FINAL REPORT
WTFRC Project #: PH-01-122 **USDA/ARS Project #:** 58-1275-0-004

Project title: Non-chemical Control of Superficial Scald

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Original Objectives: (1) Determine whether oxidation of a-farnesene to conjugated trienols in apple peel is an enzymatic reaction. (2) Clone the genes for a-farnesene synthase and, if it is proven to exist, a-farnesene oxidase (hydroxylase).

Updated Objectives: (1) Confirm putative a-farnesene synthase (a-FS) and 3-hydroxy-3-methyl glutaryl CoA reductase (HMGR) clones from apple peel by functional expression in *E. coli* or yeast cells. (2) Determine changes in the level of expression of a-FS and HMGR genes over 0 to 8 weeks of 0 °C air storage in peel tissue from untreated control and 1-MCP-treated apples. (3) Clone ethylene-responsive promoter that controls expression of the a-FS gene and possibly other genes in the a-farnesene biosynthetic pathway during the initial weeks of cold storage.

Early in 2001 we were successful in cloning a full-length putative terpene synthase gene from apple peel tissue. Because a-farnesene synthase (a-FS) is the optimal gene to target in terms of highly specific downregulation of a-farnesene synthesis, a great deal of time and effort was spent trying to achieve functional expression of this gene (and thereby show that it is definitely a-FS). Although we now have a bacterial protein expression system that yields impressive amounts of the gene product in cell lysates, enzymatic activity remains elusive. Therefore, in late 2001 and early 2002 we focused more on the cloning and characterization of genes encoding 3-hydroxy-3-methyl glutaryl CoA reductase (HMGR) isozymes, with the aim of identifying one that is dedicated mainly to the a-farnesene biosynthetic pathway and is consequently a target for downregulation. We currently have one full-length (HMG1) and two partial (HMG2 and HMG3) clones from apple peel. Our recent publication describes the functional expression of HMG1 in *E. coli* as well as the characterization of HMG1 and HMG3. For the remainder of 2002, we will continue to use peel tissue from nontreated and 1-MCP-treated apples as an experimental system to identify genes involved in a-farnesene synthesis which are specifically upregulated in resonse to ethylene early in storage. We also plan to use apple leaf gDNA to attempt to clone a promoter involved in ethylene-induced upregulation of the putative a-FS gene.

Concerning our modification of the research objectives, the hypothesis that *in vivo* oxidation of a-farnesene to conjugated trienols is enzymatic remains viable, and quite recently we began a line of research aimed at demonstrating conversion of a-farnesene to conjugated trienols in buffered peel tissue extracts and cloning cytochrome P450 genes that might encode an a-farnesene oxidase (hydroxylase) enzyme. However, there is evidence that ethylene-induced transcription and translation of HMG gene(s) is closely correlated with a-farnesene synthesis in cold-stored apples (Ju and Curry, 2000), and because we now have putative a-FS and HMG clones from apple peel tissue, as well as the new potent inhibitor of ethylene action 1-MCP, it was decided that the project should focus mainly on regulation of key genes and enzymes in the a-farnesene biosynthetic pathway by ethylene.

Progress and significant findings: A highly qualified candidate for the funded Postdoctoral Research Associate position (Steven W. Pechous) was hired in late December of 2000. Project accomplishments and findings during his 18 month tenure and over the *ca*. 2-3 month period prior to his arrival were as follows:

X Total RNA was isolated and purified from frozen peel tissue of 'Law Rome' apples that were left untreated or treated with 1-MCP at harvest then stored for 0, 4, or 8 weeks at 0 °C in air. RNA from the 4 and 8 week untreated tissue samples (period of sharp increase in a-farnesene synthesis) was used to construct an apple peel tissue cDNA library.

X Using degenerate primers based on highly conserved regions of known plant HMG genes, two clones encoding HMGR isozymes with *ca*. 80% identical amino acid sequences were obtained by 5'- and 3'-RACE (designated HMG1 and HMG3). A full-length clone of HMG1 was then obtained by screening the cDNA library. Recently, using a 3' primer based on the short partial cDNA for apple HMG2 reported in GenBank, we used 3'-RACE to obtain the entire 3' untranslated region and about 70% of the open reading frame of HMG2 from 'Law Rome' apple peel tissue.

X Using a poly-A adapter primer plus a single degenerate primer based on the most conserved region found among known plant sesquiterpene synthases and cyclases, a large fragment of a putative a-farnesene synthase clone was obtained by 3'-RACE. This was used as a probe to obtain a full-length clone from the apple peel cDNA library.

X RNA gel blot analysis showed that mRNA of the putative a-FS gene in peel tissue of untreated fruit increased 2.5-fold in the first 4 weeks of storage and remained 60% higher at 8 weeks than at harvest. In peel tissue of 1-MCP-treated fruit, however, putative a-FS mRNA had declined by 50% at 4 weeks and was barely detectable at 8 weeks of storage. By contrast, levels of HMG1 mRNA were quite high at harvest, did not change over 4 to 8 weeks of storage, and were not affected by 1-MCP treatment prior to storage.

X The putative a-FS cDNA was cloned into *E. coli* and expression of the encoded protein was optimized such that it was produced at high levels and composed 50% of the total soluble protein in bacterial cell lysates. Despite this we have not yet been able to demonstrate enzymatic activity using a broad range of assay conditions.

X Tissue cultures have been established using surface-sterilized germinating seed from 'Law Rome' apples. This tissue can be utilized to generate plants transformed with antisense constructs of the a-FS and HMG genes, with the aim of downregulation of a-farnesene synthesis.

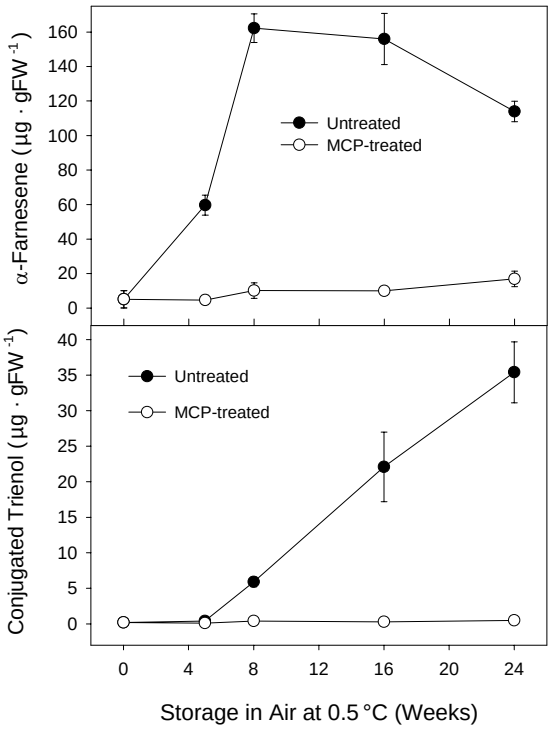
Methods: Current methods in molecular biology and biochemistry (including RNA and DNA gel blot and protein immunoblot analyses, DNA cloning and sequencing, expression of cloned apple genes in bacteria using suitable expression vectors, and enzyme assays and characterization using appropriate substrates and cofactors) were employed to examine the mechanism whereby ethylene stimulates the a-farnesene biosynthetic pathway in peel tissue of apple fruit during the first few weeks of cold storage. Apple peel HMG1 and a putative a-FS cDNA were cloned into *E. coli* or and expressed, and the encoded enzymes were partially purified and assayed. Epitope-tagged constructs of these cDNAs were cloned into *E. coli* to enable determination of protein expression levels and approximate molecular masses on immunoblots (Western blot analysis). Standard tissue culture techniques were used to establish sterile tissue cultures from germinating apple seed. Experiments were initiated to demonstrate enzymatic oxidation (hydroxylation) of a-farnesene to conjugated trienols (9*E* and 9*Z* isomers of 2,6,10-trimethyldodeca-2,7,9,11-tetraen-6-ol), the predominant *in vivo* oxidation products of a-farnesene that accumulate in apple peel during storage (Rowan et al., 1995).

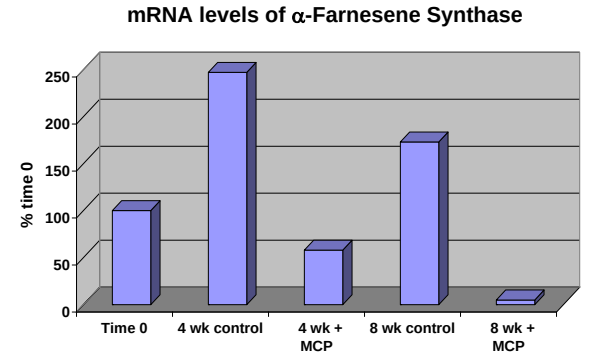
Degenerate primers based on conserved regions of plant cytochrome P450 (CYP) genes were designed to clone and amplify CYP genes from apple peel that might code for a-farnesene oxidase (hydroxylase).

Results and discussion: A cDNA library was constructed with RNA from peel tissue of highly scald-susceptible 'Law Rome' apple fruit and utilized to clone genes encoding key enzymes in the a-farnesene biosynthetic pathway. So far we have isolated one full-length and two partial cDNAs that code for three HMGR isozymes, and a full-length putative terpene synthase cDNA that likely encodes a-FS. Using these genes and the potent inhibition of a-farnesene synthesis by 1-MCP as our experimental system, we have begun to determine, at the molecular level, how ethylene induces production of a-farnesene in apples after a short duration in storage. Superficial scald has long been associated with synthesis and oxidation of a-farnesene (Huelin and Murray, 1966), and although there is some recent contradictory evidence (Rupasinghe et al., 2000), it is still generally accepted that oxidation products of a-farnesene are directly linked with development of severe scald. An understanding of the genetic and physiological controls of a-farnesene synthesis in harvested apple fruit should enable molecular genetic manipulation to dramatically reduce production of the sesquiterpene. This is clearly a long-term endeavor, but the potential benefits to both the apple industry and the consumer are great. At the very least, specific elimination of a-farnesene synthesis in fruit of a highly scald-susceptible cv. such as 'Law Rome' or 'Granny Smith' would provide proof of whether a-farnesene is required for scald induction. If it is, as many studies have indicated, it should be possible to develop new scald-resistant lines of popular commercial apple cvs. that no longer require a prestorage drench with DPA plus a fungicide. Unlike most of the genetically-modified seed crops that have been the focus of much (largely unfounded) criticism by the public and environmental groups, the transgenic apple lines would not include any foreign genes. Furthermore, specifically inhibiting a-farnesene synthesis by crippling a gene exclusively involved in the sesquiterpene pathway would not affect any other desirable trait of the fruit. To date, the closest approximation to this is prestorage treatment of apples with 1-MCP, which strongly inhibited a-farnesene synthesis and greatly reduced scald in 'Law Rome' fruit (Fig. 1; Watkins et al., 2000). However, blocking ethylene action with 1-MCP affects many aspects of fruit physiology and biochemistry, not just a-farnesene production (Fan et al., 1999a,b).

The peel tissue from control and 1-MCP-treated 'Law Rome' fruit used to generate the data in Fig. 1 was also the source of the RNA used in our molecular biology work. Pooled RNA from 4 and 8 week controls was used to construct the cDNA library from which the HMG1 and putative a-FS cDNAs were isolated. Radiolabeled gene-specific 3'-UTR fragments of these cDNAs were hybridized with peel tissue mRNA from 0-, 4-, & 8-week control and 1-MCP-treated fruit on RNA gel (Northern) blots. A large increase in transcript of the putative a-FS occurred in the initial weeks of storage in untreated fruit, whereas this mRNA dropped to very low levels in 1-MCP-treated fruit (Fig. 2). In contrast, HMG1 was expressed at the same high level regardless of storage or 1-MCP treatment (Fig. 3; Pechous and Whitaker, 2002). As well, the very low level of HMG3 transcript in 4- and 8-week control fruit indicates that expression of this HMG gene is not involved in the early burst of a-farnesene production (data not shown). These data appear to contradict a report by Ju and Curry (2000), which indicated that control of a-farnesene synthesis by ethylene is mainly a function of transcription and translation of HMG gene(s). However, Rupasinghe et al. (2001) recently reported that HMG2 is strongly upregulated by ethylene in peel tissue of 'Red Delicious' fruit, and we have now generated a cDNA encoding about two-thirds of the HMG2 from 'Law Rome.' It is also possible that ethylene induces synthesis of regulatory proteins that stimulate translation and/or activity of HMGR. Future work to purify, characterize, and construct antibodies against the HMG and putative a-FS enzymes should answer important questions about their levels in apple peel tissue at harvest and during storage, and whether post-translational modification (e.g. phosphorylation or prenylation) is required for enzymatic activity. We have achieved expression of active HMG1 enzyme in *E. coli*

(Fig. 4; Pechous and Whitaker, 2002), as well as high-level expression of the putative a-FS. Efforts to obtain full-length clones of HMG2 and HMG3 by 5'-RACE in our lab and by others (Rupasinghe et al., 2001) have so far been unsuccessful, most likely due to GC-rich regions in the 5' end of these genes. Use of thermostable DNA polymerase and a higher reaction temperature may solve this problem. Our inability to show enzymatic activity of the putative a-FS expressed in bacteria has been frustrating and collaboration with a lab highly experienced in assay of terpene synthases and cyclases is planned. Rupasinghe et al. (2000) reported that activity of a-FS in extracts of 'Red Delicious' peel tissue was lost with attempts to purify the enzyme, suggesting that, unlike most terpene synthases, a-FS requires specific cofactors or secondary proteins for activation. Fortunately, lack of demonstrated a-FS activity or full-length HMG2 and HMG3 cDNAs does not preclude efforts to transform apple tissue with antisense constructs of these genes. Because apple leaf tissue is capable of a-farnesene production, transformed plants could be tested at the seedling stage for reduced levels of a-FS or HMG mRNAs, as well as a-farnesene in the headspace volatiles (Mir and Beaudry, 1999).





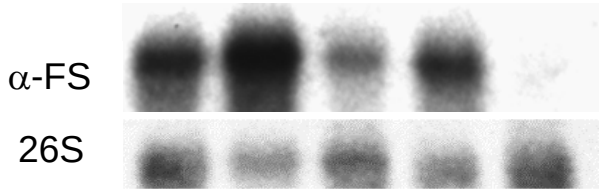
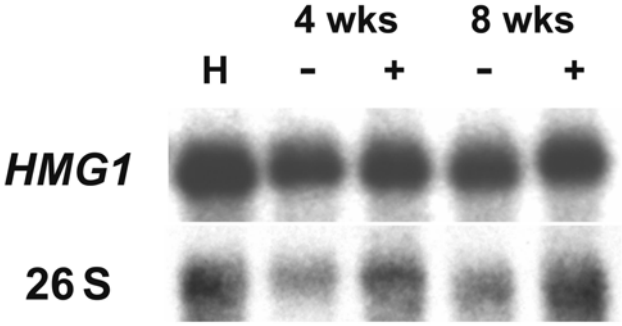


Fig. 1 Effect of 1-MCP treatment on accumulation of α-farnesene and its conjugated trienol oxidation products during storage of 'Law Rome' apples. Scald incidence was 99% in control and 1% in MCP-treated fruit.

Fig. 2 Effect of 1-MCP treatment on changes in the level of putative α-Farnesene Synthase mRNA in peel tissue of 'Law Rome' apples over 4 to 8 weeks of storage in air at 0.5 °C.



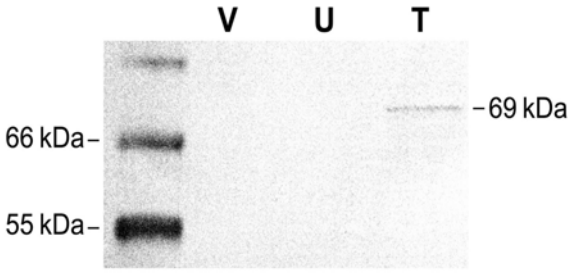


Fig. 3 Effect of 1-MCP treatment on changes in the level of HMG1 mRNA in peel tissue of 'Law Rome' apples over 4 to 8 weeks of storage in air at 0.5 °C.

Fig. 4 Protein immunoblot (Western blot) showing epitope-tagged HMG1 gene product expressed in transformed E. coli cells.

Budget: One year of funding to pay the GS-11 salary, benefits, and indirect research costs for a Postdoctoral Research Associate was awarded in August and received in October of 1999. The attempt to hire a Korean citizen to fill the position in 2000 was thwarted by the inability to secure the necessary visa. Steve Pechous, a highly qualified recent Ph.D. from Penn State, was hired in late December of 2000. Thus no additional funds were requested for 2000. A second year of funding to pay Dr. Pechous's GS-11 salary and benefits was awarded in August 2001.

Project duration: 1999-2001
Non-chemical Control of Superficial Scald
Bruce D. Whitaker
Project total – 3 years: $116,130

Year	Year 1 (1999)	Year 2 (2000)	Year 3 (2001)
Total	55,534	0	0

Yearly breakdown over project duration

Item	Year 1 (1999)	Year 2 (2000)	Year 3(2001) [§]
Salary (GS-11)	40,714	0	44,352
Benefits (+24%)	9,771	0	10,644
Services [‡]	0	0	3,800
Supplies [†]	0	0	1,800
I.R.C.*	5,049	0	0
Total	55,534	0	60,596

* Indirect research costs collected by the Beltsville Area Administration

[‡] Custom peptide antibody production ($2,600); DNA sequencing ($1,200)

[¤] Protein affinity column packing ($1,000); expression vector cloning kits and supplies ($800)

['] Increases relative to 2000 reflect cost of living adjustment to GS-11 salary

Other funding sources: Base funding for our research is provided by ARS through CRIS Project # 1275-42430-001-00D, "Quality Maintenance and Food Safety of Fresh and Fresh-cut Fruits and Vegetables," which includes 3.4 staff scientists, 1 support scientist, 3 technicians, and several other support personnel. The total allocation for FY-2001 was $1,001,210. Of this amount, $872,859 was expended on salaries and indirect research costs, leaving $128,351 for all other expenses.

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