Inhibition of ethylene-induced α-farnesene synthase gene PcAFS1 expression in ‘d’Anjou’ pears with 1-MCP reduces synthesis and oxidation of α-farnesene and delays development of superficial scald

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Abstract

Pre-storage treatment of superficial scald-susceptible apple and pear fruits with the blocker of ethylene action 1-methylcyclopropene (1-MCP) inhibits the synthesis of α-farnesene. Consequently, accumulation of α-farnesene and its conjugated trienol (CTol) oxidation products in the fruit skin is diminished, and scald is largely prevented. In cold-stored apple fruit, a marked increase in expression of AFS1, the gene encoding α-farnesene synthase (AFS), precedes the rapid accumulation of α-farnesene. A Pyrus communis L. gene encoding AFS (PcAFS1) was cloned using RT-PCR with primers based on apple AFS1 and RNA from peel tissue of ‘d’Anjou’ pears cold-stored for 33–94 days. Non-treated control and 1-MCP-treated (300 nL L−1 for 6 h at 1 °C) pears were stored at −1 °C in air for up to 216 days. PcAFS1 expression in control fruit increased sharply over the first 63 days, remained high through 123 days, fell to much lower levels from 157 to 183 days, then increased again to maximum levels at 216 days. In comparison, expression of PcAFS1 was attenuated in 1-MCP-treated fruit. PcAFS1 transcript was almost nil through 94 days, rose sharply at 123 days and reached a maximum at 183 days that was two-fold lower than the highest level in controls. Maximum concentrations of α-farnesene and CTols in control fruit occurred at 94 and 123 days, respectively, and scald incidence was 100% after 94 days. α-Farnesene and CTol levels at these time points were, respectively, 9- and 19-fold lower in 1-MCP-treated fruit, which had no scald at 157 days, 13% at 183 days and 40% at 216 days. Post-storage ripening, with the associated increases in softening, respiration and ethylene production, was fully enabled in control fruit after 63 days at −1 °C, whereas 1-MCP-treated pears failed to ripen properly even after 216 days.

Keywords: Pear fruit; Pyrus communis; α-Farnesene synthase; Ethylene; Gene expression; Superficial scald

1. Introduction

Fruit of ‘d’Anjou’ pear are highly susceptible to superficial scald, a physiological storage disorder that affects certain cultivars of apples and pears, and manifests as brown or black patches on the fruit skin that typically intensify after removal from storage. Symptoms of the disorder result from necrosis of the hypodermal cortical tissue (Bain and Mercer, 1963) and it is thought that cell damage is induced by oxidation products of the sesquiterpene (E,E)-α-farnesene (Huelin and Coggiola, 1970; Anet, 1972; Chen et al., 1990b; Rowan et al., 2001). Scald-susceptible apples and pears typically exhibit a high rate of α-farnesene synthesis shortly after they are placed in low temperature storage, which results in a marked accumulation of the sesquiterpene in the skin during the first 2–3 months (Huelin and Coggiola, 1968; Anet, 1972; Chen et al., 1990b; Whitaker et al., 1997; Bai, in press). The concentration of α-farnesene subsequently declines as its conjugated triene (CT) oxidation products increase to a maximum at about 4–6 months (Huelin and Coggiola, 1970; Anet, 1972; Chen et al., 1990b; Whitaker et al., 1997).
The primary CT oxidation products of α-farnesene that accumulate in apple epicuticular wax and peel tissue during cold storage have been identified as conjugated trienols (CTols), 9E and 9Z isomers of 2,6,10-trimethylidodeca-2,7,9,11-tetra-6-ol (Rowan et al., 1995; Whitaker et al., 1997). Application of these CTols and their corresponding hydroperoxides to apple fruit prior to storage-induced symptoms indistinguishable from naturally occurring superficial scald (Rowan et al., 2001). Pre-storage treatment of apples and pears with the antioxidants diphenylamine and ethoxyquin, respectively, inhibits oxidation of α-farnesene and largely prevents development of scald (Huelin and Coggiola, 1970; Chen et al., 1990a, 1990b; Bai, in press).

In addition, exposure of apple and pear fruit to the blocker of ethylene action 1-methylcyclopropene (1-MCP) greatly curtails α-farnesene production and markedly reduces scald incidence and severity (Fan et al., 1999; Watkins et al., 2000; Argenta et al., 2003; Bai, in press; Chen and Spotts, 2005; Lurie et al., 2005). Results from the studies with 1-MCP, as well as several previous reports (Du and Bramlage, 1994; Watkins et al., 1995; Whitaker and Solomos, 1997; Ju and Curry, 2000a), have shown that ethylene production and perception, and tissue responsiveness to ethylene, are involved in regulation of α-farnesene synthesis and induction of scald in apple and pear fruits.

In apple peel tissue, α-farnesene is synthesized almost exclusively via the mevalonic acid pathway, rather than the chloroplastic deoxyxylulose phosphate pathway (Rupasinghe et al., 2001; Ju and Curry, 2000b). The final, rate-limiting enzyme in the pathway is α-farnesene synthase (AFS), which converts farnesyl diposphate (FDP) to α-farnesene (Rupasinghe et al., 1998, 2000). We recently cloned the gene encoding AFS from peel tissue of scald-susceptible ‘Law Rome’ apple fruit (AFS1; GenBank accession number AY182241) and showed that an ethylene-dependent four-fold increase in AFS1 transcript occurred during the initial 4 weeks of storage in air at 0.5 °C (Pechous and Whitaker, 2004). In this investigation, we cloned the corresponding gene (PcAFS1; accession number DQ309034) from peel tissue of scald-susceptible ‘d’Anjou’ pear and subsequently determined its expression in non-treated control and 1-MCP-treated fruit in relation to the accumulation of α-farnesene and CTols, and the post-storage incidence of superficial scald.

2. Materials and methods

2.1. Plant material, fruit treatment and storage and tissue sampling

‘d’Anjou’ pear (Pyrus communis L.) fruit with flesh firmness of 58 ± 4N were harvested from an orchard at the Mid-Columbia Agricultural Research and Extension Center in Hood River, OR, USA, on 15 September 2004 (2 weeks after commercial maturity). Pears from 3 orchard blocks were segregated as 3 replicate lots and defect-free fruit from each lot were packed in sixteen 20-kg boxes (90 fruit per box) fitted with perforated polyethylene liners. Eight boxes of fruit from each replicate were immediately stored in air at −1 °C and >95% relative humidity (non-treated controls). The remaining 24 boxes (8 per replicate) were treated shortly after harvest with 1-MCP (SmartFresh®, AgroFresh, Spring House, PA, USA) at a concentration of 300 nL L⁻¹ in a 40 m³ airtight room at 1 °C for 6 h. The desired concentration of 1-MCP was established according to the manufacturer’s instructions; briefly, 19.2 g of SmartFresh® powder (0.14% active ingredient) was added to 320 mL of 40 °C water in a 1 L beaker and dissolved by vigorous agitation with a magnetic stir plate centered in the airtight room. Following 1-MCP treatment, the fruit were transferred to a large open room ventilated by electric fans for 24 h at 20 °C prior to storage in air at −1 °C.

Non-treated and 1-MCP-treated fruit were sampled after 9, 33, 63, 94, 123, 157, 183 and 216 days in storage. Peel tissue, including the epidermis and 2–3 mm of hypodermal cortex, was excised with a stainless steel fruit peeler from the equatorial region of 10 randomized fruit from each treatment (4 + 3 + 3 fruit from the 3 replicate boxes) and immediately frozen in liquid N₂. Pooled 30 g samples were stored at −72 °C in zip-lock bags until shipped packed in dry ice to the USDA, ARS Produce Quality and Safety Laboratory by overnight courier. Upon arrival, the peel tissue samples were stored at −80 °C until used for extraction of RNA or extraction and analysis of α-farnesene and CTols.

2.2. Measurements of firmness, respiration, and ethylene, and evaluation of scald

Parameters of fruit quality and physiology, including flesh firmness, internal ethylene concentration (IEC), rates of respiration and ethylene evolution, and scald incidence, were measured at harvest and thereafter at roughly monthly intervals of −1 °C storage for about 7 months (33, 63, 123, 157, 183 and 216 days). Measurements were made at 1 and/or 7 days after transfer to 20 °C, with the exception of IEC, which was measured directly after removal from storage (in cold fruit).

Flesh firmness was measured on 10 fruit per replicate using a fruit texture analyzer (Model GS-14, Guss Manufacturing Ltd., Strand, South Africa) with an 8 mm diameter plunger that penetrates 9 mm in 0.9 s. Two measurements were obtained per fruit on opposite sides of the equator after removal of 20 mm diameter peel discs.

Gas samples for determination of IEC were obtained from the seed cavity of submerged fruit (10 fruit per time × treatment; Bai et al., 2003) and then injected into a gas chromatograph (Shimadzu GC-9A, Kyoto, Japan) equipped with a flame ionization detector and a Porapack Q column (80/100 mesh, 3.0 mm i.d., 1.83 m long). The carrier gas was helium at a flow rate of 0.67 mL s⁻¹ and the oven temperature was 60 °C. An external standard of ethylene (1.0 µL L⁻¹) was used for calibration, as described by Chen and Mellenthin.
(1981). The limit of ethylene detection was approximately 0.01 \mu\text{L}^{-1}.

Respiration and ethylene production were determined using a flow-through system. Groups of five fruit from each replicate of the two treatments (total mass \sim 1.1 kg) were placed in a 10 L chamber at 20°C ventilated with humidified air at a flow rate of 3.33 mL s^{-1}. Gas samples were withdrawn through a septum in the system using a 1 mL gas-tight syringe and analyzed by gas chromatography (GC). Ethylene was measured with the same GC system used for IEC determination. CO₂ concentration was measured using an HP-5890A GC (Hewlett-Packard, Avondale, PA, USA) equipped with a thermal conductivity detector and a CTR1 molecular sieve/porous polymer column from Alltech Associates Inc. (Deerfield, IL, USA) (Bai et al., 2003).

Superficial scald was assessed visually in 60–70 fruit from each replicate and treatment 7 days after transfer from cold storage to 20°C. Scald incidence was defined as the percentage of pears exhibiting slight to severe scald, i.e., \geq 0.6 cm² of the fruit surface exhibiting symptoms (Chen et al., 1990b).

2.3. Extraction and quantification of α-farnesene and conjugated trienols

Hexane extraction of α-farnesene and CTols from each set of frozen pear peel tissue samples was conducted as described in Whitaker et al. (1997) with the exception that 2 g of tissue were immersed in 12 mL of hexane. Extractions were performed in duplicate. A 1.5 mL aliquot of each 2 g of tissue was immersed in 12 mL of hexane. Extracts were transferred to a 2 mL glass vial, the hexane was evaporated with a gentle stream of N₂ and the residue was dissolved in 400 \mu\text{L} of methanol. The samples were analyzed by high-performance liquid chromatography (HPLC) using a Hewlett-Packard Series 1100 HPLC system (Agilent Technologies) with a quaternary pump, autosampler and photodiode array detector (PDA). Sample vials were placed in the autosampler and 25 \mu\text{L} aliquots were injected onto a Luna 5 \mu\text{m} particle size C₁₈(2) column (250 mm long, 4.6 mm i.d.) from Phenomenex (Torrance, CA, USA) and eluted with isocratic methanol:water:acetonitrile, 90:5:5 (v/v/v), at a flow rate of 13.3 mL min⁻¹. PDA monitoring at 232 nm was used to determine levels of α-farnesene and CTols, which eluted at 13.8 and 5.8 min, respectively. HPLC-purified apple α-farnesene and CTol samples were used as external standards for quantification (Whitaker et al., 1997). Concentrations of these standards were calculated using the molar extinction coefficients \epsilon_{232nm} = 27,740 for α-farnesene (Huelin and Coggiola, 1968) and \epsilon_{269nm} = 42,500 for CTol (Amet, 1969).

2.4. RT-PCR amplification and cloning of ‘d’Anjou’ PcAFS1 cDNA

Total RNA was isolated as described by Pechous et al. (2005) with modification. Frozen peel tissue samples (0.2 g) were ground to a powder in liquid N₂ using a mortar and pestle and transferred to 15 mL screw-cap centrifuge tubes, followed by immediate addition of 0.25 mL of plant aid solution and 2 mL of lysis buffer from the RNAqueous™ RNA extraction kit (Ambion Inc., Austin, TX, USA). After vortexing and incubation for 10 min at 25°C, the samples were processed according to the manufacturer’s instructions. The resulting RNA preparations were treated with DNase (Promega) to degrade genomic DNA, extracted with phenol:chloroform, 1:1 (v/v), and total RNA was precipitated by addition of 1 volume isopropyl alcohol plus 0.1 volume of 3 M sodium acetate, pH 5.5. The precipitated RNA was pelleted by centrifugation, washed with cold 70% ethanol and resuspended in RNase-free water.

Total RNA isolated from peel tissue of ‘d’Anjou’ fruit stored for 33–94 days was pooled and cDNA produced by reverse transcription (RT) using the Thermoscript™ RT-PCR system (Invitrogen) with random hexamer primers and subsequently with random amplification of cDNA ends (3' RACE) primers or gene specific primers (GSPs), following the manufacturer’s guidelines. Degenerate primers NEG016 (5′-TTSACMY/WGCGWITTRAKAGRTGGG-3′) and NEG020 (5′-CCAYKYYGICTCYWCCA1WAAYRC-3′) (K=T+G, M=A+C, R=A+G, S=C+G, V=A+C+G, W=A+T, Y=C+T, I=deoxyinosine) were used to isolate a 230-bp fragment of PcAFS1 from cDNA produced using random hexamer primers. Nested 3' RACE was carried out using a 3′−5′ RACE kit (Ambion) to amplify the 3′ end of PcAFS1 from cDNA produced using a 3′ RACE adapter primer. In the initial PCR, NEG023 (5′-TTGAGAGACAATGGTTGGAAACCAAG-3′) was used with the Ambion 3′ outer primer, followed by a nested PCR using NEG024 (5′-CCCTCAATTGACCAAAGTGTGGGCAG-3′) and the Ambion 3′ RACE inner primer. The 5′ end of PcAFS1 was amplified using NEG021 (5′-ATGGAGTTTAGATCTACATTGCAGTCTGCTG-3′) forward and NEG026 (5′-GTGGCCACACTTTGTGTCATATTGAGG-3′) reverse primers with random hexamer-primed cDNA as template. Subsequently, using the forward and reverse GSPs NEG021 and NEG027 (5′-ACAAGACAAACTACTACTTTTTTTATTTICAT-3′), a cDNA including the complete PcAFS1 opening reading frame and 3′ untranslated region (3′ UTR) was amplified from cDNA produced by RT using pear peel tissue RNA and NEG027. PCR was performed using 1–5 \mu\text{L} of cDNA as template and was run for 35 cycles with a 55°C annealing temperature and an extension for 2 min at 68°C. The Invitrogen High Fidelity™ PCR system (Invitrogen Life Technologies, Carlsbad, CA, USA) was used to ensure full-length amplification of the ‘d’Anjou’ PcAFS1 cDNA.

2.5. Northern analysis of PcAFS1 expression

Total RNA was extracted from peel tissue of non-treated control and 1-MCP-treated ‘d’Anjou’ pears as described by Pechous and Whitaker (2004). Northern analysis was car-
Table 1
Scald incidence, internal ethylene concentration (IEC), flesh firmness, and rates of ethylene evolution and respiration in non-treated control and 1-MCP-treated pears after 0–216 days of storage in air at −1 °C and >95% relative humidity

<table>
<thead>
<tr>
<th>Storage duration (days)</th>
<th>Scald incidence (a) (%)</th>
<th>IEC (b) (μL L⁻¹)</th>
<th>Flesh firmness (c) (N)</th>
<th>Ethylene (d) (pmol kg⁻¹ s⁻¹)</th>
<th>Respiration (e) (μg kg⁻¹ s⁻¹)</th>
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<tr>
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<td>0.1</td>
<td>54ab</td>
<td>45c</td>
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Values followed by different letters (a–f) are significantly different.

a Visual evaluation 7 days after transfer to 20 °C.
b Measured directly after removal from −1 °C storage.
c Measured 1 and 7 days after transfer to 20 °C.
d Mean separation in a column by L.S.D. (P < 0.05).

ried out as described by Gapper et al. (2005), using 10 μg of total RNA. After electrophoresis on a 1.2% agarose gel, RNA was transferred in 10× SSC to nylon membranes (Hybond XL, Amersham) by downward capillary transfer (Chomczynski, 1992). After blotting, membranes were washed in 2× SSC and the RNA was fixed to membranes by UV cross-linking (GS Gene Linker UV Chamber, Bio-Rad). Double stranded DNA probes for PcAFS1 or Pc18S were prepared by random-primed labeling using the 5′–3′ polymerase activity of Klenow (Roche) according to the manufacturer’s instructions. Membranes were bathed in hybridization solution (Church and Gilbert, 1984) and hybridized with [32P]dATP-radiolabeled probes at 65 °C for 16 h. Membranes were washed sequentially for 20 min each in 2×, 1×, 0.5× and 0.1× SSC plus 1% SDS (w/v) at 65 °C. After this series of washes, Kodak Biomax MR or MS film was exposed to the membranes at −80 °C.

2.6. DNA sequencing
Automated dideoxy sequencing was performed at the Iowa State University DNA Sequencing and Synthesis Facility. Reactions were set-up using the Applied Biosystems (Foster City, CA, USA) Prism BigDye Terminator v3.1 cycle sequencing kit with AmpliTaq DNA polymerase and reactions were electrophoresed on an Applied Biosystems 3730 DNA analyzer. The programs BLASTN and BLASTP on the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov) were used to search the nucleotide and protein sequence databases.

3. Results
3.1. Scald incidence, IEC, firmness, ethylene production, and respiration rate
The incidence of superficial scald in non-treated control and 1-MCP-treated fruit was evaluated 7 days after transfer from −1 to 20 °C (Table 1). A low incidence of scald (7%) was evident in the controls after 63 days of storage, and 100% of the non-treated fruit developed slight to severe scald symptoms after 94 days and after all subsequent removals from storage. In marked contrast, 1-MCP-treated pears were free of scald for up to 157 days of storage and scald incidence reached a maximum of 40% at the end of storage (216 days).
IECs in ‘d’Anjou’ fruit were below the detection limit at harvest. In non-treated controls, the mean IEC increased steadily after 33 days, reached a maximum of 4.1 μL L⁻¹ at 157 days then leveled off for the remainder of storage. In sharp contrast, in 1-MCP-treated fruit internal ethylene was not detectable through 183 days of storage and was only 0.1 μL L⁻¹ at 216 days.
Flesh firmness, ethylene and respiration (CO₂), measurements were made to assess fruit ripening after storage at −1 °C for up to 216 days plus 1 or 7 days post-storage at
20°C. In non-treated control fruit assessed 1 day after transfer to 20°C, firmness declined significantly from 58 to 47 N during 216 days of storage, while in 1-MCP-treated fruit there was no significant loss of firmness (54 N after 216 days). In controls tested 7 days after transfer to 20°C, softening was substantial after 33 and 63 days of cold storage, and was near maximal after 94 days. By contrast, there was no loss of flesh firmness in 1-MCP-treated pears after 94 days of storage and after 216 days treated fruit still softened significantly less than controls stored for only 33 days.

When measured 1 day after transfer of control fruit from −1°C storage to 20°C, the rate of ethylene evolution increased gradually to a maximum of 15 pmol kg⁻¹ s⁻¹ after 123 days, whereas when measured 7 days post-storage, the increase was much more pronounced, reaching a maximum of about 266 pmol kg⁻¹ s⁻¹ after 183 days. Post-storage ethylene evolution in 1-MCP-treated pears was undetectable over the entire duration of cold storage.

The respiration rate (CO₂ evolution rate) of non-treated control fruit determined 1 day after removal from storage increased about four-fold (from 0.9 to 3.4 μL L⁻¹ s⁻¹) during 216 days at −1°C, whereas in 1-MCP-treated fruit there was no increase in respiration after 157 days and only a two-fold increase by 216 days. When measured 7 days after transfer to 20°C, the increase in respiration in control fruit was about 11-fold between harvest and 216 days of storage, compared with just over a 4-fold increase in 1-MCP-treated fruit, which occurred during the final 59 days of storage.

3.2. Accumulation of α-farnesene and CTols in pear peel tissue during storage

The concentration of α-farnesene in peel tissue of non-treated ‘d’Anjou’ pears increased from trace levels to ~60 mg kg⁻¹ fresh weight during the initial 33 days of storage, continued to increase sharply through 94 days to a maximum of ~200 mg kg⁻¹, leveled off at 123 days, and then declined abruptly to ~70 mg kg⁻¹ at 157 days, remaining between about 60 and 80 mg kg⁻¹ thereafter (Fig. 1A). Levels of α-farnesene were much lower in 1-MCP-treated fruit than in non-treated fruit throughout storage; about nine-fold lower at 94 days and five-fold lower at 123 days, when the concentration in 1-MCP-treated fruit reached a maximum of ~40 mg kg⁻¹. For the remainder of storage (157–216 days), the concentration ranged from about 20 to 30 mg kg⁻¹.

CTols in peel tissue of non-treated pears were scarcely detectable at 33 days but increased dramatically through 94 days, further increasing to a maximum concentration of ~90 mg kg⁻¹ at 123 days (Fig. 1B). The CTol concentration then declined 50% between 123 and 157 days, but subsequently rose to about 70–80 mg kg⁻¹ for the remainder of storage. In accord with the low levels of α-farnesene in 1-MCP-treated fruit, accumulation of CTols was delayed and markedly reduced in comparison with that in the controls. CTols were barely detectable at 94 days and the concentration at 123 days was about 19-fold lower than that in non-treated fruit. Between 94 days and the end of storage, CTols in 1-MCP-treated pears rose continuously, albeit slowly, reaching a maximum of ~17 mg kg⁻¹ after 216 days of storage.

3.3. Nucleotide and encoded amino acid sequences of PcAFS1

Sequence analysis determined that the coding region of the ‘d’Anjou’ AFS1 cDNA (PcAFS1; GenBank accession number DQ309034) is 1731 base pairs in length and encodes a predicted protein of 576 amino acids. Comparison of the coding region of PcAFS1 with known α-farnesene synthase cDNAs revealed the following identities: 99.2% identity to P. communis L. (European pear) AY566286; 99.3% identity with a partial cDNA from Pyrus pyrifolia (Burm. f.) Nak. (snow pear) AY491399; 97.6% identity to Malus domestica Borkh. (apple) ‘Law Rome’ AY182241 (Pechous and Whittaker, 2004); 97.6% identity to M. domestica ‘Royal Gala’ AY787633; 97.5% identity to M. domestica ‘White Pearmain’ AY563622; 97.3% identity to M. domestica ‘Idared’ AY523409 (Pechous et al., 2005). Comparison of the predicted protein’s amino acid sequence (GenBank accession number ABC25002) with those of other deduced α-farnesene synthase proteins from pear and apple revealed the following identities: 98.3% identity to P. communis

![Fig. 1. Concentrations of α-farnesene (A) and its conjugated trienol (CTol) oxidation products (B) in peel tissue of non-treated control and 1-MCP-treated (0.3 μL L⁻¹, 6 h at 1°C) ‘d’Anjou’ pears stored at −1°C in air for 0–216 days. Separation and quantification of α-farnesene and CTols were performed by C18-HPLC with UV monitoring at 232 and 269 nm, using HPLC-purified α-farnesene and CTol standards of known concentration as external standards. Error bars in (A) and (B) represent the standard error of replicate analyses.](image-url)
AAT70237; 98.2% identity with a partial predicted protein from *P. pyrifolia* AAS93266; 96.5% identity to *M. domestica* ‘Law Rome’ AAO22848; 96.2% identity to *M. domestica* ‘Royal Gala’ AAX19772; 96.0% identity to *M. domestica* ‘White Pearmain’ AAS68019; 95.7% identity to *M. domestica* ‘Idared’ AAS01424. Two domains critical to enzymatic function, the DDXXD divalent cation-binding domain (amino acids 326–330), common to all terpene synthases, and the RR(X8)W motif near the N-terminus (amino acids 33–43), common to the *Tps-b* and *Tps-d* gene families (Dudareva et al., 2003), are identical in all six of the complete deduced *H9251*-farnesene synthase proteins from pear and apple (accession numbers AAO22848, AAS01424, AAS68019, AAT70237, AAX19772 and ABC25002).

3.4. *PcAFS1* transcript levels in non-treated control and 1-MCP-treated fruit

Northern analysis was used to follow expression of the *α*-farnesene synthase gene *PcAFS1* in ‘d’Anjou’ pear peel tissue over the full course of storage (Fig. 2). The level of *PcAFS1* transcript in control fruit increased about three-fold during the first 63 days of storage, reaching a maximum at 123 days. On the whole, *PcAFS1* transcript levels were considerably lower in peel tissue of 1-MCP-treated compared with non-treated fruit; about 8–15-fold lower from 9 to 94 days, and more than 2-fold lower at 123 and 216 days. *PcAFS1* gene expression in treated fruit remained at basal levels during the first 94 days of storage, more than quadrupled between 94 and 123 days, and then leveled off for the remainder of storage.

Fluctuations in the abundance of *PcAFS1* transcript in non-treated control fruit were plotted in relation to changing peel tissue concentrations of *α*-farnesene and CTols over the course of storage (Fig. 3). An early increase in *PcAFS1* mRNA preceded a similar increase in *α*-farnesene; the level of *PcAFS1* transcript rose rapidly during the first 63 days of storage, whereas *α*-farnesene peaked at 94–123 days and then declined. Accumulation of CTol oxidation products of *α*-farnesene peaked at 123 days and then the concentration dipped transiently before increasing again at the end of storage, a pattern consistent with the changes in *α*-farnesene levels.

4. Discussion

Pre-storage treatment of apples and pears with the antioxidants diphenylamine (DPA) and ethoxyquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline) has been the primary means of controlling superficial scald for about 40 years (Ingle and D’Souza, 1989; Chen et al., 1990a, 1990b; Whitaker, 2004; Bai, in press). However, because these chemical treatments result in unwanted chemical residues, and fruit injury (phytotoxicity) can also occur (Ingle and D’Souza, 1989; Chen et al., 1990a; Bai, in press), alternative control measures have been investigated, most notably low or ultra-low oxygen controlled atmosphere (CA) storage (Ingle and D’Souza, 1989; Chen et al., 1993; Wang and Dilley, 2000; Ma and Chen, 2003). CA storage has the added benefit of delaying fruit ripening and softening, but it is costly and con-
ditions optimal for storage of one apple or pear variety can be injurious to or ineffective for another. Recently, pre-storage treatment of pome fruit with the blocker of ethylene action 1-MCP has emerged as a highly effective measure for scald control and maintenance of quality during long-term storage (Fan et al., 1999; Watkins et al., 2000; Argenta et al., 2003; Calvo, 2003; Arquiza et al., 2005). One caveat with regard to the pear cultivar ‘d’Anjou’ is that doses of 1-MCP required for prevention of scald can inhibit normal ripening, depending on the growing region (Bai, in press; Chen and Spotts, 2005).

A universal effect of the treatments or CA conditions used to control scald is the inhibition of synthesis and/or oxidation of α-farnesene in scald-susceptible apples and pears. DPA and ethoxyquin were shown to inhibit α-farnesene oxidation in vivo (Huelin and Coggiola, 1970; Anet and Coggiola, 1974; Chen et al., 1990b; Arquiza et al., 2005), and in some studies, DPA treatment delayed accumulation of α-farnesene in apple fruit (Huelin and Coggiola, 1968; Du and Bramlage, 1994; Whitaker, 2000). Low oxygen CA storage delayed accumulation of α-farnesene and CTols in apples (Whitaker et al., 1998; Whitaker, 2000) and pears (Chen et al., 1993; Bai, in press), and prevention of scald in ‘Granny Smith’ apples by flow-through 1.5% O2 CA was associated with nearly total suppression of ethylene production (Whitaker and Solomos, 1997). Finally, a number of studies have shown that prevention of scald in pome fruits by 1-MCP treatment is correlated with both strong inhibition of ethylene production and marked reduction in α-farnesene synthesis (Watkins et al., 2000; Bai, in press; Chen and Spotts, 2005; Arquiza et al., 2005; Lurie et al., 2005). This finding prompted investigation of whether ethylene up-regulates the expression of one or more genes in the α-farnesene biosynthetic pathway.

Although there is one report of ethylene-induced up-regulation of the 3-hydroxy-3-methylglutaryl-CoA reductase (HMG) isogene HMG2 in ‘Delicious’ apples during the first weeks of cold storage (Rupasinghe et al., 2001), studies with fruit of ‘Law Rome’ and ‘Granny Smith’ have indicated that neither HMG genes HMG1, HMG2 and HMG3 (Pechous and Whitaker, 2002; Lurie et al., 2005; Pechous et al., 2005) nor a gene encoding farnesyl diphosphate synthase (T. Solomos, personal communication) is up-regulated by ethylene. On the other hand, there is ample evidence that expression of the apple gene encoding α-farnesene synthase, AFS1, is induced by ethylene during the initial weeks of storage (Pechous and Whitaker, 2004; Lurie et al., 2005; Pechous et al., 2005). Moreover, ethylene-induced expression of AFS1 is closely correlated with α-farnesene accumulation in apple peel tissue, and AFS1 suppression by 1-MCP treatment results in a marked reduction in α-farnesene production (Lurie et al., 2005; Pechous et al., 2005).

In light of these findings, the present study was conducted to determine whether increased expression of AFS1 in response to ethylene is also involved in the abundant accumulation of α-farnesene during cold storage of highly scald-susceptible ‘d’Anjou’ pears. Our strategy for cloning ‘d’Anjou’ AFS1 using degenerate primers based on three apple AFS1 cDNAs (AY182241, AY523409 and AY563622) and four other plant α- and β-farnesene synthase nucleotide sequences (AF024615, AF529266, AF543528 and AY640154) was successful, yielding a 230-bp fragment of P. communis AFS1. Subsequent RACE PCR enabled the cloning of a complete cDNA (PeAFS1) including the 1731-bp coding region plus a 142-bp 3′ UTR. During the course of this work, another complete P. communis AFS1 cDNA was registered in GenBank (accession number AY566286), which differs in the open reading frame by only 13 of 1731 nucleotides, encodes an identical protein, and clearly represents the same gene. The coding regions of these two P. communis cDNAs bear remarkable similarity to those of AFS1 cDNAs from ‘Law Rome’, ‘White Pearmain’, ‘Idared’ and ‘Royal Gala’ apple fruit, with about 97–98 and 96–97% identity, respectively, in the nucleotide and encoded amino acid sequences. In contrast, PeAFS1 shares only about 25–40% identity with other reported plant terpene synthases and cyclases, as noted for ‘Law Rome’ apple AFS1 (Pechous and Whitaker, 2004).

Comparison of PeAFS1 transcript levels in peel tissue of non-treated control and 1-MCP-treated ‘d’Anjou’ pears clearly indicated that up-regulation of the gene early in storage is mediated by ethylene (Fig. 2). PeAFS1 expression was evident in control fruit after only 9 days at −1°C and reached close to maximum levels after 63 days, whereas in 1-MCP-treated fruit, PeAFS1 transcript was barely detectable up to 94 days and increased to relatively low levels thereafter, never exceeding 50% of the maximum level in controls. Because internal ethylene and ethylene evolution in 1-MCP-treated pears were below the limit of detection during most or all of the storage period (Table 1), it is inferred that either ethylene stimulates PeAFS1 expression at very low levels or some other factor associated with long-term low temperature storage can also elicit expression. On the whole, the patterns of AFS1 gene expression in control and treated ‘d’Anjou’ pears were similar to those in comparable ‘Law Rome’ (Pechous et al., 2005) and ‘Granny Smith’ (Lurie et al., 2005) apples, although the maximum duration of storage in the apple studies was 4–5 months. In addition, as observed in apple fruit, accumulation of α-farnesene and CTols in ‘d’Anjou’ peel tissue generally followed the pattern and magnitude of PeAFS1 expression (Figs. 1–3). The much higher rate of α-farnesene production in non-treated fruit could in part result from greater substrate availability with the onset of ripening and increased respiration (Rupasinghe et al., 2001). There was a substantial lag between the initial rise in PeAFS1 transcript and the corresponding marked accumulation of α-farnesene (Fig. 3), suggesting either a slow rate of PeAFS1 protein synthesis or substrate limitation, whereas CTol accumulation closely followed that of α-farnesene (Figs. 2 and 3), indicating rapid oxidation of the sesquiterpene. Indeed, the second peak in CTol concentration at 183–216 days with little coincident increase in α-farnesene suggests that late in storage the rates of α-farnesene synthesis and oxidation in control fruit were nearly equal. The rather surprising second sharp rise in PeAFS1 transcript levels late in storage (Fig. 2)
was not correlated with elevated ethylene production or respiration (Table 1) and warrants further investigation.

In this study, pre-storage treatment of ‘d’Anjou’ pears with 0.3 μL L⁻¹ 1-MCP greatly suppressed ethylene production and inhibited ripening for up to 7 months plus 7 days post-storage at 20°C, although a modest increase in respiration was evident between 157 and 216 days (Table 1). Moreover, scald was not completely controlled in the treated fruit, with an unacceptably high incidence of the disorder (40%) by the end of storage. Two other recent studies of the influence of 1-MCP on post-storage quality of ‘d’Anjou’ pears grown in the Mid-Columbia region of Oregon gave similar results (Bai, in press; Chen and Spotts, 2005). Bai (in press) found that in fruit treated with 0.3 μL L⁻¹ 1-MCP and stored for up to 4 months at −1°C plus 7 days at 20°C, scald was entirely prevented but the ability to ripen was also lost. As in the present study, internal ethylene was extremely low throughout storage. Chen and Spotts (2005) showed that pre-storage treatment with 1-MCP ranging from 0.05 to 0.3 μL L⁻¹ gave complete scald control after up to 6 months at 1°C, but again the fruit failed to ripen, even after exposure to 500 μL L⁻¹ ethylene for 7 days at 20 or 25°C. Lower doses of 1-MCP (0.01 and 0.02 μL L⁻¹) controlled scald for 3 months and allowed normal ripening. However, after 4 months of storage or longer, these fruit had an unacceptable incidence of scald. Much more satisfactory results of 1-MCP treatment have been reported for ‘d’Anjou’ pears grown in the Alto Valley in Argentina (Calvo, 2003) and in north central Washington (Argenta et al., 2003). In both studies, 1-MCP exposure at 0.1 μL L⁻¹ prevented scald for 7 months or more, and fruit treated with relatively high concentrations (0.4–1.0 μL L⁻¹) were able to ripen to dessert quality after 7–9 months of cold storage plus 7–15 days at 20°C.

Currently, 0.3 μL L⁻¹ is the lowest concentration of 1-MCP approved for commercial use. Although an effort was made to optimize the outcome of this storage trial, i.e., late-harvest fruit were exposed to 1-MCP relatively briefly (6h) at a low temperature (1°C), pre-storage treatment at 0.3 μL L⁻¹ did not adequately control scald or allow normal ripening. Because of the uncertainty about how ‘d’Anjou’ pears from various regions or from different harvests or growing seasons might respond to 1-MCP treatment, it appears that a molecular genetic or marker-assisted breeding approach to scald prevention in fruit of this cultivar may warrant consideration. The cloning of P. communis gene PcAFS1 reported in this study is the first step toward such an approach, providing the potential for future down-regulation of α-farnesene synthesis to prevent scald development.

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