Regulation of Ethylene Biosynthesis in Response to Pollination in Tomato Flowers[1]

Immaculada Llop-Tous[2], Cornelius S. Barry[3], and Donald Grierson*

Plant Science Division, School of Biological Sciences, The University of Nottingham, Sutton Bonington Campus, Loughborough LE12 5RD, United Kingdom

Pollination of many flowers leads to an increase in ethylene synthesis and flower senescence. We have investigated the regulation of pollination-induced ethylene synthesis in tomato (*Lycopersicon esculentum*) using flowers of the *dialytic* (*dl*) mutant, in which pollination can be manipulated experimentally, with the aim of developing a model system to study tomato flower senescence. Ethylene synthesis increased rapidly in *dl* pistils following pollination, leading to accelerated petal senescence, and was delayed in ethylene-insensitive *Never-ripe* (*Nr*) pistils. However, *Nr* pistils eventually produced more ethylene than *dl* pistils, suggesting the presence of negative feedback regulation of ethylene synthesis following pollination. *LEACS1A* expression correlated well with increased ethylene production in pollinated *dl* pistils, and expression in *Nr* revealed that regulation is via an ethylene-independent mechanism. In contrast, the induction of the 1-aminocyclopropane-1-carboxylic acid oxidases, *LEACO1* and *LEACO3*, following pollination is ethylene dependent. In addition, the expression profiles of *ACS* and *ACO* genes were determined during petal senescence and a hypothesis proposed that translocated 1-aminocyclopropane-1-carboxylic acid from the pistil may be important for regulating the initial burst of ethylene production during petal senescence. These results are discussed and differences between tomato and the ornamental species previously studied are highlighted.

Pollination leads to the onset of fruit development and the senescence of floral organs that become obsolete after pollination has occurred. In many flowers, the initial response to pollination is an early increase in ethylene production by the stigma that is often followed by increased ethylene production from ovaries and petals. The pollination-induced ethylene produced by different floral organs is responsible for coordinating pollination-associated events such as ovary growth and senescence of the perianth (for review, see Larsen et al., 1993; Woltering et al., 1994). Ethylene is synthesized by two enzymes: 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS), which catalyzes the conversion of *S*-adenosyl-L-Met into ACC, and ACC oxidase (ACO), which converts ACC into ethylene (Kende, 1993). These enzymes are encoded by multigene families in all species examined (Zarembinski and Theologis, 1994). The effect of pollination on the expression pattern of these genes has been studied in the ornamental species carnation, geranium, petunia, and orchid. Increased ethylene synthesis following pollination of these flowers is accompanied by increased *ACS* and *ACO* gene expression and elevated enzyme activities (Woodson et al., 1992; O'Neill et al., 1993; Tang et al., 1994; Tang and Woodson, 1996; Clark et al., 1997; Jones and Woodson, 1997; Bui and O'Neill 1998).

Tomato (*Lycopersicon esculentum*) has become one of the model species for studying the regulation of ethylene biosynthesis and perception. Tomato ACS is encoded by a multigene family containing at least eight members (*LEACS1A*, *LEACS1B*, and *LEACS2-7*; Zarembinski and Theologis, 1994; Oetiker et al., 1997; Shiu et al., 1998). The expression of some members of the *ACS* gene family has been investigated in fruit, roots, and leaves at different developmental stages and under various environmental conditions (Van der Straeten et al., 1990; Olson et al., 1991, 1995; Rottmann et al., 1991; Yip et al., 1992; Lincoln et al., 1993; Spanu et al., 1993; Nakatsuka et al., 1998). However, little is known about the expression of members of the *ACS* gene family in tomato flowers and only the expression of *LEACS2* has been characterized (Rottmann et al., 1991). *LEACS2* transcripts accumulate in mature and senescent anthers and in fully senescent petals, but no expression could be detected in pistils.

Four ACO genes (*LEACO1-4*) have been identified in tomato and their expression has been analyzed in response to wounding and during flower development, leaf senescence, and fruit ripening (Holdsworth et al., 1988; Barry et al., 1996, Blume and Grierson, 1998; Nakatsuka et al., 1998). The studies of Barry et al. (1996) analyzed the spatial and temporal regulation of *LEACO1, 2*, and *3* gene expression in tomato flowers during development, although the effect of pollination on the expression was not exam-

[1] This work was supported by the European Union (grant nos. FAIR–96–5069 and FAIR CT 95–0225 to D.G.).

[2] Present address: Consejo Superior de Investigaciones Científicas, Jordi Girona 18–26, 08034 Barcelona, Spain.

[3] Present address: Department of Horticultural Sciences, Texas A&M University, College Station, TX 77843–2133.

* Corresponding author; e-mail Donald.Grierson@nottingham.ac.uk; fax 44–0–115–951–6334.

ined. In addition, components of the ethylene perception and signal transduction pathway are beginning to be identified in tomato (Bleecker, 1999). The tomato *Never-ripe* (*Nr*) mutant displays ethylene insensitivity (Lanahan et al., 1994) and subsequent analysis has shown that it is defective in a member of the ethylene receptor gene family (Wilkinson et al., 1995). *Nr* plants have recently been successfully used to elucidate the role of ethylene in several processes (Aloni et al., 1998; Lund et al., 1998; Clark et al., 1999).

We have studied the physiological and molecular events associated with pollination-induced flower senescence in tomato and compared these with previous results from ornamental species. Our results show that *LEACS1A*, *LEACO1*, and *LEACO3* appear to be important for pollination-induced ethylene synthesis in pistils. *LEACS1A* is regulated independently of ethylene, whereas *LEACO1* and *LEACO3* have a strong ethylene requirement. In addition, the differential expression of the *ACS* and *ACO* gene families during petal senescence is reported.

RESULTS

The Use of the *dialytic* Mutant as a Model to Study Pollination Events in Tomato

The flowers of tomato are comprised of six anthers fused to form a cone that is attached to the petals and surrounds a single enclosed pistil. The presence of the anther cone ensures that the flowers normally undergo self-fertilization. This makes pollination studies cumbersome, as emasculation must occur to prevent self-pollination and to allow access to the stigmatic surface. In turn, emasculation often leads to wound responses and severe damage of the flower petals, rendering them useless for further investigation. To circumvent these problems, we have utilized the flowers of the *dialytic* (*dl*) mutant (Darby et al., 1977), in which individual anthers are not fused to form a cone (Fig. 1A). This phenotype has two benefits for this study. First, the stigma is readily accessible without emasculation, and second, self-pollination is easily prevented as pollen does not collect around the stigmatic surface. Apart from the altered phenotype of the anthers, no other aberrant phenotype was evident; fertilization occurred normally and the rate of flower senescence was identical to wild-type cv Ailsa Craig plants. Therefore, *dl* flowers represent an ideal model for studying post-pollination events in tomato.

Effect of Pollination on Flower Senescence

Physical changes in response to pollination were examined (Fig. 1B). Fully open *dl* flowers were hand-pollinated and collected after 24, 48, 72, and 96 h. Mock-pollinated flowers, in which the stigmatic surface was touched with a flat spatula free of pollen, were used as non-pollinated control. Whereas most

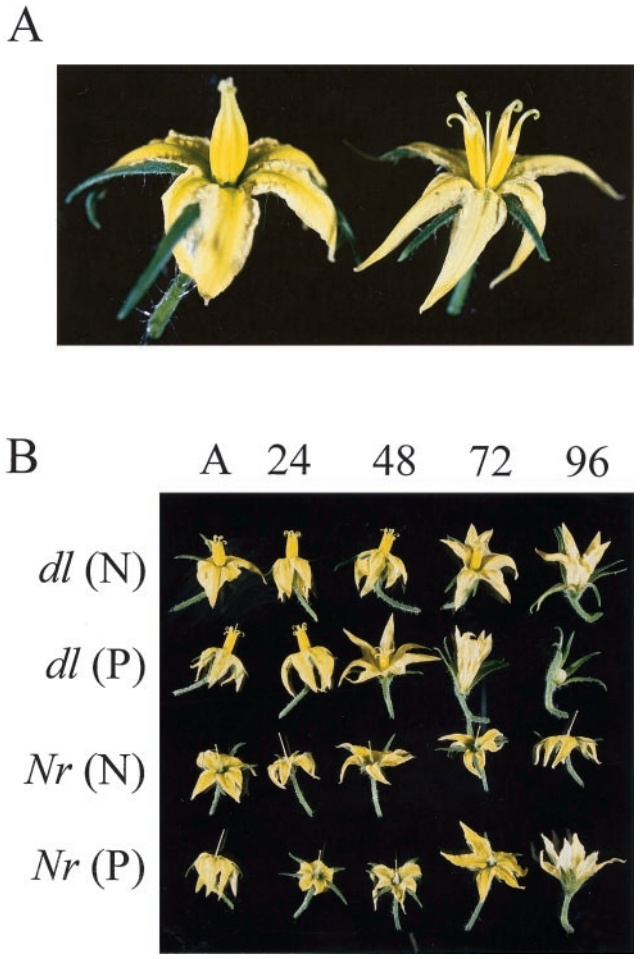

Figure 1. A, Effect of the *dl* mutation in tomato flowers. Left, Wild-type tomato flower. Right, *dl* flower, in which individual anthers are not fused to form a cone. B, Natural and pollination-induced senescence of *dl* and *Nr* tomato flowers. Non-pollinated (N) and pollinated (P) *dl* or *Nr* flowers were examined at anthesis (A) and 24, 48, 72, and 96 h after anthesis. The *Nr* flowers were emasculated 2 d prior to anthesis.

of the non-pollinated flowers showed the first symptoms of corolla senescence at 72 h after anthesis, pollinated flowers presented visible wilting symptoms within 48 h. At 72 h after pollination, the perianth was clearly degraded, and by 96 h, had abscised.

The role of ethylene in mediating flower senescence in response to pollination was examined in the *Nr* mutant (Fig. 1B). *Nr* flowers have normal wild-type flower morphology, therefore, to avoid self-pollination, the anther cone was removed 2 d before anthesis and they were pollinated when the petals were fully reflexed. Mock-pollinated *Nr* flowers failed to show any visible signs of senescence during 96 h of observation. Senescence symptoms were visible in pollinated *Nr* flowers after 96 h.

Ethylene Production in Response to Pollination

The rate of ethylene production was measured from pistils of *dl* flowers at different times after pol-

lination (Fig. 2A). Ethylene production by pistils at anthesis was approximately 150 nL g^{-1} h^{-1}. There was no detectable difference in the rate of ethylene production between mock-pollinated and pollinated pistils until 4 h post-pollination. At 4 h an increase was observed in pollinated pistils that continued to rise and peaked at 6 h after pollination when values were approximately double those measured at anthesis. The levels of ethylene decreased slowly to prepollination levels after 24 h. Pistils from non-pollinated flowers exhibited constant basal level of ethylene production during the experimental period.

The rate of ethylene production was also analyzed in *Nr* pistils (Fig. 2A). The profile and level of ethylene production in *Nr* pistils was very similar to *dl* pistils, although the peak was delayed in *Nr* pistils, occurring approximately 10 h after pollination. Concomitant with the delayed increase, the decline to prepollination levels also took longer in *Nr* pistils and the total ethylene produced during the experimental period was substantially higher than that in *dl* pistils.

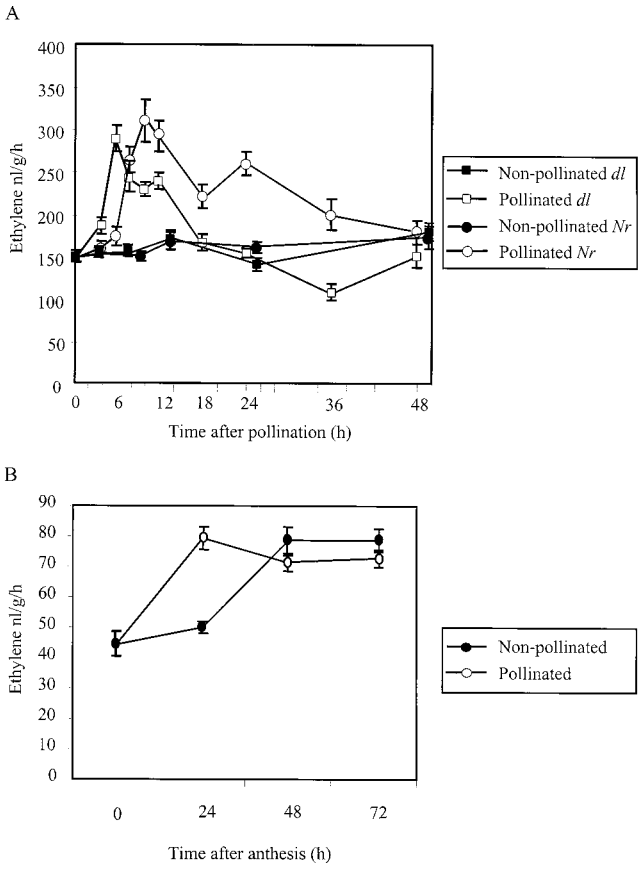

Figure 2. A, Ethylene production from pistils of *dl* and *Nr* in response to pollination. Individual pistils, isolated at anthesis (time 0) and at different times after pollination or mock-pollination, were enclosed in airtight vials and ethylene-sampled after 45 min. B, Ethylene production by petals isolated from pollinated and non-pollinated *dl* flowers. Flowers were pollinated or mock-pollinated at anthesis and collected after 24, 48, and 72 h. Isolated petals were enclosed in airtight vials for 45 min. Values represent means of at least 10 samples. Vertical bars represent SE.

To investigate if the differences in the pattern of ethylene production were associated with different characteristics of pollen tube growth, pollinated pistils from *dl* and *Nr* flowers were stained with aniline blue and callose deposits visualized by fluorescence microscopy. In both cases, pollen grains germinated between 1 and 3 h after pollination and pollen tubes reached the base of the style within 8 h of pollination.

Ethylene production by *dl* petals was also examined (Fig. 2B). In petals from non-pollinated flowers, ethylene increased from approximately 44 nL g^{-1} h^{-1} at anthesis to 79 nL g^{-1} h^{-1} at 48 h after anthesis. Pollination accelerated the onset of ethylene production by petals by approximately 24 h. In both pollinated and non-pollinated flowers, the increase in ethylene production occurred before symptoms of senescence were apparent (compare Figs. 1B and 2B). No additional increase in ethylene production was detected in association with the progress of petal senescence.

ACS and *ACO* Expression in Tomato Pistils

The abundance of eight ACS transcripts was measured by RNase protection assay (RPA) analysis in pistils of tomato flowers following pollination. The transcripts of *LEACS1A*, *LEACS2*, and *LEACS6* genes were identified in pistils of *dl* flowers, but no signal was observed with the other probes (*LEACS1B*, *LEACS3*, *LEACS4*, *LEACS5*, and *LEACS7*). *LEACS2* and *LEACS6* transcripts were present at extremely low levels in *dl* pistils and no changes were observed in response to pollination (data not shown). *LEACS1A* transcripts were detectable in *dl* pistils at anthesis and remained constant in non-pollinated flowers for 24 h, when an increase in abundance was observed (Fig. 3). In pollinated *dl* pistils, an increase in *LEACS1A* expression occurred within 4 h and persisted through to 48 h post-pollination. Pollination of *Nr* pistils induced the same changes in *LEACS1A* expression with a similar kinetic profile.

The expression of the four members of the tomato *ACO* gene family was assessed by northern-blot analysis with gene-specific probes. Our results indicated that all four *ACO* genes were expressed at constant levels in pistils from non-pollinated *dl* flowers, but they showed different regulation in response to pollination (Fig. 3). *LEACO1* mRNA levels were very low in pistils of non-pollinated flowers, but accumulated dramatically after pollination, reached a maximum at around 12 h, and declined thereafter. *LEACO3* expression also increased following pollination and showed the same pattern kinetically as *LEACO1*. Both *LEACO2* and *LEACO4* transcript abundance declined in response to pollination. The expression of *ACO* genes was examined in pistils from *Nr* flowers (Fig. 3). The increase in *LEACO1* and *LEACO3* and the decrease in *LEACO2* in response to

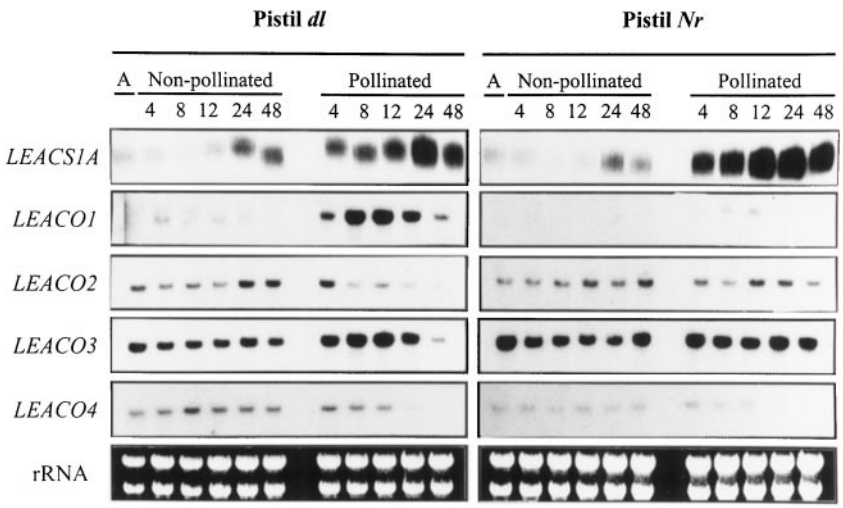

Figure 3. Accumulation of ACS and ACO transcripts in response to pollination in *dl* and *Nr* pistils. RNA was extracted from *dl* or *Nr* pistils at anthesis (A) and 4, 8, 12, 24, and 48 h after pollination. Pistils from mock-pollinated flowers (non-pollinated) were used as controls. Thirty micrograms of total RNA was hybridized to radiolabeled ACS gene-specific probes and used for RPA analysis. ACO gene expression was determined by RNA gel-blot analysis using 15 μg of total RNA. Gels were stained with ethidium bromide to ensure equal loading of the samples.

pollination did not occur in *Nr* pistils. In contrast, the pattern of expression of *LEACO4* in pistils from *Nr* flowers was identical to that observed in *dl* pistils.

Expression of *ACS* and *ACO* Genes in *dl* Petals

Petals were collected from *dl* tomato flowers at anthesis and at 4, 12, 24, 48, and 72 h after pollination. Mock-pollinated flowers were used as controls. Expression analysis of the eight members of the tomato *ACS* gene family indicated that only transcripts corresponding to *LEACS1A*, *LEACS2*, *LEACS3*, and *LEACS6* were present in petals (Fig. 4). Increased expression of *LEACS1A* and *LEACS6* was evident in petals of both non-pollinated and pollinated flowers at around 48 h post-anthesis. The expression of *LEACS2* and *LEACS3* increased at 72 h in non-pollinated flowers and 24 h earlier in pollinated flowers. All four *ACO* genes were expressed in petals, but different expression patterns were evident (Fig. 4). The expression of *LEACO1* and *LEACO3* was up-regulated in petals from non-pollinated flowers at 72 h after anthesis and 1 d earlier in petals from pollinated flowers. In contrast, the expression of *LEACO2* and *LEACO4* remained constant in all samples examined except that an increase was observed in the latter at 72 h in non-pollinated and pollinated samples.

DISCUSSION

In this study, we have examined the effect of pollination in regulating flower senescence in tomato and analyzed the role of ethylene in mediating post-pollination events. In the absence of pollination, tomato flowers showed visible signs of senescence, as indicated by initial petal curling, approximately 72 h after anthesis. This was accelerated by approximately 24 h in pollinated flowers, indicating that pollination affects the rate of senescence (Fig. 1B). The rapid rate of tomato flower senescence in the absence of pollination is different from other species that have previously been studied. For example, flower longevity in carnation, petunia, and orchids can range from a couple of weeks to several months in the absence of pollination (Nichols, 1977; Pech et al., 1987; Singh et al., 1992; Stead, 1992; O'Neill et al., 1993). In many

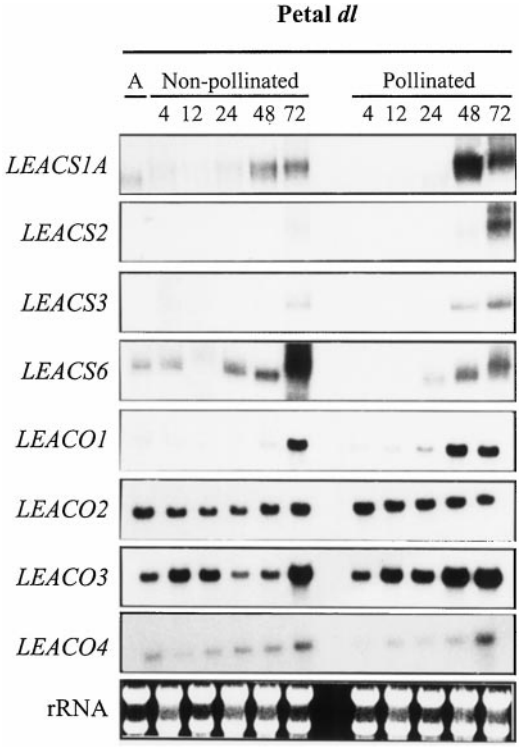

Figure 4. Analysis of the expression ACS and ACO genes in petals from *dl* flowers. Total RNA was obtained from petals collected from flowers at anthesis (A) and from pollinated and non-pollinated flowers at 4, 12, 24, 48, and 72 h after anthesis. For ACS gene expression, RPA analysis was performed using 30 μg of total RNA per sample. The accumulation of ACO transcripts was examined by RNA gel-blot analysis using 15 μg of total RNA. Gels were stained with ethidium bromide to ensure equal loading of the samples.

flowers pollination is followed by a rapid increase in ethylene production by the pistil, and this is accompanied by increased *ACS* and *ACO* expression and enzyme activity (O'Neill et al., 1993; Tang et al., 1994; Tang and Woodson, 1996; Jones and Woodson, 1997, 1999; Bui and O'Neill, 1998). Our results indicate that ethylene biosynthesis by tomato pistils starts to increase 4 h after pollination, peaks at 6 h, is elevated up to 12 h, and slowly declines thereafter. This increase in ethylene production is first detected after the pollen grains have germinated and when the pollen tubes have penetrated approximately one-quarter of the style length. This result contrasts with those reported in other flowers in which the increase in ethylene production occurs either simultaneously or before the germination of the pollen grains (Zhang and O'Neill, 1993; Larsen et al., 1995; Tang and Woodson, 1996).

In an attempt to understand the molecular basis for the increase in ethylene production following pollination of tomato pistils and to determine the role of ethylene, the expression of the genes involved in ethylene biosynthesis were analyzed in both *dl* and *Nr* pistils (Fig. 3). Pollination induced an increase in *LEACS1A* expression in *dl* pistils and exactly the same pattern was seen in *Nr* pistils, indicating that this change occurs independently of ethylene. All four *ACO* genes were expressed in *dl* pistils. *LEACO1* and *LEACO3* showed increased expression in response to pollination, whereas transcripts corresponding to *LEACO2* and *LEACO4* declined following pollination. The changes in expression of *LEACO1*, *LEACO2*, and *LEACO3* following pollination did not occur in *Nr* pistils, indicating that they are ethylene dependent. However, *LEACO4* expression was similar in both *dl* and *Nr* pistils, suggesting that expression is regulated independently of ethylene. Furthermore, increased expression of *LEACS1A*, *LEACO1*, and *LEACO3* in *dl* pistils following pollination correlated well with the increased ethylene production (compare Figs. 2A and 3).

Examples of ethylene-independent induction of *ACS* gene expression in response to pollination have previously been reported for the *Phal-ACS2* and *Phal-ACS3* genes from orchid and the *DCACS3* from carnation (Jones and Woodson, 1997, 1999; Bui and O'Neill, 1998). It has been suggested that these genes respond to primary signals derived from pollen and are, therefore, responsible for initiating ethylene synthesis in pistils. The expression pattern of *LEACS1A* suggests that it may also fall into the same category. Additionally, in orchid and carnation, secondary ethylene-dependent induction of *ACS* gene expression has been observed in pistils in response to pollination brought about by increased expression of *Phal-ACS1* and *DCACS1* and *DCACS2*, respectively (Jones and Woodson, 1997, 1999; Bui and O'Neill, 1998). In tomato, we did not detect any secondary ethylene-dependent increase in *ACS* gene expression, indicating differences occur in the regulation of ethylene synthesis between these species. The hypothesis that increased ethylene production in tomato pistils following pollination is due to elevated *LEACS1A* expression alone and not to any other secondary ethylene-dependent *ACS* expression is supported by comparison of ethylene production in *dl* and *Nr* pistils (Fig. 2A).

Disruption of ethylene perception in *Nr* did not lead to a reduction in ethylene production by pistils in response to pollination, although a delay of several hours occurred until maximal levels were attained and the total amount of ethylene produced was higher. This suggests that ethylene perception affects the timing and the extent of ethylene biosynthesis after pollination. This contrasts with previous studies using carnation and petunia flowers. Treatment of petunia styles with the ethylene action inhibitor 2,5-norbornadiene did not affect the timing and extent of ethylene production during the first 8 h after pollination, but inhibited the production of ethylene after 24 h (Tang and Woodson, 1996). In carnation the timing of ethylene production was unaltered, but the level of ethylene biosynthesis was lower in 2,5-norbornadiene- and diazocyclopentadiene-treated flowers compared to non-treated flowers (Jones and Woodson, 1997).

The delay in ethylene production in *Nr* pistils may be attributed to a reduction in total ACO protein due to reduced expression of *LEACO1* and *LEACO3* (Fig. 3). This suggests an important role for ACO in regulating the timing of ethylene synthesis in tomato pistils in response to pollination. Although maximal ethylene synthesis was delayed in *Nr* pistils, they eventually produce higher levels of ethylene than *dl* pistils due to a reduction in the rate of decline following the peak of synthesis (Fig. 2A). This suggests the operation of a negative feedback mechanism by which ethylene can auto-inhibit its own synthesis following pollination. This is consistent with the results of Wilkinson et al. (1997) which showed that ethylene-insensitive petunia flowers produced more ethylene than wild-type flowers following pollination. Higher levels of *LEACO2* and *LEACO3* transcripts in pollinated *Nr* pistils at later time points may account for this increase (Fig. 3). Alternatively, in wild-type flowers, ethylene may induce ACC conjugation or cause inactivation of either ACS or ACO, leading to feedback inhibition.

Pollination of tomato flowers results in increased ethylene synthesis and an acceleration of petal senescence. However, petal senescence occurred in the absence of pollination, but with a 24-h delay (Fig. 1B). This indicates that the floral organ is already programmed to undergo senescence and that pollination simply accelerates the process. The fact that senescence is accelerated in tomato and other flowers by pollination suggests that following pollination, sig-

nals derived from the pistil are translocated to the perianth to induce ethylene production and senescence. The nature of the signal that mediates interorgan communication is not clear. ACC or ethylene itself have been proposed to be the translocated signal in orchids (O'Neill et al., 1993; Woltering et al., 1995; Bui and O'Neill, 1998) and carnation (Reid et al., 1984; Woltering, 1990; Have and Woltering, 1997). Alternatively, other factors such as auxin, short-chain fatty acids, or electrical signals have been suggested as mobile senescence signals (Burg and Dijkman, 1967; Linskens and Spanjers, 1973; Fromm et al., 1995; Halevy et al., 1996). In tomato, it is possible that ACC may be the signal that is translocated from pistils to petals. Indirect evidence to support this hypothesis comes from the observation that ethylene synthesis increases in petals of pollinated flowers prior to a de novo increase in *ACS* gene expression (compare Figs. 2B and 4). An increase in ACC may be produced via elevated *LEACS1A* expression in pistils at between 24 and 48 h (Fig. 3) at a time when *ACO* expression (Fig. 3) and ethylene synthesis (Fig. 2) are declining. Translocated ACC may then be converted to ethylene by the relatively high ACO that is already present in petals. Subsequent maintenance of ethylene production in petals through 72 h and complete senescence may then be achieved by increased *ACS* and *ACO* expression. The role of multiple *ACS* and *ACO* genes in petals remains unclear. However, as well as senescence, other biological phenomena occur in senescent petals. These include abscission, subsequent wounding at the abscission zone, wilting, and cell death, some of which are known to be regulated, at least in part, by ethylene (He et al., 1996; O'Donnell et al., 1996; González-Carranza et al., 1998).

CONCLUSIONS

We have investigated the role of pollination in regulating flower senescence in tomato both at the physiological and molecular level. Pollination causes an increase in ethylene synthesis, an enhanced rate of senescence, and is accompanied by changes in *ACS* and *ACO* gene expression. *LEACS1A* appears to be the sole *ACS* gene responsible for increased ethylene production in pistils and is regulated in an ethylene-independent way. Disruption of ethylene perception alters the timing of ethylene production in response to pollination as a delay occurs in *Nr*. This delay may occur as a result of reduced ethylene-dependent expression of *LEACO1* and *LEACO3* early after pollination. In addition, ethylene perception is required for the decrease in ethylene production after maximum levels have been reached, indicating a negative feedback control of ethylene synthesis following pollination. These data suggest that both *ACS* and *ACO* are important for regulating ethylene synthesis in tomato in response to pollination.

MATERIALS AND METHODS

Plant Material

Tomato (*Lycopersicon esculentum* Mill.) plants homozygous for the *dl* and *Nr* mutations contained within the Ailsa Craig genetic background were grown under standard greenhouse conditions using routine horticultural practices. Fully open *dl* flowers (with petals fully reflexed) were hand-pollinated by touching the stigmatic surface with a flat spatula loaded with pollen. Mock-pollinated flowers, in which the stigma was touched with a flat spatula free of pollen, were used as non-pollinated controls. *Nr* flowers were emasculated 2 d prior to anthesis to avoid self-pollination. Pollination and mock pollination were carried out as for *dl* flowers.

Ethylene Measurements

Ethylene production by the different floral organs was measured at different times after pollination. The samples (pistils or petals) were detached and enclosed in airtight vials and incubated at 25°C for 45 min following which 1 mL of the headspace was withdrawn. Ethylene concentration in the gas sample was measured by gas chromatography using an ATI UNICAM 610 series gas chromatograph (Unicam Analytical, Cambridge, UK) linked to a PC with UNICAM 4880 chromatography data handling software (Unicam Analytical). Column specifications were: length, 150 mm; outer dimension, 6 mm; inside dimension, 4 mm; support, alumina F_1 mesh range 80 to 100. Temperatures were as follows: oven/column, 110°C; injector, 108°C; detector, 160°C.

Analysis of Pollen Tube Growth

Stigma/style from pollinated flowers were collected at 1-h intervals after pollination. Samples were prepared as described previously by Clark et al. (1997) and stained with 0.1% (w/v) aniline blue in 0.1 M K_2HPO_4. Samples were squashed on a slide with a coverslip and growth of pollen tubes was visualized using a LEITZ DMR microscope (Lieca Microsystems, Wetzlar, Germany) provided with a UV lamp. Pollen tube lengths were measured using an eyepiece graticule.

ACS and *ACO* Gene-Specific Radiolabeled Probes

Gene-specific probes for *LEACS1A*, *LEACS1B*, *LEACS5*, and *LEACS6* were PCR-amplified using primers previously described by Oetiker et al. (1997). The products were cloned into the pCR2.1 vector (Invitrogen, San Diego). *LEACS2*, *LEACS3*, *LEACS4*, and *LEACSS7* (Rottmann et al., 1991; Lincoln et al., 1993; Olson et al., 1995; Shiu et al., 1998) gene-specific probes were designed from around the 3′-untranslated region of each gene. Primer pairs were as follows: *LEACS2*, ACS2F: 5′-ttaaaagggaagaatttaatt-3′ and ACS2R: 5′-taacaatataatcgagaaag-3′ generating a probe fromnucleotides2,702to2,957;*LEACS3*,ACS3F:5′-gtcattctccaagtgggttt-3′ and ACS3R: 5′-gtagtagtttgaacatttcaag-3′ generating a probe from nucleotides 4,073 to 4,377; *LEACS4*,

ACS4F: 5′-ggagtcatgaagaacaagcac-3′ and ACS4R: 5′-aactatgttgggcccgtgct-3′ generating a probe from nucleotides 2,624 to 2,855; *LEACS7*, ACS7F: 5′-gtctagtcatgtgaaagt-3′ and ACS7R2: 5′-gcacttgtgcggtcacct-3′ generating a probe from nucleotides 4,065 to 4,335. PCR products were cloned into *Sma*I cut pBluescript II SK+ (Stratagene, La Jolla, CA) or pGEM-T Easy (Promega, Madison, WI).

LEACO1-, *LEACO2-*, and *LEACO3*-specific probes have previously been described (Barry et al., 1996). A *LEACO4*-specific probe was designed from the 3′-untranslated region of the cDNA sequence reported by Nakatsuka et al. (1998) using the following primers: ACO4F, 5′-ggacactaattaagaggattaaag-3′, and ACO4R, 5′- ccccatagagaacaacctc-3′. The resultant 137-bp fragment was cloned in to pGEM-T Easy (Promega). The identity of all clones was confirmed by DNA-sequence analysis.

Single-strand specific radiolabeled RNA probes were prepared by in vitro transcription from linear plasmid template using either T3 or T7 RNA polymerase (Promega) according to the manufacturer's instructions.

RNA Extraction and Analysis

RNA was extracted from frozen pistils and petals using the protocol described by Griffiths et al. (1999), except that the initial volume of extraction buffer was reduced to 2 mL to accommodate the reduced fresh weight of tissue used. On average, 40 flowers were used for each extraction. RNA gel-blot analysis was performed as described by Griffiths et al. (1999). RPA was used as previously described (Barry et al., 1996) with the following modifications. All hybridizations were carried out using 30 μg of total RNA and digestions with RNase ONE (Promega) were performed at 28°C for 3 h using 3 units of enzyme per reaction.

ACKNOWLEDGMENTS

We would like to thank Dr. Roger Chetelat (University of California-Davis) and Dr. Ian Taylor (University of Nottingham) for helpful discussions at the onset of this project and for providing seed stocks of the *dl* mutant. We would also like to thank Dr. Jeremy Roberts (University of Nottingham) for helpful comments on the manuscript.

Received October 29, 1999; accepted April 6, 2000.

LITERATURE CITED

Aloni R, Wolf A, Feigenbaum P, Avni A, Klee HJ (1998) The *Never ripe* mutant provides evidence that tumor-induced ethylene controls the morphogenesis of *Agrobacterium tumefaciens*-induced crown galls on tomato stems. Plant Physiol **117:** 841–849

Barry CS, Blume B, Bouzayen M, Cooper W, Hamilton AJ, Grierson D (1996) Differential expression of the 1-aminocyclopropane-1-carboxylate oxidase gene family of tomato. Plant J **9:** 525–535

Bleecker AB (1999) Ethylene perception and signaling: an evolutionary perspective. Trends Plant Sci **4:** 269–274

Blume B, Grierson D (1998) Expression of ACC oxidase promoter-GUS fusions in tomato and *Nicotiana plumbaginifolia* regulated by developmental and environmental stimuli. Plant J **12:** 731–746

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

Burg SP, Dijkman MJ (1967) Ethylene and auxin participation in pollen induced fading of *Vanda* orchids. Plant Physiol **49:** 1648–1650

Clark DG, Gubrium EK, Barrett JE, Nell TA, Klee HJ (1999) Root formation in ethylene-insensitive plants. Plant Physiol **121:** 53–59

Clark DG, Richards C, Hilioti Z, Lind-Iverson S, Brown K (1997) Effect of pollination on accumulation of ACC synthase and ACC oxidase transcripts, ethylene production and flower petal abscission in geranium (*Pelargonium* × hortorum L.H. Bailey). Plant Mol Biol **34:** 855–865

Darby LA, Ritchie DB, Taylor IB (1977) Isogenic lines of the tomato 'Ailsa Craig.' Annual Report Glasshouse Crops Research Institute, pp 168–184

Fromm J, Hajirezaei M, Wilke I (1995) The biochemical response of electrical signaling in the reproductive system of *Hibiscus* plants. Plant Physiol **109:** 375–384

González-Carranza ZH, Lozoya-Gloria E, Roberts JA (1998) Recent developments in abscission: shedding light on the shedding process. Trends Plant Sci **3:** 10–14

Griffiths A, Barry C, Alpuche-Solis AG, Grierson D (1999) Ethylene and developmental signals regulate expression of lipoxygenase genes during tomato fruit ripening. J Exp Bot **50:** 793–798

Halevy AH, Porat R, Spiegelstein H, Borochov A, Botha L, Whitehead CS (1996) Short-chain fatty acids in the regulation of pollination-induced ethylene sensitivity of *Phalaenopsis* flowers. Physiol Plant **97:** 469–474

Have A, Woltering EJ (1997) Ethylene biosynthetic genes are differentially expressed during carnation (*Dianthus caryophyllus* L.) flower senescence. Plant Mol Biol **34:** 89–97

He CJ, Morgan PW, Drew MC (1996) Transduction of an ethylene signal is required for cell death and lysis in the root cortex of maize during aerenchyma formation induced by hypoxia. Plant Physiol **112:** 463–472

Holdsworth MJ, Schuch W, Grierson D (1988) Organisation and expression of a wound/ripening-related small multigene family from tomato. Plant Mol Biol **11:** 81–88

Jones ML, Woodson WR (1997) Pollination-induced ethylene in carnation: role of stylar ethylene in corolla senescence. Plant Physiol **115:** 205–212

Jones ML, Woodson WR (1999) Differential expression of three members of the 1-aminocyclopropane-1-carboxylate-synthase gene family in carnation. Plant Physiol **119:** 755–764

Kende H (1993) Ethylene biosynthesis. Annu Rev Plant Physiol Plant Mol Biol **44:** 283–307

Lanahan MB, Yen H-C, Giovannoni JJ, Klee HJ (1994) The *Never-ripe* mutation blocks ethylene perception in tomato. Plant Cell **6:** 521–530

Larsen PB, Ashworth EN, Jones ML, Woodson WR (1995) Pollination-induced ethylene in carnation: role of pollen

tube growth and sexual compatibility. Plant Physiol **108:** 1405–1412

Larsen PB, Woltering EJ, Woodson WR (1993) Ethylene and interorgan signaling in flowers following pollination. *In* I Raskin, J Schultz, eds, Plant Signals in Interactions with Other Organisms. American Society of Plant Physiologists, Rockville, MD, pp 112–122

Lincoln JE, Campbell AD, Oetiker J, Rottmann WH, Oeller PW, Shen NF, Theologis A (1993) LE-ACS4, a fruit ripening and wound-induced 1-aminocyclopropane-1-carboxylate synthase gene of tomato (*Lycopersicon esculentum*). J Biol Chem **268:** 19422–19430

Linskens HF, Spanjers AW (1973) Changes in the electrical potential in the transmitting tissue of petunia styles after cross- and self-pollination. Incompatible Newslett **3:** 81–85

Lund ST, Stall RE, Klee HJ (1998) Ethylene regulates susceptible response to pathogen infection in tomato. Plant Cell **10:** 371–382

Nakatsuka A, Murachi S, Okunishi H, Shiomi S, Nakano R, Kubo Y, Inaba A (1998) Differential expression and internal feedback regulation of 1-aminocyclopropane-1-carboxylate synthase, 1-aminocyclopropane-1-carboxylate oxidase, and ethylene receptor genes in tomato fruit during development and ripening. Plant Physiol **118:** 1295–1305

Nichols R (1977) Sites of ethylene production in the pollinated and unpollinated senescing carnation (*Dianthus caryophyllus*) inflorescence. Planta **135:** 155–159

O'Donnell PJ, Calvert C, Atzorn R, Wasternack C, Leyser HMO, Bowles DJ (1996) Ethylene as a signal mediating the wound response of tomato plants. Science **274:** 1914–1917

Oetiker JH, Olson DC, Shiu OY, Yang SF (1997) Differential induction of seven 1-aminocyclopropane-1-carboxylate synthase genes by elicitor in suspension cultures of tomato (*Lycopersicon esculentum*). Plant Mol Biol **34:** 275–286

Olson DC, Oetiker JH, Yang SF (1995) Analysis of LE-ACS3, a 1-aminocyclopropane-1-carboxylic acid synthase gene expressed during flooding in the roots of tomato plants. J Biol Chem **270:** 14056–14061

Olson DC, White JA, Edelman L, Harkins RN, Kende H (1991) Differential expression of two genes for 1-aminocyclopropane-1carboxylate synthase in tomato fruits. Proc Natl Acad Sci USA **88:** 5340–5344

O'Neill SD, Nadeau JA, Zhang XS, Bui AQ, Halevy AH (1993) Interorgan regulation of ethylene biosynthetic genes by pollination. Plant Cell **5:** 419–432

Pech JC, Latché A, Larrigaudière C, Reid MS (1987) Control of early ethylene synthesis in pollinated petunia flowers. Plant Physiol Biochem **25:** 431–437

Reid MS, Fujino DW, Hoffman NE, Whitehead CS (1984) 1-Aminocyclopropane-1-carboxylic acid (ACC): the transmitted stimulus in pollinated flowers. J Plant Growth Regul **3:** 189–196

Rottmann WH, Peter GF, Oeller PW, Keller JA, Shen NF, Nagy BP, Taylor LP, Campbell AD, Theologis A (1991) 1-Aminocyclopropane-1-carboxylate synthase in tomato is encoded by a multigene family whose transcription is induced during fruit and floral senescence. J Mol Biol **222:** 937–961

Shiu Y, Oetiker JH, Yip WK, Yang SF (1998) The promoter of LE-ACS7, an early flooding-induced 1-aminocyclopropane-1-carboxylate synthase gene of the tomato, is tagged by Sol3 transposon. Proc Natl Acad Sci USA **95:** 10334–10339

Singh A, Evensen KB, Kao T-H (1992) Ethylene synthesis and floral senescence following compatible and incompatible pollinations in *Petunia inflata*. Plant Physiol **99:** 38–45

Spanu P, Boller T, Kende H (1993) Differential accumulation of transcripts of 1-aminocyclopropane-1-carboxylate synthase genes in tomato plants infected with *Phytophthora infestans* and in elicitor-treated tomato cell suspensions. J Plant Physiol **141:** 557–562

Stead AD (1992) Pollination-induced flower senescence: a review. Plant Growth Regul **11:** 13–20

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

Tang X, Woodson WR (1996) Temporal and spatial expression of 1-aminocyclopropane-1-carboxylate oxidase mRNA following pollination of immature and mature petunia flowers. Plant Physiol **112:** 503–511

Van der Straeten D, Van Wiemeersch L, Goodman HM, Van Montagu M (1990) Cloning and sequence of two different cDNA encoding 1-aminocyclopropane-1-carboxylate synthase in tomato. Proc Natl Acad Sci USA **87:** 4859–4863

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

Wilkinson JQ, Lanahan MB, Yen H-C, Giovannoni JJ, Klee HJ (1995) An ethylene-inducible component of signal transduction encoded by *Never-ripe.* Science **270:** 1807–1809

Woltering EJ (1990) Inter-organ translocation of 1-aminocyclopropane-1-carboxylic acid and ethylene coordinates senescence in emasculated *Cymbidium* flowers. Plant Physiol **91:** 837–845

Woltering EJ, Somhorst D, Van der Veer P (1995) The role of ethylene in inter-organ signaling during flower senescence. Plant Physiol **109:** 1219–1225

Woltering EJ, Ten Have A, Larsen PB, Woodson WR (1994) Ethylene biosynthetic genes and inter-organ signaling during flower development. *In* RJ Scott, AD Stead, eds, Molecular and Cellular Aspects of Plant Reproduction. Cambridge University Press, Cambridge, UK, pp 285–307

Woodson WR, Park KY, Drory A, Larsen PB, Wang H (1992) Expression of ethylene biosynthetic pathway transcripts in senescing carnation flowers. Plant Physiol **99:** 526–532

Yip W-K, Moore T, Yang SF (1992) Differential accumulation of transcripts for four 1-aminocyclopropane-1-carboxylate synthase homologs under various conditions. Proc Natl Acad Sci USA **89:** 2475–2479

Zarembinski T, Theologis A (1994) Ethylene biosynthesis and action: a case of conservation. Plant Mol Biol **26:** 1579–1597

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