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Expression of Ethylene Biosynthetic Pathway Transcripts in Senescing Carnation Flowers[1]

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ABSTRACT

We have examined the expression of mRNAs for *S*-adenosylmethionine synthetase (EC 2.5.1.6), 1-aminocyclopropane-1-carboxylate (ACC) synthase (EC 4.4.1.14), and the ethylene-forming enzyme (EFE) in various floral organs of carnation (*Dianthus caryophyllus*) during the increase in ethylene biosynthesis associated with petal senescence. The abundance of ACC synthase and EFE mRNAs increased and *S*-adenosylmethionine synthetase transcripts decreased concomitantly with the ethylene climacteric in senescing petals. The increase in abundance of ACC synthase and EFE mRNAs in aging flowers was prevented by treatment with the ethylene action inhibitor 2,5-norbornadiene. Furthermore, an increase in ACC synthase and EFE transcripts was detected in petals from presenescent flowers within 3 to 6 hours of exposure to 2 microliters per liter of ethylene. The increase in ethylene production by senescing petals was associated with a concomitant increase in ethylene biosynthesis in styles, ovary, and receptacle tissues. In all tissues, this increase was associated with increased activities of ACC synthase and EFE. The increase in EFE activities by all floral organs examined was correlated with increased abundance of EFE transcripts. In contrast, the level of ACC synthase mRNA, as detected by the cDNA probe pCARACC3, did not always reflect enzyme activity. The combined tissues of the pistil exhibited high rates of ACC synthase activity but contained low levels of ACC synthase mRNAs homologous to pCARACC3. In addition, pollinated styles exhibited a rapid increase in ethylene production and ACC synthase activity but did not accumulate detectable levels of ACC synthase mRNA until several hours after the initiation of ethylene production. These results suggest that transcripts for ACC synthase leading to the early postpollination increase in ACC synthase activity and ethylene production are substantially different from the mRNA for the ethylene-responsive gene represented by pCARACC3.

Ethylene biosynthesis in plant tissues is under strict metabolic regulation and subject to induction by a variety of signals including mechanical wounding, auxin, and endogenous developmental factors in senescing flowers and ripening fruit (30). The ethylene biosynthetic pathway was elucidated by Adams and Yang (1) and is Met→SAM[2]→ACC→ethylene. In this pathway, the conversion of SAM to ACC, catalyzed by ACC synthase (EC 4.4.1.14), is generally regarded as rate limiting (30). However, in certain tissues, the capacity for oxidation of ACC to ethylene by EFE limits ethylene production (30). The conversion of Met to SAM is catalyzed by SAM synthetase. In addition to its role as a substrate for ACC production, SAM serves as a methyl donor in many transmethylation reactions involving a wide range of acceptor molecules, a role that likely creates a greater demand on SAM as compared to ethylene biosynthesis (30).

Because of its role as the rate-limiting step in ethylene biosynthesis, a significant effort has been directed at the isolation and characterization of ACC synthase. Several groups have reported partial purification of the enzyme (2, 13, 21–23), and recently cDNA clones for ACC synthase have been isolated from zucchini squash (22), winter squash (14), tomato (17, 24), apple (4), and carnation (18). In several species, evidence has been presented indicating that ACC synthase is encoded by more than one gene (8, 17, 24). Not surprisingly, given the diverse stimuli leading to induction of ACC synthase, differential regulation of genes encoding this enzyme has been reported (8, 17).

In addition to ACC synthase, significant progress has been made recently in the characterization of EFE. Early progress on this enzyme was slow due to the lack of a suitable *in vitro* enzyme assay. A significant advancement was made when the expression of an antisense fruit ripening-related mRNA in transgenic tomatoes was shown to inhibit EFE activity (6). This led to the speculation that the fruit-ripening-related cDNA clone pTOM13 may encode the EFE. Indeed, this has now been confirmed by functional expression of homologous cDNAs in *Saccharomyces cerevisiae* and *Xenopus laevis* oocytes (5, 23).

The senescence of carnation petals is associated with a substantial increase in the biosynthesis of the phytohormone ethylene (3). In this organ, ethylene is responsible for inducing many of the biochemical processes leading to programmed cell death including an activation of senescence-related gene transcription (10, 11). The increase in ethylene production is the result of increased activity of ACC synthase and EFE (12, 19). The regulation of ethylene biosynthesis in senescing petals is autocatalytic in nature, *i.e.* ethylene promotes its own synthesis (3, 25). This is analogous to the situation in "climacteric" fruits, which exhibit autocatalytic ethylene production during ripening (30). Consistent with autocatalytic regulation, treatment of presenescent flowers with exogenous ethylene stimulates both ACC synthase and EFE activities, whereas interruption of ethylene action with the competitive

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[2] Abbreviations: SAM, *S*-adenosylmethionine; ACC, 1-aminocyclopropane-1-carboxylic acid; EFE, ethylene-forming enzyme; NBD, 2,5-norbornadiene.

inhibitor NBD inhibits these enzymes and reduces ethylene production in senescing petals (25). The increase in ethylene production by intact flowers during petal senescence is not confined to petals.

Other floral organs, and particularly the pistil, produce large amounts of ethylene concomitantly with petals (15). Whereas in unpollinated flowers the increase in ethylene production by various floral organs appears to occur simultaneously, pollination of flowers results in a rapid induction of ethylene production by the styles which is followed within a few hours by an increase in ethylene production by petals inducing premature senescence (15). In this case, it has been proposed that a transmissible substance, possibly ACC, moves from the pollinated stigma to the petals, leading to ethylene production and petal senescence (16). It is also possible that ethylene itself moves through the connecting tissues of the flower to induce autocatalytic ethylene in the petals, as has recently been found in orchids (28).

Research in our laboratory has focused on the regulation of ethylene biosynthesis in carnation flowers during senescence. As a first step to understanding this regulation at the molecular level, we have isolated cDNA clones from carnations for three enzymes in the ethylene biosynthetic pathway including SAM synthetase (9), ACC synthase (18), and EFE (26). In this paper, we present results of experiments in which we examined the levels of ethylene biosynthetic pathway transcripts in various floral organs and relate this to the production of ethylene.

MATERIALS AND METHODS

Plant Material

Carnation (*Dianthus caryophyllus* L. cv White Sim) flowers were harvested at anthesis from plants grown under greenhouse conditions. Stems were cut to 10-cm lengths, placed in distilled water, and held under laboratory conditions. For treatment with ethylene, flowers were placed in 24-L flow-through chambers through which ethylene and humidified air were passed. Flowers were incubated in an atmosphere of 2000 μL/L NBD (Fluka Chemical Company, Zurich, Switzerland) in enclosed 24-L chambers that were opened daily for air exchange and the NBD concentration reestablished. Flowers were pollinated with pollen from "Starlight" carnations by brushing the stigma with dehiscent anthers. Tissue samples were collected, frozen immediately in liquid nitrogen, and stored at −70°C for subsequent RNA extraction.

Ethylene Biosynthesis

Ethylene production by intact flowers and floral organs was measured by enclosing tissue in airtight chambers for 0.5 h and sampling the headspace gas for ethylene by GC using an activated alumina column and flame ionization detector. Floral organs were removed from intact flowers immediately before enclosure. EFE activity was determined *in vivo* as previously described (25) in 1 mM ACC. Tissue ACC content was determined after extraction in 80% ethanol and conversion to ethylene as previously described (25). For extraction of ACC synthase, 1 g of tissue was powdered under liquid nitrogen and extracted with 3 mL of buffer containing 100 mM Hepes-KOH (pH 8.0), 4 mM DTT, 5 μM pyridoxal phosphate, and 30% (v/v) glycerol. The extract was centrifuged at 10,000*g* and the supernatant passed through Miracloth. The extract (1 mL) was passed through a column of Sephadex G-50 (6 mL bed volume) that had been equilibrated with elution buffer (10 mM Hepes-KOH [pH 8.0], 10 μM pyridoxal phosphate, and 15% [v/v] glycerol). Protein was eluted from the column with elution buffer at a flow rate of 0.3 mL/min, and 1-mL fractions were collected. Fractions were measured for ACC synthase activity as previously described (25) in the presence and absence of 100 μM SAM to ensure no contamination of enzyme with extracted ACC.

RNA Extraction and Analysis

Total RNA was extracted from frozen tissue as previously described (28, 29), separated electrophoretically on 1% agarose gels containing 2.2 M formaldehyde, and transferred to supported nitrocellulose. The nitrocellulose filters were UV cross-linked with RNA using a controlled UV energy source (Strataliker from Stratagene). Cross-linked filters were prehybridized at 42°C for 4 h in a solution containing 50% (v/v) formamide, 5× Denhardt's solution (1× Denhardt's solution is 0.02% PVP, 0.02% Ficoll, 0.02% BSA), 0.1% SDS, 6× SSPE (1× SSPE is 0.15 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA [pH 7.4], and 100 μg/mL denatured salmon sperm DNA). Hybridization was carried out in identical buffer solution containing 5×10^5 cpm/mL denatured ^{32}P-labeled cDNA probe for 18 h. cDNA probes were labeled by random priming using Amicon kit and [^{32}P]dCTP at >3000 Ci/mmol (New England Nuclear). Following hybridization, nitrocellulose filters were washed three times at 42°C in 3× standard sodium citrate (1× standard sodium citrate is 0.15 M NaCl, 15 mM sodium citrate [pH 7.0]) and 0.1% SDS and exposed to Kodak XAR-5 film with an intensifying screen at −70°C.

RESULTS

Accumulation of Ethylene Biosynthetic Pathway Transcripts in Senescing Carnation Flowers

Carnation flowers exhibit a climacteric pattern of ethylene production beginning 5 d after harvest (3). We previously reported that this increase in ethylene production was associated with the accumulation of transcripts for ACC synthase (18). To relate the temporal pattern of ethylene production with the amount of transcripts for other ethylene biosynthetic pathway enzymes, we subjected total RNA extracted from flower petals to gel blot analysis using cDNA probes for SAM synthetase (pSAM2), ACC synthase (pCARACC3), and EFE (pSR120). The results of these hybridizations are presented in Figure 1. The increase in ethylene production that accompanied the first visible signs of petal senescence (in-rolling) was associated with a decrease in levels of mRNAs for SAM synthetase and a concomitant increase in mRNAs for ACC synthase and EFE. No transcripts for ACC synthase or EFE were detected in preclimacteric flowers.

The ethylene climacteric associated with carnation petal senescence is the result of a concomitant increase in ethylene production by several floral organs including the petals, ovary, styles, and receptacle (Fig. 2). On a fresh weight basis, styles

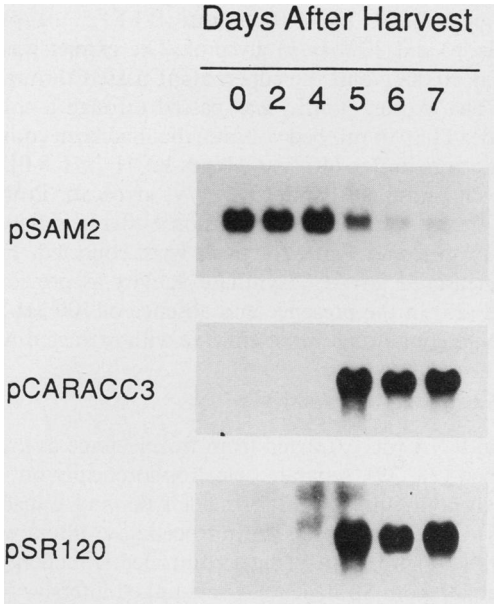

Figure 1. RNA gel blot analysis of ethylene biosynthetic pathway mRNAs in carnation flower petals after harvest. Total RNA was isolated from flower petals at various times after harvest, separated by electrophoresis in formaldehyde-agarose gels, transferred to supported nitrocellulose, and hybridized with cDNA probes for SAM synthetase (pSAM2), ACC synthase (pCARACC3), and EFE (pSR120).

exhibited the highest rate of ethylene production in climacteric flowers. To assess the roles of ACC synthase and EFE in the regulation of ethylene biosynthesis in whole flowers, the activities of these enzymes and the level of ACC were determined in various floral organs. These data are summarized in Figure 2. In preclimacteric flower petals, ethylene production was limited by low activities of ACC synthase and EFE. In contrast, styles from preclimacteric flowers contained substantial EFE activity but low levels of ACC and ACC synthase activity. Ovary tissue from climacteric flowers contained a high concentration of ACC, apparently the result of an increase in ACC synthase activity coupled with limited capacity to oxidize ACC to ethylene. Receptacle tissue, which serves as connective tissue between the gynoecium and petals, contained substantial ACC but exhibited the lowest activities of both ACC synthase and EFE of all the floral organs.

To relate ethylene biosynthetic enzyme activities with transcript levels, we subjected total RNA extracted from preclimacteric and climacteric floral organs to gel blot analysis (Fig. 3). The cDNA clone for SAM synthetase detected mRNAs in all floral organs from both preclimacteric and climacteric flowers, except in petals in which a decrease in mRNA level was seen in senescing petals from climacteric flowers. Transcripts for ACC synthase as detected by the cDNA probe pCARACC3 increased during the ethylene climacteric in all floral organs analyzed. Petal tissue from climacteric flowers contained the greatest amount of transcripts homologous to pCARACC3, followed by an easily detectable signal for ACC synthase mRNA from styles. The increase in ACC synthase transcripts in ovary and receptacle tissue during the ethylene

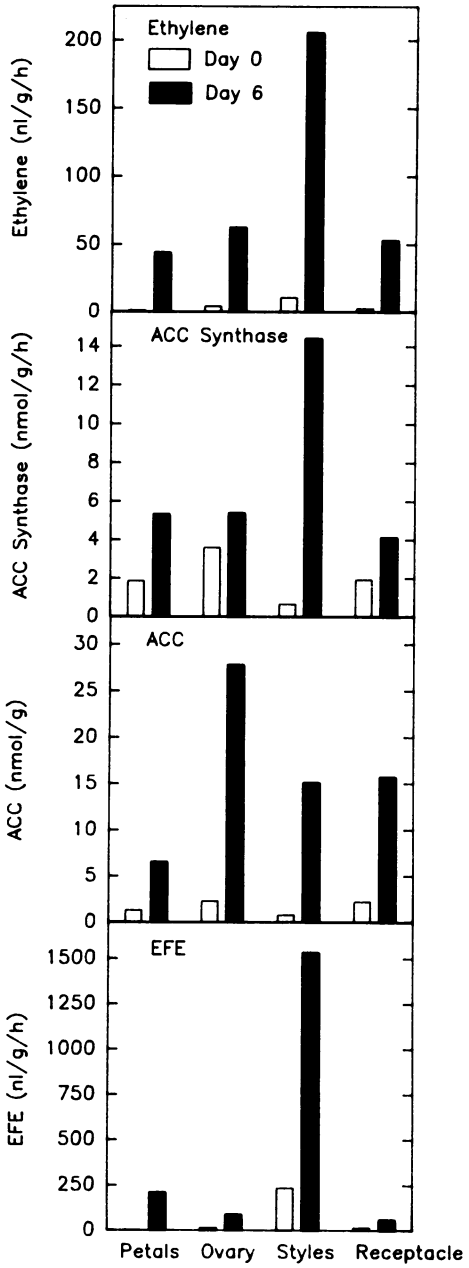

Figure 2. Ethylene biosynthesis in various floral organs from carnation. Flowers were analyzed at anthesis (day 0) and 6 d after harvest at the ethylene climacteric. Ethylene production, ACC synthase activity, ACC content, and *in vivo* EFE activity were determined in petals, ovary, styles, and receptacle tissue as described in "Materials and Methods."

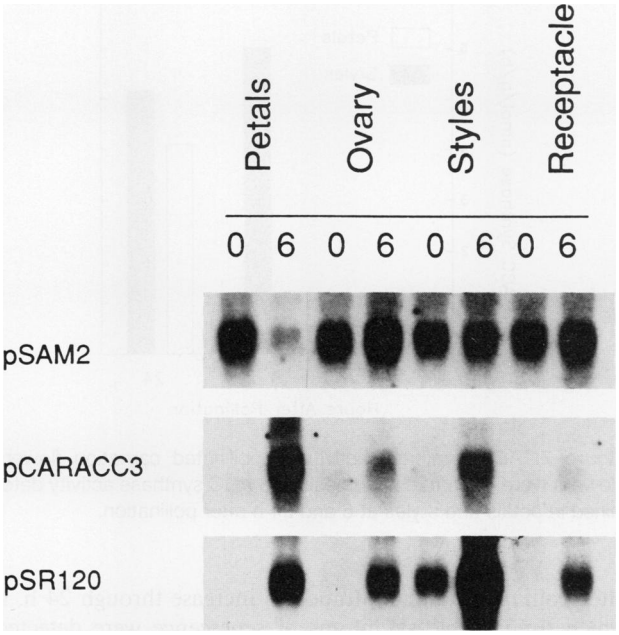

Figure 3. RNA gel blot analysis of ethylene biosynthetic pathway transcripts in various floral organs from carnation. Total RNA was extracted from floral organs the day of harvest (0) or 6 d after harvest (6) during the ethylene climacteric. Total RNA (10 μg) was separated by electrophoresis in formaldehyde-agarose gels, transferred to supported nitrocellulose, and hybridized with cDNA probes as in Figure 1.

climacteric, although detectable, was much less than in petals and styles. The level of mRNA in tissue that hybridized with the pCARACC3 probe did not necessarily reflect the measured activity of ACC synthase. For example, petal and ovary tissue from climacteric flowers contained very similar activities of ACC synthase on a protein basis, but petal tissue accumulated substantially more mRNA represented by pCARACC3. In addition, styles that exhibited the highest activity of ACC synthase exhibited a weaker hybridization signal with pCARACC3 than petals. The levels of transcripts for EFE as detected by hybridization with the cDNA clone pSR120 increased significantly in all floral tissues during the ethylene climacteric. The relative abundance of EFE transcripts reflected the activity of this enzyme as judged by the *in vivo* capacity of tissue to convert ACC to ethylene. Styles accumulated large amounts of EFE transcripts during the ethylene climacteric and exhibited high rates of ethylene production in the presence of exogenous ACC.

Role of Ethylene in Expression of Ethylene Biosynthetic Pathway Transcripts

The concomitant appearance of ACC synthase and EFE transcripts in early climacteric flower petals suggests that ethylene may play a role in the accumulation of these mRNAs. Alternatively, their expression may be a response to developmental signals independently of ethylene. To distinguish between these possibilities, flowers were incubated in an atmosphere of NBD, a competitive inhibitor of ethylene action (25). Under these conditions, flowers do not exhibit the ethylene climacteric, nor do they exhibit symptoms of senescence. Total RNA extracted from petals 6 d postharvest from flowers held in air or NBD was subjected to gel blot analysis and hybridized with ethylene biosynthetic pathway cDNA probes. Incubation of flowers in NBD prevented the accumulation of transcripts for ACC synthase and EFE as detected by cDNA probes pCARACC3 and pSR120, respectively (Fig. 4). These results suggest that the expression of ACC synthase and EFE, at least as detected by our probes, is a response to ethylene. The decrease in SAM synthetase mRNAs seen in senescing petals held in air was reduced but not completely prevented by treatment with NBD. This indicates that the decline in SAM synthetase mRNAs may be more a function of age than of ethylene.

In another experiment, we examined the influence of exogenous ethylene on the expression of ethylene biosynthetic pathway transcripts in preclimacteric carnation petals. Flowers were harvested at anthesis and incubated for various durations in 2 μL/L ethylene. Under these conditions, an increase in the abundance of ACC synthase transcripts was detected within 6 h of initiating ethylene exposure, and EFE transcripts increased in abundance within 3 h (Fig. 5). In contrast, the levels of SAM synthetase mRNA remained relatively constant in ethylene-treated flower petals until 12 h of exposure, at which time a slight reduction was seen. A detectable increase in ethylene production by flower petals was seen following 9 to 12 h of ethylene exposure (data not

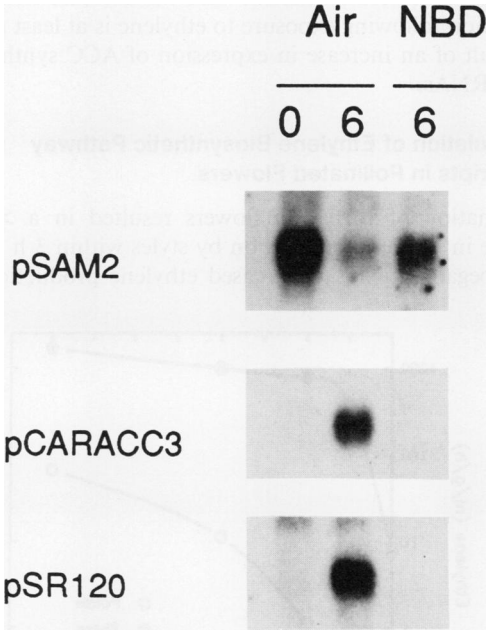

Figure 4. Influence of the ethylene action inhibitor NBD on the accumulation of ethylene biosynthetic pathway transcripts in carnation petals. Flowers were held in an atmosphere of air or 2000 μL/L NBD for 6 d after harvest (6), and total RNA was extracted from their petals. RNA (10 μg) was separated by electrophoresis in formaldehyde-agarose gels, transferred to supported nitrocellulose, and hybridized to ethylene biosynthetic pathway cDNA probes as described in Figure 1.

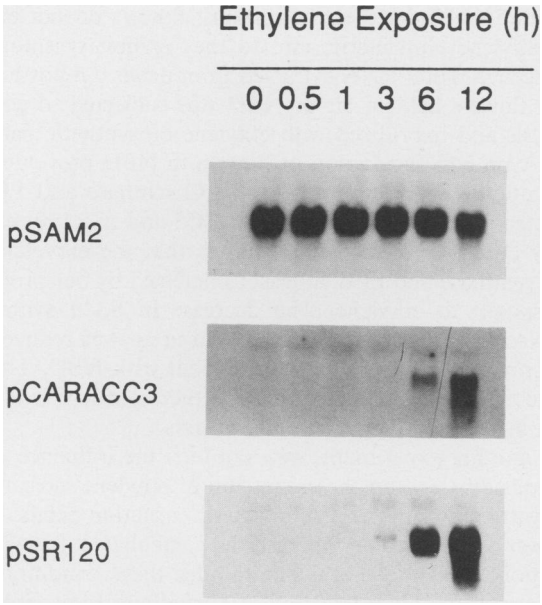

Figure 5. Accumulation of ethylene biosynthetic pathway transcripts in presenescent flowers following exposure to exogenous ethylene. Flowers were harvested and incubated in an atmosphere of 2 μL/L ethylene for various times, after which total RNA was extracted from petals. RNA (10 μg) was separated by electrophoresis in formaldehyde-agarose gels, transferred to supported nitrocellulose, and hybridized to ethylene biosynthetic pathway cDNA probes as described in Figure 1.

shown). These results indicate that the increase in ethylene production following exposure to ethylene is at least partially the result of an increase in expression of ACC synthase and EFE mRNAs.

Accumulation of Ethylene Biosynthetic Pathway Transcripts in Pollinated Flowers

Pollination of carnation flowers resulted in a >50-fold increase in ethylene production by styles within 3 h (Fig. 6). Petals began to exhibit increased ethylene production 12 h

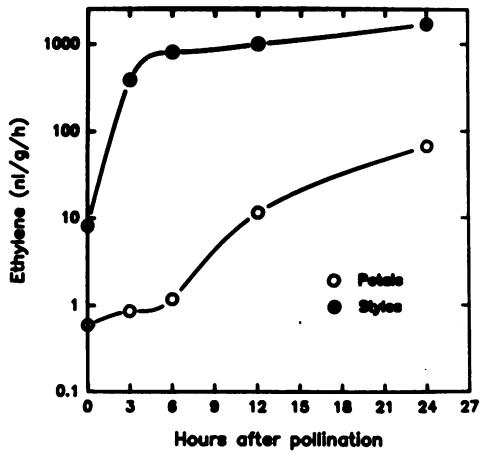

Figure 6. Ethylene production by pollinated carnation flowers. Flowers were pollinated at anthesis, and ethylene production was measured in styles and petals at various times after pollination.

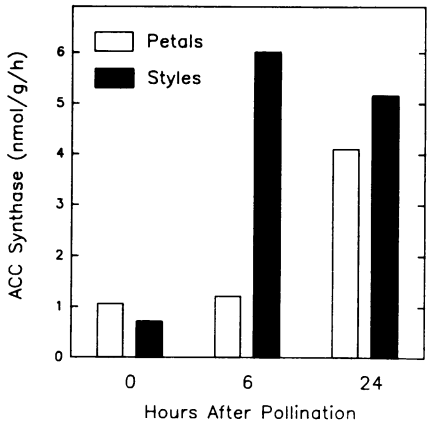

Figure 7. ACC synthase activity in pollinated carnation flowers. Flowers were pollinated at anthesis and ACC synthase activity determined in petals and styles at 6 and 24 h after pollination.

after pollination and continued to increase through 24 h, at which time visible symptoms of senescence were detected. Given the high constitutive capacity of stylar tissue to convert ACC to ethylene (Fig. 2), pollination-induced ethylene production is likely the result of increased ACC synthase activity or the presence of ACC in the pollen (3, 7). ACC synthase activity was significantly higher 6 h after pollination (Fig. 7), indicating that at least a portion of the increased ethylene produced by pollinated styles was a result of increased ACC synthase activity. In contrast with styles, petals did not exhibit increased ACC synthase activity 6 h postpollination, but by 24 h enzyme activity had increased substantially.

We examined the levels of ethylene biosynthetic pathway transcripts in styles and petals of pollinated flowers, and the data are presented in Figure 8. An increase in ACC synthase mRNAs homologous to pCARACC3 was detected in both styles and petals 12 h postpollination and continued to increase through 24 h. The maximum ACC synthase mRNA levels were seen in petals 24 h postpollination. An increase in transcripts for EFE was seen in styles within 3 h of pollination, and their abundance continued to increase through 12 h. In contrast, an increase in EFE transcripts was not detected in petals until 12 h postpollination. Taken together with rates of ethylene production and ACC synthase activity, these results indicate that ethylene production in pollinated flowers is regulated by increases in both ACC synthase and EFE activities. The rapid increase in ethylene production and ACC synthase activity seen in pollinated styles was not associated with a detectable increase in ACC synthase mRNAs homologous to pCARACC3.

DISCUSSION

We have investigated the expression of ethylene biosynthetic pathway transcripts in various floral organs of carnation. The increase in ethylene production by senescing carnation petals was associated with a dramatic increase in the abundance of mRNAs for ACC synthase and EFE. This is consistent with the *de novo* synthesis of these enzymes leading to the increase in ethylene production (12, 19). Both ACC

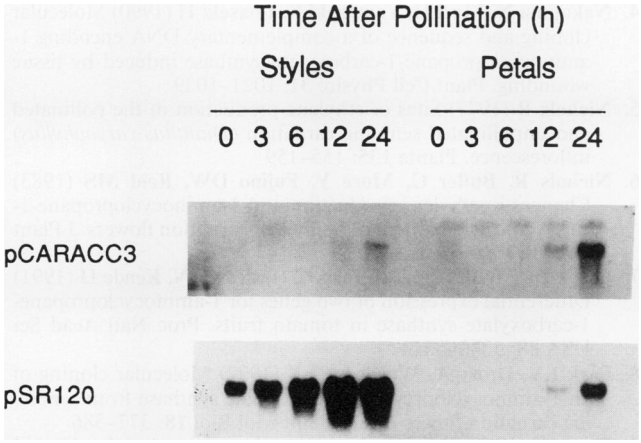

Figure 8. Accumulation of ACC synthase and EFE transcripts in pollinated carnation flowers. Flowers were pollinated at anthesis, and total RNA was extracted from petals and styles at various times after pollination. RNA (10 μg) was separated by electrophoresis in formaldehyde-agarose gels, transferred to nitrocellulose, and hybridized to cDNAs for ACC synthase and EFE as described in Figure 1.

synthase and EFE mRNAs were undetectable in presenescent petals. These data suggest that transcription of these genes likely leads to the increase in ethylene biosynthesis, which leads to petal senescence; however, a role for mRNA stability cannot be ruled out. In contrast to ACC synthase and EFE, the ethylene climacteric in petals was associated with a significant decrease in SAM synthetase mRNA levels. This decrease appeared to be specific to the senescing petals because other floral organs that exhibited a significant increase in ethylene production without showing symptoms of senescence maintained high levels of SAM synthetase mRNA.

Clearly, from these data we can conclude that the increase in ethylene production in carnation flowers does not require an increase in SAM synthetase mRNA. The decrease in SAM synthetase mRNA in petals is representative of a number of transcripts that decline during senescence (28) and may reflect an overall decrease in demand for SAM as a result of decreased macromolecule synthesis (3). Peleman *et al.* (20) previously reported strong cellular preference for SAM synthetase mRNA expression in lignifying tissue of *Arabidopsis*, indicating that in some cases SAM synthetase expression is regulated based on substrate demand. In relation to the requirement of SAM as a methyl donor, ethylene biosynthesis does not likely represent a significant demand for SAM (31).

The increase in ethylene biosynthesis in aging petals is a response to ethylene and, therefore, is referred to as autocatalytic (3, 30). An intriguing question is how the ethylene biosynthetic pathway is induced in the absence of increased ethylene? All plant tissues including preclimacteric carnation petals appear to produce a low basal rate of ethylene. One possibility is that flower petals respond to this low level of ethylene with increased ethylene production after a critical stage of responsiveness is reached. In support of this, carnation petals have been found to become more sensitive to ethylene with age as judged by the induction of senescence-related gene expression and ethylene biosynthesis (11, 29). We found that both ACC synthase and EFE transcripts accumulate in response to ethylene and treatment of flowers with the ethylene action inhibitor, NBD, prevented the accumulation of these transcripts during postharvest aging of flowers, which is consistent with autocatalytic regulation of ethylene biosynthesis in carnation flowers. This supports previous work from our laboratory, which indicated that both ACC synthase and EFE activity depend on continued perception of ethylene (25).

The increase in ethylene production by senescing carnation petals is associated with a concomitant increase in ethylene in other floral organs. By comparing the activities of ACC synthase and EFE with their respective mRNA levels, we are able to draw conclusions concerning the mechanisms regulating ethylene production in climacteric carnation flowers. In all tissues, the increase in ethylene was associated with increased ACC synthase and EFE activities. The accumulation of EFE mRNA reflected the *in vivo* enzyme activity as measured by the capacity to convert saturating levels of ACC to ethylene. However, the levels of ACC synthase mRNA did not always reflect enzyme activity.

Preclimacteric petals exhibited moderate levels of ACC synthase activity, but CARACC3 (18) did not detect ACC synthase mRNA. In addition, senescing petals contained the highest amount of ACC synthase mRNA as detected by the cDNA probe pCARACC3; however, styles contained the greatest enzyme activity of all the floral organs examined. Similarly, ovaries exhibited ACC synthase activities equal to petals in climacteric flowers, but pCARACC3 detected very low ACC synthase transcript levels.

Another inconsistency was seen in pollinated flowers, in which a large increase in ethylene production and ACC synthase activity in styles 6 h postpollination was not associated with any detectable accumulation of ACC synthase transcripts. In several cases, evidence has been presented to indicate that ACC synthase is encoded by more than one gene (8, 17, 24). This appears to be the case in carnation, for which we previously reported the detection of additional sequences in the carnation genome hybridizing with pCARACC3 at reduced stringency (18).

In tomato, two distinct cDNAs have been isolated (17, 24). Olson *et al.* (17) recently reported that these two cDNAs represent genes for ACC synthase that exhibit differential regulation. One cDNA clone represented a wound-induced transcript, whereas both cDNAs hybridized to transcripts accumulating in ripe fruit. Similarly, Nakajima *et al.* (14) found that a wound-induced ACC synthase cDNA clone from winter squash did not detect transcripts for ACC synthase accumulating in response to auxin. Furthermore, these enzymes appear to be immunologically distinct (13).

We previously reported that pCARACC3 did not detect ACC synthase transcripts in wounded leaves that exhibited substantial increases in ethylene production and ACC synthase activity (18). Our results suggest that the ACC synthase expressed in pistil tissue following pollination is significantly different from the ethylene-induced petal senescence-related transcript. The induction of ethylene in styles by pollination is thought to mimic a wound response possibly resulting from the growing pollen tube and has been proposed to involve "elicitors" similar to those found in plant-microbe interactions (7).

Given the apparent ethylene inducibility of the cloned

mRNAs for ACC synthase and EFE described in this paper, a possible scenario for pollination-induced petal senescence would be the induction of ACC synthase in styles by wound- or pollen-specific elicitors leading to increased ethylene, which upon diffusion to petals induces both ACC synthase and EFE leading to petal senescence. Isolation and characterization of additional cDNA clones for ACC synthase from carnation will allow us to address the differential regulation of this ethylene biosynthetic pathway enzyme and the role of ethylene as a signal in interorgan communication following pollination.

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