non-pollinated (dashed line, ○). A, B. Pollination of mature tobacco pistils: expression in the whole style over time (A) and in the different segments after 48 h (B). Striped bars indicate pollen tubes present in that stylar segment. C, D. Pollination of immature tobacco pistils in previously emasculated (C) and non-emasculated flowers (D). Charts represent relative values only within the same experiment; the highest value was set to 100%.

the stigma, pollen tubes continued to grow in the transmitting tissue for almost 2 cm. No further growth was observed even if ethephon was replenished several times. Bypassing the arrest of *N. maritima* pollen-tube growth was only possible if the ethephon treatment occurred prior to or simultaneously with pollination. The application of ethephon after the arrest had occurred at the stigma could not restore pollen-tube growth. In non-pollinated styles ethephon treatment accelerated corolla wilting.

Expression of the ACC oxidase and ACC synthase genes upon pollination was tested in excised flowers either treated or not treated with ethephon. In this case, even control (water-treated) non-pollinated styles had a detectable amount of ACC synthase transcript. This level was three times higher if the pistils had been treated with ethephon. Pistils pollinated with tobacco and *N. rustica* pollen had higher levels of ACC synthase mRNA than control non-pollinated pistil; pollination with *N. maritima* pollen induced a low level of expression. For all types of pollination, treatment with ethephon contributed to a higher production of this messenger (Figure 4A).

Ethephon treatment led to an increased expression of the ACC oxidase gene in the non-pollinated styles. As described in Figure 3A, pollination with tobacco or

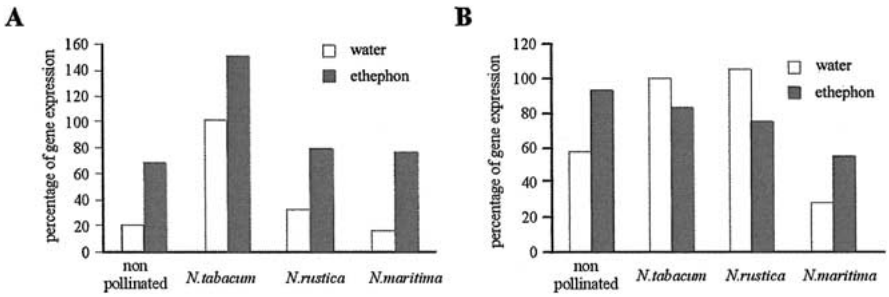

Figure 4. Level of ACC synthase and oxidase expression after different pollination in the presence and absence of ethephon. A. ACC synthase gene expression. B. ACC oxidase gene expression. Pollinations were conducted on excised flowers and the pistils were collected 24 h after pollination. The values for the expression in untreated self-pollinated pistils was set to 100% and the other points represent therefore relative values. 10 μg of total RNA were loaded on a gel.

N. rustica pollen also led to the production of a higher level of transcript than that found in the non-pollinated situation. When the pistils were treated with ethephon the amount of mRNA present after pollination was less. For *N. maritima*, pollination alone was not able to induce a high production of the ACC oxidase transcript; however treatment with ethephon increased this level significantly (Figure 4B).

Apart from ethephon, treatments with other chemicals that could either prevent or increase the production of ethylene were carried out, but no significant effect on pollen-tube growth was registered. Ethylene inhibitors (AVG or STS) had no effect on pollen-tube growth but they significantly reduced corolla wilting and senescence, while treatment with ACC (an ethylene precursor) induced no differences in the corolla. STS seemed to induce curling and callose deposition in *N. maritima* pollen tubes with no effect on total pollen-tube length. This probably reflects the effect of the silver ions on the pollen tubes, similar to what has been described for other heavy metals (Sawidis and Reiss, 1995).

Discussion

Similar to what occurs in other plant species (O'Neill, 1997), the pollination-induced increase in ethylene production in tobacco was accompanied by an increased expression of the ACC synthase and ACC oxidase genes. Upon self-pollination the levels of both transcripts increased in the tobacco style. This increase was visible a few hours after the pollen was placed on the stigma, and the level of the messenger remained high during the process of pollen-tube growth and fertilization. The regulation of expression of the genes coding for enzymes involved in ethylene biosynthesis is most likely a very effective way to control ethylene production upon pollination.

Pollen from other sources was also able to elicit an increased expression of the ethylene-related genes, thereby demonstrating that this response is not entirely specific for compatible pollination. However, it is clear that the patterns of expression differed for different types of pollination. We found a very good correlation between the type of pollen-tube growth in the tobacco style and the expression of these genes (and, consequently, of ethylene release): the further the pollen tubes grew the higher the level of the transcript (and the production of ethylene). This correlation was especially clear for ACC synthase (Figure 2A). Moreover, in a comparison of the expression of ACC synthase with the pattern of ethylene release (De Martinis *et al.*, submitted) it appeared that ACC synthase gene expression and the synthesis of ACC are the rate-limiting steps in ethylene production following pollination (O'Neill, 1997; Lindstrom *et al.*, 1999).

It is possible that ethylene release by the pistil is just a response to the wounding caused by the growing pollen tubes in the transmitting tract (Gilissen *et al.*, 1977). However, that does not seem likely in the present case. Although the effect of wounding in ethylene production is almost universal (Morgan and Drew, 1997), comparisons between self-pollen and *N. rustica* pollen up to the time of arrest of the *N. rustica* pollen tubes (around 20–24 h after pollination) showed no difference in the number and length of the pollen tubes inside the style that could account for the difference in

Clark *et al.* (1997) for geranium, where incompatible pollen, although able to reach 67% of the length of self-pollen tubes, were found to trigger only 39% of the ethylene release observed for self-pollinated pistils. According to Woltering *et al.* (1997), even though both pollination and wounding induce ethylene production and corolla senescence, the signal(s) involved in inter-organ regulation of one and the other may be, at least partially, different.

ACC oxidase controls the last step in ethylene biosynthesis. The basal level of mRNA suggests that the stylar tissue has a constitutive capacity to convert ACC to ethylene. However, because ACC oxidase has a low intrinsic catalytic power, high levels of protein are probably necessary for high ethylene production (Dong *et al.*, 1992) and, therefore, an increased expression of this gene upon pollination is also important.

As in *Petunia* (Tang and Woodson, 1996), the timing and site of ACC oxidase expression are consistent with a role of pollen tubes in the elicitation of ethylene production (Figure 3A and B). The presence of pollen tubes in the style coincided with increased levels of expression. Figure 3B suggests that the induction takes place a little before the pollen tubes reach the style, as if a signal travelled ahead of the pollen tubes. Wang *et al.* (1996) found that in tobacco another post-pollination response (poly(A) shortening of some messengers) requires but precedes the arrival of the pollen tubes, indicating that there must be a signal that anticipates the tube tips. This signal could be ethylene itself.

Tobacco flowers can be pollinated at the bud stage, but this led in all cases to limited flower development and premature senescence and abscission.

All types of pollination investigated led to an increase in the levels of ACC synthase and oxidase mRNA, whereas the slight increase in ACC oxidase expression in non-pollinated flowers was probably related to the maturation of the pistil itself or a result of emasculation. An increased accumulation of ACC oxidase transcripts in the pistil, following pollination of immature buds, had already been reported in *Petunia* (Tang and Woodson, 1996). This increase was steeper than the one observed for mature *Petunia* flowers (also observed here for tobacco) and probably reflects the requirements for a certain level of ethylene production in the post-pollination responses. In tobacco, it seems that immature pistils are not able to distinguish between different kinds of pollen since all of them could reach the bottom of the style. When no differences were observed between pollen-tube growth, no differences were detected in ACC oxidase or synthase gene expression. This can be interpreted in at least two ways: either immature pistils cannot distinguish different pollen types and therefore react similarly with ethylene being part of that reaction, or similar patterns of pollen-tube growth (because, for example, immature pistils do not produce an inhibitor) produce the same effect (wounding) in the style and lead to the same response. Probably, as suggested for *Petunia* (Tang and Woodson, 1996), there are two types of factors, one ethylene-independent associated with pollen tubes, and another ethylene-dependent associated with the pistil.

When stage 6 tobacco flowers were emasculated 24 h prior to pollination, *N. maritima* pollen tubes were arrested in almost the same way as they were in mature flowers, and ACC oxidase expression had a pattern similar to that found in non-pollinated styles, suggesting again that the presence of the pollen tubes is needed to activate gene expression. This also seems to suggest that emasculation (perhaps with the involvement of ethylene itself) accelerates pistil development in the sense that it renders it capable of distinguishing foreign pollen tubes.

Because of the differences in gene expression in the mature pistil upon different pollinations we decided to test if ethylene was just a consequence or whether it was also involved in the cause of differential pollen-tube growth. To this end we used ethephon, a substance that releases ethylene upon hydrolysis, to increase the level of ethylene in the flower. Since ethylene is a simple gaseous molecule it was expected that all tissue types could be exposed to it. The first immediate observation was that ethephon was able to induce the wilting of even non-pollinated flowers. This reinforces the idea that the differences between different pollination types (with respect to senescence) were due to different levels of ethylene produced.

Ethephon treatment increased the expression of both ACC oxidase and ACC synthase in the non-pollinated styles (Figure 4), which confirms the autocatalytic nature of ethylene biosynthesis in tobacco, as reported for *Petunia* (Tang and Woodson, 1996), although this does not take place in geranium (Clark *et al.*, 1997).

It should be noted that these experiments were conducted with excised (hence wounded) flowers, which could account for the detectable level of ACC synthase in untreated, non-pollinated styles in contrast

with that of the non-pollinated styles obtained when the experiments were conducted *in planta* (Figure 2A).

Even though there was no effect on self-pollen tube growth when the flowers were treated with ethephon, the presence of this chemical had a mild effect on *N. rustica* pollen tubes and, surprisingly, allowed *N. maritima* pollen tubes to grow further in the style. This effect of ethephon is not likely to be a direct effect on the pollen tubes, since ethephon had to be applied prior to or simultaneously with pollination to achieve further tube growth. Furthermore, ethephon did not have any effect *in vitro* on the growth of *N. maritima* pollen tubes (data not shown) or other pollen tubes (Ana Moutinho, personal communication). Ethephon, via the production of ethylene, is probably able to modify in some way the transmitting tissue in which the pollen tubes are growing. Some authors have suggested that ethylene contributes to the degeneration of the transmitting tissue cell walls by activating cell-wall degrading enzymes (Singh *et al.*, 1992). Tissue degeneration caused by ethylene could explain how in the presence of ethephon *N. maritima* pollen tubes grew more inside the style. It is obvious, however, that an effect of ethylene on the transmitting tissue is not essential for the growth of pollen tubes in the tobacco style, since no negative effect could be seen in the presence of AVG (an inhibitor of ACC oxidase) or STS (an inhibitor of ethylene receptors), as has already been observed for *Petunia* (Hoekstra and Roekel, 1988).

Pollination induces mRNA polyA shortening within 2–3 hours, and this is followed by a decline in the levels of some transmitting tract messengers, probably those no longer required following passage of the pollen tubes (Wang *et al.*, 1996). As ethephon has a similar effect, it is possible that if the product of one of these messengers was involved in the recognition or arrest of the *N. maritima* pollen tubes, in the presence of ethephon there would not be enough protein and the pollen tubes could grow unrecognized.

However ethephon acts, this effect seems to require that the pollen tubes are at least able to penetrate the transmitting tissue; when the experiment was repeated using pollen from another *Nicotiana* (*N. repanda*) whose pollen tubes did not reach the style, no effect on pollen-tube growth was observed (data not shown). In any case ethephon only facilitated pollen-tube growth in the first part of the style while there was no observable effect of ethephon on the length of the *N. rustica* pollen tube. It is interesting to note that in the presence of ethephon, *N. maritima* tubes reached the length that *N. rustica* pollen tubes usually reached.

In conclusion, although our results are not quantitative, they certainly enable us to draw some conclusions on the modulation of gene expression and on the effect of different pollen tubes on gene expression. Pollination in tobacco induces an increase in the expression of the ethylene biosynthesis genes ACC synthase and ACC oxidase, both of which are responsible for the ethylene release observed after pollination. Incongruous pollination also triggers ethylene release and increased gene expression, even though these patterns are related to how far the different pollen tubes are able to grow. Corolla wilting is also dependent on ethylene release and differs for different pollen-tube growth, suggesting that ethylene might serve to co-ordinate events that take place in the style with the senescence of other flower organs like the petals, so that abscission only takes place if the pollination is successful. Post-pollination ethylene production is regulated auto-catalytically. Wounding caused by the growing pollen tubes might be partially responsible for the ethylene produced but cannot account for the differences observed; therefore, other factors have to be involved. Apparently there is a signal preceding the pollen tubes that increases ethylene production (or at least ACC oxidase gene expression).

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