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Sesquiterpene α-Farnesene Synthase: Partial Purification, Characterization, and Activity in Relation to Superficial Scald Development in Apples

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ABSTRACT. **To decipher the relation between α-farnesene metabolism and the development of superficial scald in apples, *trans,trans*-α-farnesene synthase, the enzyme that catalyzes the conversion of farnesyl pyrophosphate to α-farnesene, was partially purified from skin tissue of 'Delicious' apples (*Malus ×domestica* Borkh.) and characterized. Total and specific activities of the enzyme were higher in the cytosolic fraction than in membrane fractions. α-Farnesene synthase was purified 70-fold from the cytosolic fraction by ion exchange chromatography and gel permeation, and the native molecular weight was estimated to be 108,000. The enzyme had optimal activity at a pH of 5.6 and absolutely required a divalent metal ion such as Mg^{2+} or Mn^{2+} for activity. It exhibited allosteric kinetics, $S_{(0.5)}$ for farnesyl pyrophosphate being 84 ± 18 μmol·L^{-1}, and a Hill coefficient (n_H) of 2.9, indicating the number of subunits to be two or three. Enzyme activity was highest between 10 and 20 °C, while 50% of the maximal activity was retained at 0 °C. In vivo α-farnesene synthase activity was minimal at harvest, then increased rapidly during 16 weeks storage in air at 0 °C, and decreased during further storage. Activity of α-farnesene synthase, α-farnesene content, and conjugated triene alcohol (the putative scald-causing oxidation product of α-farnesene) content in skin tissue were not correlated to the inherent nature of scald susceptibility or resistance in 11 apple cultivars tested.**

Superficial scald is a serious postharvest physiological disorder affecting several cultivars of apple (*Malus×domestica* Borkh.). The disorder is characterized by an uneven browning or bronzing of the skin, associated with death of the hypodermal cells, along with the development of skin wrinkling and pitting with increasing severity (Bain and Mercer, 1963). Susceptibility to scald is determined by several factors such as cultivar differences, environmental conditions that apples are exposed to during growth and development, stage of maturity at harvest, and storage atmosphere (Ingle and D'Souza, 1989). Fruit of the apple cultivars Delicious, McIntosh, Cortland, Granny Smith, Idared, Rome Beauty, and Fuji are susceptible to this disorder, whereas those of cultivars such as Empire, Gala, Mutsu, Jonagold, and Northern Spy are resistant to scald development (Emongor et al., 1994).

α-Farnesene, an acyclic sesquiterpene hydrocarbon ($C_{15}H_{24}$; [3*E*,6*E*]-3,7,11-trimethyl-1,3,6,10-dodecatetraene) which is a constituent of the skin cell layers, has been associated with the occurrence of scald. Oxidation products of α-farnesene that include intermediate hydroperoxides and conjugated trienes have been suggested to perturb membrane lipids, causing disruption of endodermal cells and thereby leading to the 'bronzed' appearance on the skin of scalded apples (Anet, 1972; Huelin and Coggiola, 1970a, 1970b). Conjugated trienes, as measured by ultraviolet (UV) spectroscopy at 258, 269, and 281 nm, progressively accumulate on the surface of apples during storage and closely correlate with the occurrence and severity of superficial scald (Chen et al., 1990; Gallerani and Pratella, 1991; Ghahramani and Scott, 1998; Huelin and Coggiola, 1970b; Meir and Bramlage, 1988). Most of the early studies have relied on direct UV absorbance measurements of crude extracts of the skin for quantification of α-farnesene and conjugated trienes. Concentrations of trienes measured by high-performance liquid chromatography (HPLC) were only 12% to 35% of the triene concentration estimated by UV spectroscopy (Rowan et al., 1995), indicating that previous analyses of apple skin washes may have seriously overestimated the triene concentrations. Rowan et al. (1995) also identified a conjugated triene alcohol [CTOL (2,6,10-trimethyldodeca-2,7*E*,9*E*,11-tetraen-6-ol)] as the major component of conjugated trienes in the apple cuticle, accounting for 88% to 95% of the total, and its 7*E*,9*Z* isomer constituting most of the remainder. CTOL also possessed UV absorption maxima at 258, 269, and 281 nm, characteristic of conjugated trienes (Spicer et al., 1993). CTOL is capable of producing scald-like symptoms on stored apples in bioassays (Brimble et al., 1994; Rowan, Personal Communication).

α-Farnesene is synthesized in apple skin tissue by the direct conversion of *trans,trans*-farnesyl pyrophosphate (FPP) (Rupasinghe et al., 1998). The step is catalyzed by a single sesquiterpene synthase enzyme, *trans,trans*-α-farnesene synthase. Interestingly, in vivo *trans,trans*-α-farnesene synthase activity was nearly three times lower in scald-developing skin tissue when compared with scald-free skin tissue (Rupasinghe et al., 1998). In addition, hexane-extractable α-farnesene content of skin tissue measured by HPLC (Rupasinghe et al., 1998; Whiting et al., 1997), and α-farnesene evolution into the headspace (Paliyath et al., 1997; Whiting et al., 1997) were 2- to 3-fold greater in scald-free sides than scald-developing sides of apples. These observations raised the question whether differences in α-farnesene levels between scald-developing and scald-free tissues are due to differences in biosynthesis of α-farnesene or its catabolism. In general, sesquiterpene biosynthesis is catalyzed by sesquiterpene cyclases or synthases (Cane, 1981; Croteau and

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Cane, 1985). Sesquiterpene synthases have been purified and characterized from several higher plants that include sage (*Salvia officinalis* L.) (Croteau and Gundy, 1984; Dehal and Croteau, 1988), patchouli (*Pogostemon cablin* Benth.) (Croteau et al., 1987), tobacco (*Nicotiana tabacum* L.) (Vögeli et al., 1990), calamondin (*Citrofortunella mitis*) (Belingheri et al., 1992), potato (*Solanum tuberosum* L.) (Zook et al., 1992), maritime pine (*Pinus pinaster* Ait.) (Salin et al., 1995), and cotton (*Gossypium hirsutum* L.) (Davis et al., 1996).

A synthetic antioxidant, diphenylamine (DPA), inhibits oxidation of α-farnesene to conjugated trienes (Chen et al., 1990; Huelin and Coggiola, 1970a, 1970b; Smock, 1955) and is used commercially to prevent the development of scald (Ingle and D'Souza, 1989). However, future use of DPA is under scrutiny because of its possible undesirable biological degradation products (Kim-Kang et al., 1998). The development of alternative control strategies requires a better understanding of the role of α-farnesene and its oxidation products in the development of superficial scald. In particular, the ability to markedly down regulate or inhibit α-farnesene biosynthesis could be used to demonstrate unequivocally the physiological role of α-farnesene in scald development and help develop alternative strategies to control superficial scald. Thus, the objective of the present study was to characterize *trans,trans*-α-farnesene synthase and examine the relation between α-farnesene metabolism and susceptibility to superficial scald development in apples.

Materials and Methods

Plant material. 'Delicious' apples were harvested at optimum maturity during the first week of Oct. 1996, from a commercial grower in Meaford, Ontario. Apples were cooled to 0 °C within 8 h of harvest and stored at 0 °C in air or in controlled-atmosphere (CA) of 3 kPa O_2 and 2.5 kPa CO_2. These apples were used for extraction, purification, and characterization of α-farnesene synthase. For monitoring α-farnesene synthase activity, and α-farnesene and CTOL contents of the skin of 'Delicious' and 'Empire' apples during storage, and comparing the activity and content among different cultivars, apples were harvested at commercial maturity from the Horticultural Research Station, Simcoe, Ont. between 14 Sept. and 18 Oct. 1997. Apples were stored as above.

Substrates and chemical reagents. Radiolabelled *trans,trans*-[1-^{3}H]farnesyl pyrophospate (FPP) (7.4×10^{14} Bq·mol^{-1}) was purchased from American Radiolabelled Chemicals (ARC), Inc., St. Louis, Mo. A standard mixture of α and β isomers of farnesene was obtained from TCI, Tokyo; standards of farnesol and farnesyl acetate were from Aldrich Chem. Co., Milwaukee, Wis.; and NAD, NADH, NADP, NADPH, ATP, and ADP were from Boehringer Mannheim, Canada Inc., Montreal, Que. Synthetic CTOL (2,6,10-trimethyldodeca-2,7*E*,9*E*,11-tetraen-6-ol) was a gift from D.D. Rowan, Horticulture and Food Research Institute Ltd., New Zealand. All other chemicals were obtained from Sigma-Aldrich Canada Ltd., Oakville, Ont.

In vitro enzyme assay. α-Farnesene synthase activity was determined by incubating the enzyme extract (200 µg of protein, unless otherwise mentioned) in a final volume of 1 mL reaction mixture containing 0.1 mol·L^{-1} MES buffer (pH 5.6), 10 mmol·L^{-1} $MgCl_2$, and 0.25 mmol·L^{-1} $MnCl_2$. Assays were carried out at 20 °C in 4.5 mL amber glass vials. The reaction was started by the addition of 50 µL of radiolabelled substrate prepared as follows. Ten microliters of authentic [1-^{3}H]FPP (ARC) was transferred to a test tube and adjusted to 1 mL final volume by adding 990 µL of 0.1 mol·L^{-1} MES buffer [pH 5.6] and shaken vigorously. Fifty microliters of this solution contained ≈25 pmol of radiolabelled FPP. After 20 min of incubation with gentle shaking, the reaction was terminated by adding 0.1 mL of 1 mol·L^{-1} KOH and 1 mL of *n*-hexane followed by vigorous mixing. Control experiments were performed with boiled enzyme in which nonenzymatic formation of α-farnesene was negligible.

The influence of several cofactors (NAD, NADH, NADP, NADPH, ATP, and ADP) were tested at 20 and 50 mmol·L^{-1} and antioxidants (ascorbic acid and a-tocopherol) at 100 to 50 mmol·L^{-1}. To create anaerobic conditions, reaction vials were flushed three times for 1 min each with pure N_2 before adding the substrate. To assess the possibility of blocking farnesol formation from FPP, three pyrophosphatase inhibitors (ammonium vanadate, ammonium molybdate, and sodium fluoride) were tested independently at concentrations of 1 to 10 mmol·L^{-1}. The experiments of enzyme activity as a function of substrate concentration and calculation of $S_{(0.5)}$ were conducted using substrate containing hot and cold FPP in a ratio of 990 Bq **:**1 nmol of cold FPP.

In vivo enzyme assay. Cylindrical pieces of tissue were removed from the equatorial regions of fruit using a cork borer (1 cm diameter), and the skin tissues (1 to 2 mm thick; 105 ± 12 mg fresh weight) were excised using a surgical blade. Two tissue disks were incubated in 4.5 mL amber glass vials containing 950 µL of 0.1 mol·L^{-1} MES buffer (pH 5.6). The vials were sealed tightly using screw caps with teflon–silicon septa. The reaction was started by adding 50 µL of radiolabelled substrate (equivalent to ≈25 pmol substrate) to the reaction vial containing skin tissues. The skin tissues were subjected to vacuum infiltration to facilitate the uptake of the radiolabelled substrate. After 1 h of incubation with gentle shaking, the reaction was terminated by adding 0.1 mL of 1 mol·L^{-1} KOH and 1 mL of *n*-hexane followed by vigorous mixing.

Separation and analysis of radiolabelled product. A one-half milliliter aliquot of the *n*-hexane extract was transferred to a test tube which contained 20 µL of a 0.05% mixture of farnesene isomers and evaporated to dryness under N_2. The residue remaining was redissolved in 50 µL of *n*-hexane and a 25 µL aliquot was spotted on a thin layer chromatography plate (TLC; silica gel G, 20 × 20 cm, LK5D, Whatman Inc., Clifton, N.J.). The plates were developed completely with hexane:ethyl ether (4:1, v/v) and dried under N_2. α-Farnesene (R_f = 0.66) was visualized in the presence of iodine vapor. The region of the plate corresponding to authentic standard was scraped, mixed with 5 mL of scintillation cocktail (Ecolume, ICN, Costa Mesa, Calif.), and radioactivity determined by liquid scintillation counting (Beckman LS6800, Beckman Canada, Inc., Mississauga, Ont.). The assay product separated by TLC and corresponding to farnesene was eluted and compared with the elution of authentic farnesene isolated by HPLC, which was again confirmed to be exclusively α-farnesene by GC–MS analysis as described by Paliyath et al. (1997).

Enzyme extraction and purification. Since in vivo synthesis of α-farnesene was found mainly in the skin rather than cortex tissue of apple fruit (Rupasinghe et al., 1998), skin tissue was used for enzyme preparation. About 200 g of outer cortical tissue (0.25 to 0.5 cm depth) including the cuticle was removed and cut into 0.25 to 0.5 cm^3 pieces and homogenized using a Polytron homogenizer (Brinkmann Instruments, Westbury, N.Y., model PT 10/35) for 1 min in 150 mL of 0.1 mmol·L^{-1} sodium phosphate buffer (pH 6.5) containing 0.25 mol·L^{-1} sucrose, 50 mmol·L^{-1} ascorbic acid sodium salt, 2 mmol·L^{-1} EDTA, 1 mmol·L^{-1} DTT, 10 mmol·L^{-1} $MgCl_2$, 1

mmol·L^{-1} PMSF, and 50 g·L^{-1} polyvinylpyrrolidone. The homogenate was filtered through four layers of cheesecloth, the filtrate was centrifuged at 750 g_n for 10 min to remove starch and debris, and the supernatant (crude extract) was used as the enzyme source. The crude extract was centrifuged at 10,000 g_n for 20 min. The resultant pellet was resuspended in 0.1 mol·L^{-1} sodium phosphate buffer (pH 6.5) containing 0.25 mol·L^{-1} sucrose, 1 mmol·L^{-1} $MgCl_2$, and 1 mmol·L^{-1} DTT (buffer A) and comprised the chloroplast/mitochondrial membrane fraction (Edward and Gardestrom, 1987). The supernatant was centrifuged again at 105,000 g_n for 60 min to yield the microsomal membrane pellet and cytosol. The pellet obtained after ultracentrifugation was resuspended as mentioned above to provide the microsomal fraction. For membrane solubilization studies, a total membrane fraction was obtained by centrifuging crude extract at 105,000 g_n for 60 min and resuspending the resulting pellets as mentioned earlier. One hundred micrograms of total membrane was incubated with either 0.5 to 5 mmol·L^{-1} CHAPS (3-[-3-cholamidopropyl] di-methylammonio-1-propanesulphonate) or 0.01% to 0.1% Triton X-100 for 60 min at 4 °C before the enzyme assay.

The cytosolic fraction was loaded on a DEAE Sephacel column (1.6 × 25 cm) previously equilibrated with buffer A at a flow rate of 0.5 mL·min^{-1}. The column was washed with 75 mL of the same buffer to remove unbound proteins. The bound proteins were eluted with 5 mL volumes of 0.1 mol·L^{-1} to 0.6 mol·L^{-1} NaCl in 0.05 mol·L^{-1} steps (0.1, 0.15, 0.2 … mol·L^{-1}). α-Farnesene synthase was estimated in each fraction and the most active fractions were pooled and used for gel permeation chromatography. All steps were carried out at 4 °C. A Waters AP-1 column (10 × 500 mm, Waters Ltd., Mississauga, Ont.) packed with Sephacryl S300-HR equilibrated with 0.01 mol·L^{-1} Tris buffer (pH 6.5) was used for size exclusion chromatography at a flow rate of 0.7 mL·min^{-1} using a HPLC system (Waters 626 LC system). α-Farnesene synthase activity was determined in 1.4 mL fractions. Protein concentrations were determined according to the method of Bradford (1976) using bovine serum albumin as the standard.

Estimation of relative molecular weight. The Sephacryl column was calibrated with a Sigma MW-GF-200 kit that consisted of the following standard markers; horse heart cytochrome C (12,400), bovine erythrocytes carbonic anhydrase (29,000), bovine serum albumin (66,000), yeast alcohol dehydrogenase (150,000), and sweet potato α-amylase (200,000). The void volume (V_o) was determined with Blue Dextran (2,000,000) using 0.01 mol·L^{-1} Tris buffer (pH 6.5). The elution profile was monitored by absorbance at 280 nm.

Analysis of a-farnesene, CTOL, methyl heptenone (MHO), and methyl heptenol (MHOL) content in the skin using HPLC. Two segments of fruit skin tissue, prepared as described for the in vivo enzyme assay, were immersed in 2 mL of *n*-hexane and kept for 24 h at 4 °C in a closed 4.5 mL amber glass vial. Hexane-soluble a-farnesene and its catabolites were analyzed by HPLC using a Nova-Pak C_{18} column (3.9 ¥ 150 mm). The absorbance maxima used for a-farnesene and CTOL were 233 and 269 nm, respectively. MHO (6-methyl-5-hepten-2-one) and MHOL (6-methyl-5-hepten-2-ol) were monitored at 210 nm, the absorbance maximum determined for these compounds. Acetonitrile (100 %) was used as the mobile phase, at a flow rate of 0.75 mL·min^{-1}. Elution times for farnesene, CTOL, MHO, and MHOL were 3.1, 2.34, 1.98, and 2.14 min, respectively. Quantification of each metabolite was performed using a standard curve prepared with the corresponding authentic compound.

Statistical analysis. Determinations of the effects of pH, buffers, metal ions, heterotrophic effectors, and temperature on α-farnesene synthase activity were conducted in triplicate and analyzed statistically using a completely randomized experimental design (CRD). Analyses of in vivo α-farnesene synthase activity, and of α-farnesene (skin and headspace) and CTOL content in the skin during storage of different apple cultivars were performed also using CRD with three replicates. Time of removal from storage and cultivar were assigned as the main factors of the factorial treatment combination. Replicates of 10 apples each were obtained randomly from three boxes (a box contained 80 to120 apples) of each specific cultivar which had been stored in air at 0 °C. All statistical analyses were done using SAS release 6.12 (SAS Inst., Inc., Cary, N.C.).

Results

α-Farnesene synthesis during in vitro reaction. α-Farnesene synthase activity was low in the various preparations analyzed. During the time-course studies of α-farnesene formation, the rate of reaction was nearly linear up to 20 min and reached a plateau at 30 min. Therefore, in further experiments the incubation period was restricted to 20 min to obtain an optimal level of α-farnesene among the reaction products. In general, the crude extract from CA-stored apples converted 0.1% to 1% of [1-^{3}H]FPP to α-farnesene. However, nearly 20% of the radiolabel from [1-^{3}H]FPP was converted to farnesol, possibly due to the action of pyrophosphatases (Croteau and Karp, 1979; Dehal and Croteau, 1988). Addition of pyrophosphatase inhibitors such as ammonium vanadate, ammonium molybdate, or sodium fluoride (1 to 10 mmol·L^{-1}) during enzyme assays (Croteau and Karp, 1979) not only substantially blocked farnesol formation, but also adversely affected α-farnesene synthase activity. Conducting the enzyme assay under anaerobic conditions or in the presence of antioxidants (ascorbic acid and α-tocopherol) either marginally improved or inhibited α-farnesene synthase activity (data not presented). This suggests that the α-farnesene formed is not spontaneously oxidized to other products and lost during separation and analysis.

Buffer and pH. The effect of pH on α-farnesene synthase activity was determined using 0.1 mol·L^{-1} acetate and sodium phosphate buffers (pH range from 4 to 8). Enzyme activity increased rapidly from pH 5.2, reached a maximum at pH 5.6 and

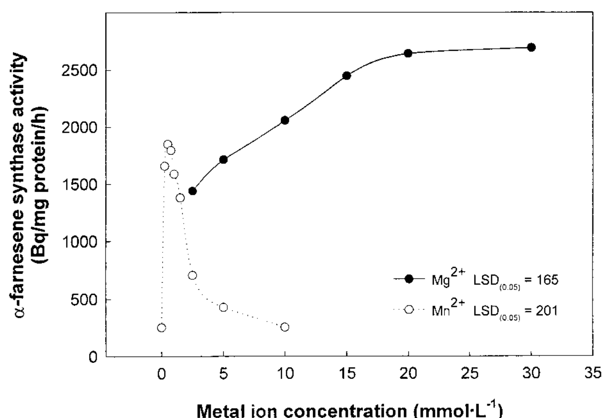

Fig. 1. Effects of $MgCl_2$ (●) and $MnCl_2$ (○) on α-farnesene synthase activity. The enzyme was extracted from skin tissue of apples stored in CA for 5 months and assayed in the presence of 100 mmol·L^{-1} MES buffer at pH 5.6. Each data point is a mean of three replicates. Each assay was provided with 1.85 × 10^4 Bq radiolabelled FPP.

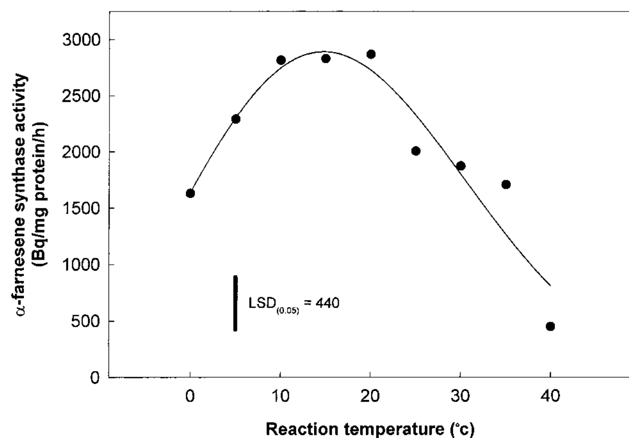

Fig. 2. Influence of incubation temperature on α-farnesene synthase activity. Enzyme was extracted from skin tissue of apples stored in CA for 5 months. The activity was assayed as described previously in the presence of 100 mmol·L^{-1} MES buffer at pH 5.6, containing 10 mmol·L^{-1} $MgCl_2$, 0.25 mmol·L^{-1} $MnCl_2$ and 5 % sucrose. Each data point is a mean of three replications.

declined thereafter, showing a typical bell-shaped activity profile. Therefore, all subsequent assays were conducted at pH 5.6. Similar properties have been reported for most other sesquiterpene synthases with pH optima ranging from 6 to 7 (Croteau and Cane, 1985; Croteau et al., 1987; Dehal and Croteau, 1988; Salin et al., 1995; Vögeli et al., 1990; Zook et al., 1992). The activity of α-farnesene synthase was compared in four different buffer systems at pH 5.6 [acetate, MES (2-[N-morpholino] ethanesulfonic acid), Tris-HCl, and sodium phosphate] each at 50 and 100 mmol•L^{-1}. The ionic strength of the buffer systems had no significant effect on enzyme activity ($P = 0.05$), but the highest activity was obtained in MES (pK_a at 25 °C = 6.1) and hence this buffer was used for subsequent enzyme assays.

Cofactor requirement—metal ions. The only cofactor required for α-farnesene synthase activity was a divalent metal ion, specifically Mg^{2+} or Mn^{2+}. The highest enzyme activity with Mn^{2+} was at 0.5 mmol•L^{-1}, and at Mn^{2+} concentrations above 0.5 mmol•L^{-1} activity was decreased (Fig. 1). α-Farnesene synthase activity increased with increasing concentration of Mg^{2+} up to 20 mmol•L^{-1} and further increases in Mg^{2+} concentration did not promote enzyme activity (Fig. 1). A relatively high activity was observed at 0.25 mmol•L^{-1} $MnCl_2$ and 10 mmol•L^{-1} $MgCl_2$ in combination.

Temperature. α-Farnesene synthase activity increased from 0 to 10 °C, reached a plateau between 10 and 20 °C, and declined rapidly above 20 °C (Fig. 2). Enzyme activity was lowered by only 50% at 0 °C compared with the activity at the optimum temperature range (10 to 20 °C) (Fig. 2). This observation supports our notion that α-farnesene biosynthesis actively occurs in apples stored at 0 to 4 °C.

Isolation and partial purification of α-farnesene synthase. The highest total activity and specific activity of α-farnesene synthase were found in the crude extract and the highest proportion of activity was confined to the cytosol rather than the membrane fraction (Table 1). Enzyme activity was lowest in the microsomal fraction, with intermediate levels in the chloroplast/ mitochondrial fraction (Table 1). The first step of purification by ion-exchange chromatography on DEAE Sephacel resulted in a 20-fold purification (Table 2, Fig. 3A). α-Farnesene synthase was eluted from the column with 0.35 mol•L^{-1} NaCl indicating that the enzyme has a relatively large negative charge. Subsequent gel permeation chromatography on Sephacryl further increased the purity of the enzyme preparation (70-fold) (Table 2, Fig. 3B). Poor recovery from this step together with the instability of partially purified enzyme restricted further purification of α-farnesene synthase to homogeneity. The most active enzyme preparations were obtained from apples removed from CA storage during the period of March to June and these apples had to be kept at 0 °C in air for an additional 1 to 2 weeks to attain maximal activity. The enzyme activity remained stable for a maximum period of 2 to 3 months only if stored as a crude extract at –80 °C. Incubation of the total membrane fraction (105,000 g_n pellet) with 0.5 to 5 mmol•L^{-1} CHAPS (3-[-3-cholamidopropyl] dimethylammonio-1-propanesulphonate) or 0.01% to 0.1% Triton X-100 did not enhance α-farnesene synthase activity in the solubilized fraction, but inhibited the activity with increasing concentration (data not presented).

Apparent molecular weight of α-farnesene synthase. The molecular weight of α-farnesene synthase was estimated using

Table 1. Distribution of α-farnesene synthase activity among different subcellular fractions of skin tissue of 'Delicious' apple. Fruit were stored 5 months in CA at 3.0 kPa O_2 and 2.5 kPa CO_2. Data is representative of three independent experiments each showing similar results.

Cellular fraction	Total protein (mg)	Total activity (Bq·h^{-1})	Specific activity (Bq·mg^{-1}·h^{-1})
Crude extract	64.4	31,045	482
Crude extract without debris	51.9	17,935	345
Chloroplast + mitochondria	19.3	5,698	295
Microsome	4.1	579	141
Cytosol	18.8	8,317	442

Table 2. Partial purification of α-farnesene synthase from skin tissue of 'Delicious' apple. The cytosol was subject to anion-exchange chromatography on DEAE-sephacel. Active fractions were pooled and subjected to gel filtration using a Sephacryl S300-HR column.

Purification step	Total protein (mg)	Total activity (Bq·h^{-1})	Specific activity (Bq·mg^{-1}·h^{-1})	Recovery (%)	Purification factor
Cytosol	91.20	11,476	126	100	---
DEAE-Sephacel	2.10	5,316	2,531	46	20
Sephacryl	0.27	2,385	8,833	21	70

standard molecular weight markers ranging from 12,000 to 200,000 (Sigma MW-GF-200 kit) and size exclusion column chromatography. Using a calibration curve of V_e/V_o against *log* molecular weight and determining the V_e/V_o of eluted α-farnesene synthase activity peak, an apparent molecular weight of 108,000 was estimated.

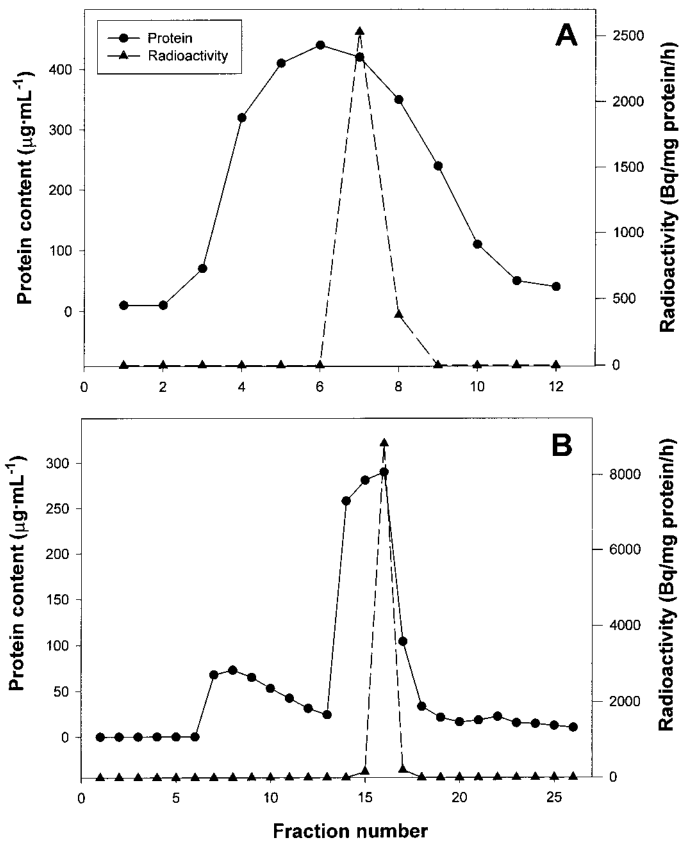

Fig. 3. Partial purification of α-farnesene synthase by ion exchange chromatography on DEAE Sephacel (**A**) and gel permeation chromatography on Sephacryl (**B**). Inset in A applies to both figures. The conditions are as described in Materials and Methods.

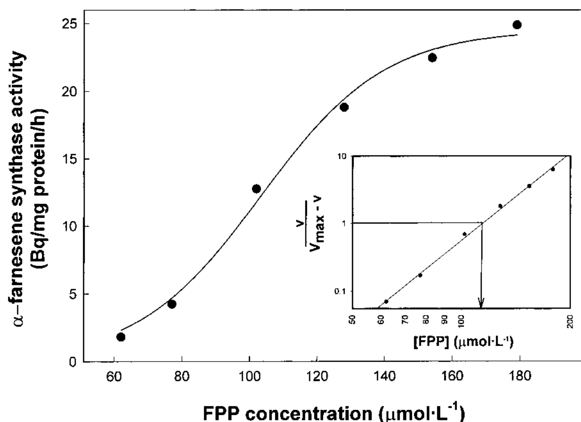

Fig. 4. Effect of increasing farnesyl phyrophosphate (FPP) concentration on initial velocity of α-farnesene synthesis. The enzyme source was a 20-fold purified cytosolic extract from CA-stored apple skin tissue. The assay was conducted in the presence of 100 mmol·L^{-1} MES buffer at pH 5.6, containing 10 mmol·L^{-1} $MgCl_2$ and 0.25 mmol·L^{-1} $MnCl_2$. The substrate contained hot and cold FPP in a ratio of 990 Bq :1 nmol. The curve is representative of three separate experiments showing similar results. The inset shows determination of $S_{(0.5)}$ and number of sub units (n_H) of native α-farnesene synthase protein using a Hill plot.

Enzyme kinetics. Partially purified α-farnesene synthase obtained after DEAE-Sephacel-chromatography exhibited a sigmoidal pattern of enzyme kinetics (Fig. 4). Substrate concentration at half maximal velocity [$S_{(0.5)}$] was 84 ± 18 μmol·L^{-1} for FPP as determined from the results of three independent estimations. A slope (Hill coefficient, n_H) of 2.9 ± 0.5 was obtained from Hill plots of *log* FPP concentration against *log* [$v/(V_{max}-v)$] (the inset of Fig. 4). These results suggest that α-farnesene synthase is an allosteric enzyme comprising two or three subunits. α-Farnesene synthase activity was not influenced by cofactors such as NAD, NADH, NADP, NADPH, ATP, and ADP (data not presented).

In vivo α-farnesene synthase activity and α-farnesene and CTOL content during storage of different cultivars. α-Farnesene was not detectable in the skin tissue of scald-susceptible 'Delicious' or scald-resistant 'Empire' fruit before attaining physiological maturity or at harvest (Fig. 5A). α-Farnesene content increased rapidly in both cultivars from 4 to 10 weeks after harvest during storage in air at 0 °C (Fig. 5A). Ten weeks after harvest, α-farnesene content was nearly 3-fold higher in 'Delicious' apples than in 'Empire', and then declined rapidly (Fig. 5A). By contrast, α-farnesene content in 'Empire' apples increased gradually during storage and showed a marginal decline after 25 weeks of storage (Fig. 5A).

α-Farnesene synthase activity was similar in 'Delicious' and 'Empire' apples (Fig. 5B). The changes in enzyme activity during

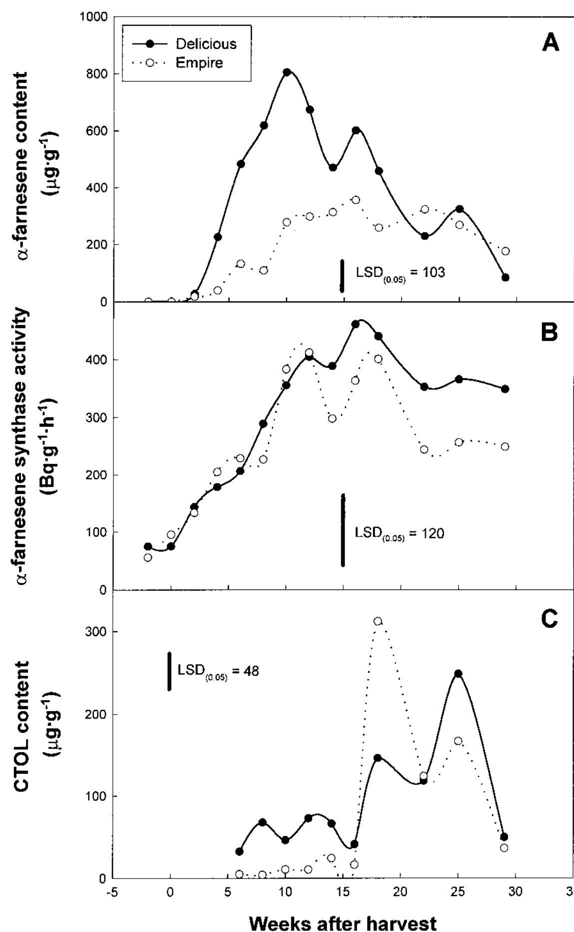

Fig. 5. (**A**) α-Farnesene content, (**B**) in vivo α-farnesene synthase activity, and (**C**) conjugated triene alcohol (CTOL) content of the skin of 'Delicious' and 'Empire' apples during air storage at 0 °C. Inset in A applies to all figures. All measurements are expressed on a fresh weight basis and each data point is a mean of three separate sets of experiments.

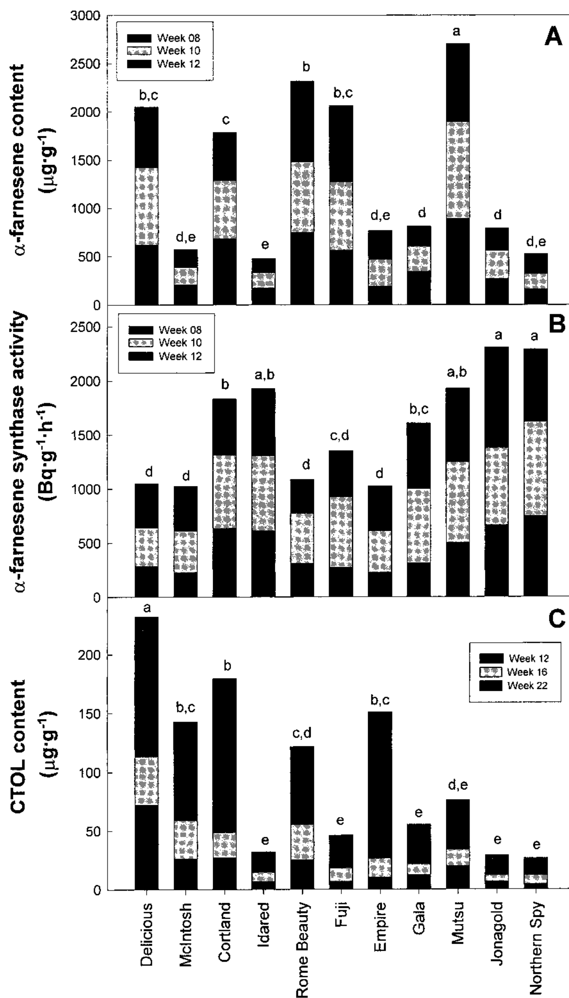

Fig. 6. (**A**) α-Farnesene content, (**B**) in vivo α-farnesene synthase activity, and (C) conjugated triene alcohol (CTOL) content of the skin of scald-susceptible (Delicious, McIntosh, Cortland, Idared, Rome Beauty), moderately scald-susceptible (Fuji), and scald-resistant (Empire, Gala, Mutsu, Jonagold, Northern Spy) cultivars of apple. Each bar, as shown in the inset, represents the cumulative value of data acquired for each cultivar at three time intervals during air storage at 0 °C. All measurements are expressed on the basis of fresh weight. Bars not marked by a common letter are significantly different ($P = 0.05$). Data from experiments conducted each week are a mean of three replicates.

storage nearly paralelled changes in α-farnesene content in the skin, except during the latter part of storage where the decline in α-farnesene content was more rapid in 'Delicious' apples than the decline in enzyme activity (Fig. 5A and B). CTOL, the putative scald-causing oxidative product of α-farnesene, was very low in the skin of 'Delicious' and negligible in 'Empire' up to 16 weeks in storage (Fig. 5C). CTOL content increased sharply in 'Empire' at week 18, but decreased thereafter (Fig. 5C). In 'Delicious', maximal levels of CTOL were observed at week 25, after which the CTOL level declined (Fig. 5C). Interestingly, the cumulative amount of CTOL produced in storage by 'Delicious' and 'Empire' was similar, regardless of their inherent susceptibility to scald.

The relation between α-farnesene synthase activity and α-farnesene levels was explored further by analysis in a wide variety of apple cultivars with differing degrees of scald-susceptibility. The susceptibility of the cultivars was confirmed by storing them in air for 4 to 6 months at 0 °C and evaluating for scald. In addition to 'Delicious' and 'Empire', four scald-susceptible (McIntosh, Cortland, Idared, and Rome Beauty), one moderately scald-susceptible (Fuji), and four scald-resistant (Gala, Mutsu, Jonagold, and Northern Spy) cultivars of apple were evaluated. Data were acquired at three selected storage intervals during which the α-farnesene and CTOL contents or α-farnesene synthase activity reached their maxima. 'Delicious', 'Cortland', 'Rome Beauty', 'Fuji', and 'Mutsu' showed a distinctively higher amount of accumulated α-farnesene in the skin compared with the other six cultivars (Fig. 6A). Interestingly, scald-resistant 'Mutsu' had the highest amount of α-farnesene (Fig. 6A). As observed previously (Fig. 5), α-farnesene synthase activity of different cultivars was not proportional to the corresponding α-farnesene content (Fig. 6A and B). For example, the enzyme activities of 'Jonagold' and 'Northern Spy' fruit were the highest, yet the accumulated α-farnesene was relatively low (Fig. 6A and B). Those cultivars that produced high amounts of CTOL were 'Delicious', 'Cortland', 'Empire', 'McIntosh', and 'Rome Beauty' (Fig. 6C). However, scald-susceptible 'Idared' had relatively low amounts of CTOL while scald-resistant 'Empire' and 'Mutsu' had relatively high amounts of CTOL (Fig. 6C). Linear regression analysis of α-farnesene content, CTOL content, and percentage scald development gave an r value of 0.23 between α-farnesene and percentage scald, and 0.56 between CTOL and percentage scald.

α-Farnesene, CTOL, MHO, and MHOL content in relation to superficial scald development. In agreement with some of our previous studies (Paliyath et al., 1997; Rupasinghe et al., 1998; Whiting et al., 1997), α-farnesene content was higher in scald-free tissue and decreased with increasing propensity to scald (Table 3). Accumulated CTOL content of scald-developing and severely scalded tissue was 50% and 30% higher, respectively, compared with scald-free tissue (Table 3). Diphenylamine (DPA) treatment did not influence α-farnesene content, but decreased CTOL content by 90% (Table 3). CA storage suppressed α-farnesene and CTOL contents by 58% and 66%, respectively, compared with air storage at 0 °C. Content of MHO did not have any relation to the severity of scald, but MHOL content was 60% and 20% higher in scald-developing and severely scalded tissue, respectively (Table 3).

Discussion

The current theory on the mechanism of superficial scald development in apple is centered around the biosynthesis and degradation of the sesquiterpene α-farnesene present in the skin tissue. However, recent work conducted in our laboratory consistently revealed that α-farnesene content and evolution in scald-free apples are three times higher than in scald-developing apples (Paliyath et al., 1997; Rupasinghe et al., 1998; Whiting et al., 1997). In the present study, we have attempted to answer some of the basic questions arising from the above observations as to whether i) the differences in α-farnesene levels in the skin tissues derived from scald-free and scald-developing apples are due to their differences in α-farnesene biosynthetic capacity or degradation, and ii) the inherent levels of α-farnesene and conjugated trienes of different apple cultivars have any relation to scald susceptibility. We have also examined the characteristics of α-farnesene synthase, which converts farnesyl pyrophosphate to α-farnesene (Rupasinghe et al., 1998). Most of the early studies on scald have relied on direct UV absorbance measurements of crude hexane extracts of the cuticular or surface region at absorption maxima of 232 nm and 269 nm for quantification of α-farnesene and conjugated trienes, respectively. We have reexamined the

Table 3. Content of hexane-extractable α-farnesene and its catabolites in skin tissue of 'Delicious' apple in relation to scald severity. Apples were stored at 0 °C in air for 17 weeks. Metabolites were extracted by immersing 20 segments of skin tissue from the equatorial region of 10 apples in *n*-hexane for 24 h. Analytical procedure was as described in materials and methods. Data are representative of two experiments each showing similar results.

Scald	Metabolite ($\mu g \cdot g^{-1}$ fresh wt)			
severity	α-Farnesene	CTOL	MHO	MHOL
Scald-free apple	531	168	57	35
Healthy tissue of scald-developing apple	508	168	58	35
Scald-developing tissue	203	260	55	58
Severely scalded apple	197	219	54	48
DPA-treated, scald-free	534	017	ND[z]	ND
CA-stored, scald-free	225	058	ND	ND

[z]ND = not determined.

level of α-farnesene and its putative metabolites such as CTOL (Rowan et al., 1995) in relation to the development of superficial scald using more accurate analytical tools such as HPLC, GC/SPME, and GC–MS.

The highest α-farnesene synthase activity was in the cytosolic fraction, though fractionation adversely affected recovery. Biosynthesis of sesquiterpenes in other plant tissues is associated with two specialized subcellular compartments, the cytosol/endoplasmic reticulum (ER) boundary (Belingheri et al., 1992; Gleizes et al., 1980) and plastids (Bernard-Dagan et al., 1982). In leaves of *Pinus pinaster* Ait., the 38,000 g_n supernatant was involved mainly in the biosynthesis of the acyclic sesquiterpene *trans*-β-farnesene, whereas cyclic sesquiterpene hydrocarbons were synthesized by the microsomal pellet (ER) (Bernard-Dagan et al., 1982). However, the presence of high levels of α-farnesene synthase activity in the supernatant does not imply that it is a cytosolic enzyme. In general, sesquiterpene synthases are rather hydrophobic and possess relatively low p*I* values (Croteau and Cane, 1985). Bernard-Dagan et al. (1982) observed that most enzymes involved in sesquiterpene biosynthesis could be solubilized easily during fractionation and that the enzyme activity is associated presumably with a membrane compartment. Therefore, it is likely that α-farnesene synthase could be bound weakly to plastid or ER membranes and dissociated during the homogenization process. To test this assumption, the 105,000 g_n pellet (total membrane) was incubated with different detergents to observe possible enhancement of α-farnesene synthase activity in the solubilized fraction. However, the enzyme activity was inhibited with CHAPS or Triton X-100. This implies that either the enzyme is not membrane-associated or the detergents adversely affected enzyme activity. However, based on high activity in the cytosolic fraction, together with a low pH optimum (5.6), it could be suggested that α-farnesene synthase is localized in the cytosol, loosely bound to the ER or in other vesicular compartments.

In common with other enzymes of sesquiterpene biosynthesis, α-farnesene synthase required divalent metal ions for its activity, with relatively more affinity for Mn^{2+} than Mg^{2+} (Belingheri et al., 1992; Cane, 1981; Dehal and Croteau, 1988; Salin et al., 1995; Vögeli et al., 1990). The presence of a divalent metal ion was an absolute requirement for this enzyme, since only a trace level of enzyme activity was detected in the absence of metal ions in the reaction mixture. A divalent metal ion is the only cofactor required for most known sesquiterpene synthases (Croteau and Gundy, 1984; Croteau and Cane, 1985). Half maximal stimulation of α-farnesene synthase occurred at a concentration of 2 to 2.5 $mmol \cdot L^{-1}$ Mg^{2+} which also reflects the physiological cytosolic levels of Mg^{2+} (Hepler and Wayne, 1985). The divalent metal ion binds to the pyrophosphate moiety of the allylic cosubstrate, so as to neutralize/shield negative charges of the pyrophosphate moiety and to make it a better leaving group in the ionization step (Chayet et al., 1984; McGarvey and Croteau, 1995). Ionization of the allylic pyrophosphate (FPP) leads to formation of a charge-stabilized allylic carbocation (Chayet et al., 1984; McCaskill and Croteau, 1997; McGarvey and Croteau, 1995) as an intermediate of sesquiterpene formation.

Unlike β-farnesene synthase (Salin et al., 1995), α-farnesene synthase in apples exhibits typical sigmoidal enzyme kinetics with increasing FPP in the reaction mixture. The estimated $S_{(0.5)}$ value was 84 ± 18 $\mu mol \cdot L^{-1}$ (mean ± SD) and the Hill coefficient (n_H) was 2.9, suggesting the number of subunits of α-farnesene synthase to be two or three. None of the dinucleotide heterotropic effectors tested influenced enzyme activity. The native molecular weight estimated for α-farnesene synthase after gel filtration was 108,000 and was similar to that estimated for *trans*-β-farnesene synthase in needles of *Pinus pinaster* Ait. (Salin et al., 1995). SDS-PAGE of purified *trans*-β-farnesene synthase shows a single band at 45,000 (Salin et al., 1995), supporting the potential dimeric or multimeric nature of functionally active farnesene synthase in apples.

The decline in α-farnesene content after attaining maximal levels during storage was more rapid than that of α-farnesene synthase activity, and was more evident in scald-susceptible 'Delicious' than scald-resistant 'Empire'. The decline in α-farnesene content and increase in CTOL is relatively stoichiometric, the increase in concentration of CTOL nearly reflecting the decline in tissue α-farnesene content. The decline in α-farnesene synthase activity could reflect a depletion of substrates and cofactors involved in the isoprenoid pathway. The increase in CTOL could result from increased peroxidation, an inherent feature of advancing senescence in fruit tissues. Therefore, the present results revealed that the decline of α-farnesene during prolonged cold storage was related to both lowered α-farnesene synthase activity as well as enhanced oxidation of α-farnesene to CTOL-like compounds. The symptoms of scald usually appeared during this period.

Comparison of scald-susceptible and scald-resistant cultivars showed that α-farnesene content in the skin of fruit does not correlate with their inherent, relative scald susceptibility. Scald-resistant 'Mutsu' produced the highest levels of α-farnesene, whereas scald-susceptible 'Idared' and 'McIntosh' had relatively low amounts of α-farnesene. To test the possibility whether varying rates of α-farnesene evolution observed were due to differences in skin permeability, headspace α-farnesene content of the 11 cultivars was measured 12 weeks after harvest and

compared with that of the corresponding α-farnesene content in the skin. The saturated α-farnesene level in the headspace was 50- to 100-fold less compared with the α-farnesene content of skin (on a fresh weight basis). As well, there was a significant high correlation ($r = 0.88$) between α-farnesene content in the skin and α-farnesene level in the headspace (data not presented). Hence, it can be suggested that poor correlation between α-farnesene content and α-farnesene synthase activity ($r = 0.05$) is not due to differences in skin permeability among the cultivars. It is possible that the regulation of α-farnesene biosynthesis in apple skin could be upstream of the isoprenoid pathway. The enzyme 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGR) is considered a primary control point for isoprenoid biosynthesis in plants and is regulated by a variety of developmental and environmental signals (McCaskill and Croteau, 1997; McGarvey and Croteau. 1995). Therefore, a lack of correlation between α-farnesene synthase activity and α-farnesene present in the skin could have resulted from differences in available FPP for this terminal enzyme of α-farnesene biosynthesis. Cultivars which produce high amounts of α-farnesene appear to produce relatively more CTOL, except 'Fuji' and 'Empire'. However, preliminary analysis on the endogenous levels of CTOL, the presumed scald-causing α-farnesene catabolite (Huelin and Coggiola, 1970a, 1970b; Whitaker et al., 1997), showed relatively poor correlation ($r = 0.56$) with the scald index of the eleven different apple cultivars tested. Similarly, Rao et al. (1998) found a poor relationship between conjugated trienes (CT258, CT281, and the C258/CT281 ratio) and scald susceptibility of 'White Angel' x 'Rome Beauty' apple selections.

Analysis of α-farnesene in relation to scald severity revealed a close relation between reduced α-farnesene content and the severity of scald, consistent with the observations from our previous studies (Paliyath et al., 1997; Rupasinghe et al., 1998; Whiting et al., 1997). The earlier evidence supporting the role of conjugated triene as the primary scald-causing agent was its close correlation to the severity of scald, and suppression of conjugated triene accumulation by DPA treatment (Huelin and Coggiola, 1970a, 1970b; Meigh and Filmer, 1969). In agreement with the above conclusion, we observed a 90% reduction in CTOL content after DPA treatment. Storage of apples under low-oxygen atmosphere (CA storage) is another means of suppressing development of scald (Ingle and D'Souza, 1989). The reduction in both α-farnesene (58%) and CTOL (66%) in CA-stored apples compared with apples stored in air indicates that low oxygen affects α-farnesene oxidation as well as general α-farnesene metabolism, implying a role for oxygen in these metabolic processes. MHO is an in vitro (Anet, 1972; Filmer and Meigh, 1971) as well as in vivo (Mir et al, 1999; Mir and Beaudry, 1999) oxidative product of α-farnesene. Recently, Mir et al. (1999) found that MHO released from DPA-treated 'Cortland' fruit peels was 8000-fold lower than from peel samples of control fruit and suggested that MHO could somehow be involved in scald development. In contrast, our analysis suggested that MHO content in the skin did not have any direct relation to the severity of scald. However, similar to CTOL, MHOL was 60% higher in scald-developing tissue, suggesting further reduction of the ketone to alcohol. MHOL has been observed to be present in the headspace volatiles of 'Jonagold' apples and was 3-fold higher in air-stored apples than in CA-stored apples (Girard and Lau, 1995). However, no MHO was detected in their analysis (Girard and Lau, 1995), suggesting that it may have been reduced to MHOL in this apple cultivar. These observations taken together with those of Buttery and Ling (1993) that tomato (*Lycopersicon esculentum* Mill.), a fruit that does not develop scald, evolves large quantities of MHO as a volatile during ripening and that MHO is a catabolite of β-carotene, another long chain isoprenoid compound, cast considerable doubt on the notion that MHO is involved directly in scald development.

The absence of α-farnesene in the skin of apples at harvest and the rapid increase in α-farnesene content and α-farnesene synthase activity during storage at 0 °C indicates that developmental regulation of α-farnesene synthase activity and α-farnesene biosynthesis took place following detachment of mature fruit from the tree and/or possible induction by low temperature stress. Similar to the present results, it has been observed previously that hexane-extractable α-farnesene content progressively increases during the first 2 months of storage at 0 °C and declines thereafter (Huelin and Murray, 1966; Meigh and Filmer, 1969; Meir and Bramlage, 1988; Whitaker et al., 1997). Interestingly, the level of α-farnesene in the skin of lime (*Citrus latifolia* Tanaka), mandarin (*Citrus reticulata* Blanco), and grapefruit (*Citrus paradisi* Macf), which do not develop scald, also increases during 4 to 6 weeks storage at 0 °C (Yuen et al., 1995). In our study, α-farnesene synthase was active at low temperatures and retained 50% of the activity in crude apple skin extract at 0 °C compared with its maximum activity between 10 to 20 °C.

Comparison of the α-farnesene content of 'Delicious' apples stored at 0 °C and 5 °C revealed that α-farnesene biosynthesis is 1.5- to 3-fold higher at 0 °C than at 5 °C at 8 and 10 weeks after harvest (data not presented). Wills et al. (1975) found that α-farnesene levels of 'Dwarf Cavendish' bananas (*Musa acuminata* Colla) can be induced by low temperature storage. As well, in 'Anjou' pears (*Pyrus communis* L.) that contain similar levels of α-farnesene as in apples, α-farnesene levels can be increased 6-fold by holding fruit for 3 d at 0 °C compared with those stored at 10 °C (Rupasinghe, Paliyath and Murr, unpublished data). Recently, α-farnesene has been identified as a major volatile induced by insect herbivory in cotton (*Gossypium hirsutum* L.) (Paré and Tumlinson, 1997). These results suggest the possibility that α-farnesene is a low temperature or stress-induced secondary metabolite. This would support the earlier notion by Watkins et al. (1995), who designated scald as a typical chilling injury. Scald development has also been related to the production of free radicals through α-farnesene catabolism. Under chilling conditions, disruption of electron flow in the mitochondria could lead to the generation of superoxide radicals (Purvis et al., 1995). Thus, the generation of free radicals and active oxygen species could serve as a potential primary cause(s) for the development of superficial scald. Antioxidants such as α-tocopherol (Anet, 1974; Barden and Bramlage, 1994; Gallerani et al., 1990; Meir and Bramlage, 1988), and ascorbic acid and flavanols (Albrigo and Childers, 1970; Barden and Bramlage, 1994) are associated with resistance to scald development. Recently, Rao et al. (1998) found higher activities of the H_2O_2-degrading enzymes, guaiacol-peroxidases and catalases in scald-resistant selections of 'White Angel' x 'Rome Beauty' apples. Therefore, in apples, scald susceptibility or resistance could depend primarily on the generation of active oxygen species and/or the ability of the natural antioxidant defense system to effectively scavenge free-radicals generated during postharvest storage.

In conclusion, our results do not support the hypothesis that scald susceptibility of apple cultivars is related directly to the amount of α-farnesene produced and its oxidation to conjugated trienes and other metabolites. Instead they support the idea that

conversion of α-farnesene to CTOL occurs potentially through a free radical-mediated oxidation as a secondary event of scald development, and that the inherent ability of the fruit tissue to counteract or cope with oxidative stress plays a significant role in determining scald susceptibility or resistance in apples.

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