FINAL PROJECT REPORT **Year 2/2**
WTFRC Project #: **WSU ARC Project #: 3055-3799**

Project title: Apple Scald/Lenticel Disorder Biochemistry

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OBJECTIVES

OVERALL OBJECTIVE. Determine the role of farnesyl protein transferase in signaling the peel cell death associated with scald development. Using antibodies to human and tomato FPT, we have identified presence of the protein in apples. In light of research findings coupled with molecular modeling studies, oxidation of FPT due to farnesene radicals may contribute to cell-death scald symptoms. Our research in the first year concentrated on the role of organic radicals (farnesene oxidation products) as destructive analogs of FPP that kill FPT, interfering with cellular signaling, causing cell death, and producing the symptoms of superficial scald. However, our experimental results from year one indicated a need to further examine FPT activity in light of the documented role of ethylene in the development of scald, and the possibility that inactivation of FPT could putatively prevent scald.

- Examine the effect of specific inhibitors of FPT on scald development in 'Granny Smith' apples. These molecules are currently undergoing clinical trials to treat cancer in humans.
- Characterize apple FPT. Once sufficient quantities of apple FPT are available, examine FPT for activity and substrate specificity in order to develop specific blockers/enhancers of FPT as a new scald treatment. NOTE: this is an ambitious undertaking and will take more time than the project duration.
- Determine the role of antioxidants, natural or synthetic, in protection of native FPT in apple skin.
- Using spintrap molecules, characterize the nature and amount of free radical species present during the scald-inducing reactions.

SIGNIFICANT FINDINGS

2002-2003

'Granny Smith' apples from the 2002 harvest were treated with 1-MCP, and stored under RA (control) or CA conditions. After 8 months storage, apples were placed at 19ºC. At one (D1) and 7 days (D7), volatiles were measured (Fig. 1) and α-farnesene oxidation products in apple peel were quantified. Apples from each treatment were kept for an additional 33 days at 19ºC, with last sampling at 40 days (D40).

- Increased production of farnesene in apple disks treated with an FPT inhibitor, showing the shunting of unused FPP into farnesene production.

- Western blot indicating consistent expression of FPT in CA and RA fruit, suggesting that FPT is not destroyed in apple peel under either storage condition.
- Farnesene production in CA stored fruit at D1 was similar to D1 RA fruit, but decreased slowly and at D40 was equivalent to RA production at D7. CA-MCP treated fruit did not produce any significant amount of farnesene until D40 (Fig.1).
- Ethylene production increased while farnesene production decreased. D40 CA-MCP fruit was approximately that of D1 RA controls (Fig. 1).
- The major farnesene radical species with a retention time of 5.7 increased over time in both CA and CA-MCP fruit, and at D40 in CA-MCP fruit was equal to RA fruit at D1 and D7 (Fig. 1).
- Conjugated trienol concentration decreased in RA controls, and increased in both CA and CA-MCP fruit, although MCP-treated fruit concentrations remained very low (Fig. 1).
- After 40 days at 19ºC, CA-MCP apples were not scalded (data not shown).

2003-2004

Apples were harvested on 9/12/03 and 10/6/03 and treated with either FPTI, ascorbigen antioxidant at 2500 ppm, or 1-MCP at 0.5 ppm, and stored for 7-8 months in either RA or CA conditions, at which time apples were removed from storage and placed at 19ºC. After 7 days at 19ºC, apples were evaluated for scald, respiration rate, ethylene and volatile production, conjugated trienol and radical concentration, and peel sampled for protein extraction. Apples from each treatment remained at room temperature in the laboratory for observation of scald progression and development of other treatment-related physiological phenomena.

- On western blot, the FPT protein was present in all treated samples (Fig. 2).
- Scald score was 3.5, 2.9, 2.7, 0.33, 0.067, and 0, in RA (control) apples, ascorbigen-treated apples, FPTI-treated apples, RA-MCP apples, CA apples, and CA-MCP apples, respectively (Fig. 3). Over time, FPTI treated apples exhibited cortical softening as well as considerable progression of scald. RA-MCP and CA apples showed some scald that continued to develop over time when held at room temperature (data not shown).
- Over 7 days, the mean respiration rate was 15%, 78%, 74%, and 85% less than RA controls for FPTI, RA-MCP, CA, and CA-MCP apples, respectively (Fig. 3). The mean respiration for RA controls was 40 ml kg/hr CO_2 production. This was likely due to the apoptotic effect of FPTI on electron transport as described by Suzuki *et al.* (1998).
- Ethylene and farnesene production were highest in CA stored apples, lowest in CA-MCP apples (Fig. 3). Volatile production in FPTI apples was similar to controls.
- MCP-RA apples produced a small amount of ethylene, while farnesene production was similar to FPTI and control apples (Fig. 3).
- Conjugated trienols were highest in CA stored controls (harvest 10/6/03) and FPTI-treated apples, and lowest in MCP-treated apples, both RA and CA stored (Fig. 3). Radical content was highest in apples treated with FPTI (Fig. 3). Radical content was also relatively high in CA-stored apples, followed by RA controls .

RATIONALE

The actual chemical mechanisms that govern scald development are slowly being characterized. Superficial scald, perhaps the most severe physiologic disorder known to occur in stored apples, has been circumstantially associated with oxidative stress. Since Murray *et al*. (Murray *et al.*, 1964) reported the presence of α-farnesene in the peel of apple fruit, it has been a major focus of scald research, and it has been suggested that its auto-oxidation products directly induce the development of this storage disorder (Anet, 1969; Rowan *et al.*, 1995; Rowan *et al.*, 2001). However, results of these studies have been inconclusive. Synthesized from trans, trans-farnesyl pyrophosphate

(FPP) by the action of the enzyme trans, trans-α-farnesene synthase (Rupasinghe *et al.*, 1998), farnesene is endogenously produced by the fruit throughout the storage period. The plant hormone ethylene, involved in many physiological and developmental processes (Abeles, 1985) has also been implicated in the development of scald.

Farnesyl protein transferase (FPT) has become a major focus of study in the medical community because it has been implicated in the development of several forms of human cancer (Haluska *et al.*, 2002). The enzyme FPT first binds FPP and then binds a protein to which it transfers the farnesyl moiety of FPP. The farnesyl moiety is hydrophobic and targets the protein to a membrane or other hydrophobic region, e.g. the hydrophobic domain of another protein (Marshall, 1993; Haluska *et al.*, 2002). Atomic resolution studies of the reaction path of FPT indicate that a new substrate molecule (FPP) must bind to displace the farnesylated protein (Long *et al.*, 2002). Thus, inhibition of substrate binding with an inhibitor molecule would prevent release of product and effectively inactivate FPT. Recent research into the role of FPT in the cell determined that FPT is necessary for the activation of all of the major mitogen activated protein kinases (MAPKs) that are involved in many cell signaling pathways (Vervenne *et al.*, 2002). Moreover, it has been established that ethylene signaling occurs through a MAPK pathway (Ouaked *et al.*, 2003). Constitutive ethylene response 1 (CTR1) is a member of this MAPK pathway and physically interacts with the integral membrane ethylene receptor, ethylene response 1 (ETR1) in plants (Clark *et al.*, 1998).

It is well-documented that apples harvested late, after the onset of the climacteric, are more resistant to scald. Moreover, apples treated with an ethylene releasing agent such as ethephon show a decrease in the occurrence of scald (Couey, 1973). Conversely, it has also been shown that treatment with MCP, an ethylene action inhibitor, reduces scald (Fan and Mattheis, 1999). We hypothesized that ethylene and FPT act in tandem to produce scald in apples. In the presence of ethylene (the signal) and FPT, the activator of CTR1, senescense is accelerated, farnesene is produced at lower levels, staying in the skin longer, where it is oxidized into radicals that destroy cell membranes and cause cell death, i.e., scald. In the absence of ethylene, as occurs in CA storage, metabolism is slowed and most of the pool of FPP is shunted to farnesene production, which in itself does not produce scald, and the production of damaging farnesene radicals is reduced, attenuating the symptoms of scald.

RESULTS

Results of this research support ethylene signaling and α-farnesene auto-oxidation products as components of scald. The presence of increased levels of conjugated trienols and radicals in FPTI-treated apples indicates increased farnesene production during storage. Scald was present in FPTI-treated apples and was accompanied by softening of cortical tissue that was not apparent in other treatments. Thus FPT inhibition appeared to induce apoptosis (cell death) in apple tissue below the peel. Respiration in FPTI-treated apples was decreased relative to controls, likely due to the effects of FPTI-induced release of cytochrome c from mitochondria and activation of caspase 3 in the presence of farnesyl protein transferase inhibitors (Suzuki *et al.*, 1998). Hence, FPT appears to play an important role in apple postharvest physiology. Interestingly, western blot analysis of FPT showed the presence of protein in FPTI-treated apples, thus it appears that inhibition does not lead to protein destruction. Decreased respiration in other treatments was likely attributable to a preclimacteric physiological state.

RA-MCP-treated apples were slow to scald, but scald did eventually develop, and we predict that CA-MCP apples will also scald. This may be due to synthesis of new ethylene receptors, functioning of other ethylene receptors with less affinity for MCP, or cell membrane damage from ROS. Alternatively, another ethylene signaling pathway may be involved.

FPT activation of CTR1 (farnesylation) remains equivocal. Specific screening for farnesylated components of apple cell membranes, possible cloning of apple CTR1, and further investigation of ethylene signaling pathways in other model species will be needed for a final determination.

CA stored MCP-treated apples did not scald, and all other indicators of scald development were also low. The combination of CA-MCP treatment is at present the best alternative to DPA. However, MCP does impact ripening, and studies to address texture, flavor, and aroma volatile production are needed to determine the best marketing strategy.

We have determined that apples treated with an FPT inhibitor scald similarly to RA stored controls, but additionally exhibit deep tissue softening resembling symptoms of soft scald. This study also documented intact FPT protein remains in the peel of apples treated with an FPT inhibitor, thus inhibition does not lead to destruction of the protein. We showed that ascorbigen has little value as an anti-scald, antioxidant treatment, and found that apples treated with MCP and stored under RA conditions will eventually scald through unknown mechanisms, perhaps functioning of ethylene receptors with a lower affinity for MCP, another ethylene signaling pathway, or turnover of ethylene receptors. Most significantly, CA-stored, MCP-treated apples retain physiological youth, and studies addressing texture, flavor, and aroma volatile development are recommended to determine an effective marketing strategy for these apples.

Potential applications

The availability of a "free-radical detector" system may enable future postharvest engineers to apply machine vision and robotic handling systems to sort apples for "scaldability". It would be possible to segregate fruit for DPA or other antioxidant treatment, thus lowering storage and marketing costs, not to mention the benefit to certified organic producers.

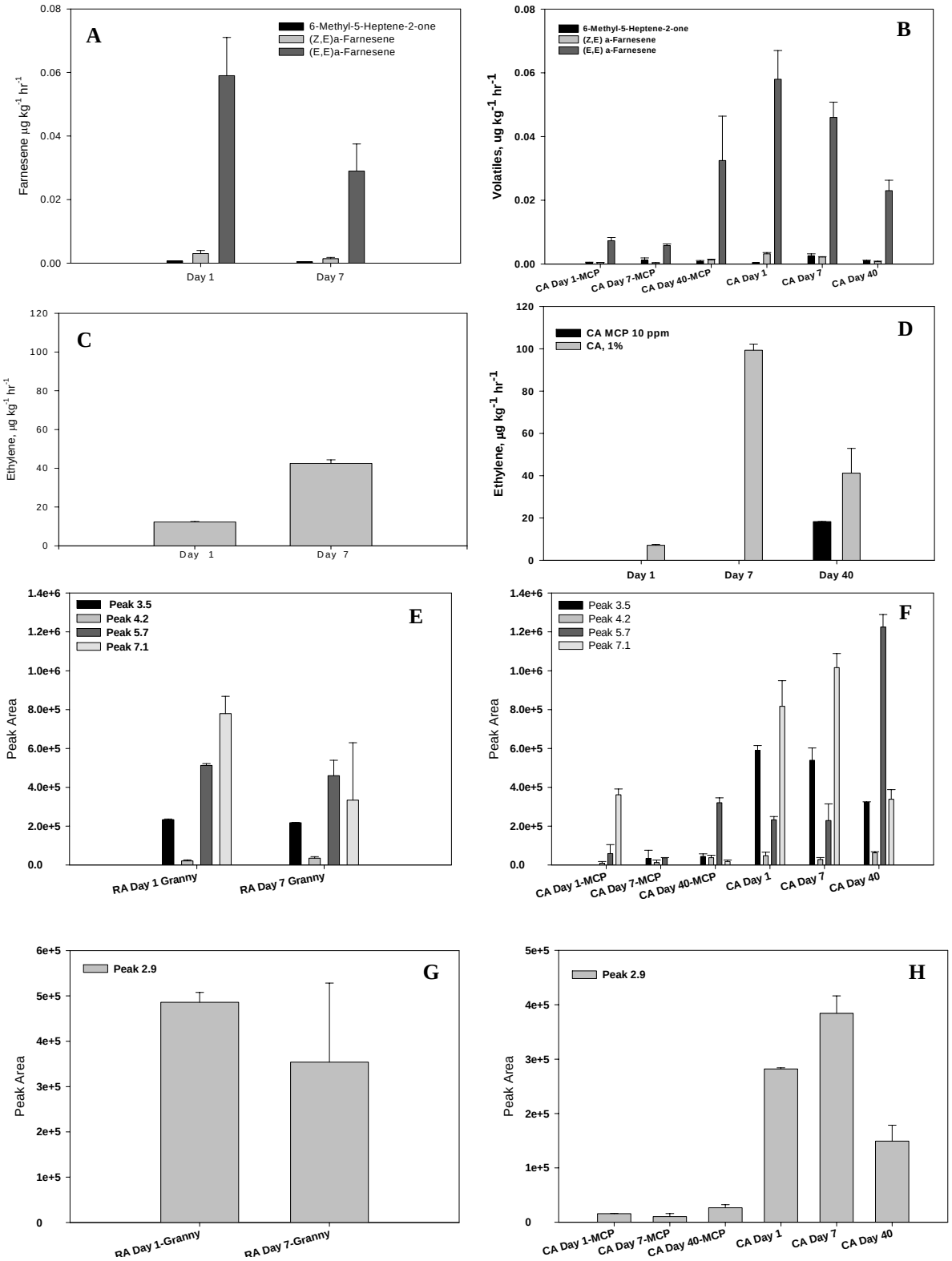

Fig.1. 'Granny Smith' apples stored for 8 months at 0ºC. Farnesene production in RA controls (A), CA-stored apples (B); ethylene production in RA (C), or CA-stored apples (D); farnesene oxidative radical concentration in RA (E), or CA (F) stored apples; conjugated trienol content in RA (G), or CA (H) stored apples. CA stored apples were either treated with 10 ppm 1-MCP prior to storage, or kept as CA controls. Measurements were taken at 1, 7, and 40 days in CA apples, 1 and 7 days in RA apples.

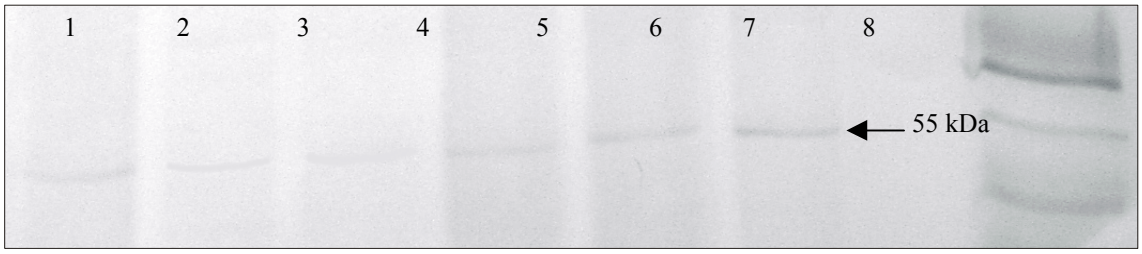

Fig. 2. Western blot following 7-8 months storage of treated 'Granny Smith' peel protein extracts hybridized with Rabbit Anti-Rat FPT followed by Donkey Anti-Rabbit IgG. Lanes: 1, RA; 2 RA-MCP; 3, 2500 ppm ascorbigen; 4, FPTI; 5, CA; 6, CA-MCP; 7, human FPT standard, (not shown); 8, MW marker. Samples were taken after 7 days at 19ºC.

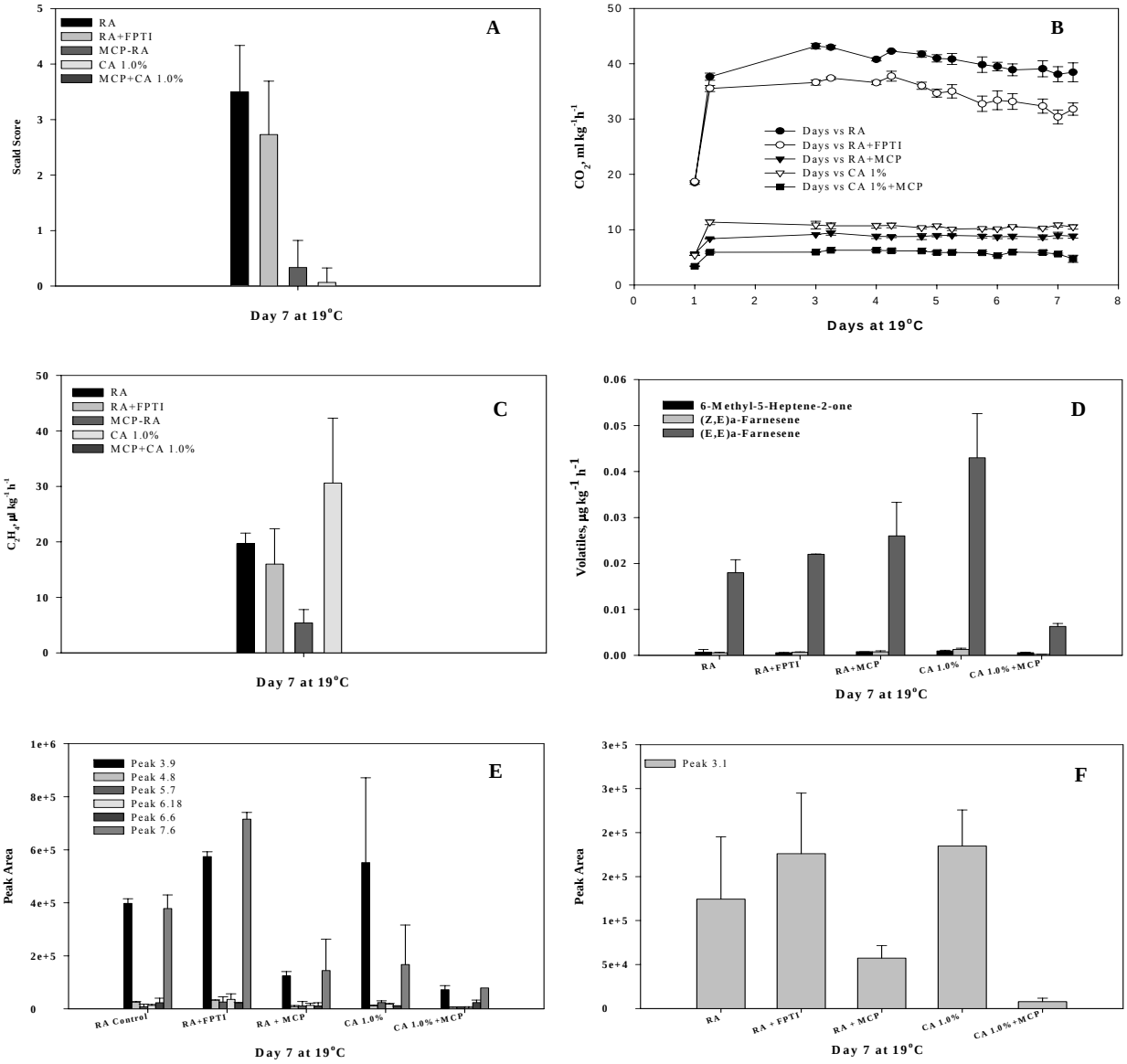

Fig. 3. Scald score (A), respiration rate (B), ethylene production (C),farnesene production (D), peel radical content (E), and peel conjugated trienol content (F) in 'Granny Smith' apples treated with MCP, or FPTI, stored under RA or CA conditions for 7-8 months, and placed at 19ºC.

Budget justification

Margo Haines, Postdoctoral Research Associate, was employed in October 2001 to devote her efforts to this work. Funding was required to continue her work, other salary was for partial support of Scott Mattinson, Postharvest Physiology Research Associate. Additional support for his salary came from WSU and USDA Special grant funds. Supplies category included: motor pool vehicle for sample gathering ($116/trip) reagent chemicals, operation of CA storage facility, mass spectroscopy and NMR facilities use. New information has required us to become proficient in molecular biology; use of these tools are expensive due to the vast amounts of consumable supplies required. Miscellaneous funds were requested for chemical consultation services with Professor Natale, publication, graphics and computer support. Wages were for part-time student helpers.

Budget
Project Duration: **2 years (2003-2004)**
Project total: **$117,052**

Item	Year 1 (2003)	Year 2 (2004)	**Total**
Salaries			
0.2 FTE DS Mattinson	6,638	6,804	**13,442**
1.0 FTE Margo Haines	26,713	27,247	**53,960**
Benefits 40%/42%	12,675	14,165	**26,840**
Wages	4,500	4,500	**9,000**
Benefits 9%	405	405	**810**
Equipment	0	0	**0**
Supplies	6,000	6,000	**12,000**
Travel	0	0	**0**
Miscellaneous	500	500	**1,000**
Total	57,431	59,621	**117,052**

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