

Short chain oligogalacturonides induce ethylene production and expression of the gene encoding aminocyclopropane 1-carboxylic acid oxidase in tomato plants

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Oligogalacturonic acids (OGAs), derived from plant cell wall pectin, have been implicated in a number of signal transduction pathways involved in growth, development and defense responses of higher plants. This study investigates the size range of OGAs capable of inducing ethylene synthesis in tomato plants, and demonstrates that in contrast with many other effects, only short chain OGAs are active. Oligomers across a range of DP from 2–15 were separated and purified to homogeneity by QAE-Sephadex anion exchange chromatography using a novel elution system. The OGAs were applied to tomato plants and assayed for their ability to induce ethylene gas release and changes in steady state levels of mRNA encoding the ethylene forming enzyme aminocyclopropane-1-carboxylic acid oxidase (ACO). The study demonstrated that only OGAs in the size range of DP4–6 were active both in eliciting ACO expression and in the production of ethylene.

Key words: aminocyclopropane 1-carboxylic acid oxidase/ethylene/oligogalacturonides/tomato/wounding

Introduction

Fragments of plant cell wall polysaccharides are known to elicit a range of effects in plants (reviewed in Ryan and Farmer, 1991; Darvill *et al.*, 1992; Fry *et al.*, 1993). In particular, oligogalacturonic acids (OGAs) derived from pectins show hormone-like effects, such as the inhibition of auxin-induced stem elongation (Branca *et al.*, 1988), stimulation of flowering (Marfa *et al.*, 1991), and the inhibition of root formation (Bellincampi *et al.*, 1993). In addition to these developmental events, OGAs have also been shown to induce defense-related changes, including the induction of proteinase inhibitor gene expression (Bishop *et al.*, 1984; Farmer *et al.*, 1991), expression of pathogenesis-related genes such as chitinase and β 1–3 glucanase (Davis and Hahlbrock, 1987; Broekaert and Peumans, 1988), and phytoalexin production (Hahn *et al.*, 1981; Walker-Simmons *et al.*, 1983; Jin and West, 1984).

Despite this broad range of activity in many different plant species, relatively little is known of the transduction pathway(s) linking OGAs to their end-effects. As a focus for analyzing these pathways and the specificity of OGA action, it is essential to define the size of the structure that is recognized by the plant and may interact with a surface receptor. For many responses, oligomers with a degree of polymerization (DP) in excess of 10

have been shown to be active (for reviews, see Darvill *et al.*, 1992; Van Cutsem and Messiaen, 1994). This has generally been attributed to the ability of the OGAs of DP > ~9 to form multiligomer complexes with Ca²⁺ (Kohn, 1975, 1987). However, there are some reports in which small-sized oligomers, incapable of forming Ca²⁺ complexes, have also been shown to induce effects (Campbell and Labavitch, 1991a,b). For example, early studies on the wound response of tomato plants showed that individual OGAs of DP 2–6 were capable of inducing the accumulation of protease inhibitor proteins (Bishop *et al.*, 1984), and recently in the same experimental system, a mixture of small-sized OGAs was found to elicit ethylene production (O'Donnell *et al.*, 1996).

Given these observations we decided to analyze the bioactivity of small OGAs in more detail to determine whether there was a specific size requirement for the production of ethylene and whether ethylene biosynthesis in this experimental system was associated with the upregulation of the a gene encoding the ethylene-forming enzyme, aminocyclopropane 1-carboxylic acid oxidase (ACO). This report shows that both of these events are triggered by a specific narrow size-range of short chain OGAs.

Results

Preparation of OGAs using a volatile buffer system

Investigations into the use of a volatile elution buffer system were begun with the aim of reducing losses of material during desalting. Such losses were especially great with the high concentrations of imidazole salt (up to 750 mM) needed to separate the larger OGAs using the method of Davis *et al.* (1986). The ammonium formate gradient not only gave excellent separation of the mixture of larger OGAs (Figure 1a) but the buffer was easily removed by freeze drying. This, therefore, completely eliminated losses of material due to desalting. The new method gave purified homogeneous size fractions of OGAs up to DP 15. The purity and size of the OGAs isolated in the pooled fractions of each peak was assessed by thin layer chromatography (TLC, see Figure 1b) and, for pools 3–7, by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (see Figure 1c). Each pool contained an OGA of the corresponding DP (i.e., pool 1 contained GalA, pool 2 contained DP 2, etc.) with no evidence of oxidation at the reducing-terminus or 4,5 unsaturation due to β -elimination at the nonreducing terminus, as has been observed previously (Darvill *et al.*, 1992).

Ethylene production in response to OGAs

Initial results (Simpson and Ashford, unpublished observations) using individual OGAs of DP 1–10 suggested that OGAs of DP 4, 5, and 6 were the most effective elicitors of ethylene production. These experiments were performed at a concentration of 1mg/ml in the bioassay. To ensure that this was not a result

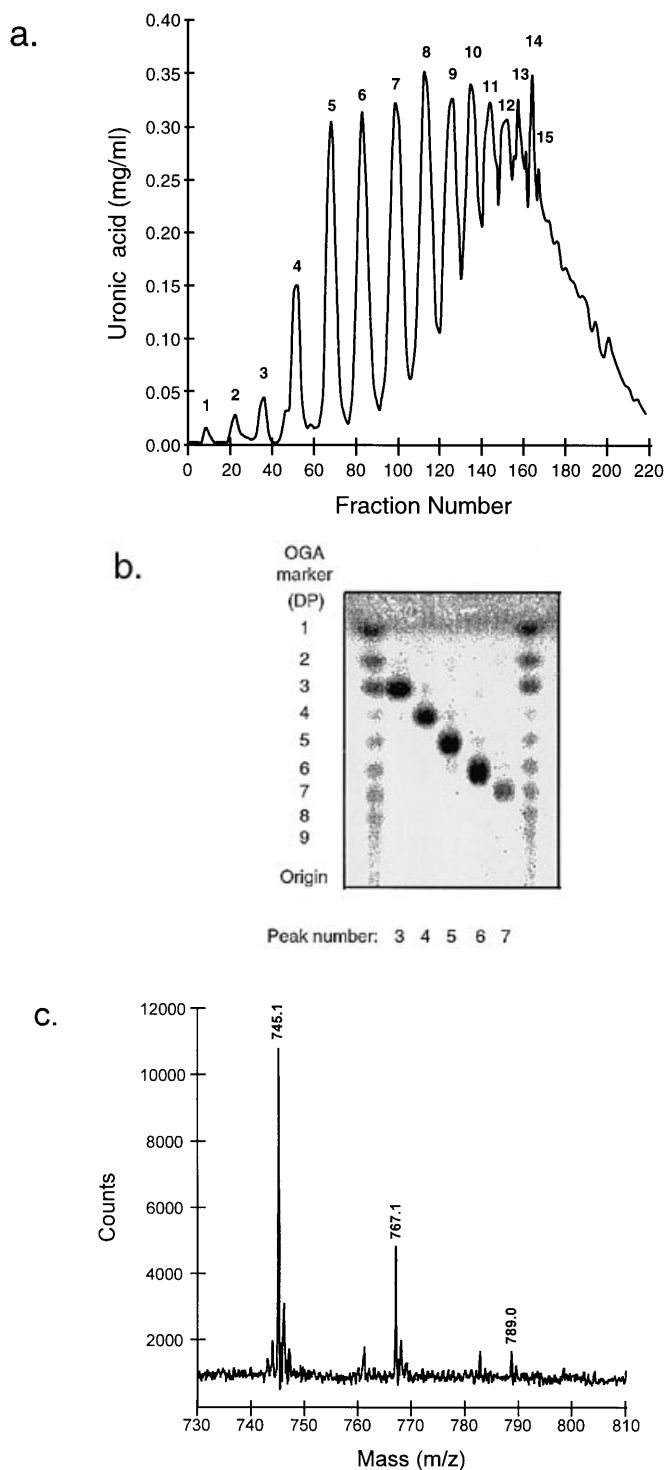


Fig. 1. Separation and characterization of individual OGA oligomers. (a) OGAs obtained by digestion with polygalacturonase and precipitation with ethanol were separated by QAE-Sephadex anion exchange chromatography using an ammonium formate gradient as described in *Materials and methods*. Individual fractions were assayed for their uronic acid content, and each peak pooled, freeze dried and examined by TLC to determine the size of the OGA present. Numbers above the peaks represent the DP of the OGA eluted. (b) Chromatogram showing TLC of 20 μ g of pooled fractions 3–7 compared to OGA size standards. (c) Positive ion MALDI mass spectrum of OGA DP 4 obtained as described in *Materials and methods*. The mass-annotated peaks correspond to Na^+ adducts of the free acid form of GalA_4 (745.1) and its mono- (767.1) and di- Na^+ salt (789.0). Unlabeled peaks are produced by the corresponding potassium adducts.

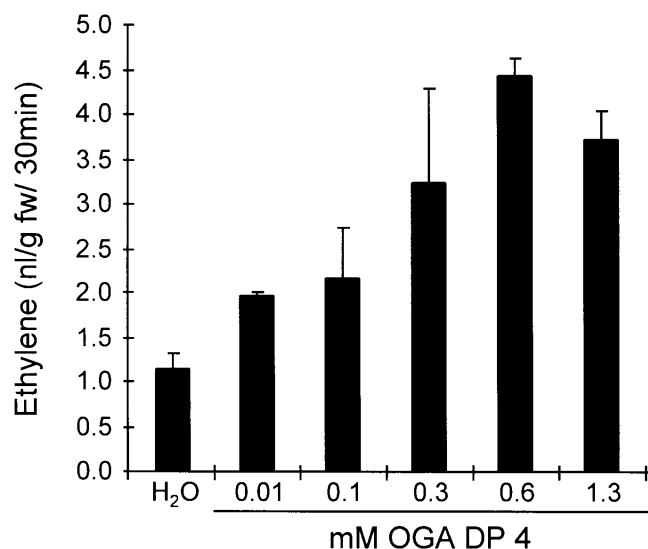


Fig. 2. Dose response of ethylene production with increasing concentrations of OGA DP 4. Data shown is the mean of three experiments.

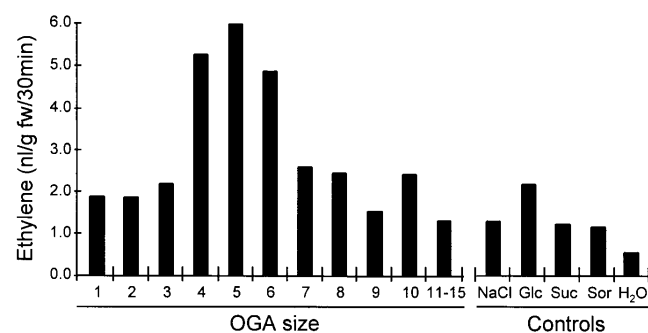


Fig. 3. Ethylene production by a range of OGA fragment sizes. Ethylene production elicited by OGAs with DP 1–15 was compared with several controls, each at 0.6mM and pH 6.5. Glc, glucose; Suc, sucrose; and Sor, sorbitol. The data shown is the mean of at least two experiments.

of a bias toward the smaller OGAs because of their higher relative molar concentration, a dose response experiment for ethylene production was performed using the OGA of DP 4 (Figure 2). A concentration of 0.6 mM was shown to give the highest level of ethylene production. All further bioassay experiments were therefore performed at this concentration of each OGA. In the case of the larger OGAs (DP 11–15) in experiments where they were applied to the plants as a mixture, the molar concentration was calculated for the median OGA (i.e., DP 13).

The ethylene produced by a range of OGA sizes is shown in Figure 3. This reveals that OGAs with DP 4, 5, and 6 reproducibly induced the highest level of ethylene and that DP1–3 and DP >7 were ineffective as elicitors.

*Expression of *aco* in response to OGAs*

Total RNA was extracted from tomato leaves at 1 h after treatment with the individual OGAs and probed with an ACO cDNA. As shown in Figure 4a, expression is specifically induced by OGAs of DP 4, 5, and 6, with little or no effect seen with any other fragment size. Equal loading of the extracted RNA was confirmed

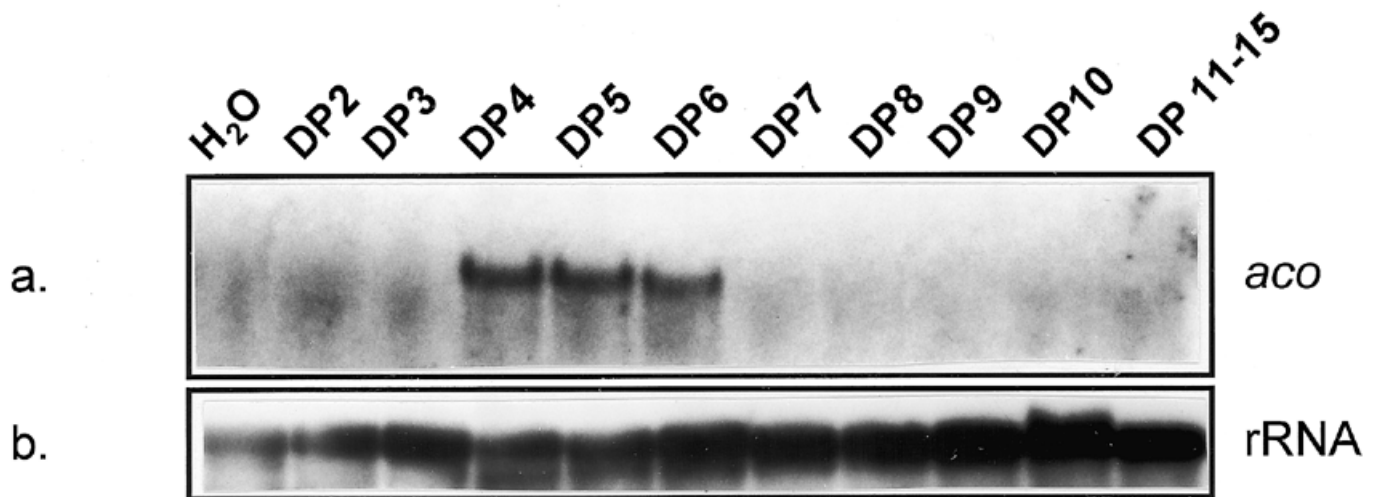


Fig. 4. Accumulation of aminocyclopropane 1-carboxylic acid oxidase (*aco*) mRNA as a result of treatment with a range of purified OGAs. (a) Increased *aco* transcription was assayed by Northern blot analysis and hybridizing total RNA with a 32 P-labeled pTOM13 probe. (b) The blot shown in (a) was stripped and rehybridized with a ribosomal RNA probe.

by stripping the blot pictured and reprobing with a ribosomal RNA probe (Figure 4b).

Discussion

This study shows that a defined size-range of small chain OGAs induce ethylene production and *aco* expression in tomato plants. The pentasaccharide exhibits the greatest activity. While it is well known that application of OGAs to plants can lead to a wide range of effects, their bioactivity is generally discussed in the context of the ability of large OGAs (DP >9) to form intermolecular “egg box” complexes that bind Ca^{2+} as bridging ions to stabilize the multimer structure (Ryan, 1987; Darvill *et al.*, 1992; Reymond *et al.*, 1995). Shorter OGAs are unable to form such structures with Ca^{2+} . The results presented here now indicate that Ca^{2+} complexing is not necessarily a requirement for OGA activity, and therefore there is no minimum size requirement for biological activity of OGAs, in contrast to the view postulated by Reymond *et al.* (1995).

Our findings are in good agreement with earlier studies. For example, Bishop *et al.* (1984) showed that proteinase inhibitor 1 accumulation could be induced by purified OGAs of DP 2–6, and maximally by the hexagalacturonide. More recently O’Donnell *et al.* (1996) reported that a mixture of OGAs of DP 1–8 induced ethylene production. These authors also showed that ethylene was an essential requirement for the induction of proteinase inhibitor 2 (*pin2*) gene expression. In addition, Cambell and Labavitch (1991a,b) also clearly demonstrated that a mixture of shorter OGAs (DP <12) were more potent as elicitors of ethylene production than a mixture of larger sizes. Despite this range of examples demonstrating an effect of shorter OGA mixtures on the induction of ethylene it was possible that the bioactivity only occurred in the larger oligomers present in the mixture. Only by purifying and characterizing individual OGAs could this question be answered. For example, in our own studies we have used a commercially available trigalacturonic acid (Sigma) in the tomato plant bioassay and found that at 1 mg/ml it induced the production of 4.7 nl/g fw/30 min ethylene. This value was more than twice that obtained with the same concentration of our purified OGA DP 3. TLC analysis of the commercial trimer

subsequently showed that it contained larger components in addition to OGA DP 3. These contaminants could therefore lead to a false understanding of the bioactivity of the OGA trimer. Our results clearly show that in this experimental system OGAs of DP 4, 5, and 6 induced maximal ethylene production and only these oligomer sizes were able to induce *aco* mRNA accumulation.

In this report, individual oligomers were applied to tomato plants through the transpiration stream, and since they are known to be mobile in the xylem (MacDougall *et al.*, 1992), presumably were transported via the apoplastic route to the leaves, where their effects were produced. Oligogalacturonides are charged and hydrophilic, properties implying that they would remain in the extracellular space of the leaf and not cross the hydrophobic barrier of the plasma membrane (PM). Given that their application to the plant leads to ethylene production, and that the enzymes and metabolites involved in ethylene synthesis are cytoplasmic or contained within the vacuole (Guy and Kende, 1984; Bouzayen *et al.*, 1990), the transduction pathway must cross the PM. A protein in the PM of tomato leaf cells that is phosphorylated in response to OGAs has been identified (Farmer *et al.*, 1991; Jacinto *et al.*, 1993; Reymond *et al.*, 1995; 1996) and has been shown to bind OGAs (Reymond *et al.*, 1996). However, the phosphorylation response only occurs with large OGAs (DP at least 13) and therefore this “receptor” is probably not involved in the perception of small OGA signal molecules described in this report. Previously, application of a small size-range mixture of OGAs (DP 2–9) to tomato leaf mesophyll cells was shown to induce an immediate depolarization of the membrane potential (Thain *et al.*, 1990). This change in ion transport across the PM might represent an early event in the transduction pathway of the OGA signal in the leaf. The ethylene produced by exogenous OGAs in tomato plants is transient (O’Donnell *et al.*, 1996), with kinetics and amplitude identical to the “ethylene burst” characteristic of stress ethylene observed on wounding and during the early stages of plant–pathogen interactions (Saltveit and Dilley, 1978). Although OGAs have been suggested to be endogenous stress signals, there is no direct evidence as yet of their involvement *in planta*. Pathogens and pests are known to release hydrolases, including pectinases (Collmer and Keen, 1986; Cervone *et al.*, 1989a), capable of digesting plant cell walls. Plant polygalacturonase inhibitor

proteins induced during pathogenesis are thought to regulate the time-course and extent of pectinase activity (Cervone *et al.*, 1989b), but the presence of free OGAs at challenge sites has not as yet been detected (Darvill *et al.*, 1992).

The ability of OGAs of DP 4, 5, and 6 to elicit *aco* mRNA accumulation and maximal ethylene production represents one of the few examples where galacturonide elicitor activity is only seen over a very tight range of fragment sizes. This could in part be because previous experiments showing the biological activity of OGAs have relied on the use of heterogeneous mixtures of OGAs. Such an approach, however, would fail to detect any variation in the biological activity of individual OGAs in these responses. Taken together with the growing body of evidence that specific OGA size ranges exert very different biological activities (Darvill *et al.*, 1992), particularly in the field of plant defense responses, the need to examine the action of individual oligomer lengths becomes paramount.

Materials and methods

Plant material

Tomato seeds (*Lycopersicon esculentum* Mill cv. MoneyMaker) were sown in 7.5 cm pots in Levingtons Universal compost and cultivated in a controlled environment: 16 h, 22°C day; 8 h, 18°C night; 70% relative humidity under Warm White fluorescent lights providing 200 $\mu\text{E}/\text{m}^2/\text{s}$.

Isolation of individual oligogalacturonic acids

Polygalacturonic acid (PGA), derived from citrus pectin (Sigma) was deesterified, and freeze dried. Deesterified PGA was dissolved in dH_2O (5 mg/ml) and heated to 37°C, then 0.03 mU/mg of *Aspergillus niger* polygalacturonase (Sigma) was added. After incubation for 1 h the digestion was stopped by heating to 100°C. For the isolation of oligomers with a DP from 1–8, the digested PGA was repeatedly dialyzed against H_2O and the dialysate concentrated by vacuum evaporation. The pooled dialysate was then separated by anion exchange chromatography on a QAE-Sephadex A-25 matrix (Pharmacia, 2.5×60 cm) equilibrated with 0.125 M imidazole HCl buffer pH 7.0 (Davis *et al.*, 1986). Fractions (8 ml) were assayed for their uronic acid content by the *m*-phenyl phenol method (Blumenkrantz and Asboe-Hansen 1973) using galacturonic acid as a standard. Individual peaks were pooled and de-salted on a 500 ml column of Sephadex G-25 matrix (Pharmacia), equilibrated, and eluted with dH_2O . For the isolation of oligomers with a DP of 5–15 the digested PGA was first selectively precipitated with ethanol and sodium acetate (Spiro *et al.*, 1993). The precipitate was redissolved and separated by QAE-Sephadex chromatography using a shallow gradient of ammonium formate buffer (pH 9.0). The sample was applied to a QAE column as described above except equilibrated with 50 mM ammonium formate pH 9. OGAs were eluted using a linear gradient running from 250 mM to 600 mM ammonium formate pH 9 at a flow rate of 2 ml/min (total volume 4 l). Fractions (8 ml) were assayed for their uronic acid content. Individual peaks were pooled, diluted 1:1 with dH_2O , and freeze dried twice to remove the ammonium formate. The size and purity of the individual OGA oligomers eluted in each peak was determined by TLC and MALDI mass spectrometry. OGA oligomers were resolved on silica gel plates (Whatman) developed in butanol-formic acid- H_2O (33:50:17, v/v/v; Koller and Neukom, 1964) and visualized by spraying with 0.5% carbazole,

5% H_2SO_4 in ethanol and heating for 10 min at 110°C. For mass spectrometry, samples (125 pmol) were mixed on the target with 2,5-dihydroxybenzoic acid and allowed to dry. The target spots were then recrystallized from 0.5 μl ethanol (Harvey, 1993). Positive ion MALDI mass spectra were recorded with a PerSeptive Biosystems Voyager Elite time-of-flight mass spectrometer (nitrogen laser, 337 nm) operating in the reflectron mode. The delayed-extraction ion source was operated with a 75 ns delay, the extraction voltage was 20 kV and the grid voltage was set at 65%.

Tomato plant bioassay

Twenty-one day old plants were excised and their stems incubated for 30 min in H_2O or 0.6 mM solution of the individual OGA (pH 6.5), before transfer to water for the remainder of the incubation period (O'Donnell *et al.*, 1996). Plants were maintained under constant light and at a temperature of 22°C. Leaf material was harvested after 1 h.

Extraction of total RNA and Northern analysis

Total RNA was prepared using a modification of the method described by Logemann *et al.* (1987). Frozen plant tissue (0.5–1 g) was crushed with a pestle and mortar, placed in a 15 ml plastic tube and 0.75 ml each of 8 M guanidine HCl (pH 7.0) buffer and Tris-phenol (pH 8.0) were added. This mixture was homogenized with the microprobe of a Polytron homogenizer (Kinematica Gmb., Switzerland) for 20–30 s. The homogenate was centrifuged for 10 min at 4°C. The upper phase was reextracted with Tris-phenol and centrifuged. This procedure was repeated until the interface was clear. RNA was precipitated with 0.1 ml 1 M acetic acid and 0.3 ml ethanol overnight at -20°C. The resultant precipitate was collected by centrifugation at 1.7×10^4 g for 10 min at 4°C. The pellet was washed twice with 3 M sodium acetate buffer pH 5.5, and once with 70% ethanol, centrifuging for the same period each time. The pellet was dried and redissolved in 40–60 μl dH_2O . Agarose gels 1.4% (w/v) were made in 1 \times MOPS, containing 0.66 M formaldehyde and 0.2 $\mu\text{g}/\text{ml}$ ethidium bromide (Sambrook *et al.*, 1989). Samples were heated at 100°C for 2 min prior to loading, and gels were electrophoresed at a maximum of 7.5 V/cm at 4°C in 1 \times MOPS. Formaldehyde was removed from the gel by washing in 1.5 M NaCl, 0.14 M sodium citrate pH 7.0 (10 \times saline-sodium citrate (SSC)) twice for 10 min. RNA was then transferred on to Hybond-N membrane (Amersham), as described by Sambrook *et al.* (1989), and then cross linked onto the membrane by baking at 80°C for 2 h.

Prehybridization was carried out overnight at 42°C in 50% (v/v) deionized formamide, 5 \times SSC, 1 \times Denhardt's solution, 0.5% (w/v) sodium dodecyl sulfate, 0.25 M sodium pyrophosphate pH 6.5, 100 $\mu\text{g}/\text{ml}$ sheared herring sperm. Hybridization was carried out in the same solution overnight at 42°C, with the addition of a cDNA corresponding to pTOM13 (*aco*) labeled with (α - ^{32}P)dATP. Membranes were washed sequentially in 5 \times SSC, 1 \times SSC, and 0.2 \times SSC, each for 20 min at 42°C, and then exposed to x-ray film.

Measurement of ethylene production

Leaf material harvested from six plants was placed in gas tight vessels (20 ml) and incubated under constant light conditions at 22°C for 30 min prior to the removal and analysis of 1 ml of gas from the head space. Ethylene accumulation was measured by gas chromatography as described by Peck and Kende (1995). Gases

were separated on a Shimadzu GC14B series gas chromatograph with a flame ionization detector using an Alumina PLOT column (30 m × 0.53 mm). The flow rate of the carrier gas (nitrogen) was 4 ml/min.

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Abbreviations

ACO, aminocyclopropane 1-carboxylic acid oxidase; DP, degree of polymerization; MALDI, matrix-assisted laser desorption/ionization; OGA, oligogalacturonic acid; PGA, polygalacturonic acid; PM, plasma membrane; SSC, saline-sodium citrate; TLC, thin layer chromatography.

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