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ORIGINAL ARTICLE

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Cloning and functional expression of an (*E*,*E*)-α-farnesene synthase cDNA from peel tissue of apple fruit

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Abstract Increased production of terpenes and many other aroma-related volatiles occurs with the onset of ripening in apple (*Malus domestica* Borkh.) fruit. The gaseous plant hormone ethylene plays a key role in the induction of volatile synthesis, but the mechanism is not yet understood. Using a degenerate primer based on a short conserved sequence shared by several sesquiterpene synthases, reverse transcription–polymerase chain reaction with RNA isolated from peel tissue of 'Law Rome' apples yielded an approx. 800-bp gene fragment. This was used to screen a cDNA library generated from the peel tissue mRNA. A full-length terpene synthase (TS) cDNA 1,931 nucleotides long was isolated. The 1,728-bp open reading frame encodes a protein 576 amino acids long with a molecular mass of 66 kDa. Sequence analysis of the apple TS showed it to be most similar to several monoterpene synthases. Oddly, the TS includes an RR(X_8)W motif near the N-terminus that is common among monoterpene synthases but it lacks the plastid transit peptide sequence typically associated with genes of that group. Expression of the apple TS gene in *Escherichia coli* gave *myc*-epitope-tagged and untagged proteins estimated at approx. 68 and approx. 66 kDa, respectively. In assays of sesquiterpene synthase activity, with farnesyl diphosphate as substrate, the untagged bacterially expressed TS gene product synthesized (*E*,*E*)-α-farnesene almost exclusively. In monoterpene synthase assays, with geranyl diphosphate as substrate, the untagged apple TS produced only (*E*)-β-ocimene, albeit at much reduced levels. Addition of a C-terminal *myc* tag appeared to completely prevent production of soluble protein under all of the expression conditions tested. This is the first report of an (*E*,*E*)-α-farnesene synthase gene (*AFS1*; GenBank accession number AY182241) from a flowering plant. RNA gel blots showed that *AFS1* transcript increased about 4-fold in peel tissue of apple fruit during the first 4 weeks of storage at 0.5°C. In contrast, when fruit were treated at harvest with 1-methylcyclopropene, a blocker of ethylene action, *AFS1* mRNA declined sharply over the initial 4 weeks of cold storage, and fell to nearly undetectable levels by 8 weeks.

Keywords Apple fruit · (*E*,*E*)-α-Farnesene · Ethylene regulation · *Malus* · Sesquiterpene biosynthesis · Superficial scald

Abbreviations AA: Amino acid · DPA: Diphenylamine · EI: Electron impact · FDP: Farnesyl diphosphate · FS: (*E*,*E*)-α-Farnesene synthase · GC–MS: Gas chromatography–mass spectrometry · GDP: Geranyl diphosphate · IPTG: Isopropyl β-D-1-thiogalactopyranoside · 1-MCP: 1-Methylcyclopropene · ORF: Open reading frame · RACE: Rapid amplification of cDNA ends · RT–PCR: Reverse transcription–polymerase chain reaction · TS: Terpene synthase · UTR: Untranslated region

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Introduction

The tissues of apple fruit release an array of volatile terpenes after wounding and during climacteric ripening (Brown et al. 1966; Romani and Ku 1966; Cunningham et al. 1986; Mattheis et al. 1991). These compounds contribute to the characteristic aroma of apple fruit and flowers and can act as antifeedant defense compounds as well as insect pheromones. Prime examples among the major constituents of ripe apple fruit volatiles are the homoterpene 4,8-dimethyl-1,3(*E*),7-nonatriene (DMNT), an antifeedant and attractant of predatory insects, and the sesquiterpene (*E*,*E*)-α-farnesene, a

lepidopteran attractant and oviposition inducer (Sutherland and Hutchins 1972; Wearing and Hutchins 1973; Landolt et al. 2000; Bengtsson et al. 2001). Apple leaves and fruit emit the monoterpenes (*β*)-linalool and (*E*)-*β*-ocimene at different phenological stages (Bengtsson et al. 2001), whereas (*E*,*E*)-α-farnesene is the predominant terpene produced during storage of apple fruit (Huelin and Murray 1966; Meigh and Filmer 1969; Anet 1970; Anet and Coggiola 1974).

Oxidation products of (*E*,*E*)-α-farnesene are hypothesized to be the causal agents of superficial scald, a serious physiological disorder that occurs in fruit of susceptible apple cultivars after several months of cold storage and intensifies after warming to market temperature (Huelin and Coggiola 1970; Ingle and D'Souza 1989; Rowan et al. 1995). Production of (*E*,*E*)-α-farnesene occurs in the epidermal and/or adjacent hypodermal cell layers (Rupasinghe et al. 1998), and the sesquiterpene can accumulate to high levels in the natural epicuticular coating of scald-susceptible apples during the first several weeks of storage (Huelin and Coggiola 1968; Whitaker et al. 1997, 1998). After about 2 months of storage, (*E*,*E*)-α-farnesene declines with a concomitant rise in its primary oxidation products, conjugated trienols, which are 9*E* and 9*Z* isomers of 2,6,10-trimethydodeca-2,7,9,11-tetraen-6-ol (Rowan et al. 1995; Whitaker et al. 1997). Further oxidative degradation of these compounds yielding free radicals and toxic volatiles is thought to cause damage to, and eventual necrosis of, the hypodermal cell layers (Anet and Coggiola 1974; Mir et al. 1999; Whitaker and Saftner 2000; Rowan et al. 2001). This results in development of slightly sunken brown or black patches on the surface of injured fruit, the symptoms of superficial scald.

The antioxidant compound diphenylamine (DPA) has been used commercially for many years to control scald on cold-stored apples (Ingle and D'Souza 1989; Mir and Beaudry 1999; Whitaker 2000). Early experiments showing that DPA inhibited oxidation of (*E*,*E*)-α-farnesene in vivo and in vitro and largely prevented scald development lent support to the α-farnesene oxidation–scald induction hypothesis (Huelin and Coggiola 1970; Anet and Coggiola 1974). The most compelling recent evidence supporting the hypothesis was the demonstration by Rowan et al. (2001) that treatment of 'Granny Smith' apples with synthetic conjugated triene alcohol and hydroperoxide oxidation products of α-farnesene induced symptoms indistinguishable from "natural" superficial scald. An important question concerning α-farnesene production in apples is what triggers the dramatic increase in synthesis and accumulation shortly after the fruit are placed in cold storage. Several studies indicated that the gaseous plant hormone ethylene plays a key role (Watkins et al. 1993; Du and Bramlage 1994) and that the inhibition of (*E*,*E*)-α-farnesene synthesis and scald by low oxygen atmospheres is associated with greatly reduced ethylene production (Whitaker and Solomos 1997). More recently, experiments comparing scald-susceptible apples that were untreated or treated at harvest with 1-methylcyclopropene (1-MCP), an irreversible blocker of ethylene receptor sites, have shown unequivocally that ethylene perception is critical for induction of both (*E*,*E*)-α-farnesene production and scald development (Fan et al. 1999; Rupasinghe et al. 2000a; Watkins et al. 2000).

Because DPA treatment leaves unwanted chemical residues on the fruit, restricts export markets, and creates environmental concerns, we are currently pursuing a long-range molecular genetic strategy for control of scald by reduction of (*E*,*E*)-α-farnesene synthesis in scald-susceptible apples. The success of this strategy will rely on our ability to identify, clone, and characterize key genes involved in α-farnesene biosynthesis and its regulation by ethylene. There is clear evidence indicating that production of (*E*,*E*)-α-farnesene in apple fruit occurs via the mevalonic acid (MVA) pathway (Ju and Curry 2000; Rupasinghe et al. 2001). Since this pathway is also utilized in the synthesis of a number of other isoprenoids, such as phytosterols, dolicols, and ubiquinones (Hartmann et al. 2000), the optimal target for gene knockout or suppression to specifically block (*E*,*E*)-α-farnesene production is that encoding (*E*,*E*)-α-farnesene synthase (FS), the final, rate-limiting enzyme that converts farnesyl diphosphate to (*E*,*E*)-α-farnesene. Here we report the cloning and functional expression of an FS cDNA, *AFS1*, from peel tissue of 'Law Rome' apples, and furthermore show that *AFS1* expression is substantially enhanced by ethylene during the initial weeks of cold storage when a marked increase in α-farnesene production occurs.

Materials and methods

Plant materials, fruit treatment and storage, and tissue sampling

'Law Rome' apple (*Malus domestica* Borkh.) fruit were harvested at commercial maturity (mean internal ethylene concentration 1.2 μl/l) from the Cornell University orchards at Lansing, NY. Young developing leaves (laminae approx. 2–3 cm long) were collected from 'Law Rome' trees growing in a commercial orchard owned by the Rice Fruit Company in Gardners, PA. On the day of harvest, one group of fruit was exposed to 2 μl/l 1-methylcyclopropene (1-MCP) for 7 h at 22°C in a sealed container. Non-treated control fruit were sealed in an identical container for the same duration without 1-MCP. All fruit were subsequently stored for 0, 4, or 8 weeks at 0.5°C in air at approx. 65% relative humidity. Apple peel tissue (including the skin and approx. 3 mm of hypodermal tissue) was excised and frozen in liquid N_2. Both fruit peel and leaf tissue samples were stored at −80°C until used for RNA or DNA extraction.

Molecular biology supplies, chemicals, and solvents

Degenerate and gene-specific primers, PCR reagents (including Platinum *Taq* and Elongase polymerase mixes), the λZipLox cDNA library cloning system, and the 3′ and 5′ RACE (rapid amplification of cDNA ends) kits were all purchased from Life Technologies (Rockville, MD, USA). The pET-3d expression vector was obtained from Novagen (Madison, WI, USA), and the BL-21 DE3 (RIL) chemically competent *E. coli* were obtained from Stratagene (La Jolla, CA, USA). Unless stated otherwise, all

chemicals were of reagent grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). Organic solvents were purchased from JTBaker through VWR (West Chester, PA, USA).

RNA and genomic DNA extraction, and cDNA library construction

Because of the polysaccharide content of apple fruit tissue, a hot-phenol RNA extraction protocol was used to isolate apple peel RNA (per Clontech protocol # PT1354–1 from the Extract-A-Plant RNA isolation kit). One exception to the protocol was the precipitation of RNA in 2 M LiCl at 4°C overnight, rather than 70°C for 1 h. Total RNA was resuspended in diethylpyrocarbonate-treated deionized water and quantified spectrophotometrically (Warburg and Christian 1942). Total RNA was used for RT–PCR and RNA gel blotting, whereas RNA for cDNA library construction was purified to mRNA using the Quickprep micro mRNA purification kit from Amersham/Pharmacia (Piscataway, NJ, USA). Isolation of genomic DNA from young apple leaf tissue was reported previously (Pechous and Whitaker 2002). The cDNA library was constructed as described by Smith et al. (1998) using pooled mRNA isolated from peel tissue of non-treated fruit stored for 4 and 8 weeks and the λZipLox cDNA library construction kit from Life Technologies.

Degenerate RT–PCR

RNA from peel tissue of non-treated fruit stored for 4 and 8 weeks was combined and reverse-transcribed, and the cDNA produced was used as template for degenerate PCR. Superscript II reverse transcriptase (Gibco–BRL) with a poly-T first-strand synthesis primer was used to generate cDNA from 2 μg total RNA (1 μg each of 4- and 8-week samples) per the manufacturer's instructions. The initial PCR reaction included the 3′ RACE poly-T adapter primer along with a degenerate 5′ primer based on a small region of homology among sesquiterpene synthases (Fig. 1). The degenerate primer sequence corresponds to 5′-GANMGNYTiCCiGAiTAYATGAARAT-3′, where i = deoxyinosine, N = A + T + C + G, M = A + C, Y = C + T, and R = A + G. The initial PCR reaction was carried out with 300 nM primers, 1.5 mM $MgCl_2$, and 200 μM dNTPs. Two μl of RT reaction products was used as template and 2.5 units of *platinum Taq* DNA polymerase (Life Technologies) was added. Thirty cycles of PCR were performed at 94°C melting, 42°C annealing, and 68°C extension temperatures, with a 2-min initial melting step at 94°C and a final 5-min extension at 68°C. The initial PCR product (200 ng) served as template in a second PCR reaction. This reaction included a universal adapter primer along with the degenerate primer, and an annealing temperature of 55°C rather than 42°C, which was designed to increase product specificity.

Fig. 1 Most conserved region of three sesquiterpene synthase genes from dicotyledonous plants. The *arrow* and *white type with black highlighting* indicate the 9-AA sequence selected for design of the degenerate PCR primer used in initial PCR amplification of an approx. 800-bp fragment of the apple (*Malus domestica*) peel *AFS1* cDNA. GenBank accession numbers: AF171216—vetispiradiene synthase (*Lycopersicon esculentum*), L04680—aristolochene synthase (*Nicotiana tabacum*), AF024615—(*E*)-*β*-farnesene synthase (*Mentha piperita*)

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AF171216 (tomato)   KQELSEVSRWWKDLDFVTTLPYARDRAVECYFWTMGVYAEPQYSQARVML
L04680   (tobacco)  KQELAQVSRWWKDLDFVTTLPYARDRVVECYFWALGVYFEPQYSQARVML
AF024615  (mint)    KEELSQLSRWWNTWNLKSKLPYARDRVVEAYVWGVGYHYEPQYSYVRMGL

AF171216 (tomato)   AKTIAMISIVDDTFDAYGIVKELEVYTDAIQRWDISHIDRLPDYMKISYK
L04680   (tobacco)  VKTISMISIVDDTFDAYGTVKELEAYTDAIQRWDINEIDRLPDYMKISYK
AF024615  (mint)    AKGVLICGIMDDTYDNYATLNEAQLFTQVLDKWDRDEAERLPEYMKIVYR

AF171216 (tomato)   ALLDLYDDYETELSKDGRSDVVHYAKERMKEIVRNYFVEAKWFIEGYMPP
L04680   (tobacco)  AILDLYKDYEKELSSAGRSHIVCHAIERMKEVVRNYNVESTWFIEGYTPP
AF024615  (mint)    FILSIYENYERDAAKLGKSFAAPYFKETVKQLARAFNEEQKWVMERQLPS
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Cloning into propagation and expression vectors

The secondary degenerate PCR product was gel-purified using a Qiagen gel-purification kit and cloned into a pGEM-T vector (Promega). The resulting plasmid construct containing the putative terpene synthase (TS) PCR product was transformed into chemically competent DH5α *E. coli.* Individual clones containing the pGEM-T vector plus inserts were sequenced at the Iowa State University DNA Sequencing and Synthesis Facility. The sequences of all inserts were identical. One of these clones was used to screen the cDNA library generated from apple peel mRNA with the λZipLox cDNA library construction kit from Life Technologies. This kit utilizes the pZL1 plasmid cloning vector within the λZipLox phage vector. Sequencing of positive pZL1-containing recombinant DH10B (ZIP) clones led to the isolation of a full-length putative TS cDNA. Concurrent experiments employing the 5′ RACE system (Gibco–BRL) and the original PCR product yielded an identical full-length clone.

One of the library clones was used as a PCR template to amplify the TS coding region with restriction sites added at either end to facilitate subsequent in-frame cloning into the pET-3d expression vector. The upstream primer, with the sequence 5′-ATATACCATGGAATTCAGAGTTCA-3′, incorporated the *Nco*I site of the vector as well as the ATG start codon for the TS. Two different downstream primers were used, one incorporating a *myc* epitope (amino acid sequence EQKLISEENL) at the C-terminal end for immunodetection and the other lacking the epitope tag. A *Bam*HI restriction site was engineered into both 3′ primers. The sequence of the 3′ primer including the *myc* tag was 5′-GCCGGATCCTTACAGATCCTCTTCTGAGATGAGTTTTTGTTCGTTTACAAGAAGTT-3′ and the sequence of the primer lacking the epitope tag was 5′-GCCGGATCCTTAGTTTACAAGAGGTT-3′. PCR was performed as stated above, with 500 pg of the pZL1 plasmid containing the full-length putative TS clone serving as the template.

The PCR products were digested to completion with *Nco*I and *Bam*HI, and ligated overnight into the similarly digested pET-3d expression vector. The resulting recombinant plasmids were transformed into BL-21 DE3 (RIL) chemically competent *E. coli* cells, and individual colonies were selected for expression and sequencing analyses. The BL-21 DE3 (RIL) *E. coli* are designed with a plasmid containing eukaryotic arginine, isoleucine, and leucine codons, which are rare in prokaryotes but common in eukaryotic organisms. These rare codons enable optimal expression of the introduced eukaryotic gene in *E. coli.*

Growth of cultures, and induction and analysis of expression

Pilot expression in E. coli

An individual bacterial colony containing the *myc*-epitope-tagged TS plasmid construct was applied to Luria-Bertani (LB) plates containing 100 μg/ml ampicillin and 50 μg/ml chloramphenicol.

Individual colonies from the LB plates were used to inoculate 5-ml portions of LB broth containing 100 μg/ml ampicillin plus 50 μg/ml chloramphenicol. These starter cultures were grown overnight at 37°C on an orbital shaker at 225 rpm. A second group of 5-ml sample cultures was inoculated with 250 μl of starter culture and grown overnight at 37°C. Cells from these sample cultures were pelleted by centrifugation and prepared for Western blotting with 1× SDS–PAGE loading dye according to the cloning vector instructions.

PAGE and Western blots

The level of induction of the *myc*-tagged TS was initially determined via SDS–PAGE, followed by protein immunodetection on Western blots. Specifically, 20 μl of supernatant from the 1× SDS–PAGE samples was loaded onto Novex 4–20% gradient gels and the proteins were separated according to the manufacturer's instructions. The polyacrylamide gel was then stained with Simply Blue Safestain (Invitrogen) per the manufacturer's protocol. For Western blotting, an identical polyacrylamide gel was run, the separated proteins were transferred to nitrocellulose membranes, and the expressed enzyme tagged with the *myc* epitope was detected using mouse-derived anti-*myc* primary antibodies (Invitrogen) and goat:anti-mouse alkaline phosphatase-linked secondary antibody (Sigma).

Northern and Southern blots

Northern and Southern blots of apple fruit peel RNA and leaf genomic DNA, respectively, were performed according to the methods in Sambrook et al. (1989), with slight modifications, as described in Pechous and Whitaker (2002). The radiolabeled probe used on both RNA and DNA gel blots consisted of the TS 3′ untranslated region (UTR; 150 nucleotides) along with the C-terminal 222 nucleotides of the TS open reading frame (ORF).

Sesquiterpene and monoterpene synthase assays

Five-ml portions of LB media supplemented with 100 μg/ml ampicillin and 50 μg/ml chloramphenicol were inoculated with BL-21 DE3 (RIL) *E. coli* colonies containing the empty pET-3d expression vector, the pET-3d–FS4 (untagged TS) construct, or the pET-3d–FSD (*myc*-tagged TS) construct. Cultures were grown overnight at 37°C in an incubator with an orbital shaker operating at 225 rpm. The next day, 2.5-ml portions of the stationary-phase cultures were used to inoculate 50 ml of LB-chlor liquid medium, and the new cultures were placed on an orbital shaker at 37°C for 1.5 h. At this time, 100 μl was removed from each culture and placed on ice for subsequent protein analysis, and isopropyl *β*-D-1-thiogalactopyranoside (IPTG) was added to each culture to a final concentration of 1.7 mM. The 50-ml cultures were placed back on the shaker at 20°C for 6 h, after which 30-μl aliquots were withdrawn for protein analysis and the bulk of the cells were collected by centrifugation (6,000 *g* for 10 min). Cell pellets were resuspended in 2 ml of the appropriate reaction buffer, which for monoterpene synthase assays was 50 mM Tris/HCl (pH 7.5), 500 mM KCl, 1 mM $MnCl_2$, 5 mM dithiothreitol, 0.05% (w/v) $NaHSO_3$, and 10% (v/v) glycerol, and for sesquiterpene synthase assays was 10 mM Na_2HPO_4, 1.8 mM NaH_2PO_4 (pH 7.3), 140 mM NaCl, 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.05% (w/v) $NaHSO_3$, and 10% (v/v) glycerol (Bohlmann et al. 1997). Assays to determine the pH optimum for sesquiterpene synthase activity were conducted over a pH range of 5.0 to 8.0 at pH-unit increments of 0.5. Aliquots of the pH-7.3 sodium phosphate assay buffer listed above were adjusted down to the pH 5.0–7.0 increments with phosphoric acid and up to the pH 7.5–8.0 increments with NaOH. Bacterial cultures were split into seven equal aliquots before pelleting, followed by resuspension and lysis in the seven different pH buffers. For all TS assays, the resuspended cells were chilled on ice and sonicated twice for 10 s at maximum power using a Fisher Model 300 sonic dismembrator fitted with a microprobe. The lysed cells were centrifuged at 14,000 *g* for 25 min, and 1.5 ml of cleared lysate was added to a 12-ml screw-cap test tube with a Teflon-lined cap. Next, 25 μg of substrate [19.2 μM farnesyl diphosphate (FDP) or 22.9 μM geranyl diphosphate (GDP)] was added to start the reaction, except for the pH-optimum assays, in which 10 μg of FDP (7.6 μM) was added. The reaction mix was briefly agitated by hand and overlaid with 1 ml of pentane to trap volatile products. The reaction was allowed to continue at 30°C for 4 h (20 min in pH-optimum assays), at which time 10 μl of 2 M KOH was added to stop enzymatic activity. One ng of R-(+)-limonene (from Sigma–Aldrich) in 1 μl of pentane was added to the pH-optimum assay extracts as an internal standard. Reaction products were extracted from the mix three times with 1.5 ml of pentane. Solvent extracts were pooled and carefully concentrated under a gentle stream of nitrogen until the pentane volume was approx. 100 μl.

TS reaction products were analyzed by gas chromatography–mass spectrometry (GC–MS). A 1-μl aliquot of the concentrated pentane extract was injected into a Shimadzu GCMS GP5050A system fitted with a 30-m CyloSil-B chiral capillary column (0.25 μm film thickness, 0.25 mm i.d.) from Agilent Technologies. The carrier gas was hydrogen at 40 cm/s, and injection was splitless with the injector temperature at 240°C. Column oven temperature was held at 55°C for 1 min then increased linearly to 180°C at 5°C/min. The retention times and electron impact (EI) ionization mass spectra of reaction products from the monoterpene and sesquiterpene synthase assays were compared with those of authentic standards of the monoterpenes geraniol, R-(+)-limonene, linalool (racemic), and (*E*)- and (*Z*)-*β*-ocimene, and the sesquiterpenes nerolidol (racemic) and (*E*,*E*)-*α*-farnesene. Geraniol, limonene, linalool, and nerolidol standards were from Sigma–Aldrich. A sample of synthetic ocimene composed predominantly of the (*E*)-*β* and (*Z*)-*β* isomers was kindly provided by International Flavors and Fragrances, Union Beach, NJ, USA. The two major isomers in the sample were purified by high-performance liquid chromatography (HPLC) on a 5-μm-particle-size Luna C18(2) column (250 mm long, 4.6 mm i.d.; Phenomenex, Torrence, CA, USA) using a Hewlett-Packard 1100 Series HPLC system with photodiode array detection (Agilent Technologies). A 100-μl aliquot of the ocimene sample was dissolved in 1.5 ml of methanol and injected in 30-μl portions. With an isocratic mobile phase composed of methanol (75%), acetonitrile (12.5%), and water (12.5%) at a constant flow rate of 0.8 ml/min, the two major ocimene isomers eluted as a pair of fused peaks with retention times of 14.7 and 14.9 min, and UV absorbance maxima at 232 and 236 nm, respectively. The HPLC fraction eluting from 14.3 to 15.2 min was collected from a series of five HPLC separations and the pooled eluate was extracted with pentane to yield the sample used as a GC–MS standard. Analysis of this preparation by GC with flame ionization detection (GC–FID) on a Hewlett-Packard 5890 gas chromatograph fitted with a 30-m SPB-1 capillary column (0.20 μm film thickness, 0.25 mm i.d.) from Supelco, Bellefonte, PA, USA (column oven isothermal at 60°C, injector 120°C, detector 200°C, and helium as the carrier gas at a head pressure of 35 kPa), showed that it was composed of 65.3% (*E*)-*β*-ocimene (retention time 11.53 min) and 31.5% (*Z*)-*β*-ocimene (retention time 10.69 min). A second ocimene HPLC fraction enriched in the (*E*)-*β* isomer (87% by GC–FID) was obtained by repeated collection of the eluate from 14.3 to 14.8 min. After pentane extraction and evaporation of the solvent under a gentle stream of N_2 while cooling on dry ice, the ocimene was dissolved in 0.8 ml of $CDCl_3$ and analyzed by proton nuclear magnetic resonance (^{1}H-NMR) spectrometry on a Bruker QE 300 MHz NMR spectrometer. The ^{1}H-NMR spectrum was acquired deuterium locked at 25°C and chemical shift values were assigned relative to the frequency of residual $CHCl_3$ externally referenced to trimethylsilane. The NMR data obtained for the major component in the ocimene HPLC sample matched those reported for the (*E*)-*β*-ocimene isomer by Ohloff et al. (1964). In particular, the doublet of doublets at 6.35 ppm from the single proton on C2 indicated the *E* (*trans*) configuration and the triplet at

2.82 ppm from the pair of protons on C5 indicated the β configuration (1,3,6-octatriene, 3,7-dimethyl). Concerning the authentic (*E*,*E*)-α-farnesene standard, Anet (1970), with an examination of all six possible isomers of α- and β-farnesene by UV, IR, NMR, and GC–MS, demonstrated unequivocally that the isomers of farnesene produced in apple fruit are (*E*,*E*)-α and (*Z*,*E*)-α in a ratio of about 300:1. Thus, a sample of 99% pure (*E*,*E*)-α-farnesene was isolated from a hexane extract of peel tissue excised from cold-stored 'Law Rome' apples by a combination of Florisil column chromatography and C_{18} RP–HPLC as described by Whitaker et al. (1997).

Results

Degenerate-primer PCR, gene isolation, and sequencing

The first step in the strategy devised to clone the apple FS gene was to design a set of degenerate PCR primers based on the most conserved region of the deduced amino acid (AA) sequences among the reported sesquiterpene synthase genes from dicotyledonous plants (Fig. 1). These primers were used along with a 3′ poly-T adapter primer for 3′ RACE using cDNA reverse-transcribed from apple peel tissue RNA as the template. Because of the generally low degree of sequence homology among plant TS enzymes, and the fact that at the time of primer design none of the TS genes in GenBank was from a species in the Rosaceae, it seemed that the likelihood of success with this approach was not great. To improve the odds, the RNA used for both the initial RT reaction and construction of a cDNA library was isolated from peel tissue of 'Law Rome' apples that were stored at 0.5°C in air for 4–8 weeks. These fruit were known to be producing α-farnesene at a high rate (Watkins et al. 2000).

The initial degenerate PCR product appeared as a smeared band on a 1% agarose gel. Subsequent optimization via nested PCR with a 3′ adapter primer greatly enhanced product specificity and yielded a sharp band estimated to be approx. 800 bp on the basis of its migration. Multiple clones of this PCR product were sequenced and all were found to be identical. This PCR-amplified gene fragment was used to screen an apple peel cDNA library and several full-length cDNA clones were obtained, each with 1,931 nucleotides. Sequencing of the inserts yielded a TS homologue with an ORF of 1,728 nucleotides encoding a protein 576 amino acids in length, a 53-bp 5′ UTR, and a 150-bp 3′ UTR. The deduced AA sequence of this putative FS (Fig. 2, AFS1, GenBank accession no. AAO22848) shares the greatest degree of homology with a variety of monoterpene synthases, including a (−)-β-pinene synthase (accession no. AAM53945), a γ-terpinene synthase (AAM53943), and a (+)-limonene synthase (AAM53944) from *Citrus limon* (lemon), which have 41–42% AA identity and 59–61% AA similarity (positives). A geraniol synthase (CAD29734) from *Cinnamomum tenuipilum* (cinnamon) and an isoprene synthase (AAQ16588) from *Populus tremuloides* (quaking aspen) also have 42% identity and 60% similarity to AFS1 (Fig. 2). Among the sesquiterpene synthases reported in GenBank, the closest homology is with a linalool/nerolidol synthase (CAD57106) from *Fragaria ananassa* (strawberry; 35% identity, 58% positives) and a (+)-d-cadinene synthase (AAF74977) from *Gossypium hirsutum* (cotton; 33% identity, 55% positives). Analysis of the N-terminal 50 AA encoded by the apple TS gene using the ChloroP chloroplast-targeting sequence analysis program (Emanuelsson et al. 1999) indicated the absence of an organelle-targeting sequence. The encoded protein appears to lack approx. 20 amino acids that are present in the N-terminal chloroplast-targeting region of monoterpene synthases. Thus, the product of this TS gene is most likely localized in the cytosol. The deduced amino acid sequence includes the DDXXD divalent cation-binding domain common to all TS enzymes (Fig. 2; $AA^{326-330}$), as well as an $RR(X_8)W$ motif often found in the N-terminal portion of monoterpene synthases (Fig. 2; AA^{33-43}).

Southern and Northern blots

The DNA gel blot (Southern blot) obtained using the 3′ UTR plus the C-terminal portion of the TS ORF as a probe indicated that there is one copy of the gene per haploid apple genome (data not shown). An RNA gel blot (Northern blot) in which the same probe used in the Southern analysis was used with total RNA extracted from peel tissue of non-treated and 1-MCP-treated fruit stored for 0, 4, or 8 weeks indicated that transcription of the TS gene is regulated by ethylene (Fig. 3). Specifically, 1-MCP treatment at harvest resulted in nearly a total decline in TS transcript between 0 and 8 weeks of storage, whereas in non-treated fruit the level of TS transcript increased about 4-fold during the initial 4 weeks of storage and remained higher at 8 weeks than at harvest.

Western blot and PAGE analysis of TS expression

The full-length ORF from the TS cDNA was inserted into the pET-3d vector and expressed in BL-21 DE3 (RIL) cells both with and without a *myc*-epitope tag. The expression protocol resulted in production of substantial amounts of TS protein with both the tagged and untagged constructs, as indicated by prominent immunoreactive bands on a Western blot (data not shown) and Coomassie-stained bands on a polyacrylamide gel (Fig. 4). However, PAGE analysis also showed that expressed TS protein was present in the soluble cytosolic fraction from bacteria with the untagged construct but absent in the cytosol from cells with the *myc*-tagged construct (Fig. 4). On the basis of their migration relative to standard protein markers, the molecular masses of the untagged and tagged TS proteins were estimated to be about 66 kDa and 68 kDa, respectively.

Fig. 2 Amino acid sequence alignment of AFS1 (accession no. AAO22848) and five related plant terpene synthase (TS) proteins, including a geraniol synthase from *Cinnamomum tenuipilum* (TS2—accession no. CAD29734), a (−)-*β*-pinene synthase from *Citrus limon* (TS3—accession no. AAM 53945), an isoprene synthase from *Populus tremuloides* (TS4—accession no. AAQ 16588), an *α*-farnesene synthase from *Pinus taeda* (TS5—accession no. AAO61226), and an (*E*)-*β*-farnesene synthase from *Mentha piperita* (TS6—accession no. AAB95207). A BLAST search on GenBank showed that TS2, TS3, and TS4 share the greatest homology with AFS1, whereas TS5 and TS6 are functionally related in that they produce isomers of the sesquiterpene farnesene. Sequences were aligned using ClustalW and the alignments are coded as follows: *black highlighting* conserved, *dark gray highlighting* mostly conserved, *light gray highlighting* slightly conserved, *no highlighting* not conserved

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AFS1    1   MEFRVHLQADN--------EQKIFQNQMKPEPEASYLINQ-------------RRSAN
TS2     1 MALQMIAPFPSSF----LPNPRHRLAAHGLTHQKCVSKHISCSTT-TPTYSTTVPRRSGN
TS3     1 MALNLLSSIPAACNFTRLSLPLSSKVNGFVPPITRVQYHVAASTTPIKPVDQTIIRRSAD
TS4     1 MATELLCLHRPISLTHKLFRNPLPKVIQATPLTLKLRCSVSTEN-VSFSETETETRRSAN
TS5     1   MSSLAVDDAE-----------------------------------------RRVGD
TS6     1 MATNGVVISCLREVR---------------------------------------PPMTK

AFS1   38 YKPNIWKNDFLDQSLISKYDGDEYRKLSEKLIEEVKIYIS----AET----MDLVAKLEL
TS2    56 YKPSIWDYDFV-QSLGSGYKVEAHGTRVKKLKEVVKHLLK----ETD-----SSLAQIEL
TS3    61 YGPTIWSFDYI-QSLDSKYKGESYARQLEKLKEQVSAMLQ----QDNKVVDLDTLHQLEL
TS4    60 YEPNSWDYDYL-LSSDTDESIEVHKDKAKKLEAEVRREIN----NEK----AEFLTLLEL
TS5    16 YHPNLWDDALI-QSLSTPYGASPYRDVAEKLIGEIKEMFASISIEDGDDEICYFLQRLWM
TS6    21 HAPSMWTDTFSNFSLDDKEQ-QKCSETIEALKQEARGMLMA---------ATTPLQQMTL

AFS1   90 IDSVRKLGLANLFEKEIKEALDSIAAIESDN-------LGTRDDLYGTALHFKILRQHGY
TS2   106 IDKLRRLGLRWLFKNEIKQVLYTISSDNTS--------IEMRKDLHAVSTRFRLLRQHGY
TS3   116 IDNLHRLGVSYHFEDEIKRTLDRIHNKNT------------NKSLYATALKFRILRQYGY
TS4   111 IDNVQRLGLGYRFESDIRRALDRFVSSGGFD-------GVTKTSLHGTALSFRLLRQHGF
TS5    75 IDNVERLGISRHFENEIKAAMEDVYSRHWSDKGIACGRHSVVADLNSTALAFRTLRLHGY
TS6    71 IDTLERLGLSFHFETEIEYKIELINAAEDDG-----------FDLFATALRFRLLRQHQR

AFS1  143 KVSQ-DIFGRFMDEKGTLEN---HHFAHLKGMLELFEASNLGFEGED-ILDEAKASLTLA
TS2   158 KVST-DVFNDFKDEKGCFKP---SLSMDIKGMLSLYEASHLAFQGET-VLDEARAFVSTH
TS3   164 NTPVKETFSRFMDEKGSFKS--SSHSDDCKGMLALYEAAYLLVEEESSIFRDAKSFTTAY
TS4   164 EVSQ-EAFSGFKDQNGNFLE---NLKEDIKAILSLYEASFLALEGEN-ILDEAKVFAISH
TS5   135 SVCS-DVFKIFQDQKGEFACSADQTEGEIKGILNLLRASLIAFPGER-ILQEAEIFATTY
TS6   120 HVSC-DVFDKFIDKDGKFEES---LSNNVEGLLSLYEAAHVGFREER-ILQEAVNFTRHH

AFS1  198 LRD----SGHICYPDSNLSRDVVHSLELPSHRRVQWFDVKWQINAYEKDIC-------RV
TS2   213 L------MDIKENIDPILHKKVEHALDMPLHWRLEKLEARWYMDIYMREEG--------M
TS3   222 LKEWVIEHDNNKHDDEHLCTLVNHALELPLHWRMPRLEARWFIDVYENGPH--------M
TS4   219 LK-----ELSEEKIGKELAEQVSHALELPLHRRTQRLEAVWSIEAYRKKED--------A
TS5   193 LK-----EALPKIQGSRLSQEIEYVLEYGWLTDLPRLETRNYIEVLAEEITPYFKKPCMA
TS6   175 LEG-----AELDQSPLLIREKVKRALEHPLHRDFPIVYARLFISIYEKDDS--------R

AFS1  247 NATLLELAKLNFNVVQAQLQKNLREASRWWANLGIADNLKFARDRLVECFACAVGVAFEP
TS2   259 NSSLLELAMLHFNIVQTTFQTNLKSLSRWWKDLGLGEQLSFTRDRLVECFFWAAAMTPEP
TS3   274 NPILLELAKVDFNIVQAVHQENLKYASRWWKKTGLGENLNFVRDRIVENFMWTVGEKFEP
TS4   266 NQVLLELAILDYNMIQSVYQRDLRETSRWWRRVGLATKLHFARDRLIESFYWAVGVAFEP
TS5   248 VEKLLKLAKIEFNLFHSLQQTELKHLSRWWKDSGFAQ-LTFTRHRHVEFYTLASCIAMEP
TS6   222 DELLLKLSKVNFKFMQNLYKEELSQLSRWWNTWNLKSKLPYARDRVVEAYVWGVGYHYEP

AFS1  307 EHSSFRICLTKVINLVLIIDDVYDIYGSEEELKHFTNAVDRWDSRETEQLPECMKMCFQV
TS2   319 QFGRCQEAVAKVAQLIIIIDDIYDVYGTVDELELFTNAIDRWDLEAMEQLPEYMKTCFLA
TS3   334 QFGYFRRMSTMVNALITAVDDVYDVYGTLEELEIFTDAVERWDATAVEQLPHYMKLCFHA
TS4   326 QYSDCRNSVAKMFSFVTIIDDIYDVYGTLDELELFTDAVERWDVNAINDLPDYMKLCFLA
TS5   367 KHSAFRLGFAKLCYLGIVLDDIYDTYGKMEELELFTAAIKRWDTSTTECLPEYMKGVYMA
TS6   342 QYSYVRMGLAKGVLICGIMDDTYDNYATLNEAQLFTQVLDKWDRDEAERLPEYMKIVYRF

AFS1  367 LYNTTCEIAREIEEENGWNQVLPQLTKVWADFCKALLVEAEWYNKSHIPTLEEYLRNGCI
TS2   379 LYNSINEIGYEILKEEGRN-VIPYLRNTWTELCKAFLVEAKWYSSGCTPTLEEYLQTSWI
TS3   394 LRNSINEMTFDALRDQGVDIVISYLTKAWADICKAYLVEAKWYNSGYIPPLQEYMENAWI
TS4   386 LYNTINEIAYDNLKDKGEN-ILPYLTKAWADLCNAFLQEAKWLYNKSTPTFDDYFGNAWK
TS5   367 FYDCVNEMARQAEKTQGWD-TLDYARKTWEALIDAFMEEAKWISSGYVPTFQKYLDNGKV
TS6   342 ILSIYENYERDAAK-LGKSFAAPYFKETVKQLARAFNEEQKWVMERQLPSFQDYVKNS-E

AFS1  427 SSSVSVLLVHSFFSITHEGTKEMADFLHKNEDLLYNISLIVRLNNDLGTSAAEQERGDSP
TS2   438 SIGSLPMQTYVFALLGKNLAPESSDFAEKISDILRLGGMMIRLPDDLGTSTDELKRGDVP
TS3   454 SIGATVILVHANTFTANPITKEGLEFVKDYPNIIRWSSMILRFADDLGTSSDELKRGDVH
TS4   445 SSSGPLQLIFAYFAVVQNIKKEEIENLQKYHDIISRPSHIFRLCNDLASASAEIARGETA
TS5   426 SFGYRAATLQPILTLDIPLPLHILQEIDFPSSFNDLASSILRLRGDICGYQAERSRGEQA
TS6   400 KTSCIYTMFASIIPGLKSVTQETIDWIKSEPTLATSTAMIGRYWNDTSSQLRESKGGEML

AFS1  487 SSIVCYMR-EVNASEETARKNIKGMIDNAWKKVNGKCFTTNQVPFLSSFMNNATNMARVA
TS2   498 KSIQCYMH-EAGVTEDVARDHIMGLFQETWKKLN--EYLVESS-LPHAFIDHAMNLGRVS
TS3   514 KSIQCYMH-EAGVSEGEAREHINDLIAQTWMKMNRDRFGNPHF-VSDVFVGIAMNLARMS
TS4   505 NSVSCYMR-TKGISEELATESVMNLIDETWKKMNKEKLG--GSLFAKPFVETAINLARQS
TS5   486 SSISCYMKDNPGSTEEDALSHVNAMIGDKIPEFNWEFMKPSKAPISS--KKYAFDILRAF
TS6   460 TALDFHMK-EYGLTKEEAASKFEGLVEETWKDINKEFIATTNYNVGREIAITFLNYARIC

AFS1  546 HSLYKDGD----GFGDQEKGPRTHILSLLFQPLVN---------------
TS2   554 YCTYKHGDGFSDGFGDPGSQEKKMFMSLFAEPLQVDEAKGISFYVDGGSA
TS3   572 QCMYQFGDGH--GCG-AQEITKARVLSLFFDPIA----------------
TS4   562 HCTYHNGD----AHTSPDELTRKRVLSVITEPILPFER------------
TS5   544 YHLYKYRD----GFSIAKIETKKLVMRTVLDPVPM---------------
TS6   519 EASYSKTDG---DAYSDPNVAKANVVALFVDAIVF---------------
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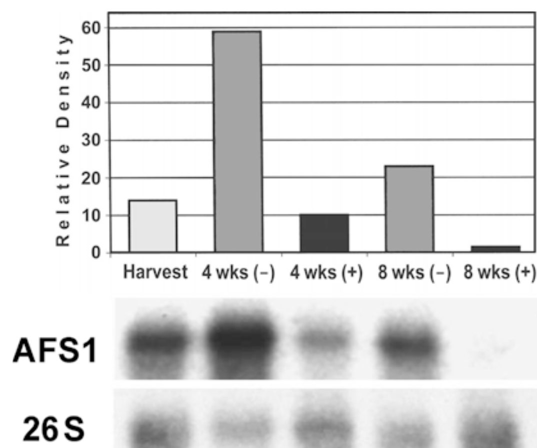

Fig. 3 Northern blot analysis of *AFS1* transcript from peel tissue of 'Law Rome' apple fruit at harvest and after storage for 4 or 8 weeks at 0.5°C in air. The (+) RNA samples were isolated from fruit treated prior to storage with 2 μl/l 1-MCP, an ethylene response inhibitor, and the (–) RNA samples were isolated from control fruit not exposed to 1-MCP but otherwise handled as the 1-MCP-treated fruit were. The *AFS1* gene-specific radiolabeled probe was a DNA fragment consisting of the 150-bp 3′ UTR plus 222 bp of the C-terminal coding region. The bar graph depicts the data from densitometry scans of the *AFS1* mRNA and 26S rRNA loading control autoradiogram bands; relative density values equal the ratio of *AFS1*:26S × 10

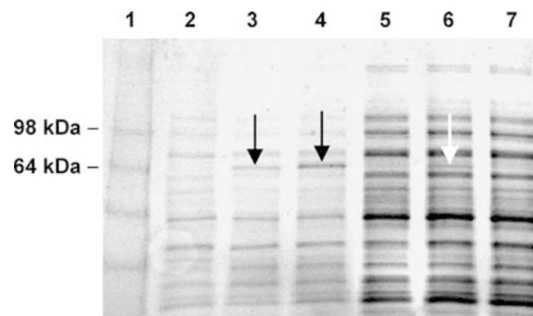

Fig. 4 Coomassie-stained SDS–glycine 4–10% polyacrylamide gel of bacterial cell lysates, including whole cells and the soluble cytosolic fraction. *Lane 1* Molecular mass standards. *Lanes 2–4* Whole-cell lysates of bacteria housing the empty vector, untagged construct, and *myc*-tagged construct, respectively. *Lanes 5–7* Soluble cytosolic fraction from cells housing the empty vector, untagged construct, and *myc*-tagged construct, respectively. *Arrows* indicate the presence of expressed apple AFS1 protein; note that the band for the *myc*-tagged product at approx. 68 kDa is prominent in whole-cell lysates (*lane 4*) but absent in the soluble fraction (*lane 7*)

Enzyme assays

Under the sesquiterpene assay conditions, the untagged TS protein, when present as a soluble enzyme, produced the acyclic sesquiterpene (*E*,*E*)-α-farnesene almost exclusively (Fig. 5). Other sesquiterpenes present in the assay extract in trace amounts were identified as (*Z*,*E*)-α-farnesene (Fig. 5a), and (*E*)-nerolidol and β-farnesene (data not shown). Levels of these minor sequiterpenes were at least two orders of magnitude lower than that of (*E*,*E*)-α-farnesene, and it is not certain that their production was enzymatic. However, it is noteworthy that the ratio of the major (*E*,*E*)-α-farnesene isomer to the minor (*Z*,*E*)-α-farnesene isomer was about 200:1 in both the untagged-TS assay extract and in the α-farnesene sample isolated from peel tissue of stored 'Law Rome' apples (Fig. 5a,b). This value is fairly close to the ratio of 300:1 reported by Anet (1970) for isomers of α-farnesene from 'Granny Smith' apples. Within the limits of detection, there were no sesquiterpene products in assays performed with the soluble cytosolic fraction from bacterial cells including either the empty expression vector or the *myc*-tagged TS construct. Assays to determine the pH optimum for sesquiterpene synthesis by the untagged soluble TS were conducted over the range of pH 5.0–8.0 at increments of 0.5 pH units. Within this pH range, (*E*,*E*)-α-farnesene was the only sesquiterpene produced in appreciable amounts. Maximal activity was observed at pH 7.0 and above. Activity declined below pH 7.0 and at pH 5.0–5.5 it was reduced by ≥50% (data not shown).

As observed in the sesquiterpene synthase assays, the only reaction that clearly yielded a monoterpene product was the one utilizing the cytosolic fraction from bacteria expressing the untagged TS construct. The soluble untagged TS protein produced a small amount of an acyclic monoterpene identified by the NIST '02 GC–MS EI mass spectral library as an isomer of ocimene (Fig. 6a). The GC retention time (12.03 min) and the EI mass spectrum of this monoterpene were essentially identical to those of an authentic standard of (*E*)-β-ocimene (Fig. 6a–d). Low levels of geraniol and two linalool isomers, identified by comparison of their GC retention times and EI mass spectra with those of authentic standards (data not shown), were detected in pentane extracts from all the monoterpene synthase assays regardless of the construct (Fig. 6a). Possibly these monoterpene alcohols were produced by dephosphorylation of GDP, either nonenzymatically or by a bacterial diphosphatase (Croteau and Cane 1985; Rupasinghe et al. 2000b). The levels of (*E*)-β-ocimene produced with GDP as substrate were at least two orders of magnitude lower than those of (*E*,*E*)-α-farnesene produced with a comparable amount of FDP as substrate. No (*E*)-β-ocimene or other monoterpene was produced when the soluble untagged TS was incubated with GDP in the sesquiterpene synthase assay buffer.

Discussion

The presence of an enzyme that converts FDP to (*E*,*E*)-α-farnesene in the outermost tissues of apple fruit was predicted when the sesquiterpene was first discovered in the waxy epicuticular coating of stored apples (Huelin and Murray 1966). Rupasinghe et al. (1998) were able to demonstrate this enzyme activity in crude extracts of apple peel tissue, and subsequently achieved partial purification and characterization of the FS from 'Delicious' apples (Rupasinghe et al. 2000b). There were,

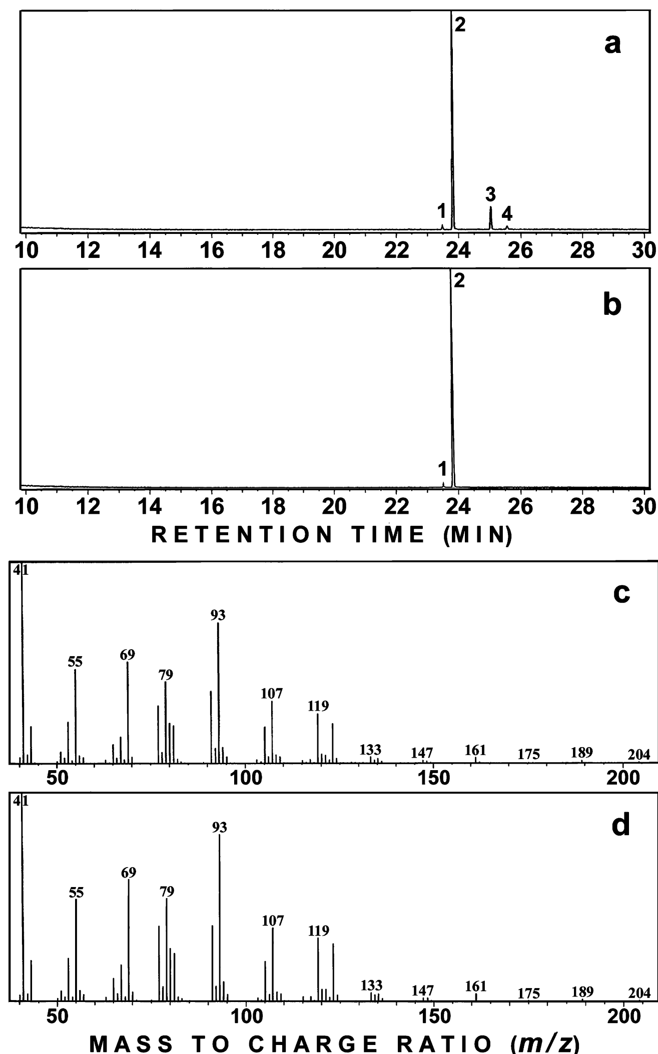

Fig. 5 a,b GC–MS total ion chromatograms of reaction products from a sesquiterpene synthase assay of bacterially expressed AFS1 (**a**) and an HPLC-purified (*E*,*E*)-α-farnesene standard from peel tissue of cold-stored 'Law Rome' apple fruit (**b**). *Peak 1* (retention time 23.50 min) and *peak 2* (retention time 23.83 min) in both chromatograms were identified by the NIST ′02 GC–MS EI mass spectral library as (*Z*,*E*)- and (*E*,*E*)-α-farnesene, respectively, in accord with the report by Anet (1970) for farnesene isomers in apple fruit. *Peaks 3* and *4* in **a** are non-terpene bacterial contaminants that were present in all TS assay extracts (data not shown). **c** GC–MS EI mass spectrum of the predominant sesquiterpene product of bacterially expressed AFS1 (*peak 2* in **a**). **d** GC–MS EI mass spectrum of (*E*,*E*)-α-farnesene isolated from peel tissue of cold-stored 'Law Rome' apple fruit (*peak 2* in **b**)

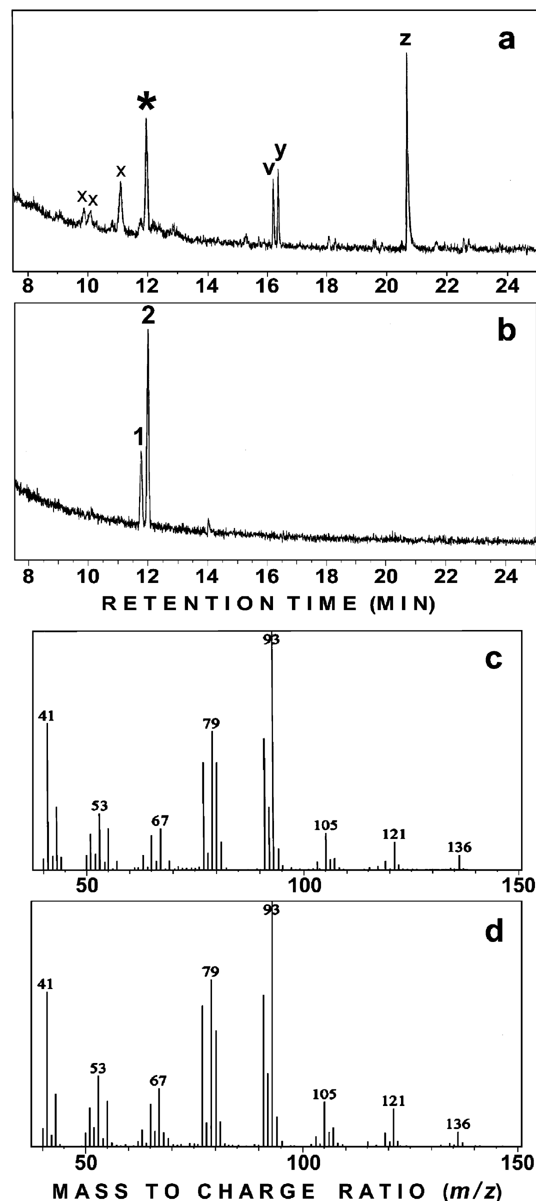

Fig. 6 a GC–MS total ion chromatogram of reaction products from a monoterpene synthase assay of bacterially expressed AFS1. The peak with a retention time of 12.03 min and marked with an *asterisk* (*) was the only monoterpene product of AFS1. *Peaks v* and *y* were identified as linalool isomers and *peak z* as geraniol by comparison of their retention times and mass spectra with those of authentic standards (data not shown). These three compounds, as well as three non-terpene contaminants (marked *x*), were present in pentane extracts from all monoterpene synthase assays, including those with the cytosolic fraction from bacteria housing the empty expression vector (data not shown). **b** GC–MS total ion chromatogram of an authentic standard including (*Z*)-β-ocimene (*peak 1*, retention time 11.79 min) and (*E*)-β-ocimene (*peak 2*, retention time 12.03 min). **c** GC–MS EI mass spectrum of the peak marked with an *asterisk* (*) in **a**, the only detectable monoterpene product of AFS1. **d** Matching GC–MS EI mass spectrum of authentic (*E*)-β-ocimene (*peak 2* in **b**)

however, limitations to this classical enzymological approach. In particular, activity was low and was lost altogether with attempts to purify the apple FS to homogeneity. Moreover, nonspecific diphosphatases in the apple tissue extracts cleaved much of the radiolabeled FDP substrate to farnesol, and addition of diphosphatase inhibitors (vanadate, molybdate, and fluoride) inhibited FS activity as well. In light of these difficulties, we have undertaken a molecular genetic approach to isolating and characterizing the FS gene and its encoded enzyme from apple.

The combined use of RNA extracted from peel tissue of cold-stored apple fruit exhibiting a high rate of (*E*,*E*)-α-farnesene production and a degenerate primer based on a conserved region of known sesquiterpene synthases

ultimately yielded a full-length putative TS cDNA. Functional expression of the untagged coding region in *E. coli* showed unequivocally that the cDNA encodes an FS, which, unlike many plant TS enzymes, makes this one sesquiterpene product almost exclusively.

Fruit of the apple cultivar Law Rome that were used in this work are quite susceptible to the storage disorder superficial scald and accumulate high concentrations of (*E*,*E*)-α-farnesene, and subsequently its conjugated trienol oxidation products, during low-temperature storage in an air atmosphere (Watkins et al. 2000). For apples from a number of scald-susceptible cultivars, pre-storage treatment with diazocyclopentadiene or 1-MCP, potent blockers of ethylene action, greatly reduced the incidence and severity of scald, as well as the accumulation of (*E*,*E*)-α-farnesene and its oxidation products (Gong and Tian 1998; Fan et al. 1999; Rupasinghe et al. 2000a; Watkins et al. 2000). The results of our Northern analysis of the levels of *AFS1* transcript in peel tissue of non-treated and 1-MCP-treated 'Law Rome' apples are consistent with these observations. In non-treated controls, *AFS1* mRNA more than quadrupled in the first 4 weeks of storage and remained elevated after 8 weeks, whereas in 1-MCP-treated fruit, *AFS1* mRNA declined substantially in the first 4 weeks and was scarcely detectable after 8 weeks (Fig. 3). We conclude that ethylene-induced expression of *AFS1* likely plays a key role in the burst of (*E*,*E*)-α-farnesene synthesis during the initial 8 weeks of low-temperature storage.

The deduced AA sequence of the protein encoded by *AFS1* shares no more than 43% identity with the TS enzymes included in GenBank. Overall, the AFS1 AA sequence has greater homology with an array of monoterpene synthases than with any sesquiterpene synthase. One facet of this closer similarity to monoterpene synthases is the presence of an RR(X_8)W motif in the N-terminal region, which to our knowledge has been reported in only one other sesquiterpene synthase, an α-farnesene synthase recently cloned from loblolly pine (Phillips et al. 2003). The RR(X_8)W motif is a characteristic feature of two monoterpene subfamilies of TS enzymes, *Tps-b* and *Tps-d*, and is known to be involved in the reaction mechanism of these enzymes, although it is not universally required for monoterpene synthase activity (Dudareva et al. 2003). It can now be inferred that the RR(X_8)W motif probably plays a role in the reaction mechanism of two sesquiterpene synthases that yield the same acyclic product, (*E*,*E*)-α-farnesene. The sesquiterpene synthase in GenBank most similar to AFS1 is also a monoterpene synthase; a linalool/nerolidol synthase from strawberry (accession no. CAD57106) that lacks the RR(X_8)W motif but does include a plastid targeting sequence and an RR pair in the N-terminal region (note: this sequence was not retrieved in a BLAST search of AFS1 because it is part of a patent application and is listed as an "unnamed protein"). In this case, the degree of homology between the two TS enzymes may reflect the relatively close taxonomic relationship of the two species. Finally, neither the α-farnesene synthase from loblolly pine nor the (*E*)-*β*-farnesene synthase from mint (accession nos. AAO61226 and AAB95209, respectively) has much sequence similarity to AFS1 (33% and 29% AA identity, respectively). Hence, a high degree of homology is clearly not required for similar or even identical enzymatic activity.

In a series of experiments early in our investigation, *myc*-tagged and untagged AFS1 expressed protein in bacterial inclusion-body fractions was urea-denatured, purified, and renatured prior to assay of enzymatic activity. Both the *myc*-tagged and untagged enzyme preparations yielded very low levels of the sesquiterpene alcohol, (*E*)-nerolidol (data not shown). Improper refolding of the inclusion-body enzymes is one explanation for the (low level) synthesis of nerolidol rather than (*E*,*E*)-α-farnesene. An exhaustive screening of expression conditions with the *myc*-tagged *AFS1* construct failed to establish a protocol that yielded soluble *myc*-tagged enzyme. Consequently, it is not know whether the C-terminal epitope tag has any influence on either enzymatic activity or the sesquiterpene product(s).

Cloning of the *AFS1* cDNA has provided a new tool for testing the validity of the (*E*,*E*)-α-farnesene oxidation–scald induction hypothesis, which has prevailed for more than 30 years (Huelin and Coggiola 1970) but remains unproven. Opponents of the hypothesis have raised the question of cause and effect, i.e., are (*E*,*E*)-α-farnesene oxidation products truly the cause of scald development, or are they merely one manifestation of unbridled active oxygen species and free-radical reactions that lead to tissue injury and necrosis (Rupasinghe et al. 2000b). Molecular genetic manipulation to achieve marked suppression or knockout of *AFS1* expression in a highly scald-susceptible apple cultivar such as 'Law Rome' should now be possible, and ultimately could prove or disprove a direct role of (*E*,*E*)-α-farnesene oxidation in the induction of superficial scald. If suppression of *AFS1* is shown to prevent scald, this technology could be applied to create new scald-resistant apple lines, eliminating the need to treat fruit with DPA plus a fungicide prior to low-temperature storage. In addition, because α-farnesene attracts codling moth larvae and adult females (Sutherland and Hutchins 1972; Landolt et al. 2000; Bengtsson et al. 2001) and stimulates oviposition by the latter (Wearing and Hutchins 1973), suppression of *AFS1* could have the secondary benefit of reducing infestation by this serious insect pest.

Conversely, it is probable that ethylene-induced production of (*E*,*E*)-α-farnesene in apple has some beneficial function. It is noteworthy that apple branches produce this sesquiterpene upon wounding (Bengtsson et al. 2001), most likely in response to wound ethylene, and as mentioned previously, (*E*,*E*)-α-farnesene is the predominant terpene produced in harvested apple fruit (Huelin and Murray 1966; Meigh and Filmer 1969). Moreover, we have shown that expression of *AFS1* is tightly regulated and that the enzyme encoded by *AFS1*,

unlike many TS enzymes, makes essentially one product. One interpretation of these findings is that some manner of evolutionary pressure has ensured that (*E*,*E*)-α-farnesene is produced in apple tissues under certain conditions of stress or at a specific stage of development, as was recently proposed for a valencene synthase gene from citrus fruit (Sharon-Asa et al. 2003). Two possible and not necessarily mutually exclusive roles for (*E*,*E*)-α-farnesene are that it acts as a defense compound or that it serves as an attractant for animals that eat the ripe fruit then disperse the seed. Concerning the defense function, (*E*,*E*)-α-farnesene emitted as a consequence of *Psylla* infestation of pear trees was found to be a potent attractant of predatory anthocorid insects (Scutareanu et al. 1997). In addition, 6-methyl-5-hepten-2-one, a volatile oxidation product of (*E*,*E*)-α-farnesene (Whitaker and Saftner 2000), was shown to attract parasitoid wasps to bean plants infested with aphids (Du et al. 1998).

Finally, we and others have observed a high degree of natural variation in the production of (*E*,*E*)-α-farnesene among fruit of different apple cultivars (Huelin and Coggiola 1968; Ingle and D'Souza 1989; Whitaker et al. 1997, 1998; Rupasinghe et al. 1998). Whether this variation is a function of differences in transcription and/or translation of *AFS1* and other genes encoding enzymes of the (*E*,*E*)-α-farnesene biosynthetic pathway will be the subject of further investigation in our laboratory. We also intend to clone and analyze the *AFS1* promoter, which should lead to an understanding of how ethylene regulates expression of this gene.

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