Expression of α -Farnesene Synthase *AFS1* and 3-Hydroxy-3-methylglutaryl-coenzyme A Reductase *HMG2* and *HMG3* in Relation to α -Farnesene and Conjugated Trienols in ‘Granny Smith’ Apples Heat or 1-MCP Treated to Prevent Superficial Scald

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ABSTRACT. Untreated control, 1-methylcyclopropene (1-MCP)-treated, and heated fruit of the superficial scald-susceptible ‘Granny Smith’ cultivar of apple [*Malus sylvestris* (L.) Mill. var. *domestica* (Borkh.) Mansf.] were compared with respect to scald incidence, internal ethylene concentration (IEC), α -farnesene metabolism, expression of the genes *AFS1*, which encodes α -farnesene synthase, the final, rate-limiting enzyme in the α -farnesene biosynthetic pathway, and *HMG2* and *HMG3*, which encode isozymes of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, the proposed rate-limiting enzyme in the mevalonate pathway of isoprenoid synthesis. The incidence of scald in untreated ‘Granny Smith’ apples after 16 weeks at 0 °C plus 1 week at 20 °C was 100%; 1-MCP treatment prevented scald development, whereas heat treatment delayed and reduced scald development. 1-MCP also inhibited both α -farnesene and IEC, suggesting that ethylene induces transcription of key genes involved in α -farnesene biosynthesis. Heat treatment reduced levels of α -farnesene and its oxidation products, conjugated trienols (CTols), but not to the extent of 1-MCP. Internal ethylene concentrations in heated apples did not differ from those in the controls. In both control and heated fruit, a sharp increase in *AFS1* mRNA during the first 4 weeks of storage preceded an increase in α -farnesene and a subsequent increase in CTols. *AFS1* transcript was absent from 1-MCP-treated apples for the first 10 weeks of storage, and even at 16 weeks was lower than in heated and untreated control fruit. Levels of the *HMG2* and *HMG3* transcripts varied during storage and among treatments, and were not correlated with the incidence of scald. *HMG2* mRNA transcript accumulation was low at harvest and increased in abundance during storage in all treatments, with the greatest increase occurring in 1-MCP-treated fruit. In contrast, *HMG3* transcript was constitutively present at all storage times, although it too was slightly more abundant in 1-MCP-treated fruit.

It is widely accepted that synthesis and oxidation of the sesquiterpene α -farnesene play a central role in development of superficial scald, a physiological storage disorder of apples and pears (*Pyrus communis* L.) (Anet 1972a, 1972b; Huelin and Coggiola, 1968; Huelin and Murray, 1966; Ingle and D’Souza, 1989). This physiological disorder manifests as browning of the apple peel, and is thought to be induced or exacerbated by the oxidation products of α -farnesene (Rowan et al., 2001; Whitaker et al., 2000). The primary in vivo oxidation products of α -farnesene that accumulate in apple epicuticular wax and peel tissue during cold storage have been identified as conjugated trienols (CTols) (Rowan et al., 1995; Whitaker et al., 1997). Accumulation of high levels of CTols is closely correlated with the subsequent appearance of scald symptoms, whereas high levels of α -farnesene itself are less well correlated with scald (Anet, 1972b; Huelin

and Coggiola, 1968, 1970). This may be in part attributed to the fact that during low-temperature storage α -farnesene content peaks and then declines due to its oxidation to CTols before the disorder is manifested (Huelin and Coggiola, 1968; Whitaker et al., 1997, 1998).

Exposure of apple fruit to the blocker of ethylene action, 1-methylcyclopropene (1-MCP), greatly curtails α -farnesene production and reduces scald incidence and severity (Fan et al., 1999; Rupasinghe et al., 2000a; Shacham et al., 2003; Watkins et al., 2000). Results from the studies with 1-MCP, as well as several previous reports (Du and Bramlage, 1994; Ju and Curry, 2000a; Watkins et al., 1993, 1995; Whitaker and Solomos, 1997), have shown that ethylene production and perception, and tissue responsiveness to ethylene, are involved in regulation of α -farnesene synthesis and induction of scald in apple fruit.

Hot air treatment has been shown to effectively delay scald development for a period of up to 4 months in air storage and at least 8 months in controlled-atmosphere storage (Lurie et al., 1990). The retardation of scald appearance was attributed to the inhibitory effect that heating has on many processes, including ripening and production of ethylene and volatiles (Fallik et al., 1997; Lurie and Klein, 1990). The production of α -farnesene is

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also delayed in heat-treated apples, as is the accumulation of its oxidation products (Shacham et al., 2003).

Studies using radiolabeled precursors (Rupasinghe et al., 2001) or a statin inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) (Ju and Curry, 2000b) have shown that in apple peel tissue α -farnesene is synthesized via the mevalonic acid pathway. The final, rate-limiting enzyme in the pathway is α -farnesene synthase, which converts farnesyl diphosphate (FDP) to α -farnesene (Rupasinghe et al., 1998, 2000b). Recently, two cDNAs encoding this enzyme (designated *AFSI*) have been cloned from 'Law Rome' and 'Idared' apple peel tissue (Pechous and Whitaker, 2004; Pechous et al., 2004). In addition, one complete and two partial cDNAs encoding isozymes of HMGR (designated *HMG1*, *HMG2*, and *HMG3*) have been cloned from one or more apple cultivars (Pechous and Whitaker, 2002; Rupasinghe et al., 2001). Among the *HMG* genes, *HMG1* was constitutively expressed at high levels in fruit of both 'Delicious' (Rupasinghe et al., 2001) and 'Law Rome' (Pechous and Whitaker, 2002), and therefore is unlikely to be specifically involved in sesquiterpene synthesis. In the present study, utilizing the 'Law Rome' *AFSI*, *HMG2*, and *HMG3* cDNAs as probes, we compared the expression of their respective genes during storage of 'Granny Smith' apples that were untreated (controls) or treated before storage with either 1-MCP or heat.

Materials and Methods

PLANT MATERIAL AND EXPERIMENTAL PROCEDURE. 'Granny Smith' apples were harvested from a commercial orchard in the Jerusalem hills in early October for three seasons. Fruit starch content at harvest was between 2 and 3, based on the Cornell chart where 1 = full starch and 8 = no starch (Blanpied and Silsby, 1992), indicating that they were preclimacteric and suitable for long-term storage. The apples were sorted for uniformity and absence of blemishes, and then divided into three lots of 500 fruit each. One lot was placed in plastic boxes directly into 0 °C air storage (control), the second was heated in air for 4 d at 38 °C then placed in 0 °C storage (heat, Klein and Lurie, 1990), and the third was treated with 1 $\mu\text{L}\cdot\text{L}^{-1}$ 1-MCP for 18 h at 20 °C then placed in 0 °C storage. One box of fruit per treatment was removed every 2 weeks for evaluation as described below. For 1-MCP treatment, fruit in plastic field boxes were placed in a 0.74-m³ plastic container, which was sealed for 18 h at 20 °C after addition of a test tube containing 1.04 g of the formulated 1-MCP product (SmartFresh, 0.14% a.i.; Rohm and Haas Co., Rome) dissolved in 20 mL of 40 °C water.

At each time of removal from storage, peel disks were taken 2 h after removal from 10 fruit from each treatment for measurement of α -farnesene and CTols (described below). Additional peel samples were frozen in liquid nitrogen and stored at -80 °C for subsequent preparation of RNA. Five fruits per treatment were also taken for determination of internal ethylene concentration (IEC). At 2-week intervals, 30 fruit from each treatment were held 1 week at 20 °C and then evaluated for superficial scald, which appeared as a brown discoloration of the peel. Scald was determined as a percentage of the fruit having this brown discoloration, no matter how much of the peel surface was affected.

ANALYSIS OF α -FARNESENE AND CONJUGATED TRIENOLS. Five 1-cm disks were removed from a strip of peel taken from the equatorial region of each apple (10 apples total). These were placed in test tubes containing 5 mL of hexane for 10 min. The extracts were agitated and filtered, and the absorbance was read

at 232, 281, and 290 nm. Content of α -farnesene and CTols per square centimeter of apple peel was calculated using extinction coefficients of 29,000 for α -farnesene (nm 232) and 25,000 (nm 281–290) for CTols (Anet and Coggiola, 1974).

INTERNAL ETHYLENE DETERMINATION. IECs were measured by taking 1-mL samples of internal gas from the fruit drawn into a syringe through a hypodermic needle inserted into the core cavity of each fruit. This sample was injected into a gas chromatograph (model 3300; Varian, Walnut Creek, Calif.) with a 2-m packed alumina column and a FID detector. The carrier gas was helium at 40 mL $\cdot$ min⁻¹; the injector port, oven, and detector were held at 100, 80, and 180 °C, respectively.

PREPARATION OF RNA AND ANALYSIS OF TRANSCRIPT ABUNDANCE. Total RNA was extracted from 2 g of apple peel tissue as described by Lopez-Gomez and Gomez-Lim (1992). Fifteen micrograms of total RNA were separated on a 1% agarose/formaldehyde gel and blotted to nylon membrane filters. RNA blots were prehybridized and hybridized in formamide buffers at 42 °C as described in Sambrook et al. (1989). Final wash of RNA blots was carried out in 0.5 $\times$ SSC (sodium chloride/sodium citrate), 0.1% SDS at 60 °C. cDNAs of *AFSI* (Pechous and Whitaker, 2004; GenBank accession no. AY182241), *HMG2* ('Law Rome' homologue of 'Delicious' *HMG2*, accession no. AF316112), and *HMG3* (accession no. AY043491; both from Pechous and Whitaker, 2002) were radioactively labeled using the random priming method and used as probes. To measure loading variations, blots were re-hybridized with the 25S ribosomal RNA (rRNA) gene probe. RNA blots were developed using a BAS reader (Fuji, Tokyo).

STATISTICAL ANALYSIS. Scald incidence, α -farnesene, and CTol determinations are combined results from experiments performed during three seasons. The ethylene measurements and the analysis of transcript abundance of *AFSI*, *HMG2*, and *HMG3* are from the third season. Results are presented as means $\pm$ SD.

Results

By week 8 of storage, 25% of control apples had slight scald symptoms, whereas apples from heated and 1-MCP treatments were scald free (Fig. 1). By week 10, all of the control apples had developed scald. Heated apples were scald free until week 14, whereas 1-MCP-treated apples developed no scald over the duration of the experiment.

α -Farnesene levels were already quite high in control apples by week 6 and continued to increase through week 12, after which they declined (Fig. 2A). Levels in heated apples increased at a slower rate and remained lower than those in control apples until week 16. 1-MCP-treated apples maintained very low levels of α -farnesene from 6 to 16 weeks. Control fruit had high levels of CTols at 8 weeks (when visible scald was first detected), whereas their accumulation was delayed and more gradual in heated apples (Fig. 2B). In 1-MCP-treated apples, CTol levels began to increase slightly only after 12 weeks. Over 16 weeks of storage, maximum levels of α -farnesene and CTols in heated fruit were $\approx$ 70% and 50%, respectively, of those determined for control fruit.

IEC was very low in 1-MCP-treated apples at all times of observation (Fig. 3). Control apples and heated apples had low IEC at harvest and after 2 weeks of storage. From 4 weeks of storage onwards their IEC increased progressively as storage time was extended. There were no differences in IEC between the fruit from these two treatments.

The transcript of α -farnesene synthase, *AFSI*, was not detectable at harvest or immediately after 1-MCP or heat treatment, (Fig.

Fig. 1. Superficial scald development in 'Granny Smith' apples following storage at 0 °C for 6 to 16 weeks. At each time of removal from storage, 30 fruit from each treatment were held at 20 °C for 7 d and then scored for scald incidence. The data points are the average values from three seasons. Standard deviation is indicated with vertical bars.

4). However, by 4 weeks of storage *AFS1* mRNA was abundant in both control and heat-treated apples, and a high level was maintained throughout the 16 weeks of storage. In 1-MCP-treated apples the transcript began to appear only on week 12, and then at a level much lower than that in control and heated fruit. Transcripts of the two genes for 3-hydroxy-3-methylglutaryl-coenzyme A reductase, *HMG2* and *HMG3*, were present in apples of all treatments at harvest. The transcript for *HMG2* was initially at a

very low level and gradually became more abundant as storage time increased. *HMG2* transcript appeared from week 12 on to be more abundant in 1-MCP-treated than in control or heated fruit. *HMG3* appeared to be constitutively expressed; transcript levels did not change substantially during the time that the apples were in storage. As observed for *HMG2*, 1-MCP treatment appeared to slightly stimulate expression of *HMG3*.

Discussion

For about 40 years the apple industry has controlled superficial scald by prestorage treatment of susceptible fruit with the antioxidant DPA, which retards the autoxidation of α -farnesene (Anet and Coggiola, 1974; Huelin and Coggiola, 1968, 1970). This treatment appears to have other beneficial effects on fruit physiology, including reduced rates of respiration and ethylene evolution (Du and Bramlage, 1994; Lurie et al., 1989; Whitaker, 2004). A negative aspect of the DPA drench treatment is that a fungicide must be included to limit postharvest decay. In addition, some countries have proscribed the use of DPA. Hence, this has prompted the search for alternative methods of scald control. A promising recent development in this effort was the demonstration that prestorage treatment of apples with 1-MCP, a blocker of ethylene receptor sites, greatly diminished α -farnesene production and largely prevented scald (Fan et al., 1999; Rupasinghe et al., 2000a; Shacham et al., 2003; Watkins et al., 2000). Another method for controlling scald is a prestorage heat treatment, which also inhibits ethylene production (Lurie and Klein, 1990). These

two methods demonstrated close correlation between ethylene perception or production and the increase in α -farnesene synthesis shortly after apples are placed in low-temperature storage.

Recently the possibility that ethylene regulates transcription and translation of genes in the α -farnesene biosynthetic pathway has been investigated. Complete or partial cDNAs have been cloned that encode three HMGR isozymes, a farnesyl diphosphate synthase, and an (*E,E*)- α -farnesene synthase (Whitaker, 2004). Of these genes, the ethylene-dependent up-regulation of 'Delicious' *HMG2* (Rupasinghe et al., 2001) and 'Law Rome' *AFS1* (Pechous and Whitaker, 2004) during the first several weeks of storage coincides with the marked increase in α -farnesene synthesis during this interval. In the present study, *HMG2* transcript increased during storage, but in contrast with the previous study with 'Delicious' apples, the greatest increase was in fruit treated with 1-MCP, in which ethylene perception and production were inhibited (Fig. 4). Comparison of *AFS1* expression in fruit of 'Idared', a scald-resistant cultivar, and 'Law Rome', a scald-susceptible cultivar, showed that although upregulation by ethylene occurred in both cultivars, the level of *AFS1* transcript was always substantially higher in 'Law Rome' than in 'Idared' (Pechous et al., 2004). In our study with scald-sensitive 'Granny

Fig. 2. Concentrations of α -farnesene (A) and its conjugated trienol oxidation products (B) in peel tissue of control, heat-treated and 1-MCP-treated 'Granny Smith' apples stored at 0 °C for up to 16 weeks. Peel tissue was sampled from 6 to 16 weeks of the storage period at 2-week intervals. The data were collected over three seasons, and at each time of removal from storage 10 fruit were sampled for each treatment. Standard deviation is indicated with vertical bars.

Fig. 3. Internal ethylene concentration in 'Granny Smith' apples following storage at 0 °C for 0 to 16 weeks. At each removal time 10 fruit were sampled for each treatment. Standard deviation is indicated with vertical bars.

Smith' apples, the level of *AFS1* transcript was extremely low in 1-MCP-treated apples, which did not develop scald, and similarly high in control and heat-treated fruit, which developed scald after 8 and 16 weeks, respectively (Fig. 4).

The temporal pattern and level of α -farnesene accumulation during storage did not completely correlate with the expression of *AFS1* in the variously treated fruit. Transcript of *AFS1* was equally high in heated and control fruit after 4 weeks of storage, and was maintained at a similar level during later removals. Despite this, scald developed after only 8 weeks in controls, but not until 14 weeks in heated fruit (Fig. 1). This may in part be attributed to the slower accumulation of α -farnesene in heated compared with control apples (Fig. 2). In the study of 'Idared' and 'Law Rome' apples, however, the level of *AFS1* transcript correlated well with α -farnesene production (Pechous et al., 2004). It can be inferred from this that in these two cultivars translation of *AFS1* mRNA

and consequent α -farnesene synthase activity were proportional to the abundance of *AFS1* mRNA, although other pathway enzymes and the levels of substrates may also influence the rate of α -farnesene production (Rupasinghe et al., 2001). However, in the present study, the *AFS1* transcript level may not be linearly correlated with the α -farnesene synthase enzyme level, particularly in the heat-treated fruit. It has been found previously that in heated fruit transcripts of various enzymes accumulate without a concomitant rise in enzyme activity (Lurie et al., 1996). Generation of α -farnesene synthase specific antibodies and immunoblot analysis of apple peel tissue proteins will be required to evaluate properly translation versus transcription of *AFS1*.

After 6 weeks of storage the concentration of α -farnesene was over 4-fold greater in control than in heated fruit, and from 8 to 14 weeks the levels of α -farnesene in heated apples averaged $\approx 67\%$ of that in the controls (Fig. 2). By comparison, the concentration of conjugated trienols in heated vs. control fruit was relatively low, with levels in heated apples averaging $\approx 30\%$ of those in controls over 8 to 14 weeks. Thus, the proportion of α -farnesene that undergoes autooxidation during storage appears to be greater in control than in heated fruit. This discrepancy was even more evident in the comparison of scald-prone 'Law Rome' and scald-resistant 'Idared' apples (Pechous et al., 2004). Autooxidation of α -farnesene is thought to involve a free radical-mediated reaction, which increases dramatically once a threshold level of oxidized α -farnesene accumulates (Anet, 1974). The relatively high level of conjugated trienol accumulation in peel tissue of control fruit may result from a more rapid depletion of natural antioxidants and/or lower ascorbate peroxidase activity in these fruit (Shacham et al., 2003).

Previous studies have shown a correlation between levels of conjugated triene oxidation products of α -farnesene in cold-stored apples and scald severity after removal of the fruit from storage (Anet and Coggiola, 1974; Huelin and Coggiola, 1970; Rowan et al., 2001; Watkins et al., 1993; Whitaker and Solomos,

Fig. 4. RNA gel blot comparison of *AFS1*, *HMG2*, and *HMG3* transcript levels in peel tissue of control, heat-treated, and 1-MCP-treated 'Granny Smith' apples stored at 0 °C for up to 16 weeks. Ribosomal RNA is shown at the bottom to indicate equal loading of the lanes. Abbreviations are: HV = harvest; W = weeks; C = control fruit; H = heat-treated fruit; M = 1-MCP-treated fruit.

1997). Accumulation of a high concentration of α -farnesene is relatively poorly correlated with subsequent development of severe scald, indicating that the onset and rate of α -farnesene oxidation are critical to the development of superficial scald (Anet, 1972a, 1974; Anet and Coggiola, 1974). Nevertheless, rapid α -farnesene synthesis early in storage typically results in the relatively early accumulation of high levels of conjugated trienols in fruit of scald-susceptible cultivars (Watkins et al., 2000; Whitaker and Solomos, 1997; Whitaker et al., 1998). The effect of heat treatment is a temporary inhibition of both ethylene synthesis and α -farnesene accumulation, while 1-MCP has a much stronger and longer lasting effect, inhibiting ethylene perception and production and α -farnesene accumulation. The inhibition of α -farnesene biosynthesis by 1-MCP is at the level of transcript abundance of *AFS1*, whereas heat treatment does not affect transcript abundance. However, heat treatment retards the onset of both α -farnesene synthesis and oxidation, delaying the appearance of scald symptoms on the fruit. The two treatments were effective in delaying scald development in 'Granny Smith' apples; heat treatment for at least 12 weeks, and 1-MCP for at least 16 weeks, in cold storage.

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