Plant Molecular Biology **28**: 93–103, 1995.

Molecular cloning of an ozone-induced 1-aminocyclopropane-1-carboxylate synthase cDNA and its relationship with a loss of *rbcS* in potato (*Solanum tuberosum* L.) plants

Carl D. Schlagnhaufer[1], Richard E. Glick[2], Richard N. Arteca[1,*] and Eva J. Pell[2]
[1] *Department of Horticulture (* author for correspondence) and* [2] *Department of Plant Pathology and Environmental Resources Research Institute, The Pennsylvania State University, University Park, PA 16802, USA*

Received 13 October 1994; accepted in revised form 15 February 1995

Key words: 1-aminocyclopropane-1-carboxylic acid (ACC), ACC synthase, ethylene, potato, ozone, *Solanum tuberosum*

Abstract

Acute or chronic exposure of potato plants to ozone (O_3) induces ethylene production. We isolated a 1586 bp cDNA (*pOIP-1*) encoding 1-aminocyclopropane-1-carboxylate (ACC) synthase from a cDNA library constructed with mRNA extracted from O_3-treated leaves. The clone has a 1365 bp open reading frame and a 221 bp trailing sequence. The active site found in all ACC synthases and 11 of the 12 amino acid residues conserved in aminotransferases are found in *pOIP-1*. Northern analysis showed that the mRNA encoding ACC synthase was detectable 1 h after the onset of O_3 exposure, and the message increased over time as did ethylene production. Concurrent with the increased ACC synthase mRNA was a decrease in the message for the Rubisco small subunit (*rbcS*) with no change in the large subunit (*rbcL*). When the plants were treated with aminooxyacetic acid (AOA), both ethylene production and level of ACC synthase transcript were inhibited. The decline in *rbcS* was also inhibited by AOA suggesting a correlation between ethylene production and loss of *rbcS*. Based on nuclear run-on studies it appears that the increase in ACC synthase mRNA may result from O_3-induced transcriptional activity.

Introduction

There are many stresses, both biotic and abiotic, which are associated with an increase in the rate of foliar senescence [18]. Ozone (O_3) is a phytotoxic chemical that is thought to induce accelerated foliar senescence in susceptible plant species [22]. Symptoms associated with O_3 stress include premature leaf yellowing and abscission. Ozone accelerates loss of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) [6], decreases levels of Rubisco mRNA [24], and in-

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number L20634.

creases ethylene production [15, 21, 24, 28], all of which are indications of leaf senescence.

Ethylene production is involved in many plant physiological processes including stress responses and has frequently been shown to be associated with foliar senescence [1]. There are numerous reports in the literature showing that biotic stresses due to bacteria, fungi, insects, nematodes or viruses and abiotic stresses, both natural and anthropogenic in origin stimulate ethylene production. Studies using non-enzymatic model systems initially suggested that methionine is a precursor of ethylene biosynthesis in plant tissues. This conclusion has since been confirmed by tracer experiments using radioactive methionine in a variety of plant tissues [33]. Since AdoMet is involved in numerous pathways, the key regulatory step in the ethylene biosynthetic pathway is the conversion of AdoMet to 1-aminocyclopropane-1-carboxylic acid (ACC) which is catalyzed by ACC synthase.

When potato plants (*Solanum tuberosum* L. cv. Norland) are subjected to acute or chronic exposure of O_3, there is an increase in the ethylene precursor, ACC [24]. The increases in ACC synthase activity, noted in many developmental and inducible systems, in many cases, result from increases in expression of mRNA for ACC synthase. Using antisense RNA, Oeller *et al.* [19] were able to inhibit the expression of ripening-induced mRNAs from ACC genes resulting in the inhibition of ethylene production. Expression studies using cDNAs from a variety of plant tissues show that increased ACC synthase activity is often correlated with increases in expression of mRNA for ACC synthase [13]. A multigene family for ACC synthase genomic sequences has been reported in zucchini [10], tomato [25], *Arabidopsis* [16, 30] mung bean [4], rice [34] and potato (van der Straeten, personal communication). There are also numerous reports showing differential expression of the ACC synthase genes in a variety of plant systems suggesting an important regulatory mechanism in many physiological processes [13].

Picton *et al.* [20] reported that transgenic tomato plants underproducing ethylene had delayed foliar senescence. More recently, Lanahan *et al.* [14] showed that transgenic tomato plants overproducing ethylene did not show increased foliar senescence, leaving the question remaining how ethylene involved is in the senescence process. In this study we have successfully cloned a cDNA encoding ACC synthase from the foliage of O_3-treated potato plants, and have demonstrated that O_3 induces expression of mRNA for ACC synthase. We have also found that there appears to be a correlation between the induction of ethylene and the loss of *rbcS* mRNA, suggesting that ethylene may play a key role in stress-induced foliar senescence.

Materials and methods

Plant material and treatment

Solanum tuberosum L. cv. Norland plants were grown in a greenhouse with supplemental light and charcoal-filtered air. Ozone exposures were performed when the plants were 4–5 weeks old with about 14 leaves. Plants were treated in continuous stirred tank reactors [8] with charcoal-filtered air or 0.30 μl/l O_3 for 4 h or as otherwise designated. For inhibitor experiments, plants were sprayed with 100 μM AOA and 0.1% Tween 20 or distilled H_2O and 0.1% Tween 20 one h prior to the O_3 exposure. Unless otherwise indicated, after exposure the fifth leaf from the apex, which was fully expanded, was cut from the plant at the base of the petiole. The excised leaf was sealed for 2 h in a 250 ml bottle with the base of the petiole immersed in 25 ml of distilled water. The ethylene produced was measured by gas chromatography under the conditions outlined [2]. Leaf 4, which was still expanding, was removed from the plant, frozen in liquid N_2 and stored at −80 °C until analyzed.

Construction of cDNA library

A 15 g portion of potato leaves taken from plants treated with O_3 was ground to a fine powder in liquid N_2. The RNA was extracted by resuspend-

ing the powder in 50 ml of 25 mM sodium citrate (pH 7) containing 4 M guanidine thiocyanate, 50 mM EDTA, 0.5% Sarkosyl (w/v), 100 mM 2-mercaptoethanol and 1.5 M CsCl. The extract was filtered through two layers of Miracloth and centrifuged at 27000 × *g* for 15 min. The clarified supernatant was layered onto a 5.7 M CsCl cushion and centrifuged at 85000 × *g* for 24 h. The RNA pellet was washed in 70% ethanol and resuspended in DEPC-treated water [5]. The solution was extracted once with phenol/chloroform (1:1), once with chloroform and the RNA recovered by ethanol precipitation. Poly(A)$^+$ RNA was purified by oligo-dT cellulose according to Sambrook *et al.* [27]. Double-stranded cDNA was prepared using Amersham's cDNA Synthesis System Plus kit (Amersham, Arlington Heights, IL). A 0.8% agarose gel was run, dried and autoradiographed to evaluate the size and quality of the cDNA. Amersham's cDNA Cloning System λgt11 kit was used for preparation of the library. Recombinant phage DNA was packaged and *Escherichia coli* strain Y1090 was infected.

Generation of PAC-1 by RNA-PCR

A 200 ng portion of total RNA from O_3 treated leaves was reverse-transcribed with 2.5 U of moloney murine leukemia virus (MMLV) reverse transcriptase in a reaction mixture of 10 mM Tris-HCl pH 8.3 containing 50 μM KCl, 5 mM $MgCl_2$, 1 μM of each dNTP, 1 U RNase inhibitor and 0.75 μM of oligonucleotide primer *OLE-4* [3]. The reaction proceeded for 30 min at 42 °C then the mixture was heated to 99 °C for 5 min to inactivate the reverse transcriptase. The cDNA produced was then amplified with 2.5 U AmpliTaq DNA Polymerase in a reaction mixture of 10 mM Tris-HCl pH 8.3 containing 2 mM $MgCl_2$, 50 μM KCl, 0.1% gelatin, 0.2 mM of each dNTP, and 0.15 μM of *OLE-5* and *OLE-4* [3] in a total volume of 100 μl. The PCR parameters were 1 min template denaturation at 94 °C, 1 min primer annealing at 48 °C and 2 min primer extension at 72 °C for 45 cycles.

The products of the PCR reaction were further amplified using a second set of oligonucleotide primers *OLE-5* and *OLE-6* [3]. The cDNA originally obtained was incubated with 2.5 U AmpliTaq DNA Polymerase in a reaction mixture of 10 mM Tris-HCl pH 8.3 containing 1.5 mM $MgCl_2$, 50 μM KCl, 0.2 mM of each dNTP, 0.1% gelatin and 0.2 μM of each primer in a total volume of 100 μl. The PCR parameters were 1 min template denaturation at 94 °C, 1 min primer annealing at 54 °C and 2 min primer extension at 72 °C for 30 cycles. The PCR products were analyzed on 0.8% agarose gels and visualized with ethidium bromide [27]. *PAC-1*, a 1098 bp cDNA ACC synthase fragment, was obtained from this preparation.

Library screening and subcloning

Filters were prehybridized at 60 °C in prehybridizion solution (6 × SSC, 5 × Denhardt's reagent, 0.1% SDS, 100 μg/ml denatured fragmented salmon sperm DNA) for 4 h. Hybridizations were performed overnight at 65 °C using 1×10^6 cpm/mL of *PAC-1* labelled with α-^{32}P-dCTP by the random priming method. Hybridized filters were washed at room temperature in 2 × SSC, 0.1% SDS for 15 min, then 2 times in 0.2 × SSC, 0.1% SDS at 65 °C for 15 min each. The dried filters were then exposed to Kodak XAR-5 X-ray film with one intensifying screen overnight at −80 °C. Individual positive plaques were purified by several rounds of plating and hybridization. The insert cDNA from positive plaques was excised with *Bam* HI and ligated into pBluescript (*SK*$^+$) (Stratagene, La Jolla, CA). The ligation mixtures were used to transform *E. coli* DH5α. Transformants were selected on LB plates containing ampicillin (50 μg/ml) and X-gal (0.033% w/v). Plasmid DNA was isolated using the alkaline lysis method [20].

DNA sequence analysis

DNA was sequenced by the dideoxy chain termination method [26] using Sequenase Version

2.0 (United States Biochemical, Cleveland, OH). Universal and reverse M13 sequencing primers were used (United States Biochemical, Cleveland, OH). DNA sequence analysis was facilitated by the use of the Intelligenetics Molecular Biology package installed on a SUN 3/50 computer. The percentage homology between ACC synthase amino acid sequences from the GenBank database with our gene was accomplished using a Clustal method with PAM 250 residue weight table and a MegAlign program (DNASTAR, Madison, WI).

Northern analysis

Samples of total RNA (10 μg) were separated by electrophoresis in a 1.2% agarose gel containing 2.2 M formaldehyde and transferred to a nylon membrane (Hybond-N; Amersham, Arlington Heights, IL). The filters were hybridized at 42 °C for 16 h in 6× SSC, 5× Denhardt's solution, 0.5% SDS, 50% formamide and 100 μg/ml denatured salmon sperm DNA using a α-^{32}P-dCTP-labelled probe. The probes used in this study were as follows: a 900 bp *Pst* I fragment of a cDNA (*rbcS*) for the potato Rubisco small subunit [32], a 900 bp *Bam* HI/*Eco* RI fragment of a Rubisco large subunit gene (*rbcL*) from potato [7] and a 1360 bp *Spe* I fragment *pOIP-1* for ACC synthase mRNA. After hybridizion, the nylon membrane was washed two times in 2× SSC, 0.1% SDS for 15 min at room temperature followed by a 20 min wash in 0.2× SSC, 0.1% SDS at 65 °C and a 15 min wash in 0.1× SSC, 0.1% SDS at 65 °C. The membrane was exposed to Kodak XAR-5 X-ray film at −80 °C for 24–48 h with two intensifying screens. The blots were rehybridized with a pea ribosomal gene [11] to ensure equal amounts of RNA were loaded in each lane.

Genomic Southern analysis

Genomic DNA was extracted from the leaves of potato plants which had been held in the dark for 3 days. Ten grams of tissue was ground to a fine powder in liquid N_2, then stirred for 15 min in 50 ml of 100 mM Tris-HCl pH 8 containing 250 mM NaCl, 100 mM EDTA, 1% Sarkosyl (w/v), plus 1% 2-mercaptoethanol (v/v). The mixture was centrifuged for 15 min at 27000 × *g*, and the supernatant was filtered through two layers of Miracloth. The solution was extracted once with 50 ml of phenol/chloroform (1:1) and once with 50 ml of chloroform. The nucleic acids were recovered by isopropanol precipitation. The DNA was resuspended in TE buffer (10 mM Tris-HCl pH 8, 5 mM EDTA) and purified by centrifugation through CsCl (1 g/ml). The DNA (10 μg) was digested with *Bam* HI, *Eco* RV and *Sac* I. After the DNA fragments were fractionated on a 0.6% agarose gel they were transferred to a nylon membrane. The blot was then prehybridized, hybridized, and washed as previously described for northern analysis.

Preparation of nuclei

After O_3 treatment the sixth leaf was assayed for ethylene, and the fourth and fifth leaves were frozen in liquid N_2 and stored at −80 °C until analyzed. Ethylene production by the fourth and fifth leaves was previously determined to be similar. *In vitro* transcription in nuclei isolated from frozen tissue was comparable to nuclei isolated from fresh tissue. The isolation of nuclei was done at 4 °C using autoclaved glassware and sterile solutions. Nuclei were prepared from 10 g of leaf tissue, which was ground to a powder in liquid N_2 with a mortar and pestle. The powdered tissue was suspended in 10 volumes of nuclei isolation buffer (NIB) (10 mM MES pH 6, 0.75 M sucrose, 10 mM NaCl, 5 mM EDTA, 0.5 mM spermidine, 20 mM 2-mercaptoethanol, and 0.25% nonidet P-40). The frozen powder was stirred in a beaker at 4 °C until thawed and the suspension was filtered through 4 layers of sterile cheesecloth, 100, 50, 20 μm nylon mesh and centrifuged at 1000 × *g* for 5 min at 4 °C in a HB-4 rotor. The nuclei were gently resuspended in a small volume of NIB without P-40. Buffer was added to a final volume of 30 ml, and the nuclei were centrifuged as above.

The nuclei were resuspended in 0.5 ml NIB without P-40 and a 10 μl aliquot was removed, mixed with acetocarmine and counted with a hemocytometer. The remaining nuclei were diluted to 30 ml with nuclei resuspension buffer (NRB) (50 mM Tris pH 8, 5 mM $MgCl_2$, 75 mM KCl, 0.2 mM EDTA, 25% glycerol, 2 mM DTE) and centrifuged as above. The nuclei were resuspended in a minimum (known) volume of NRB. Aliquots of 10^6 (for DNA quantitation) and 7.5×10^6 (for nuclear run-on assays) were stored at −80 °C until analyzed. The nuclear DNA was quantified as described by Wanner and Gruissem [31]. The aggregation of nuclei in NRB made it necessary to count nuclei while still in NIB. There was a direct correlation between the number of nuclei and DNA content of the nuclei preparations.

Nuclear run-on transcription

Reintiation of nuclei transcription was done essentially as described by Wanner and Gruissem [31] except 7.5×10^6 nuclei (ca. 40 μg of DNA) were used with 125 μCi of α-^{32}P-UTP (3000 Ci/mmol) in a final volume of 200 μl, unlabelled UTP was omitted from the reaction mix, and transcription reaction were incubated at 27 °C. DNA was digested with 10 units of RQ1 RNase-free DNase (Promega, 1000 units/ml) and transcripts were not chromatographed on a spin column. Ethanol precipitated transcripts were resuspended with 100 μl of hybridization solution.

Southern blot analysis of plasmid DNA was prepared by agarose gel electrophoresis of 5 μg of *Bam* HI-digested *pOIP-1*. The gel was blotted onto a nylon membrane by alkaline transfer [27]. The filter was prehybridized in 6× SSPE, 10× Denhardt's reagent, 1% (w/v) *n*-lauroylsarcosine, 100 μg/ml salmon sperm DNA, 50% (v/v) formamide for 2 h at 42 °C. Hybridization used the same solution (without Denhardt's reagent and 50 μg/ml salmon sperm DNA) to which labelled transcripts were added. After hybridization at 42 °C for 16–24 h, the filter was washed in 2× SSPE, 0.85% (w/v) *n*-lauroylsarcosine for 15 min at room temperature, 2× SSPE, 0.85% (w/v) *n*-lauroylsarcosine for 30 min at 65 °C, and 0.1× SSPE, 0.85% (w/v) *n*-lauroylsarcosine for 30 min at 65 °C. The filter was autoradiographed at −80 °C with two intensifying screens.

Results

Isolation of ACC synthase cDNA

Using degenerate oligonucleotides corresponding to several conserved regions found in previously reported ACC synthase sequences, we used RNA-PCR to reverse-transcribe and amplify an 1098 bp cDNA from the total RNA fraction extracted from O_3-treated potato plants. After subcloning and transformation, we screened the bacterial colonies and found nine which had plasmids with inserts of the predicted length. Nucleotide sequencing revealed that the cDNA (*PAC-1*) had a high degree of homology with sequences reported for ACC synthase.

In an effort to clone a full length cDNA we used *PAC-1* to screen a cDNA library constructed from the poly(A)$^+$ RNA of O_3-treated plants. After screening 300000 pfu we isolated 3 positives. The largest cDNA (*OIP-1*) was subcloned into pBluescript (*SK*$^+$) and the plasmid was used to transform *E. coli* DH5α.

The nucleotide and deduced amino acid sequence of the cDNA (*pOIP-1*) is shown in Fig. 1. The clone is 1586 bp long with an 1365 bp open reading frame and a 221 bp trailing sequence. The absence of the initiator *ATG* signal indicates that the clone is not full length. Comparison with other ACC synthase clones suggests that our clone is lacking sequence encoding for the first ca. 30 amino acids and any leader sequence. The open reading frame encodes 455 amino acids including the active site of ACC synthase. It is reported that among the different aminotransferases there are 12 conserved amino acids [10] and 11 are present in all reported ACC synthases [3]. These 11 amino acid residues are also conserved in *pOIP-1* (Fig. 1).

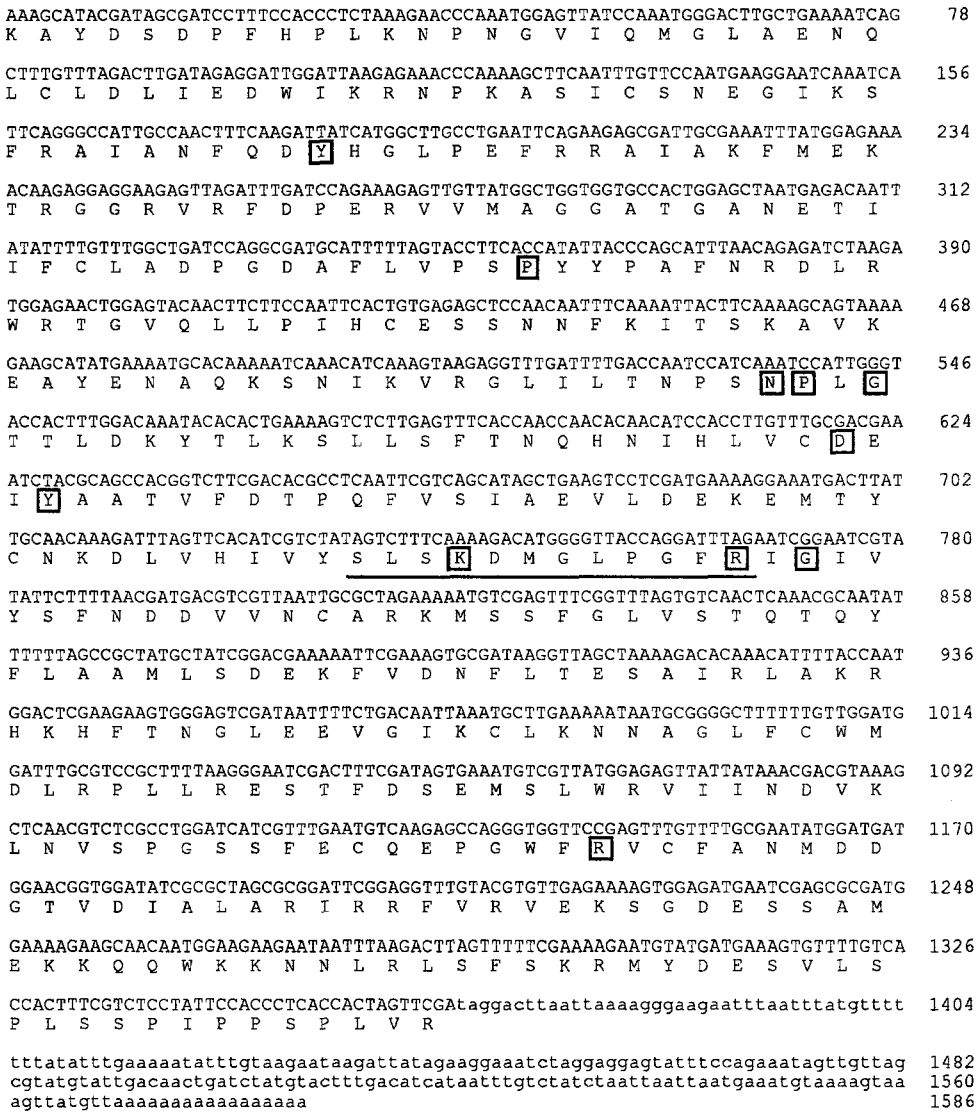

Fig. 1. The complete nucleotide and deduced amino acid sequences for *pOIP-1*. The active site is underlined and the eleven conserved residues found in aminotransferases are boxed.

Genomic Southern blot analysis

Southern analysis shows that each of the digests (*Sac* I, *Eco* RV, *Bam* HI) yielded a single band with molecular weight of ca. 2.01, 2.01, and 1.89 (Fig. 2), respectively, when hybridized with the *Sac* I-*Eco* RV restriction fragment of *OIP-1* (Fig. 2). Concurrent with the digests we also ran ca. 1, 2, 4, and 8 copies of *pOIP-1*. By comparing the intensity of the digest bands with the intensity of the *OIP-1* bands, we estimate that *OIP-1* exists as 1 possibly 2 copies per haploid genome (Fig. 2).

Expression of OIP-1, rbcS *and* rbcL *in response to* O_3

Using *Spe* I-cut *pOIP-1* as a probe, we evaluated expression of the ACC synthase message in re-

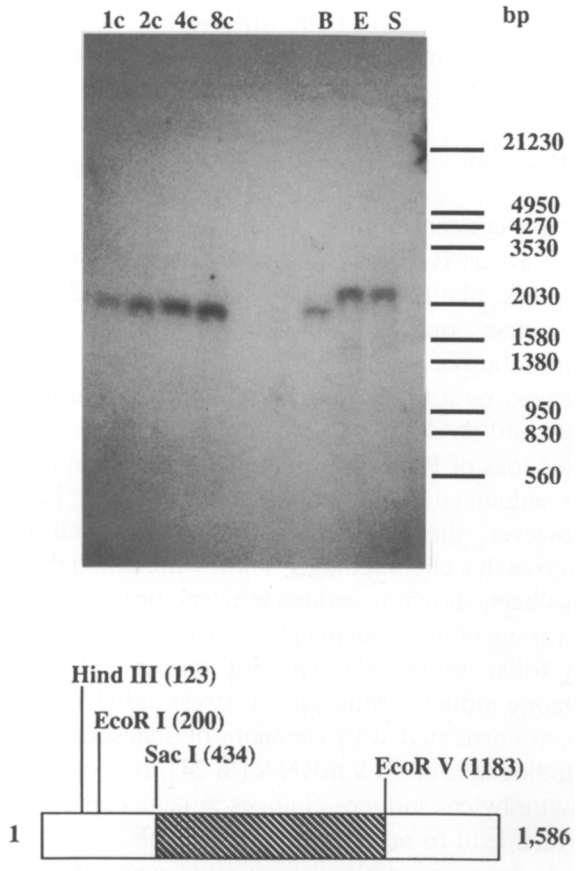

Fig. 2. Southern analysis of one-copy (1c), two-copy (2c), four-copy (4c), or eight-copy (8c) of *pOIP-1* and potato leaf genomic DNA (10 μg) cut with *Sac* I (S), *Eco* RV (E), or *Bam* HI (B). Partial restriction map of *pOIP-1*. The *Sac* I-*Eco* RV fragment used as a probe in the Southern analysis is represented by the dashed box.

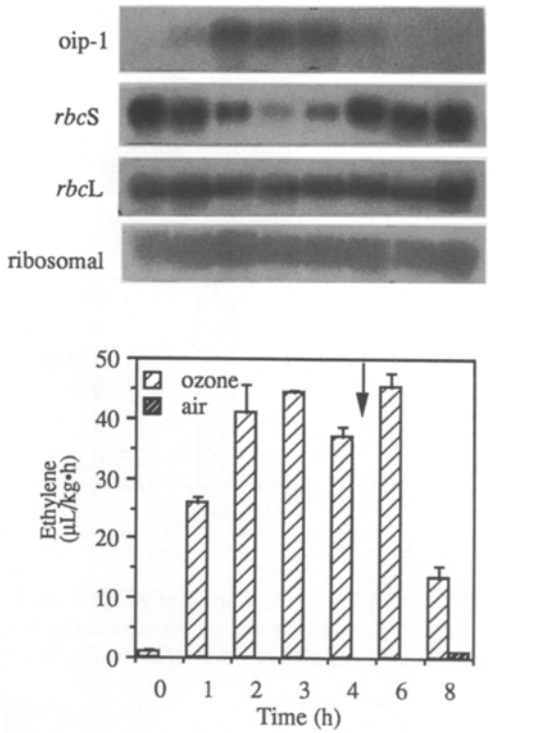

Fig. 3. Expression of *OIP-1*, *rbcS*, *rbcL* mRNA, and ethylene production in response to O_3 over time. Arrow indicates time at which O_3 exposure was ended. Ethylene values represent the means of three replications ± SE.

sponse to acute (0.30 μl/l) exposure to O_3. In untreated plants message was absent; however, low levels of message were detected after 1 h of O_3 exposure with a corresponding increase in ethylene production from 0 to 25 μl kg^{-1} h^{-1}. Both the levels of ethylene and message increased dramatically after 2 h, and only small changes were noted after 3 and 4 h of fumigation (Fig. 3). After 4 h of exposure the O_3 was removed. Within 2 h after the removal of O_3, there was a sharp decrease in ACC synthase message although ethylene production remained constant. Within 4 h we could not detect any ACC synthase message and ethylene production decreased dramatically (Fig. 3).

As ethylene increased there was a corresponding decrease in *rbcS* mRNA (Fig. 3). When the O_3 was removed, there was a rapid recovery of *rbcS* associated with a decrease in ACC synthase expression, but the reduction in ethylene production was somewhat slower. Additionally, there was little or no change in the level of *rbcL* or ribosomal (used as a control) mRNA in response to O_3.

We further investigated the association of ethylene and *rbcS* mRNA by treating the plants with the ethylene biosynthesis inhibitor AOA prior to exposure to O_3. Ethylene production and the ACC synthase message were suppressed (Fig. 4). With the decrease in ethylene, there was also reduced loss of *rbcS* mRNA.

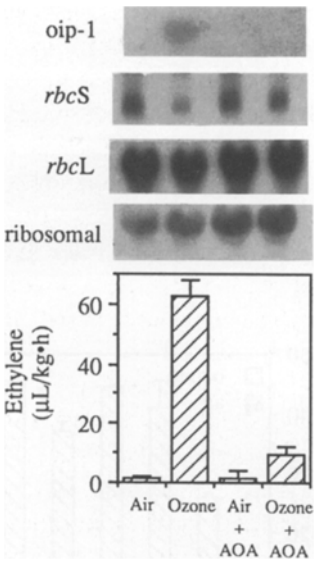

Fig. 4. Effect of AOA on the expression of *OIP-1*, *rbcS*, *rbcL* mRNA, and ethylene production in response to O_3. Ethylene values represent the means of three replications ± SE.

Nuclear run-on

Transcripts produced by nuclei isolated from control tissue did not hybridize with *OIP-1*. However, there was hybridization of transcripts produced by nuclei isolated from O_3-treated tissue with *OIP-1* (Fig. 5), thereby providing evidence that the induction of the mRNA for ACC synthase by ozone is transcriptionally regulated.

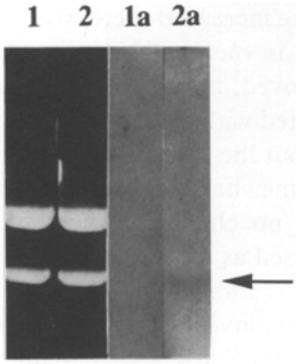

Fig. 5. Nuclear run-on assay of *pOIP-1*. Lanes 1 and 2, gel electrophoresis of *Bam* HI cut *pOIP-1*. Lane 1a, autoradiogram of *Bam* HI cut *pOIP-1* probed with transcripts produced by nuclei isolated from control tissue. Lane 2a, autoradiogram of *Bam* HI-cut *pOIP-1* probed with transcripts produced by nuclei isolated from O_3-treated tissue.

Discussion

At low concentrations, O_3 can cause visible injury and a variety of physiological responses in susceptible plant species (see for review, [12]). A response commonly associated with O_3 is the promotion of accelerated foliar senescence as evidenced by a reduction in the net photosynthetic rate and levels of Rubisco protein [23]. Accelerated loss of Rubisco has been attributed, in part, to enhanced degradation of the protein [23]; however, the actual mechanism by which O_3 causes this effect remains unknown. Although O_3 has been shown to induce ethylene production in a variety of test systems [1] the role which it plays in foliar senescence remains unclear [14, 20]. Ozone-induced emission of stress ethylene has been correlated with elements of senescence including loss of *rbcS* mRNA [9, 24]. It is possible that ethylene induces changes in gene expression which lead to accelerated senescence.

In many inducible systems ethylene production is the result of an accumulation of the ethylene precursor ACC which is produced from AdoMet via the highly labile enzyme ACC synthase. This step is generally accepted to be rate limiting in the ethylene biosynthetic pathway [33]. In many inducible systems ACC synthase activity results from the *de novo* synthesis of a highly unstable mRNA for ACC synthase allowing for the rapid regulation of ethylene production in these systems [1]. When potato (*Solanum tuberosum* L. cv. Norland) plants are subjected to acute or chronic O_3 exposure, ACC levels in the tissue increase resulting in a stimulation of ethylene production [9, 24]. Large increases in ethylene resulting from acute or chronic O_3 exposures are coupled with large decreases in Rubisco mRNA prior to loss of Rubisco protein [9, 24].

In order to better understand the relationship between O_3-induced ethylene production and the loss in *rbcS* message in potato plants we isolated *OIP-1*, an O_3-induced ACC synthase cDNA in

potato (Fig. 1). It is now generally accepted that ACC synthase is encoded by a multigene family, and the ACC synthase polymorphism may be due to the evolution of a family of proteins with different enzymatic properties in order to efficiently utilize AdoMet in different tissues during plant development or in response to stress conditions [25]. We compared *OIP-1* with other ACC synthase amino acid sequences from the GenBank database using Clustal method with PAM 250 residue weight table and a MegAlign program (DNASTAR, Madison, WI). *OIP-1* has 43 to 93% homology at the amino acid level for ACC synthases found in other species reported thus far. The highest degree of homology (92%) was found with tomato ACC synthase cDNA clones *ptACC2* [25] and *pcVV4A* [29]. The *ptACC2* mRNA is expressed in ripening fruits and is induced by wounding of non-ripened fruits. The *pvVV4A* mRNA is expressed at low levels in ripening fruit; however, its expression is increased by wounding, thereby suggesting that the genes encoding these mRNAs are involved in stress-induced accelerated senescence. Interestingly, *OIP-1* shows a greater degree of homology to the deduced amino acid sequences of *ptACC2* [25] and *pvVV4A* [29] than the amino acid sequences encoded by 3 ACC synthase genes from potato recently reported (Van der Straeten, personal communication).

The active site for ACC synthase has been identified in all plant species studied [13]. The amino acid sequence of the dodecapeptide in *OIP-1* is SLSKMGLPGFR which is similar to other ACC synthase active sites reported [13]. It has been suggested that ACC synthase could be an aminotransferase since it contains 11 of 12 amino acid residues conserved in aminotransferases [10], *OIP-1* also contains all eleven amino acid residues found in other ACC synthases reported thus far (Fig. 1).

In this study we evaluated the effects of O_3 on ethylene, *OIP-1*, *rbcS* and *rbcL* over time during a 4 h exposure to O_3 and up to 4 h following removal. Acute O_3 exposure caused a rapid increase in the mRNA for ACC synthase resulting in high levels of ethylene. The increased levels of mRNA for ACC synthase and ethylene were sustained for the duration of the O_3 exposure (Fig. 3). Associated with the rapid increase in ACC synthase mRNA is a dramatic transitory reduction in *rbcS* mRNA previously shown by Reddy *et al.* [24]. The *rbcS* message is lower throughout O_3 exposure. Time periods when *rbcS* levels show the greatest reduction viz. 2, 3, and 4 h of O_3 exposure coincide with the times of greatest *OIP-1* expression. The magnitude of reduction in *rbcS* during this time interval varied between the three replicate samples and over the 3 h time period, but was always significantly lower than levels before or after exposure to O_3. When O_3 is removed there is a rapid decline in ACC synthase message which is associated with a parallel increase in *rbcS* mRNA, also shown by Reddy *et al.* [24]. The rapid increase/decrease in ACC synthase mRNA is in agreement with results previously reported in other test systems [13] and suggests a quick 'turn-on/turn-off' mechanism of induction and rapid turnover of ACC synthase induced by O_3. We are showing for the first time that the O_3-induced increase in ACC synthase message occur as a result of a change in transcription based on nuclear run-on studies (Fig. 5). The elevated levels of ethylene production were sustained somewhat longer than the message for ACC synthase. The extended emission of ethylene after mRNA for ACC synthase is no longer detectable which may reflect a saturation of the ACC oxidase present and build-up of ACC in the tissue. It has been shown that the ethylene biosynthetic inhibitor AVG has the ability to prevent O_3 induced damage in plants [17, 28]. In this study the loss of *rbcS* message was reduced when plants were treated with the ethylene biosynthetic inhibitor AOA prior to O_3 exposure (Fig. 4). Also, there was a substantial inhibition of ACC synthase message and ethylene, further showing a correlation between the amount of ethylene produced and *rbcS* mRNA loss. The implications of chronic O_3 exposure to emission of ethylene, reduction of *rbcS* message and accelerated foliar senescence is currently being studied in our laboratory [9].

Acknowledgements

Contribution No. 284, Dept. Horticulture, The Pennsylvania State University. This research was supported in part by EPRI contract RP-8011-01 and NRICGP 93-37100-8921. We are grateful to Ms Judith Sinn, Ms Jeannette Arteca and Mr Tzann-Wei Wang for technical assistance.

References

1. Abeles FB, Morgan PW, Saltveit Jr ME: Ethylene in Plant Biology, 2nd ed. Academic Press, San Diego, CA (1992).
2. Arteca RN: Stimulation of auxin induced ethylene production in mung bean hypocotyl segments by 5,5'-dithiobis-2-nitrobenzoic acid. Plant Cell Physiol 23: 309–314 (1982).
3. Botella JR, Arteca JM, Schlagnhaufer CD, Arteca RN, Phillips AT: Identification and characterization of a full length cDNA encoding for an auxin-induced 1-aminocyclopropane-1-carboxylate synthase from etiolated mung bean hypocotyl segments and expression of its mRNA in response to indole-3-acetic acid. Plant Mol Biol 20: 425–436 (1992).
4. Botella JR, Arteca JM, Schlagnhaufer CD, Arteca RN, Phillips AT: Identification of two new members of the 1-aminocyclopropane-1-carboxylate synthase-encoding multigene family in mung bean. Gene 123: 249–253 (1993).
5. Chirgwin JM, Przbyla AE, MacDonald RJ, Rutter WJ: Isolation of biologically active ribunocleic acid from sources enriched in ribunocleases. Biochemistry 18: 5294 (1979).
6. Dann MS, Pell EJ: Decline of activity and quantity of ribulose bisphosphate carboxylate/oxygenase and net photosynthesis in ozone-treated potato foliage. Plant Physiol 91: 427–432 (1989).
7. Enyedi AJ, Pell EJ: Comparison of the *rbcL* gene of two potato cultivars with differential sensitivity to ozone. Plant Physiol 99: 356–358 (1992).
8. Enyedi AJ, Eckardt NA, Pell EJ: Activity of ribulose bisphosphate carboxylate/ oxygenase from potato cultivars with differential response to ozone stress. New Phytol 122: 493–500 (1992).
9. Glick RE, Schlagnhaufer CD, Arteca RN, Pell EJ: Loss of *rbcS* mRNA induced by ozone is correlated with ethylene production. Plant Physiol 105: 107 (1994).
10. Huang PL, Parks JE, Rottmann WH, Theologis A: Two genes encoding 1-aminocyclopropane-1-carboxylate synthase in zucchini (*Cucurbita pepo*) are clustered and similar but differentially regulated. Proc Natl Acad Sci USA 88: 7021–7025 (1991).
11. Jorgenson RA, Cuellar RE, Thompson WF: Modes and tempos in the evolution of nuclear encoded ribosomal RNA genes in legumes. Carnegie Inst Wash Yearb 81: 98–101 (1982).
12. Kangasjarvi J, Talvinen J, Utrianen M, Karjalainen R: Plant defense systems induced by ozone. Plant Cell Environ 17: 783–794 (1994).
13. Kende H: Ethylene biosynthesis. Annu Rev Plant Physiol Plant Mol Biol 44: 283–307 (1993).
14. Lanahan MB, Yen HC, Giovannoni JJ, Klee HJ: The *neverripe* mutation blocks ethylene perception in tomato. Plant Cell 6: 521–530 (1994).
15. Langebartels C, Kerner K, Leonardi S, Schraudner M, Trost M, Heller W, Sandermann H Jr: Biochemical plant response to ozone. I. Differential induction of polyamine and ethylene biosynthesis in tobacco. Plant Physiol 95: 882–889 (1991).
16. Liang X, Abel S, Keller JA, Shen NF, Theologis A: The 1-aminocyclopropane-1-carboxylate synthase gene family of *Arabidopsis thaliana*. Proc Natl Acad Sci USA 89: 11046–11050 (1992).
17. Melhorn H, Wellburn AR: Stress ethylene formation determines plant sensitivity to O_3. Nature 327: 417–418 (1987).
18. Nooden LD: The phenomena of senescence and aging. In: Schulte-Hostede S, Darrall NM, Blank LW, Wellburn AR (eds) Air Pollution and Plant Metabolism pp. 1–50. Elsevier Applied Science, London (1988).
19. Oeller PW, Min-Wong L, Taylor LP, Pike DA, Theologis A: Reversible inhibition of tomato fruit senescence by antisense RNA. Science 254: 437–439 (1991).
20. Picton S, Barton S, Bouzayen M, Hamilton A, Grierson D: Altered fruit ripening and leaf senescence in tomatoes expressing an antisense ethylene-forming enzyme transgene. Plant J 3: 469–481 (1993).
21. Pell EJ: Secondary metabolism and air pollutants. In: Schulte-Hostede S, Darrall NM, Blank LW, Wellburn AR (eds) Air Pollution and Plant Metabolism, pp. 189–205. Elsevier Applied Science, London (1988).
22. Pell EJ, Dann MS: Multiple stress-induced foliar senescence and implications for whole-plant longevity. In: Mooney HA, Winner WE, Pell EJ (eds) Response of Plants to Multiple Stresses, pp. 189–205. Academic Press, San Diego, CA (1991).
23. Pell EJ, Eckardt NA, Glick RE: Biochemical and molecular basis for impairment of photosynthetic potential. Photosyn res 39: 453–462 (1994).
24. Reddy GN, Arteca RN, Dai Y-R, Flores HE, Negm FB, Pell EJ: Changes in ethylene and polyamines in relation to mRNA levels of the large and small subunits of ribulose bisphosphate carboxylate/oxygenase in ozone-stressed potato foliage. Plant Cell Environ 16: 818–826 (1993).
25. Rottmann WR, Peter GF, Oeller PW, Keller JA, Shen NF, Nagy BP, Taylor LP, Campbell AD, Theologis A: 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 (1991).
26. Sanger F, Nicklen S, Coulson AR: DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74: 5463–5467 (1977).
27. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).
28. Taylor GE Jr, Ross-Todd BM, Gunderson CA: Action of ozone on foliar gas exchange in *Glycine max* L. Merr: a potential role for endogenous stress ethylene. New Phytol 110: 301–307 (1988).
29. Van der Straeten D, Van Wiemeersch L, Goodman HM, Van Montagu M: Cloning and sequence of two different cDNAs encoding 1-aminocyclopropane-1-carboxylate synthase in tomato. Proc Natl Acad Sci USA 87: 4859–4863 (1990).
30. Van der Straeten D, Rodrigues-Pousada RA, Villarroel R, Hanley S, Goodman HM, Van Montagu M: Cloning, genetic mapping and expression analysis of an *Arabidopsis thaliana* gene that encodes 1-aminocyclopropane-1-carboxylate synthase. Proc Natl Acad Sci USA 89: 9969–9973 (1992).
31. Wanner LA, Gruissem W: Expression of the tomato rbcS gene family during development. Plant Cell 3: 1289–1303 (1991).
32. Wolter FP, Fritz CC, Willmitzer L, Schell J, Schreier PH: rbcS genes in *Solanum tuberosum*: conservation of transit peptide and exon shuffling during evolution. Proc Natl Acad Sci USA 85: 846–850 (1988).
33. Yang SF, Hoffman NE: Ethylene biosynthesis and its regulation in higher plants. Annu Rev Plant Physiol 35: 155–189 (1984).
34. Zarembinski TI, Theologis A: Anaerobiosis and plant growth hormones induced two genes encoding 1-aminocyclopropane-1-carboxylate synthase in rice (*Oryza sativa* L.). Mol Biol Cell 4: 363–373 (1993).