FINAL REPORT
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Project title: Regulation of Ethylene-induced Farnesene Synthesis to Control Scald

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Objectives: (1) Obtain complete genomic clones plus promoter regions of apple peel genes encoding HMGR isozymes HMG2 and HMG3 and determine whether expression of these genes is regulated by ethylene; (2) Demonstrate function of the gene product and obtain a genomic clone of the ethylene-responsive promoter of the putative apple α-Farnesene Synthase gene; (3) Determine correlation among ethylene-induced transcription of genes encoding enzymes in the α-farnesene synthetic pathway, accumulation of α-farnesene during storage, and the incidence and severity of scald development; (4) Transform apple shoots with antisense constructs of HMG2, HMG3, and/or α-FS genes and test transgenic apple plants for suppression of α-farnesene production.

We previously cloned a full-length terpene synthase gene from ‘Law Rome’ apple peel tissue, which we believed was likely to encode an α-farnesene synthase (α-FS). Because *α-FS* is the optimal gene to target for highly specific inhibition of α-farnesene synthesis, a great deal of time and effort was spent trying to express this gene in bacteria and obtain a soluble protein product with enzymatic activity. Several weeks ago we were successful, and cells of *E. coli* containing the putative apple *α-FS* cDNA were induced to express an enzyme that efficiently catalyzed the conversion of farnesyl diphosphate (FDP) to (*E*,*E*)-α-farnesene, the isomer of α-farnesene that is produced naturally in apple peel tissue. This showed unequivocally that the ‘Law Rome’ terpene synthase gene encodes an α-FS. Using primers based on the the ‘Law Rome’ *α-FS* cDNA, the corresponding *α-FS* cDNA from ‘Ida Red’ apple was quickly obtained by reverse transcription of peel tissue mRNA. This established a good experimental system for comparison of ethylene-induced *α-FS* gene expression and α-farnesene production in fruit of a highly scald-susceptible cultivar (Law Rome) and fruit of a very scald-resistant cultivar (Ida Red).

An apple gene encoding a 3-hydroxy-3-methyl glutaryl CoA reductase (HMGR) isozyme has also been targeted as a likely control point in the α-farnesene biosynthetic pathway. Previous work in our laboratory and in two other labs has identified three apple genes, *HMG1*, *HMG2*, and *HMG3*, encoding different HMGR isozymes. On the basis of studies with other plant species, it is probable that one of the apple HMG genes is largely dedicated to the branch pathway involved in α-farnesene synthesis. Data from Northern blot analysis of gene transcript levels in peel tissue of apples at harvest and after 4-16 weeks of 0 °C storage indicated that *HMG2* expression is induced by ethylene and therefore the HMG2 is most likely to be the isozyme involved in α-farnesene synthesis (Rupasinghe et al. 2001). Many attempts to obtain a full-length cDNA of ‘Law Rome’ *HMG2* by RT-PCR and 5'-RACE failed due to a very long GC-rich region in the 5' end of the sequence, but quite recently we were able to use a genome walk strategy to obtain a complete genomic clone of *HMG2*.

Primers based on this genomic clone were used to generate two overlapping cDNA fragments that can be combined to yield the full-length *HMG2* cDNA for bacterial expression studies. The genomic *HMG2* also includes a long stretch of the upstream 5'-flanking region that should encompass most or all of the promoter and the hypothesized ethylene regulatory element (ERE). Cloning and further study of this promoter will help to elucidate how ethylene induces expression of the gene.

Progress and significant findings: Project accomplishments and findings during the past 12 months (06/02 – 06/03) were as follows:

- A genome walk strategy was employed to obtain a full-length genomic clone of the *HMG2* gene from 'Law Rome' apple. The clone also includes about 1.5 kb of the 5'-flanking region of the *HMG2* start codon, and therefore should include most, if not all, of the promoter sequence. Using 5' and 3' primers based on the genomic sequence, two large cDNA fragments that encompass the entire gene were obtained by 3'- and 5'-RACE. In the last week, RT-PCR with 'Law Rome' mRNA and thermostable enzymes appears to have yielded a full-length *HMG2* cDNA. If not, the two large cDNA fragments will be spliced to produce a complete cDNA.

- Efforts to optimize conditions for functional expression of the putative apple *α-FS* cDNA in *E. coli* were finally successful about one month ago. The soluble terpene synthase produced by the bacterial cells was shown to efficiently catalyze conversion of the farnesyl diphosphate (FDP) substrate to (*E*,*E*)-α-farnesene. Only trace amounts of other terpene products were detected, and incubation with geranyl diphosphate (GDP) as substrate yielded relatively small amounts of (*E*)-β-ocimene, the monoterpene counterpart of the sesquiterpene α-farnesene. Further biochemical characterization (pH optimum, etc.) of the bacterially expressed α-FS is in progress.

- Using 5' and 3' primers based on the full-length 'Law Rome' *α-FS* cDNA and total RNA extracted from 'Ida Red' apple peel tissue, a full-length cDNA of the 'Ida Red' *α-FS* was obtained. 'Law Rome' is a highly scald-susceptible apple, whereas 'Ida Red" fruit are quite scald resistant. Preliminary results from RT-PCR analysis indicated that expression of the *α-FS* gene is about 5-fold higher at harvest and after 4 weeks of 0 °C storage in 'Law Rome' compared with 'Ida Red' apples, which is consistent with the marked difference in scald susceptibility.

- A repeat experiment was conducted with 'Law Rome' apples harvested from the Cornell University orchards. As observed with fruit from the same location two years ago, RT-PCR analysis of *α-FS* mRNA levels in peel tissue of non-treated and 1-MCP-treated apples showed that expression of the *α-FS* gene increased at least twofold in non-treated control fruit but declined substantially in 1-MCP-treated fruit during the first 4 weeks of air storage at 0 °C. This confirms the finding that *α-FS* gene expression is greatly enhanced by ethylene in cold-stored fruit and is likely a key control point in the marked increase in α-farnesene synthesis early in storage.

- A plasmid containing a "sense" (normal 5' to 3' orientation) construct of the 'Law Rome' α-FS cDNA driven by the CaMV 35S promoter was introduced into *Agrobacterium* for transformation of *Arabidopsis* and apple tissue. Transgenics of *Arabidopsis* are currently being selected and will be tested for the acquired ability to synthesize α-farnesene.

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 α-farnesene biosynthetic pathway in peel tissue of apple fruit during the first few weeks of cold storage. An untagged construct of the 'Law Rome' peel tissue putative *α-FS* cDNA

was expressed in *E. coli*. Assays of the soluble terpene synthase from lysed bacteria were conducted at pH 7.3 with farnesyl diphosphate (FDP) as the substrate for sesquiterpene synthesis, and at pH 7.5 with geranyl diphosphate as the substrate for monoterpeene synthesis. The divalent cation cofactor in both assays was Mg^{2+}. Analysis of the terpene products was performed by GC-MS using a 30 m CycloSil B chiral capillary column that efficiently separates terpene enantiomers. Biochemical characterization of the 'Law Rome' α-FS was initiated to determine pH and temperature optima, divalent cation requirements (Mg^{2+} versus Mn^{2+}), and potential specific inhibitors. Collaborators at Cornell University provided peel tissue from scald-susceptible 'Law Rome' and scald-resistant 'Ida Red' apples that were untreated or treated with 2 µL/L 1-MCP and stored for 0 to 24 weeks at 0 °C in air. Primers based on the 'Law Rome' α-FS cDNA sequence and 5' and 3' RACE were used with peel tissue RNA from 'Ida Red' apples to obtain a complete 'Ida Red' α-FS cDNA (open reading frame). A genome walk strategy was utilized to obtain a full-length genomic clone of 'Law Rome' *HMG2* that includes the 5'-flanking promoter region. Primers based on the genomic sequence and RT-PCR with highly thermostable enzymes were used to produce what appears to be a full-length *HMG2* cDNA (not yet sequenced).

In collaboration with postharvest plant physiologists at Cornell University and the Volcani Center in Israel, experiments were initiated last year to evaluate the correlation between inhibition of α-farnesene synthesis following prestorage heat or 1-MCP treatment and reduction or prevention of scald in 'Granny Smith' apples. Northern blot analysis and quantitative RT-PCR are currently being used to examine the expression of genes in the α-farnesene pathway in relation to α-farnesene production and eventual development of scald in 'Granny Smith' fruit stored at 0 °C in air.

Conditions for 'Law Rome' tissue culture were established and tissue is currently available for transformation experiments. A sense construct of the *α-FS* cDNA driven by the CaMV 35S promoter has been inserted into the pBI 121 vector in *Agrobacterium* for transformation of apple tissue to conduct cosuppression experiments. Seed of *Arabidopsis* plants infected with *Agrobacterium* containing this vector and construct are currently being screened for transformants, which will eventually be tested for the acquired ability to produce α-farnesene.

Results and discussion: Two primary objectives of the project were realized in the past few weeks: (1) functional expression of the 'Law Rome' terpene synthase cDNA in *E. coli*, which demonstrated that the gene encodes an α-farnesene synthase, and (2) isolation of a complete genomic clone of 'Law Rome' *HMG2*, the gene encoding the isozyme of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) that is most likely to initiate the α-farnesene biosynthetic pathway (Rupasinghe et al., 2001). Successful completion of this phase of the research was essential for attainment of the long-range goals of the project. Using the *α-FS* and *HMG2* genes and the inhibition of α-farnesene synthesis by 1-MCP as our experimental system, we have begun to determine, at the molecular level, how ethylene induces production of α-farnesene in apples after a short duration in cold storage.

Superficial scald has long been associated with synthesis and oxidation of α-farnesene (Huelin and Murray, 1966), and although there is some contradictory evidence (Rupasinghe et al., 2000a), it is still generally accepted that oxidation products of α-farnesene are directly linked with development of severe scald. The most compelling evidence in support of the hypothesis is 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. In addition, experiments comparing untreated control and 1-MCP-treated scald-susceptible apples have repeatedly shown that ethylene perception is critical for induction of both α-farnesene production and scald development (Fan et al., 1999b; Rupasinghe et al., 2000b; Watkins et al., 2000).

An understanding of the genetic and physiological controls of α-farnesene synthesis in harvested apple fruit should enable molecular genetic manipulation to dramatically reduce production of the sesquiterpene. Alternatively, comparison of the *α-FS* and *HMG* genes and their regulation in highly

scald-susceptible and scald-resistant apple cultivars (e.g., ‘Law Rome’ and ‘Ida Red,’ respectively) should identify unique genetic markers that could be used in a conventional breeding program to select potentially scald-resistant lines at the seedling stage of development. With either approach, this is clearly a long-term endeavor, but the potential benefits to both the apple industry and the consumer are great. Specific elimination of α-farnesene synthesis in fruit of a highly scald-susceptible cultivar such as ‘Law Rome’ or ‘Granny Smith’ would provide proof of whether α-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 cultivars that no longer require a prestorage drench with DPA plus a fungicide. Transgenic apple lines with antisense or RNAi suppression of *α-FS* or *HMG2* would not include any foreign genes. Furthermore, specifically inhibiting α-farnesene synthesis by crippling a gene exclusively involved in the sesquiterpene pathway would not affect any other desirable trait of the fruit. The closest approximation to this is prestorage treatment of apples with 1-MCP, which strongly inhibited α-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 α-farnesene production (Fan et al., 1999a,b). One additional, very useful outcome of this research will be a better understanding of how ethylene induces the expression of genes involved in production of volatiles and other ripening- and senescence-related processes. At present, there is very little known about the final steps of the ethylene signal transduction cascade, in which specific DNA-binding proteins recognize ethylene-response elements in the gene promoter and induce or suppress transcription of the gene (Deikman, 1997; Deikman et al., 1998).

Both *myc*-epitope-tagged and untagged constructs of the ‘Law Rome’ putative *α-FS* cDNA were expressed in *E. coli*. Protein immunoblot (Fig. 2) and SDS-PAGE (Fig. 3) analyses showed protein bands at ~67 kDa and ~65 kDa for the epitope-tagged and untagged terpene synthases, respectively. Immunoblots revealed that, under a broad range of induction conditions, the epitope-tagged expressed protein was always sequestered in inclusion bodies and was not present in the soluble fraction released by lysis of the bacterial cells (Fig. 2). An effort was made to recover active enzyme by urea denaturation of isolated inclusion bodies followed by slow renaturation. Under these conditions, the renatured epitope-tagged protein produced very small amounts of the sesquiterpene alcohol (*E*)-nerolidol from the FDP substrate. Fortunately, it was eventually established that induction of bacterial expression of the untagged *α-FS* construct at 20 °C for 4 h with addition of 1.7 mM IPTG yielded significant amounts of the soluble expressed protein (Fig. 3). When the untagged soluble enzyme was assayed at pH 7.3 with FDP as substrate, it yielded relatively large amounts of one predominant sesquiterpene product with a GC retention time of 23.83 min (Fig. 4). GC-MS analysis with separation on a chiral capillary column that gives good resolution of terpene isomers showed that the major product was (*E*,*E*)-α-farnesene (Fig. 5), the naturally-occurring α-farnesene isomer in apple peel tissue. Thus, we now have unequivocal proof that the ‘Law Rome’ terpene synthase gene that is upregulated in fruit peel tissue in response to ethylene during the initial weeks of 0 °C storage is an α-farnesene synthase.

Given this information, primers based on the ‘Law Rome’ *α-FS* cDNA were used to clone a corresponding *α-FS* cDNA from scald-resistant ‘Ida Red’ apple. Not surprisingly, the proteins encoded by the ‘Law Rome’ and ‘Ida Red’ *α-FS* cDNAs differ by only a few conserved amino acid substitutions. An important next step toward understanding induction of α-farnesene synthesis in apple peel tissue by ethylene and low temperature will be to use primers based on the *α-FS* cDNA sequences to isolate the corresponding genomic clones that include the entire promoter and ethylene-response element in the 5'-flanking region. Inasmuch as the open reading frames of the ‘Law Rome’ and ‘Ida Red’ *α-FS* genes are nearly identical, differences in their promoters and expression of the genes are likely to play a key role in the markedly different rates of α-farnesene production in fruit of the two cultivars. In support of this proposal, preliminary data from RT-PCR analysis of *α-FS*

transcript levels in peel tissue from fruit at harvest and after 4 weeks at 0 °C indicated that *α-FS* mRNA was about 4- to 5-fold higher in 'Law Rome' compared with 'Ida Red' (data not shown).

A complete genomic clone of 'Law Rome' *HMG2* was also obtained in the course of our work this past year and the corresponding cDNA will soon be available for bacterial expression studies (as in Pechous and Whitaker, 2002). The next step will be to determine definitively, by quantitative RT-PCR and/or Northern blot analysis, whether *HMG2* transcript levels are significant and increase in response to ethylene in the initial weeks of cold storage. It is also possible that ethylene induces synthesis of regulatory proteins that stimulate translation of *HMG2* message and/or activity of the HMG2 enzyme. Future work to purify, characterize, and construct antibodies against the HMG2 and α-FS enzymes should answer important questions about their levels in apple peel tissue at harvest and during storage, and whether post-translational modification of HMG2 (e.g. phosphorylation or prenylation) is required for optimal enzymatic activity.

The stage is set for *Agrobacterium*-mediated transformation of apple tissue with antisense or RNAi constructs of the *α-FS* and *HMG2* genes. Regeneration of stable transgenic plantlets will likely be the most difficult and time-consuming task in continuing this line of research. Because apple leaf tissue is capable of α-farnesene production, transgenic plants could be tested at the seedling stage for reduced levels of *α-FS* or *HMG2* mRNAs, as well as α-farnesene in the headspace volatiles (Mir and Beaudry, 1999). If we are able to generate stable transgenic plants of a highly scald-susceptible cultivar such as 'Law Rome' or 'Granny Smith' that exhibit a 90-100% reduction in α-farnesene synthesis, we will definitely follow through (undoubtedly as a collaborative effort) with the long-range processes of grafting to appropriate root stock and growing trees to the fruit-bearing stage.

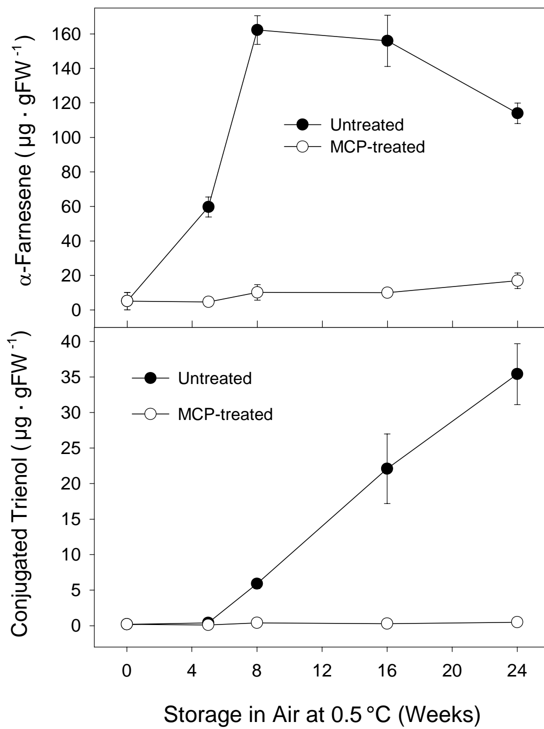


Fig. 1 Effect of 1-MCP on accumulation of α-farnesene and its conjugated trienol oxidation products during storage of 'Law Rome' apples. Scald incidence was 99% in control and 1% in 1-MCP-treated fruit.

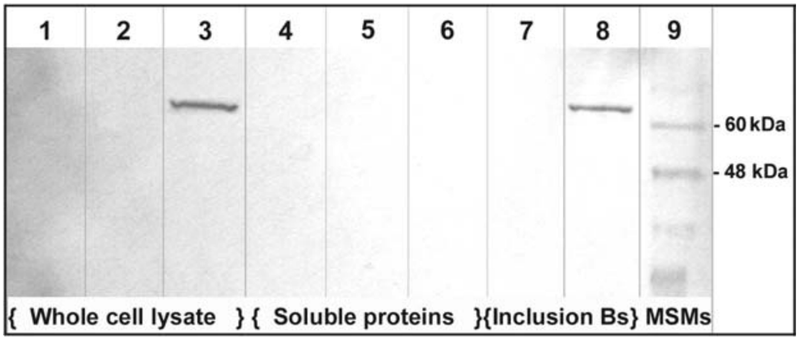


Fig. 2 Immunoblot (Western blot) of epitope-tagged *α-FS* gene product expressed in transformed E. coli cells. Lanes 1 & 4 = empty vector controls; lanes 2, 5 & 7 = untagged construct; lanes 3, 6 & 8 = *myc*-tagged construct; lane 9 = molecular size markers (MSMs). Note that the *myc*-tagged protein was detected in whole cells and inclusion bodies, but not in the soluble fraction from cell lysates.

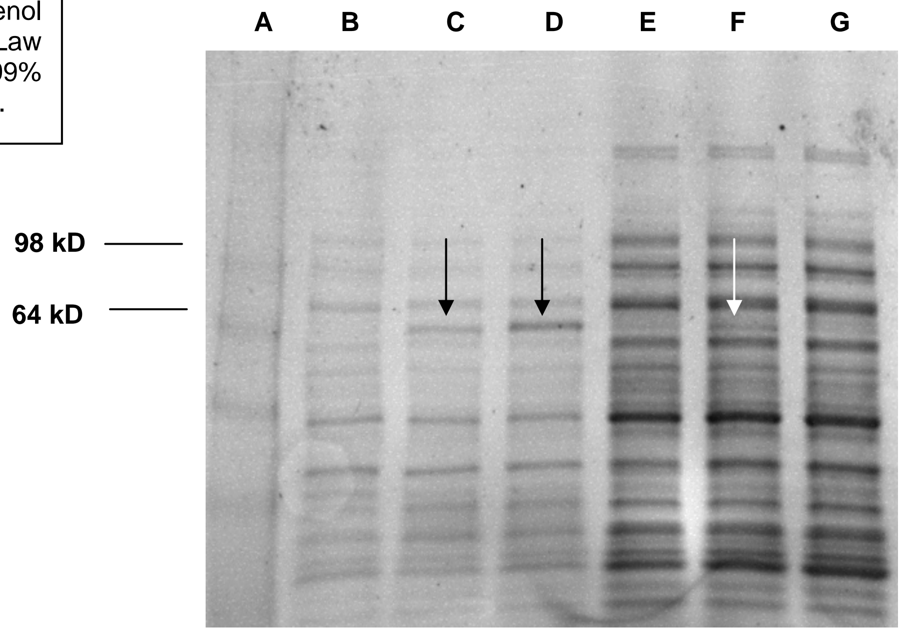


Fig. 3 Coomassie-stained SDS-PAGE of bacterial proteins derived from whole cells (B, C, D) or the soluble fraction from cell lysates (E, F, G). Lane A = molecular size markers; lanes B & E = empty vector controls; lanes C & F = untagged construct; lanes D & G = *myc*-epitope-tagged construct. Black arrows show substantial levels of the untagged and epitope-tagged proteins in whole cells. White arrow shows that only the untagged protein was present in the soluble cell lysate fraction.

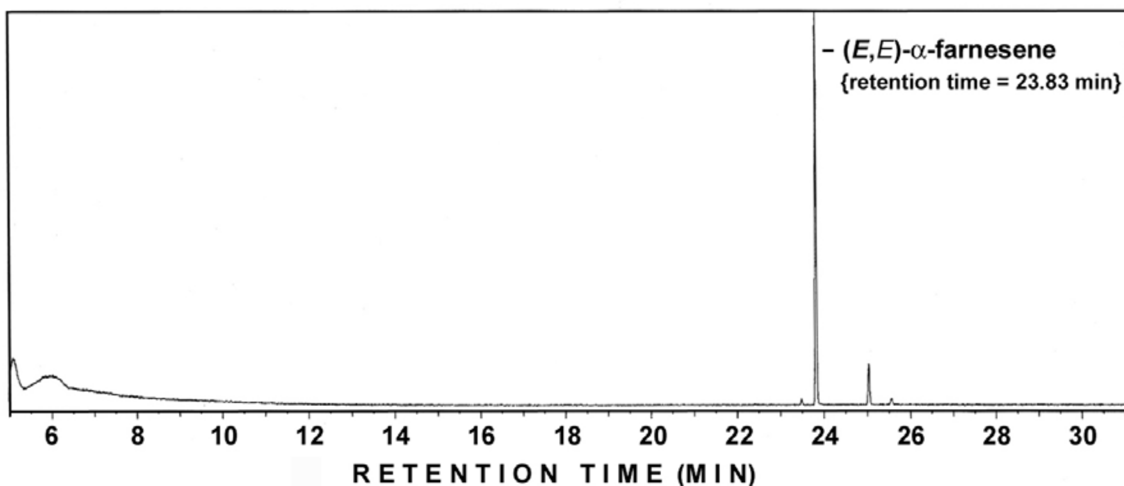
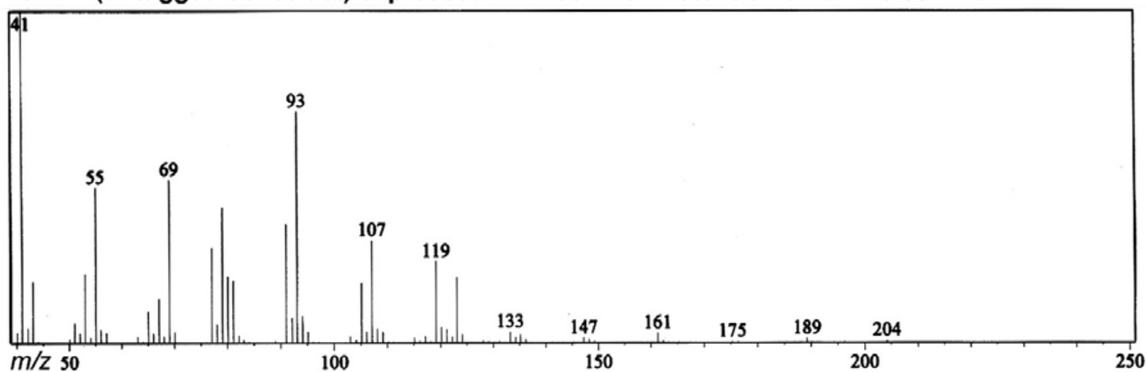


Fig. 4 GC-MS total ion current (TIC) chromatogram of sesquiterpene products of the ‘Law Rome’ terpene synthase showing (*E*,*E*)-α-farnesene as the predominant constituent.

EI mass spectrum of predominant sesquiterpene product of 'Law Rome' terpene synthase cDNA (untagged construct) expressed in *E. coli*: GC retention time = 23.83 min



EI mass spectrum of (*E*,*E*)-α-farnesene {3,7,11-trimethyl-1,3,6,10-dodecatetraene} from NIST mass spectra library: Formula = C15H24 Molecular Mass = 204

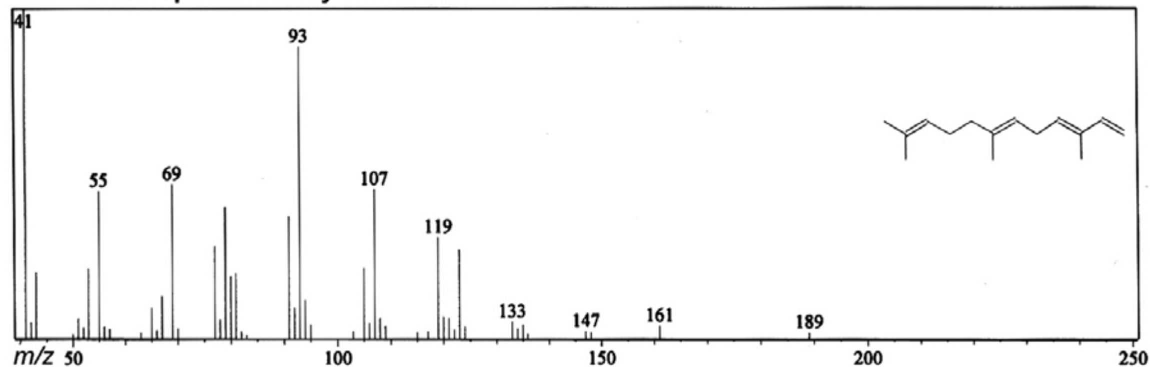


Fig. 5 Comparison of GC-MS EI mass spectra of predominant ‘Law Rome’ terpene synthase product from the FDP substrate and NIST library entry for (*E*,*E*)-α-farnesene.

Budget: One year of funding to pay ~91% of the GS-11 salary for postdoctoral Research Associate Steve Pechous was awarded in September, 2002 and received in May 2003.
Regulation of Ethylene-induced Farnesene Synthesis to Control Scald
Bruce D. Whitaker
Project duration: 2002-2003
Amended budget request

Item	Year 1 (2002)	Year 2 (2003)
Salary (GS-11-02)	45,000*	0
Benefits (+24%)	0	0
Total	45,000	0

* Represents ~91% of GS-11-02 salary with locality pay = $49,566

Other funding sources: Base funding for our research is provided by ARS through CRIS Project # 1275-43000-007-00D, "Quality Maintenance and Food Safety of Fresh and Fresh-cut Fruits and Vegetables," which includes 3 staff scientists, 4 technicians, and several other support personnel. The total allocation for FY-2002 was $1,002,263. Of this amount, $911,615 was expended on salaries and indirect research costs, leaving $90,648 for all other expenses.

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