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Differential Expression of Three Members of the 1-Aminocyclopropane-1-Carboxylate Synthase Gene Family in Carnation[1]

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We investigated the expression patterns of three 1-aminocyclopropane-1-carboxylate (ACC) synthase genes in carnation (*Dianthus caryophyllus* cv White Sim) under conditions previously shown to induce ethylene biosynthesis. These included treatment of flowers with 2,4-dichlorophenoxyacetic acid, ethylene, LiCl, cycloheximide, and natural and pollination-induced flower senescence. Accumulation of ACC synthase transcripts in leaves following mechanical wounding and treatment with 2,4-dichlorophenoxyacetic acid or LiCl was also determined by RNA gel-blot analysis. As in other species, the carnation ACC synthase genes were found to be differentially regulated in a tissue-specific manner. DCACS2 and DCACS3 were preferentially expressed in styles, whereas DCACS1 mRNA was most abundant in petals. Cycloheximide did not induce increased accumulation of ACC synthase transcripts in carnation flowers, whereas the expression of ACC synthase was up-regulated by auxin, ethylene, LiCl, pollination, and senescence in a floral-organ-specific manner. Expression of the three ACC synthases identified in carnation did not correspond to elevated ethylene biosynthesis from wounded or auxin-treated leaves, and there are likely additional members of the carnation ACC synthase gene family responsible for ACC synthase expression in vegetative tissues.

The gaseous plant hormone ethylene plays an important regulatory role in growth and development. In plant tissues ethylene production typically is low, but, increases at developmental stages such as ripening and senescence and in response to mechanical and environmental stresses (Yang and Hoffman, 1984). In higher plants ethylene is synthesized from Met via the following route: Met→*S*-adenosylmethionine→ACC→ethylene (Adams and Yang, 1979). The conversion of *S*-adenosylmethionine to ACC, which is catalyzed by the enzyme ACC synthase, represents one of the rate-limiting reactions in the biosynthesis of ethylene, and the induction of ethylene biosynthesis has been shown to require de novo synthesis of ACC synthase (Yang and Hoffman, 1984; Kende, 1989).

Recently, the cloning of ACC synthase genes from a number of different species has demonstrated that the enzyme is encoded by a multigene family, the members of which are differentially regulated in a tissue-specific manner by a variety of signals, including auxin treatment, wounding, anaerobiosis, ripening, senescence, and Li^+ (Kende, 1993). Although there is evidence for the regulation of ACC synthase at the posttranscriptional level (Chappell et al., 1984; Felix et al., 1991, 1994; Spanu et al., 1994), expression studies indicate that the induction of ACC synthase activity is most often the result of the increased accumulation of ACC synthase mRNAs (Kende, 1993; Zarembinski and Theologis, 1994).

Highly divergent ACC synthase multigene families have been identified and characterized in Arabidopsis (Liang et al., 1992, 1996; Van der Straeten et al., 1992), tomato (Olson et al., 1991; Rottmann et al., 1991; Yip et al., 1992; Lincoln et al., 1993; Oetiker et al., 1997), mung bean (Botella et al., 1992a, 1992b, 1993; 1995; Kim et al., 1992, 1997), zucchini (Huang et al., 1991), rice (Zarembinski and Theologis, 1993), potato (Destefano-Beltran et al., 1995; Schlagnhaufer et al., 1995), and orchid (Bui and O'Neill, 1998). The Arabidopsis gene family includes at least five members whose expression is differentially induced by hormones, developmental cues, Li^+, and the protein-synthesis inhibitor CHX (Liang et al., 1996). Four of the ACC synthase genes in tomato have been shown to be differentially regulated in fruit and hypocotyls during ripening by wounding and auxin treatment (Yip et al., 1992). ACC synthase mRNAs are also induced during flower senescence and following pollination in carnation and orchid (Park et al., 1992; Woodson et al., 1992; O'Neill et al., 1993; Jones and Woodson, 1997; Bui and O'Neill, 1998). Few studies of other species have included the investigation of ACC synthase expression in floral tissue. We were interested in the differential regulation of ethylene biosynthesis in carnation floral organs during senescence. We show that three members of the carnation ACC synthase gene family are differentially expressed in floral organs in response to various chemical stimuli, pollination, and senescence.

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Abbreviations: CHX, cycloheximide; NBD, 2,5-norbornadiene.

MATERIALS AND METHODS

Plant Material

Greenhouse-grown carnation (*Dianthus caryophyllus* L. cv White Sim) plants were used in all experiments. Flowers were harvested at anthesis, when the styles had fully elongated. Stems were recut to 10 cm, placed in deionized water, and held in the laboratory.

Flower Treatments

For treatment with ethylene, flowers were sealed in a 24-L chamber and ethylene was injected to yield a final concentration of 10 μL L^{-1}. For NBD treatments, liquid NBD was injected onto filter paper in a 24-L chamber to yield a concentration of 2500 μL L^{-1} after volatilization. Intact carnation flowers and leaves were treated with a number of known inducers of ACC synthase. These inducers included 50 mM LiCl, the synthetic auxin 2,4-D (100 μM), and 25 μM CHX, an inhibitor of protein synthesis. Intact flowers were held in a solution containing the various treatments for 24 h. Ethylene production from individual flower organs was then determined. Treatments also included pollinated and naturally senescing flowers. Flowers were pollinated by brushing cv White Sim stigmas with freshly dehisced cv Starlight anthers (Larsen et al., 1995). Flower organs were collected 12 and 24 h after pollination.

Harvested flowers were also left in a solution of water on the laboratory bench, and flower organs were assayed 6 d after harvest, when the petals were inrolling. A more detailed study of pollinated flowers included styles collected from flowers at various times from 1 to 48 h after pollination. Leaves were treated with LiCl and 2,4-D by placing the cut bases in the respective solutions. After the leaves were treated for 24 h, ethylene production was determined. Leaves also were wounded with a steel brush, and ethylene production was measured after 2 h.

Ethylene Measurements

Individual styles, ovaries, receptacles, and petals isolated from intact flowers after treatment were enclosed in 6-mL vials with a rubber septum. Following a 15-min incubation period, 1-mL gas samples were withdrawn from the vials and analyzed for ethylene using a gas chromatograph (Varian, Sugarland, TX) equipped with an activated alumina column and a flame-ionization detector. Leaves were sealed in 25-mL vials for 30 min for ethylene determination. Each experiment utilized a replication of at least six flowers or leaves per treatment, and the graphed values represent the mean ± SE ethylene production for the replications. All experiments were conducted a minimum of three times with similar results.

RNA Extraction and Gel-Blot Analysis

Treated carnation tissue was frozen in liquid N_2 and stored at −80°C until being used for RNA extraction. Total RNA from carnation tissue was extracted as described by Lawton et al. (1990) and quantified spectrophotometrically. Ten micrograms of total RNA was separated by electrophoresis through a 1% (w/v) agarose gel containing 2.2 M formaldehyde. The separated RNAs were transferred to membranes (Nytran, Schleicher & Schuell) and cross-linked with a controlled UV light source (Stratalinker, Stratagene). Membranes were prehybridized and hybridized as previously described (Jones et al., 1995). Membranes were hybridized for 20 h at 42°C with 5×10^5 cpm mL^{-1} ^{32}P-labeled cDNA. Membranes were washed in 2× SSC (1× SSC is 0.15 M NaCl and 15 mM sodium citrate, pH 7.0) and 0.1% SDS for 15 min at room temperature, followed by 15 min at 55°C, and then 15 min in 0.2× SSC and 0.1% SDS at 55°C. Blots were exposed to Kodak XAR-5 film at −80°C for 5 d using a single intensifying screen. Blots were used for multiple hybridizations by stripping in boiling 0.1% SDS.

Probes used for the detection of ACC synthase were either partial cDNAs, including the coding region, or gene-specific probes containing the 3′ untranslated regions of the three ACC synthase cDNAs. Probes containing the coding regions included a 1250-bp fragment of DCACS1 (Park et al., 1992), a 1175-bp PCR clone, DCACS2 (Henskens et al., 1994), and a 1515-bp PCR clone, DCACS3. ACC synthase gene-specific probes were constructed utilizing restriction sites at the periphery of the 3′ end of the coding region to give inserts approximately 250 to 300 bp in length that represented the 3′ untranslated regions of these ACC synthase cDNAs. The DCACS1–3′ probe corresponds to bp 1662 to 1950 from DCACS1 (previously called caracc3, accession no. M66619) and DCACS3–3′ corresponds to bp 1268 to 1515 from DCACS3 (accession no. AF049137). The PCR clone DCACS2 isolated by Henskens et al. (1994, accession no. X66605) does not include the 3′ untranslated region of the cDNA.

To construct the gene-specific probe for DCACS2 the 3′ end of this ACC synthase cDNA was amplified by reverse-transcriptase PCR. A sense primer specific to the DCACS2 sequence and a nonspecific oligo(dT)-antisense primer were utilized by reverse-transcriptase PCR of pollinated style total RNA to clone the 3′ end of DCACS2 (data not shown). The PCR clone was identified based on sequence identity to the original DCACS2 clone. The nucleotide sequence data for the PCR-amplified 3′ end of DCACS2 will appear in the database under the accession no. AF049138. The gene-specific probe DCACS2–3′ corresponds to bp 429 to 709. To demonstrate equal loading of RNA samples, membranes were reprobed with rRNA (Goldsbrough and Cullis, 1981).

Specificity of Gene-Specific Probes

Approximately 10 ng of cDNA representing the coding regions and 3′ untranslated regions of DCACS1, DCACS2, and DCACS3 were electrophoresed through a 1% agarose gel. The gel was depurinated for 10 min in 0.25 M HCl, denatured for 30 min in 0.5 M NaOH and 1.5 M NaCl, and neutralized for 30 min in 1 M Tris (pH 8.0) and 1.5 M NaCl. The DNA was transferred to membranes and cross-linked with a controlled UV light source, as described above.

Membranes were probed with the DCACS1, DCACS2, and DCACS3 cDNA clones and the gene-specific probes DCACS1–3′, DCACS2–3′, and DCACS3–3′. Prehybridization, hybridization, and washing were carried out under the conditions described above for the RNA gels, with the addition of a 15-min wash in 0.2× SSC and 0.1% SDS at 65°C.

RESULTS

Homology among the Carnation ACC Synthases

DCACS3 shares the highest amino acid identity (83.7%) with the carnation ACC synthase DCACS2, whereas amino acid identity between DCACS3 and DCACS1 is only 66.7% (Table I). The high homology between DCACS2 and DCACS3 made it difficult to distinguish between the transcripts of DCACS2 and DCACS3 when using the full-length clones as probes for RNA gel-blot analysis under high-stringency conditions (Fig. 1). When probes were constructed that included only the 3′ untranslated regions of the cDNAs, each probe detected only its corresponding cDNA. Whereas nucleotide identity of the coding regions of DCACS2 and DCACS3 was 79.2%, the homology between the 3′ untranslated regions of DCACS2 and DCACS3 was only 42.7% (Table I).

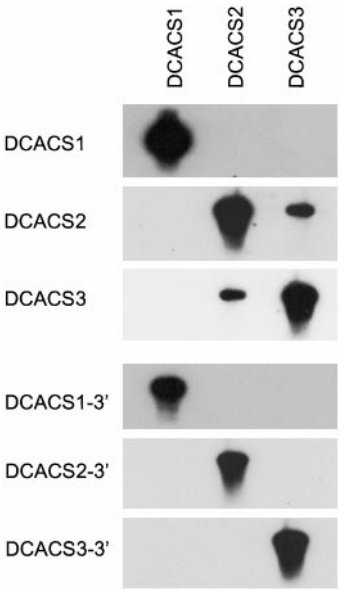

Figure 1. DNA gel-blot analysis showing the specificity of the ACC synthase gene-specific probes. Each lane contained 10 ng of DNA corresponding to the full-length cDNA of DCACS1, DCACS2, and DCACS3. The blot was hybridized with the full-length ^{32}P-labeled cDNA probes DCACS1, DCACS2, and DCACS3 and probes consisting of the 3′ untranslated region of the cDNA of DCACS1–3′, DCACS2–3′, and DCACS3–3′.

Differential Expression of ACC Synthase in Carnation Floral Organs

Intact carnation flowers were treated with a number of known inducers of ACC synthase to characterize the differential regulation of the three members of the ACC synthase gene family that have been identified in carnation. Intact flowers were placed in aqueous solutions of 25 μM CHX, 50 mM LiCl, 100 μM 2,4-D, or water. After 24 h of treatment floral organs were removed from the treated flowers for ethylene analysis. Floral organs also were assayed from intact flowers treated with 10 μL L^{-1} ethylene for 24 h, from pollinated flowers 12 or 24 h after pollination, and from naturally senescing flowers 6 d after harvest.

The patterns of expression of the three ACC synthase genes could be determined using gene-specific probes in the styles, ovaries, receptacles, and petals in response to the various treatments. RNA gel-blot analysis revealed that the carnation ACC synthases were differentially regulated in a tissue-specific manner. All three ACC synthase transcripts were detected in styles in response to various treatments, but DCACS2 and DCACS3 were preferentially expressed (Fig. 2). Only DCACS3 mRNAs could be detected at low levels in the control/untreated styles, and expression of DCACS3 was enhanced by all of the treatments except CHX. Pollination, flower senescence, and treatment of carnation flowers with exogenous ethylene and LiCl resulted in increased levels of stylar ethylene production. Expression of DCACS2 was induced in styles by Li, ethylene, pollination, and senescence. DCACS1 mRNAs exhibited an increase in senescing styles that was equivalent to levels of DCACS2 and DCACS3 transcripts, but lesser increases in DCACS1 mRNAs were observed following treatment with LiCl or ethylene or after 24 h of pollination.

Auxin treatment of flowers induced elevated ethylene production in ovaries that was greater than all other treatments except 24 h of pollination (Fig. 3). In ovaries ACC synthase transcripts were primarily those of DCACS3. DCACS1 transcripts were detected in ovaries by the gene-specific probes only following treatment with ethylene. Very low levels of DCACS2 transcripts were detectable in all samples except control and CHX. Basal levels of ACC synthase mRNAs corresponding to DCACS3 were detected in untreated ovaries but did not appear to be enhanced by any of the treatments, despite elevated ethylene production from these ovaries. DCACS3 mRNA levels decreased below the constitutive level detected in untreated ovaries in ethylene-treated, 12-h-pollinated, and senescing ovaries.

Table I. *Comparison of the percentage of nucleotide identity and percentage of amino acid identity (in parentheses) between the coding regions (cds) and the percentage of nucleotide identity between the 3′ untranslated regions (3′ UTR) of the carnation ACC synthases DCACS1, DCACS2, and DCACS3*

ACC Synthase	DCACS1	DCACS2	DCACS3
		%	
DCACS1 cds	X	68.77 (65.77)	66.64 (65.87)
DCACS2 cds	68.77 (65.77)	X	79.21 (83.73)
DCACS3 cds	66.64 (65.87)	79.21 (83.73)	X
DCACS1 3′ UTR	X	43.23	38.03
DCACS2 3′ UTR	43.23	X	42.71
DCACS3 3′ UTR	38.03	42.71	X

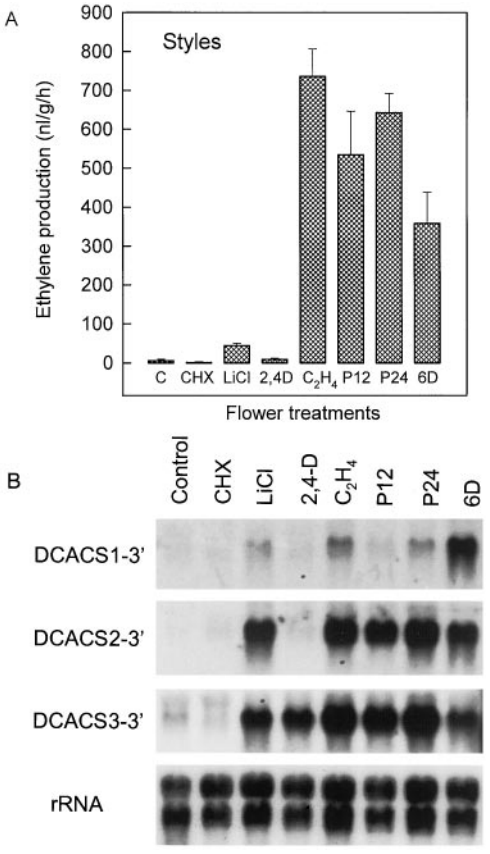

Figure 2. Ethylene production and expression of ACC synthase in carnation styles in response to various inducers of ACC synthase activity. Flowers were treated with water as a control (C) or with 25 μM CHX, 50 mM LiCl, 100 μM 2,4-D, or 10 μL L^{-1} ethylene for 24 h, at which time styles were collected for analysis. Styles were also collected from pollinated flowers 12 and 24 h after pollination (P12 and P24, respectively) and from senescing flowers 6 d after harvest (6D). A, Ethylene production by the styles. Each bar consisted of the average ± SE of styles from six flowers. B, Accumulation of ACC synthase mRNAs in styles following treatment. Each lane contained 10 μg of total RNA. Blots were hybridized with the gene-specific ACC synthase probes DCACS1–3′, DCACS2–3′, and DCACS3–3′ and with an rRNA probe. ACC synthase blots were exposed to film for 5 d.

In receptacles, despite the induction of ethylene biosynthesis by 2,4-D, ethylene, pollination, and senescence, only treatment with 2,4-D resulted in a substantial increase in ACC synthase mRNAs (Fig. 4). These mRNAs corresponded to all three ACC synthases. Very low levels of ACC synthase transcripts were also detected in all samples by the DCACS2 and DCACS3 gene-specific probes.

In contrast to styles, petals showed preferential expression of DCACS1 (Fig. 5). Ethylene-treated, 2,4-D-treated, 24-h-pollinated, and senescing petals all produced elevated levels of ethylene, and both DCACS1 and DCACS2 mRNAs accumulated in response to these treatments. In all of these treatments, DCACS1 transcript abundance was greater than DCACS2. An increase in DCACS3 mRNAs was detected in petals only in response to 2,4-D.

Pollination-Induced Expression of ACC Synthase in Carnation Styles

Inhibition of pollination-induced ethylene production by pretreating carnation styles with aminoethoxyvinylglycine, an inhibitor of ACC synthase, suggests that early-pollination-induced ethylene requires ACC synthase activity in the style (Woltering et al., 1993). To identify the ACC synthase gene responsible for early-pollination-induced ethylene in carnation styles, RNA gel-blot analysis was performed on styles from 1 to 48 h after pollination using the gene-specific probes. RNA gel blots showed enhanced accumulation of DCACS3 transcript as early as 1 h after pollination (Fig. 6). Induction of DCACS2, the other ACC synthase previously shown to be preferentially expressed in styles, was delayed to 6 h after pollination. DCACS1 transcripts were detected at much lower levels and were not induced until 24 h after pollination. To identify which

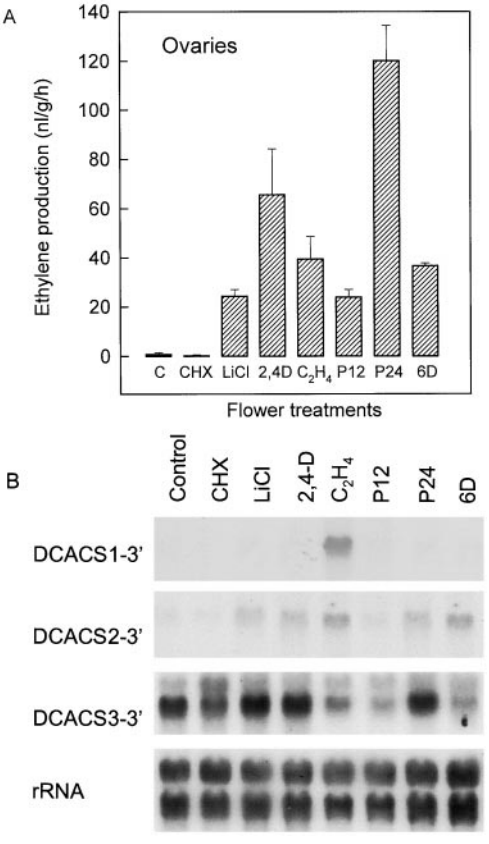

Figure 3. Ethylene production and expression of ACC synthase in carnation ovaries in response to various inducers of ACC synthase activity. Flowers were treated with water as a control (C) or with 25 μM CHX, 50 mM LiCl, 100 μM 2,4-D, or 10 μL L^{-1} ethylene for 24 h, at which time ovaries were collected for analysis. Ovaries were also collected from pollinated flowers 12 and 24 h after pollination (P12 and P24, respectively) and from senescing flowers 6 d after harvest (6D). A, Ethylene production by the ovaries. Each bar consisted of the average ± SE of six ovaries. B, Accumulation of ACC synthase mRNAs in ovaries following treatment. Each lane contained 10 μg of total RNA. Blots were hybridized with the gene-specific ACC synthase probes DCACS1–3′, DCACS2–3′, and DCACS3–3′ and with an rRNA probe. ACC synthase blots were exposed to film for 5 d.

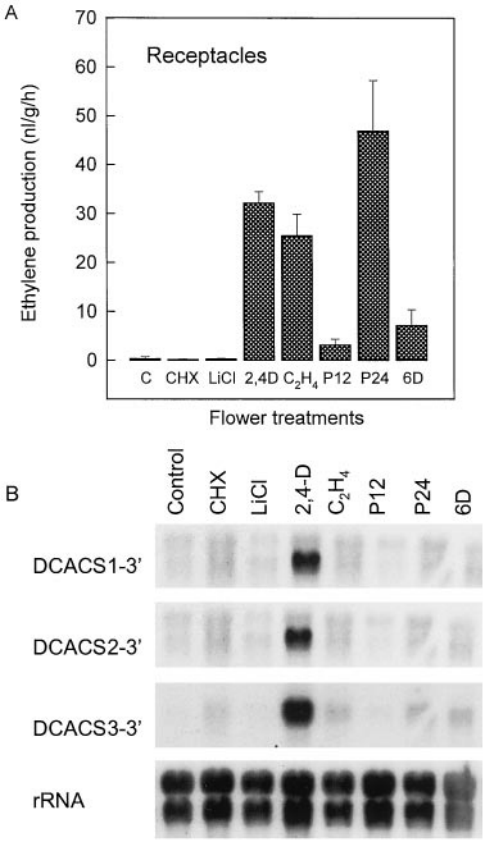

Figure 4. Ethylene production and expression of ACC synthase in carnation receptacles in response to various inducers of ACC synthase activity. Flowers were treated with water as a control (C) or with 25 μM CHX, 50 mM LiCl, 100 μM 2,4-D, or 10 μL L^{-1} ethylene for 24 h, at which time receptacles were collected for analysis. Receptacles were also collected from pollinated flowers 12 and 24 h after pollination (P12 and P24, respectively) and from senescing flowers 6 d after harvest (6D). A, Ethylene production by the receptacles. Each bar consisted of the average ± SE of six receptacles. B, Accumulation of ACC synthase mRNAs in receptacles following treatment. Each lane contained 10 μg of total RNA. Blots were hybridized with the gene-specific ACC synthase probes DCACS1–3′, DCACS2–3′, and DCACS3–3′ and with an rRNA probe. ACC synthase blots were exposed to film for 5 d.

genes were directly induced by pollination and which were induced by subsequent ethylene production, flowers were placed in an atmosphere of 2500 μL L^{-1} NBD, an inhibitor of ethylene action, immediately after pollination. NBD treatment did not block the enhanced accumulation of DCACS3 transcripts but completely inhibited the induction of both DCACS1 and DCACS2 in pollinated styles. In NBD-treated flowers, DCACS3 mRNAs continued to increase in styles until 12 h after pollination, after which time the transcript abundance decreased.

Expression of ACC Synthase in Vegetative Tissue

To determine whether the three ACC synthases identified from carnation were flower specific, RNA gel-blot analysis was conducted to determine expression of the ACC synthase mRNAs in leaves. Leaves were treated with LiCl or 2,4-D or wounded to induce ethylene biosynthesis. Although all treatments induced ethylene, treatment of isolated leaves with 2,4-D for 24 h resulted in the greatest increase in ethylene production (Fig. 7). No ACC synthase transcripts were detected in control leaves by the three probes, and, despite induction of ethylene biosynthesis by all of the treatments, only LiCl treatment resulted in the induction of DCACS2 mRNAs in leaves.

DISCUSSION

As has been discovered in many species, members of the ACC synthase multigene family in carnation are differentially regulated in a tissue-specific manner. The carnation ACC synthases were found to be induced by auxin, LiCl,

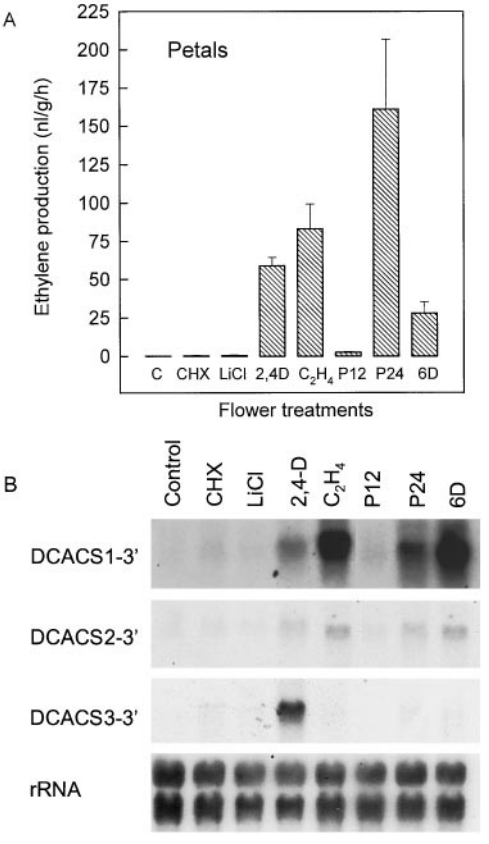

Figure 5. Ethylene production and expression of ACC synthase in carnation petals in response to various inducers of ACC synthase activity. Flowers were treated with water as a control (C) or with 25 μM CHX, 50 mM LiCl, 100 μM 2,4-D, or 10 μL L^{-1} ethylene for 24 h, at which time petals were collected for analysis. Petals were also collected from pollinated flowers 12 and 24 h after pollination (P12 and P24, respectively) and from senescing flowers 6 d after harvest (6D). A, Ethylene production by the petals. Each bar consisted of the average ± SE of petals from six flowers. B, Accumulation of ACC synthase mRNAs in petals following treatment. Each lane contained 10 μg of total RNA. Blots were hybridized with the gene-specific ACC synthase probes DCACS1–3′, DCACS2–3′, and DCACS3–3′ and with an rRNA probe. ACC synthase blots were exposed to film for 5 d.

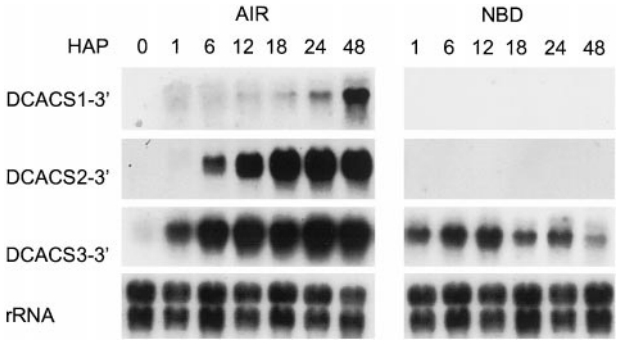

Figure 6. Expression of ACC synthase in styles from pollinated flowers treated with NBD or air. Each lane contained 10 μg of total RNA. Blots were hybridized with the gene-specific ACC synthase probes DCACS1–3′, DCACS2–3′, and DCACS3–3′ and with an rRNA probe. ACC synthase blots were exposed to film for 5 d. HAP, Hours after pollination.

ethylene, senescence, and pollination. No induction or enhancement of ACC synthase genes was detected when protein synthesis was inhibited by CHX, although induction of ACC synthase by CHX has been demonstrated in many other species (Zarembinski and Theologis, 1994). Among treatments, expression of DCACS2 and DCACS3 was primarily localized to the gynoecium, whereas DCACS1 was more abundantly expressed in petals. In styles and petals transcript levels of one or more of the ACC synthase genes were up-regulated by treatments that increased ethylene production, although there was not always a good correlation between ethylene production rates and transcript levels. Despite high levels of ethylene evolution following most treatments in ovaries, receptacles, and leaves, these tissues had little up-regulation of ACC synthase mRNAs, as detected by the three gene-specific probes.

Auxin is a well-documented inducer of ethylene biosynthesis, and at least one member of each ACC synthase gene family identified to date has been shown to be induced or enhanced in response to treatment with auxin (Huang et al., 1991; Nakagawa et al., 1991; Botella et al., 1992b; Kim et al., 1992; Yip et al., 1992; Zarembinski and Theologis, 1993; Abel et al., 1995; Destefano-Beltran et al., 1995). Some of these genes, including ACS4 from Arabidopsis (Abel et al., 1995), LEACS3 from tomato (Yip et al., 1992), and VRACS6 from mung bean (Yoon et al., 1997), are specifically induced by auxin, whereas other auxin-responsive ACC synthase genes are induced by other stimuli as well (Huang et al., 1991; Lincoln et al., 1993; Botella et al., 1995). This study failed to identify a single auxin-regulated member of the carnation ACC synthase gene family, because all three genes were induced by auxin in various organs. DCACS3 was the only ACC synthase to be up-regulated in styles, receptacles, and petals in response to treatment with 2,4-D. The up-regulation of DCACS3 mRNAs by 2,4-D in the petals when there was little regulation by ethylene suggests that DCACS3 is an auxin-responsive gene.

In 1984, Boller discovered that Li^+ greatly enhanced ACC synthase activity in tomato fruit, and it was Li^+- and IAA-enhanced accumulation of ACC synthase in zucchini fruits that made it possible to isolate the first ACC synthase cDNA (Sato and Theologis, 1989). Recent expression studies identified ACC synthase genes that are induced by Li^+. These Li-inducible genes include ACS5 in etiolated Arabidopsis seedlings (Liang et al., 1992), CPACS1 in zucchini fruit (Huang et al., 1991), and OSACS1 and OSACS3 in rice (Zarembinski and Theologis, 1993). Plants grown under normal conditions do not contain Li^+, but it is one of the strongest inducers of ACC synthase activity in plants (Liang et al., 1996). Li^+ inhibits the activity of inositol-phosphate phosphatases, but the molecular mechanism of action of ACC synthase induction in plants is not well understood (Liang et al., 1996). In carnation flowers the induction of ACC synthase by Li^+ was very tissue specific, with induction of all three ACC synthase genes only in the styles.

DCACS1 is a senescence-related ACC synthase that is regulated by ethylene (Park et al., 1992; Woodson et al.,

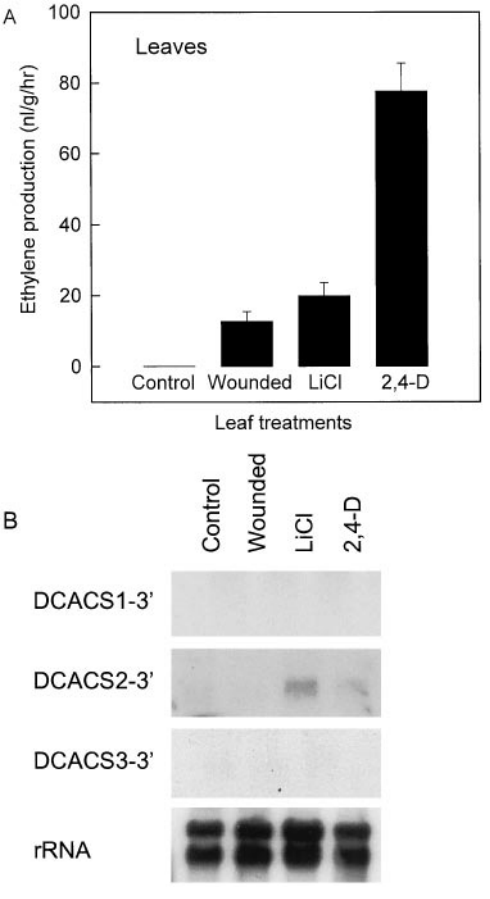

Figure 7. Ethylene production and ACC synthase expression in carnation leaves. Leaves were wounded with a wire brush or treated with 50 mM LiCl or 100 μM 2,4-D. A, Ethylene production by control leaves, wounded leaves 2 h after wounding, and LiCl- or 2,4-D-treated leaves 24 h after treatment. Bars represent the averages ± SE of six leaves. B, Accumulation of ACC synthase mRNAs in control, wounded, and treated leaves. Each lane contained 10 μg of total RNA. Blots were hybridized with the gene-specific ACC synthase probes DCACS1–3′, DCACS2–3′, and DCACS3–3′ and with an rRNA probe. ACC synthase blots were exposed to film for 5 d.

1992). Studies by ten Have and Woltering (1997) found tissue-specific expression of the ACC synthase genes in carnation, with DCACS1 predominantly expressed in senescing petals and DCACS2 preferentially expressed in the gynoecium. We have shown that the recently identified ACC synthase DCACS3 cross-hybridizes with DCACS2. Using gene-specific probes, we confirmed that DCACS1 is predominantly expressed in petals, whereas both DCACS2 and DCACS3 are preferentially expressed in the gynoecium during ethylene-induced and natural flower senescence. Although similar regulation of DCACS2 and DCACS3 by ethylene and senescence were observed in the style, only DCACS2 transcripts were detected in senescing petals. The absence of DCACS3 transcripts in senescing petals and the abundance of constitutive levels of DCACS3 in ovaries suggests that the expression of DCACS3 may be more specific to the gynoecium than that of DCACS2.

In styles DCACS2 and DCACS3 transcripts correlated well with ethylene biosynthesis. This is consistent with the transcriptional regulation of ACC synthase and de novo synthesis of the ACC synthase enzyme leading to increased ethylene production. In petals the patterns of expression and ethylene production were not so easy to interpret. Whereas 24 h after pollination petals were producing the most ethylene, ethylene-treated and senescing petals had the largest accumulation of DCACS1 transcripts. Although ACC synthase is generally considered to be regulated at the level of transcription, regulatory mechanisms at the post-transcriptional and -translational levels may be equally important means of regulating the production of ACC and ethylene in many systems. Evidence for this type of regulation has been demonstrated at the level of mRNA splicing (Olson et al., 1995), processing of the C terminus of the enzyme (Li and Mattoo, 1995), and posttranslational modification of the enzyme by phosphorylation (Felix et al., 1994; Spanu et al., 1994). Further experiments are necessary to determine whether posttranscriptional processing of ACC synthase is involved in the regulation of ethylene biosynthesis in carnation flowers. Considering that five or more ACC synthase genes have been identified in Arabidopsis, tomato, and mung bean (Liang et al., 1996; Kim et al., 1997; Oetiker et al., 1997; Yoon et al., 1997), it is likely that the three ACC synthase genes described in this paper represent only a few of the members of the carnation ACC synthase gene family. When gel blots of pollinated and senescing petal RNA are probed with the full-length DCACS1 probe (cDNA clone no. 403; Park et al., 1992), the patterns of expression are more closely correlated with ethylene levels (M.L. Jones, unpublished data). These data support the existence of additional ACC synthase genes with sequence homology to the senescence-related ACC synthase DCACS1.

Pollination accelerates ethylene biosynthesis and coordinates developmental changes that occur during the natural senescence of flowers (Stead, 1992). Like senescence in unpollinated flowers, pollination also induces preferential accumulation of DCACS1 in petals and DCACS2 and DCACS3 in the gynoecium. In styles pollination-induced ethylene can be defined temporally by three distinct peaks (Larsen et al., 1995; Jones and Woodson, 1997). The time points 12 and 24 h correspond to the second and third peaks of ethylene production from the style, respectively. We previously reported that ACC synthase mRNAs detected by the full-length DCACS2 probe increased in styles by 1 h after pollination (Jones and Woodson, 1997).

Using gene-specific probes, we differentiated between DCACS2 and DCACS3 transcripts and have shown in Figure 6 that early increases in ethylene biosynthesis following pollination were the result of DCACS3 gene expression, whereas the expression of DCACS2 and DCACS1 corresponded to the second and third peaks of ethylene production, respectively. Pollination-induced expression of DCACS1 and DCACS2 in styles was prevented by NBD, indicating that these genes were transcriptionally regulated by ethylene. We previously showed that the stylar ethylene produced within the third peak and a portion of the second peak is autocatalytic and can be prevented by treatment of the flower with NBD (Jones and Woodson, 1997). Pollination-induced expression of DCACS3 was independent of ethylene action; therefore, DCACS3 represents a pollination-responsive gene.

Recently, Bui and O'Neill (1998) showed similar regulation of ACC synthase in the gynoecium of orchid. They showed that early-pollination-induced ethylene biosynthesis from the gynoecium was the result of pollination-responsive ACC synthases (Phal-ACS2 and Phal-ACS3), and autocatalytic ethylene production from the flower resulted from the expression of an additional ACC synthase gene (Phal-ACS1). In orchids auxin contained in the pollinia has been proposed as the primary pollination signal, and the application of auxin to the stigma duplicates post-pollination events, including ethylene production, flower fading (Curtis, 1943; Burg and Dijkman, 1967), ovary growth, and ovule differentiation (O'Neill et al., 1993; Zhang and O'Neill, 1993). Consequently, both pollination-responsive ACC synthase genes identified in orchid were induced by application of auxin to the stigma. The ethylene-independent regulation of DCACS3 by pollination and its responsiveness to 2,4-D suggest that auxins may also play a role in postpollination development and signaling in carnation flowers.

In the ovary the induction of ethylene biosynthesis did not correlate well with ACC synthase gene expression. Basal levels of DCACS3 mRNAs were detected in ovaries but were not enhanced by any of the treatments, whereas DCACS2 mRNAs were only slightly up-regulated by various treatments including pollination and senescence. In contrast, ACC oxidase mRNAs have been shown to be highly up-regulated by pollination and senescence in the carnation ovary (Woodson et al., 1992; Jones and Woodson, 1997). Large increases in DCACO1 mRNAs can be detected in pollinated and senescing ovaries by exposing blots for only 18 h, whereas 5-d exposures are required for detection of ACC synthase transcripts (Jones and Woodson, 1997). The comparatively low levels of ACC synthase transcripts in carnation ovaries have led us to propose that ACC transport to the ovary is necessary for the sustained increases in ethylene biosynthesis induced by pollination and senescence (Jones and Woodson, 1997).

Similar to the basal level of DCACS3 detected in carnation gynoecia, low steady-state levels of LEACS2 have been shown in green tomato fruits (Yip et al., 1992). Yip et al. (1992) suggested that this low level of LEACS2 expression in green tomato fruits is responsible for the system I ethylene (Yang and Hoffman, 1984) produced in preclimacteric fruits. Although the basal levels of DCACS3 transcripts detected in carnation gynoecia may be responsible for the low levels of ethylene produced by these organs, the constitutive levels of DCACS3 mRNAs detected in control ovaries seem relatively high compared with the low levels of ethylene production measured. Untreated ovaries from preclimacteric flowers have very low levels of ACC oxidase activity compared with the levels of ACC synthase activity, and ACC oxidase mRNAs have not been detected in these ovaries by RNA gel-blot analysis (Woodson et al., 1992; Jones and Woodson, 1997; ten Have and Woltering, 1997). This evidence suggests that ACC oxidase limits ethylene production in preclimacteric ovaries. Although ACC synthase is often considered to be the rate-limiting step in ethylene biosynthesis, activity of the enzyme ACC oxidase has also been shown to limit ethylene biosynthesis in flowers and fruits (Yang and Hoffman, 1984).

DCACS3 mRNA abundance in ovaries from ethylene-treated, 12-h-pollinated, and 6-d-senescing flowers was actually lower than basal levels in control ovaries, indicating the presence of negative regulation or repression of ACC synthase transcripts. ten Have and Woltering (1997) showed that treating carnation flowers with exogenous ethylene resulted in increases in ACC synthase mRNAs in the ovary by 3 h, but this accumulation then decreased after 18 h of exposure. These experiments also suggested that there is a negative regulation or inhibition of ACC synthase by ethylene in the ovary. A detailed study involving multiple exposure times and ethylene concentrations will be needed to more accurately assess the regulation of DCACS3 by ethylene in carnation ovaries.

As was observed with carnation ovaries, the induction of ethylene biosynthesis by various treatments did not correlate well with ACC synthase transcripts in leaves and receptacles. In these tissues the three members of the carnation ACC synthase gene family described in this paper were insufficient to catalyze the observed ethylene biosynthesis. Although ethylene biosynthesis in the absence of ACC synthase transcripts could be the result of ACC transport from other parts of the flower, as has been suggested for ovaries, reports of increased ACC synthase activity in senescing receptacles provide evidence for de novo synthesis of ACC in the receptacle and suggest that an ACC synthase gene responsible for ethylene biosynthesis in the receptacle tissue remains to be identified (Woodson et al., 1992).

The phytohormone ethylene is associated with carnation flower petal senescence, and auxin treatment of flowers has also been shown to induce ethylene biosynthesis and petal senescence (Sacalis, 1989). In contrast, both ethylene and auxin cause increases in the fresh weight of the ovary similar to those following pollination (Nichols, 1971). Following pollination, ethylene biosynthesis is induced in styles, ovaries, and petals, but the results of ethylene action in these floral organs is dramatically different. Whereas ethylene induces senescence of the petals and styles, organs that have now completed their function in pollen reception, the ovary is now induced to grow and develop into a fruit. Considering their differing roles in reproduction, it is not unexpected that ethylene biosynthesis would be differentially regulated within the individual organs of a flower.

Using gene-specific probes we have identified differential patterns of ACC synthase gene expression in carnation flowers and leaves. The differential regulation of the members of the carnation ACC synthase gene family following pollination and senescence will be useful in determining the role of ACC and ethylene in interorgan communication within the flower. In addition, the expression of the pollination-responsive DCACS3 can be used to help identify the primary pollination signal or pollen factor responsible for inducing ACC synthase immediately after pollination in the style and setting into motion the subsequent postpollination events. Except for moderate accumulation of DCACS2 mRNAs in response to LiCl treatment of leaves, the three carnation ACC synthases appeared to be flower specific. Experiments that resulted in increased ethylene biosynthesis but no detectable increases in ACC synthase mRNAs suggested that the carnation ACC synthase gene family has additional unidentified members that are not detected by the gene-specific probes. To gain a complete understanding of the regulation of ethylene biosynthesis during flower senescence, it will be necessary to identify these members and compare their expression patterns with ACC synthase and ACC oxidase enzyme activity, as well as expression of the ACC oxidase genes.

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