Expression of 1-Aminocyclopropane-1-Carboxylate Oxidase during Leaf Ontogeny in White Clover

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We examined the expression of three distinct 1-aminocyclopropane-1-carboxylic acid oxidase genes during leaf ontogeny in white clover (Trifolium repens). Significant production of ethylene occurs at the apex, in newly initiated leaves, and in senescent leaf tissue. We used a combination of reverse transcriptase-polymerase chain reaction and 3′-rapid amplification of cDNA ends to identify three distinct DNA sequences designated TRACO1, TRACO2, and TRACO3, each with homology to 1-aminocyclopropane-1-carboxylic acid oxidase. Southern analysis confirmed that these sequences represent three distinct genes. Northern analysis revealed that TRACO1 is expressed specifically in the apex and TRACO2 is expressed in the apex and in developing and mature green leaves, with maximum expression in developing leaf tissue. The third gene, TRACO3, is expressed in senescent leaf tissue. Antibodies were raised to each gene product expressed in Escherichia coli, and western analysis showed that the TRACO1 antibody recognizes a protein of approximately 205 kD (as determined by gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis) that is expressed preferentially in apical tissue. The TRACO2 antibody recognizes a protein of approximately 36.4 kD (as determined by gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis) that is expressed in the apex and in developing and mature green leaves, with maximum expression in mature green tissue. No protein recognition by the TRACO3 antibody could be detected in senescent tissue or at any other stage of leaf development.

The plant hormone ethylene is an important regulator of several physiological processes in higher plants (Abeles et al., 1992) and also functions as a mediator of responses to external stimuli, such as wounding, flooding, and pathogen invasion (Kende, 1993). The biosynthetic pathway of the hormone in higher plants has now been characterized (Adams and Yang, 1979) and two committed enzymes in the pathway, ACC synthase (EC 4.4.1.14) and ACC oxidase (EC 1.4.3), have been identified (Yang and Hoffman, 1984; Theologis, 1992; Kende, 1993). ACC synthase is recognized as the rate-determining step in the ethylene-biosynthesis pathway, and many inducers are proposed to act by stimulation of this enzyme (Yang and Hoffman, 1984; Theologis, 1992; Kende, 1993). The enzyme is known to be coded for by a multigene family in several plant species, with many of these genes cloned from a wide variety of tissues and in response to a variety of stimuli (Flurh and Mattoo, 1996).

In contrast, the ability of most plant tissues to convert ACC to ethylene was interpreted originally as evidence that the regulation of ACC oxidase is not a major control point of ethylene biosynthesis (Yang and Hoffman, 1984). More recently, two significant advances have provided the foundation for more detailed biochemical analysis of the enzyme. The first was the expression of a cDNA, designated pTOM13, coding for the putative ethylene-forming enzyme (Hamilton et al., 1990) in yeast (Hamilton et al., 1991), and another highly homologous cDNA, pHPTOM5, in Xenopus laevis oocytes (Spanu et al., 1991). In both of these studies the transformants could convert ACC to ethylene and the trans isomer of the ACC analog 1-amino-2-ethylcyclopropane-1-carboxylic acid to ethylene in preference to the cis isomer.

The second significant advance was the demonstration that, because the amino acid sequence of pTOM 13 is similar to the enzyme flavanone 3-hydroxylase, complete recovery of enzyme activity in vitro from melon fruit could be achieved using factors shown to preserve the activity of the hydroxylase (Ververidis and John, 1991). ACC oxidase has now been purified to homogeneity and characterized from apple fruits (Dong et al., 1992; Dupille et al., 1993; Pirrung et al., 1993) and partially purified and characterized from a range of tissues, including apple (Fernandez-Maculet and Yang, 1992; Kuai and Dilley, 1992), avocado (McGarvey and Christoffersen, 1992), pear (Vioque and Castellano, 1994), and citrus (Dupille and Zacarias, 1996).

In concert with these biochemical studies, genes coding for ethylene have now been cloned from a wide variety of tissues in many plants (Flurh and Mattoo, 1996). Of particular significance is the view that the expression of the ACC oxidase gene family, like that of ACC synthase genes, is highly regulated in plants and constitutes an extra tier of control of ethylene biosynthesis. Differential expression of ACC oxidase genes has been observed in orchid flowers (Nadeau et al., 1993), mung bean epicotyls (Kim and Yang, 1994), petunia floral tissues (Tang et al., 1994; Tang and Woodson, 1996), broccoli floral tissue (Pogson et al., 1995), tomato (Barry et al., 1996) and melon leaf tissues (Lasserre

Abbreviations: RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; UTR, untranslated region.

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et al., 1996, 1997), carnation floral tissues (ten Have and Woltering, 1997), sunflower seedling tissue (Liu et al., 1997), geranium floral tissue (Clark et al., 1997), and leaf tissue of Nicotiana glutinosa (Kim et al., 1998). However, compared with fruit and floral tissue, fewer studies have been undertaken of the regulation of ACC oxidase gene expression during leaf development (John et al., 1995; Barry et al., 1996; Lasserre et al., 1996; Bouquin et al., 1997; Kim et al., 1998), even though ethylene is considered an important regulator of leaf ontogeny in higher plants (Osborne, 1991).

In this study we used the stoloniferous growth habit of white clover (Trifolium repens) as a system to provide leaf tissue at all developmental stages in a perfectly replicated fashion. Three distinct DNA sequences with homology to ACC oxidase were generated using RT-PCR and 3’ RACE, and Southern analysis confirmed that these sequences are complementary to distinct genes. We show here that these three genes are differentially expressed during leaf ontogeny in white clover. We also report the expression of these genes with respect to ethylene evolution, ACC oxidase enzyme activity, and protein accumulation and then interpret the results as further evidence for the highly coordinated regulation of ACC oxidase expression in plants.

**MATERIALS AND METHODS**

**Plant Material and Tissue Sampling**

Stock plants of white clover (Trifolium repens L. genotype 10F; AgResearch Grasslands, New Zealand) were grown under natural light in 8-L planter bags in a greenhouse maintained at a minimum temperature of 18°C (day) and 12°C (night) and vented at 25°C (day) and 18°C (night). Apical cuttings with two or three nodes were taken from these stock plants, and all leaves were excised at the petiole/stolon junction except the youngest fully emerged leaf. The cuttings were placed with the basal node buried in a bark/pumice potting mix, and as the stolon grew, the apex was trained to direct growth over a dry matrix provided by white polythene sheeting. At weekly intervals, any outgrowths from the axillary buds were excised to maintain a single (unbranched) stolon. Once we achieved a consistent program of leaf development from initiation at the apex to senescence over 16 to 20 nodes, we harvested the leaf tissue. For tissue-sampling purposes, leaf 1 was designated as the first (unfolded) leaf protruding clearly from its sheath, and the apex was defined as all tissue distal to the leaf 1 node.

**Determination of Leaf Chlorophyll**

Chlorophyll determinations were made essentially using the method of Moran and Porath (1980). Up to 400 mg of freshly harvested leaf material was immersed in 5 mL of cold (4°C) N,N-dimethylformamide, the mixture was incubated in darkness at 4°C for 48 h, and the A664.5 and A647 of the extractant were determined. We calculated the chlorophyll concentrations as described by Insk bee and Bloom (1985).

**Measurements of Ethylene Evolution**

Individual attached leaves ranging from nodes 2 to 16 were enclosed in 30-mL plastic containers with a slot cut to accommodate the petiole. Apex and leaf 1 tissues were enclosed in a 16.5-mL plastic container. The petiole (or stolon for the apex) was held in place with petroleum jelly, the containers were sealed, and after 1 h, a 1-mL gas sample was removed and the concentration of ethylene measured using a gas chromatograph (model 10570+, Photovac, Markham, Ontario, Canada). We used the method of Lizada and Yang (1979) to measure the ACC content.

**Nucleic Acid Isolations**

Genomic DNA was isolated from leaf tissue using a method modified from that of Junghans and Metzlaff (1990). Leaf tissue from node 3 or 4 (1.6 g) was ground to a fine powder in liquid nitrogen and added to 10 mL of lysis buffer (50 mM Tris-HCl, pH 7.6, containing 100 mM NaCl, 50 mM EDTA, and 0.5% SDS) and 10 mL of 100 mM Tris-buffered phenol, pH 8.0. After the sample was shaken vigorously, 5 mL of chloroform:isoamyl alcohol (24:1) was added, the mixture was shaken further, the cell debris was pelleted by centrifugation at 10,000g for 10 min at 4°C, and the supernatant was extracted again with phenol and chloroform:isoamyl alcohol. The aqueous phase was obtained by centrifugation at 10,000g for 10 min at 4°C; 0.67 volume of isopropanol was added to precipitate the nucleic acids; the precipitate was collected by centrifugation at 10,000g for 10 min at 4°C; and the pellet was washed with 80% (v/v) ethanol and then dried at 40°C for 5 min. The pellet was resuspended in 4 mL of 10 mg mL−1 RNase in 10 mM Tris-HCl, pH 8.0, containing 100 mM NaCl and 1 mM EDTA, and incubated at 37°C for 25 min before extraction with 2 mL of phenol and 2 mL of chloroform:isoamyl alcohol. The aqueous fraction was obtained by centrifugation at 10,000g for 10 min at 4°C; the nucleic acids were precipitated with 2.5 volumes of 99.7% (v/v) ethanol and then dried at 40°C for 5 min. The precipitate was collected by centrifugation at 10,000g for 10 min at 4°C; and the supernatant was extracted again with phenol and chloroform:isoamyl alcohol. The aqueous phase was obtained by centrifugation at 10,000g for 10 min at 4°C; the nucleic acids were precipitated with 2.5 volumes of 99.7% (v/v) ethanol; the precipitate was collected by centrifugation at 10,000g for 10 min at 4°C; and the pellet was washed with 80% (v/v) ethanol, dried, and then resuspended in 500 μL of sterile ultrapure water (Milli-Q, Millipore). A260 was used to quantify DNA.

RNA was extracted essentially as described by Van Slogteren et al. (1983). Frozen tissue was powdered under liquid nitrogen and added to 5 volumes of 100 mM Tris-HCl, pH 8.0, containing 100 mM LiCl, 10 mM EDTA, and 1% (w/v) SDS made up as a 1:1 ratio with 100 mM Tris-buffered phenol, pH 8.0. After vortexing and the addition of 2.5 volumes of chloroform/isoamyl alcohol (24:1), the extract was incubated at 50°C for 15 min. After centrifugation at 5000g for 30 min at 4°C, RNA was precipitated from the supernatant by overnight incubation in a final concentration of 2 M LiCl at 4°C. The RNA was collected by centrifugation as described before, extracted once more with chloroform:isoamyl alcohol, and ethanol precipitated, and the pellet was dried and then resuspended in water. To isolate poly(A⁺) mRNA, the PolyATract system (Promega) was used according to the manufacturer’s instructions. A260 was used to quantify poly(A⁺) mRNA.
RT-PCR Amplification and 3’ RACE

To amplify putative ACC oxidase-coding sequences, first-strand cDNA synthesis using total RNA isolated from apical and leaf tissues was performed with RT (Superscript RNase-H, Boehringer Mannheim) and a 17-mer oligo(dT)-primer, according to the instructions supplied with the product. PCR amplification was achieved using a high-fidelity system (Expand, Boehringer Mannheim) with one round of 30 cycles (1 min at 92°C, 1 min at 42°C, 1 min at 72°C, with a final extension for 10 min at 72°C) using a thermal cycler (PTC-200, MJ Research, Watertown, MA) and ACOF1 and ACOR1 as primers (Table I). One-hundred microliters was used as the reaction volume and, at the conclusion of the first round, 1.0 µL was removed and used as template in a second PCR round with identical amplification conditions but using ACOF2 and ACOR2 as primers (Table I). PCR-generated sequences were T/A cloned into pCRII vectors (Invitrogen, San Diego, CA) and sequenced using an automated DNA sequencer (model 377, PRISM, Applied Biosystems). The database was searched using Blast-N (Altschul et al., 1990). Sequence alignments (Align-Plus, Science & Educational Software, Durham, NC) compared putative ACC oxidase sequences generated by RT-PCR.

To amplify the 3’ UTRs from each distinct ACC oxidase gene identified after RT-PCR of the coding region (described above), first-strand cDNA synthesis was done as described previously but using an ADAP primer (Table I) with a 20-mer oligo(dT)-extension. PCR amplification was first used to clone the 3’ UTR with a portion of the reading frame so that the corresponding 3’ UTR could be identified. PCR amplification conditions were as described previously, except for an annealing temperature of 55°C. One or two rounds of PCR amplification were performed with primers specific for each ACC oxidase gene (Table I). PCR-generated sequences were cloned and sequenced as described above. To obtain 3’-UTR-specific sequences, one round of PCR amplification was performed (annealing at 55°C) using each reading frame 3’-UTR sequence cloned in pCRII as a template and using the primers specific for each ACC oxidase gene listed in Table I.

Sequence Phylogeny

A majority-rule consensus tree (neighbor-joining/observed-distances/100-bootstraps) was built using the computer program PAUP (Phylogenetic Analysis Using Parsimony, version 4.0, Sinaur Associates, Sunderland, MA). Sequences used to construct the tree are identified by accession number in the legend to Figure 6.

Southern and Northern Analyses

For genomic Southern analysis, DNA (20-µg aliquots) was first digested with 100 units of HindIII, EcoRI, or XbaI

<table>
<thead>
<tr>
<th>Table 1. Primer sequences for the amplification of the coding and 3’ UTRs of TRACO1, TRACO2, and TRACO3</th>
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<tbody>
<tr>
<td><strong>Gene</strong></td>
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<td>---------------------------------------------------------------</td>
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<tr>
<td><strong>Part 1 (Primers used for amplification of coding region)</strong></td>
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<td>First round</td>
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<td>Second round</td>
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<tr>
<td><strong>Part 2 (Primers used for amplification of each coding region and 3’ UTR)</strong></td>
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<tr>
<td>First round</td>
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<td>Second round</td>
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<td><strong>Part 3 (Primers used for amplification of gene-specific 3’ UTRs)</strong></td>
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<td>First round</td>
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<tr>
<td><strong>Part 4 (Primer used on the complementary strand for amplification of the coding region and 3’-UTR amplification and the gene-specific 3’ UTR)</strong></td>
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</table>
in a total volume of 200 μL for 16 h at 37°C, at which time another 50 units was added and the digestion was continued for another 4 h. The digested DNA was precipitated with the addition of 30 μL of 8 M ammonium sulfate and 1 mL of ethanol and incubated for 15 min at −80°C. The precipitated DNA was collected by centrifugation at 13,000g for 15 min at 4°C. The pellet was washed with 75% (v/v) ethanol, dried, and resuspended in 20 μL of gel-loading buffer (0.2% [w/v] SDS, 10% [v/v] glycerol, 0.01% [w/v] bromphenol blue, and 0.02 μL EDTA, pH 8.0). The digested DNA was separated through a 0.8% (w/v) agarose gel with TAE (40 μm Tris-acetate and 2 mm EDTA, pH 8.0) as a running buffer for 4 h at 100 V, stained in 1% (w/v) ethidium bromide for 20 min, depurinated in 0.25 M HCl for 30 min, denatured in 0.4 M NaOH and 3 M NaCl for 1 h, and then washed in transfer buffer (8 μM NaOH and 3 M NaCl) for 15 min.

The DNA was transferred onto a nylon membrane (Hybond N+, Amersham) for 16 h using the downward alkaline-cappillary method of Chomczynski (1992). After transfer, the DNA was cross-linked onto the membrane with a UV cross-linker (Stratalinker 2400, Stratagene), and the membrane was neutralized in 50 μM sodium phosphate buffer, pH 7.5. DNA probes (3'-UTR sequences) were produced by PCR amplification as described previously, and each fragment was randomly labeled with [α-32P] dATP using a DNA-labeling system (Megaprime, Amersham) according to the manufacturer’s instructions. After purification through microcolumns (ProbeQuant G-50, Amersham), the denatured labeled probes were added to membranes bathed in hybridization solution (0.25 M sodium phosphate, pH 7.2, 7% [w/v] SDS, 1% [w/v] BSA, and 1 mm EDTA, pH 8.0; Church and Gilbert, 1984) and hybridized for 16 h at 65°C. Membranes were washed at 65°C for 20 min in 20 μL sodium phosphate, pH 7.2, 0.5% (w/v) SDS, 0.5% (w/v) BSA, and 1 mm EDTA, pH 8.0, and then for 20 min in 2× SSPE (0.36 μM NaCl, 20 mm sodium phosphate, and 2 mm EDTA) and 0.1% (w/v) SDS, then for 20 min in 0.2× SSPE and 0.1% (w/v) SDS, and finally for 30 to 60 min in 0.1× SSPE and 0.1% (w/v) SDS. After washing, membranes were exposed to Kodak XAR-5 film at −70°C.

Each 3’ UTR was also subjected to Southern analysis. Fifty nanograms of each sequence was separated for 90 min through a 1.2% (w/v) agarose gel and then immediately transferred to a nylon membrane for 4 h, and the membrane was probed and washed.

For northern analysis, 1.0 μg of poly(A+) mRNA was denatured in 2.2 M formaldehyde by heating at 70°C for 10 min and then cooling on ice; the denatured RNA was separated for 5 h at 80 V through a 1.0% (w/v) agarose gel, using 20 mm Mops, 50 mm sodium acetate, 10 mm EDTA, and 2.23 M formaldehyde as the running buffer. At the conclusion of electrophoresis, the separated RNA was transferred to a nylon membrane for 4 h, and the membrane was again probed and washed.

Antibody Production and Western Analysis

The reading frame of the three TRACO sequences was obtained by PCR amplification using two primers: 5’-

GGATTCAAGCNTGYSANAAYTGGGGH-3’ to provide a 5’-EcoRI site and 5’-GGCAAGCTTCTCGAGATCG-3’ to provide a 3’-HindIII site. The PCR-generated fragment was then directionally cloned into the EcoRI/HindIII polylinker sites on the expression vector pPROEX-1 (Life Technologies), and the construct was transformed into the Escherichia coli strain TB-1 (Life Technologies). Induction with isopropylthio-β-galactoside and purification of the recombinant protein using nickel-based affinity chromatography was according to the protocol from the manufacturer (Life Technologies). Amino acid sequencing of trypsin-generated fragments, as described by Watson et al. (1998) confirmed that the amino acid sequence of the recombinant protein matched the translated sequence of each reading frame, and SDS-PAGE confirmed that the recombinant proteins were the size calculated from the translated sequence (data not shown).

To produce antibodies, 250 μg of the TRACO2 recombinant protein (for rabbits) and 100 μg of the TRACO1 and TRACO3 recombinant proteins (for rats) were dissolved in PBS, emulsified with an equal volume of Freund’s complete adjuvant, and immunized through several sites on each animal’s back. Booster injections with the protein emulsified in Freund’s incomplete adjuvant were administered at monthly intervals over 3 months.

SDS-PAGE was carried out essentially as described by Laemmli (1970). Western blotting and antibody detection were achieved using the method of Towbin et al. (1979) as described by McManus et al. (1994). Whole sera from both rat and rabbit were used at a dilution of 1:1000, with alkaline phosphatase-linked secondary antibodies used for primary antibody detection. The intensity of recognition was determined using image analysis. To achieve this, the developed membranes were imaged using a videocamera (model DXC-3000P, Sony, Tokyo), and the image was captured on a personal computer using a color frame grabber (Visionplus, Imaging Technology, Inc., Bedford, MA). Two images were captured for each sample, one of the membrane and one of the background baseboard. The background image was smoothed using a 25- × 25-pixel moving average, and for each band of product deposition the maximum pixel and two minimum values were found. The minima mark the front and back edges of the band and the pixel values between front and back are summed to give the integrated density.

To determine the molecular mass of the immune-recognized proteins, 8% to 15% or 10% to 20% polyacrylamide gradient gels were used with the method outlined by Hames and Rickwood (1981).

ACC Oxidase Enzyme Assays

Leaf tissue, previously frozen in liquid nitrogen and powdered, was extracted in 5 volumes of ice-cold 100 mm Tris-HCl, pH 7.5 containing 10% (v/v) glycerol, 2 mm DTT, and 30 mm ascorbate. The slurry was filtered through Miracloth (Calbiochem) and centrifuged at 12,000g for 15 min at 4°C. Solid ammonium sulfate was added to the supernatant to give a final saturation of 30%, and the salt was dissolved while keeping the mixture ice-cold (approxi-
imately 30 min). After the sample was centrifuged at 12,000g for 10 min at 4°C, solid ammonium sulfate was added to the supernatant to give a final saturation of 90%, and the salt was dissolved while keeping the mixture ice-cold (approximately 60 min). The precipitated protein was then collected by centrifugation at 12,000g for 15 min at 4°C. The pellet was dissolved in 50 mM Tris-HCl, pH 7.5, containing 2 mM DTT, 30 mM ascorbate, and 10% (v/v) glycerol, and the solution was desalted using Sephadex G-25 (Amersham) equilibrated in the same buffer.

The protein concentration in these extracts was measured using the method of Bradford (1976). To measure ACC oxidase activity, 0.2-mL aliquots of the enzyme preparation were warmed to 30°C, mixed with 0.8 mL of pre-equilibrated (30°C) reaction buffer in 4.5-mL-capacity vacuum tubes (Vacutainer, Becton-Dickinson) to give a final concentration of 50 mM Tris-HCl, pH 7.5, containing 1 mM ACC, 10% (v/v) glycerol, 2 mM DTT, 30 mM ascorbate, 50 µM FeSO₄, and 30 mM NaHCO₃. The tubes were sealed and shaken at 30°C for (usually) 20 min, and then 1.0 mL of the gas phase was removed and the ethylene content was determined with a gas chromatograph (model GC-8A, Shimadzu, Kyoto, Japan).

RESULTS
Changes in Chlorophyll Content, Ethylene Evolution, and ACC Content during Leaf Development

Rooting at a single node and the subsequent outgrowth of the stolon over a dry matrix produces the full program of leaf development along the stolon from initiation at the apex through maturation, senescence, and necrosis (Fig. 1A). It is known that, if the root primordium at a node is in contact with a moist substratum, it will grow out as a nodal root (Thomas, 1987). In the field, this occurrence has a large impact on development in the white clover plant, with the natural senescence of old sections of the stolon proceeding in waves that are interrupted by the presence of strongly rooted nodes (Sackville-Hamilton and Harper, 1989). The use of this growth system, in which root initiation is inhibited, produces plants in which the number of leaves attached to the stolon reaches a constant number as the production rate is balanced by the senescence rate.

As an indicator of leaf maturity, total chlorophyll (chlorophyll a and b) content was determined for each leaf (Fig. 1B). In the example shown, an increase in chlorophyll content was observed in leaves excised from the apex to the

Figure 1. A, Stages of leaf development along a single stolon of white clover. B, Total chlorophyll (●), ethylene evolution (○), and ACC content (▲) determined from leaves excised from single stolons identical to that shown in A. Results are mean values ± se; n = 5. FW, Fresh weight.
node to leaf 3 (the developing leaves), and then a constant (maximum) content was observed in leaves excised from nodes 4 to 9 (leaves 4–9, mature green leaf tissue). In leaves excised from nodes 10 to 16 (leaves 10–16), chlorophyll content decreased, causing green/yellow and then yellow leaf tissue. No measurements were made from desiccated yellow or clearly necrotic tissues.

In vivo, two stages of significant ethylene production were observed (Fig. 1B). The first was observed at the apex, which failed to reach a minimum value by leaf 3. Minimum evolution of ethylene was observed from leaves 4 to 10, broadly coinciding with the mature-green leaf stage, after which a second stage of significant ethylene evolution was observed. Here the rate of ethylene production gradually increased to leaf 16 (Fig. 1B) and decreased again only in necrotic tissue (data not shown). ACC content remained constant in developing and mature-green tissues and then increased in senescent tissue (leaves 11–16, Fig. 1B), in concert with significant senescence-associated ethylene production.

Isolation of TRACO1, TRACO2, and TRACO3 Gene Sequences

RT-PCR was used to amplify approximately 800-bp cDNA sequences from the apex, leaf 4 (mature green), leaf 9 (onset of senescence), and leaf 14 (senescent) using primers designed to conserve domains within ACC oxidase genes sequenced from several other plant species. Sequencing of clones from each tissue revealed three distinct sequences with homology to other ACC oxidase genes in the database, and these were designated TRACO1, TRACO2, and TRACO3. To produce gene-specific probes, 3'RACE was used to amplify the 3'-UTR sequences, since significant sequence diversity in ACC oxidase genes occurs within this UTR (Fluhr and Mattoo, 1996).

The deduced amino acid sequences from the three ACC oxidase genes amplified from white clover are shown as a comparison with a consensus sequence devised from 23 other ACC oxidases (Kadyrzhanova et al., 1997) (Fig. 2). Comparison of the RT-PCR-generated sequences with the consensus revealed that the translated white clover sequence makes up the majority of the reading frame. The amino acid sequence is complete to the carboxy terminus but begins at the conserved Ala, 27 amino acids downstream of the initial Met.

The homology comparison at the nucleotide level of the three ACC oxidase DNA sequences in white clover leaf tissue is shown in Table II, with homology values ranging from 75% to 84% for the regions of the coding frame amplified by RT-PCR. Comparison of the 3' UTRs revealed much greater sequence divergence within the 3' UTR. The nucleotide sequences corresponding to each 3' UTR from the three ACC oxidase DNA sequences from white clover were also compared (Fig. 3). In TRACO1 and TRACO2, single near-upstream elements and far-upstream elements could be identified, whereas two elements of each are observed in TRACO3. Near-upstream elements and far-upstream elements are proposed to direct processing at the

Table II. Nucleotide homology values between reading frame ACO sequences from white clover generated by RT-PCR and the corresponding 3' UTRs generated by 3'RACE

<table>
<thead>
<tr>
<th>Reading Frame</th>
<th>TRACO1</th>
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<th>TRACO3</th>
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<td>84</td>
<td>—</td>
<td>55</td>
<td>59</td>
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</table>

Figure 2. Alignment of the deduced ACC oxidase amino acid sequences from white clover with a consensus (CONS.) ACC oxidase sequence compiled by Kadyrzhanova et al. (1997). Uppercase letters in the consensus sequence indicate complete agreement within the 23 ACC oxidases compared, and lowercase letters represent the most frequently occurring residue. Regions of complete alignment of the white clover sequences with the consensus sequence are boxed.
poly(A⁻) site situated near the near-upstream element motif (Rothnie, 1996). The occurrence of more than one near- and far-upstream element motif in the 3' UTR of TRACO3 suggests that differential processing of TRACO3 mRNA may occur. Furthermore, during the course of PCR of the 3' UTR regions of TRACO3, two additional truncated sequences (indicated by arrows in Fig. 3), each with an attendant poly(A⁻) tail, were amplified (data not shown). These truncated forms were not observed during the PCR amplification of the 3' UTR regions from TRACO1 and TRACO2.

The specificity of each (full-length) 3'-UTR sequence as a probe for use in molecular analysis was confirmed by Southern analysis (Fig. 4). Each 3' UTR probe hybridized only to its corresponding sequence under the hybridization and washing conditions used.

Genomic Southern analysis was used to confirm that each 3'-UTR sequence was complementary to distinct genes (Fig. 5). Genomic DNA was digested with EcoRI, HindIII, and XbaI, and the hybridization pattern was compared using each 3' UTR as a probe. Markedly different hybridization patterns were observed for each probe, confirming that each of the ACC oxidase DNA sequences generated from white clover by RT-PCR and 3' RACE are complementary to distinct genes. White clover is an allotetraploid, comprising two distinct diploid genomes (Williams, 1987). Therefore, multiple banding patterns can arise through polymorphisms at alleles on one or both genomes.

**Phylogenetic Comparison of White Clover ACC Oxidase Sequences**

A phylogenetic tree was reconstructed from an alignment of the deduced amino acid sequence from the white clover ACC oxidases with other ACC oxidases in the database (Fig. 6). TRACO2, ACO1 from bean (PV, accession no. AF053354), and TRACO3 clustered together strongly, with the closest relationship between TRACO3 and the bean ACO1. Other leaf-senescence-associated ACC oxi-
expression of TRACO1, TRACO2, and TRACO3 during Leaf Development

Gene-specific probes comprising the 3' UTRs were used in northern analysis to determine the constitutive expression of each ACC oxidase gene (Fig. 7). TRACO1 is expressed almost exclusively in the apex, with a much lower intensity of hybridization discernible in leaf 1 and no detectable hybridization (using this method) in leaf 2 or any other leaf along the stolon. TRACO2 is detectable in the apex, shows maximal expression in leaves 1 to 2, and then gradually decreases in intensity such that no discernible expression can be observed by leaf 11. The expression of TRACO3 is clearly detectable first in leaf 8, reaching maximum expression in leaves 13 to 16. The expression of TRACO1, TRACO2, and TRACO3 in leaves excised from nodes older than 16 was not undertaken.

Changes in ACO Enzyme Activity and Protein Accumulation during Leaf Development

In vitro, ACO enzyme activity was detected in the apex and increased to reach a maximum in leaves 4 to 9, after which the activity steadily declined (Fig. 8A). The pattern of ACO activity observed contrasts with the trend of ethylene evolution that is virtually undetectable in leaves 4 to 9 but then increases again (Fig. 1B).

Antibodies were produced to translated proteins from each ACO gene, and western analysis was used to determine the accumulation of each protein (Fig. 8B). Antibodies raised against TRACO1 recognized a protein of approximately 205 kD (as determined by gradient SDS-PAGE), the accumulation of which occurs predominantly in the apex, and then declines until it is undetectable (using this method) after leaf 8. Antibodies raised against TRACO2 recognized a protein of 36.4 kD (as determined by gradient SDS-PAGE), the accumulation of which broadly matched the detectable ACC oxidase activity observed in Figure 8A. The pattern of ACO accumulation detected by TRACO2 is seen most clearly using image analysis to quantify signal intensity (Fig. 8C).

Figure 6. Phylogenetic analysis of ACC oxidase amino acid sequences from white clover and other ACC oxidases in the database. A majority rule consensus tree (neighbor-joining/observed-distances/100-bootstrap) was reconstructed using the computer program PAUP. Bootstrap values are shown and the accession number of each sequence is provided in the parentheses. ● Leaf senescence-associated sequence. AT: Arabidopsis thaliana; CS1, 2, and 3: Cucumis sativus, Cs-ACO1, 2, and 3; PV: Phaseolus vulgaris; PHO1, 2, and 3: Pelargonium hortorum, ACO1, 2, and 3; HA1, 2, and 3: Helianthus annus, ACCO1, 2, and 3; NT: Nicotiana tabacum; MD: Malus domestica; PS: Pisum sativum; VR1 and 2: Vigna radiata; pVR-ACO1 and 2; AC: Actinidia chinensis; CM1, 2, and 3: Cucumis melo, CM-ACO1, 2, and 3; BP: Betula pendula; LE1, 2, and 3: Lycopersicon esculentum, ACO1, 2, and 3; PHY1, 3, and 4: Petunia hybrida, ACO1, 3, and 4; OS: Oryza sativa.

dases are indicated in Figure 6: LE1, ACO1 from tomato (Barry et al., 1996), and CM1, CM-ACO1 from melon (Lasserre et al., 1996). However, TRACO3 is more closely related to TRACO2 than to these other leaf-senescence-associated sequences. TRACO1 is most closely related to pPE8 (PS, accession no. P31239) from pea (Peck et al., 1993), and the nearest neighbor to this grouping is pVR-ACO1 from mung bean (VR1, accession no. U06046; Kim and Yang, 1994).
Antibodies raised against TRACO3 did not recognize any proteins in the leaf extracts examined (data not shown). Weak recognition by TRACO3 antibodies (determined by a significantly longer development time in an alkaline phosphatase substrate) was observed from a 36.4-kD protein that had the identical accumulation pattern as that observed when using antibodies raised against TRACO2 (data not shown). These observations suggest that the antibodies raised against TRACO2 and TRACO3 recognize the same protein, and each antibody recognizes the protein product of the other three ACO gene products obtained using expression vectors in E. coli.

**DISCUSSION**

In this study we have shown that three distinct ACC oxidase genes are expressed differentially during leaf ontogeny in white clover. The maximal expression of two of these genes coincides with the two peaks of ethylene evolution observed. The expression of TRACO1 is predominantly in the apex, whereas the expression of TRACO3 almost precisely matches the increase in ethylene evolution during leaf senescence.

Ethylene evolution from the apex has been reported in several plant species (for review, see Osborne, 1991), with some consensus that in dicotyledonous plants the role of the hormone is to limit cell expansion in younger leaves (Osborne, 1991; Kieber et al., 1993; Lee and Reid, 1996). However, to our knowledge, this is the first report of apex-specific ACC oxidase gene expression. A scanning electron microscope study of the apex of white clover revealed that it is a complex tissue comprising the apical meristem, leaf primordia, and an axillary bud at the third node (Thomas, 1987). As yet we cannot say which of these tissues that comprise the apex specifically express TRACO1, but tissue localization of expression using in situ hybridization should provide significant clues regarding the role of ethylene. The use of a phylogenetic tree has placed the apex-specific sequence closest to pPE8, a cDNA clone isolated from pea seedling shoot tissue (Peck et al., 1993). The next nearest neighbor was pVR-ACO1, which is constitutively expressed in hypocotyl, leaf, and stem tissues from mungbean seedlings (Kim and Yang, 1994). However, it was not reported in either study whether these sequences were expressed specifically in apical tissues.

Ethylene evolution from senescent leaves is now well documented, and senescent leaves of many species can convert ACC to ethylene (Roberts et al., 1985; Osborne, 1991). However, there are fewer reports of ACC oxidase gene expression during leaf senescence. In tomato leaves ethylene evolution increases as senescence proceeds, an increase that coincides with an increase in transcript accumulation of the ACC oxidase gene ACO1 (John et al., 1995).

More recently, a second ACO gene, ACO2, was shown to be expressed at the onset of leaf senescence (Barry et al., 1996). A similar pattern in which two genes of ACC oxidase are differentially expressed in mature-green and senescent leaf tissue was also reported for melon (Lasserre et al., 1997). The differential pattern of gene expression observed in white clover leaves is similar to these species. However, the construction of a phylogenetic tree has revealed that the white clover senescence-associated transcript (TRACO3) is more closely related to TRACO2 than sequences expressed in senescent leaf tissue from other species. TRACO3 is most closely related to PV-ACO1, a cDNA cloned from bean seedling tissues whose expression is regulated by light (Pidgeon et al., 1997); the authors did not report whether this sequence is also expressed in senescent tissue.

The examination of ACO gene expression has been extended in white clover with the measurement of corresponding ACC oxidase activity in vitro during leaf ontogeny. Detectable ACC oxidase activity coincides more closely with TRACO2 gene expression. Some ACO activity is observed in the apex, but in leaves from nodes 13 to 16, where the expression of TRACO3 is induced, there is no concomitant increase in detectable enzyme activity in vitro. Leaf tissues from many species have been shown to convert ACC to ethylene, evidence that an ACC-dependent ethylene-forming system is functional in vivo (Osborne, 1991). However, we are not aware of any studies in which ACO activity has been demonstrated in vitro in senescent leaf extracts.

![Figure 8](https://www.plantphysiol.org)
To determine the pattern of ACO protein accumulation (which may be independent of detectable enzyme activity), antibodies were raised to each gene product. In these experiments, the antibodies raised against TRACO2 identified an ACC oxidase protein of 36.4 kD, which is within the size range (35–41 kD) reported for ACC oxidase proteins from other plant species (Dong et al., 1992; Dupille et al., 1993; Pirrung et al., 1993; Rombaldi et al., 1994), and the relative accumulation of the protein determined by western blotting matched the measurable ACO activity in vitro. The TRACO1 antibody recognized a protein of approximately 205 kD that was expressed predominantly in the apex. Clearly, a protein of 205 kD cannot be transcribed from a 1.35-kb mRNA transcript, although we cannot exclude the possibility that the protein may be highly anomalous in terms of its migration using SDS-PAGE. However, the 205-kD protein does warrant further investigation because, although proteins of this size that exhibit ACC oxidase activity have not been characterized previously, the protein-accumulation pattern coincides with TRACO1 gene expression, and neither the serum raised against TRACO2 nor the one raised against TRACO3 recognized this protein. We are currently using immunoaffinity approaches to purify a sufficient quantity of this protein for further analysis.

In this study we were not able to demonstrate protein accumulation by antibody staining in the senescent leaf tissue that coincides with TRACO3 transcript accumulation. However, using hydrophobic and ion-exchange column chromatography, preliminary purification of ACO from senescent leaf tissue of white clover revealed a protein with ACO activity that was distinct from an isoform purified from mature-green leaf tissue (D. Gong and M.T. McManus, unpublished data). The apparent unmasking of significant activity in senescence extracts after hydrophobic column chromatography suggests that the activity of the enzyme may be regulated quite differently in senescent tissue. Although we have examined variables such as cofactor requirements and pH optima in senescent extracts and found no significant difference compared with mature-green extracts, such differential regulation of ACO activity has been reported from experiments with corn and sunflower seedlings, in which the occurrence of organ-specific enzymes with different substrate requirements has been demonstrated (Finlayson et al., 1997).

The results presented here add to the growing number of studies demonstrating that the expression of the ACC oxidase gene family is highly regulated during plant development. Furthermore, they show that the two peaks of ethylene production during leaf ontogeny coincide with the expression of distinct ACC oxidase genes. It has been shown that in bean ethylene can induce the differentiation of its own target cell class (McManus et al., 1998). Given that the ethylene produced at each leaf developmental stage in white clover induces separate responses (modulation of leaf growth in the apex and regulation of senescence in mature tissues), it is interesting to speculate further that regulation of the biosynthesis of ethylene may be intimately linked to its competence to respond to it. A study of the molecular basis for the control of transcription for each member, like the one done in melon (Lasserre et al., 1997), will play an important part in establishing such a link.

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